Effect of L-arginine and guanidinium chloride (GdmCl) on the unfolding and refolding of hen egg-white lysozyme (HEWL)

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Zusammenfassung

L-Arginin ist ein für die Faltung von Proteinen *in vitro* häufig verwendetes Additiv, das die Proteinaggregation unterdrückt und dadurch die Ausbeute an nativem Protein erhöht. Neben Arginin werden auch andere Additive zur Verbesserung der Effizienz bei der Proteinrückfaltung verwendet, aber Arginin ist eines der wirksamsten Faltungsadditive.

Obwohl der faltungsverbessernde Effekt von Arginin seit einer Patentveröffentlichung im Jahre 1985 bekannt ist, wurde der Wirkungsmechanismus bisher nicht näher charakterisiert. Daher hatte diese Arbeit das Ziel, den Wirkungsmechanismus von Arginin zu charakterisieren. Arginin wurde mit Guanidiniumchlorid (GdmCl) verglichen, das auch eine Guanidingruppe besitzt. In höheren Konzentrationen wirkt GdmCl denaturierend. Der Wirkmechanismus beider Additive wurde im Konzentrationsbereich 0-2 M charakterisiert, wobei 2 M die Löslichkeitsgrenze von Arginin darstellt.

Der Einfluß beider Additive auf die Rückfaltung und Entfaltung von Lysozym, sowie die Wirksamkeit von andern zu Arginin strukturell ähnlichen Verbindungen wurde bestimmt.

Einfluß auf die Rückfaltung von Lysozym

Beide Additive (Arginin und GdmCl) sind imstande, die Rückfaltung von Lysozym sehr erfolgreich zu verbessern. In Gegenwart dieser Additive kann eine Rückfaltungsausbeute von nahezu 100% erreicht werden (bei einer Additivkonzentration von mehr als 0,5 M). Die Aggregation wird durch beide Substanzen vollständig unterbunden.

Um den Einfluß auf die Faltungs- und Aggregationskinetik zu untersuchen, wurden Rückfaltungskinetiken in Gegenwart verschiedener Arginin- und GdmCl-Konzentrationen gemessen. Die Daten wurden nach dem kinetischen Modell von Hevehan und De Bernardez Clark (1997) ausgewertet, das die Berechnung getrennter Geschwindigkeitskonstanten für Faltung und Aggregation erlaubt. Die Ergebnisse belegen, daß beide Additive die Geschwindigkeitskonstanten für Aggregation in ähnlicher Weise beeinflussen. In Gegenwart von mehr als 0,5 M GdmCl erniedrigt sich die Geschwindigkeitskonstante für die Faltung sehr stark, wobei sich in Gegenwart von 0,25 M-0,5 M Arginin die Geschwindigkeitskonstante für die Faltung leicht erhöht. Beide Additive verändern das Verhältnis zwischen den Geschwindigkeitskonstanten für Faltung und Aggregation dahingehend, daß Intermediatprotein nur durch die Faltungsreaktion verbraucht wird. In Gegenwart höherer GdmCl-Konzentrationen überwiegt der denaturierende Effekt von GdmCl, was die Entfaltung von

Intermediat- und nativem Protein erleichtern, und daher die Geschwindigkeitskonstante für die Faltung erniedrigt.

Daher wurden Kinetiken der Intermediatbildung in Gegenwart beider Additive verglichen. Faltungsintermediate sind instabil und lassen keine detaillierte strukturelle Charakterisierung zu. Die Anwesenheit solcher Intermediate kann aber mit dem Fluoreszenzfarbstoff 1-Anilino-8-Sulfonsäure (ANS) nachgewiesen werden. ANS-Fluoreszenzkinetiken wurden in Abwesenheit und Anwesenheit von 1 M Arginin und GdmCl gemessen. Die beobachteten Kinetiken zeigen keinerlei Unterschiede, woraus zu schließen ist, dass die Bildung von Faltungsintermediaten nicht durch Additive beeinflußt wird. Das deutet darauf hin, daß die Additive nicht die Bildung von Intermediaten beeinflussen, sondern die Faltungs- und Aggregationsprozesse, die nach der Bildung von Intermediaten stattfinden. Bei der Rückfaltung von Lysozym kommt es zu einer Konkurrenz zwischen Faltung und Aggregation, welche die Additive zugunsten der Faltung beeinflussen.

Um die vollständige enzymatische Aktivität zu erreichen, müssen während der Rückfaltung alle vier Disulfidbrücken in Lysozym korrekt ausgebildet werden. Um dies zu ermöglichen, muß ein für die entsprechenden Bedingungen geeignetes Thiol-Reaktionssystem verwendet werden (*Oxido Shuffling System*). Es besteht aus einer reduzierenden und oxidierenden Sulfhydrylkomponente. Hier wurde 2 mM Dithiothreitrol (DTT) und 5 mM oxidiertes Glutathion (GSSG) für die Rückfaltung von Lysozym verwendet.

Nach Zugabe des *Oxido Shuffling Systems* zu nativem Lysozym konnten keine Veränderungen in der Sekundär-, Tertiärstruktur und Aktivität festgestellt werden. In Gegenwart des *Oxido Shuffling Systems* verringert sich die thermodynamische Stabilität von Lysozym, was durch die Messung von CD Temperaturentfaltungskurven bestimmt wurde. In Gegenwart des *Oxido Shuffling Systems* und von 1 M GdmCl kommt es zur weiteren Destabilisierung, wohingegen die Anwesenheit von 1 M Arginin zu einer Erhöhung der Stabilität führt. Diese Versuchsbedingungen erlauben es, Schlußfolgerungen auf den Einfluß von Arginin während des Rückfaltungsprozesses zu ziehen. Während eines Rückfaltungsprozesses bildet sich Sekundär- und Tertiärstruktur von zunächst vollständig denaturiertem Protein aus. Denaturiertes Protein ist konformell sehr flexibel, was dazu führt, daß es sehr anfällig für Thiol-Austauschreaktionen ist. Während Temperaturentfaltungsmessungen entfaltet sich das Protein langsam und wird daher mehr und mehr anfällig für Thioloxidations- und Reduktionsreaktionen. Arginin erhöht die thermodynamische Stabilität von Lysozym unter diesen Bedingungen. Die Versuchsbedingungen (Temperaturentfaltung von Lysozym in Gegenwart von Thiolverbindungen) sind vergleichbar mit dem Rückfaltungsprozeß von Lysozym mit dem Unterschied, daß

zu Beginn des Rückfaltungsprozesses Lysozym vollständig denaturiert ist. Arginin verändert nicht die thermodynamische Stabilität von nativem Lysozym.

Proteinaggregate bilden sich während der Rückfaltung äußerst schnell aus und sind daher nicht für spektroskopische Charakterisierungen oder kinetische Messungen zugänglich. Der Einfluß von Arginin und GdmCl auf große, vollständig ausgebildete Aggregate wurde bestimmt, indem Aggregate mit Puffern, die verschiedene Arginin- und GdmCl-Konzentrationen enthielten, in Verbindung gebracht wurden und anschliend die Konzentration an löslichem Lysozym bestimmt wurde. In Gegenwart von Arginin konnte keinerlei Resolubilisierung von aggregiertem Protein festgestellt werden, wohingegen GdmCl Aggregate teilweise auflösen konnte (bei Konzentration über 2,5 M kommt es zur vollständigen Auflösung).

Arginin muß zu Beginn des Rückfaltungsprozesses anwesend sein muß, um das Gleichgewicht zwischen Faltungs- und Aggregationsreaktion zu verändern. In einem weiteren Versuch wurden Additive erst nach einigen Minuten zugegeben, um zu bestimmen, ob die Aggregation verringert wird, wenn die Rückfaltung bereits begonnen hat. GdmCl kann die Aggregation bis zu 30 min nach Reaktionsbeginn verringern, wohingegen Arginin während der ersten 3 min anwesend sein muß. Im Vergleich mit Intermediatkinetiken zeigt sich, daß Arginin nur solange die Aggregation verringern kann, bis Faltungsintermediate vollständig ausgebildet sind. Dies deutet darauf hin, daß Arginin mit Faltungsintermediaten interagiert, da es nur während der Ausbildung von Intermediaten den Aggregationsprozeß beeinflußen kann. Für GdmCl deuten die Daten darauf hin, daß es wahrscheinlich Intermediate und Aggregate resolubilisiert, und so Aggregation vermindert.

Einfluß auf die Entfaltung von Proteinen

Während der Rückfaltung von Lysozym ist die Aggregation eine sehr schnelle Reaktion, die nicht abgestoppt werden kann. Aggregate werden sofort nach Beginn der Rückfaltungsreaktion sichtbar.

Typischerweise sind einige der Aggregate durch Disulfidbrücken miteinander vernetzt, was die Zugabe reduzierender Reagenzien erfordert wenn Aggregate komplett aufgelöst werden sollen. Der Aggregationsprozeß kann nicht abgestoppt werden, da sich Intermediate sofort nach Erniedrigung der GdmCl Konzentration ausbilden, und diese erst nach und nach vollständig reagieren.

Aufgrund dieser Tatsache ist es sehr schwierig, den Einfluß von Additiven auf den Aggregationsprozeß zu bestimmen. Wegen dieser Nachteile wurde ein geeigneter langsamer, kontrollierbarer Aggregationsprozess verwendet, um den Einfluss von Additiven auf die Aggregationsreaktion zu bestimmen. Wenn

Dithiothreitol (DTT) zu nativem Lysozym zugegeben wird, kommt es zur vollständigen Aggregation nach einer Lag-Phase. Dieser Aggregationsprozeß kann jederzeit durch Alkylierung freier Sulfhydrylgruppen unterbrochen werden, was darauf hindeutet, daß die Aggregation nach der Reduktion von Disulfidbrücken stattfindet. Die beobachteten Aggregationskinetiken können am besten durch einen sigmoiden Kurvenlauf angenähert werden. Es kann daher angenommen werden, daß dieser Aggregationsprozeß durch einen oder mehrere Intermediatzustände verläuft. Beide Additive unterdrücken die Aggregation.

Dem Aggregationsprozeß geht ein Aktivitätsverlust voran, was darauf hindeutet, daß Aktivität als Folge der reduzierten Disulfidbrücken verloren geht. Da alle vier Disulfidbrücken für die volle Aktivität erforderlich sind, deutet eine verringerte Aktivität auf eine (teilweise) Reduzierung von Disulfidbrücken hin, bevor dann der Aggregationsprozess beginnt. Der Aktivitätsverlust kann am besten mit einer exponentiellen Funktion beschrieben werden.

Für den Aktivitätsverlust wurden Unterschiede zwischen beiden Additiven festgestellt. In Gegenwart von unterschiedlichen GdmCl-Konzentrationen wurden keinerlei Unterschiede in der Kinetik des Aktivitätsverlusts gemessen, wohingegen in Anwesenheit von höheren Argininkonzentrationen der Aktivitätsverlust langsamer stattfindet und in Gegenwart von 1 M Arginin etwas Aktivität erhalten bleibt. Da der Aktivitätsverlust direkt von der Reduktion der Disulfidbrücken abhängig ist, bedeutet dies, daß Arginin die Geschwindigkeitskonstante der Reduktionsreaktion verringert. Die Reduktionsgeschwindigkeit hängt nicht von der GdmCl-Konzentration ab, sondern ist konstant – sie ist genauso schnell wie in Abwesenheit von Additiven.

Um zu beurteilen, wie die Sekundär- und Tertiärstruktur in Gegenwart von Additiven und DTT beeinflußt wird, wurden CD-Spektren gemessen. In der Gegenwart von Arginin (und DTT) bleibt die native Sekundär- und Tertiärstruktur von Lysozym erhalten, wohingegen in Gegenwart von GdmCl die Sekundär- und Tertiärstruktur verloren geht.

Das Auftreten von hydrophoben Entfaltungsintermediaten wurde mittels ANS-Fluoreszenz ermittelt. Keine Intermediate (und nachfolgend auch keine Aggregation) wurde in Gegenwart von mehr als 0,5 M Arginin oder GdmCl beobachtet, bei geringeren Konzentrationen wurden Entfaltungsintermediate beobachtet.

Die Überlagerung der Intermediat- und Aggregationskinetiken zeigt, daß Intermediate vor dem Auftreten von Aggregaten nachgewiesen werden können. Bei Zugabe von mehr als 0,5 M Arginin oder GdmCl wurden keine Entfaltungsintermediate (durch ANS-Fluoreszenz) oder und den nachfolgenden Aggregationsprozeß. Die Überlagerung der Aggregations- und ANS-Kinetiken

vermittelte wertvolle Erkenntnisse über den Mechanismus, mit dem die beiden Additive die Aggregation unterdrücken.

In Abwesenheit von Arginin und GdmCl und in Gegenwart von 0,25 M GdmCl überlagern sich beide Kinetiken sehr gut, was bedeutet, daß sich die Aggregation zur selben Zeit erhöht wenn sich Intermediate ausbilden. In Anwesenheit von 0,25 M Arginin erfolgt die Entstehung von Intermediaten in einem ähnlichen Zeitraum wie zuvor beschrieben, jedoch erfolgt die Aggregation verzögert und findet nicht mehr synchron mit dem Auftreten von Intermediaten statt. Dies deutet darauf hin, dass Arginin zwar nicht die Kinetik der Ausbildung von Intermediaten beeinflußt, aber deren Aggregation verzögert bzw. vollständig unterbindet. GdmCl verhindert die Aggregation dadurch, daß alle auftretenden Spezies – Intermediate und Aggregate gleichermaßen – entfaltet oder thermodynamisch destabilisiert werden.

Struktur-Wirkungsbeziehung von Arginin

Bis jetzt wurde der Wirkungsmechanismus von Arginin und GdmCl beschrieben, wobei strukturelle Besonderheiten dieser Verbindungen nicht in Betracht gezogen wurden. Um die Rolle der verschiedenen funktionellen Gruppen in Arginin zu bestimmen, wurde der Einfluß strukturell ähnlicher Substanzen auf Rückfaltung und Aggregation ermittelt.

Als erstes wurde D-Arginin mit L-Arginin verglichen. Es wurden keine Unterschiede in der Wirksamkeit zwischen D- und L-Arginin festgestellt, was zeigt, daß keinerlei stereospezifischen Effekte eine Rolle spielen.

Da die stark positive Ladung der Guanidingruppe in Arginin für die Wirksamkeit eine Rolle spielen könnte, wurde die stark negativ geladenen Aminosäure Glutamat verwendet. Neben Lysozym wurde auch BSA (*bovine serum albumin*) eingesetzt, da Lysozym im verwendeten Puffer positiv geladen ist, wohingegen BSA negativ geladen ist. Dies ermöglicht es, den Einfluß der Ladung auf die Aggregation zu bestimmen. Es zeigt sich, daß Arginin die Aggregation beider Proteine unterdrückt. Im Gegensatz dazu erhöht Glutamat die Aggregation beider Proteine. Die Wirksamkeit von Arginin hängt daher nicht vom isolelektrischen Punkt des Proteins ab. Die Versuche mit Ornithin belegen, daß die Geometrie der Guanidingruppe in Arginin für den auf die Rückfaltung förderlichen Einfluß verantwortlich sein muss. Anstelle der Guanidingruppe besitzt Ornithin eine Aminogruppe. Ornithin verhindert die Proteinfaltung. Das zeigt, daß ähnlich wie bei Glutamat eine andere funktionelle Gruppe als die Guanidingruppe nicht die Proteinfaltung verbessert. Die Geometrie oder Ladungsverteilung der Guanidingruppe muss für den Effekt verantwortlich sein.

Schließlich wurde die Wirksamkeit von stärker zu Arginin verwandten Verbindungen bestimmt. L-Homoarginin, das eine zusätzliche Methylengruppe in der Seitenkette besitzt, fördert die Rückfaltung von Lysozym bis zu einer Konzentration von 1 M, unter höheren Konzentrationen nimmt die Aktivität stark ab. Ein ähnliches Bild ergab sich für Agmatin, das decarboxyliertes Arginin ist, und das ebenfalls die Faltung bis zu einer Konzentration von 1 M fördert und dann verringert, allerdings verringert es auch die Aktivität von nativem Lysozym. Agmatin verringert jedoch die Aggregation, was zeigt, dass es den Proteinfaltungsprozess positiv beeinflußt.

Um die Bedeutung der Amino- und Carboxylgruppe in Arginin zu bestimmen, wurde die Wirksamkeit von Poly-L-Arginin auf die Ruckfaltung von Lysozym ermittelt. Da die Amino- und Carboxylgruppen in Poly-L-Arginin in Peptidbindungen involviert sind, können sie nicht die Wechselwirkungen mit Lysozym beeinflussen. Poly-L-Arginin übt einen negativen Effekt auf die Lysozymaktivität aus. Da Poly-L-Arginin stärkere Wechselwirkungen mit Lysozym eingeht, war es schwierig, Schlußfolgerungen zu ziehen.

Abschließende Bemerkungen

Die vorliegende Arbeit befasst sich mit dem Einfluß von Arginin und GdmCl auf die Proteinfaltung und –entfaltung von Lysozym. Einige Schritte zu Beginn des Rückfaltungsprozesses finden sehr schnell statt und sind daher nicht der genaueren Analyse zugänglich. Einige ungewöhnliche Eigenschaften von Arginin wurden jedoch sichtbar, z.B. daß Arginin nur während der ersten Minuten nachdem die Rückfaltung begonnen wurde, wirksam ist. Im Gegensatz dazu ist die Wirksamkeit von GdmCl weniger zeitabhaengig. Mit der Charakterisierung des reduktiven Entfaltungsprozesses von Lysozym konnte der Einfluß beider Additive auf die Aggregation bestimmt werden. Der Aggregationsprozeß ist dabei sehr langsam und kann jederzeit abgestoppt werden, was sehr hilfreich war um einen genaueren Einblick auf die Wirkung von Arginin und GdmCl zu gewinnen.

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1. Introduction

1.1. Protein folding

The result of a particular protein folding process is biologically active protein in its native conformation. During refolding, a denatured, unstructured polypeptide chain (Tanford, 1968) undergoes formation of secondary, super-secondary, tertiary, and quaternary structure (in case of oligomeric proteins; Jaenicke, 1987, 1999) through non-covalent and covalent interactions (Jaenicke, 1996, 1999). Typically, secondary structure formation is completed within several hundred milliseconds, while formation of the structural features of higher order may take up to several hours. With the exception of small single domain proteins, proteins usually fold through intermediates. Usually the fast formation of a population of hydrophobic intermediates is observed immediately after folding has been initiated. This hydrophobic collapse is called burst. Intermediates are partially structured and usually not as compact as the native protein. Usually, they are not kinetically stable and therefore it is difficult to characterize them.

Protein folding may not be entirely successful in which case the protein is biologically inactive and either aggregated or soluble. Both cases limit the yield of biologically active protein. A specialized case of aggregation is the formation of fibrils, which are also associated with certain diseases (BSE, Alzheimer's Disease, Huntington's Disease, Senile Systemic Amyloidosis (SSA)). The difference is that fibrils usually form from native proteins, while amorphous aggregates originate from refolding of denatured protein (Fink, 1998; Kelly *et al.*, 1997; Serpell, 2000). Fibrils are sometimes referred to as ordered aggregates to differentiate them from amorphous aggregates.

The reason why proteins fold is of thermodynamic nature. Every possible conformation has a certain Gibbs Free Energy. The native protein has the lowest Free Energy, while the denatured protein has the highest Free Energy. Entropy is reduced during the folding process because the degree of conformational freedom decreases. The thermodynamic gradient between unfolded and native conformations is the driving force which enables protein folding. However, thermodynamic parameters do not explain why some proteins fold faster than others. The thermodynamic description of protein folding does not describe folding pathways. Levinthal showed through a simple calculation that a random search through the conformational space is not the possible way of protein folding. Levinthal (Levinthal, 1968) describes this through a simple model: each amino acid of a hypothetical protein can only occur in three different conformational angles. The conversion time between each conformation is assumed to be one picosecond (10^{-12} s; the shortest possible conversion time). A polypeptide chain of 150 amino acids would have $3^{150}=10^{68}$ possible conformations to search, which would require in the order of 10^{48} years (10^{56}

seconds). This simple calculation shows that folding kinetics can not be determined by a random search through all conformations.

A good visualization of folding pathways in combination with thermodynamic aspects of protein folding has been accomplished by folding funnels (Chan and Dill, 1998; Fig. 1-1). The wide opening on top of the funnel represents the unfolded protein, which is highly flexible, resulting in a large number of available conformations. The plane created by the *x*- and *y*-axis represents the possible number of conformations, showing that the unfolded protein on top of the funnel has the largest degree of conformational freedom. As the protein folds, the possible number of conformations is reduced, resulting in just one final folded conformation at the bottom of the funnel. As seen in Fig. 1-1, there are other minima near the native state, which are local minima but do not have the same low free energy as the native state. In the middle, several peaks are grouped around the center. These peaks represent the activation energy of different pathways which lead to native protein, along the rugged folding landscape. Several pathways exist, which all lead to correctly folded protein, but each pathway has different kinetic parameters.

During actual protein folding experiments, intermediates are frequently observed. Through the use of appropriate fluorescent dyes like 1-anilino-8-naphtalene sulfonic acid (ANS; Stryer, 1965) it has been shown that folding intermediates are highly hydrophobic. Upon refolding hydrophobic amino acids form hydrophobic clusters (local zones of high hydophobicity). Following the "hydrophobic collapse" the native structure forms through further structurization of the protein.

In the last decades, the *molten globule* state (Kuwajima, 1989; Arai and Kuwajima, 2000) has been extensively characterized as an accessible folding intermediate. It was first found in α-lactoglobulin (Kuwajima, 1989). Subsequent experimental data showed that many proteins fold by forming a molten globule-like state. Characteristic for a *molten globule* are the presence of: near-native secondary structure, but not fully native tertiary structure.

A *molten globule* must also bind ANS, which is an indicator that hydrophobic amino acid side chains are exposed to the solvent. The hydrodynamic radius is larger than in the native protein (Kuwajima, 1989). Ptitsyn and coworkers (1990) investigated folding of many globular proteins, and monitored folding kinetics. In all cases they found formation of a molten globule. Thus they proposed that formation of a molten globule is mandatory for folding of globular proteins. However, the significance of the molten globule state for protein folding pathway continues to be highly controversial (Creighton, 1997).

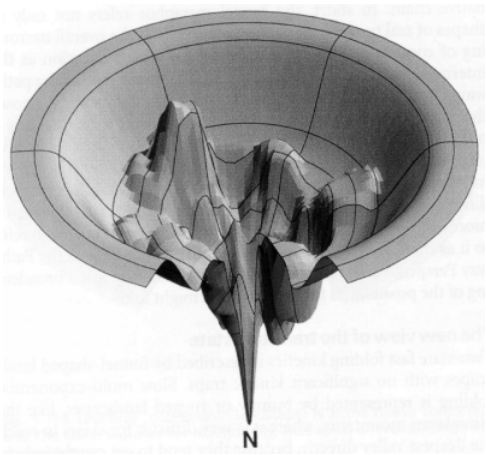


Fig. 1-1: Folding funnel. Minima at the bottom represent (left) the native state and (right) a molten globule state. Figure adapted from Chan and Dill (1998).

The interpretation of ANS fluorescence data requires caution, because it has been reported that ANS itself can induce formation of ANS binding intermediate(s) (Kamen and Woody, 2001). It has also been shown that ANS can suppress aggregation during thermal unfolding of carbonic anhydrase (Kundu and Guptasarma, 1999).

Protein folding intermediates can only be detected while the refolding process is taking place. In order to characterize population of intermediates, stopped-flow instrumentation capable of recording fast kinetics needs to be utilized. Stopped-flow equipment allows recording of folding processes in the millisecond range.

In the last couple of years examples of protein folding processes have been described, which are kinetically controlled (Baker and Agard, 1994). Examples include folding of proteases which require their pro-regions to fold efficiently. In absence of their pro-sequence folding rates of these proteins are dramatically reduced. Other proteins fold simultaneously through several pathways which have different kinetic rates, because their activation energies are different.

Some proteins can be converted into alternative states under certain conditions. In these cases, they exhibit tertiary and/or secondary structure different from the native protein. Lowering pH is one factor which may lead to formation of alternative states of some proteins. This has been extensively described for transthyretin and other amyloidogenic proteins (Lai *et al.*, 1996; Serpell, 2000; Fink, 1998), which are more prone to fibril formation in acidic conditions. Another example are immunoglobulins, which form different conformations under acidic conditions (Buchner *et al.*, 1991).

1.2. Technical aspects of protein refolding processes

Through progress in molecular biology in the 1970s overexpression of recombinant proteins in bacteria and other host organisms has become possible. Although eukaryotic hosts can be used to enable post-translational modification, prokaryotic cells are used far more frequently. The overexpression of proteins in bacteria can result in incorrectly folded, non-functional insoluble inclusion body protein (Fig. 1-2), which is accumulated inside bacterial cells. IR spectra of inclusion body proteins show that the protein has a high content of secondary structure (Przybycien *et al.*, 1994; Fink, 1998; Seshadri *et al.*, 1999). Strategies can be pursued to decrease formation of inclusion bodies, but they may be regarded as advantageous, since they contain target protein in a relatively pure and highly concentrated form. Soluble target proteins require extensive purification after harvesting. Inclusion bodies contain primarily target protein. In order to recover biologically active protein from inclusion bodies, inclusion bodies need to be isolated and solubilized, for which chaotropic reagents such as guanidinium chloride (GdmCl) or urea are used. In cases where the target protein contains disulfide bonds, reducing agents need to be added to completely dissolve the inclusion body protein and to assure that incorrectly formed disulfide bonds are reduced and may be reformed correctly during the refolding process. Protein from the insoluble inclusion bodies is now dissolved and denatured, from which it must be refolded to recover active soluble protein (Rudolph and Lilie, 1996; Rudolph *et al.*, 1999; De Bernardez Clark, 2001).

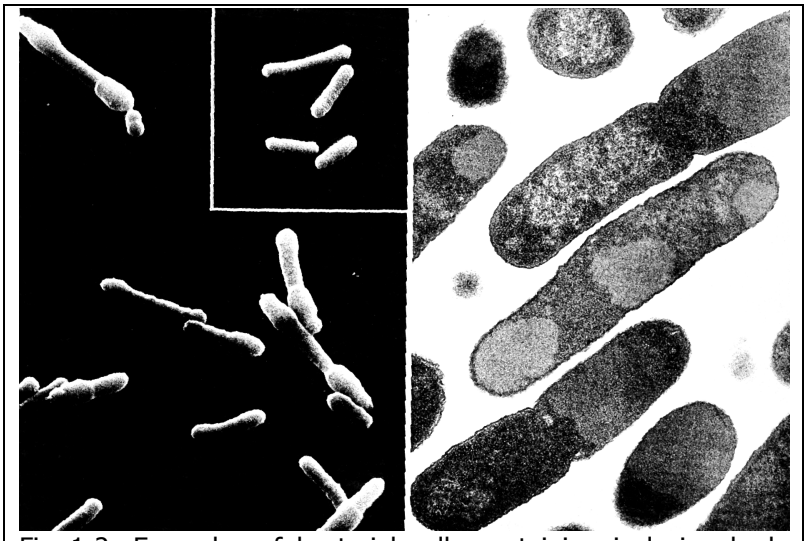


Fig. 1-2: Examples of bacterial cells containing inclusion body protein. Typically, inclusion bodies form on both ends of the cells. Adapted from Old and Primrose (1994).

Protein refolding is initiated when concentration of denaturant (GdmCl or urea) is lowered. This can be accomplished by diluting the highly concentrated solution of denatured protein into refolding buffer. Another way of lowering the denaturant

concentration is dialysis or diafiltration. In addition, size-exclusion chromatography ("refolding chromatography") can be used for buffer exchange, resulting in removal of denaturant.

Formation of aggregates is a major problem during protein refolding. The more the protein becomes diluted during the refolding step, the less prone it is to aggregation. Unfortunately, a high dilution to initiate refolding is not practical for industrial processes, because it results in a large volume of solution, which needs to be concentrated afterwards. Additional purification steps may also be needed which are much more expensive for larger volumes. Therefore, the challenge is to develop protein refolding processes which work at high protein concentrations. The following strategies have been shown to be efficient for suppression of aggregation (De Bernardez Clark *et al.*, 1999):

- High dilution of denatured protein solutions, which as mentioned above is impractical as it requires a concentration step of the highly dilute protein solution afterwards.
- Step addition of denatured protein (Rudolph and Fischer, 1990). Denatured protein is added in successive aliquots, which are given enough time to refold before the next aliquot is added. Since correctly folded protein is present when the next aliquot is added, aggregation is less likely to occur.
- Immobilization of unfolded protein on a matrix and subsequent refolding of protein while bound to the matrix (Stempfer *et al.*, 1996a, 1996b). Denatured protein is bound to a column, utilizing ionic interactions. For this purpose the sequence for a poly–histidine or poly–arginine tag is added to the target gene sequence. Refolding is initiated while protein is bound to the column. This suppresses aggregation, because protein-protein interactions leading to aggregation are minimized. After the refolding step, correctly folded protein is eluted from the column.
- Use of specific antibodies to prevent accessibility of aggregation sites (Katzav-Gozansky *et al.*, 1996). Specific antibodies are directed towards aggregation sites to prevent protein-protein interactions which lead to aggregation. Binding to folding intermediates, however, should not be too tight, otherwise formation of native protein is inhibited.
- Aggregation is mediated through hydrophobic side chains, which are exposed to solvent and form patches. The patches interact, leading to formation of aggregates. Mutating the target protein in a way that breaks up the hydrophobic patches is another strategy to suppress aggregation. Knappik and Plückthun (1995) and Nieba and coworkers (Nieba *et al.*, 1997) have demonstrated the feasibility of this method.
- Application of hydrostatic pressure in the order of 1-3 kbar can be used to dissociate proteins without disrupting protein structure (Schade *et al.*, 1980). Higher hydrostatic pressure can be used to unfold proteins and to prevent aggregation. This has been shown for a wide variety of proteins (Schmid *et*

al., 1979; Gorovits and Horowitz, 1998; Foguel *et al.*, 1999; St John *et al.*, 1999; Webb *et al.*, 2000; St John *et al.*, 2001; Randolph *et al.*, 2002).

- Aggregation can be suppressed by using fusion constructs with hydrophilic proteins or peptides. The fusion peptide or protein should be completely folded when refolding of the target protein is initiated. The fusion peptide or protein should not inhibit the refolding process of the target protein. This strategy has been successfully implemented for refolding of insulin-like growth factor I by using the immunoglobulin binding domain from staphylococcal protein A (Samuelsson *et al.*, 1991). Some proteins do not fold well without their pro-sequence, which may be seen as an application of this strategy by nature. Examples are zymogens or rh-pro-NGF (*nerve growth factor*; Rattenholl *et al.*, 2001). In this case the pro-sequence consists of 103 amino acids, while the mature NGF consists of 120 amino acids. In absence of the pro-sequence only 0.5% of protein folds, while in presence of the pro-sequence approximately 35% refolding yield was achieved. The role of the pro-sequence is not clearly understood but it was speculated that it may act as a scaffold for the formation of the cystine knot in the center of the NGF molecule.
- Addition of molecular chaperones can also improve folding yields (Schwarz *et al.*, 1996). Molecular chaperones interact unspecifically with unfolded proteins, especially at hydrophobic sections of the unfolded protein and promote formation of native protein (Hartl, 1996). Although chaperones appear to be universally applicable to decrease aggregation, their successful use remains an exception in comparison to other strategies mentioned here. For the *in vitro* application of chaperones, the most frequently used is the bacterial GroEL/GroES system. The lens protein α-crystallin also has chaperone activity and was used by Ganea and Harding to facilitate refolding of glyceraldehyde-3-phosphate dehydrogenase (Ganea and Harding, 2000). Bettelheim and coworkers (1999) showed that α-crystallin reduces formation of aggregates as monitored by dynamic light scattering. Cyclodextrin can be used to provide similar effects as chaperones, since cavities of cyclodextrin provide exclusion from the solvent (Leung *et al.*, 2000).
- Addition of low molecular additives to the refolding buffer (Rudolph and Lilie, 1996; De Bernardez Clark *et al.*, 1999). Suitable low molecular weight additives which suppress aggregation are added when refolding is initiated. Folding additives play a crucial role in the development of cost-efficient processes, since they allow folding to take place at relatively high concentrations (Fig. 1-3), and they are universally applicable and inexpensive in comparison to previously mentioned strategies.

Prolyl cis/trans isomerase catalyzes the cis-trans transition of prolyl residues, which may be rate-limiting for folding of some proteins. Disulfide oxidoreductases can be used to facilitate formation of correct disulfide bonds.

Cysteine residues of cytosolic proteins are usually reduced, while extracellular proteins usually have oxidized cysteine residues.

ADDITIVES USED TO INCREASE YIELD OF CORRECT FOLDING AND PREVENT AGGREGATION

Additive	Recommended concentration	References[a]
L-Arginine hydrochloride	0.4–0.8 *M*	1, 2
Polyethylene glycol (PEG, 3350 MW)	0.1–0.4 g/liter	3, 4
Nondenaturing concentrations of		5, 6
Urea	≤2.0 *M*	
Gdm/Cl	≤1.0 *M*	
Methylurea	1.5–2.5 *M*	1
Ethylurea	1.0–2.0 *M*	1
Formamide	2.5–4.0 *M*	1
Methylfomamide	2.0–4.0 *M*	1
Acetamide	1.5–2.5 *M*	1
Ethanol	Up to 25% (v/v)	7
n-Pentanol	1.0–10.0 m*M*	8
n-Hexanol	0.1–10.0 m*M*	8
Cyclohexanol	0.01–10.0 m*M*	8
Tris	≥0.4 *M*	9
Na_2SO_4 or K_2SO_4	0.4–0.6 *M*	10
Glycerol	20–40% (v/v)	10
Sorbitol	20–30% (v/v)	10
α-Cyclodextrin	20.0–100.0 m*M*	11
Lauryl maltoside	ca. 0.06 mg/ml	12
Cetyltrimethylammonium bromide	ca. 0.6 mg/ml	13
CHAPS	10.0–60.0 m*M*	8, 14
Triton X-100	ca. 10.0 m*M*	8
Dodecyl maltoside	2.0–5.0 m*M*	15
Mixed micelles	Depending on compounds used	16, 17

[a] (1) R. Rudolph, S. Fischer, and R. Mattes, U.S. Patent 5,593,865 (1997); (2) J. Buchner and R. Rudolph, *Bio/Technology* **9,** 157 (1991); (3) J. L. Cleland, S. E. Builder, J.-R. Swartz, M. Winkler, Y. Chang, and D. I. C. Wang, *Bio/Technology* **10,** 1013 (1992); (4) J. L. Cleland, C. Hedgepeth, and D. I. C. Wang, *J. Biol. Chem.* **267,** 13327 (1992); (5) G. Orsini and M. E. Goldberg, *J. Biol. Chem.* **253,** 3453 (1978); (6) S. E. Builder and J. R. Ogez, U.S. Patent, 4,620,948 (1986); (7) K. R. Hejnaes, S. Bayne, L. Nørskov, H. H. Sørensen, J. Thomsen, L. Schäffer, A. Wollmer, and L. Skriver, *Prot. Engin.* **5,** 797 (1992); (8) D. B. Wetlaufer and Y. Xie, *Prot. Sci.* **4,** 1535 (1995); (9) D. Ambrosius and R. Rudolph, U.S. Patent 5,618,927 (1997); (10) U. Michaelis, R. Rudolph, M. Jarsch, E. Kopetzki, H. Burtscher, and G. Schumacher, U.S. Patent 5,434,067 (1995); (11) N. Karuppiah and A. Sharma, *Biochem. Biophys. Res. Commun.* **211,** 60 (1995); (12) S. Tandon and P. Horowitz, *Biochim. Biophys. Acta.* **955,** 19 (1988); (13) S. Tandon and P. Horowitz, *J. Biol. Chem.* **262,** 4486 (1987); (14) N. Cerletti, G. K. McMaster, D. Cox, A. Schmitz, and B. Meyhack, U.S. Patent 5,650, 494 (1997); (15) J. Stockel, K. Doring, J. Malotka, F. Jahnig, and K. Dornmair, *Eur. J. Biochem.* **248,** 684 (1997); (16) G. Zardeneta and P. M. Horowitz, *J. Biol. Chem.* **267,** 5811 (1992); (17) G. Zardeneta and P. M. Horowitz. *Anal. Biochem.* **218.** 392 (1994).

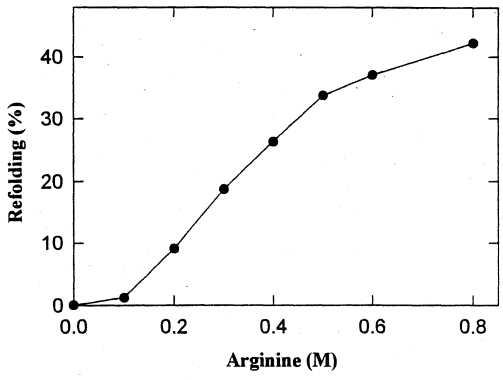
Fig. 1-3: Commonly used folding additives. Figure adapted from DeBernardez Clark *et al.*, 1999.

Since most proteins require correctly formed disulfide bonds to be active, and inclusion body protein usually is not correctly disulfide bonded, suitable measures need to be taken during refolding. Metal ion catalyzed air oxidation has been

used to promote disulfide bond formation (Ahmed *et al.*, 1975). However, air oxidation is limited by mass transfer of oxygen into aqueous solutions and usually yields low activities due to formation of incorrect disulfide bonds. Prolonged exposure to air may also lead to modifications of certain amino acid side chains (*i.e.* oxidation), thus reducing protein activity. A more practical and thus more common used strategy is the use of 'oxido shuffling systems'.

An oxido shuffling system promotes initial oxidation of free sulfhydryl groups as well as disulfide rearrangement for the formation of correct disulfide bonds. Oxido shuffling systems consist of an oxidizing and a reducing sulfhydryl component.

Mixtures of reduced (GSH) and oxidized (GSSG) glutathione are used mostly frequently, but the pairs cysteine/cystine, cysteamine/cystamine, 2-mercaptoethanol/2-hydroxyethyl disulfide can also be used (De Bernardez Clark *et al.*, 1999). The molar ratio of reduced to oxidized thiol component usually is in the range of 1:1 to 10:1. Protein disulfides can also be formed after formation of mixed disulfides with glutathione or sulfonation of protein thiols (Rudolph *et al.*, 1997).



Effect of L-arginine on the refolding of rh-tPA (U. Opitz, Ph.D. Thesis, University of Regensburg, Germany, 1988). rh-tPA as the mixed disulfide derivative with glutathione was refolded by dilution in 0.1 *M* Tris–Cl, 1 m*M* EDTA, 1 mg/ml BSA, 1 m*M* GSH, pH 8.5, at a protein concentration of 3 mg/ml. The folding yield as percentage of the total amount of protein was determined after 17 hr of renaturation at 25°.

Fig. 1-4: Effect of arginine on the refolding of recombinant tPA. Adapted from De Bernardez Clark *et al.*, 1999.

L-arginine was discovered to be an efficient folding additive when added during refolding of tissue plasminogen activator (tPA) to suppress auto-proteolysis. Surprisingly not only auto-proteolysis was suppressed, but also the yield of native protein strongly increased (R. Rudolph, private communication; Fig. 1-4). This discovery lead to the first description of arginine as protein refolding additive in a patent entitled *"Verfahren zur Aktivierung von t-PA nach Expression in Prokaryonten"* ("Process for activation of t-PA after expression in procaryotic cells") (Rudolph *et al.*, 1985a, 1985b).

Subsequently, ability of arginine to increase refolding yields was tested with other proteins and found to be satisfactory (Buchner and Rudolph, 1991; Brinkmann *et al.*, 1992; Lin and Traugh, 1993). The original patent for renaturation of tPA was extended to process patents, claiming all protein renaturation processes using arginine as refolding additive (Rudolph and Fischer, 1990; Rudolph *et al.*, 1995). Since application of arginine is not limited to proteins with certain properties, it is frequently used because of its high efficiency.

Fig. 1-3 shows a table of commonly used refolding additives. Although many additives have been described as protein refolding enhancers, they often are only efficient for one particular protein. Folding additives can be categorized depending on the concentration needed to promote activity. Surfactants and detergents are effective at very low concentrations, while salts, amino acids, and solvents are effective at higher concentrations, sometimes up to their solubility limit.

1.3. Effect of solvent additives on the stabilization of protein structure

Storage of proteins in solvent additives has been common practice for decades, but mechanistic aspects involved have never been characterized. As cosolvents for the storage of proteins mostly sucrose, glycerol, and other polyols are used. These additives need to be applied in relatively high concentrations to be active. Typically, concentrations of 1 to 4 M are needed to efficiently protect proteins during storage.

The need for high cosolvent concentrations to observe the effects indicates that specific protein-cosolvent interactions are likely not responsible for the stabilization effect, since otherwise small concentrations of cosolvent would be enough to achieve this effect. Interaction of cosolvents with proteins has been extensively characterized by Timasheff and coworkers using a sophisticated dialysis equilibrium method (Arakawa *et al.*, 1990; Bhat and Timasheff, 1992; Kita *et al.*, 1994; Lin and Timasheff, 1996; Timasheff, 1992a, 1992b, 1993, 1995; Xie and Timasheff 1997a, 1997b, 1997c). This technique allows for determination of preferential binding, utilizing high-precision densimetry and differential refractometry. As result, the cosolvent concentration inside and outside the dialysis bag is determined. This measurement allows to perform affinity measurements of one particular cosolvent in relation to a protein. In those cases where cosolvent becomes more concentrated inside the dialysis bag, the effect is called *preferential binding,* in those cases where the concentration is reduced inside the dialysis bag, the effect is called *preferential hydration* (Fig. 1-5). Binding stochiometries of most cosolvents from the perspective of the protein

are negative. This means that cosolvents are excluded from around the protein and enriched in the outside. Appearance of preferential exclusion in the system of protein and cosolvent means that the chemical potential of protein and ligand becomes more positive (increase of free energy), which makes the interaction thermodynamically unfavorable.

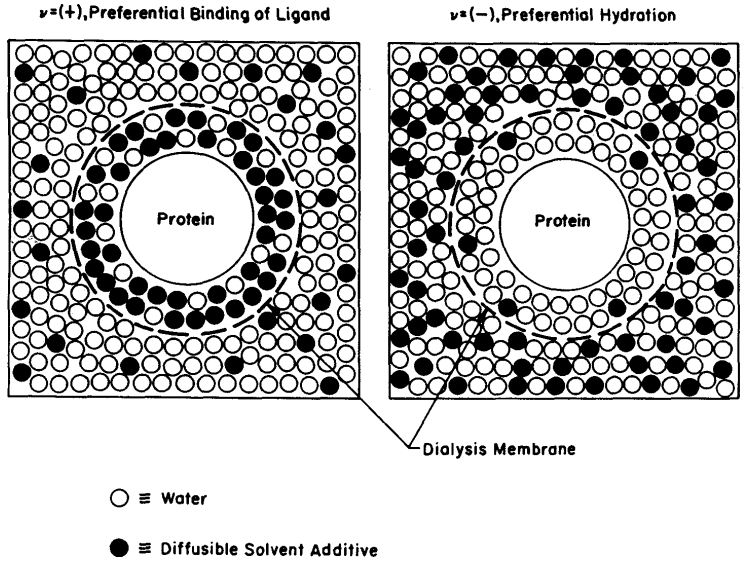


Fig. 1-5: Schematic representation of preferential binding and preferential hydration. Adapted from Timasheff (1992a).

When a protein becomes denatured, the surface volume increases, because the protein is less compact than in the native, correctly folded state. The greater surface volume requires a higher amount of preferential exclusion of the protein. As mentioned above, preferential exclusion of cosolvent increases free energy of the whole system. According to the principle of Le Chatelier, the system tries to move towards the lower free energy (Fig. 1-6). In this case this means that the equilibrium is driven towards a smaller exclusion volume. The smallest exclusion volume is observed for the native protein. This explains why cosolvents, which experimentally show preferential exclusion, stabilize the native state and suppress unfolding of the protein. The salting-out effect of some compounds works in a similar way. The salted-out precipitates with protein are excluded from water, because contact areas between protein molecules are preferable by providing preferential exclusion.

Two basic types of mechanisms contribute to cosolvent exclusion. Mechanism (1): interactions are determined by solvent properties. Proteins remain inert towards the additive and only presents a surface. Interactions between the protein and cosolvent are not affected by changes in the pH or changes of the concentration of cosolvent. This type of interaction is caused by steric exclusion of water from the protein surface or through effects caused by increased surface tension.

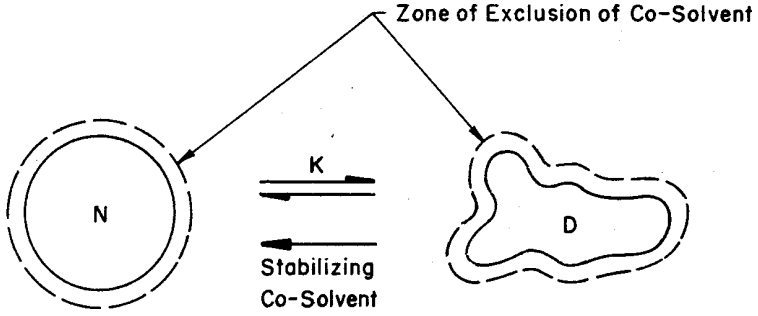


Fig. 1-6: Visualization of the zone of exclusion, which becomes bigger when the protein is unfolded. Adapted from Timasheff (1992a).

Mechanism (2): the chemical nature of the protein surface determines the interactions – attractive as well as repulsive. The effects of most cosolvents cannot be exclusively assigned to either mechanism, but show characteristics of both.

For mechanism (1), two categories of cosolvents can be identified, one of which is steric exclusion. Another category is cosolvents which perturb the surface tension.

Steric exclusion of water from the protein surface is observed as result of size differences between water and cosolvent (Fig. 1-7). Instead of water, cosolvent binds to the protein, thus water is excluded from the protein surface.

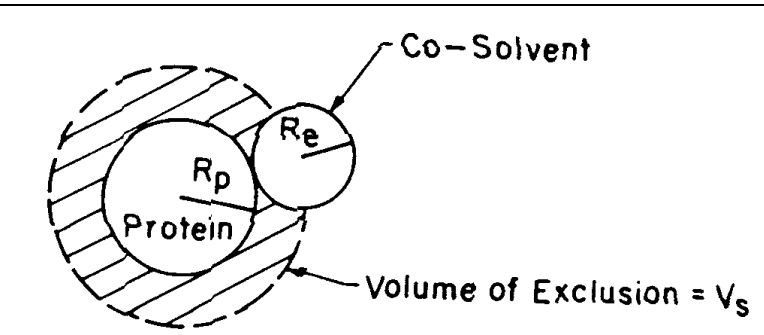


Fig. 1-7: Effect of cosolvents on the volume of exclusion. Adapted from Timasheff (1992a).

One example of a cosolvent acting by steric exclusion is polyethylene glycol (PEG). Due to size of cosolvent molecules, they are not able to penetrate the protein structure and are limited in their approach to center-to-center distance of protein and cosolvent leading to *preferential hydration*. Exclusion by surface tension is based on interactions between water and cosolvent molecules. Many small molecules perturb the surface tension of water. A cosolvent increasing

surface tension of water will be depleted in the surface layer of water. In the dialysis equilibrium experiment this behavior manifests as negative binding stochiometry. Substances interacting in this manner are mostly sugars, amino acids and salts. This type of mechanism is by far most common.

Another class of cosolvents consists of those in which prefential exclusion is determined by chemical interactions between solvent components and exposed groups in the solvent. The last category of cosolvents did not involve chemical interactions between solvent and protein, this class of cosolvents involves some which interact with solute molecules. Possible mechanisms in this category include solvophobicity or repulsion from charges on the protein surface. Again, this leads to preferential exclusion as cause for stabilizing the native state: non-polar interactions between protein and cosolvent are entropically less favorable than contact with water, because non-polar interactions will increase the entropy. One cosolvent identified to act through this mechanism is glycerol. Glycerol shows some affinity for polar groups of proteins, but the overall observed effect of glycerol is preferential exclusion.

Some salts, which are well known for their ability to salt-out proteins, have the effect of stabilizing proteins in solution. The mechanism behind this effect is preferential exclusion. Although the overall effect of a cosolvent is preferential exclusion it may still bind to the protein surface in a weak interaction, resulting overall in preferential exclusion. Through combinations of different ions and counterions it has been determined that each ion contributes separately. A good measure for strength has been published over 100 years ago by Hofmeister (Hofmeister, 1888). The published data describe capability of cations and anions to salt-out proteins. The data set is commonly called "Hofmeister series". The ability to preferentially exclude water *increases* in the following order:

Anions: chlorine < acetate < sulfate.
Cations: guanidinium < (magnesium, calcium, barium) < sodium.

Although the effect on stabilization of the native protein structure can be well explained by above mentioned effects, it is not possible to predict the effect on denatured proteins, since they are not accessible to characterization without addition of cosolvents (denaturants). If a cosolvent is inert towards the protein surface, meaning that no direct interactions occur, preferential exclusion of water from the protein surface will be the only observed effect, stabilizing the protein. It is not possible to make a reliable prediction on how a given cosolvent will affect the equilibrium between native and denatured protein. However, direct interactions between protein and cosolvents may occur, which may be stronger than the above mentioned effects.

In order for a cosolvent to denature proteins, specific interactions between protein and cosolvent must be responsible for its denaturing effect.

Although denaturing properties of some chemicals have been known for a long time, mechanistic details concerning specific interactions between protein and cosolvent leading to denaturation have not been described so far.

Co-solvent	*Protein*	*Apparent binding of co-solvent to protein*			*Preferential hydration of protein*	
		g/g protein	*mol/mol protein*	*Ratio*[a]	*g H_2O/g protein*	*Reference*
1 M Sucrose	Tubulin	−0.106	−38.0	0.69	0.243	2
4.1 M Glycerol	Tubulin	−0.127	−149	neg	0.154	3
1 M Sucrose	RNase A	−0.190	−7.6	0.53	0.437	2
2 M Glucose	BSA (pH 6.0)	−0.099	−37.4	0.72	0.212	4
3.4 M Glycerol	BSA (pH 5.8)	−0.113	−83.4	neg	0.212	5
2 M Glycine	BSA (pH 6.1)	−0.069	−62.2	0.88	0.416	6
2 M Betaine	BSA (pH 6.1)	−0.125	−72.5	—	0.428	6
2 M Sodium glutamate	BSA (pH 7.0)	−0.171	−68.8	0.72	0.417	7
2 M Lysine HCl	BSA (pH 6.0)	−0.123	−45.8	0.49	0.248	7
1 M Lysine HCl	BSA (pH 6.0)	−0.047	−17.4	0.44	0.222	7
1 M Arginine HCl	BSA (pH 5.7)	−0.028	−9.09	0.35	0.114	unpublished
1 M Sodium glutamate	BSA (pH 7.0)	−0.088	−35.5	0.84	0.477	7
1 M NaCl	BSA (pH 5.6)	−0.0145	−16.8	0.64	0.243	9
1 M Na_2SO_4	BSA (pH 5.6)	−0.074	−35.4	0.78	0.524	9
1 M NaO_2CCH_3	BSA (pH 5.6)	−0.027	−22.4	—	0.312	9
1 M $MgSO_4$	BSA (pH 4.5)	−0.047	−26.5	0.55	0.388	9
1 M $MgCl_2$	BSA (pH 4.5)	−0.0040	−2.8	0.13	0.041	9
1 M $MgCl_2$	BSA (pH 3.0)	−0.0158	−11.1	0.53	0.162	10
1 M Gdm_2SO_4	BSA (pH 4.5)	−0.052	−16.4	0.38	0.211	11
2 M Gdm_2SO_4	BSA (pH 4.5)	−0.184	−58.0	0.69	0.316	11
1 M $GdmO_2CCH_3$	BSA (pH 5.6)	−0.010	−5.8	—	0.077	11
1 M GdmCl	BSA (pH 4.5)	0.025	17.5	neg	−0.239	11
3.9 M MPD (50%)	RNase A (pH 5.8)	−0.943	−109	neg	1.031	12
3.9 M MPD (50%)	RNase A (pH 2.0)	−0.531	−61.6	neg	0.602	12
0.4 M (30%) PEG_{1000}	β-Lactoglobulin (pH 2.0)	−0.165	−3.04	neg	0.411	13

[a] Ratio of the experimentally determined value of the effect of the co-solvent on the chemical potential of the protein to that calculated from the effect of the co-solvent on the surface tension of water.

Fig. 1-8: Properties of common additives used to denature and precipitate proteins. In the third column the molar binding ratio of cosolvent to protein is described. While arginine hydrochloride has a negative ratio, guanidinium hydrochloride has a positive ratio, indicating that it binds to proteins. The table also lists preferential hydration of excipients. Adapted from Timasheff and Arakawa (1996).

In Fig. 1-8 properties of frequently used additives are shown. Binding ratioes of arginine and guanidinium hydrochloride are shown. While arginine has a negative binding ratio, GdmCl has a positive. A negative ratio indicates that cosolvent does not become enriched around the protein (preferential hydration), while a positive value indicates that the cosolvent binds to the protein (preferential binding). While arginine has a positive value for preferential hydration, GdmCl has a negative value. This indicates that water density around the protein increases slightly in presence of protein and decreases with GdmCl present.

1.4. Hen egg-white lysozyme (HEWL)

Hen egg-white lysozyme (HEWL, EC 3.2.1.17, Fig. 1-9) was used as model protein throughout this thesis. It is a relatively small protein of 129 amino acids with a molecular weight of 14 kDa. Its three dimensional structure was the first to be resolved by X-ray diffraction (Blake *et al.*, 1965). Although it is monomeric, it shows characteristics of a two-domain protein during refolding.

The four α-helices form the "α-domain", which shows independent folding characteristics when monitored by NMR spectroscopy (Matagne *et al.*, 1998; Dobson *et al.*, 1994; Miranker *et al.*, 1991; Radford *et al.*, 1992a, 1992b). The short, triple-stranded β-sheet forms the "β-domain" which has its own independent kinetic behavior during refolding.

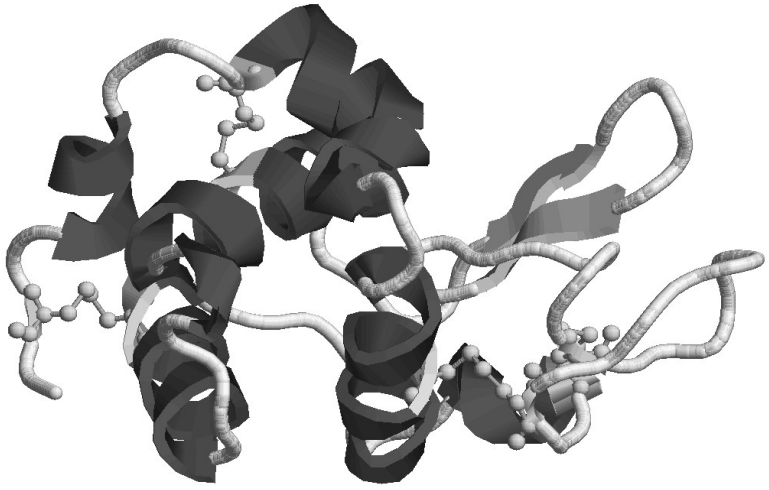


Fig. 1-9: Three-dimensional structure of hen egg-white lysozyme (HEWL). Its four disulfide bonds are shown as ball and stick. The picture was created using the PDB file 193L (Vaney *et al.*, 1996) and the program Rasmol (Bernstein, 2000). Although the figure only shows a two-stranded β-sheet, it contains a very short third strand.

Both domains form a cleft, into which substrate binds. After publication of the X-ray structure in 1965, Phillips (1967) proposed a mechanism based on binding sites of a hexaglycosidic model substrate. He based his suggestions for binding sites from the published three-dimensional structure. In order to bind to the active center, the fourth substrate residue needs to convert into a distorted conformation. Following cleavage, Phillips proposed existence of a stable oxocarbenium ion. Alternatively, Koshland (1953) proposed a general mechanism of hydrolases, which involves formation of a covalent intermediate between an amino acid side chain within protein and substrate.

Only recently existence of a covalent intermediate during hydrolysis of substrate has been shown. The revised enzymatic mechanism of lysozyme unifies the substrate distortion mechanism proposed by Phillips and formation of a covalent intermediate proposed by Koshland (Vocadlo *et al.*, 2001).

1.5. HEWL refolding

The refolding process of lysozyme has been extensively characterized. Depending on whether disulfide bonds are reduced or not, the folding reaction differs substantially in its result and time scale.

Lysozyme contains four disulfide bonds, which make a significant contribution to the stability of the native structure, and which are essential for full enzymatic activity (Denton and Scheraga, 1991; Acharya and Taniuchi, 1976, 1977, 1978, 1982). Refolding of non-reduced lysozyme is completely reversible.

If disulfide bonds are not reduced, folding is very fast and requires use of stopped-flow equipment to monitor formation of secondary and tertiary structure. The different folding rates of the α and β domains were characterized by NMR and optical spectroscopy (Kiefhaber, 1995; Dobson *et al.*, 1994; Miranker *et al.*, 1991; Radford *et al.*, 1992a, 1992b). During refolding of lysozyme, various intermediates form. Some intermediates may act as a trap leading to a "slow track" folding pathway.

Refolding from denatured-reduced lysozyme (oxidative refolding) is very slow compared to non-reducing conditions. Depending on the conditions, refolding takes minutes to hours (Epstein and Goldberger, 1963; Fischer *et al.*, 1993). In addition to native, fully active protein, aggregates also form in this pathway if no measures are taken to address this problem. In addition to formation of secondary and tertiary structure, disulfide bonds need to form. Formation of disulfide bonds has been enabled by air oxidation (Epstein and Goldberger, 1963), or by addition of suitable oxido shuffling components (Goldberg *et al.*, 1991, De Bernardez Clark *et al.*, 1998; Maachupalli-Reddy *et al.*, 1997). Addition of an oxido shuffling system strongly accelerates the refolding process.

Stochiometry of the oxido shuffling system determines the maximum yield (Hevehan and De Bernardez Clark, 1997; Fig. 1-10). Refolding yields also depend on the protein concentration (Hevehan and De Bernardez Clark, 1997; Figs. 1-11, 1-12). Low protein concentrations lead to higher relative yields, because aggregation decreases when the protein concentration decreases.

Fig. 1-10 shows the maximum refolding yields of 0.1 mg/mL lysozyme in presence of various oxidized glutathione (GSSG) concentrations. As reducing

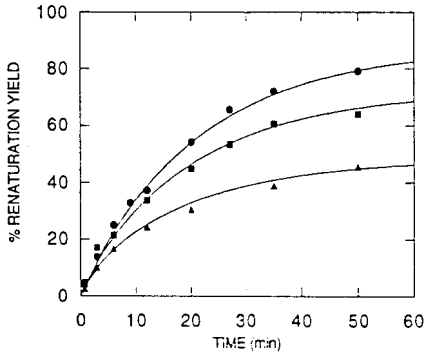
component of the oxido shuffling system 2 mM dithiothreitrol (DTT) was used. To decrease loss of activity due to aggregation, 0.5 M GdmCl was added.

[GSSG] (mM)	renaturation yield (%)	[GSSG] (mM)	renaturation yield (%)
1.0	3.70	5.0	84.0
3.0	73.5	10.0	8.40

[a] Renaturation conditions were 0.1 mg/mL lysozyme, 50 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, variable GSSG, and 0.5 M GdmCl (pH 8, 22 °C).

Figure 1-10: Maximum refolding yields in dependency of GSSG. Adapted from Hevehan and De Bernardez Clark (1997).

Similarly, refolding kinetics of lysozyme in dependency of various protein concentrations are shown in Fig. 1-11. 0.5 M GdmCl was provided to decrease aggregation. The optimum oxido shuffling system of 2 mM DTT and 5 mM GSSG was used to enable best possible recovery of activity. The achievable yields of lysozyme refolding are dependent on the lysozyme concentration (Fig. 1-12). An increase in the protein concentration from 0.17 mg/mL to 0.6 mg/mL leads to a 50% decrease of the refolding yield.

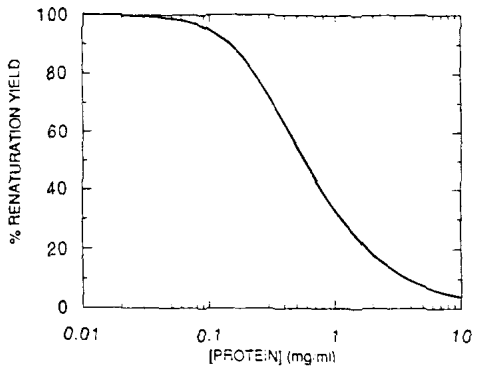


Effect of protein concentration on the kinetics of the oxidative renaturation of lysozyme. Renaturation was initiated by rapidly diluting reduced denatured lysozyme into renaturation buffer. Final conditions were 0.5 M GdmCl, 2 mM DTT, and 5 mM GSSG in 50 mM tris-HCl and 1 mM EDTA (pH 8, 22 °C), and (●) 0.17 mg/mL lysozyme, (■) 0.30 mg/mL lysozyme, (▲) 0.60 mg/mL lysozyme.

Figure 1-11: Refolding kinetics of lysozyme in dependency of different protein concentrations. Adapted from Hevehan and De Bernardez Clark (1997).

Simulated refolding yields are shown in Fig. 1-12. Hevehan and De Bernardez Clark (1997) developed a kinetic model to calculate folding and aggregation rates in dependency of protein concentration. While aggregation at protein concentrations between 0 and 0.1 mg/mL does not significantly decrease the

yield, aggregation at higher protein concentrations has a marked effect on refolding yield.



Predicted renaturation yield as a function of lysozyme concentration. Calculated using eq 2, which assumes third-order kinetics for the aggregation pathway and instantaneous formation of the intermediate I in Figure 1. Valid for final folding conditions of 0.5 M GdmCl, 50 mM tris-HCl, and 1 mM EDTA (pH 8, 22 °C) and 2 mM DTT and 5 mM GSSG.

Figure 1-12: Simulated HEWL refolding yields under optimum conditions of 2 mM DTT/5 mM GSSG and 0.5 M GdmCl. Adapted from Hevehan and De Bernardez Clark (1997).

Goldberg and coworkers (Roux *et al.*, 1997) addressed the question whether secondary structure forms first during lysozyme refolding, thus enabling formation of disulfide bonds, or if disulfide bonds form first and thus act as scaffold for secondary structure. To distinguish both possibilities, they compared refolding kinetics of denatured-reduced and reduced lysozyme with those of lysozyme mutants lacking certain disulfide bonds. To prevent aggregation which would make analysis by optical spectroscopy more difficult, the authors chose to add 0.5 M GdmCl. Under those conditions 80% of the activity could be recovered. Analysis of the kinetics by far-UV CD spectroscopy indicated that the refolding takes place in three distinct phases. Besides far-UV CD spectroscopy the authors used intrinsic fluorescence, which only detected occurrence of biphasic folding kinetics. Using far-UV CD influence of disulfide bond formation was determined to attribute the portion of disulfide bond formation on the signal. The data indicated that secondary structure and disulfide bonds form simultaneously. A short, but detectable lag phase was detected, which indicates the presence of intermediate(s). The following model for the refolding of lysozyme was proposed:

$$U \rightarrow I_1 \rightarrow I_2 \rightarrow N$$

By comparing amplitudes, it was estimated that I_1 has approximately 20% native secondary structure, while I_2 has been formed when the CD signal is in

equilibrium. At this point the protein has approximately 70% activity. This coincides with observations previously described in the literature of 3 S-S lysozyme, which has 70-80% activity. These data suggest that formation of disulfide bonds goes hand in hand with formation of secondary structure.

In a later publication Goldberg and coworkers (Roux *et al.*, 1999) focused on the relationship of 3 S-S and 4 S-S species during lysozyme refolding. To characterize partially disulfide bonded protein, they alkylated free thiol groups and analyzed the modified protein by mass spectrometry. To explain why 3 S-S species were detectable after a long period of refolding time, the authors used a double-labeling technique. Even after several hours 3 S-S intermediates were detected, which were not detected in earlier studies. Explanations for this observation include (1) 3 S-S lysozyme consists mainly of aggregates, which were removed in previous studies (2) there exists an equilibrium between 3 S-S and 4 S-S species (3) protein in earlier studies consisted of a mixture of both intermediates, but was not detected, and (4) the formation of 3 S-S represents a stable off-pathway.

Characterizations of the 3 S-S/4 S-S mixture revealed that 3 S-S is soluble in refolding buffer (0.5 M GdmCl), but not without GdmCl. Hypothesis (1) was ruled out by splitting a batch of protein of which one aliquot was centrifuged, but not the other. To rule out an equilibrium between both species (2) native lysozyme was incubated with "disulfide interchange catalyst", then labeled and analyzed. The resulting protein did not contain any 3 S-S intermediates, ruling out the equilibrium hypothesis. To test whether 3 S-S protein becomes trapped in the pathway (3), aliquots were either labeled directly, allowed to refold for an additional 24 hours, or were supplemented with additional reduced and oxidized glutathione to facilitate thiol exchange reactions (4).

The results indicate that 3 S-S species disappeared only in the third sample. The data let the authors propose a revised model for the refolding of lysozyme:

$$U \rightarrow I_0 \rightarrow I_1 \rightarrow I_2 \rightarrow N$$
$$\qquad\qquad\qquad\qquad\quad \searrow$$
$$\qquad\qquad\qquad\qquad\quad I_2' \text{ (3 S-S)}$$

Dobson and coworkers characterized the oxidative refolding pathway of lysozyme (van den Berg *et al.*, 1999a; 1999b). In order to monitor the folding process without need for stopped-flow equipment, they slowed down the refolding process with 2 M urea. They found that the intermediate lacking the disulfide bond between C76–C94 becomes accumulated during refolding. The C76-C94 disulfide bond is important to link both domains in lysozyme together. It remains to be shown if refolding conditions chosen by Dobson and coworkers actually correspond to refolding conditions in absence of urea. The chosen concentration of 2 M decreases aggregation significantly. It is not clear if the identified des[76-

94] disulfide intermediate becomes the main intermediate under conditions where a significant amount of the protein aggregates.

Laurents and Baldwin (1997) investigated unfolding of lysozyme in 6 M GdmCl at 10 °C using NMR. At this temperature unfolding takes between two and three hours. To investigate the unfolding process, the signal of nitrogens in the indole system of tryptophanes were monitored by NMR. Upon denaturation, the signal disappears and a common signal at 10.06 ppm appears. Since deuterated solvents are used, the proton signal disappears alltogether when tryptophanes become exposed to deuterated solvent. By using far-UV CD spectroscopy the authors detected one kinetic phase, which confirms that the unfolding process is highly cooperative.

By using intrinsic fluorescence (with specific excitation for tryptophanes), the authors were able to detect the unfolding process as a biphasic process. The reaction is dominated by a slow major and a fast minor phase. Because two of the five tryptophanes which gave a signal in 1H-NMR exchange hydrogens within the dead time of the experiment, only three tryptophanes could be used to monitor the unfolding process. Exchange kinetics indicate that no dry molten globule forms before the slow process begins. The authors observed a burst reaction at the beginning, which seems to be attributable to some of the tryptophan residues.

Overall, their studies indicate that the non-core residues unfold first before the rest of the protein loses structure. All usable tryptophan markers in this study were located in the α-domain, therefore no information about unfolding properties of the β-domain could be obtained.

1.6. Thesis objectives

L-arginine has been extensively used to facilitate protein refolding. Mechanistic effects of arginine have never been characterized in detail. Motivation for this work was to explain effects of arginine on protein folding. While the observed effect of arginine is to suppress aggregation, a wide variety of thermodynamic or kinetic aspects of folding may be influenced by arginine, in turn resulting in suppression of aggregation.

During oxidative refolding of lysozyme, competition between folding and aggregation is observed. Intermediates are formed, which either refold to native protein or aggregate. At high protein concentrations, aggregation is favored, because it has a reaction order ≥ 2, while the folding pathway, which leads to the formation of native, biologically active protein, is a first order reaction. The following kinetic parameters may be influenced by arginine:

- *increased folding rates;* an increase in folding rates makes the pathway to native protein faster in comparison with the aggregation process. This would consume intermediate(s) faster, leaving less protein accessible for the aggregation pathway.
- *decreased aggregation rates;* on the molecular level aggregation processes involve interaction of at least two protein molecules. If aggregation and folding rates under a given condition were the same, the aggregation process would consume more of the intermediate(s) at a given time interval than the folding process, because of the reaction order of >2 of the aggregation process. Additives may specifically decrease the aggregation rate, while not affecting the folding rate. If this effect is observed, protein folding intermediate(s) should become accumulated to a greater extent in presence of additives as they are not consumed by the aggregation process as fast as they otherwise would.

The following thermodynamic parameters could be influenced by arginine, resulting in higher refolding yields:

- *stabilization of the native state;* by stabilizing the native state arginine can shift the equilibrium towards native protein.
- *increased aggregate solubility;* by dissolving aggregated protein the otherwise irreversible aggregation reaction becomes (partially) reversible. The dissolved aggregated protein can then enter the folding pathway.
- *modification of solvent properties;* aggregation occurs when hydrophobic amino acid residues are exposed to a polar solvent. Under those conditions protein-protein intermolecular interactions are favored, resulting in aggregated protein. If polarity of the solvent is changed by a co-solvent, aggregation may not be as favorable.

Since proteins vary in molecular weight, geometry, quaternary structure, charges and other parameters, it is frequently observed that suitable refolding conditions which are efficient for refolding of one protein are not suitable for another protein. Many effects, which result in decreased aggregation and facilitated protein refolding are based on specific protein-additive interactions, which do not allow for generalization. For this thesis, all experiments were performed with hen egg-white lysozyme (HEWL). This protein is relatively inexpensive and easily available, which was important since some experiments required high amounts of protein. The choice of doing all experiments with just one protein has advantages and disadvantages. It is, however, a suitable strategy to develop a hypothesis about the effects of arginine with one protein and then to test it with other proteins and either confirm or decline this hypothesis.

Primary focus of this work is to understand the role that L-arginine hydrochloride plays in modulating the competition between folding and aggregation. The effect

of guanidinium hydrochloride (GdmCl) was chosen as second forms of study, because it also contains a guanidinium group (Fig. 1-13). GdmCl is mainly used as chaotropic reagent to denature proteins and to dissolve aggregates and inclusion body protein. In order to have the effects on proteins, it usually needs to be applied at higher concentrations. However, at lower concentrations (0-2 M) it may facilitate protein folding of some proteins, as it does in the case of lysozyme. Other additives were used to address the role of individual functional groups.

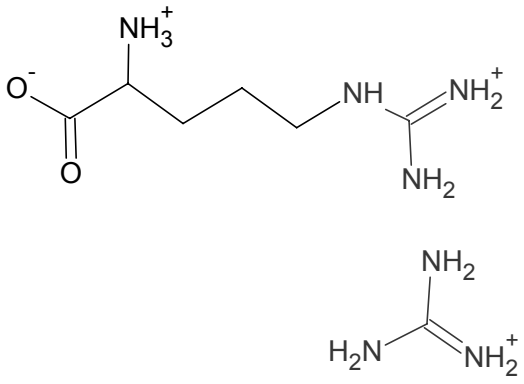


Fig. 1-13: Structure of L-arginine (top) and GdmCl (bottom).

2. Materials and Methods

2.1. Instrumentation

The following instruments were used:

UV/VIS DAD spectrophotometer	Hewlett-Packard	HP8452A
Fluorescence spectrometer	Hitachi	F-4500
CD spectrometer	Jasco	J-710
Dynamic light scattering instrument	Protein Solutions	DynaPro 99
MALDI-TOF mass spectrometer	Applied Biosystems	Voyager DE Pro
UV/VIS microtiterplate reader	Molecular Devices	SpectraMax

2.2. Chemicals

The following chemicals were obtained in the highest available purity:

Hen egg-white lysozyme (3x crystallized)	Sigma	St. Louis, MO
Dithiotheitrol (DTT)	Sigma	St. Louis, MO
5,5-Dithio-bis-2-nitrobenzoic acid (DTNB)	Sigma	St. Louis, MO
Gluthathione, oxidized (GSSG)	Calbiochem	San Diego, CA
Iodoacetic acid (IAA)	Sigma	St. Louis, MO
Tris solution, 1 M, pH 8.0	Fisher	Pittsburgh, PA
EDTA solution, 0.1 M	Fisher	Pittsburgh, PA
Guanidinium hydrochloride (GdmCl)	Fisher	Pittsburgh, PA
L-Arginine hydrochloride	Sigma	St. Louis, MO
1-Anilino-8-naphthalene sulfonic acid (ANS)	Sigma	St. Louis, MO
Ethanol (EtOH)	Sigma	St. Louis, MO
Dexocholic acid (DOC)	Sigma	St. Louis, MO
Trichloroacetic acid 100% solution (TCA)	Sigma	St. Louis, MO
Micrococcus lysodeikticus cells	Sigma	St. Louis, MO
Acetic acid, glacial	Fisher	Pittsburgh, PA
Na_2HPO_4	Fisher	Pittsburgh, PA
NaH_2PO_4	Fisher	Pittsburgh, PA
K_2HPO_4	Fisher	Pittsburgh, PA
KH_2PO_4	Fisher	Pittsburgh, PA
Coomassie Plus protein reagent	Pierce	Rockford, IL
Agmatine sulfate	Sigma	St. Louis, MO
D-Arginine hydrochloride	Sigma	St. Louis, MO
L-Homoarginine hydrochloride	Sigma	St. Louis, MO
L-Ornithine hydrochloride	Sigma	St. Louis, MO
Poly-L-arginine hydrochloride	Sigma	St. Louis, MO

2.3. Solutions

The following paragraphs list solutions used throughout this thesis:

Tris-EDTA (TE) buffer: TE buffer was prepared from 1 M tris stock solution, pH 8, and 100 mM EDTA stock solution. The final concentrations were 50 mM tris, 1 mM EDTA.

2 M arginine in TE buffer (50 mL): 21.07 g arginine were dissolved in water then 500 µL 100 mM EDTA and 1 mL 1 M tris solution pH 8 were added to provide the buffer components for TE buffer. After adjusting the pH to 8 with NaOH, the volume was adjusted to 50 mL with DI water.

8 M GdmCl in TE buffer (1 L): 764.2 g GdmCl were dissolved in water then 10 mL 100 mM EDTA stock solution and 20 mL 1 M tris stock solution pH 8 were added, after adjusting the pH to 8, the volume was adjusted to 1000 mL and the solution was stirred overnight.

2.4. Methods

Protein spectroscopy was performed as described in the review by Schmid (1996). For performing temperature thermotransition measurements, the review by Pace *et al.* (1996) was used.

Determination of lysozyme concentration

Lysozyme concentration was determined by measuring the absorbance at 280 nm. An extinction coefficient of 2.63 mL mg^{-1} cm^{-1} was used for native lysozyme and 2.37 mL mg^{-1} cm^{-1} for denatured lysozyme (Saxena and Wetlaufer, 1970).

For Coomassie based protein assays, a ready-to-use reagent from Pierce was used. The assay was performed with microtiter plate assays, using 10 µL sample, to which 300 µL reagent was added. It was calibrated by using a lysozyme stock solution of known concentration, and showed a linear response in the concentration range 0.2-0.8 mg/mL.

Quantitation of aggregates

Throughout this thesis, aggregated protein is defined as precipitable protein harvested by centrifugation at 13,000 rpm for 5 min. Besides harvesting insoluble aggregates, this centrifugation may also precipitate aggregate precursors. Such precursors are detectable by MALDI-TOF mass spectrometry (data not shown). After centrifugation the supernatant was discarded and the

pellet was washed with TE buffer, followed by another centrifugation step. After removing the supernatant, the pellet was dissolved in 8 M GdmCl. If disulfide bond formation was enabled, 10 mM DTT was added to 8 M GdmCl solution to dissolve disulfide bridged aggregates.

Lysozyme activity measurements

The assay for the determination of lysozyme activity is based on the lysis of *Micrococcus lysodeiktikus* cells. Loss of turbidity is measured over time (Hevehan and De Bernardez Clark, 1997). Lyophilized cells were suspended in 67 mM phosphate buffer pH 6.2 to an approximate concentration of 0.15 mg/mL. Activity was determined by addition of 10 µL of sample to 1 mL of cell suspension. Changes in turbidity at 450 nm were recorded for 30 seconds after a 30 second waiting time. The recorded data were used to calculate a linear regression function, of which the slope was used to calculate the concentration of active protein in relation to a freshly prepared solution of native lysozyme of known concentration.

CD spectroscopy

CD spectroscopy was performed with a Jasco J-710 CD spectrophotometer. Cuvettes of 1 cm pathlength were used for near-UV CD spectra in the wavelength range 260-310 nm. For far-UV (190-260 nm) CD spectra a pathlength of 0.1 mm was used. All spectra shown represent the average of three scans, which were acquired with a scanning rate of 20 nm/min and a response time of 16 s. Temperature transitions were performed using a Peltier heater accessory. Sealed cuvettes were used to prevent evaporation of samples. The heating rate was 10 K/hr. Temperature transitions were evaluated as described by Pace and coworkers (1996): first, a linear regression function of the region where the protein is completely folded (y_F) and a second one where the protein is completely unfolded (y_U), were calculated. The regression functions are extrapolated over the entire temperature range (Fig. 2-1). The fraction of unfolded/folded for each data point is calculated as following:

$$\frac{y_U}{y_F} = \frac{(y - y_F)}{(y_U - y_F)}$$

in which y_U and y_F represent the extrapolated sections of unfolded and folded protein, respectively, and y represents the actual data point at a given temperature. As a result of this calculation $\frac{y_U}{y_F}$ has the following range:

$0 < \frac{y_U}{y_F} < 1$. In order to calculate the transition midpoint, the following function was used to describe the sigmoid curve:

$$y = \frac{1}{1 + \exp(-\frac{x - T_m}{a})}$$

in which T_m stands for the transition midpoint, x for temperature, y for the data points as described above, and a a constant which describes the cooperativity of the unfolding process.

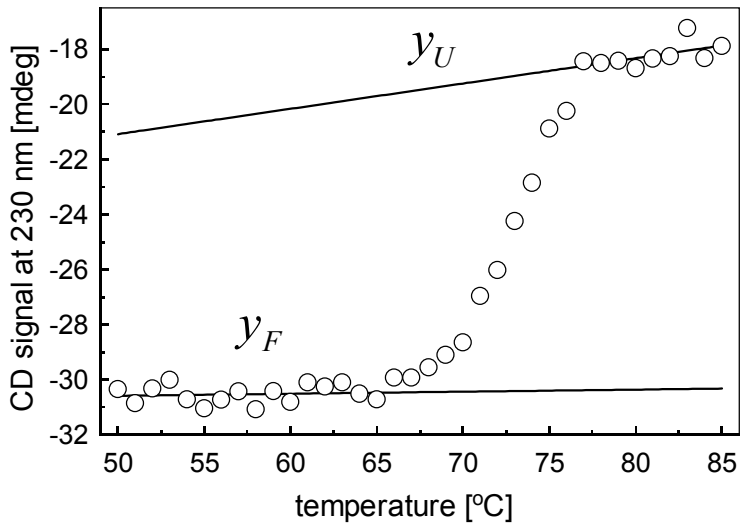


Fig. 2-1: Normalization of a temperature transition data set.

Light scattering kinetics

Aliquots of 10 µL denatured-reduced lysozyme (20 mg/mL lysozyme in 8 M GdmCl with 200 mM DTT) were added to 990 µL buffer containing the respective concentration of arginine or GdmCl and 2 mM DTT and 5 mM GSSG. Therefore the final concentration of lysozyme was 0.2 mg/mL. Samples were mixed by inversion prior to recording the optical density at 450 nm for 5 min. The dead time of this experiment was approximately 10 seconds. A single exponential regression function was used to fit the data. The exponent of this function was used as kinetic constant.

Fluorescence spectroscopy

Fluorescence spectroscopy was performed with a Hitachi F-4500 fluorescence spectrometer. Samples were measured in rectangular quartz cuvettes from Hellma (Plainview, NY) of a pathlength 1 cm. For measurements of intrinsic

fluorescence, an excitation wavelength of 290 nm was used and the fluorescence emission was recorded from 310-400 nm. For ANS-binding measurements an excitation wavelength of 350 nm was used. The fluorescence emission in this case was scanned from 380-600 nm. The following specific parameters of the F-4500 were set for the measurements: the dynode voltage was set to 700 V, the excitation slit width to 10 nm and the emission slight width to 10 nm. A scan rate of 240 nm/min was used, which results in data point spacing of 3 nm. The following equation was used to calculate the center of spectral mass:

$$I = \frac{\sum I(\lambda)\lambda}{\sum \lambda}$$

Dynamic light scattering

Dynamic light scattering (DLS) was used to determine hydrodynamic radius of lysozyme under various conditions. It was performed with a DynaPro 99 instrument (Protein Solutions-Aviv, Lakewood, NJ). Protein concentrations of 1 mg/mL were used. A micro cuvette with a volume of 12 μL and a pathlength of 0.15 cm was used for the measurements. Since the calculated hydrodynamic radius depends on viscosity and refractive index, corrections for solvents other than water were needed. Viscosity and refractive index data for arginine were provided by Protein Solutions (Charlottesville, VA), for GdmCl data by Nozaki (1972) and Kwahara and Tanford (1966) were used.

Protein precipitation

Protein was precipitated by addition of a 20-fold volume excess of cold, absolute ethanol (EtOH). Usually 2 mL cold EtOH were added to 100 μL sample. After storing the sample at –20 °C for at least one hour it was centrifuged for 10 min at 13,000 rpm. The supernatant was discarded, and the pellet was washed with 1.4 mL cold EtOH. After another centrifugation step, the supernatant was discarded and the pellet was allowed to dry before being dissolved in 40 µL of 8 M GdmCl.

HEWL refolding conditions

When lysozyme is denatured and reduced, an oxido shuffling system has to be present. Although most refolding experiments were performed with an oxido shuffling system consisting of 2 mM DTT and 5 mM GSSG, some were done with 2 mM DTT and 4 mM GSSG, which does not result in significantly different results (Fig. 3-5). Typically, a 20 mg/mL denatured-reduced lysozyme solution was created by weighing in 30 mg of lysozyme, which was dissolved in 1.5 mL 8 M GdmCl. Then 30.84 mg DTT were weighed into a separate tube. Now 1 mL of the previously dissolved lysozyme in 8 M GdmCl was pipetted into the tube

containing DTT and thoroughly mixed. The denatured-reduced protein solution was allowed to react at least one hour before use. The remainder of the lysozyme solution was used to determine the concentration by measuring the absorbance at 280 nm after diluting 100-fold. GSSG was added to the refolding buffer at a final concentration of 5 mM. In experiments usually 990 μL of refolding buffer was used, to which 10 μL of denatured-reduced protein was added, followed by rapid mixing. This resulted in final concentrations of 0.2 mg/mL lysozyme, 2 mM DTT, and 5 mM GSSG.

Determination of free sulfhydryl groups (Ellman assay)

An 8 mg/mL 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) was prepared in 8 M GdmCl. Because the protein concentration at which a signal could be detected was lower than at which the refolding experiments were conducted, concentration of samples was required. For concentration, cold ethanol precipitation was utilized.

Protein concentration was determined by dissolving 10 μL of the concentrated protein in 990 μL 8 M GdmCl and measurement of absorbance at 280 nm. For the Ellman assay 20 μL sample were used to which 50 μL DTNB reagent were added, followed by addition of 930 μL 8 M GdmCl. After incubating for 15 min, the absorbance was measured at 412 nm. The assay was calibrated against a solution of L-cysteine utilizing the known absorbance coefficient of 13,800 M^{-1} cm^{-1} of the DTNB reaction product in 8 M GdmCl. By dividing the molaritiy of free sulfhydryl groups by the molarity of protein, the number of free sulfhydryl goups was calculated.

Quenching of free sulfhydryl groups

In order to quench formation of disulfide bonds, free sulfhydryl groups were either alkylated with iodoacetic acid (IAA) or pH of the solution was lowered to 5. In cases when IAA was used, 207.9 mg IAA were dissolved in 1 mL 1 N KOH, to which 1 mL 1 M tris pH 7 was added. In order to have a sufficient concentration of IAA present, a suitable excess needed to be added. Since the IAA solution was 50 mM and most times the concentration of sulfhydryl groups did not exceed 20 mM, 10% (v/v) of the 50 mM IAA solution was added. In situations when free sulfhydryl groups needed to be preserved for consecutive reactions, the pH was dropped by adding 50% (v/v) of 1 M acetic acid, pH 5. Excess oxido shuffling components were removed by dialysis.

SDS-PAGE

SDS-PAGE was performed using the Invitrogen/Novex cassette system. It consists of readily usable gels, matching sample loading buffers, and running

buffers. The best suited separation system for separating oligomers of lysozyme was the 4-12% NuPAGE gel system, which was used with 3-(N-morpholino) propane sulfonic acid (MOPS) running buffer. The high amount of 20 μg protein per well was used to detect small amounts of oligomers. This was accomplished by mixing 30 μL of a 2 mg/mL sample with 10 μL 4x sample buffer. Samples were not boiled in order not to dissociate oligomers. Samples were run for 75 min with the current limited to 40 mA per gel. Typically, this resulted in a voltage of 120-150 V. Gels were stained with the highly sensitive Collodial Blue Stain from Invitrogen.

MALDI-TOF

For MALDI-TOF α-4-hydroxy cinnamic acid was used as matrix in a concentration of 12 mg/mL in 30:20:20:1 acetonitrile:methanol:water:TFA. Typically 40 µL sample were mixed with 20 µL 3% TFA and 80 µL matrix solution, of which 1 µL was deposited on the appropriate target. After thorough drying in the dark typically 50 spectra were acquired. The presence of arginine or GdmCl above 100 mM made the acquisition of spectra impossible and required removal of these additives. This was done by cold ethanol precipitation.

3. Results

3.1. Effect of arginine and GdmCl on native lysozyme

Near-UV CD spectra of native lysozyme under various conditions

The following experiments will clarify whether arginine or GdmCl have an effect on the structure of native lysozyme (at room temperature). Ideally the effect of arginine or GdmCl on folding intermediates should be characterized because the additives affect the competition between folding and aggregation. Since folding intermediates are not kinetically stable, they are not accessible for spectroscopic characterization. Instead, the effect of both additives and oxido shuffling system on native lysozyme was characterized.

Fig. 3-1 shows near-UV CD spectra of 0.2 mg/mL native lysozyme in the absence of additives, in presence of 1 M arginine and 1 M GdmCl. Another set of spectra was measured in presence of oxido shuffling system and absence and presence of additives. Samples were equilibrated for 24 hours prior to the acquisition of spectra.

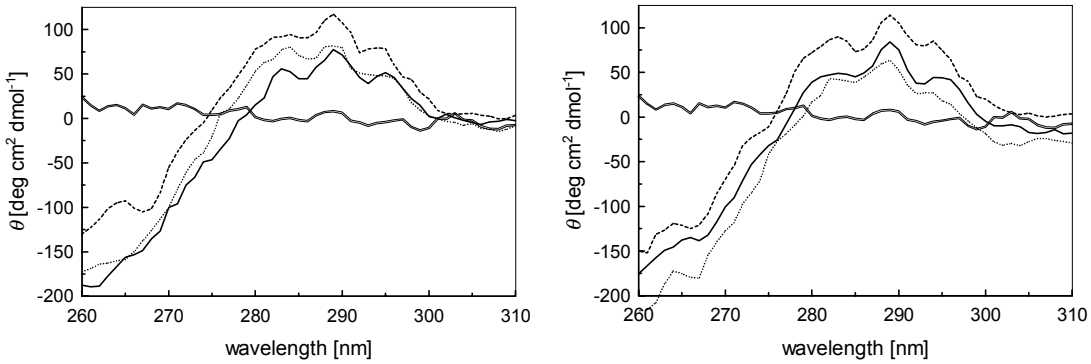


Fig. 3-1: Near-UV CD spectra of native lysozyme, (**left**) without oxido shuffling system, (**right**) with oxido shuffling system. (**—**) No additive, (·····) 1 M arginine, (---) 1 M GdmCl, and (=) denatured lysozyme in 8 M GdmCl.

The resulting data show that none of the spectra are significantly different from native lysozyme, indicating that in presence of additives and/or oxido shuffling system no significant changes in the tertiary structure occur. Only small differences in the amplitude of some of the spectra were detected, which is an indicator that small changes in the environment of aromatic amino acid side chains are induced.

For comparison the spectrum of denatured lysozyme (lysozyme in 8 M GdmCl) was superimposed. It is a relatively flat signal, which indicates that all tertiary structure has disappeared.

Far-UV CD spectra of native lysozyme

Similarly, far-UV CD spectra under the conditions mentioned above were acquired to determine whether the oxido shuffling system and/or additives alter the secondary structure of native lysozyme. In Fig. 3-2 far-UV CD spectra of 0.2 mg/mL native lysozyme in the absence and presence of 1 M arginine and GdmCl and oxido shuffling system (2 mM DTT and 4 mM GSSG) are shown. Due to strong absorbance of arginine and GdmCl below 215 nm data could only be recorded above this wavelength. To allow for sufficient equilibration before the measurement was taken, samples were incubated for 24 hours prior to the measurement. The spectra show that there are no significant differences between spectra in the presence and absence of oxido shuffling system and also in the absence of additives and 1 M GdmCl. The only spectra, which are slightly different from the rest are those in presence of arginine. Under these conditions the amplitude in the wavelength range 215-225 nm is slightly increased, which is a characteristic of increased α–helix content. A numerical evaluation of these differences was performed through CD deconvolution by using the program CDNN (Böhm *et al.*, 1991).

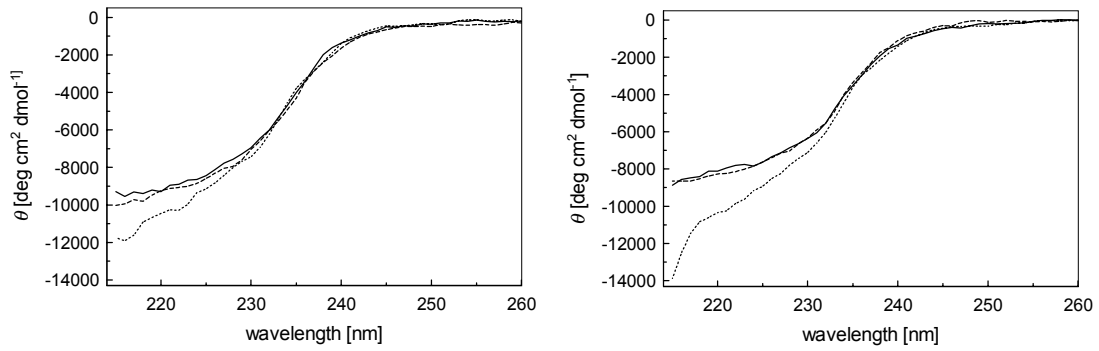


Fig. 3-2: Far-UV CD spectra of native lysozyme, (**left**) without oxido shuffling system, (**right**) with oxido shuffling system. (**—**) No additive, (·····) 1 M arginine, and (---) 1 M GdmCl.

The results of the CD deconvolution (Tab. 3-1) confirm that the presence of the oxido shuffling system does not lead to significant changes in the secondary structure content. The presence of 1 M GdmCl does not result in alterations either. In presence of 1 M arginine an increased content of α–helix was detected, which is gained through slightly reduced β-sheet and random coil contents.

Arginine absorbs strongly in the far-UV range and has a strong characteristic spectrum. Since 1 M arginine was used, but the concentration of lysozyme was ~14 μM, the lysozyme signal interferes with the signal of arginine. Therefore the

detected differences in presence of 1 M arginine may not be caused by changes in the lysozyme secondary structure, but rather artifacts due to the presence of arginine. Additionally, CD deconvolution methods are prone to errors (typically 5-10%), because certain amino acid side chains absorb and thus interfere in the far-UV wavelength range, resulting in unusually shaped far-UV CD spectra which are extremely difficult to deconvolute.

Tab. 3-1: Secondary structure content of 0.2 mg/mL lysozyme in the absence and presence of 1 M additive and oxido shuffling system, which was 2 mM DTT and 4 mM GSSG. Data were normalized to 100% secondary structure content.

	without OXS			with OXS		
	No add.	1 M Arg	1 M GdmCl	no add.	1 M Arg	1 M GdmCl
% α-helix	22	31	24	26	33	27
% β-sheet	25	20	24	22	18	22
% turn	19	18	19	18	17	19
% coil	34	32	34	34	31	33

Fluorescence spectra and activity measurements

To further confirm results obtained by far- and near-UV CD spectroscopy that the secondary and tertiary structures of lysozyme are not altered by the presence of additives and/or oxido shuffling system, intrinsic fluorescence spectra were acquired. As done with CD spectroscopy, spectra of native lysozyme in presence of 1 M arginine or GdmCl and in the absence and presence of the oxido shuffling system were measured.

Tab. 3-2: Center of spectral mass [nm] of native lysozyme with and without additives and oxido shuffling system. Excitation wavelength was 290 nm, fluorescence emission was scanned from 310-400 nm.

	no add.	1 M Arg	1 M GdmCl
Without OXS	348.0	349.2	347.8
With OXS	349.0	350.1	348.9

Samples were equilibrated for 24 hours prior to the measurements. The data show (Tab. 3-2) that there are only minimal differences in the center of spectral mass, suggesting that no significant changes of the tertiary structure of HEWL occur when additives and/or oxido shuffling system are present.

Data obtained from optical spectroscopy indicate that secondary and tertiary structure of lysozyme is not altered when additives and/or oxido shuffling system are present. Samples were allowed to equilibrate for 24 hours to provide sufficient equilibration time. The most sensitive indicator for changes in the protein structure is protein activity, because it detects changes which are not detectable by optical spectroscopy.

Thermostability of lysozyme in presence of arginine and GdmCl and oxido shuffling system

As mentioned in the introduction, additives might influence either kinetic or thermodynamic parameters. The following experiments investigate the effect of both additives on the thermodynamic stability of lysozyme. Because temperature unfolding experiments are subject to kinetic influences (a protein incubated at elevated temperatures below the transition midpoint may unfold over time), the experimental conditions were kept constant for all experiments. Thermostability measurements are used to determine whether a certain condition has a stabilizing or destabilizing effect on a particular protein. Here, it was investigated whether additives and the oxido shuffling system can influence the thermodynamic stability of native lysozyme. Additives and the oxido shuffling system may or may not stabilize native lysozyme, which becomes visible through changes in the transition midpoints upon heating. A stabilizing effect leads to a higher transition midpoint, while destabilization by a certain condition is visible through a lower transition midpoint. In addition, a condition may not have an effect at all, leaving the transition midpoint unchanged. In the preceding figures and tables properties of native lysozyme were shown in the presence and absence of additives and oxido shuffling system to detect differences in structure and activity. At room temperature none were found.

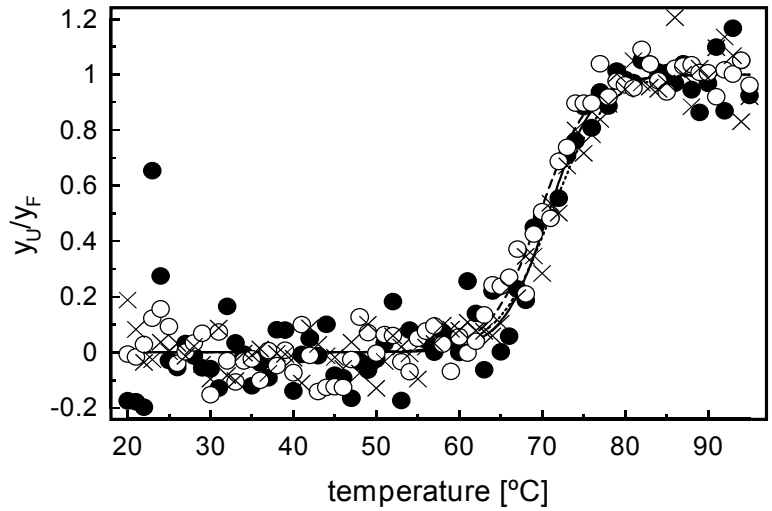


Fig. 3-3: Temperature transitions of 0.2 mg/mL lysozyme in presence of different concentrations of arginine. The CD signal was recorded at 280 nm. From the CD measurements, the signal was normalized and data sets were fitted to a sigmoid regression function. (●) 0 M arginine, (X) 0.5 M arginine, and (○) 1 M arginine.

During thermostability measurements, the increased temperature progressively unfolds the protein, which may provide some information on what additives do while a protein refolds. Although temperature-induced unfolding is the reverse

process to protein refolding, thermotransitions may give some insights into the effect of additives and the oxido shuffling system during protein refolding, in which the protein is first unfolded and then undergoes refolding. At room temperature the presence of the oxido shuffling system does not affect activity (Tab. 3-5).

Thermotransitions were measured using far- and near-UV CD spectroscopy to determine the transition midpoint for loss of secondary and tertiary structure, respectively. In cases in which both temperatures coincide, this indicates that no unfolding intermediates become populated, while a discrepancy indicates that unfolding intermediate(s) are populated. In cases in which an unfolding intermediate is populated, the near-UV transition has a lower transition midpoint than the far-UV transition, indicating that tertiary structure becomes altered first before the protein loses secondary structure and is completely unfolded.

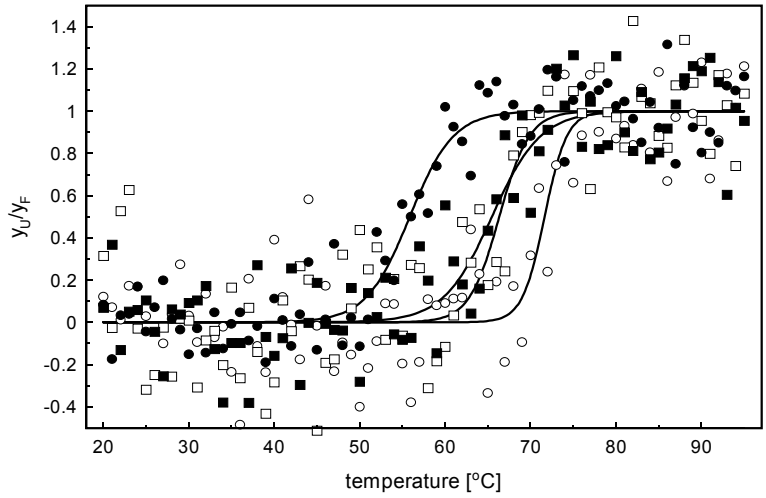


Fig. 3-4: Temperature transitions of 0.2 mg/mL lysozyme in presence of different concentrations of GdmCl. The CD signal was recorded at 280 nm. From the CD measurements, the signal was normalized and data sets were fitted to a sigmoid regression function. (○) 0 M GdmCl, (□) 0.5 M GdmCl, (■) 1 M GdmCl, and (●) 2 M GdmCl.

In Fig. 3-3 the effect of arginine on the thermodynamic stability of HEWL is shown. The addition of arginine does not affect the position of the transition, therefore it is indicative that arginine does not affect the thermodynamic stability of HEWL. In Fig. 3-4 the effect of GdmCl on the thermodynamic stability of HEWL is shown. GdmCl decreases the thermodynamic stability of HEWL, which is visible through lowering of the transition midpoint. In Fig. 3-5 the transition midpoints for HEWL in presence of GdmCl and arginine are shown. While GdmCl decreases the thermostability, arginine does not affect it at all.

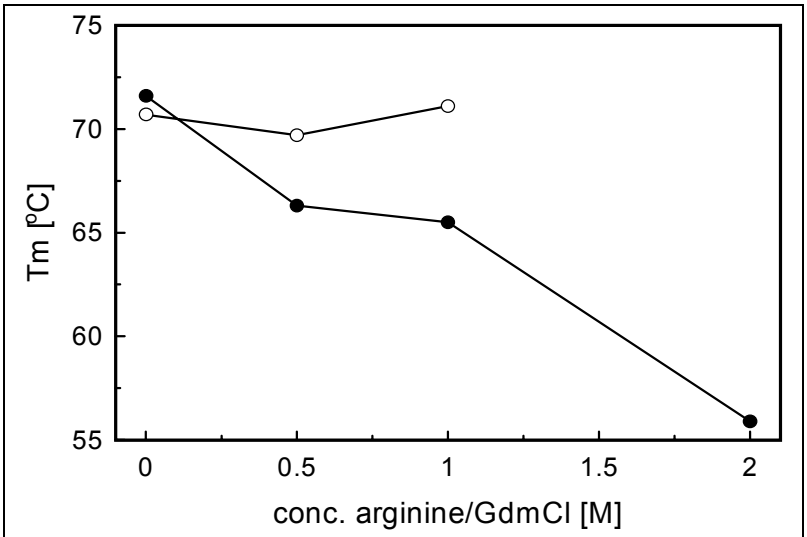


Fig. 3-5: Transition midpoints calculated from data shown in Figs. 3-3 and 3-4. The transition midpoint was calculated as outlined in chapter 2. (○) arginine and (●) GdmCl.

To further elucidate the effect of both additives on HEWL, thermotransitions of 0.2 mg/mL native lysozyme by near-UV CD spectroscopy at 288 nm under various conditions were measured: in the absence and presence of arginine and GdmCl and oxido shuffling system. The presence of oxido shuffling components was chosen, because at room temperature these components do not affect the activity of HEWL (Tab. 3-5), but at higher temperatures these components may affect the activity of HEWL, because the protein structure becomes more flexible at elevated temperatures, thus disulfide bonds may be more accessible for oxido shuffling system components to reduce.

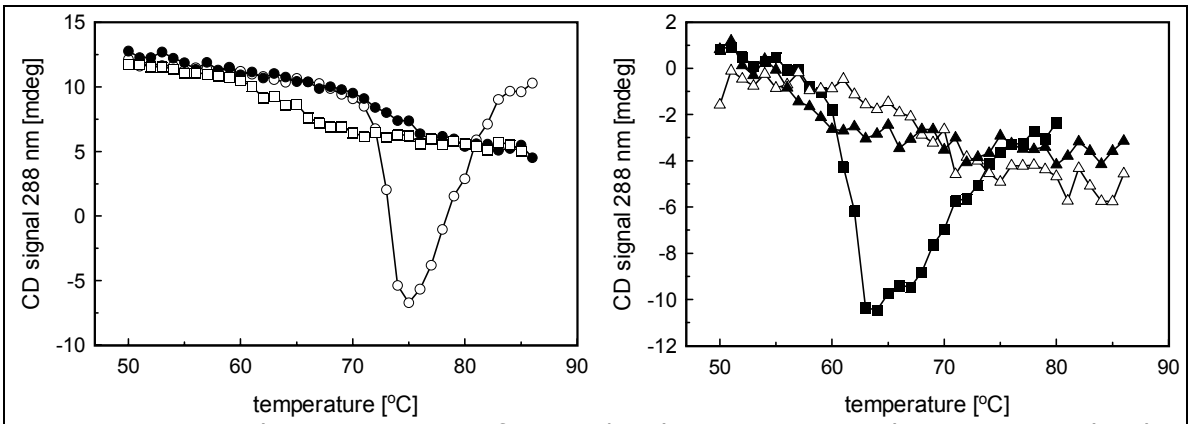


Fig. 3-6: Near-UV thermo-transitions of 0.2 mg/mL lysozyme measured at 288 nm under the following conditions: left diagram – without OXS (○) no additive, (●) 1 M arginine, (□) 1 M GdmCl; right diagram – in presence of the oxido shuffling system (2 mM DTT and 5 mM GSSG) (■) no additive, (Δ) 1 M arginine, and (▲) 1 M GdmCl

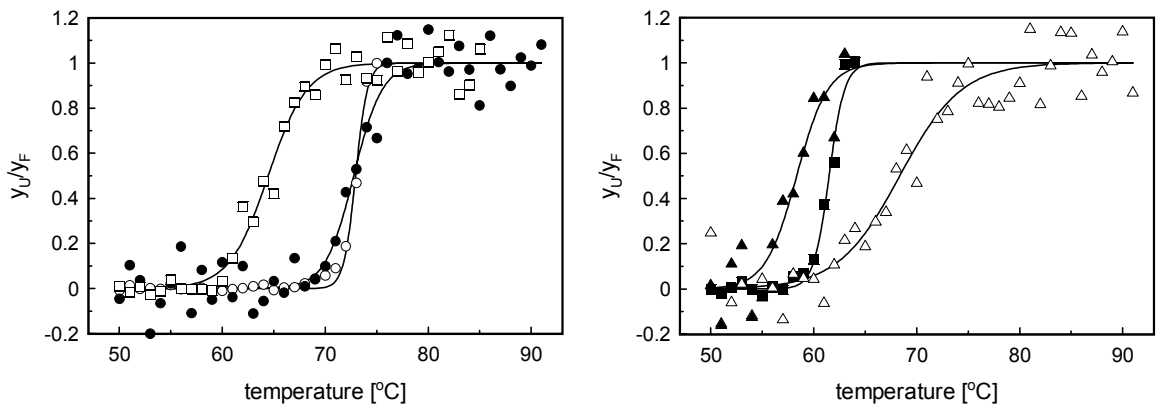


Fig. 3-7: Normalized near-UV thermotransitions of data shown in Fig. 3-6: left diagram – without OXS (○) no additive, (●) 1 M arginine, (□) 1 M GdmCl; right diagram – with OXS (■) no additive, (△) 1 M arginine, and (▲) 1 M GdmCl.

The wavelength of 288 nm for near-UV transitions was chosen because the lysozyme near-UV CD spectrum exhibits a strong peak at 288 nm (Fig. 3-6). Some of the thermotransitions show a V-shaped characteristic. Fig. 3-6 is important to orient the resulting signals. Folded protein has a positive signal at 288 nm, which declines as the protein unfolds. Thus a decrease of the signal is expected during thermal unfolding of lysozyme at this wavelength. As expected, the signal decreases as the protein unfolds, but increases afterwards, which appears to be related to the formation of aggregates. Samples which exhibited this type of result had visible aggregates. For the calculation of transition midpoints, the signal increasing section of the measurement was excluded, as it does not represent the unfolding process.

Fig. 3-8 shows far-UV CD temperature transitions at 230 nm. These measurements were performed in addition to near-UV CD ones to identify if HEWL proceeds through formation of an intermediate, indicative by discrepancies between near- and far-UV transition midpoints. Coinciding data indicate that the protein loses tertiary and secondary structure simultaneously and no unfolding intermediates are formed.

The data of all measurements were normalized as described by Pace (1997). The normalized transitions are shown in Figs. 3-7 and 3-9.

Since arginine has a strong CD signal at 230 nm, additionally to the thermotransition of lysozyme in arginine, a thermotransition with arginine, but no lysozyme, was measured to be used as a reference. This measurement was subtracted from the thermotransition with protein to increase the visibility of the transition, which appears very small in midst of the large signal caused by arginine. This strategy resulted in a transition midpoint, which was not significantly different from the near-UV measurement, although it has a slightly higher error than the other measurements, which did not exhibit this problem.

Lysozyme aggregated under all conditions investigated, making all the thermotransitions irreversible.

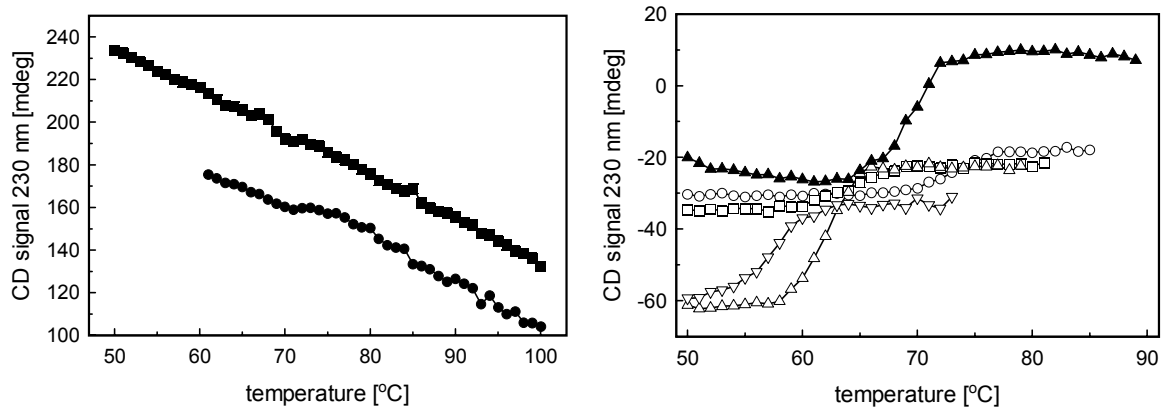


Fig. 3-8: Far-UV thermo-transitions of 0.2 mg/mL lysozyme measured at 230 nm under the following conditions: left diagram – without OXS (●) 1 M arginine, (■) 1 M arginine without protein; right diagram – without OXS (○) no additive, (□) 1 M GdmCl; with OXS (△) no additive, (▲) 1 M arginine, and (▽) 1 M GdmCl.

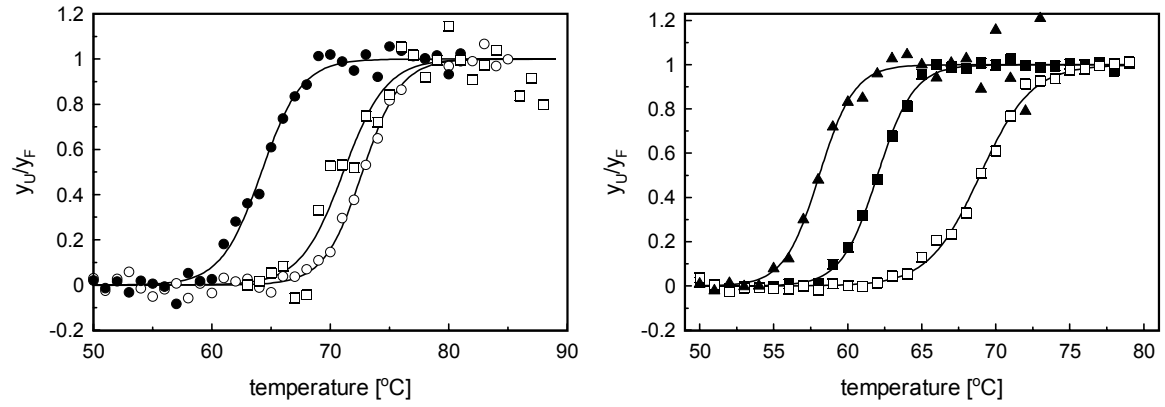


Fig. 3-9: Normalized far-UV thermotransitions of data shown in Fig. 3-8: left diagram – without OXS (○) no additive, (●) 1 M GdmCl, (□) 1 M arginine; right diagram – with OXS (■) no additive, (△) 1 M arginine, and (▲) 1 M GdmCl.

Tab. 3-3 summarizes the results. In the absence of oxido shuffling system, the presence of 1 M GdmCl leads to a lower transition midpoint, while arginine does not affect the thermodynamic stability of HEWL. Transition midpoints of the corresponding far- and near-UV thermotransitions coincide well, showing that there are no unfolding intermediates populated.

In presence of oxido shuffling system (2 mM DTT, 5 mM GSSG) the transition midpoint temperature of native lysozyme is significantly decreased (–11 °C). At room temperature no differences in spectra in the absence and presence of oxido shuffling system were found. This shows that the oxido shuffling system is responsible for thiol exchange reactions at higher temperatures, making lysozyme less stable. At elevated temperatures, HEWL gradually unfolds,

exposing more and more of its disulfide bonds leading to higher reactivity than at room temperature, resulting in thiol exchange reactions. In presence of GdmCl and oxido shuffling system the transition midpoint was decreased further showing that GdmCl further destabilizes lysozyme under these conditions, probably by facilitating unfolding of HEWL. In presence of oxido shuffling system and 1 M arginine an increase in the transition midpoint (+7 °C) was observed.

Tab. 3-3: transition midpoints of far- and near-UV CD temperature transitions of 0.2 mg/mL lysozyme in the absence of additives and the presence of 1 M arginine or GdmCl and in the absence and the presence of oxido shuffling system, which is 2 mM DTT and 5 mM GSSG. Transition midpoint temperatures are shown in °C.

	Far-UV CD			Near-UV CD		
	no add.	1 M arg.	1 M GdmCl	no add.	1 M arg.	1 M GdmCl
Without OXS	72.7	71.1	64.2	72.9	72.8	64.5
with OXS	62.0	68.9	58.1	61.5	68.3	58.3

Data shown in Tab. 3-3 suggest that arginine is able to stabilize partially unfolded lysozyme, which has undergone a conformational change at higher temperatures in presence of oxido shuffling system. Cuvettes with samples were filled completely with minimal headspace between solution and stopper to reduce evaporation and air oxidation of the solutions. This indicates that arginine might thermodynamically stabilize lysozyme under refolding conditions, when a significant population of partially unfolded protein molecules are present in the samples.

Influence on solubility

Additives are usually supplied with the buffer in which the refolding process is performed. In cases in which aggregation is not completely suppressed, aggregates form in the refolding buffer to significant sizes and become visible as insoluble precipitant. In the experiment to be described next the influence of additives on the solubility of fully-grown aggregates was investigated. Aliquots of 10 μL 20 mg/mL denatured-reduced lysozyme were diluted into 990 μL TE buffer to initiate aggregation. Under these conditions lysozyme aggregates to about 50% (Figs. 3-15, 3-16). No GSSG was supplied in the TE buffer to prevent formation of disulfide bonds in the aggregates, which would further reduce their solubility, because the presence of GSSG enables the free thiol groups to become oxidized. Besides the introduction of intrachain disulfide bonds, this oxidation step may lead to interchain disulfide bonds, resulting in formation of disulfide-bonded aggregates. In oxidative refolding experiments usually some of the aggregates are disulfide bonded, making it necessary to add a small concentration of DTT besides GdmCl to dissolve them. Aliquots of denatured-reduced lysozyme were allowed to aggregate for 24 hours, after which they were harvested by centrifugation. After washing the aggregates with TE buffer and

another centrifugation step, 1 mL additive of the appropriate concentration in TE buffer at pH 8.0 was added to the pellet and thoroughly mixed.

After incubating for 24 hours, the amount of remaining aggregates was determined by separating supernatant and pellet and determining the amount of protein left in the pellet. For this process, samples were first centrifuged. The supernatant was discarded, and the pellet was washed with TE buffer, and then dissolved with 1 mL 8 M GdmCl. By measuring the absorbance at 280 nm the percentage of remaining aggregates was calculated.

The data show (Fig. 3-10) that the presence of arginine does not significantly affect the solubility of aggregates. At 2 M arginine about 10% of the aggregates become dissolved. GdmCl dissolves aggregates more efficiently, leading to complete dissolution of aggregates above a concentration of 2 M GdmCl. In the concentration range 1-2 M GdmCl, it strongly increases the percentage of dissolved aggregates. This indicates that arginine has very little effect on grown aggregates. It may interact with partially grown aggregates, which are not yet very dense and thus easier accessible to arginine, which does not dissolve fully-grown aggregates. This experiment led to the question how arginine and GdmCl affect the solubility of native protein. An increase in solubility of native protein in presence of additives may facilitate the refolding process by affecting the mass action law towards formation of native protein.

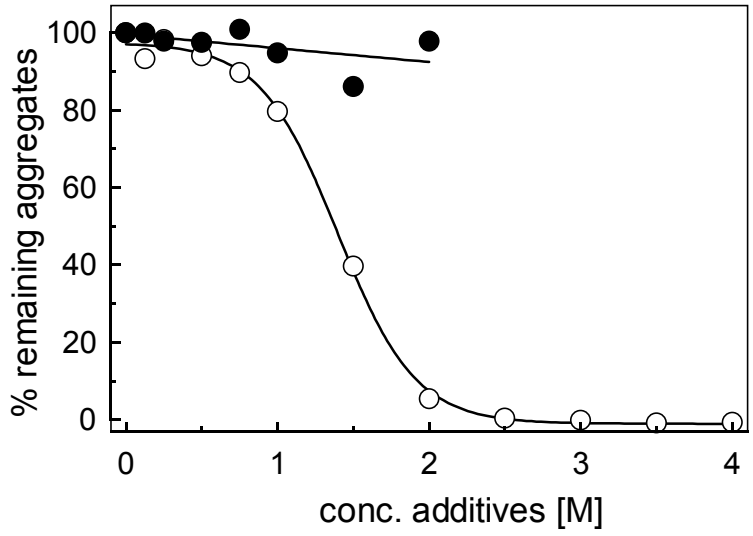


Fig. 3-10: Capability of (●) arginine and (○) GdmCl to dissolve aggregates. Since the solubility limit of arginine is 2 M, data were not extrapolated beyond 2 M. Aggregates were exposed to the appropriate additive concentration for 24 hours before the percentage of remaining aggregates was determined.

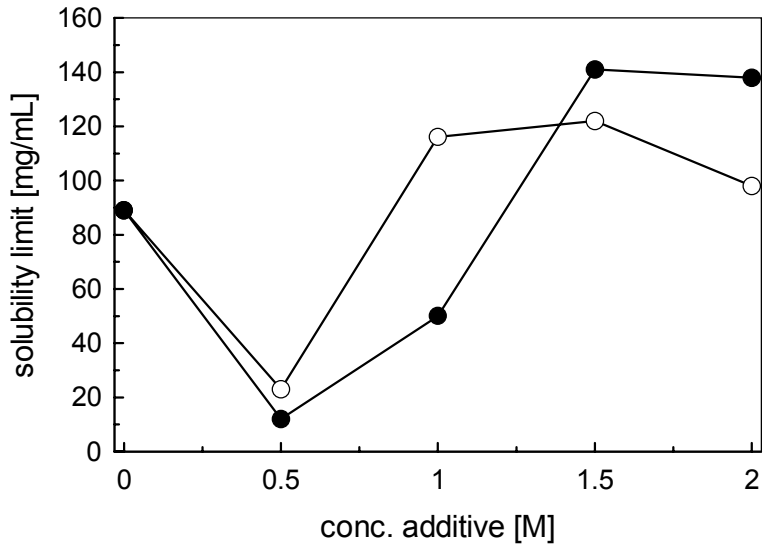


Fig. 3-11: Solubility of native lysozyme in presence of various concentrations of (○) arginine or (●) GdmCl. Solid lysozyme was added until the solution became turbid. At this point, the samples were centrifuged. The solubility limit was detected by measurement of the protein concentration in the supernatant (after appropriate dilution).

In Fig. 3-11 the results of solubility determinations are shown. In presence of 0.5 M of both additives the solubility is lower than in the absence of additives. Above 0.5 M solubility increases above the value determined for 0 M additive. As previously shown data indicate, HEWL refolds with 100% yield in presence of 0.5 M arginine, thus changes in the solubility of the native protein do not appear to affect the refolding process.

To determine the effect on solubility of denatured-reduced HEWL, a solution of 17 mg/mL HEWL in 8 M GdmCl with 200 mM DTT was dialyzed against buffer solutions with a pH of 4.0 and various concentrations of arginine or GdmCl. The low pH was chosen to keep HEWL unfolded and soluble upon removal of GdmCl. Limiting to this experiment is that HEWL does not remain fully unfolded in the dialysis buffer, but the experiment gives some insights into the effect of solubilization of denatured protein. After dialysis the protein solutions were centrifuged to remove insoluble protein and particulates. The amount of soluble protein was determined by measurement of the absorbance at 280 nm.

The data are shown in Fig. 3-12. In presence of GdmCl solubility significantly increases to 10 mg/mL, while in presence of arginine solubility increases to 0.8 mg/mL. Because GdmCl solubilizes aggregates (Fig. 3-10) in the concentration range 1-2 M, data in Fig. 3-12 show the effect of GdmCl on dissolving aggregates in addition to dissolving denatured protein. When the effect of both additives on fully grown aggregates was described (Fig. 3-10) it

turned out that 1 M GdmCl affected solubility in a similar way as 1 M arginine, resulting in only slight dissolution of aggregates.

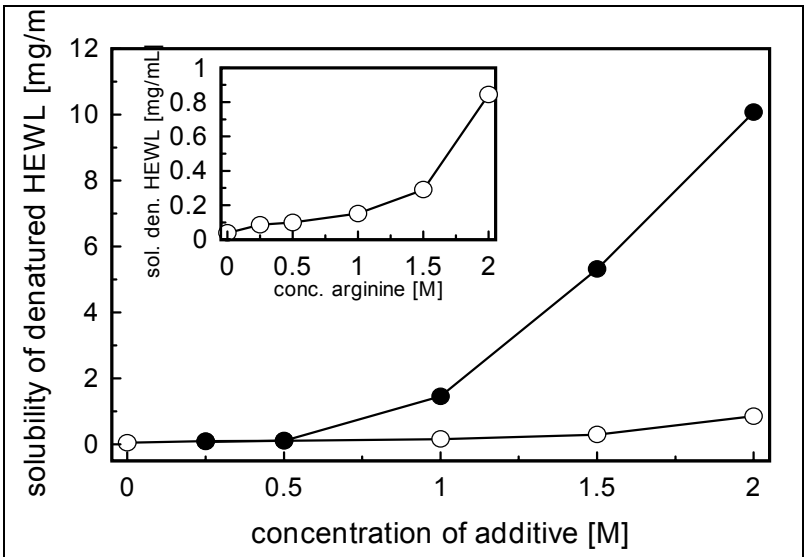


Fig. 3-12: Solubility of denatured lysozyme in presence of (○) arginine or (●) GdmCl. A solution of 17 mg/mL lysozyme was denatured and reduced with 8 M GdmCl and 200 mM DTT. The solution was then dialyzed against a solution containing the indicated additive concentrations at pH 4. Solubility was determined by measuring A_{280} of the supernatant after centrifugation.The inset shows the effect of arginine.

In summary data shown in Fig. 3-12 indicate that arginine and GdmCl may affect refolding of proteins by solubilizing denatured protein, but this effect appears to be rather weak in comparison to dissolution of aggregates (Fig. 3-10). Arginine efficiently promotes refolding of HEWL at 0.5 M. At this concentration solubility of denatured protein appears nearly unaffected by arginine, thus the effects leading to suppression of aggregation by arginine must be mainly based on other effects.

3.2. Effects of arginine and GdmCl on HEWL refolding

This section describes the effects of arginine and GdmCl on HEWL refolding in comparison to the absence of additives. Prior to the experiments HEWL is unfolded with GdmCl, and the disulfide bonds are reduced with DTT. This denatured-reduced protein is prone to aggregation and therefore a good model system for the effects of arginine and GdmCl on protein refolding.

Comparison of activity kinetics with tertiary structure formation kinetics

Fig. 3-13 illustrates the differences in the formation of tertiary structure - observed by near-UV CD spectroscopy - with recovery of activity. The conditions

were 0.2 mg/mL lysozyme and 0.5 M arginine, which was added to completely suppress aggregation, and with oxido shuffling system (2 mM DTT and 5 mM GSSG). The signal heights were normalized to values between 0 and 1. The superimposed curves show that the equilibrium value of the CD signal is reached after approximately 10 min, while the recovery of activity reaches its equilibrium after approximately 60 min under these conditions. The data show that during refolding of lysozyme tertiary structure forms first, because the near-UV CD signal reaches its equilibrium before activity does. Near-UV CD spectroscopy was chosen for this experiment, because it is less affected by absorbance of buffer components.

These data indicate that the formation of disulfide bonds requires a relatively long period of time compared to the formation of secondary and tertiary structure, which is completed within a few minutes upon initiation of refolding.

Tab. 3-4: Calculated halftimes of tertiary structure formation and activity (data from Fig. 3-13).

Parameter	Halftime [min^{-1}]
Activity	16
Tertiary structure formation	2

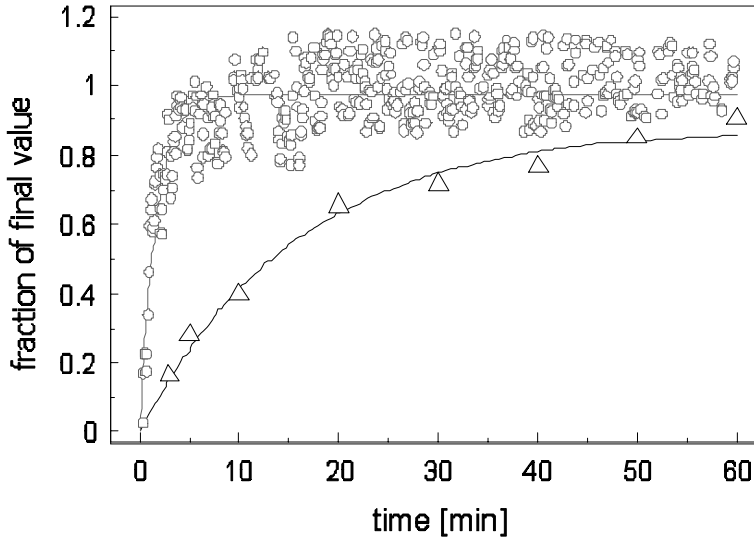


Fig. 3-13: Tertiary structure and activity formation during the refolding of 0.2 mg/mL lysozyme in presence of 2 mM DTT and 5 mM GSSG and 0.5 M arginine. (○) Formation of tertiary structure monitored by CD at 292 nm, (Δ) activity. Ordinate values represent normalized signal values.

The data shown in Fig. 3-13 were fitted to a single exponential regression function:

$$y = a(1 - e^{-bx}),$$

where a represents the amplitude and b the rate (Tab. 3-4).

The same kinetic measurement was performed with far-UV CD spectroscopy at 220 nm, indicative of formation of secondary structure. In these cases no time-dependent change of the signal could be detected, indicating that secondary structure formation is completed within the dead time of the experiment, which is between 10-20 sec. This experiment illustrates that while spectroscopic methods reveal formation of secondary and tertiary structure of lysozyme, they cannot be linked directly to activity, which provides the most conclusive information about the folding status of lysozyme.

If the formation of disulfide bonds from denatured-reduced protein is suppressed or under sub-optimal conditions (*i.e.* sub-optimal concentrations of oxido shuffling system), activity recovery of the protein is significantly lower than under ideal conditions. Fig. 3-14 demonstrates the relationship between activity and free sulfhydryl groups. While free sulfhydryl groups become rapidly oxidized upon refolding of HEWL, activity recovery requires a longer period of time to proceed. This indicates that after rapid oxidation of sulfhydryl groups thiol shuffling needs to occur to form the native version of the disulfide bonds, enabling activity recovery.

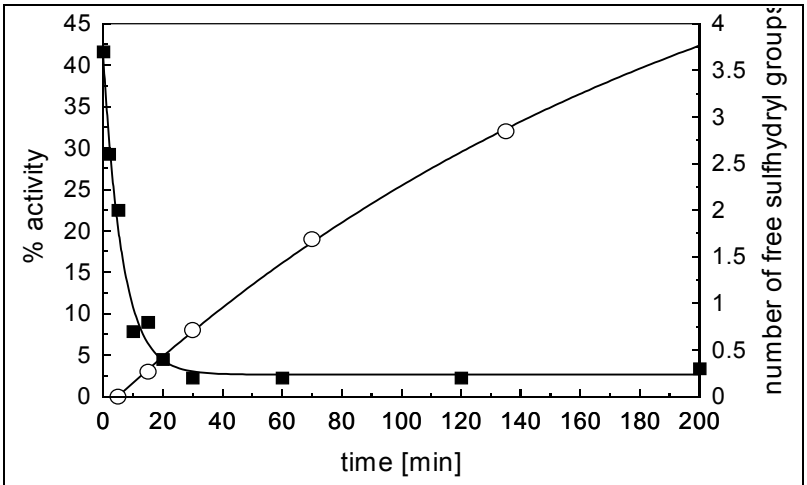


Fig. 3-14: Relationship of activity recovery and free sulfhydryl groups during refolding of 0.2 mg/mL HEWL. Samples were quenched at the indicated times with hydrochloric acid and rapidly dialyzed to prevent reoxidation. Free sulfhydryl groups were determined utilizing Ellman reagent. (○) Activity, (■) free sulfhydryl groups.

Refolding yields in presence of arginine and GdmCl

To determine the maximum recovery of activity when lysozyme is refolded with and without arginine or GdmCl, refolding experiments in presence of different

concentrations of arginine and GdmCl were allowed to proceed for 24 hours to allow complete reaction of thiol exchange reactions.

Fig. 3-15 shows the effect of arginine and Fig. 3-16 the effect of GdmCl on the refolding process of lysozyme. Both figures show the recovered activity and aggregation after 24 hours of refolding time.

The oxido shuffling system used was 2 mM DTT and 5 mM GSSG. This system leads to the recovery of 100% activity when 0.5 M GdmCl is present (Maachupalli-Reddy *et al.*, 1997; Fig. 3-18). Since native lysozyme loses activity when stored at room temperature for 24 hours (Tab. 3-5), activity data were prorated to give a more realistic view of possible refolding yields. Both additives facilitate the recovery of activity. The obtained data show a similar effect of both additives. Within 0-0.5 M additive the activity increases to the maximum value, which is between 90-100% activity. Aggregation similarly declines within this concentration range, above 0.5 M aggregation becomes completely suppressed.

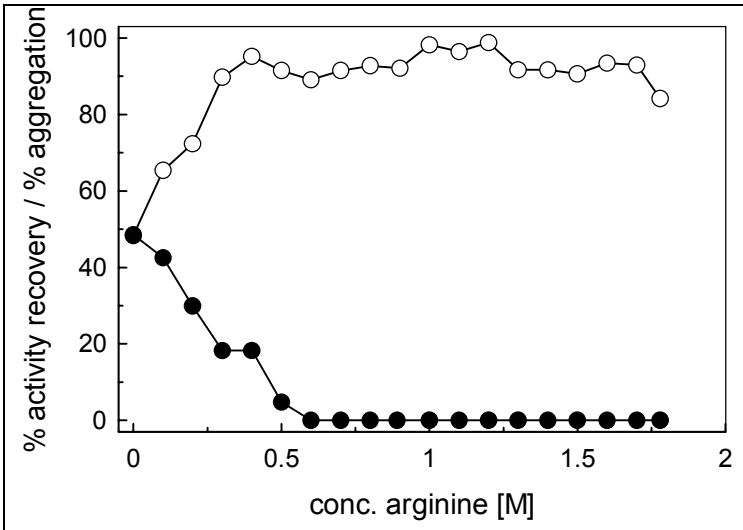


Fig. 3-15: Refolding of lysozyme in presence of arginine. The (○) recovered activity is shown as well as (●) the amount of aggregated protein after 24 hours. Refolding took place at 2 mM DTT and 5 mM GSSG with a protein concentration of 0.2 mg/mL lysozyme.

The observed suppression of aggregation does not depend on the presence of GSSG (data not shown), because aggregation is also suppressed by additives in the absence of GSSG.

Correct disulfide bonds could also be formed after the formation of secondary and tertiary structure is completed, when GSSG is added at a substantially later time after refolding was initiated (Fig. 3-17).

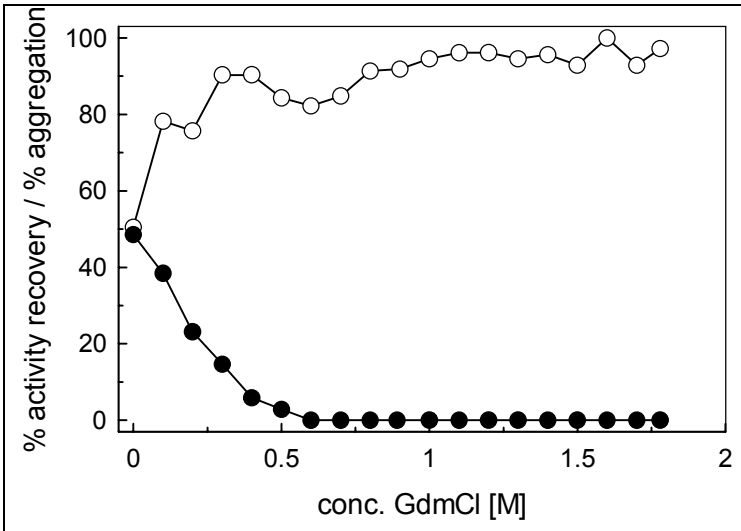


Fig. 3-16: Refolding of lysozyme in presence of GdmCl. The (○) recovered activity is shown as well as (●) the amount of aggregated protein after 24 hours. Refolding took place at 2 mM DTT and 5 mM GSSG with a protein concentration of 0.2 mg/mL lysozyme.

The data indicate that with just TE buffer being added the amount of recoverable activity rapidly declines, while in presence of 1 M arginine or GdmCl up to approximately six hours a significantly percentage of activity can be recovered.

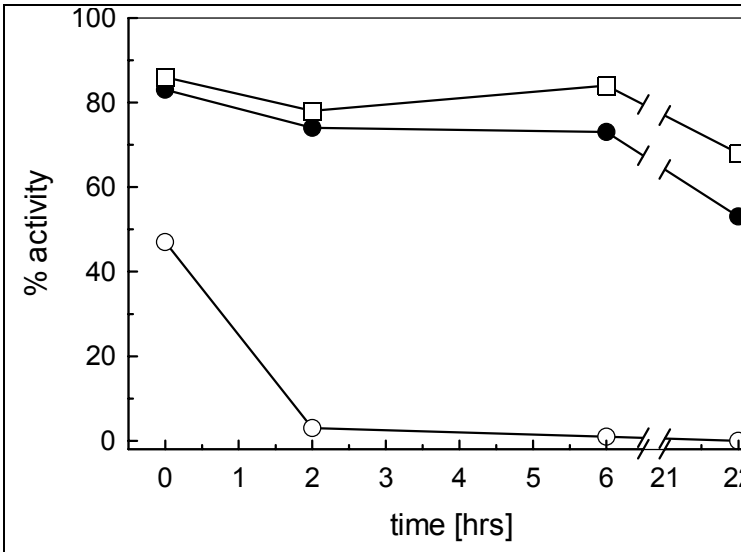


Fig. 3-17: Effect of GSSG when added after the formation of secondary and tertiary structure is complete. Lysozyme was allowed to refold for the indicated times without GSSG, after which it was added to a final concentration of 5 mM. Activity measurements were performed three hours after addition of GSSG to allow for formation of disulfide bonds. The data are shown for (o) no additive, (●) 1 M arginine, and (□) 1 M GdmCl.

This indicates that partially folded protein species do not proceed as fast into the aggregation pathway in presence of these additives as they would otherwise do. Also, once aggregates start to form, they become inaccessible to buffer, and thus later added GSSG cannot form disulfide bonds. Between GdmCl and arginine, GdmCl is slightly more efficient in facilitating the formation of disulfide bonds.

Effect of the GSSG concentration on HEWL refolding

The composition of the thiol-disulfide exchange system, also known as oxido shuffling system (OXS), greatly affects the yield of a refolding experiment. Hevehan and De Bernardez Clark (1997) investigated how various concentrations of DTT and GSSG may affect lysozyme refolding yields. The following experiment illustrates the effect of various gluthathione (GSSG) concentrations on the maximum recovery of activity, while the concentration of DTT is kept constant at 2 mM.

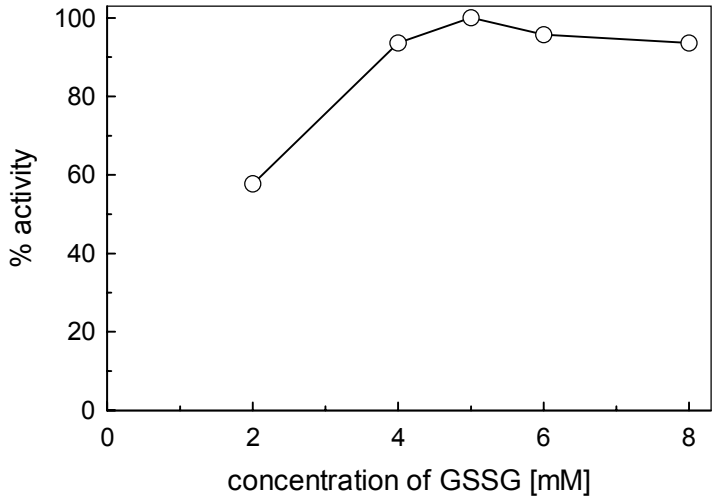


Fig. 3-18: Protein refolding yields of 0.2 mg/mL lysozyme under various oxido shuffling conditions. The DTT concentration was kept constant at 2 mM DTT, while the effect of different GSSG concentrations on activity is shown here. In order to completely suppress aggregation, 1 M arginine was provided.

In order to determine the optimum GSSG concentration for refolding of lysozyme, recovered activity was determined as a function of various GSSG concentrations. Aggregation was completely suppressed by addition of 1 M arginine. The concentration of DTT was kept constant at 2 mM, which results from the hundredfold dilution of the 20 mg/mL lysozyme, 200 mM DTT solution of denatured-reduced lysozyme. The concentration of GSSG was varied from 2 to 8 mM. The protein concentration during the refolding process was 0.2 mg/mL. In Fig. 3-18 the recovered activity after 24 hours is shown which varies from 60%

at 2 mM GSSG to 100% activity at 5 mM GSSG. This experiment demonstrates that no significant differences occur when lysozyme is refolded with 4 mM GSSG instead of 5 mM GSSG, since some of the experiments included in this section were performed with 4 mM GSSG, while the majority was performed with 5 mM GSSG during the refolding step.

Intrinsic loss of activity

In order to determine the influence of the oxido shuffling system components on the stability of native lysozyme, native lysozyme was incubated with and without oxido shuffling system for 24 hours, after which the activity was determined in comparison to a freshly prepared lysozyme solution of similar concentration (Tab. 3-5).

In the absence of oxido shuffling system the achievable yield is 80%, whereas freshly made lysozyme solution from lyophilized protein has 100% activity by definition. This indicates that lysozyme loses some of its activity when incubated overnight at room temperature. This loss of protein activity may be caused by oxidation and/or formation of microaggregates. In presence of oxido shuffling system lysozyme does not lose a higher percentage of activity than by just incubating it overnight, indicating that prolonged incubation periods with oxido shuffling system components do not influence the activity of native lysozyme. To reflect achievable protein yields, data which refer to refolding time spans of 24 hours were corrected for this loss of activity.

Tab. 3-5: activity of 0.2 mg/mL native lysozyme when incubated with oxido shuffling system (OXS) for 24 hours. Activity was measured against a freshly prepared lysozyme solution.

Sample	% Activity
Without OXS	80
With OXS	81
Solution of fresh lysozyme	100

In addition data in Tab. 3-5 provide evidence that oxido shuffling system components do not alter the activity of lysozyme at room temperature. Activity is the most sensitive indicator of structural changes and chemical degradation. Optical spectroscopy usually cannot detect chemical modifications of a protein.

Effect of arginine and GdmCl on folding and aggregation kinetic rates

To study the influence of additives on refolding kinetics, kinetics of lysozyme refolding in the absence and presence of folding additives were recorded. Since recovery of activity depends on formation of disulfide bonds, activity at a given

time can be arrested by alkylation of free sulfhydryl groups. This was done in the following kinetic experiment.

In the absence of arginine or GdmCl between 40-50% of the total protein amount aggregated, while in presence of 0.25 M arginine or GdmCl approximately 20% aggregation was observed (Figs. 3-15, 3-16). With 0.5 M arginine or GdmCl aggregation is completely inhibited. Similarly in the absence of arginine or GdmCl approximately 50% activity was recovered, while the presence of 0.25 M arginine or GdmCl leads to approximately 70% activity recovery, and at 0.5 M arginine or GdmCl the protein becomes fully active. In order to determine the influence of arginine and GdmCl on folding and aggregation kinetic rates, the kinetics of recovery of activity in presence of both additives were measured.

The refolding conditions were 0.2 mg/mL lysozyme with 2 mM DTT and 5 mM GSSG and 0, 0.25, 0.5, and 1 M arginine or GdmCl. Since refolding kinetics are very pH-dependent, great care was taken to adjust the pH to 8.0 ± 0.1. The pH in the refolding buffer needs to be slightly alkaline because the reactive group during the formation of disulfide bonds is the thiolate anion (which forms when a sulfhydryl group becomes deprotonated). Even under slightly acidic conditions sulfhydryl groups may become protonated and thus kinetic rates may become altered.

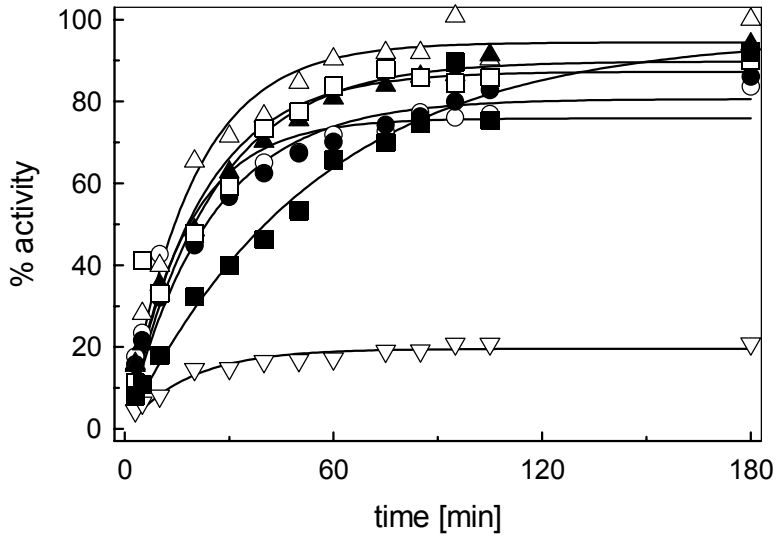


Fig. 3-19: Refolding kinetics in presence of (▽) 0 M, (○) 0.25 M arginine, (●) 0.25 M GdmCl, (△) 0.5 M arginine, (▲) 0.5 M GdmCl, (□) 1 M arginine, and (■) 1 M GdmCl. The protein concentration was 0.2 mg/mL, the oxido shuffling system was 2 mM DTT and 5 mM GSSG.

To acquire activity kinetics, the formation process of disulfide bonds needs to be quenched by alkylation of free sulfhydryl groups by IAA.

Within the time period studied (0-3 hours) recovery of activity reaches its equilibrium, except in presence of 1 M GdmCl. In the absence of additives, only 20% of the activity is recovered, the remainder becomes aggregated. The purpose of this experiment was to calculate kinetic folding rates and to derive aggregation rates from it. With exception of kinetics in presence of 1 M GdmCl, all refolding rates appeared of similar order of magnitude.

Folding and aggregation rates were calculated from data shown in Fig. 3-19 utilizing the model by Hevehan and De Bernardez Clark (1997). One advantage of this model is that it allows the calculation of aggregation rates, which are usually not accessible through experiments. Because an ongoing aggregation process cannot be arrested, it is not possible to reliably determine aggregation kinetics. Usually indirect markers for aggregation, such as light scattering are used.

The model by Hevehan and De Bernardez Clark assumes that folding proceeds from the unfolded protein (U) through an intermediate (I) with a kinetic constant k_1. This step is not rate-limiting. The pathways starting from (I) are rate-limiting (Fig. 3-20). Aggregation is a process of higher reaction order, because it involves the formation of oligomers from monomers. The reaction rate of the aggregation pathway was assigned k_3, and the reaction order is ≥ 2. In order to allow an analytic solution of the differential equation system, the reaction order of the aggregation process was set to 3. The folding pathway (k_2) is a first order reaction.

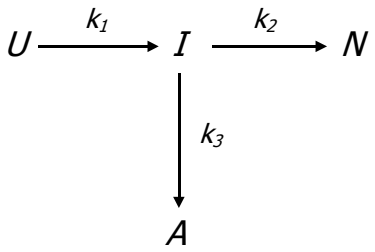


Fig. 3-20: Kinetic protein refolding model, adapted from Hevehan and De Bernardez Clark (1997). (*U*) represents the unfolded protein, (*I*) folding intermediates, (*A*) aggregates, and (*N*) native protein. The formation of intermediates (k_1) is not the rate-limiting step. The folding rate is described through k_2, the aggregation rate through k_3, which are rate-limiting.

The yield of native protein (y) over time can be described by the following equation (Hevehan and De Bernardez Clark, 1997):

$$y = \psi[\arctan\sqrt{(1+\psi^2)\exp^{2k_2 t} - 1} - \arctan(\psi)], \quad (1)$$

where ψ in (1) is defined as

$$\psi = \sqrt{\frac{k_2}{k_3 U_0^2}}. \quad (2)$$

U_0 is the protein concentration of the unfolded protein at $t=0$. The final yield of protein can be calculated as time approaches infinity by:

$$y = \psi[\frac{\pi}{2} - \arctan(\psi)]. \quad (3)$$

Utilizing non-linear regression, the respective refolding rates can be calculated from activity kinetics. In Fig. 3-21 the calculated folding and aggregation rates (from data in Fig. 3-19) are shown.

The calculated data (Fig. 3-21) show that in presence of both additives lower aggregation rates were observed than in the absence. While aggregation rates decline similarly for both additives between 0-0.5 M, they become stagnant for higher concentrations of arginine. Above ≥0.5 M GdmCl aggregation rates continue to decline. At 1 M a significant difference in the aggregation rates between arginine and GdmCl were observed. While in presence of arginine aggregation rates have become stagnant, they continue to decline in presence of GdmCl, indicating that concentrations of more than 1 M GdmCl may further reduce the aggregation rates.

The calculated folding rates (k_2) show a different concentration-dependent behavior for both additives. While refolding rates in presence of 0.25 M and 0.5 M GdmCl are only slightly increased, they decrease significantly in presence of 1 M GdmCl. In presence of arginine the folding rates show an increase of approximately 50%, which only marginally decline above 0.5 M arginine. To elucidate the relationship between folding and aggregation rates, the following term was plotted against additive concentration: $\sqrt{k_2 / k_3}$ (Fig. 3-21, inset). The resulting diagram indicates that in presence of GdmCl the ratio of $\sqrt{k_2 / k_3}$ increases beyond 1 M GdmCl, while this ratio achieves its maximum in presence of 0.5 M arginine and then slightly declines. While these data do not take into consideration that higher concentrations of GdmCl may unfold lysozyme and thus folding rates significantly decline, the data indicate how additives affect both rates in relationship to each other.

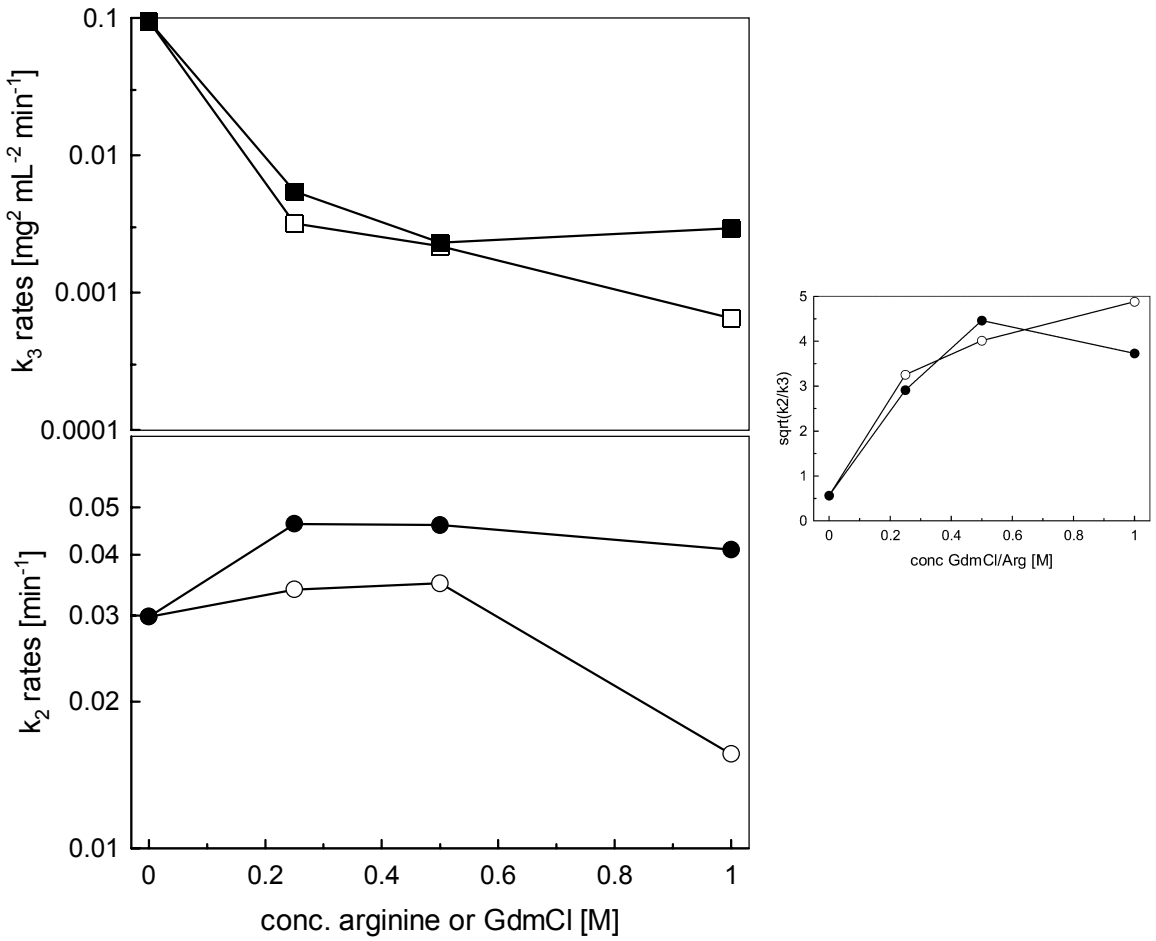


Fig. 3-21: Calculated refolding and aggregation rates from refolding kinetics in presence of arginine and GdmCl. (**top**) aggregation rates (□) k_3 GdmCl, and (■) k_3 arginine, (**bottom**) protein folding rates (○) k_2 GdmCl (●) k_2 arginine. (**Inset**) $\sqrt{k_2 / k_3}$ vs. additive concentration for (○) GdmCl and (●) arginine.

The data show that aggregation rates decline in presence of both additives, while GdmCl does not affect folding rates in the lower concentration range (0-0.5 M), but partially inhibits folding at higher concentrations (>0.5 M). Since GdmCl is well known for its denaturing effect on proteins, it is not surprising that higher GdmCl concentrations counteract folding as seen here. Surprisingly arginine slightly increases the refolding rates of lysozyme.

This shows that both additives have a different effect on the kinetic competition between folding and aggregation for lysozyme.

In Fig. 3-22 light scattering rates are shown in presence of various concentrations of arginine or GdmCl. Lysozyme at 0.2 mg/mL was allowed to refold in presence of 2 mM DTT and 5 mM GSSG. In presence of both additives light scattering rates decline, leading to near zero values above 0.5 or 0.6 M.

While static light scattering gives an overall estimate of the occurrence of aggregation through detection of subvisible particles, it does not provide detailed information about size distribution or absolute particle count, thus a few large particles may suggest a higher percentage of aggregation. Thus no coincidence with derived aggregation rates (as shown in Fig. 3-21) is expected. While the shape of both signals is different, the information from both methods is that aggregation rates decrease with increasing additive concentrations, and reach a minimum above 0.5 or 1 M.

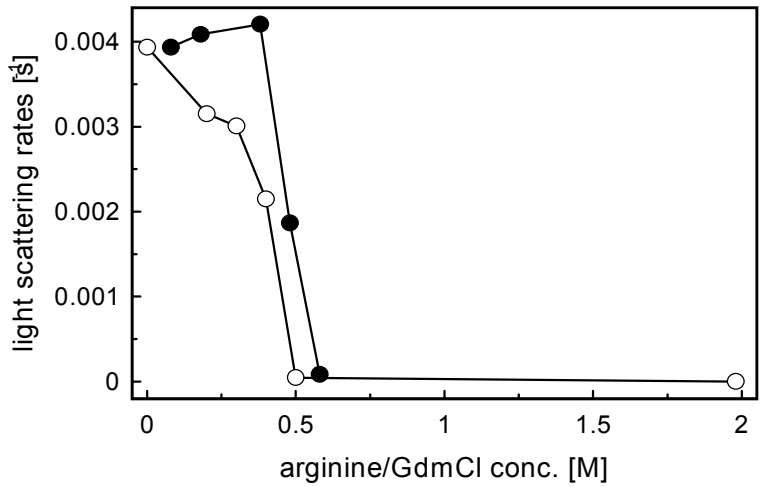


Fig. 3-22: Kinetic constants of light scattering (measured as optical density at 450 nm) in dependency of (○) arginine or (●) GdmCl concentrations.

ANS binding kinetics

Previous data showed the effect of arginine and GdmCl on folding and aggregation kinetics. The next set of data will describe the effect both additives have on the formation of hydrophobic, ANS-binding intermediates.

In order to determine the amount of populated folding intermediates, 8-anilino-1-sulfonic acid (ANS) was used. ANS is frequently used to detect the presence of folding intermediates, which are more hydrophobic than native protein. ANS only fluoresces when exposed to a hydrophobic environment. Since folding intermediates differ from the native protein by exposing hydrophobic side chains, the presence of ANS fluorescence is an indicator of hydrophobic folding intermediates. For the data shown in Fig. 3-23, lysozyme was refolded with and without additives. While the refolding process was taking place, aliquots of the samples were quenched with IAA to alkylate free sulfhydryl groups, followed by addition of an aliquot of ANS. While the presence of free sulfhydryl groups is expected to decrease rapidly upon initiation of refolding, alkylation was performed to ensure that thiol exchange reactions are completely quenched. The spectra were recorded immediately afterwards to ensure that no alterations of

the sample took place. The alkylation of free sulfhydryl groups by IAA does not interfere with ongoing changes in the protein structure, and was specifically added to prevent formation of disulfide bonds which can affect the stability of partially folded HEWL molecules.

The unfolding conditions were 0.2 mg/mL lysozyme with 2 mM DTT and 5 mM GSSG. After the appropriate time a solution containing 500 mM IAA and 0.2 mM ANS was added to the samples to alkylate free sulfhydryl groups, resulting in the final concentrations of 50 mM IAA and 20 μM ANS. Fluorescence spectra were acquired immediately after the quenching step. The samples were excited at 350 nm and fluorescence spectra were recorded between 380 and 600 nm. The fluorescence intensity at 480 nm was used to quantify ANS binding.

Because it was shown that the presence of ANS during protein refolding can induce ANS binding intermediates (Kamen and Woody, 2001) or the suppression of aggregation (Kundu and Guptasarma, 1999), the strategy pursued here (addition of ANS at the appropriate time instead of having ANS present throughout the whole experiment) helps to prevent the occurrence of artifacts.

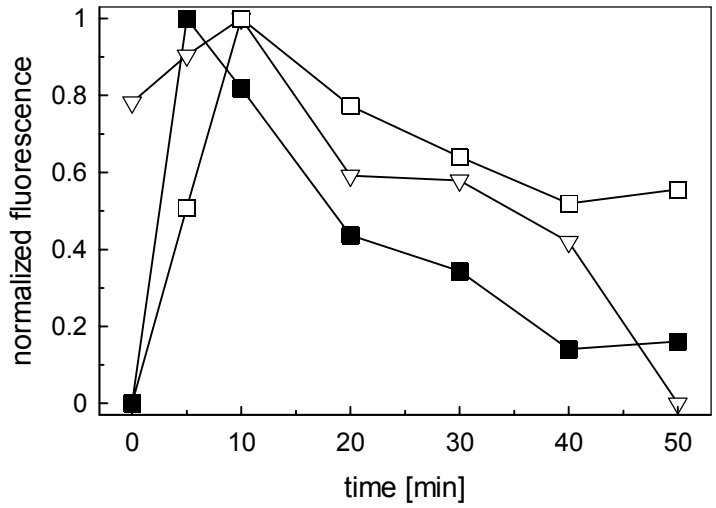


Fig. 3-23: Normalized ANS binding kinetics under various conditions. The protein concentration was 0.2 mg/mL, and the oxido shuffling system was 2 mM DTT and 5 mM GSSG, 20 μM ANS were added just before the data were recorded. (∇) 0 M additive, (□) 1 M arginine, and (■) 1 M GdmCl.

Since the presence of arginine or GdmCl in the refolding buffer alters fluorescence properties of ANS, it is neither possible to quantify the absolute amount of ANS-binding intermediates nor to compare the population of ANS-binding intermediates between samples in different buffers. For the experiment shown in Fig. 3-23 no absolute fluorescence intensities are shown because of

these limitations. Instead, raw data were normalized to values between 0 and 1 to compare the occurrence of maxima and minima.

The data show (Fig. 3-23) that within the first 5-10 min after refolding has been initiated, ANS fluorescence of all samples increases, reaching a maximum around 5-10 min, followed by a decline. This coincides with previously pulished data (Fischer *et al.*, 1993). The measured signal indicates presence of partially folded hydrophobic intermediates, which are the only species that bind ANS, causing fluorescence of ANS. In presence of 1 M arginine or 1 M GdmCl fluorescence is low at the t=0 time point. In the absence of additives, the initial fluorescence signal is very high (80% of the maximum), indicating that intermediates form within the dead time of the experiment. After 50 minutes, the signal has declined to its minimum. In presence of 1 M arginine the fluorescence signal at t=0 is very low, but increases to its maximum at t=10 min. Afterwards the fluorescence intensity declines, but does not reach zero after 50 min. In presence of 1 M GdmCl the minimum fluorescence is detected at t=0, the maximum after 5 min. The data show that intermediates in presence of 1 M arginine are populated for a longer period of time than in the absence of additives and 1 M GdmCl.

Effect of the additives on aggregation when added after refolding was initiated

The experiment shown in Fig. 3-10 investigated the effect of additives on the solubility of fully-grown aggregates, which had 24 hours to grow. In this case the addition of arginine did not result in significant solubilization of aggregates, while concentrations of more than 1 M GdmCl were efficient in dissolving aggregates. Since aggregation involves a polymerization of aggregation precursors to full-sized aggregates, the influence of additives on these aggregation precursors was investigated in the next experiment.

In the experiment described here arginine or GdmCl to a final concentration of 1 M were added 2, 10, or 30 minutes after refolding had been initiated. This assured not only formation of aggregation precursors, but also that the intermediate-forming step proceeded without the influence of additives (Fig. 3-23).

Refolding was initiated with a protein concentration of 0.4 mg/mL. After a necessary dilution step in the experiment the concentration declines to 0.2 mg/mL by addition of additive solution. In order to assure substantial formation of aggregates, final protein concentrations above 0.2 mg/mL were required, because at 0.1 mg/mL final lysozyme concentration no substantial amounts of aggregates would have formed. The solutions of arginine or GdmCl were added after the appropriate times and did not contain GSSG. After addition of arginine, GdmCl or TE buffer solutions, samples were allowed to completely aggregate for 24 hours. After this time, aggregates were harvested by

centrifugation. The supernatant was discarded. The pellet was washed with TE buffer, followed by centrifugation to remove entrapped native protein. After centrifugation the supernatant was discarded and the pellet was dissolved with 1 mL 8 M GdmCl. Based on the known protein concentration of pre-aggregated lysozyme the amount of aggregated protein was calculated.

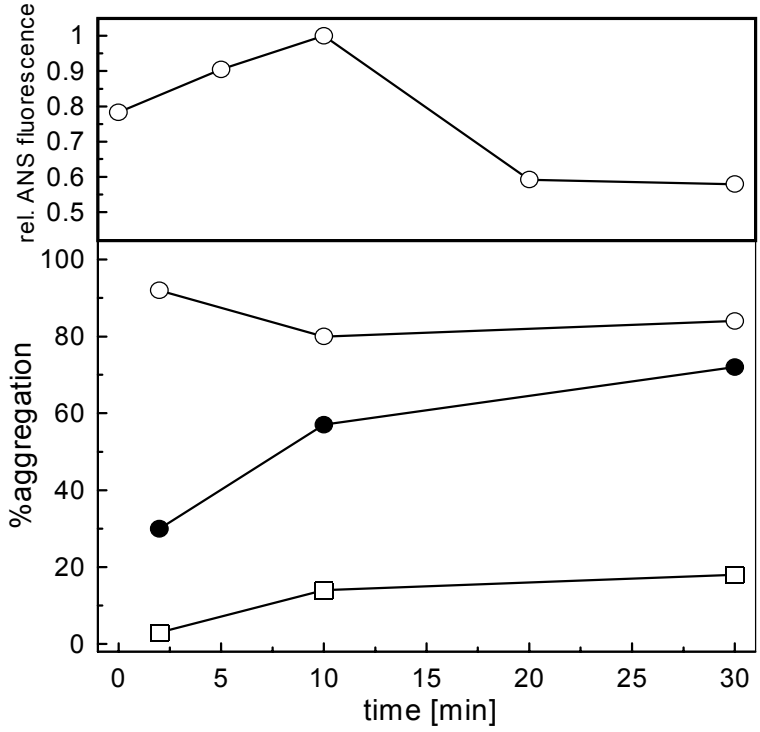


Fig. 3-24: (**Bottom**) Percentage of aggregates which form after (o) no additive, (●) 1 M arginine, or (□) 1 M GdmCl was added at the indicated times; (**top**) trace of ANS fluorescence kinetics shown for comparison (Fig. 3-23).

The data show (Fig. 3-24) that by addition of TE buffer formation of aggregated protein only slightly becomes affected. In presence of 1 M GdmCl aggregates can become dissolved even when GdmCl is added at a relatively late stage of the process. In presence of 1 M arginine aggregation can be decreased during the first two minutes of the aggregation process. If addition of arginine occurs after 10 min, the effect greatly diminishes. Partially folded protein molecules were detectable after 2 min and reached their peak occurrence after 10 min (Fig. 3-23). The experiment shown here suggests that arginine loses its highest efficiency when added after 10 min. This means that arginine cannot prevent formation of aggregates once the maximum amount of partially unfolded protein molecules has been populated. Arginine has to be present prior to completion of ANS binding intermediate formation to be able to inhibit aggregate formation. GdmCl can revert aggregation of not fully grown aggregates to a certain degree, thus it is not critical that it is present when the refolding process is initiated.

3.3. Effects of arginine and GdmCl on HEWL unfolding

In the process of preparing denatured-reduced lysozyme for oxidative refolding experiments, DTT was added to a solution of native lysozyme in TE buffer, which resulted in the formation of aggregates. The solution had a turbid appearance indicative of aggregated protein. Aggregation was found to occur after a lag phase. This effect had been described before by Denton and Scheraga (1991) who described this observation while producing des-[C^{6}-C^{127}]-lysozyme, but referred to it as "precipitation". They chose relatively low concentrations of DTT to slowly reduce lysozyme. When the solution became turbid, they used it to indicate the formation of partly reduced lysozyme and interrupted the reduction process. Joseph and Nagaraj (1992) used a similar experimental setup and described that lysozyme exposes hydrophobic groups upon reduction by DTT. Two and a half hours after the reduction process was initiated ANS fluorescence increased. They observed a more significant increase in ANS fluorescence at lower pH, but one needs to be cautious when comparing ANS spectra measured at different pH as the fluorescence properties of ANS depend on the pH of the solution.

This aggregation process has the potential to be very useful in the characterization of the effect of arginine and GdmCl on aggregation, because aggregation occurs much slower than during the refolding process. The aggregation process of lysozyme when refolded is extremely fast, and aggregates appear instantaneously, while during reductive unfolding described here a relatively long time is needed for aggregates to become visible.

To confirm that the insoluble material, which formed during reductive unfolding of lysozyme, was indeed aggregated and not precipitated, it was attempted to dissolve it in TE buffer with application of vigorous shaking. The material did not dissolve, indicating that it is indeed aggregated protein, and not precipitated as claimed by Denton and Scheraga (1991). It is soluble in 8 M GdmCl, which was utilized to quantify the amounts of aggregated protein.

To show that the aggregation process is caused by (partial) reduction of disulfide bonds, samples with 0.2 mg/mL lysozyme were allowed to become reduced by exposure to 20 mM DTT for 10 minutes (at this point samples were not turbid), before free sulfhydryl groups were quenched with an excess of iodoacetic acid (IAA). Subsequently no turbidity was observed. This suggests that the aggregation process becomes initiated by reduction of disulfide bonds. IAA reacts with free sulfhydryl groups by alkylating them and release of hydrogen iodine (HI). Because this reaction is irreversible, a complete stop of all thiol exchange reactions is accomplished. An excess amount of IAA needs to be added to ensure that thiol groups of the protein and DTT become alkylated. The alkylation thus

inactivates DTT. The data suggest that the aggregation is caused by (partial) reduction of disulfide bonds in lysozyme.

Fig. 3-25 shows a comparison of aggregation kinetics of 0.2 mg/mL and 2 mg/mL lysozyme in presence of 20 mM DTT. While both samples initially have very similar kinetics, the 2 mg/mL sample reaches the point of full aggregation at an earlier time point than the one at 0.2 mg/mL. This coincides with visual observations: it took approximately 15 min for a 2 mg/mL sample and 45 min for a 0.2 mg/mL sample to appear turbid.

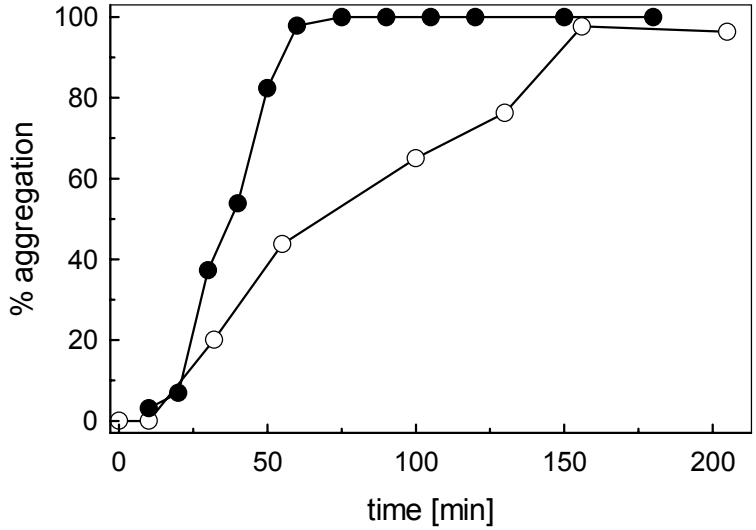


Fig. 3-25: Comparison of the unfolding aggregation kinetics at (○) 0.2 mg/mL and (●) 2 mg/mL lysozyme. At a given time free sulfhydryl groups were quenched with IAA, followed by immediate centrifugation. The supernatant was discarded, while the pellet was washed twice with buffer. Then the pellet was dissolved with an aliquot of 8 M GdmCl. From the concentration of dissolved aggregates the overall amount of aggregation was calculated.

Following partial reduction of disulfide bonds, the protein is prone to aggregation. During the aggregation process, DTT is present in substantial excess compared to protein thus the reduction of disulfide bonds is not expected to be rate-limiting or dependent on the protein concentration. The aggregation process following the reduction of disulfide bonds, however, has a reaction order of at least 2 since aggregation is a multi-molecule process involving the interaction of several molecules, and thus depends on the protein concentration. Therefore higher protein concentrations increase aggregation rates as Fig. 3-25 shows.

In the previous chapter the effect of arginine and GdmCl on the refolding of lysozyme was shown. Both additives decrease aggregation, thus increasing

refolding yields. The reduction of disulfide bonds through DTT described here leads to the formation of aggregated protein. This raises the question whether both aggregation processes are sufficiently similar so that arginine and GdmCl may decrease aggregation. To address this question various concentrations of either arginine or GdmCl were added to a solution of native lysozyme prior to the addition of DTT. As the data show (Fig. 3-26) both additives indeed decrease aggregation very efficiently. Concentrations below 0.8 M of both additives partially suppress aggregation, above 1 M it is completely suppressed. Data in Fig. 3-26 represent aggregates formed in samples after 24 hours. In presence of 1 M arginine no aggregation was detectable after 24 hours, but samples became turbid after 4 days (data not shown). This illustrates that although arginine strongly decreases the aggregation rate, it does not completely suppress aggregation.

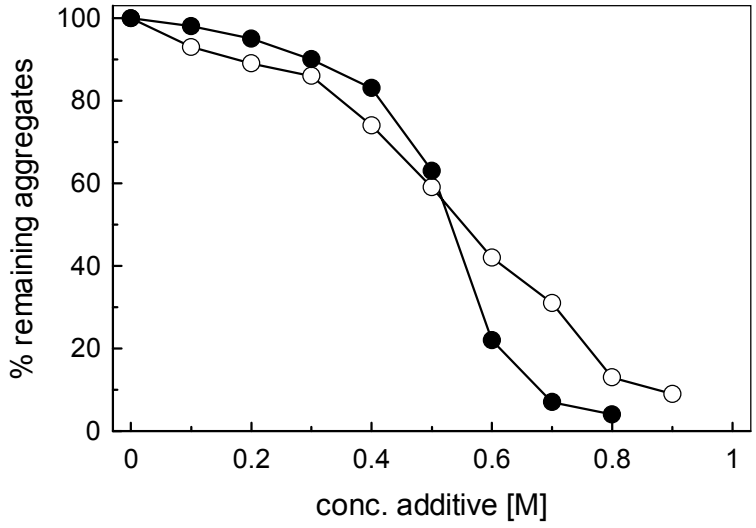


Fig. 3-26: Effect of arginine and GdmCl on aggregation of 0.2 mg/mL lysozyme and 20 mM DTT when the indicated concentration of additives was present during the 24 hour period. (○) Arginine, (●) GdmCl.

Samples with 1 M GdmCl do not become turbid after prolonged incubation times. This indicates that the mechanism by which arginine and GdmCl suppress aggregation may be different.

The excess of 20 mM DTT in solution ensures that the protein remains reduced even after longer incubation times. Upon removal of DTT the protein is prone to air oxidation, which may result in the formation of intrachain and interchain disulfide bonds. Disulfide bonds between protein chains can result in disulfide bonded aggregates, which do not become dissolved by the presence of 8 M GdmCl alone, but require addition of DTT to reduce these interchain disulfide bonds. Upon reducing disulfide bonds in lysozyme DTT becomes oxidized. The so

formed oxido shuffling system consists of reduced and oxidized DTT, which is a very weak oxidizer. Thus the effect of reducing DTT dominates, resulting in reduced lysozyme (Shin and Scheraga, 2000).

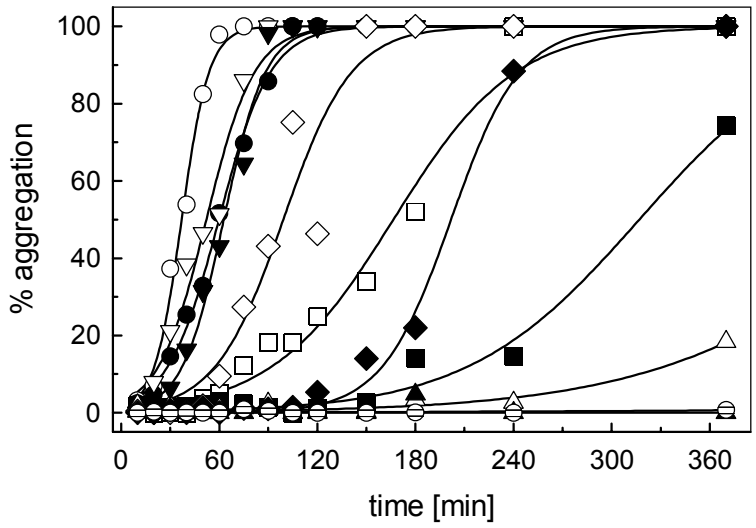


Fig. 3-27: Aggregation kinetics of 2 mg/mL native lysozyme when incubated with 20 mM DTT under the following conditions: (○) no additive, (●) 0.05 M arginine, (□) 0.25 M arginine, (■) 0.5 M arginine, (Δ) 0.75 M arginine, (▲) 1 M arginine, (∇) 0.05 M GdmCl, (▼) 0.25 M GdmCl, (◇) 0.5 M GdmCl, (◆) 0.75 M GdmCl, and (⊖) 1 M GdmCl.

So far, it has been shown that arginine and GdmCl can decrease the final aggregation amount. Thus it was of great interest to determine how arginine and GdmCl affect aggregation kinetics. Thus aggregation kinetics in presence of different concentrations of arginine or GdmCl were recorded (Fig. 3-27). All kinetics are characterized by a distinctive lag phase. The length of the lag phase varies depending on the additive concentration provided. Generally, the higher the concentration of additive, the longer the observed lag phase.

To characterize the different aggregation processes, aggregation halftimes were calculated by applying sigmoid regression functions to the data shown in Fig. 3-27:

$$y = \frac{1}{1 + e^{-\frac{x - T_{1/2}}{a}}},$$

where y is the observed amount of aggregated protein at a given time, $T_{1/2}$ the aggregation halftime (y=0.5) and a is related to the slope of the transition

region. Aggregation halftimes for each of the conditions shown in Fig. 3-27 were calculated and the resulting halftimes are shown in Fig. 3-28.

The data indicate that arginine has a strong influence on the aggregation halftime and significantly increases it, this means that it reduces the apparent aggregation rate. Although data in Fig. 3-27 suggest that arginine increases the aggregation halftime indefinitely above 0.75 M, samples containing 1 M arginine aggregated after 3 days, showing that arginine may delay aggregation, but not completely inhibits it.

The aggregation halftime is not significantly influenced by the presence of GdmCl at concentrations between 0 and 0.5 M GdmCl. At 0.75 M GdmCl an increased halftime was observed, which is still only half as long as in presence of 0.75 M arginine. Concentrations of more than approximately 0.8 M GdmCl (Fig. 3-27) completely suppress aggregation, which may be due to the fact that with increasing GdmCl concentrations a higher percentage of aggregates may become dissolved. The comparison of these data with halftimes in presence of arginine show that even low concentrations of arginine do affect the halftimes significantly, leading to a strong correlation (almost linear in the concentration range investigated) between arginine concentration and observed halftime.

On the other hand, the correlation between GdmCl concentration and halftime appears to be more exponential (Fig. 3-28), indicating that a higher concentration of GdmCl is necessary to affect the aggregation kinetics. The data indicate that the underlying mechanism by which the two additives suppress aggregation is different.

Although arginine significantly delays the aggregation process at 2 mg/mL HEWL, it is not able to suppress aggregation indefinetely at this concentration. Arginine can indefinetely inhibit aggregation of 0.2 mg/mL HEWL. Therefore the following experiments were performed at 0.2 mg/mL lysozyme. This protein concentration also complements the refolding experiments shown in the previous chapter, which were all done at 0.2 mg/mL.

To determine the effect of arginine and GdmCl on aggregation and activity over time, activity and aggregation kinetics with 0.2 mg/mL lysozyme were acquired. Activity and aggregation were determined from the same sample. At a given time point, free sulfhydryl groups in the samples were alkylated with IAA after which samples were centrifuged immediately. Activity was determined from the supernatant. The amount of aggregation was determined from the pellet. Aggregation and activity kinetics were determined in presence of 0, 0.25, 0.5, and 1 M arginine and GdmCl.

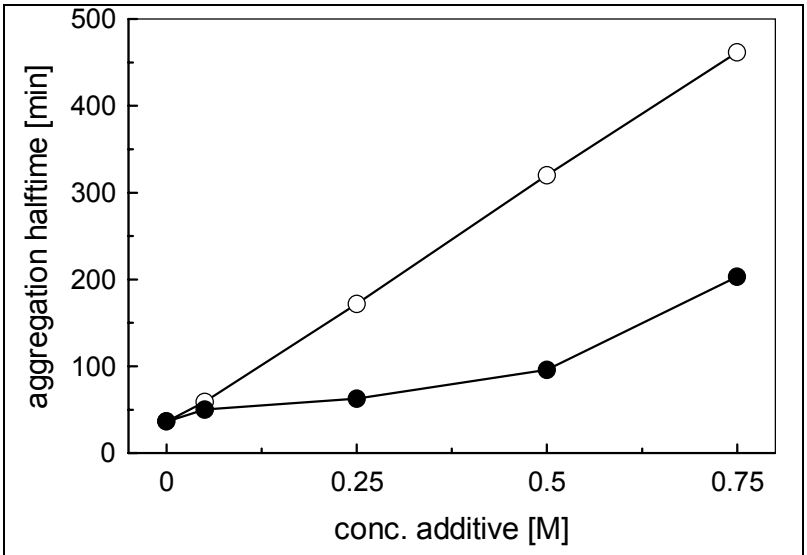


Fig. 3-28: Calculated aggregation halftimes of data shown in Fig. 3-27 for (○) arginine and (●) GdmCl.

For better clarity, data were arranged as follows: in Fig. 3-29 the activity and aggregation kinetics in the absence of arginine and GdmCl are shown, in Fig. 3-30 data in presence of arginine is shown, and in Fig. 3-31 the same for GdmCl is shown.

The data in the absence of additives (Fig. 3-29) indicate that the loss of activity correlates well with an increase in aggregation, showing that lysozyme loses activity as aggregation progresses. Lysozyme becomes completely aggregated under these conditions. It also loses all enzymatic activity over the course of the experiment.

Activity and aggregation kinetics in presence of 0.25, 0.5, and 1 M arginine are shown in Fig. 3-30. Within the timeframe of the experiment (0-4 hours) aggregation was only detectable in presence of 0.25 M arginine.

In presence of 0.25 M arginine aggregation occurs after a long lag phase of approximately 60-90 minutes. The maximum amount of aggregation at this arginine concentration is reached within the duration of the experiment. Activity measurements reveal that activity declines depending on the arginine concentration used. Although activity does not decline as fast as in the absence of additives, the presence of arginine does not completely protect the protein from reduction by DTT.

The loss of activity occurs faster in presence of lower concentrations of arginine. In presence of 1 M arginine only approximately 10% activity loss occurs over a period of 4 hours, while in presence of 0.25 and 0.5 M arginine approximately 70% and 50% loss of activity, respectively, occur over the 4 hour period.

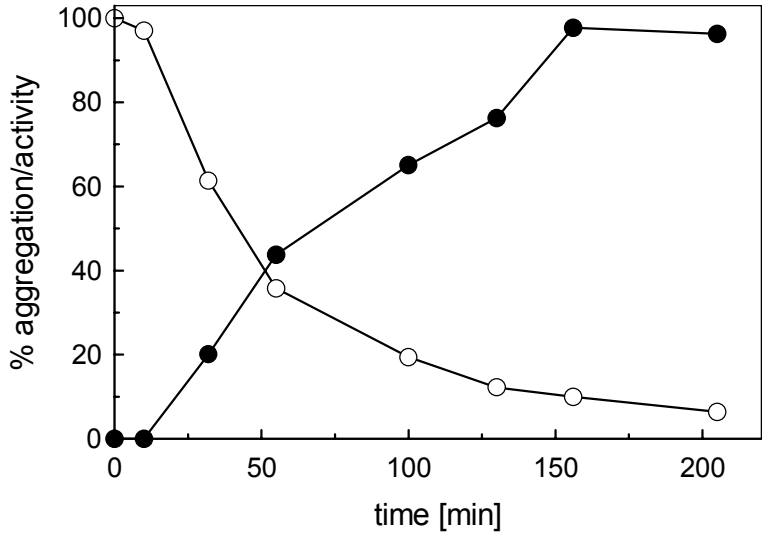


Fig. 3-29: Aggregation and activity kinetics in presence of 0.2 mg/mL lysozyme and 20 mM DTT in the absence of additives (○) activity and (●) aggregation.

This shows that arginine is capable of diminishing the effects caused by reduction with DTT. Higher arginine concentrations provide a better protection against reduction.

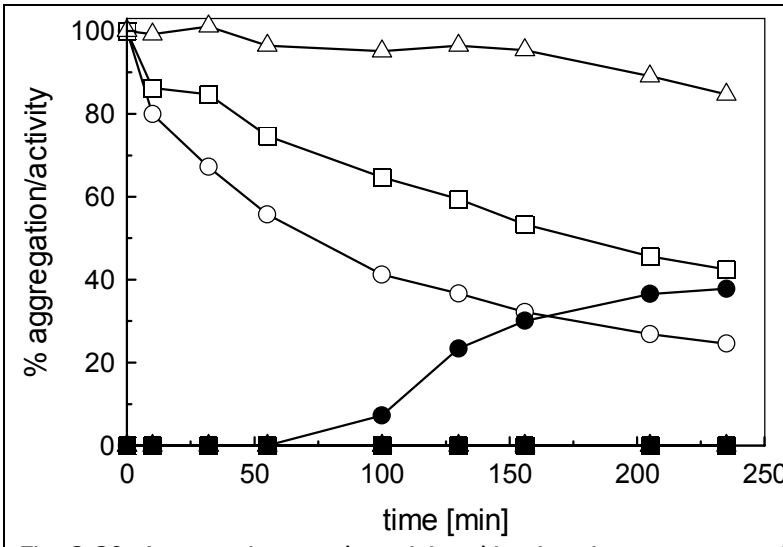


Fig. 3-30: Aggregation and activity kinetics in presence of 0.2 mg/mL lysozyme and 20 mM DTT and 0.25 M arginine: (○) activity, (●) aggregation; 0.5 M arginine: (□) activity, (■) aggregation; and 1 M arginine: (Δ) activity, (▲) aggregation.

Similarly to data in presence of arginine, aggregation was only detectable in presence of 0.25 M GdmCl (Fig. 3-31). In presence of 0.5 and 1 M GdmCl no

aggregation was detectable neither within the 4 hour period of the experiment nor during prolonged incubation periods.

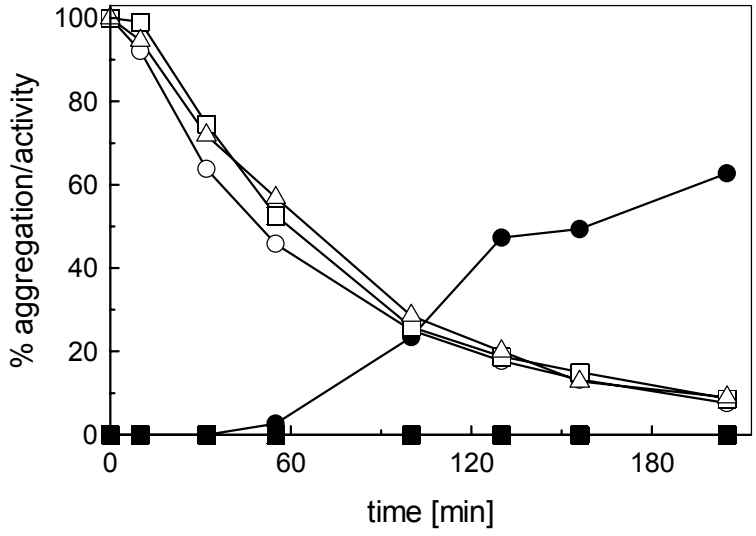


Fig. 3-31: Aggregation and activity kinetics in presence of 0.2 mg/mL lysozyme and 20 mM DTT and 0.25 M GdmCl: (○) activity, (●) aggregation; 0.5 M GdmCl: (□) activity, (■) aggregation; and 1 M GdmCl: (Δ) activity, (▲) aggregation.

This indicates that aggregation is completely suppressed by GdmCl concentrations above 0.5 M. Similarly to the previously shown data of arginine, activity kinetics were measured, too. The data show (Fig. 3-31) that the rate of loss of activity is not affected by the presence of GdmCl. Within the time period of the experiment, activity declines to approximately 10% of the original value. The presence of GdmCl does not alter activity kinetics, showing that GdmCl does not influence the reduction of disulfide bonds by DTT. This is in contrast to arginine, which influences the reduction rates of disulfide bonds and help to preserve some activity.

The activity kinetics of the data shown in Figs. 3-29, 3-30, and 3-31 were evaluated by calculating exponential regression functions. From these exponential regression functions, halftimes were calculated (time at which the sample has 50% activity left). Fig. 3-32 shows the calculated halftimes, which confirm that GdmCl does not affect the activity loss kinetics. The halftimes remain constant in presence of GdmCl. In presence of arginine, however, halftimes are markedly affected. The presence of arginine leads to longer halftime values, indicating that arginine protects the protein against loss of activity. The decrease of activity may not be linked directly to aggregation, because lysozyme may become reduced, leading to declining activity, but without aggregating. The data suggest that this occurs in samples with 0.5 M and 1 M

GdmCl, which do not aggregate but also have significantly decreased activity values.
In order to confirm that aggregation was caused by reduction of disulfide bonds, the number of free sulfhydryl groups was determined after 24 hours in the absence of additives and also in presence of 1 M arginine and 1 M GdmCl.

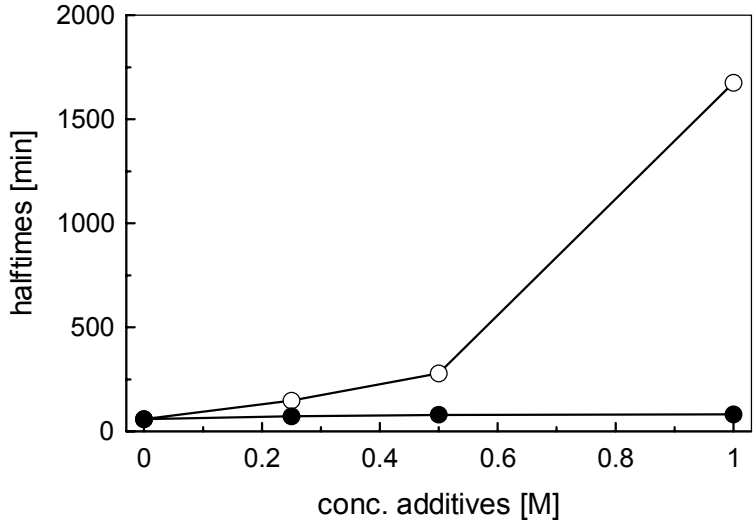


Fig. 3-32: Calculated halftimes of loss of activity of data shown in Figs. 3-30, 3-31, and 3-32. Halftimes are unaffected by (●) GdmCl, but strongly increase in presence of (○) arginine.

To determine the number of free sulfhydryl groups, IAA could not be used as it covalently reacts with free thiol groups. Instead the reduction process was arrested by lowering the pH to 5 using 1 N HCl. This prevents any thiol reactions, because at pH<5 thiolate ions become protonated and thus non-reactive. Samples were then dialyzed several times to remove excess DTT and additives (especially arginine, which interferes with DTNB measurements, resulting in formation of a yellow reaction product). In order to have a sufficiently high protein concentration for the Ellman assay samples were concentrated through a precipitation step with cold EtOH. Afterwards the precipitant was dissolved with a small volume of 8 M GdmCl. One undiluted aliquot was used for the Ellman assay, another was diluted and used to determine the protein concentration (by measuring the absorbance at 280 nm).

The data (Tab. 3-6) show that in presence of 1 M arginine only one disulfide bond becomes reduced. This coincides well with activity measurements, which showed that lysozyme loses some activity when reduced by DTT in presence of 1 M arginine. In presence of 1 M GdmCl or in the absence of additives lysozyme becomes fully reduced, resulting in very low activity values. This indicates that arginine prevents the protein from reduction, while in presence of GdmCl and in the absence of additives no protection against reduction exists.

To elucidate the consequences of reduced or non-reduced disulfide bonds, samples were incubated with 20 mM DTT and 1 M arginine, 1 M GdmCl, or just TE buffer, then dialyzed against 1 M arginine, 1 M GdmCl, or TE buffer, respectively, to remove excess DTT. Then far- and near-UV spectra were measured to analyze differences in the secondary and tertiary structure (Figs. 3-33, 3-34).

Tab. 3-6: Number of reduced disulfide bonds determined by Ellman assay. 0.2 mg/mL native lysozyme was incubated with either 1 M arginine or 1 M GdmCl in TE buffer prior to the addition of DTT to a final concentration of 20 mM. Samples were incubated for 24 hours, and then dialyzed three times against a 1 M solution of either arginine or GdmCl. The protein concentration was measured by absorbance at 280 nm. Native lysozyme is shown for comparison.

	% Activity	Number of reduced disulfide bonds
Arginine + DTT	40	1
GdmCl+ DTT	7	4
TE + DTT	7	3.6
TE	100	0

Far-UV CD spectra of the samples show (Fig. 3-33) that in presence of 1 M GdmCl during the reduction process lysozyme has a smaller signal amplitude in the wavelength range 240-215 nm. A signal loss in the wavelength range 230-210 nm is especially characteristic for losses of α-helix content of proteins. The spectra indicate that lysozyme loses some of its secondary structure under these conditions.

In presence of 1 M arginine the secondary structure appears to be very similar to that of native lysozyme, indicating that arginine facilitates the preservation of secondary structure under these conditions. CD deconvolution was not used here, because spectra could only be acquired down to 215 nm. This reduces the precision with which the deconvolution can be performed. Also, arginine has a very strong far-UV CD signal. The resulting spectra suffer from a lower signal-to-noise ratio than other spectra.

Near-UV CD spectra (Fig. 3-34) indicate that the tertiary structure of HEWL becomes completely disrupted in presence of 1 M GdmCl during the reduction by DTT. This spectrum is very similar to the one of denatured lysozyme in 8 M GdmCl (Fig. 3-1). In presence of arginine the shape of the near-UV CD spectrum is very similar to the one of native lysozyme. The only difference is that peaks appear which have a slightly higher amplitude compared to native lysozyme. Since the shape of the spectrum does not change, it indicates that the tertiary structure is not altered by the presence of 1 M arginine, which corresponds well

with the observation that activity does not decline significantly in presence of 1 M arginine.

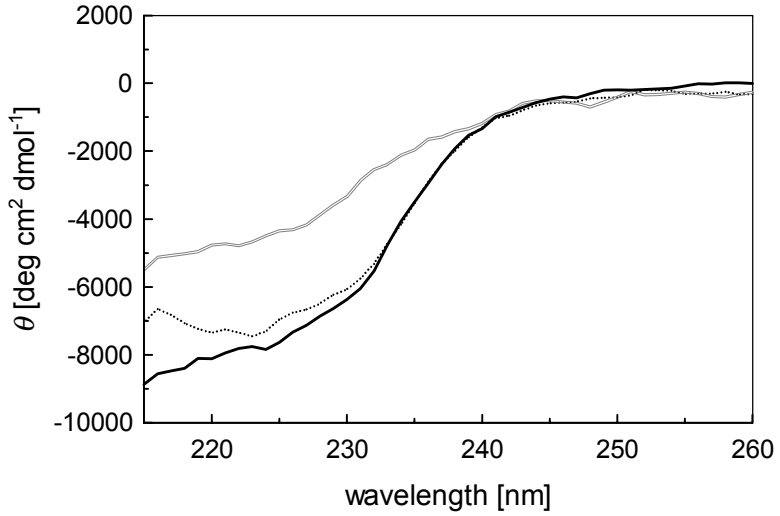


Fig. 3-33: Far-UV CD spectra of 0.2 mg/mL lysozyme incubated with 20 mM DTT for 24 hours, then excess DTT was removed by dialysis prior to measurement. (—) native lysozyme, (…) samples treated with 1 M arginine, and (=) samples treated with 1 M GdmCl.

ANS fluorescence kinetics of lysozyme, which was in the process of becoming reduced by DTT, were acquired after alkylating free sulfhydryl groups at a given time and subsequent addition of ANS. The actual fluorescence spectra were acquired immediately after addition of IAA and ANS to avoid the influence of artifacts. This strategy also enables simultaneous comparison of samples with different buffer conditions, which originate from the same protein and DTT solution. The experiments were performed with 0.2 mg/mL lysozyme. The data (Fig. 3-35) show that in the absence of arginine or GdmCl, ANS-binding intermediates become detectable after approximately 30 minutes. The maximum fluorescence value is reached after 2 hours.

In presence of 0.25 M arginine, ANS-binding intermediates become detectable at a similar time (approximately 30 minutes) than in the absence of additives. Since the fluorescence signal does not allow the quantitation of intermediates because different buffer substances affect the fluorescence signal, data were evaluated for the onset of increasing fluorescence signals. The relevance of these data is that intermediates at 0 M and 0.25 M arginine form after 30 min, while the onset of ANS-binding intermediates in presence of 0.25 M GdmCl occurs after 60 min. The kinetics do not reach their equilibrium fluorescence intensity within the duration of the experiment. The major difference with the other experiments is that the formation of ANS-binding intermediates starts significantly later. In presence of more than 0.5 M arginine or GdmCl no increase in the fluorescence signal over time was detected, indicating that no hydrophobic intermediates

become populated under these conditions. This is supported by the data in Figs. 3-30 and 3-31 showing that no aggregation was detectable under these conditions.

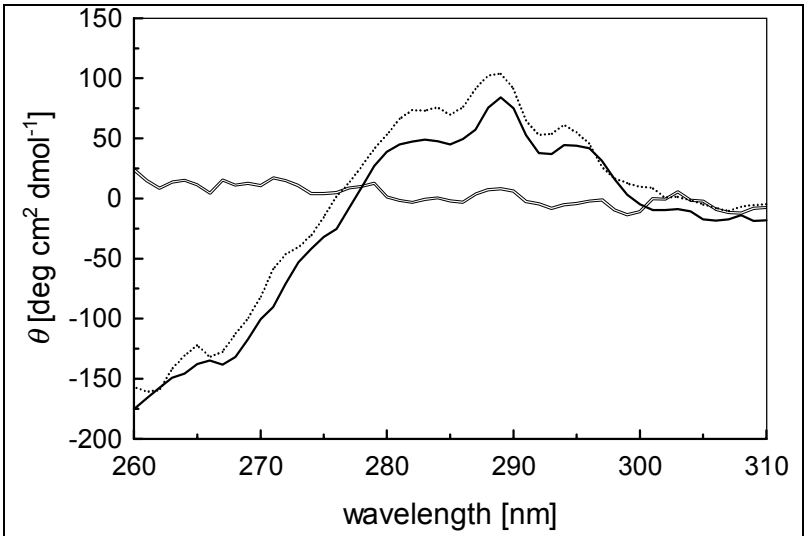


Fig. 3-34: Near-UV CD spectra of 0.2 mg/mL lysozyme incubated with 20 mM DTT for 24 hours, then excess DTT was removed by dialysis prior to measurement. (—) native lysozyme, (…) samples treated with 1 M arginine, and (=) samples treated with 1 M GdmCl.

The data suggest that following the population of ANS-binding intermediates aggregates start to form, because one driving force for aggregates to form is the burial of exposed hydrophobic groups from the buffer, which can be accomplished by association. Samples which do not show an increase in ANS-fluorescence over the course of the experiment also do not aggregate.

To gather additional information on the properties of ANS binding intermediates, a titration with ANS was performed. The results are shown in Fig. 3-36. While the relative fluorescence intensity is different for every sample, in the absence of arginine or GdmCl the signal increases to approximately 50 μM, above which it declines. The declining signal may be the result from ANS co-precipitating with subvisible aggregates. The ANS-binding intermediate containing samples are prone to aggregation and very likely start forming precipitates. When ANS is added to protein samples during a fluorescence temperature transition, the ANS fluorescence signal first rises and then declines, similar to the results observed here. This is attributed to protein forming aggregates, which subsequently coprecipitate with ANS. In presence of 1 M arginine or 1 M GdmCl the fluorescence signal increases beyond 50 μM ANS, which marked the turning point for the sample containing no arginine or GdmCl. This indicates that in presence of these additives no aggregates form and thus no ANS precipitation was observed. While this experiment cannot conclusively show that in presence of

arginine or GdmCl a larger amount of ANS binding intermediates exist, it indicates that the population of ANS binding intermediates is less prone to precipitation in presence of additives.

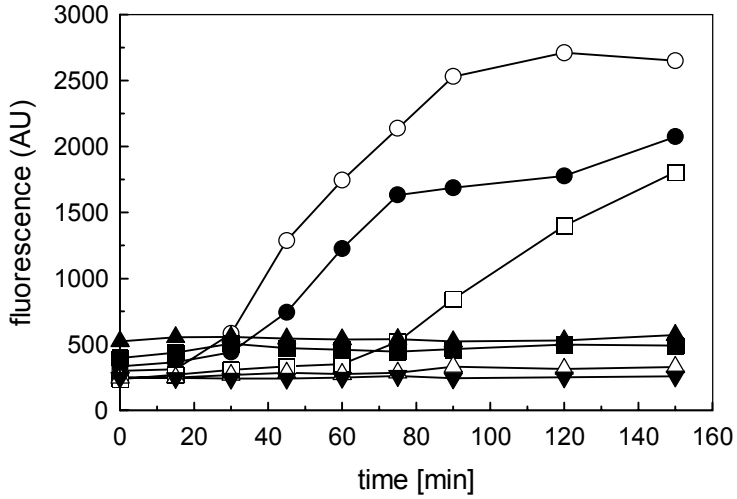


Fig. 3-35: ANS-binding kinetics of 0.2 mg/mL lysozyme and 20 mM DTT. After the indicated time, samples were quenched with iodoacetic acid and then ANS was added. (○) No additive, (●) 0.25 M arginine, (□) 0.25 M GdmCl, (■) 0.5 M arginine, (Δ) 0.5 M GdmCl, (▲) 1 M arginine, and (▼) 1 M GdmCl.

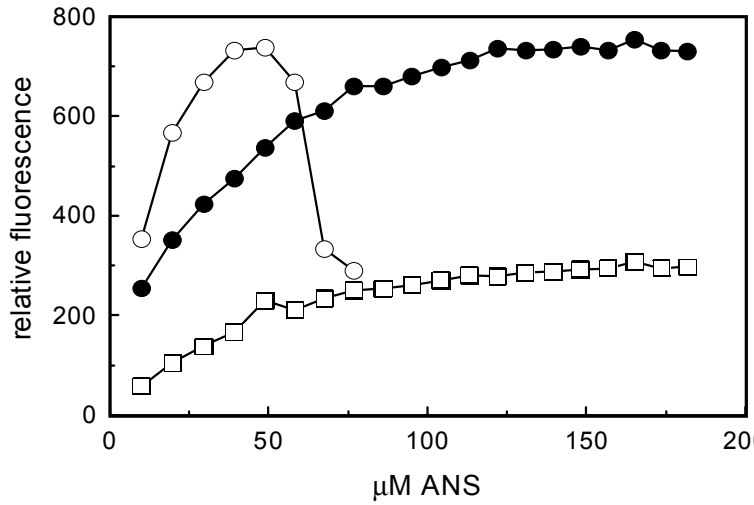


Fig. 3-36: ANS-binding titration of 0.2 mg/ml lysozyme reduced by 20 mM DTT. After 90 min samples were quenched with iodoacetic acid, then TE buffer, 2 M arginine, or 2 M GdmCl were added to a final concentration of 1 M. Titration was performed by addition of aliquots of a 20 mM ANS solution. Data were corrected for the increased volume. (○) No additive, (●) 1 M arginine, and (□) 1 M GdmCl.

Previously, aggregation and activity (Figs. 3-29, 3-30, 3-31), as well as ANS binding kinetics (Fig. 3-35) were shown. In order to identify mechanistic aspects

of the aggregation process, activity, aggregation (Figs. 3-29, 3-30, 3-31), and ANS binding kinetics (Fig. 3-35) were superimposed. Since concentrations of more than 0.5 M arginine or GdmCl do not result in aggregation or formation of ANS-binding intermediates, data from these experiments were not included in this analysis. Data from the above mentioned experiments were superimposed for absence of additives (Fig. 3-37), 0.25 M arginine (Fig. 3-38), and 0.25 M GdmCl (Fig. 3-39).

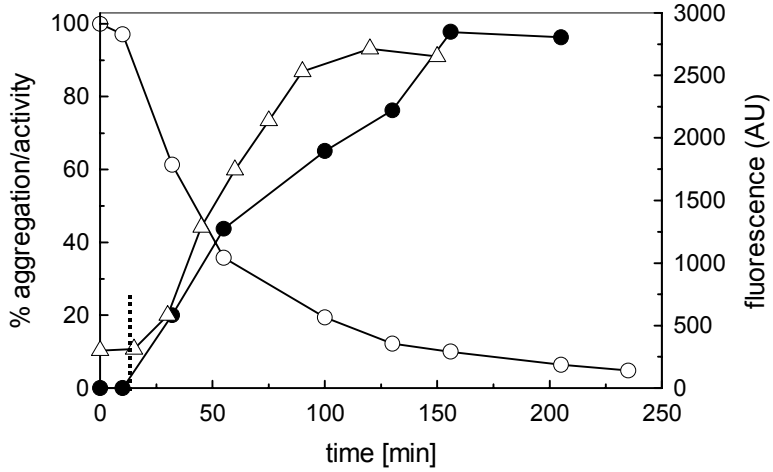


Fig. 3-37: Superimposed activity, aggregation, and ANS-binding kinetics in the absence of additives (○) activity, (●) aggregation, and (Δ) ANS-binding kinetics. The dashed line in the bottom left corner of the diagram shows the onset of aggregation and ANS binding.

When native lysozyme is incubated with 20 mM DTT and without additives (Fig. 3-37) it begins to lose activity shortly after initiation of the experiment. Also, ANS binding intermediates form shortly after the experiment has been initiated. ANS-binding intermediates and aggregates become populated at the same time (after 15 min), both kinetics coincide well, suggesting that hydrophobic intermediates become populated and aggregate immediately.

In presence of 0.25 M arginine and 20 mM DTT (Fig. 3-38) lysozyme rapidly loses activity, but approximately 30% activity remains after four hours, while in the absence of additives almost no activity remains after four hours.

While ANS binding intermediates in the absence of additives were observed after approximately 20 min, they are populated after 40 min in presence of 0.25 M arginine. Aggregated protein does not become detectable at the same time ANS-binding intermediates are, but after 50-100 min. This shows that in presence of 0.25 M arginine a significant time delay between the occurrence of ANS binding intermediates and aggregated protein exists.

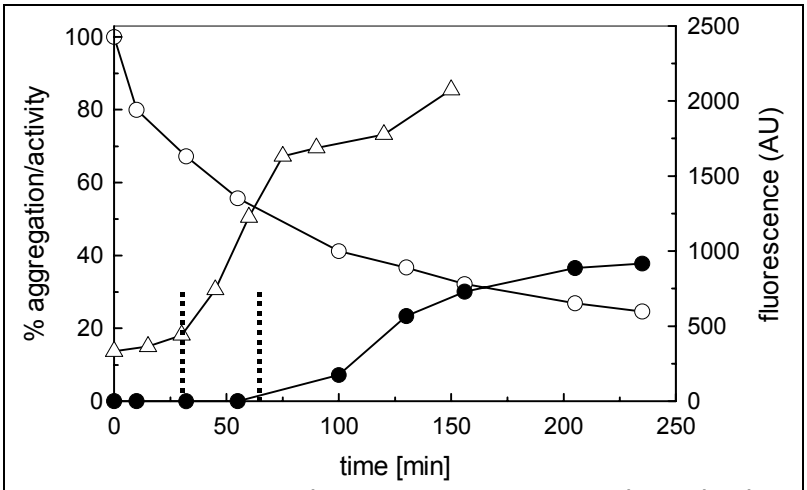


Fig. 3-38: Superimposed activity, aggregation, and ANS-binding kinetics in presence of 0.25 M arginine (○) activity, (●) aggregation, and (Δ) ANS-binding kinetics. The dashed lines in the left bottom corner show (**left**) the onset of aggregation and (**right**) ANS binding. Unlike the other conditions, a discrepancy was observed.

This is in contrast to the observations in Fig. 3-37 (no additives present) in which the formation of ANS binding intermediates always coincides with the formation of aggregated protein. The data suggest that, although ANS binding intermediates have formed, they do not aggregate immediately as seen in the absence of additives or 0.25 M GdmCl (Fig. 3-39). This means that arginine is able to keep ANS-binding HEWL intermediates in solution.

Activity and aggregation kinetics in presence of 0.25 M GdmCl and 20 mM DTT show (Fig. 3-39) that activity declines almost immediately upon initiation of unfolding. After four hours almost no activity remains (<10%). This is very similar to what is observed in the absence of additives. The onset of aggregation was observed after approximately 55 minutes. In the absence of additives, this time was approximately 20 minutes. This means that the protein starts to aggregate at a later time than in presence of 0.25 M GdmCl. The formation of ANS binding intermediates was detected after 55 minutes, showing that there is no difference in the formation of ANS binding intermediates and onset of aggregation.

The effect of GdmCl can be summarized as causing delayed population of ANS binding intermediates and formation of aggregates. The presence of >0.25 M arginine slowed down the decrease of activity, but GdmCl does not affect this process. The data suggest that GdmCl does not affect the reduction process of lysozyme, it facilitates the denaturation of intermediates and aggregates, thus influencing the formation of ANS binding intermediates and aggregated protein. Although 0.25 M GdmCl is not capable of fully dissolving grown aggregates

(Fig. 3-10), it efficiently delays the formation of new aggregates under the conditions described here.

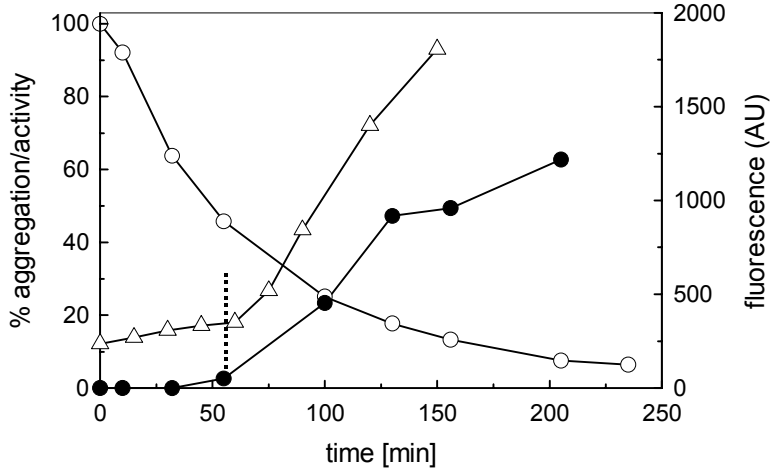


Fig. 3-39: Superimposed activity, aggregation, and ANS-binding kinetics in presence of 0.25 M GdmCl (○) activity, (●) aggregation, and (Δ) ANS-binding kinetics. The dashed line in the left bottom corner shows the onset of aggregation and ANS binding, which coincides very well.

Kinetic data of the unfolding of lysozyme in presence of arginine strongly suggest that arginine makes lysozyme less prone to reduction by DTT, because the decrease of activity occurs at a significantly lower rate. While ANS fluorescence increases similarly to the other conditions (indicative of unfolding intermediates), the intermediates are less prone to aggregation. This was seen through aggregation kinetics. In the absence of either arginine or GdmCl aggregation kinetics appear synchronized to ANS fluorescence kinetics. In presence of arginine a discrepancy in the onset of aggregation vs. ANS fluorescence was observed. Additionally a higher percentage of activity remains at the end of the process. Native lysozyme with arginine present does not show any significant differences in the near- and far-UV CD spectra (Figs. 3-1, 3-2) compared to those of native lysozyme, indicating that arginine does not significantly affect the tertiary and secondary structure of HEWL.

In addition to CD data, changes in the intrinsic fluorescence of native lysozyme were monitored in dependency of different arginine and GdmCl concentrations. Native lysozyme was incubated for 24 hours in the indicated arginine or GdmCl concentrations before the fluorescence spectra were recorded. An excitation wavelength of 290 nm was chosen, which only excites tryptophanes. The fluorescence emission signal was recorded from 310-400 nm. To evaluate the spectra, the fluorescence intensity at 340 nm was chosen, because spectra do not exhibit any changes in the peak maximum wavelength. The data are shown in Fig. 3-40. In presence of GdmCl, the fluorescence signal does not change within the concentration range shown here (0-2 M). The unfolding midpoint

concentration of lysozyme is 3.7 M GdmCl (data not shown) therefore at the concentrations below the transition midpoint used here no change in the signal was expected. The fluorescence signal in presence of arginine decreases with increasing concentrations of arginine, reaching the signal minimum at about 0.5 M arginine.

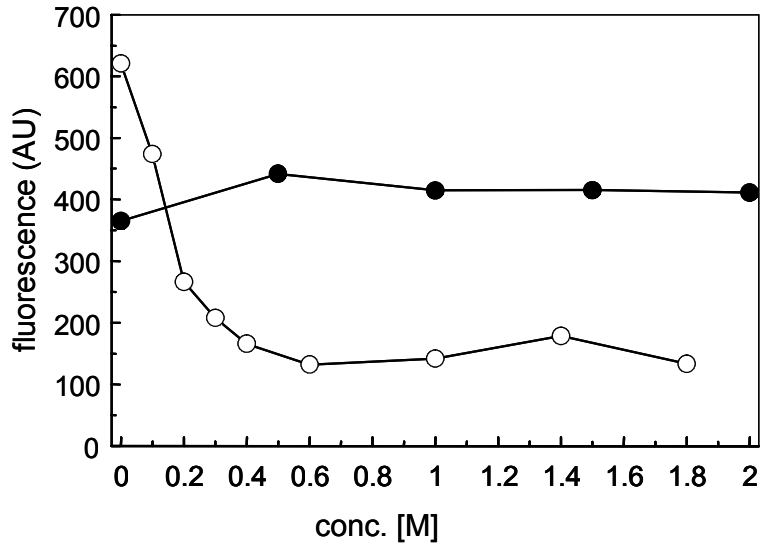


Fig. 3-40: Intrinsic fluorescence of 100 µg/ml native lysozyme in presence of 0-2 M (○) arginine and (●) GdmCl. Excitation wavelength was 290 nm, the intensity of the fluorescence signal at 340 nm is shown here.

Increasing fluorescence signals are usually observed when proteins unfold and when as consequence the distance between aromatic amino acids increases. The increased distance leads to less quenching of the signal.

Decreasing fluorescence would therefore indicate the opposite, that the protein refolds and the distance between aromatic amino acids becomes smaller. The data shown here suggest that in presence of arginine a higher percentage of the fluorescence signal becomes quenched which would be the result of higher compaction of the hydrophobic protein core. Another explanation of the decreased fluorescence emission might be that soluble lysozyme microaggregates become dissolved by increasing concentrations of arginine, thus leading to inner filter effect (oversaturation of fluorophore leads to declining fluorescence signals). A third reason for decreasing fluorescence emissions might be that arginine quenches the fluorescence signal by absorbing at this wavelength. To exclude this effect, an absorbance spectrum of 2 M arginine was recorded from 240-350 nm. This wavelength range covers the excitation as well as the emission wavelength of the previously shown data. The absorbance spectrum of a 2 M arginine solution is shown in Fig. 3-41. It absorbs below 240 nm, followed by a steady decrease of the absorbance to almost zero at

280 nm. These data support the hypothesis that the declining fluorescence in Fig. 3-40 is not caused by the presence of arginine, but rather by changes in the protein-solvent interactions.

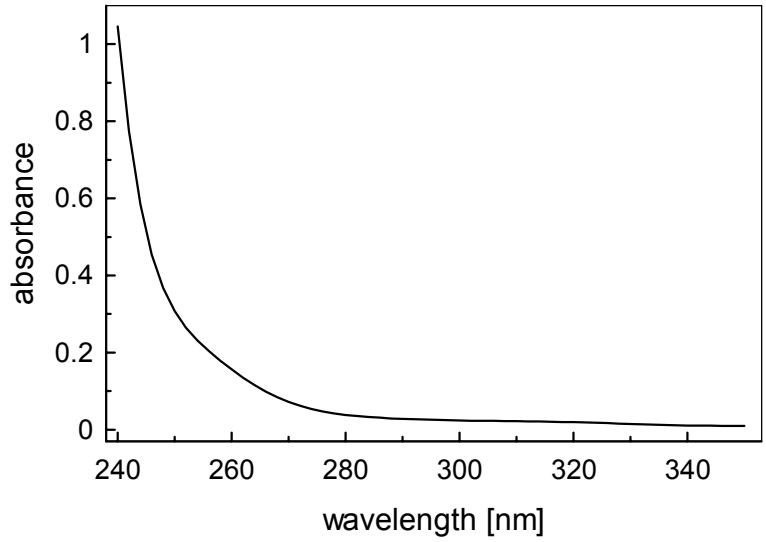


Fig. 3-41: Absorbance spectrum of 2 M arginine. At the excitation wavelength used in Fig. 3-40 (290 nm), arginine does not absorb.

In order to acquire additional experimental evidence on how lysozyme may be affected by the presence of additives, the hydrodynamic radius of lysozyme in presence of various concentrations of arginine and GdmCl was determined by dynamic light scattering (DLS).

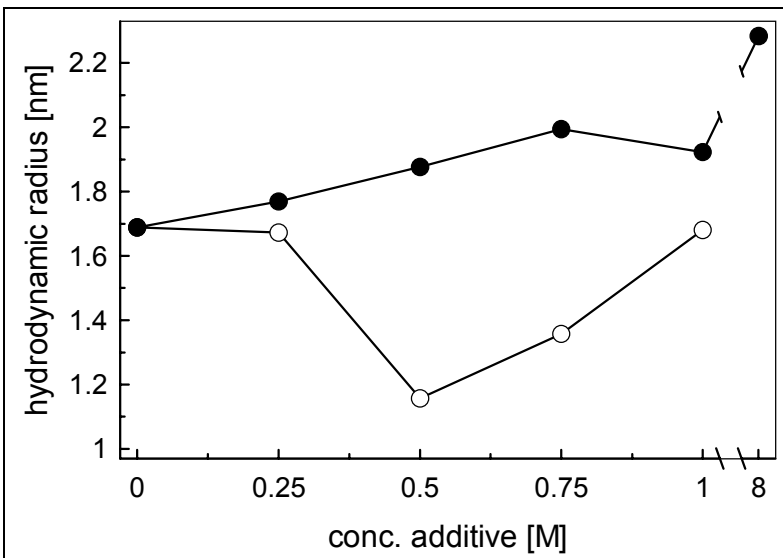


Fig. 3-42: Hydrodynamic radius of native lysozyme determined by dynamic light scattering in dependency of various concentrations of (○) arginine and (●) GdmCl.

Since viscosity and refractive index of the solvent strongly influences calculations of the hydrodynamic radius, solvent properties have to be carefully accounted for. As the data show (Fig. 3-42) a smaller than native hydrodynamic radius was observed in the concentration range 0.5-0.75 M arginine. The hydrodynamic radius remains nearly unaffected by 0.25 M and 1 M arginine. Within the concentration range 0-1 M GdmCl the hydrodynamic radius slightly increases compared to native lysozyme. This is consistent with other data, showing that GdmCl does *not* unfold or alter the activity of native lysozyme if lysozyme is exposed to these low concentrations of GdmCl. The data show that in presence of arginine the hydrodynamic radius of lysozyme becomes smaller. The smallest determined hydrodynamic radius was 1.2 nm (in presence of 0.5 M arginine), which is 30% smaller than that of native lysozyme. Since it is not very likely that lysozyme becomes 30% more compact, the data may either indicate errors in the determinations of viscosity and refractive index or an effect of arginine on the hydration layer(s) around lysozyme.

Determination of the solubility limit of lysozyme in various concentrations of arginine (Fig. 3-11) show that the solubility limit of native lysozyme is lower than average at 0.5 M arginine. To determine the solubility limit, lyophilized lysozyme was added to solutions of various additive concentrations and vortexed, followed by further addition of lyophilized protein until precipitation was observed. Using the known absorbance coefficient for lysozyme, the concentration of the protein solution was determined. Concentrations of more than 0.5 M arginine lead to an increased solubility limit, whereas the solubility limit at 0.5 M arginine is much lower than in the absence of additives (Fig. 3-11).

Although the presence of 0.5 M GdmCl did not lead to a decreased hydrodynamic radius of lysozyme, the solubility limit is significantly lower, even lower than in presence of 0.5 M arginine. If the above assumptions are true and arginine really affects the hydration patterns of lysozyme, it may lead to reduced solubility of lysozyme. A definitive answer to the effect of arginine on lysozyme conformation can be made by small angle X-ray scattering (SAXS), which allows the detection of actual particle dimensions. The fact that lysozyme is less prone to reduction in presence of arginine supports the idea that arginine may have an influence on hydration patterns around the protein and influences its solubility.

To detect reduced disulfide bonds, MALDI-TOF was utilized. To 0.2 mg/mL lysozyme 20 mM DTT was added to initiate aggregation. After 0, 20, 50, and 180 min an aliquot of the solution was quenched with IAA. To remove excess reagents and buffer substances from the samples and to concentrate them, protein was precipitated with cold ethanol.

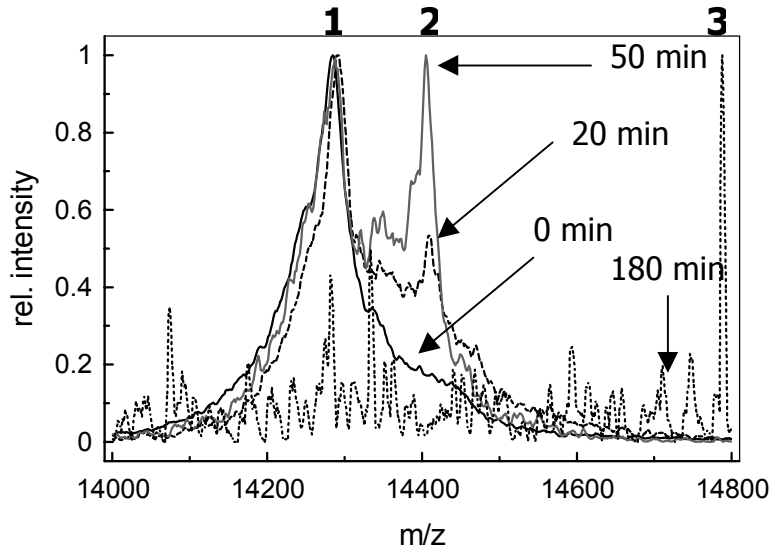


Fig. 3-43: MALDI-TOF spectra of reductive unfolded lysozyme in the absence of additives. Aggregation of 0.2 mg/mL lysozyme was initiated with 20 mM DTT. At the indicated times, an aliquot of the solution was quenched with 50 mM IAA. Samples were purified and concentrated through cold ethanol precipitation. Data were normalized to account for different protein concentrations. The amount of soluble protein after 180 min is very low, thus a high level of noise is visible in the spectrum. **1** completely oxidized lysozyme, **2** lysozyme with one disulfide bond reduced, **3** lysozyme with four disulfide bonds reduced.

After evaporation of the ethanol samples were dissolved in water and analyzed by MALDI-TOF. Upon initiation of aggregation the recoverable amount of protein strongly decreases, thus spectra were normalized in Fig. 3-43.

The data show (Fig. 3-43) that within the first 50 minutes of the process a second peak emerges, of which the mass difference corresponds to one reduced disulfide bond. After 50 minutes this peak reaches the same relative intensity as nonreduced lysozyme. After 180 minutes the largest peak was detected at 14,750 Da. This peak corresponds to four (fully) reduced disulfide bonds. These data together with the data in Fig. 3-37 indicate that upon reduction of two or more disulfide bonds hydrophobic intermediates are formed resulting in immediate aggregation.

3.4. Activity and aggregation in presence of arginine analogue compounds

While in the previous chapters the effects of arginine and GdmCl on refolding and unfolding of lysozyme were characterized, the structure-function relationship of arginine and GdmCl was not addressed. Thus in this chapter the efficiency to

promote refolding and/or decrease aggregation of arginine-analogue substances will be compared to arginine to elucidate which functional groups contribute to the effects of arginine. While this approach neglects the influence of the substances on partially folded or unfolded lysozyme species, it allows for a faster, more superficial screening of compounds. Ultimately the line between "good" and "bad" additives will be drawn on how they perform on activity recovery and aggregation.

Arginine is generally considered the most polar amino acid, because of its partition coefficient determined between an octanol and water phase. Arginine is almost exclusively distributed in the aqueous phase, thus considered highly polar. While partition coefficients calculated this way for non-amino acids accurately describe hydrophobicity they do not deliver satisfying results for amino acids in peptides or proteins, because the carboxyl and amino groups are involved in the formation of peptide bonds and thus affect polarity differently from free amino acids. In arginine, the π-electrons of the guanidinium group are isolated, because the three methylene groups, which are in between the C_α-atom, do not allow mesomeric stabilization of the guanidinium group. This means that the positive charge of the guanidinium group remains isolated in the side chain of arginine.

Because of the shortcomings of partition coefficients, a statistical approach can be pursued as alternative. The hydrophobicity of residues is derived from amphipatic helices. So-called helical wheels are created by plotting the amino acid residues of an α-helix in a circular manner aligning each residue in a 100° angle from the neighboring residue. By aligning residues this way usually results in the alignment of polar residues on one side and non-polar residues on the other. A helix showing these characteristics is called amphipatic.

Reviewing several statistical hydrophobicity scales calculated from amphipatic helices, Cornette *et al.* (1987) calculated an optimized hydrophobicity scale. Their optimized scale shows a high correlation to previously published scales. To their surprise arginine was found in some instances on the hydrophobic side of amphipatic helices. This statistical approach does not take into consideration actual interactions between arginine and other residues, meaning that arginine may be located in hydrophobic areas of an amphipatic helix, but it may not be involved in van der Waals interactions. Arginine may form salt bridges with negatively charged amino acids.
In the following section, the effect of several components on activity recovery and aggregation will be evaluated. Depending on the properties of the substances investigated, this collection of data may or may not be complete for the respective substance.

First, the effect of D-arginine (Fig. 3-44) on the recovery of activity was compared to the effects of L-arginine. Since proteins are composed of L-amino acids and L-arginine has been used for the experiments, D-arginine was used to elucidate if stereospecific interactions play a role in the efficiency of L-arginine. If D-arginine does not show similar effects to L-arginine, it would indicate that stereospecific interactions may play a major role in the effects of L-arginine. If on the other hand its efficiency is similar to L-arginine, no stereospecific interactions between lysozyme and L-arginine are responsible for the effects.

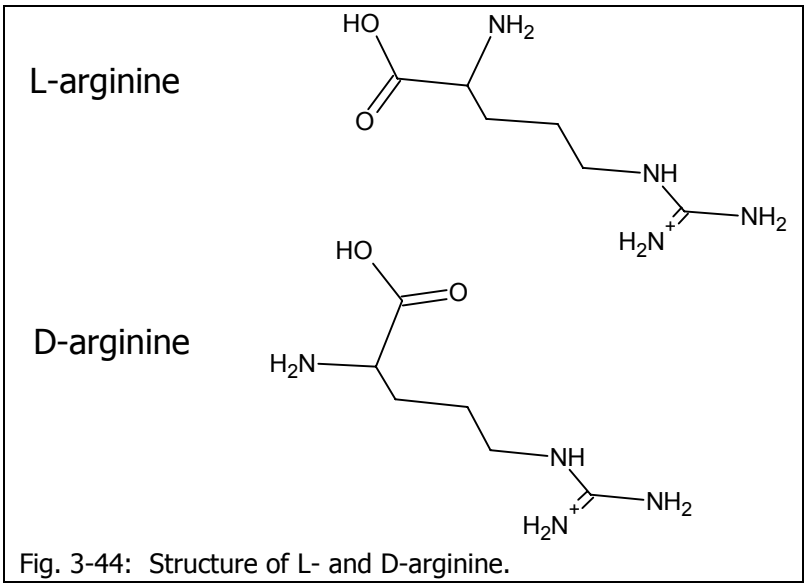


Fig. 3-44: Structure of L- and D-arginine.

The data of activity recovery in presence of D-arginine (Fig. 3-45) indicate that activity recovery in presence of D-arginine is virtually indistinguishable from L-arginine within the experimental error. Between 0 and 0.5 M the percentage of recovered activity rises to the maximum of approximately 100% activity, thus the activity recovery profile of both stereoisomers is very similar. The coinciding data of both stereoisomers indicate that the effect of L-arginine on protein refolding is not stereospecific, and the D-stereoisomer has the same effect.

L-arginine has a very strong far-UV CD signal, which makes the evaluation of spectra more challenging, because great care needs to be taken for proper subtraction of baseline reference spectra. In order to simplify the measurement of far-UV CD spectra in presence of arginine, it was attempted to prepare a racemic mixture of L- and D-arginine. For this purpose a 1 M stock solution of each stereoisomer was prepared. When equal volumes of both stereoisomers were mixed together, heavy precipitation was observed, which did not redissolve upon addition of water. This indicates that these stereoisomers may react with each other, resulting in a reaction product with low solubility in water. Because of this, it was not possible to prepare a DL-arginine solution, and CD measurements were performed in 1 M L-arginine.

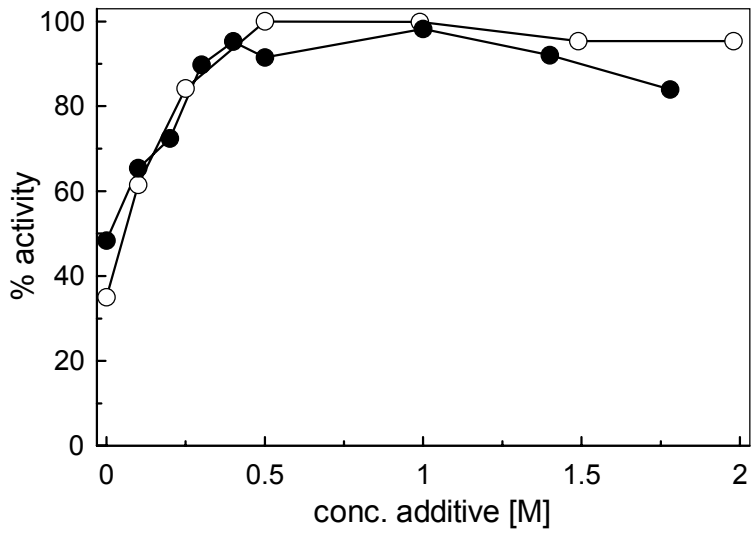


Fig. 3-45: Activity recovery in presence of (●) L-arginine and (○) D-arginine.

The arginine side chain carries a strongly positive charge mainly from the guanidinium group. The pK_a value of the guanidinium group is 12.5, making it positively charged at pH 8, at which all the refolding and unfolding experiments took place. At pH 8 the C_α carboxyl group is deprotonated, while the C_α amino group is protonated. Overall, arginine has one positive net charge at pH 8.

Since the effects of arginine may be based on attraction or repulsion of functional groups, the following experiment was designed to address whether charges of folding additive and protein play a role in inhibiting aggregation. Lysozyme has a *pI* (isoelectric point) of 9.3 or 11.1, depending on whether disulfide bonds are reduced or oxidized. At pH 8, HEWL is positively charged. So far, only the effects of a positively charged additive (arginine) on a positively charged protein (lysozyme) were investigated. To investigate the effect of opposite charges, combinations of positively and negatively charged additives and proteins were used. As at pH 8 negatively charged protein bovine serum albumin (BSA) was used, which has a *pI* of 5.6, and therefore is negatively charged at pH 8. Since BSA has no enzymatic activity, aggregation was determined. As negatively charged additive glutamic acid was chosen (Fig. 3-46). The side chain of glutamic acid is not as long as the one of arginine, but because the experiment focused on charges, this shortcoming was tolerated.

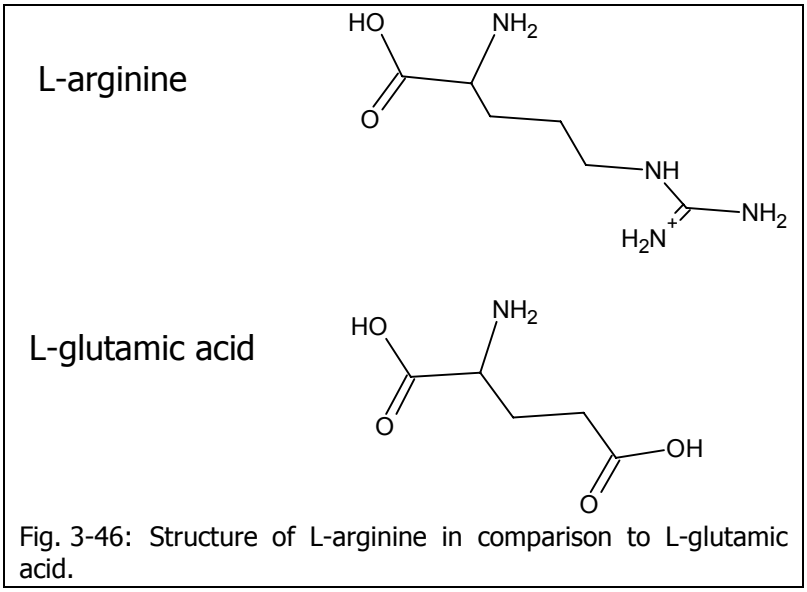


Fig. 3-46: Structure of L-arginine in comparison to L-glutamic acid.

At pH 8, the C_α carboxyl group of glutamic acid is deprotonated and so is the carboxyl group of the side chain, which results in glutamic acid having a single negative charge at pH 8. Aggregation data for all combinations of additive and protein were acquired. Data over the whole concentration range 0 to 2 M were acquired. Both proteins were denatured and reduced with 8 M GdmCl and 200 mM DTT.

The results show (Fig. 3-47) that glutamic acid does not suppress aggregation for either one of the two proteins, regardless of their charge at pH 8. Glutamic acid promotes aggregation, because the percentage of aggregation increases with increasing concentrations of glutamic acid is. In the absence of both additives, lysozyme aggregates approximately 60%, while BSA aggregates approximately 40%. In presence of arginine the percentage of aggregation steadily declines, in presence of ≥0.5 M arginine aggregation declines to nearly 0%. In presence of glutamic acid, however, aggregation increases to 80-100% for lysozyme in presence of ≥0.25 M glutamic acid, while for BSA the percentage of aggregation increases more continually until it reaches 100% at ≥1.5 M glutamic acid. Since the effect of glutamic acid does not depend on the isoelectric point of the protein, the data suggest that the observed effects of arginine and GdmCl may not be based on attractive or repulsive interactions. Furthermore, the observed effects of glutamic acid and arginine do not depend on the isoelectric point of the proteins, because the same effect is observed in both proteins. On the other hand the data show that the effect of arginine is independent of the protein's isoelectric point, making it a widely usable folding additive for proteins, regardless of their overall charge. Shiraki and coworkers (Shiraki *et al.*, 2002) demonstrated that arginine was the best suited amino acid to prevent heat-induced as well as refolding-induced aggregation. For the latter they used carboxylated denatured lysozyme. They also studied the effect of

arginine on proteins with different pI (using heat-induced aggregation), and arginine was the additive with the highest degree of suppression of aggregation.

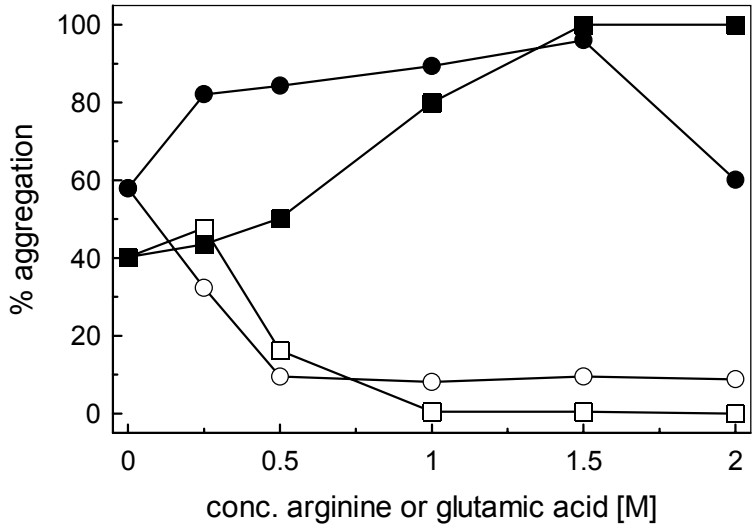


Fig. 3-47: Aggregation in presence of 0.2 mg/mL lysozyme and BSA and various concentrations of glutamic acid and arginine. (○) HEWL — arginine; (●) HEWL — glutamic acid; (□) BSA — arginine, and (■) BSA — glutamic acid.

In L-arginine the guanidinium group is separated from the C_α-functional groups by three methylene groups. These methylene groups do not support mesomeric stabilization of charges. Thus the number of methylene groups between the guanidinium group and the Cα-groups is not expected to have an effect, because the guanidinium group is not expected to interact with other functional groups. To investigate this matter, the effect of an additive with an additional methylene group was investigated. Instead of three methylene groups between the guanidinium group and the α-carbon atom, L-homoarginine has four (Fig. 3-48). This may lead to a slightly different orientation of the functional groups in relationship to each other.

The results show (Fig. 3-49) that L-homoarginine indeed promotes recovery of activity, but only below 1 M. While the influence on aggregation was not quantified, the appearance was recorded. Over the whole concentration range 0-2 M samples were not turbid. This indicates that above 1 M when activity recovery was low aggregation is suppressed by this additive. The effect of homoarginine on native lysozyme was determined to check any influences on the activity of native protein. Native, never unfolded lysozyme was dissolved in a solution of 2 M homoarginine in TE buffer. The activity of lysozyme in relationship to protein dissolved in TE buffer was determined. No significant difference was determined, indicating that homoarginine above 1 M must negatively affect the refolding process.

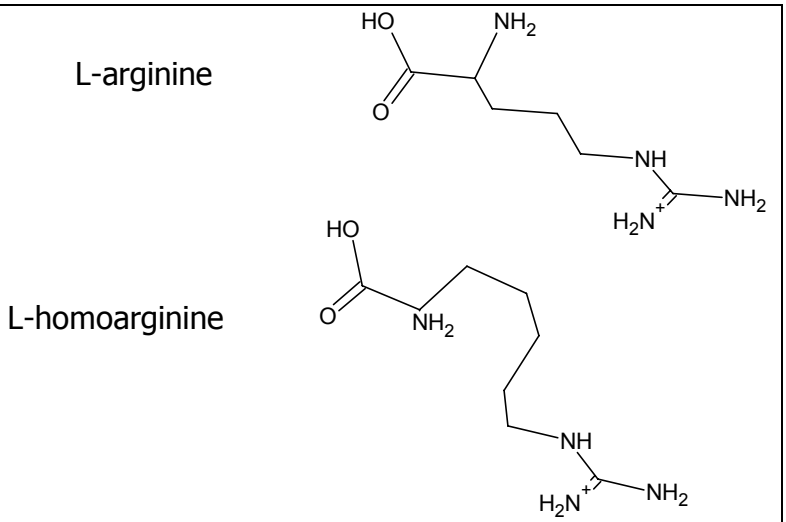


Fig. 3-48: Structure of L-arginine and L-homoarginine.

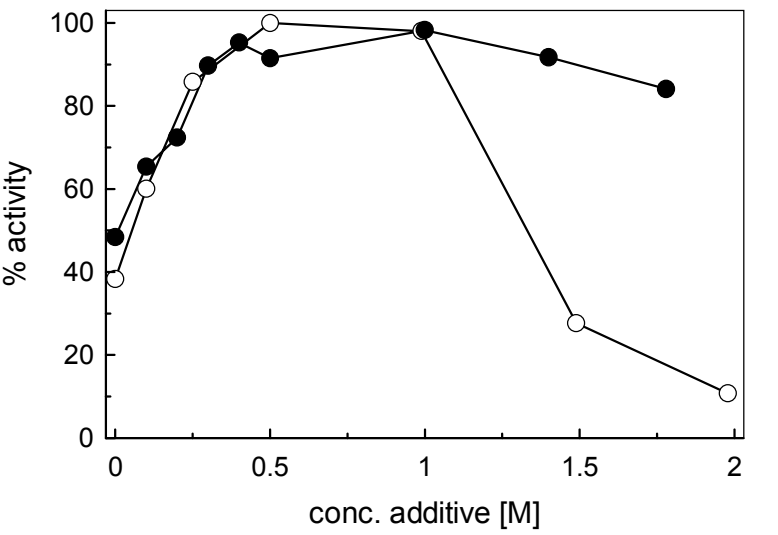


Fig. 3-49: Activity recovery of 0.2 mg/mL lysozyme in presence of (○) L-homoarginine and (●) L-arginine.

The difference to arginine is that activity recovery is lower above 1 M. Reasons for this may be that homoarginine may thermodynamically stabilizes folding intermediates, thus intermediates are locked and cannot fold to the native state. Another reason may be that homoarginine may denature HEWL above 1 M, and thus aggregation is suppressed while at the same time aggregation is completely inhibited.

In order to determine the responsibility the carboxyl and amino group play in arginine for facilitating refolding, the effect of an arginine analogue chemical without carboxyl and amino groups on the recovery of activity and aggregation

was determined. Poly-L-arginine was chosen for this purpose, which was obtained as a polypeptide with 30-85 arginine residues. The structure is shown in Fig. 3-50.

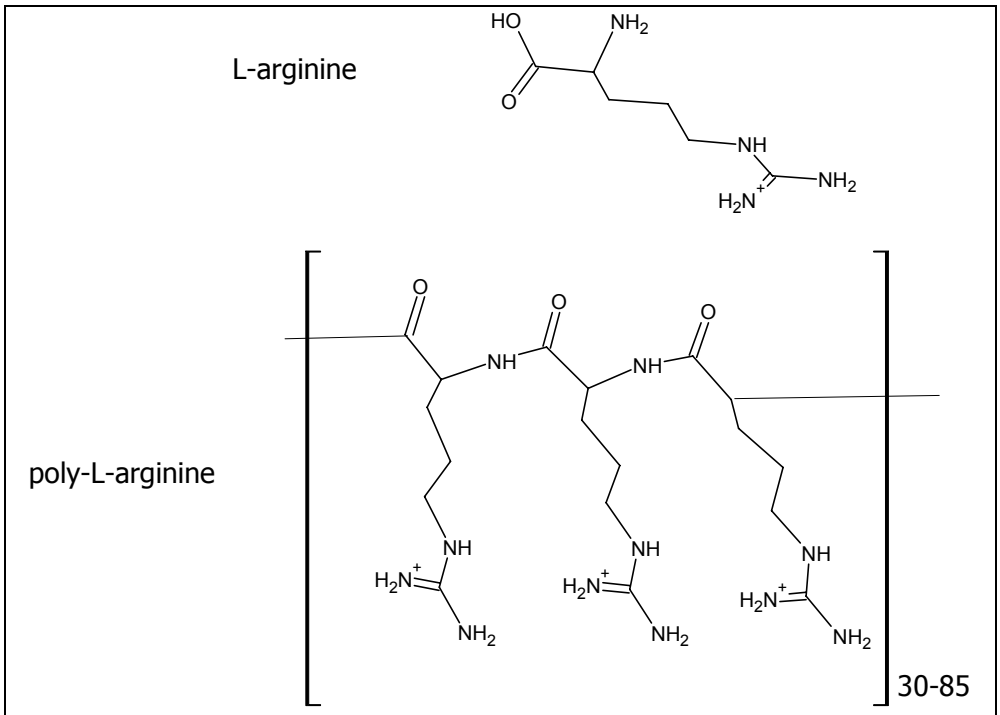


Fig. 3-50: Structure of L-arginine and poly-L-arginine. The molecular range of poly-L-arginine was from 5 to 15 kDa.

The solubility of poly-L-arginine in TE buffer was determined to be at least 10 mg/mL. A stock solution of 10 mg/mL (approximately 2 mM) poly-L-arginine was used for the experiments.

Since polypeptides may form secondary structure, which makes them more likely to interact with proteins, the far-UV CD spectrum of poly-L-arginine was acquired. The presence of a polypeptide with existing secondary structure may inhibit protein folding by interacting with folding intermediates. Because secondary structure areas in proteins become stabilized through other secondary structure areas in the neighborhood (supersecondary structure), a polypeptide may interact with folding intermediates. If the interactions are not too strong, the folding process will continue, but will become slowed down by the presence of the polypeptide. If the interactions are very strong, the folding process becomes arrested because the interactions with the polypeptide inhibit formation of native interactions within the intermediate. The far-UV CD spectrum of poly-L-arginine indicates (Fig. 3-51) that it does not form secondary structure in aqueous solution. This was concluded in comparison to far-UV CD spectra of "pure" secondary structure components (Schmid, 1996). (CD deconvolution of this spectrum was not performed, because CD deconvolution programs work by

correlation of spectra of known secondary structure with the spectrum to be analyzed. Currently available programs do not contain random coil polypeptide spectra in their reference data sets. To apply these algorithms to a random coil polypeptide spectra such as poly-L-arginine does not yield meaningful data.)

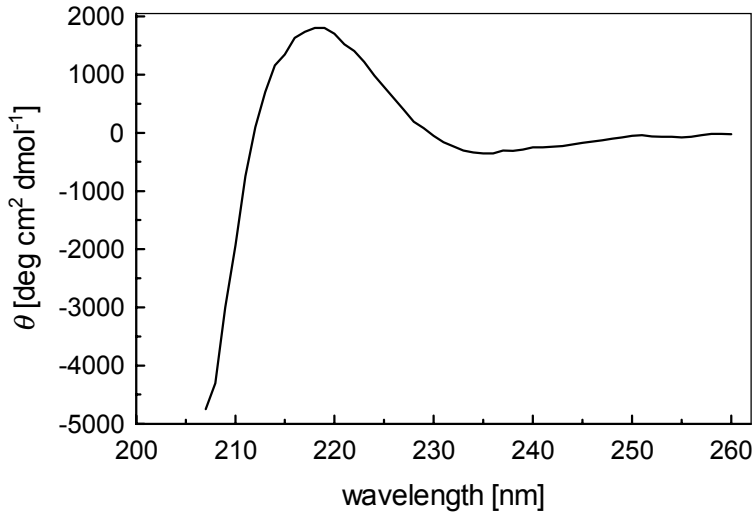


Fig. 3-51: Far-UV CD spectrum of poly-L-arginine. The spectrum shows the characteristics of a random coil, which indicates that poly-L-arginine does not form any secondary structure.

The effect of poly-L-arginine on activity and aggregation during lysozyme refolding is shown in Fig. 3-52. The conditions were 0.2 mg/mL lysozyme, the oxido shuffling system was 2 mM DTT and 5 mM GSSG. Poly-L-arginine was used in concentrations of 0-100 mM arginine equivalent (0-10 mg/mL). Although the molecular weight of poly-L-arginine is very heterogeneous, the average concentration is very low compared to the other additives analyzed here. To compare these data to arginine, the concentration equivalent to arginine was calculated by considering that the average poly-L-arginine molecule consists of 67.5 residues. The data show (Fig. 3-52) that in presence of poly-L-arginine no activity was recovered. Small concentrations of poly-L-arginine appear to completely inhibit the recovery of activity. Because no difference between different concentrations of poly-L-arginine was detected, these results indicate that very small concentrations of poly-L-arginine are sufficient to completely inhibit recovery of activity. Aggregation is not significantly affected by the presence of poly-L-arginine. Between 0 and 25 mM arginine equivalent aggregation is slightly enhanced, above which it is slightly decreased. Because of these unexpected results, the effect of poly-L-arginine on native lysozyme was evaluated. 0.2 mg/mL lysozyme was dissolved in a 10 mg/mL solution of poly-L-arginine in TE buffer. Activity was totally inhibited in presence of poly-L-arginine, indicating that poly-L-arginine very likely binds to the active center of lysozyme, leading to total inhibition. Dilution of the solution to 1 mg/mL did not lead to

recovery of activity, indicating that the binding is very likely of stochiometric nature. Aggregation slightly decreases at higher poly-L-arginine concentrations. This may be caused by poly-L-arginine binding to folding intermediates, thus reducing aggregation. The effects observed by poly-L-arginine on HEWL refolding were strongly affected by the polypeptide nature of poly-L-arginine. Better molecules suited to elucidate the effect of functional groups would have been short arginine peptides (*i.e.* di- or tri-arginine). The obtained results are too much influenced by the size of the peptide used in the experiments. Short arginine peptides were not commercially available and could not be evaluated.

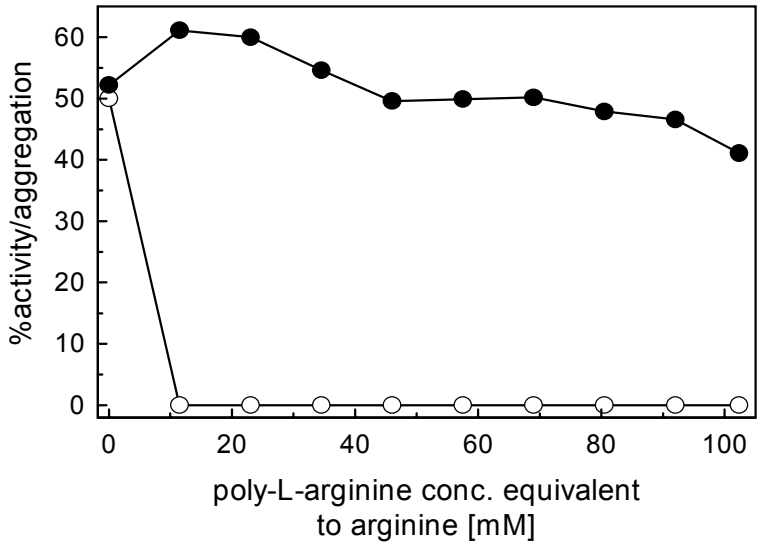


Fig. 3-52: (○) Activity and (●) aggregation of 0.2 mg/mL lysozyme with 2 mM DTT and 5 mM GSSG in presence of poly-L-arginine. Poly-L-arginine concentrations were calculated in relationship to L-arginine.

Agmatine chloride is decarboxylated arginine (Fig. 3-53). This molecule provides a possibility to elucidate the effect of functional groups in arginine on HEWL refolding. Agmatine was not available as chloride salt, but sulfate. Since a preliminary experiment indicated that the presence of sulfate ions negatively affects the recovery of HEWL activity, the anion in agmatine sulfate needed to be exchanged by chloride. Counterions may significantly affect the result of folding processes, thus it was very important to use agmatine chloride instead of sulfate to be able to compare the results to the other additives.

The ion exchange reaction was performed by addition of calcium chloride to agmatine sulfate, resulting in agmatine chloride and insoluble calcium sulfate (Agm: agmatine):

$$(Agm)_2SO_4 + CaCl_2 \rightarrow 2\ AgmCl + Ca_2SO_4\downarrow$$

Upon addition of calcium chloride the formation of calcium sulfate was observed by formation of heavy precipitation. Calcium sulfate was removed by centrifugation. The data show (Fig. 3-54) that agmatine chloride efficiently suppresses aggregation. In comparison to arginine, the effects of agmatine are nearly identical. Aggregation is completely suppressed above 0.5 M agmatine chloride, very similarly to arginine. The recovery of activity is not quite as efficient as in presence of arginine.

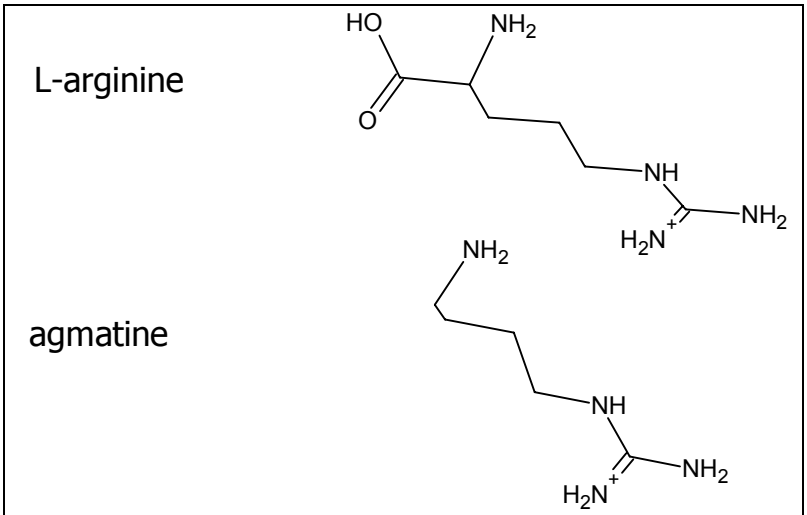


Fig. 3-53: Structure of L-arginine in comparison to agmatine chloride (decarboxylated arginine). Agmatine chloride has no chiral atoms.

Activity increases to 80% in presence of 0.5 M and 0.7 M agmatine chloride, above which the percentage of recovered activity declines. Between 0.7 M and 1.8 M agmatine chloride the percentage of recovered activity steadily declines, resulting in slightly lower activity in presence of 1.8 M agmatine chloride than at 0 M. Overall agmatine chloride decreases aggregation as efficiently as arginine and facilitates refolding, but above 0.7 M it decreases the refolding yields. This behavior is similar to homoarginine. Because the effects of agmatine chloride on lysozyme refolding may be dependent on the effect it has on native lyszoyme, the activity of native lysozyme in presence of various concentrations of agmatine chloride are shown in Fig. 3-55.

While activity of native lysozyme is affected between 0-0.5 M agmatine chloride, the refolding data in Fig. 3-54 indicate that recovery of activity is slightly facilitated by agmatine chloride. Above approximately 1 M agmatine chloride recovery of activity declines which is consistent with Fig. 3-55, which indicates that activity is significantly reduced at these concentrations of agmatine chloride.

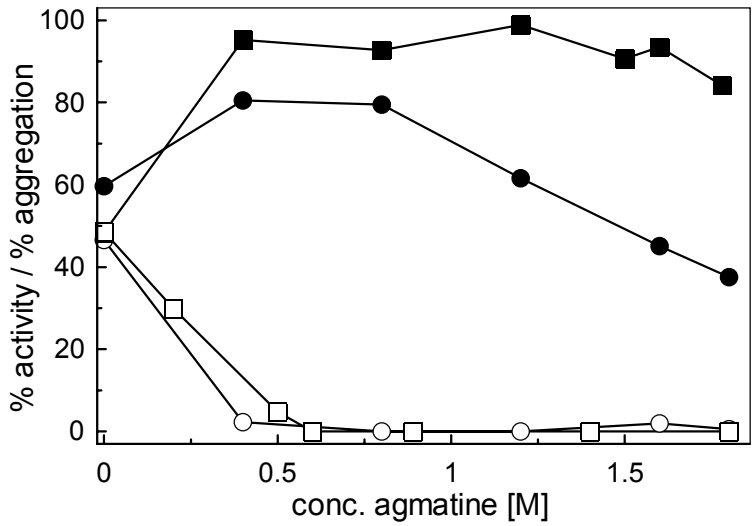


Fig. 3-54: (●) Activity and (○) aggregation of 0.2 mg/mL HEWL refolding in presence of agmatine chloride. Agmatine is decarboxylated arginine. L-arginine (■) activity and (□) aggregation are shown for comparison.

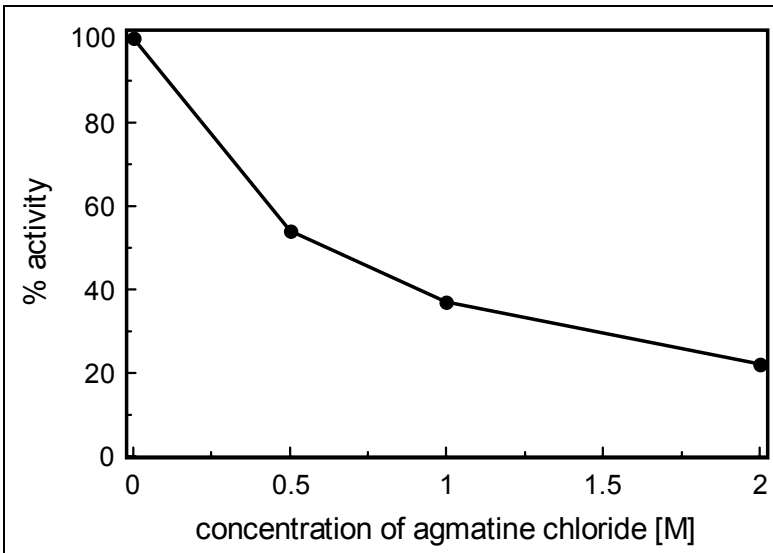


Fig. 3-55: Effect of agmatine chloride on native lysozyme.

In chapter 3.3. it was shown that reductive aggregation of lysozyme can be initiated by addition of DTT. Arginine and GdmCl decrease aggregation during this process. Although the presence of both additives leads to decreased amounts of aggregated protein, in presence of arginine some of the activity is preserved, while in presence of GdmCl lysozyme becomes completely inactive, which indicates that arginine prevents reduction of disulfide bonds by DTT while GdmCl does not affect the reduction process. The difference of both additives allows to distinguish "arginine-like" additives from "GdmCl-like", depending on whether activity is preserved or not in this process. The effect of agmatine

chloride was compared to arginine and GdmCl this way. Samples with 0.2 mg/mL lysozyme, 20 mM DTT, and 1 M agmatine chloride, arginine, or GdmCl were incubated overnight at room temperature. After 24 hours the percentage of aggregation and the remainder of activity was determined.

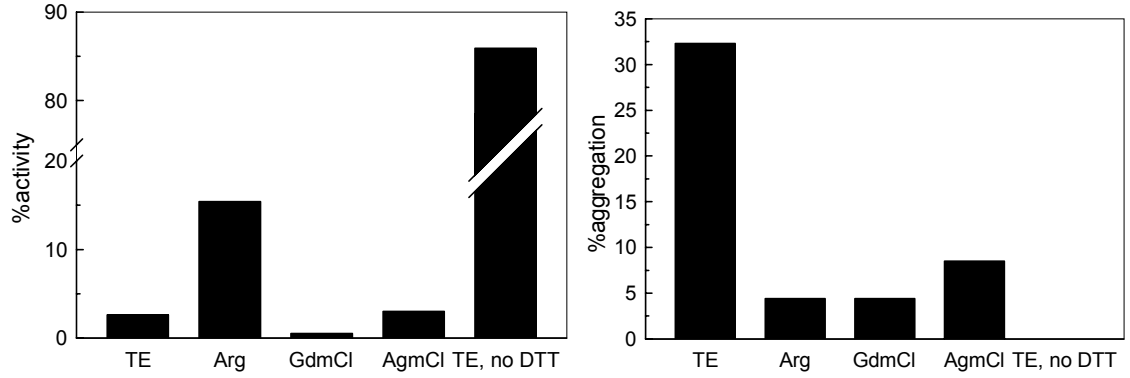


Fig. 3-56: Effect of various additives on the suppression of aggregation. 0.2 mg/mL lysozyme was incubated with 20 mM DTT and the following conditions for 24 hours: TE buffer (**TE**), 1 M arginine (**Arg**), 1 M GdmCl (**GdmCl**), 1 M agmatine chloride (**AgmCl**), and native lysozyme in TE buffer without DTT (**TE, no DTT**). (**left**) effects on activity, (**right**) effects on aggregation.

In Fig. 3-56 the results are shown. The left diagram indicates activity after 24 hours, while the right diagram shows the effect on aggregation. In the absence of additives lysozyme almost fully aggregates, while only a small percentage of activity remains (<5%) after 24 hours. In presence of 1 M arginine <5% aggregation was measured, while approximately 15% activity was detected. While the presence of 1 M arginine does not completely prevent the protein from reduction, it still preserves a significantly higher percentage of activity than in the absence of additives. In presence of 1 M GdmCl almost no activity was detectable, while approximately 5% aggregation was detected. In presence of 1 M agmatine chloride approximately 10% aggregation was detected, which is higher than in presence of 1 M arginine or 1 M GdmCl. This indicates that agmatine may be less denaturing than GdmCl, because the percentage of aggregation is higher than in presence of GdmCl. In comparison to no additive present aggregation is lower in presence of agmatine. This indicates that while arginine and GdmCl are more efficient at suppressing aggregation than agmatine, agmatine may be a weaker denaturant than GdmCl. A similar percentage of activity than in the absence of additives was measured, which appears to be the result of the following two effects which were observed for agmatine: on one hand agmatine did suppress aggregation (Fig. 3-54), thus a higher percentage of activity than in the absence of additives would be expected; on the other hand agmatine affects the activity of native lysozyme at 1 M (Fig. 3-55), thus a lower activity in presence of agmatine would be expected. It appears that both effects in this situation lead to a zero net effect. Aggregation on the other hand becomes affected by agmatine, leading to reduced aggregation.

The effects of L-ornithine on lysozyme aggregation and refolding were investigated. Arginine and ornithine are metabolically related through the urea cycle. By hydrolysis of the guanidinium group of arginine, urea is formed which is subsequently secreted. The result is ornithine (Fig. 3-55), which is a non-proteinogenic amino acid. Because of their metabolic relationship, the effects of ornithine on folding and aggregation were compared to arginine.

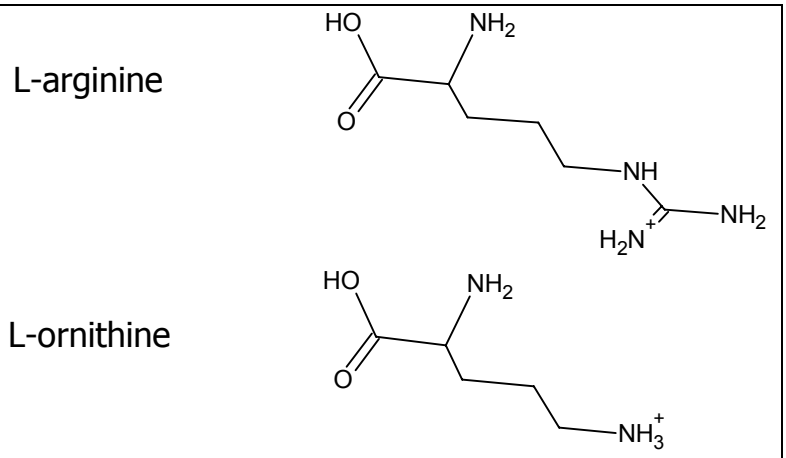


Fig. 3-57: Structure of L-arginine and L-ornithine.

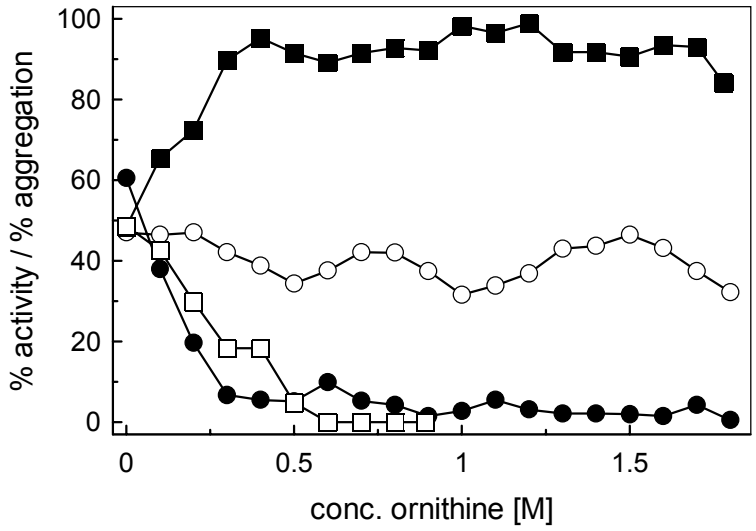


Fig. 3-58: Effects of L-ornithine on lysozyme folding and aggregation in comparison to L-arginine. The conditions were 0.2 mg/mL lysozyme and 2 mM DTT/5 mM GSSG. L-ornithine: (●) activity and (○) aggregation, L-arginine: (■) activity and (□) aggregation.

The resulting activity and aggregation data show (Fig. 3-58) that ornithine does not decrease aggregation. Over the concentration range investigated here (0-2 M ornithine), the percentage of aggregated protein remains at a constant level of approximately 45%. Within the same concentration range arginine decreases

aggregation completely. The percentage of recovered activity steadily declines with increasing concentrations of ornithine. Between 0 M and 0.5 M ornithine the percentage of activity declines to nearly 0%, above 0.5 M the activity remains at 0%. The activity of native lysozyme does not decline in presence of 2 M ornithine, thus the observed effect cannot be attributed to the influence of ornithine on native lysozyme.

Overall ornithine does not promote lysozyme refolding, because it suppresses recovery of activity, which is apparent through lower activities than in the absence of ornithine. Ornithine does not affect aggregation, because the percentage of aggregated protein remains at a constant level, regardless of the ornithine concentration applied. This indicates that the guanidinium group in arginine is essential for the observed effects of arginine. Ornithine also interactions with lysozyme intermediates in such a way that it prevents the recovery of activity.

3.5. Discussion

The presence of arginine and GdmCl among other folding additives at concentrations up to 1 M during a protein refolding process enables the refolding of proteins, which otherwise would aggregate and lead to reduced yields. The overall effect of arginine and GdmCl on protein refolding is to decrease the amount of aggregation. During a refolding process, the formation of disulfide bonds needs to be addressed if the protein has disulfide bonds. Typically, the biological activity of a protein depends on the presence of native disulfide bonds. Thus, the use of a suitable oxido shuffling system to form disulfide bonds during refolding is required.

In the case of lysozyme an oxido shuffling system needs to be present for the formation of its four disulfide bonds. Although air oxidation was used in the earlier work by Epstein and Goldberger (1963), use of a thiol-disulfide exchange (oxido shuffling) system overcomes limitations of air oxidation and allows this process to take place more rapidly and efficiently. The stochiometry of the oxido shuffling system used influences the achievable yield and needs optimization for each individual protein. For the oxidative refolding of lysozyme the optimum stochiometry for the use of DTT as reducing component of the oxido shuffling system was determined by De Bernardez Clark and coworkers (Hevehan and De Bernardez Clark, 1997; Maachupalli-Reddy *et al.*, 1998; Fig. 3-18). The use of DTT has the advantage that it does not have to be removed prior to initiation of the refolding experiment. The previously described method to produce denatured-reduced lysozyme (van den Berg et al., 1999a) has the disadvantage that in order to remove DTT a refolding step is initiated, which results in aggregated protein, which is then washed and dissolved in acidic buffer. The method used here ensures that HEWL remains fully reduced until refolding is

initiated. For the experiments shown here usually 2 mM DTT and 5 mM GSSG were used, this combination enables the recovery of 100% activity (Fig. 3-18).

Chang and Li (2002) demonstrated that upon denaturation and enabling of thiol exchange reactions lysozyme forms three major intermediates which contain non-native disulfide bonds. They demonstrated that lysozyme forms disulfide isomers in the absence of oxidizing thiol components. Thus data shown in Fig. 3-14 are consistent with their result, indicating that lysozyme becomes rapidly oxidized by formation of non-native disulfide bonds. These non-native disulfide bonds subsequently undergo disulfide shuffling reactions leading to fully native protein.

The recovered activity in presence of arginine and GdmCl is very similar (Fig. 3-15, 3-16), which raises the question as to whether both additives act by the same underlying mechanism. The evaluation of refolding kinetics (Fig. 3-19) and the use of a kinetic model, which allows the separate calculation of folding and aggregation rates (Fig. 3-21) revealed that the effect on aggregation rates is indeed very similarly for concentrations of up to 0.5 M. The effect on folding rates, however, differs. Between 0 and 0.5 M, GdmCl does not have a significant effect, above 0.5 M GdmCl a decrease of the folding rates was observed (Fig. 3-21). Arginine has the opposite effect – it slightly increases the folding rates between 0-1 M.

Since the effect on folding rates may be influenced by effects on formation rates of folding intermediates, ANS fluorescence binding kinetics were monitored and used as an indicator of partially unfolded protein species. The data demonstrate (Fig. 3-23) that additives do affect the formation rates of hydrophobic intermediates. Hydrophobic intermediates form within the deadtime of the experiment in the absence of additives, but in presence of arginine or GdmCl this process becomes slower, resulting in a peak after 5-10 min. Overall the formation of intermediates does not appear to be the rate-limiting step. The kinetic model used for the evaluation of folding kinetics reflects this result (Fig. 3-20).

Thermostability measurements provide information about thermodynamic stability of proteins under different conditions. GdmCl destabilizes native lysozyme, while arginine does not have a significant effect (neither stabilizes nor destabilizes native lysozyme). Lin and Timasheff (1996) measured thermotransitions of RNase A in presence of 0.25 M, 0.5 M, and 0.75 M L-arginine and found that it had a destabilizing effect on this protein. Similarly, Taneja and Ahmad (1994) measured thermotransitions of cytochrome c and found that arginine had a destabilizing effect. The degree to which arginine destabilizes a protein varies with the protein's properties. In case of lysozyme, arginine did not act as a destabilizer, while it destabilizes RNase A. The

differences in these results highlight that some of the results shown here are specific for lysozyme and different results may be observed with other proteins. In another study Arakawa and Tsumoto (2003) characterized the effect of arginine on heat-induced unfolding of RNase A and lysozyme. In the absence of arginine samples became turbid. The presence of arginine makes the temperature transitions reversible, while not affecting the transition midpoint. From this, they concluded that arginine cannot only bind to unfolded protein, which would lower the transition midpoint, but must bind to native and unfolded protein. This also means that arginine cannot facilitate the refolding, and the effects must be based on suppression of aggregation. Thermotransitions of HEWL were also performed in presence of the oxido shuffling system. This was utilized to simulate conditions under which lysozyme refolding takes place. Under elevated temperatures lysozyme (partially) unfolds, so the presence of oxido shuffling system simulates the situation of HEWL refolding. Under refolding conditions partially folded HEWL is present and oxido shuffling system. The slow increase of temperature assures a slow increase of partially folded intermediates, thus the response by additives and oxido shuffling system can be studied under these conditions. Without reduction of disulfide bonds HEWL refolding is completely reversible, while refolding of denatured-reduced HEWL results in competition between folding and aggregation. Thus the addition of oxido shuffling components allows thiol-disulfide exchange to occur, simulating refolding conditions. Although activity at room temperature in presence of oxido shuffling system does not decline (Tab. 3-5), it destabilizes lysozyme if present during thermotransitions. At room temperature neither structure (Figs. 3-1, 3-2, Tab. 3-2) nor activity (Tab. 3-5) of native lysozyme is affected by the presence of oxido shuffling components. At room temperature the native protein is compact, making it less prone to reduction by the oxido shuffling system. At higher temperatures the hydrodynamic radius of lysozyme increases (Fig. B-1 in Appendix B), which demonstrates that the protein structure becomes more flexible at higher temperatures, facilitating the thiol-disulfide exchange reactions, leading to destabilization of native lysozyme. Under these conditions arginine stabilizes lysozyme, resulting in a significant increase of the transition midpoint. In presence of arginine the oxido shuffling system components may have a significantly reduced impact on lysozyme, indicating that arginine influences the accessibility of thiols to the protein core.

Unlike results observed in presence of arginine, thermal unfolding of lysozyme in presence of GdmCl does not result in aggregation, probably due to the fact that GdmCl dissolves aggregates very well. Although 1 M GdmCl is not capable of dissolving very large and compact aggregates, it may facilitate solubilization of aggregates, which are in the process of growing, because those aggregates are much smaller in size and more accessible to the buffer. During thermal unfolding, aggregates form gradually, thus in the buffer provided GdmCl can dissolve them. Although at least a concentration of 2.5 M GdmCl is required to completely

dissolve fully-grown aggregates (Fig. 3-10) and only 10-15% of those aggregates are dissolved by 1 M GdmCl, the data show that arginine does not have the capability to dissolve them. Aggregates usually do not have a homogeneous size but contain a certain size distribution. The experiment was done with aggregates, which had 24 hours time to grow without formation of disulfide bonds, thus the medium aggregate size was likely to be very large. Once aggregates have reached a certain dimension they cannot be penetrated by solvent as efficiently. The visible growth process of aggregates is as follows: aggregation starts with a turbid solution, in which distinctive particles become visible over time. When the growth process has proceeded for a significant time big flakes are visible, which float in a clear surrounding solution. To harvest those aggregates, centrifugation was used, which also compresses the material and removes most of the buffer, resulting in a compact precipitant.

The addition of 1 M arginine after refolding has been initiated shows (Fig. 3-24) that arginine does not have the ability to suppress or dissolve already formed aggregates when it is added after 10 min. Protein folding intermediates complete to form after 10 min, which may be an indication that arginine can only inhibit the formation of aggregation while intermediates form. GdmCl, on the other hand, does increase the refolding yield when added after refolding has been initiated, but not to the extent when added at the beginning of the refolding process. A reason for this may be that upon initiation of refolding immediately some disulfide-bonded aggregates form, which cannot be dissolved by GdmCl.

GdmCl is thought to exhibit preferential binding towards proteins (chapter 1.3.), which means that the water density around the protein surface becomes lower. The denaturing effect of GdmCl can be explained by prefential binding, as water is expelled from the protein surface. Similarly, preferential binding of GdmCl to protein aggregates may explain the dissolution.

Arginine, on the other hand, is thought to exhibit preferential hydration towards proteins, meaning that the water density around the protein surface is increased. The data shown here suggest that oxido shuffling components do have less access to the protein surface in presence of arginine. This indicates that in presence of arginine the protein surface becomes less accessible to these components, while arginine at the same time does not directly bind to the protein.

While GdmCl can at least partially dissolve some aggregates, arginine does not have this capability. GdmCl is efficient when added to the refolding buffer after the refolding process has been started. Arginine does not significantly decrease the amount of aggregation when added after the folding process has been initiated (Fig. 3-24). Arginine can only decrease the amount of aggregation when supplied to the buffer prior to the peak occurrence of ANS-binding intermediates.

This is an indication that arginine must be present when folding intermediates form, probably to prevent the exposure to solvent. GdmCl appears to strictly affect solubility of aggregates, thus it (partially) reverses the aggregation process, and its effects are not related to folding intermediates.

The way kinetic properties become affected by the additives can be summarized as shown in Fig. 3-59. In the absence of additives aggregation rates are in an order of magnitude leading to competition between folding and aggregation. In presence of arginine the formation of intermediates takes places at a lower kinetic rate as well as aggregation rates which are lower than in the absence of additives. Folding rates are higher than in the absence of additives (Fig. 3-21). Some accumulation of intermediate protein was observed (Fig. 3-23). In presence of arginine the ANS binding fluorescence signal did not decline as seen in absence of additives or presence of 1 M GdmCl, indicating that intermediates are present for a longer period of time. In presence of GdmCl aggregation rates drop, and so do folding rates. GdmCl has a denaturing effect on proteins, which is affects protein folding by slowing down these processes. At higher GdmCl concentrations the protein eventually becomes completely denatured and folding rates approach zero.

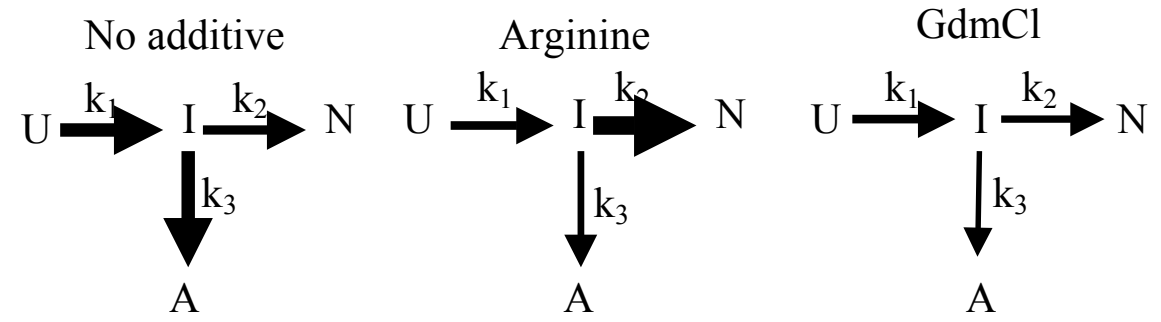


Fig. 3-59: Effect of additives on lysozyme refolding. Thick arrows represent large constants, while thin arrows represent declination of rates.

Besides investigating the effect of folding additives on lysozyme refolding, a reductive unfolding process was explored for its capability to shed light on the role folding additives play during aggregation. The reduction of the disulfide bonds in lysozyme leads to significant destabilization of the protein, resulting in complete aggregation. This process was characterized here and emphasizes that reduction of the disulfide bonds of lysozyme leads to aggregation. This shows that disulfide bonds have a major responsibility for the stability of lysozyme. During the aggregation process a sufficient excess of DTT provides a large pool of (partially) reduced lysozyme molecules, which subsequently aggregate when interactions occur. High protein concentrations lead to a higher probability of such events, leading to increased aggregation rates. It has been shown that this aggregation process is dependent upon reduction of disulfide bonds. The process

can be aborted at any time by alkylation of free sulfhydryl groups, which was utilized to characterize aggregation kinetics.

Since arginine and GdmCl have been found to profoundly affect the competition between folding and aggregation during refolding of lysozyme, the effect of these additives on the reductive aggregation process was investigated. While GdmCl at concentrations above 0.5 M completely inhibits aggregation, arginine at similar concentrations suppresses aggregation for at least 24 hours, but aggregation was observed after extended incubation periods, indicating that it does not permanently inhibit aggregation, but reduces kinetic rates. Measurements of activity and aggregation kinetics (Figs. 3-29, 3-30, 3-31) show that even in presence of 1 M arginine some decline of activity occurs (Fig. 3-30), even when no aggregation was detectable within the duration of the experiment, indicating that disulfide bonds become slowly reduced in presence of arginine. GdmCl facilitates the decline of activity by facilitating protein unfolding (Fig. 3-31). The denatured and (partially) reduced protein resulting from the presence of DTT would be very prone to aggregation, but low concentrations of GdmCl are sufficient to prevent aggregation by dissolving aggregates, thus the activity of lysozyme samples is very low while at the same time no aggregates were detectable.

CD spectra of lysozyme in presence of arginine and GdmCl (Figs. 3-33, 3-34) confirm the results, because CD spectra indicate that the tertiary structure of lysoyzme in presence of GdmCl becomes significantly disrupted, while it does not become disrupted when arginine is present (Fig. 3-34). The data show that arginine prevents or reduces the impact of reduction on lysozyme, resulting in insufficient amounts of reduced lysozyme for a sustained aggregation process, while GdmCl does facilitate the reduction of lysozyme by DTT through destabilization of the tertiary structure. It suppresses aggregation by assisting the solubilization of partially reduced lysozyme.

The binding of ANS to protein folding intermediates is frequently used to detect presence of such intermediates, which often are of the molten-globule type. Monitoring ANS fluorescence kinetics permits the detection of such intermediates and comparison to different buffer conditions. Because lysozyme undergoes complete aggregation in the reductive unfolding process described here it was expected that ANS fluorescence may be a suitable probe for the detection of unfolding intermediates, which are precursors for aggregates due to their nature of solvent-exposed hydrophobic groups. ANS-binding kinetics, indeed, show that aggregation kinetics coincide well with ANS-binding kinetics (Figs. 3-37, 3-38, 3-39). This indicates that the ANS fluorescence signal is indicative of an intermediate state which is prone to aggregation.

In the absence of arginine or GdmCl the kinetics of ANS-binding coincide well with the respective aggregation kinetics (Fig. 3-37). Above 0.5 M arginine or GdmCl no ANS-binding and no aggregation were detectable, confirming that formation of ANS-binding intermediates is a prerequisition for aggregate formation. Because >0.5 M arginine or GdmCl did not yield aggregates, aggregation and ANS-fluorescence kinetics were closely compared for 0.25 M arginine and GdmCl and compared to measurements without any additives.

ANS-binding kinetics in presence of 0.25 M arginine (Fig. 3-38) show that unfolding intermediates form at a similar time compared to absence of additives. This indicates that arginine does not affect the initial reduction process of disulfide bonds. This may appear to be in contrast to the activity kinetics which indicate that lysozyme activity was retained for a longer period of time when arginine was present. While the increase in ANS fluorescence is indicative of the reduction process, no direct correlation between fluorescence intensity and number of free disulfide bonds can be made. Lysozyme activity declines when the protein becomes reduced, but partially reduced lysozyme may have some activity, especially species with one or two reduced disulfide bonds. Arginine may prevent the protein from reduction of all four disulfide bonds, but may not inhibit partial reduction, leading to an equilibrium of partially reduced lysozyme molecules which have some enzymatic activity. The maximum of ANS fluorescence was detected after 75-90 min, at which time almost no aggregation was observed. This shows that although lysozyme intermediates are highly abundant at this time, arginine either prevents their aggregation or their progression into a stage at which aggregation is inevitable.

In presence of 0.25 M GdmCl an increase in the ANS fluorescence kinetics was recorded after approximately 50 min (Fig. 3-39), which is significantly later than in the other two cases discussed here. Aggregation kinetics and increase of ANS fluorescence coincide well, indicating that once HEWL becomes partially reduced, it becomes committed to aggregation and no disruption of the process occurs.

Data in Figs. 3-40, 3-42 indicate that lysozyme may either become more compact or hydration patterns of the protein may change when arginine is present. While it is less likely that HEWL becomes more compact in presence of arginine, because proteins are already relatively compact in their native conformation, a more reasonable explanation may be that arginine may affect the hydration shell, leading to decreased fluorescence in presence of arginine. One consequence of this effect is that the disulfide bonds of lysozyme are less prone to reduction in presence of arginine. GdmCl does not have such an effect on HEWL, because activity kinetics are not affected by the presence of GdmCl.

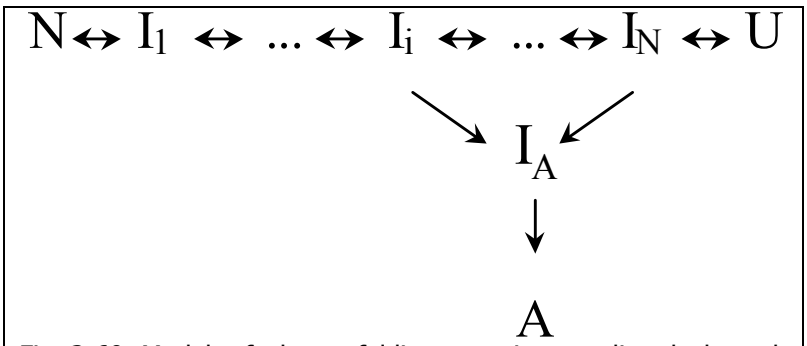


Fig. 3-60: Model of the unfolding reaction mediated through multiple intermediates. (***N***) native protein, (***I*$_{1...N}$**) unfolding intermediates, (***I*$_A$**), unfolding intermediate irreversibly committed to aggregation, (***A***) aggregates, and (***U***) unfolded protein. Intermediates from I_i on are prone to aggregation.

In presence of 1 M arginine the lowest decline of activity over time was observed. Although the decline was significantly lower than in the absence of arginine or GdmCl or in presence of 0.25 M or 0.5 M arginine, addition of 1 M arginine could not prevent reduction of activity for more than 72 hours, indicating that arginine can delay, but not completely inhibit reduction of disulfide bonds in lysozyme and thus formation of unfolding intermediates.

The shape of aggregation kinetics can be best approximated by a sigmoid regression function. This usually indicates involvement of one or more intermediates in the process. Two kinetic models may explain the process:

In the first model, reduction of lysozyme may result in formation of several unfolding intermediates (Fig. 3-60), which are in equilibrium with each other. A similar mechanism was first proposed by Goldberg and coworkers (1991) for aggregates observed during refolding of lysozyme. In this model native protein progresses from the native state *N* to intermediate states I_1 to I_N, resulting in fully unfolded protein *U*, which is soluble. The equilibria are shifted towards the intermediate I_N. As consequence some protein becomes trapped in the irreversible pathway leading to I_A, resulting in formation of aggregates (*A*). The different intermediate states in the model may correspond to lysozyme with different disulfide bonds reduced. The increase of ANS fluorescence was observed with a lag phase, which is an indication that ANS-binding species may correspond to intermediates, which are on the pathway towards I_N. Numeric simulations show that sigmoid-shaped kinetics can only be obtained in presence of several intermediates in between native and unfolded protein (Fig. 3-61).

In presence of 20 mM DTT activity declines nearly exponentially, which indicates that this step may be a first order reaction (Figs. 3-29, 3-30). In the multistep

unfolding model shown in Fig. 3-60 the decline in activity is represented as initial unfolding step $N \leftrightarrow I_1$.

$$\frac{d(N)}{dt} = -k_{1f}N + k_{1r}I_1$$

$$\frac{d(I_1)}{dt} = k_{1f}N - (k_{1r} + k_{2f})I_1 + k_{2r}I_2$$

$$\frac{d(I_2)}{dt} = k_{2f}I_1 - (k_{2r} + k_{3f})I_2 + k_{3r}I_3$$

$$\frac{d(I_3)}{dt} = k_{3f}I_2 - (k_{3r} + k_{4f})I_3 + k_{4r}A$$

$$\frac{d(A)}{dt} = k_{4f}I_3$$

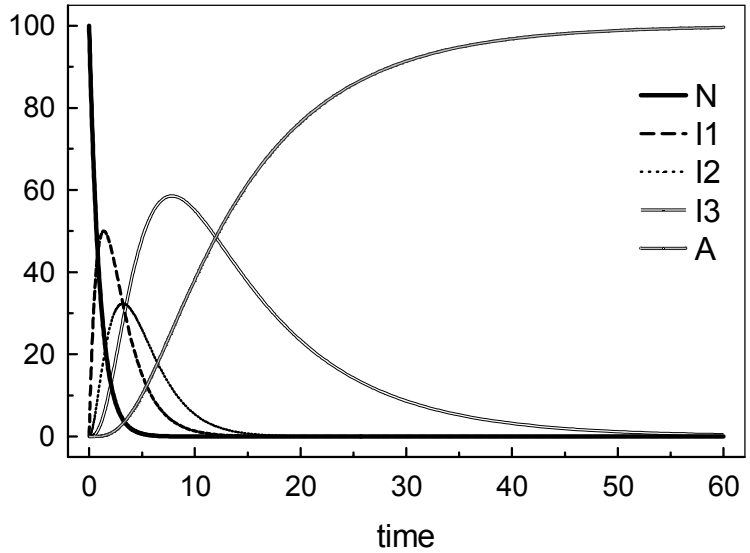


Fig. 3-61: (**Top**) Differential equation system, and (**bottom**) corresponding diagram showing the occurrence of different species of a differential equation system which describes the multiple intermediate kinetic model (Fig. 3-60). The underlying assumptions of this model are that the intermediates form in sequential order $N \rightarrow I_1 \rightarrow I_2 \rightarrow I_3 \rightarrow A$. With exception of the last step $I_3 \rightarrow A$ all intermediates are in equilibrium with each other. (Diagram computed with the program POLYMATH.)

The reduction of one disulfide bond leads to reduction of lysozyme activity (Denton and Scheraga, 1991). Shortly afterwards ANS-binding intermediates become accumulated, which correspond to $I_2 \ldots I_N$. In presence of arginine (≥0.25 M), decline of activity occurs at a slower rate than in absence of arginine (Fig. 3-30). As experimental data show, with increasing arginine concentrations activity declines at a lower rate. Additionally, lower aggregation rates were

observed in presence of arginine, indicating that a lower percentage of intermediates become trapped in the aggregation pathway. At concentrations ≥0.5 M arginine aggregation is completely inhibited, indicating that arginine prevents intermediates $I_2 \ldots I_N$ from entering the aggregation pathway. ANS fluorescence kinetics (Fig. 3-35) support these findings, because the fluorescence signal reaches a plateau after approximately 75 minutes, followed by formation of aggregates. This indicates that the ratio of intermediates on the aggregation pathway changes to a distribution of $I_1 > I_2 > \ldots > I_i > \ldots I_N$, thus a critical concentration of intermediates at which aggregation becomes detectable is not accumulated.

In presence of GdmCl the effects on the reduction steps are quite different: the decline of activity does not depend on the GdmCl concentration. This indicates that GdmCl may not affect the initial reduction step reflected as $N \leftrightarrow I_1$ in the model. Because the amount of aggregated protein declines in presence of GdmCl, this is an indication that GdmCl may interact with HEWL by keeping the intermediates $I_2 \ldots I_N$ solubilized, which otherwise would be prone to aggregation.

Since GdmCl does not reduce the amount of lysozyme going into the pathway $I_2 \ldots I_N$, its effect on reducing aggregation appears to be by solubilizing reduced and unfolded protein, which otherwise would aggregate. GdmCl drives these intermediates $I_2 \ldots I_N$ towards U, completely unfolded protein. Again, ANS data support the findings of this model (Fig. 3-37). The formation of ANS binding intermediates does not reach a plateau, and aggregates form synchronously with increased ANS fluorescence.

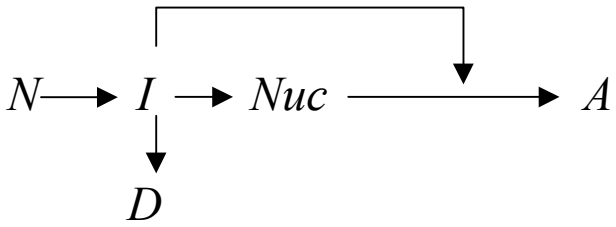


Fig. 3-62: Model of an aggregation process mediated through a nucleus. (*N*) native protein, (*I*) intermediate(s), (*Nuc*) nucleus, (*D*) denatured and reduced protein, and (*A*) aggregated protein. Adapted from Speed *et al.*, 1997.

Another suitable model to explain the appearance of sigmoid kinetics is the nucleation-propagation model (Speed *et al.*, 1997; Fig. 3-62). In the nucleation-propagation aggregation process (Fig. 3-62), aggregation takes place through formation of a nucleus. A nucleus is formed from monomers and acts as "seed" for the propagation phase. Once the nucleus has reached its critical size, the

process turns into the propagation phase, which is characterized by significantly increased growth rates. Formation of the nucleus is very slow and is rate-limiting in this model. In the aggregation process described here, reduction of disulfide bonds in lysozyme may correspond to the $\mathrm{N}\rightarrow\mathrm{I}$ reaction. Once lysozyme is fully or partially reduced (*I*), it may be forming the nucleus. Because no conclusive determination which species may bind ANS is possible, occurrence of ANS fluorescence after a lag phase and in comparison to activity kinetics suggest that partially reduced lysozyme species bind ANS. Growth of the nucleus is rate-limiting, after which the propagation process consists of addition of monomers to the nucleus.

The nucleus forming reaction $I \rightarrow Nuc$ is reversible, the equilibrium is slightly biased towards formation of the nucleus. This results in very slow growth of the nucleus. During formation of the nucleus aggregation is not detectable, because the nucleus is not yet above the critical size at which it becomes insoluble. When the process switches to the propagation phase, size of the associated molecules increases rapidly and aggregates become detectable. Experimental data coincide with this model as follows: the first step involves the formation of partially reduced protein *I*. Partially reduced protein is consumed by formation of the nuclei. The nucleus formation step represents an equilibrium between $I \xleftrightarrow{} Nuc$, with the nucleus slowly growing. Once the nucleation phase is complete, I becomes consumed by the propagation process, which is fast and irreversible. The critical step in this process is formation of the nucleus.

In presence of arginine, experimental data indicate that the initial process $N \rightarrow I$ occurs at decreased rates, as shown through activity kinetics (Fig. 3-30). This indicates that arginine may affect the nucleation process, because monomers oligomerize slower to the critical size of the nucleus. ANS-binding kinetics, which are a suitable probe for the formation of intermediates, indicate that in presence of 0.25 M arginine formation of intermediates occurs at a slower rate, while concentrations of ≥0.5 M arginine inhibit formation of intermediates completely. In comparison to data acquired in absence of arginine and GdmCl data indicate that the propagation phase may also be affected by arginine, because after a population of intermediate protein has formed, the formation of aggregates does not take place synchronously. Experimental data do not allow a distinction whether arginine affects both processes or only the nucleation process.

In presence of GdmCl the intermediate protein becomes fully denatured-reduced. GdmCl does not influence the rate at which lysozyme becomes reduced. Since the presence of GdmCl does not alter loss of activity, formation of intermediates was expected to be unaffected, too, but ANS binding kinetics (Fig. 3-35) show that no ANS binding intermediates were detected for ≥0.5 M GdmCl. In presence of 0.25 M GdmCl ANS binding intermediates were detected, but their formation rate was significantly lower compared to rates in presence of 0.25 M arginine or

in absence of additives. In presence of 0.25 M GdmCl competition between the pathway leading to nucleus and fully denatured-reduced protein occurs, especially since GdmCl has the ability to dissolve aggregate precursors (Fig. 3-10). Another way in which GdmCl facilitates formation of fully reduced and denatured protein is through dissolution of aggregates during the propagation phase. In absence of GdmCl intermediate protein is added to the growing nucleus. In presence of sufficiently high concentrations of GdmCl the propagation process becomes partially reversible, leading to an overall slower propagation process.

Although both additives are similarly efficient in suppressing aggregation, their underlying mechanisms are different. In presence of GdmCl unfolding and reduction of native lysozyme is not affected, thus leading to almost complete loss of activity. Despite complete loss of activity, aggregation is efficiently suppressed because GdmCl facilitates solubilization of unfolding intermediates. Arginine on the other hand inhibits the reduction of disulfide bonds, leading to inhibition of aggregation and preservation of protein activity. Although activity was not preserved indefinitely, by reducing the amount of intermediate protein, arginine leads to decreased aggregation rates. In presence of 0.25 M arginine, reduction of disulfide bonds was not completely suppressed, thus leading to formation of partially folded, ANS binding intermediates, which did not aggregate immediately. This shows that arginine keeps these partially folded intermediates soluble. Protein becomes retained in the early stages of reduction.

Timasheff and coworkers (Arakawa *et al.*, 1990; Bhat and Timasheff, 1992; Kita *et al.*, 1994; Lin and Timasheff, 1996; Timasheff, 1992a, 1992b, 1993, 1995; Xie and Timasheff 1997a, 1997b, 1997c) investigated the distribution of cosolvent molecules around proteins. They argued that the denaturing effect of some cosolvents is based on preferential binding of cosolvent to the protein. The effect of cosolvents can be differentiated as preferential hydration or exclusion. Arginine is among the cosolvents which cause preferential hydration, leading to a relatively lower concentration of arginine around the protein than in the surrounding space.

In presence of arginine the reduction rate of disulfide bonds of lysozyme is lower, which indicates that arginine does provide an access barrier for DTT and therefore reduction does not take place as fast as in absence of arginine.

After characterizing the effects of arginine and GdmCl on refolding and aggregation processes of lysozyme, the ability to decrease aggregation and promote refolding of several other additives was characterized. Effects of a given compound on aggregation and recovery of activity resemble two different effects, because an additive may decrease aggregation while not promoting recovery of activity, resulting in soluble, but inactive protein. Purpose of this

study was to elucidate which functional groups are relevant for the effects of arginine. For this purpose, the approach of comparing the effects with arginine was chosen.

While some of the substances were as efficient as arginine and GdmCl in promoting recovery of activity, they had this effect only in a very limited concentration range. In presence of agmatine and homoarginine only concentrations of 0.5-1 M promote activity, in contrast to arginine and GdmCl which facilitate refolding of HEWL over a concentration range 0.5-2 M.

A comparison of the effects of D-arginine with L-arginine show that both substances have the same efficiency, indicating that effects of arginine are not based on stereospecific interactions between arginine and protein. It instead indicates that geometry and arrangement of functional groups in arginine, their charges and/or distances are responsible for the effect of arginine on HEWL.

Some of the investigated substances have a guanidinium group (homoarginine, poly-L-arginine, and agmatine), which has a positive charge throughout a very large pH range. The positive charge is stabilized by mesomery within the guanidinium group. This raises the question if the sign of the charge of the guanidinium group is important, meaning if a negatively charged functional group of similar geometry would be as efficient as guanidinium. It would be highly desirable to use a compound of similar geometry but with a strong negative charge for this purpose. Unfortunately, no molecule with these properties was available. To still elucidate role of charges on formation of aggregates, an experiment with glutamic acid as negatively charged model additive was performed. Besides obvious geometric differences between the guanidinium group and carboxyl group (in glutamic acid), the guanidinium group acts as a hydrogen bond donor at pH 8, while the carboxyl group acts as an acceptor. To examine pairs of equal and opposite charges, BSA was used in this experiment in addition to HEWL. As the data show, glutamic acid does not suppress aggregation but instead facilitates it regardless of the charge of the protein. This shows that the effects of arginine are not based on charge-charge interactions between arginine and protein, but instead involve more complex interactions.

Similarly, ornithine which contains one amino group in the side chain does not facilitate recovery of activity, although it carries a positive charge at pH 8. Although no literature value for the pK_a value of the side chain could be obtained, the structural similarity of ornithine to lysine makes the assumption reasonable that the amino group of both amino acids have a similar pK_a, thus it can be assumed that the amino group is protonated at pH 8. The inability of ornithine to promote refolding (Fig. 3-58) demonstrates that a potential folding

additive must contain a guanidinium group to promote refolding of HEWL. Ornithine does not affect aggregation, while glutamic acid promotes it.

Since additives without a guanidinium group do not facilitate refolding of lysozyme, molecules with guanidinium groups, which have closer structural similarity to arginine were investigated. Among substances investigated was L-homoarginine, which has four methylene groups between the guanidinium group and the C_α groups (Fig. 3-48). Homoarginine promotes recovery of activity to 1 M, above it suppresses recovery of activity while still inhibiting aggregation. L-homoarginine may have strong molecule to molecule interactions at high concentrations that may keep it from interacting with the protein, thus it does not facilitate protein refolding at higher concentrations.

Among the compounds investigated poly-L-arginine is more unusual, because it consists of approximately 30-85 arginine residues. Small concentrations of poly-L-arginine suppress recovery of activity. Since a structured polypeptide is more likely to form specific interactions with the protein and thus may promote aggregation by specific interactions leading to co-aggregation, a far-UV CD spectrum of poly-L-arginine was acquired to determine whether it has secondary structure or not, and the CD data show (Fig. 3-51) that it does not. Despite absence of secondary structure poly-L-arginine inhibits recovery of activity, indicating that strong interactions between poly-L-arginine and HEWL occur. This was demonstrated by measuring activity of native lysozyme in presence of poly-L-arginine, which strongly inhibits it, thus indicating that it interacts with the active center of lysozyme. Aggregation on the other hand is not affected by the presence of poly-L-arginine, except >4 mg/mL which may be caused by interactions with lysozyme folding intermediates, thus slightly reducing aggregation.

Agmatine (Fig. 3-53) has an α-amino group and a γ–guanidinium group. At pH 8 the guanidinium group is positively charged. The isoelectric point of the α-amino group could not be retrieved from literature, but the amino group is expected to be positively charged at pH 8, because geometrically similar positioned amino groups in other amino acids have isoelectric points between 9 and 11. In presence of agmatine chloride activity is successfully recovered, although not quite as efficient as in presence of arginine. Aggregation decreases in a very similar way to arginine. The difference between the two additives is that concentrations of more than 1 M agmatine lead to lower percentages of recovered activity while with arginine no decline of recovered activity was observed. Activity of native lysozyme declines in presence of agmatine, which explains the reduced recovery of activity above 1 M. During unfolding experiments (initiated with DTT) agmatine proved to be less effective than GdmCl or arginine, because activity after 24 hours was lower than in presence of arginine or GdmCl. While activity of native protein was significantly reduced at

this concentration, agmatine inhibits aggregation, thus results appeared to be the composite of both effects. Also, aggregation was increased in presence of agmatine than in presence of the other two additives.

Tab. 3-7: Effect of the substances on activity and aggregation.

Additive	Activity	Aggregation	Effect on activity of native protein
L-arginine	Promotes 0-2 M	Decreases 0-2 M	No effect
D-arginine	Promotes 0-2 M	Decreases 0-2 M	Not determined
L-glutamic acid	Not determined	Increases 0-2 M	Not determined
L-homoarginine	Promotes 0-1 M, then decreases	Decreases 0-2 M	No effect
Poly-L-arginine	Inhibits strongly	Not affected	Inhibits
Agmatine	Promotes 0-1 M, then decreases	Decreases 0-2 M	Inhibits moderately <1M, then strongly
Ornithine	Inhibits	Not affected	No effect

Tab. 3-7 summarizes the effects of investigated additives. While additives with a guanidinium group and the exception of polyarginine generally promote lysozyme refolding at least to 1 M, they decline and usually completely suppress aggregation above 0.5 M. Poly-L-arginine is an exception here, because it is a peptide and inhibits strongly the activity of native lysozyme. The non-arginine additives with a guanidinium group do not promote lysozyme refolding over the whole concentration range 0-2 M but only to 1 M. Above 1 M these additives may stabilize folding intermediates, resulting in inhibition of the folding pathway.

Additives, which do not consist a guanidinium group may or may not have an effect on the recovery of activity and formation of aggregates. Since the effects show a great variability depending on functional groups present, functional groups besides a guanidinium group determine how effective the additives affect folding and aggregation.

In this thesis, the effects of arginine and GdmCl on lysozyme refolding were characterized. The apparent effect of both additives is very similar. While the chosen buffer and additive conditions do not affect structure and activity of native lysozyme, the presence of oxido shuffling system decreases the thermodynamic stability of native lysozyme. The effects of arginine and GdmCl appear as follows: while arginine is not capable to interact with aggregates once they have formed, arginine appears to interact with HEWL when folding intermediates are formed. The interaction results in declined formation of aggregates. The effects of GdmCl, on the other hand, appear to dissolute aggregates. Because GdmCl concentrations below the unfolding concentration are efficient in facilitating refolding, the low concentrations of GdmCl may act by destabilizing HEWL besides dissolving aggregates. Low concentrations of GdmCl decrease the transition midpoint of proteins in thermal unfolding studies. Similarly, presence of low concentrations of GdmCl during refolding processes will result in higher concentrations of unfolded protein than in its absence.

4. Summary and future work

L-arginine is a frequently used additive for protein refolding processes. It suppresses aggregation and strongly increases refolding yields. Arginine is effective for almost all proteins. Among other folding additives, arginine is one of the most potent ones. The effects of arginine were described for the first time in a patent application in 1985, yet the underlying mechanism by which arginine enhances refolding yields has never been explained. Thus the purpose of this work was to elucidate the mechanism by which arginine enhances refolding and inhibits the formation of aggregates by looking at kinetic as well as thermodynamic effects. The effects of arginine were compared to guanidinium chloride (GdmCl): like arginine, GdmCl has a guanidinium group, but denatures proteins at higher concentrations. The effects of both additives were investigated in the concentration range 0-2 M, with 2 M being the solubility limit of arginine. The effects of arginine and GdmCl were characterized in three different areas or processes: refolding of lysozyme, unfolding of it, and structure-function relationship of functional groups in folding additives.

Refolding of HEWL

Arginine and GdmCl are efficient promotors of lysozyme refolding while efficiently suppressing aggregation. The presence during HEWL refolding leads to nearly 100% recovered activity and complete suppression of aggregation (above 0.5 M arginine). To elucidate kinetic effects, activity kinetics in presence of various concentrations of the additives were acquired. Utilizing the model by Hevehan and De Bernardez Clark (1997), kinetic constants for folding and aggregation were derived from the data. The resulting kinetic constants indicate that both additives very effectively suppress aggregation. The observed decline of aggregation rates is very similar for both additives, but above 0.5 M GdmCl is more efficient in reducing aggregation rates than arginine. Concentrations of more than 0.5 M GdmCl significantly decreases folding rates, while in presence of 0.25 M-0.5 M arginine folding rates are slightly increased, which indicates that while arginine and GdmCl may have a similar effect on aggregation rates, GdmCl decreases refolding rates whereas arginine increases them. As consequence, both additives affect partitioning between folding and aggregation, resulting in folding pathway being the only pathway folding intermediates are consumed. At higher concentrations of GdmCl denaturing effects of GdmCl affect folding rates, facilitating unfolding of intermediates as well as native protein. Next, the effect of additives on folding intermediates was investigated. Folding intermediates are not stable and thus not accessible for comprehensive biophysical characterization, but can be detected by using the dye 1-anilino-8-naphtalene sulfonic acid (ANS). ANS fluorescence kinetics were acquired in absence and presence of 1 M arginine or GdmCl. Kinetics in presence of arginine indicate that ANS-binding intermediates populate relatively fast after initiating of refolding and

remain present for a long period of time while in presence of GdmCl intermediates populate and declines. Lysozyme needs to have four correctly formed disulfide bonds to be fully active. Changes in disulfide bonds result in partial or total loss of activity. To enable recovery of activity while the refolding process is going on, a suitable thiol exchange system needs to be present which is optimized for the appropriate folding conditions. A combination of 2 mM DTT and 5 mM GSSG enables recovery of 100% activity. Presence of the oxido shuffling substances does not impact structure or activity of lysozyme at room temperature. However, combination of the substances thermodynamically destablizes lysozyme as shown by thermal unfolding measurements. Addition of GdmCl further decreases the transition midpoint, while arginine increases it. The conditions are different to native, correctly folded protein, which is relatively compact. Conditions chosen here resemble refolding conditions, when the protein is denatured prior to initiation of the refolding process. Under temperature transition conditions, proteins slowly unfold by temperature. This leads to partial loss of tertiary and secondary structure, which makes proteins more susceptible to thiol exchange reactions, resulting in decreased thermal stability. Arginine stabilizes lysozyme under these conditions.

Aggregation occurs extremely fast when lysozyme is being refolded, thus it is not accessible for thorough characterization and kinetic measurements. Aggregation is irreversible under these conditions. To verify influence of arginine and GdmCl on aggregates, aggregates were exposed to various concentrations of arginine or GdmCl. Arginine does not increase solubility of aggregates, but GdmCl partially solubilizes aggregates (and completely dissolves aggregates above 2.5 M). In another experiment effect of both additives was studied when HEWL was allowed to refold without the additives for several minutes. The experiment indicates that arginine can only influence aggregation when it is added before the maximum amount of folding intermediates has formed. This indicates that arginine must interact with folding intermediates or pre-intermediate protein molecules to inhibit formation of aggregates. This appears to be different from GdmCl which appears to destabilize native and intermediate lysozyme and solubilizes aggregates. Since aggregates grow from oligomers to large molecular weights, effect of low concentrations of GdmCl (<2.5 M) appears to be to solubilize aggregate oligomers and thus prevent aggregation. At higher concentrations of GdmCl, folding rates decline because GdmCl destabilizes native protein as well, thus folding rates decline above 1 M.

Effect on unfolding of HEWL

During refolding of lysozyme, aggregation occurs as fast reaction, which cannot be interrupted by quenching of ongoing reactions. Aggregates are observed instantaneously when refolding is initiated. Some of the aggregates become disulfide bonded. Alkylation of free sulfhydryl groups does not interrupt the ongoing aggregation process. This makes it difficult to gain insights into the effect of additives on aggregation.

Because of these disadvantages, a slow, highly controllable aggregation process was utilized. When DTT is added to native lysozyme, a slow aggregation process becomes initiated. After a lag phase the solution becomes turbid. This aggregation process can be interrupted by alkylation of free sulfhydryl groups, indicating that aggregation is caused by reduction of disulfide bonds. Aggregation kinetics can be best described by a sigmoid function, indicating that the aggregation process involves formation of intermediate(s). Arginine and GdmCl efficiently decrease aggregation when applied during this process. Preceding aggregation, loss of activity occurs. Since full activity of lysozyme depends on correctly formed disulfide bonds, this indicates that (partial) reduction of disulfide bonds occurs first which then leads to aggregation. Decrease in activity can be best approximated by a single exponential regression function, indicating that declining activity appears to be a first order reaction. For the decline in activity, differences between the additives were observed. In presence of arginine, depending on the concentration, a slower decline of activity was observed. Since loss of activity is induced by reduction of disulfide bonds, this indicates that arginine decreases the rate at which reduction of disulfide bonds occurs. In presence of GdmCl the decline of activity does not depend on the GdmCl concentration but is constant – it occurs as fast as in absence of additives. To analyze how presence of arginine and GdmCl affect the structure of lysozyme under incubation with DTT, CD spectroscopy was utilized to analyze secondary and tertiary structure. While native secondary and tertiary structure is preserved in presence of arginine (and DTT), the protein loses all secondary and tertiary structure in presence of GdmCl. The occurrence of hydrophobic unfolding intermediates was monitored by using ANS. While no ANS-binding intermediates (and subsequently no aggregation) were observed above 0.5 M arginine or GdmCl, ANS-binding intermediates were observed at 0 M and 0.25 M arginine or GdmCl. Superimposition of aggregation and ANS-fluorescence kinetics indicates that the occurrence of hydrophobic intermediates is related to aggregation, suggesting that the populated hydrophobic intermediates are committed to aggregation. At concentrations of 0.5 M arginine or GdmCl or above ANS-fluorescence and aggregation were not observed. Superimposition of aggregation and ANS-fluorescence data revealed important information about the mechanism how arginine and GdmCl decrease aggregation. In absence of arginine and GdmCl and in presence of 0.25 M GdmCl both kinetics coincide well, meaning

that aggregation and ANS-fluorescence kinetics show an increase at the same time. In presence of 0.25 M arginine, an increase in ANS-fluorescence was observed at the same time than in absence of additives. Aggregation kinetics, however, revealed that aggregation did not become detectable at the time when ANS-fluorescence showed an increase, there was a time delay observed. This shows that although arginine does not affect formation of intermediates, it delays the aggregation process of the ANS-binding intermediates. The data indicate that arginine must affect interactions between solvent and protein, because the rate of reduction by DTT is significantly declined in presence of arginine.

Structure-function relationship of folding additives

During discussion of the effects of arginine and GdmCl, the structure-function relationship of the additives was not considered. Other, structurally related components were not included. To elucidate the role of functional groups in arginine, effect of to arginine or GdmCl structurally related substances on aggregation and activity was determined.

First, D-arginine was compared to L-arginine. No difference was detected, indicating that no stereospecific effects are involved in the effects of arginine. Since the strong positive charge present in the guanidinium group of arginine may be responsible for the effects, an additive with a negative charge was investigated. Glutamic acid was chosen for this purpose. Additionally to lysozyme, the effects on bovine serum albumin (BSA) were studied. While arginine is positively charged at pH 8, BSA is negatively charged, making it possible to elucidate whether charge-related effects are responsible. Arginine suppresses aggregation of both proteins, indicating that its efficiency does not pedend on the isoelectric point of the protein. Glutamic acid, on the other hand, facilitates aggregation of both proteins. The reason why glutamic acid did not facilitate the refolding of lysozyme may be its negative charge or different geometry of the carboxyl group in comparison to the guanidinium group. By using ornithine as folding additive, which has a positively charged amino group, data suggest that the geometry of the guanidinium group accounts for the effects of arginine, and not charge alone. Unlike glutamic acid, ornithine does not affect aggregation, but its presence leads to decreased activity.

The data suggest that the geometry of the guanidinium group rather than its charge is responsible for the effects. The data prompted for a closer look at additives containing a guanidinium group, since ones without did not promote refolding. L-homoarginine, which contains an additional methylene group in the side chain, efficiently promotes activity to 1 M, above the amount of recovered activity declines. A similar effect was observed for agmatine, which is decarboxylated arginine. In contrast to homoarginine, agmatine affects activity

of native lysozyme. To elucidate the role of the α-amino and α-carboxyl group in arginine, the effect of poly-L-arginine on lysozyme refolding was investigated. Since the amino and carboxyl groups in poly-L-arginine are involved in peptide bonds, they cannot affect protein-solvent interaction. Poly-L-arginine does not affect aggregation. The effect on lysozyme refolding could not be determined because it inhibits activity of native lysozyme.

In summary compounds without a guanidinium group do not promote refolding of lysozyme. Charge and geometry of this functional group are imperative for facilitating protein refolding. Arginine derivatives with or without additional functional groups do not promote lysozyme refolding over the concentration range 0.5-2 M. Some of the substances promote refolding from 0.5-1 M, above they suppress aggregation but inhibit protein refolding.

The work described here investigates effects of arginine and GdmCl on lysozyme refolding and unfolding. Initial refolding processes take place very fast and are not accessible for characterization. Not surprisingly, a not too detailed picture about the effects of arginine and GdmCl on HEWL emerged from refolding experiments. Unusual properties of arginine became noticeable, for instance arginine is only effective during the first couple of minutes after folding was initiated. The application of GdmCl on the other hand is not time-critical. A reductive unfolding process leading to aggregation proved to be extremely helpful in evaluating effects of both additives on aggregation. This aggregation process is very slow in comparison to the refolding aggregation. It also can be stopped at a given time which is not possible with the refolding-aggregation process. It was possible to identify interactions between additives, folding intermediates and aggregates, shedding some light on the effects.

Future work

Future work should be directed towards characterization of interactions between lysozyme (un)folding intermediates, arginine, and solvent. Some of previously shown data suggest that arginine may directly interact with partially folded lysozyme molecules. In presence of DTT lysozyme becomes reduced, leading to aggregation. This process is preceded by a strong decline in activity. When arginine is added prior to addition of DTT, lysozyme retains activity, indicating that the presence of arginine affects the reduction process. Reduction of disulfide bonds and subsequent loss of activity occurs much slower than in absence of additives. This indicates that there may be an interaction between lysozyme and arginine, reducing solvent accessibility of DTT to HEWL. Also, presence of thiol exchange components during thermotransitions resulted in destabilization of lysozyme. While GdmCl further destabilizes lysozyme, arginine stabilized lysozyme under those conditions.

Future experiments should address solvent-additive-protein interactions. These characterizations should be performed in absence and presence of arginine and GdmCl, since arginine and GdmCl act by different mechanisms. Data generated in presence of GdmCl did not indicate that GdmCl affects lysozyme-solvent interactions. Timasheff and coworkers have characterized interactions of cosolvents on proteins using a highly sensitive method for the determination of concentrations of cosolvent around proteins. According to their measurements, arginine leads to preferential hydration, meaning that the presence of arginine leads to an increase of water density around proteins. It is not clear if their results are applicable to folding intermediates, because they performed the studies with native proteins only. Arginine might interact either directly with the protein or with the hydration layer around it, alternating the protein-solvent interface. To shed light on the interactions, suitable experimental techniques should be engaged, which are capable to detect weak, non-covalent interactions between protein and cosolvents. Suitable techniques may include isothermic titration calorimetry (ITC) and ^{1}H NMR. ITC is capable of thermodynamically determine binding energies of a ligand to a receptor. Constant volumes of ligand are added to the receptor while the heat flow is monitored by the calorimeter. The resulting small changes of enthalpy are recorded by the instrument. For the situation here, small aliquots of arginine are added to a lysozyme solution. In case there is an interaction between lysozyme and arginine, the enthalpy signal will change and allow for determination of the binding enthalpy. If arginine affects the hydration layer around lysozyme, ITC may not result in detectable changes of the enthalpy, because then no direct binding of arginine to lysozyme may be observed. The changes in enthalpy may be too small to be measurable with this technique. This technique is experimentally less cumbersome than NMR, and therefore may be used prior to NMR. The use of proton NMR can conclusively determine whether solvent-lysozyme interactions become modified by the presence of arginine. This can be done through proton exchange experiments. For a proton exchange experiment, the rate of deuterium-proton exchange is measured. Arginine will be used completely deuterated so that it does not interfere with the deuterium-hydrogen exchange between protein and solvent, although the deuterium atoms in deuterated arginine will become exchanged, too. Proton NMR allows the differentiation of signals from lysozyme and arginine. For the setup of a suitable experiment, the protein needs to become completely deuterated before the experiment is initiated. For experiments in which arginine will be present, deuterated arginine needs to be used. Upon initiation of the experiment, non-deuterated water is added, which initiates exchange of deuterium atoms by protons. NMR measurements are capable to specifically detect protons in their environment, thus it can detect the rate at which deuterium atoms in lysozyme become exchanged by protons. To determine influence of additives like arginine on this process, deuterated additives are added. Upon initiation of proton exchange, arginine becomes exchanged, too. NMR allows a selective determination of rate constants, thus

exchange of arginine deuterium atoms do not affect measurements of protein exchange rates. Comparison of exchange rates in absence and presence of arginine will show whether solvent accessibility of lysozyme will decrease in presence of arginine.

Experiments reported here were done exclusively with lysozyme, which was chosen because of its commercial availability. Some of the experiments required larger amounts of protein for. The choice of just one protein for all experiments had the advantage that all work was focused on effects of arginine rather than on the explanation of protein to protein differences. While arginine does not destabilize native lysozyme, it has been reported to destabilize other proteins. This indicates that the effect of arginine may be dependent on the protein used. Future work should address the question how arginine promotes refolding of other proteins with different properties (monomeric and oligomeric proteins, different molecular weights, different numbers of disulfide bonds etc.). Besides the above mentioned highly sophisticated methods the choice of other proteins may allow for a more comprehensive biophysical characterization of refolding or unfolding processes. A similar reduction-aggregation behavior as described for lysozyme was observed for BSA, while reduction of insulin does not result in aggregation (L. Russell, private communication). It would be expected that arginine suppresses aggregation for proteins which aggregate upon reduction of their disulfide bonds.

To further elucidate the role of functional groups in arginine, efficiency of other compounds containing one or more guanidinium group(s) on the refolding of lysozyme should be investigated. Substances without a guanidinium group did not promote refolding of lysozyme. This may require custom synthesis of compounds. To determine whether the effect of two or three guanidnium groups may be additive, efficiency of di- or tri- guanidinium alkane compounds should be determined. This may either be di-arginine, linked through a peptide bond, or 1,3-diguanidinium-propane. Effect of agmatine (decarboxylated arginine) and homoarginine indicate that location of the α-amino and α-carboxyl group significantly affects the efficiency of a particular compound. Data from compounds which have the same functional groups as arginine but do not have an amino or carboxyl group on the α-carbon atom may shed light on the hypothesis drawn from refolding experiments in presence of homoarginine and agmatine. Work in this field may lead to discovery of more potent folding additives than arginine, although data so far indicate that arginine provides the best balance of interactions between various functional groups.

Some characterizations could only be performed with native lysozyme, because the folding process cannot be arrested while it is going on. The properties of intermediates and native protein are different, thus measurements on native protein may not be a suitable model for all effects of arginine. Since folding

intermediates of lysozyme are not kinetically stable, only a limited set of methods can be utilized to characterize interactions between arginine and folding intermediates. Intermediates populated during lysozyme refolding are not accessible without utilizing stopped-flow instrumentation. Even by utilizing this technique, intermediates will not be available for a thorough biophysical characterization. Stopped-flow measurements will provide information about kinetic stability of folding intermediates and whether arginine affects stability of such intermediates. Another possibility may be partially reduced lysozyme, which forms ANS-binding unfolding intermediates. Those intermediates are highly prone to aggregation, but keeping them at a low concentration may delay the aggregation process long enough for thorough biophysical characterizations and stability measurements. Those characterizations will shed light on the question of how arginine interacts with folding (or unfolding) intermediates, and how it affects the aggregation process. For those measurements NMR spectroscopy may also be used to aquire more comprehensive data on how the refolding (or unfolding) process becomes modified by arginine, and thus will allow characterization of interactions in great detail.

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Appendix

A. Abbreviations

AgmCl _____ agmatine hydrochloride (decarboxylated arginine)
ANS _____ 1-anilino-8-naphthalene sulfonic acid
Arg _____ L-arginine hydrochloride
BSA _____ bovine serum albumin
CD _____ circular dichroism
Da _____ Dalton, 1 atomic unit
DAD _____ diode array detector
DI _____ deionized
DLS _____ dynamic light scattering
DOC _____ deoxycholic acid
DTNB _____ 5,5-dithio-bis-2-nitrobenzoic acid (Ellman reagent)
DTT _____ dithiothreitrol
EDTA _____ ethylen diamine tetra acetic acid
EtOH _____ ethanol
GdmCl _____ guanidinium hydrochloride
GSH _____ glutathione, reduced
GSSG _____ glutathione oxidized
HEWL _____ hen egg-white lysozyme
HPLC _____ high-performance liquid chromatography
IAA _____ iodoacetic acid
MALDI _____ matrix-assisted laser desorption ionization
MOPS _____ 3-(N-morpholino) propane sulfonic acid
NMR _____ nuclear magnetic resonance
OXS _____ oxido shuffling system
PAGE _____ polyacrylamide gel electrophoresis
RP _____ reversed phase
rpm _____ rotations per minute
SDS _____ sodium dodecyl sulfate
TCA _____ trichloroacetic acid
TFA _____ trifluoroacetic acid
TOF _____ time of flight
tPA _____ tissue plasminogen activator
tris _____ tris(hydroxymethyl)aminomethane
TE _____ tris-EDTA buffer (50 mM tris, 1 mM EDTA)
UV/VIS _____ ultraviolett-visible
pI _____ isoelectric point (of a protein)

B. Dynamic light scattering data

Dynamic light scattering was used to investigate changes in the hydrodynamic radius of lysozyme in dependency of various additive concentrations. While these measurements were performed at room temperature, the capability of the DLS instrument Dyna Pro 99 (Protein Solutions) to measure at elevated temperatures was not utilized. In order to test the feasibility of measuring unfolding processes by changes in the hydrodynamic radius, temperature transition measurements by monitoring the hydrodynamic radius of lysozyme (Fig. B-1) and BSA (Fig. B-2) were performed. The data show that changes in the hydrodynamic radius were detectable.

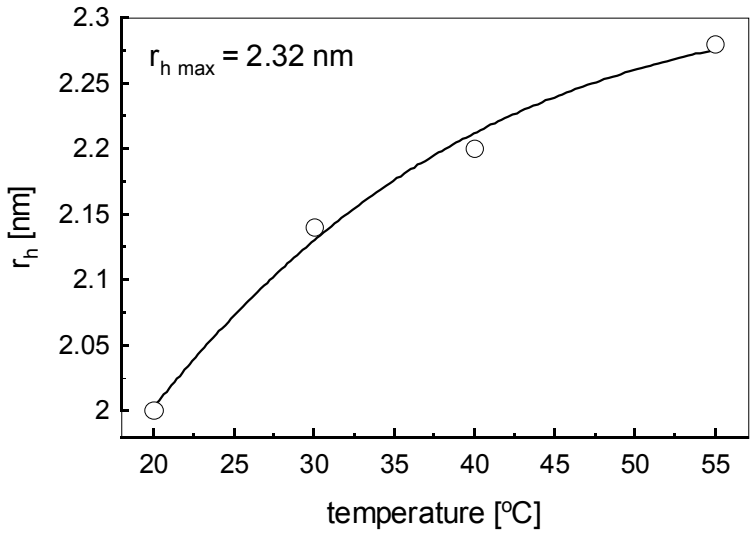

Fig. B-1: Temperature dependency of the hydrodynamic radius of lysozyme (1 mg/mL). While no distinctive transition is visible, the data suggest that the hydrodynamic radius gradually increases up to a maximum of 2.32 nm, which corresponds to the radius of the completely unfolded protein.

In case of monomeric lysozyme (Fig. B-1) a gradual increase of the hydrodynamic radius was observed, which can be best described by an exponential function. Although it was not possible to acquire data up to 95 °C, because heavy aggregation occurs which interferes with the measurements, it is obvious that the hydrodynamic radius increases gradually to an approximate maximum of 2.3 nm.

BSA (bovine serum albumin) is a tetrameric protein of the geometry $\alpha_2\beta_2$ and a molecular weight of 67 kDa. Unlike lysozyme the increase in hydrodynamic radius is very pronounced (Fig. B-2) and exhibits a sigmoid shaped behavior. The difference to lysozyme is probably caused by dissociation of subunits in BSA. The unfolding of individual subunits may not be detectable by a sharp transition – as

seen in lysozyme – but rather by a gradual transition. The large increase in the hydrodynamic radius may be characteristic for a multidomain protein.

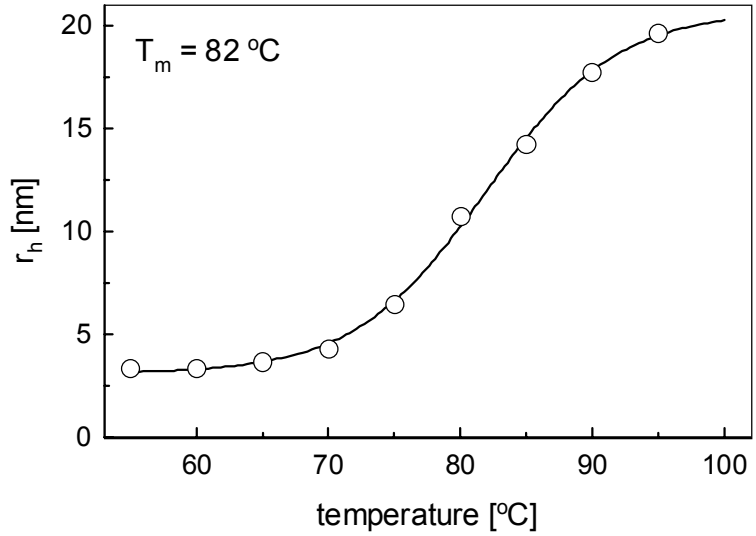

Fig. B-2: Hydrodynamic radius of 1 mg/mL BSA (bovine serum albumin) in dependency of temperature. While monomeric lysozyme did not show a distinctive transition, BSA does.

These data demonstrate that dynamic light scattering may be a valuable tool to characterize unfolding processes of proteins. Like other methods used for the detection of temperature-induced unfolding processes, it is important that the temperature raise is controlled automatically, since the reproducibility of measurements is compromised by manual temperature control. The instrument used here allows for such automated measurements.

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Hiermit erkläre ich an Eides statt, daß ich mich mit der vorliegenden Arbeit erstmals um die Erlangung des Doktorgrades bewerbe und daß diese Arbeit selbständig und nur unter Benutzung der angegebenen Quellen und Hilfsmittel erstellt wurde. Ich versichere weiterhin, daß ich die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche gekennzeichnet habe.

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