“Strategies for Production of a Recombinant Single Chain Antibody Fragment in *Escherichia coli*”

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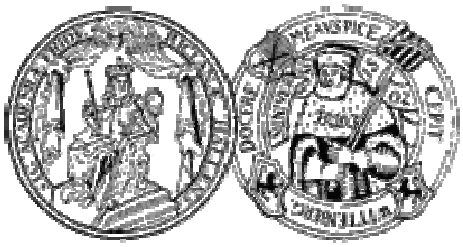

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Abstract

Recombinant expression of antibody fragments in *Escherichia coli* (*E. coli*) is often hampered by low expression, inefficient translocation and aggregation in the periplasm. Aggregation problem during *in vitro*-renaturation is also a limiting factor, when antibody fragments expressed in bacterial cytosol as inclusion bodies (IB's). The objective of this work was to achieve and optimise periplasmic production, and to explore novel strategies for *in vitro*-renaturation from cytoplasmic IB's.

For the study, the single chain antibody fragment against the hapten oxazolone (scFvOx) was chosen as the model system. Periplasmic expression was achieved under the control of the *lac* promoter. The expression of scFvOx was sustained, and not harmful to the host. Efficient translocation of the product to the periplasm was achieved, no periplasmic IB's were detected, and the soluble scFvOx was biologically active. As expected, in a laboratory scale bioreactor, the duration of production phase was directly related to the rate of specific growth (μ_{set}), and product degradation was observed at late cultivation phases in both cases. In contrast to the *lac* promoter system, *T7* promoter-driven expression led to very high level expression of the product, adversely affecting cell physiology. In addition, co-expression of DsbA (disulfide bond forming enzyme A) and its active site variants were not beneficial for the production of scFvOx.

Renaturation of scFvOx from cytoplasmic IB's was evaluated with methylimidazolium chloride-derived Ionic Liquids (IL's). The IL's were found to suppress aggregation and enhance renaturation yield. Among the other IL's tested, N-hydroxyethyl pyridinium chloride was found to promote renaturation, whereas N-ehtyl-N'methylimidazolium anion variants with phosphate and sulphate were not beneficial.

Urea-induced denaturation was less reversible at pH 8.5 than at pH 7.0. The protein was most stable between pH 6.0 and 7.0 while pH values below 5.0 or above 8.0 were destabilising. At pH 2.0, an acid-induced state, characterised by a blue shift in fluorescence, high percentage of secondary structure, and apparent thermodynamic stability, was observed. The pH dependency of renaturation of scFvOx, be it from the reduced denatured protein or from the glutathione modified mixed disulfide, reflected the pH-dependent stability of the native state. Higher renaturation yields were obtained between pH 6.0 and 7.0 than at pH 8.5.

For the renaturation of the glutathione-modified mixed disulfide of scFvOx, four aromatic thiols were tested as novel redox buffers. Three of them significantly enhanced the

renaturation yield of scFvOx at lower pH as compared to renaturation in the presence of glutathione. Of the thiol compounds, thiosalicylic acid stimulated the apparent rate of renaturation by a factor of two.

The findings from these studies might be helpful for the optimisation of the periplasmic production of antibody fragments as well as for establishing optimised renaturation protocols for inclusion body material at physiological pH.

Zusammenfassung

Der Erfolg der rekombinanten Expression von Antikörper-Fragmenten im bakteriellen Periplasma kann z.B. durch zu kleine Mengen an produziertem Protein, durch ineffiziente Translokation , und durch Präzipitation im Periplasma eingeschränkt werden. Werden Antikörperfragmente hingegen im bakteriellen Zytosol als unlösliche Einschlusskörper (inclusion bodies) produziert, können Aggregationsprobleme während der Rückfaltung zum limitierenden Faktor werden.

Ein Thema dieser Doktorarbeit war die Etablierung und Optimierung der Produktion eines Einzelketten-Antikörper-Fragmenten gegen das Hapten Oxazolon (scFvOx) im Periplasma. Weiterhin sollten neue Rückfaltungsstrategien für dieses Modellprotein nach seiner Expression in zytoplasmatische *inclusion bodies* untersucht werden.

Es ist gelungen, scFvOx im Periplasma zu produzieren. Das Protein wurde effizient in das Periplasma befördert. Die Expression unter Kontrolle des *lac*-Promoters verlief kontinuierlich und beeinträchtigte die Physiologie des Wirtsorganismus nicht negativ. Es konnten keine *inclusion bodies* in den Zellen nachgewiesen werden. Das periplasmatisch exprimierte scFvOx war biologisch aktiv.

Der Effekt der Promotorstärke auf die Expression wurde untersucht. Der Einsatz des sehr sträken *T7* Promotors führte zu sehr hohen Produktbildungsraten, wodurch es zu einer negativen Beeinträchtigung der Zellphysiologie kam. Die Koexpression Disulfideisomerase DsbA und von zwei ihrer aktiven Zentrum mutierten Varianten erwies sich entgegen den Erwartungen als nicht hilfreich für die Produktion von scFvOx.

Im Bioreaktor wurde bei einer spezifischen Wachstumsrate (μ_{set}) von 0,06 h^{-1} eine längere Produktionsphase von scFvOx erreicht als bei einer spezifischen Wachstumsrate von 0,12 h^{-1}. In den späten Kultivierungsphasen kam es in beiden Fällen zum Verlust des Produktes.

Weiterhin wurde die Rückfaltung von scFvOx aus zytoplasmatischen *inclusion bodies* untersucht. Zu diesem Zweck wurden die Einflüsse von ionischen Flüssigkeiten (IL), aus der Klasse der von N-Alkyl-N'-Methylimidazoliumchloride auf die Rückfaltung getestet. Diese IL unterdrückien die Aggregation und führen somit zu einer erhöhten Rückfaltungsausbeute. Weitere IL mit verschiedenen Kationen und Anionen wurden ebenfalls getestet. N-Hydroxyethylpyridiniumchlorid erwies sich als effektives Additiv für die Rückfaltung von

scFvOx, während N-Alkyl-N'-Methylimidazoiumderivate mit Dialkylphosphat- bzw. Ethylsulfat-Anionen weniger förderlich für die Renaturierung waren.

Die Stabilität von scFvOx gegenüber chemischer Denaturierung und pH wurde untersucht. Die Stabilität des Proteins war zwischen pH 6,0 und pH 7,0 am größten. Die chemische Denaturierung bei pH 8,5 war weniger reversibel als bei pH 7,0. Bei pH 2,0 konnte ein säureinduzierter Konformationszustand beobachtet werden. Dieser Zustand wird charakterisiert durch einen hohen Sekundässtruktur-anteil und apparente thermodynamische Stabilität.

Die pH-Abhängigkeit der Renaturierung von scFvOx ausgehend sowohl von reduziert denaturiertem Protein als auch vom gemischten Disulfid mit Glutathion spiegelt die pH-abhängige Stabilität des Proteins wieder. Die höchsten Ausbeuten der Rückfaltung wurden dabei zwischen pH 6,0 und 7,0 erzielt.

Weiterhin wurden aromatische Thiolverbindungen als neuartige Redoxreagentien für die oxidative *in vitro*-Rückfaltung von scFvOx getestet. Die untersuchen aromatischen Thiole verbesserten mit einer Ausnahme die Ausbeuten der Renaturierung gegenüber der Rückfaltung in Gegenwart von Glutathion. Während Thiosalicylsäure die Rate der Renaturierung um das zweifache beschleunigte, wurde ein solcher Effekt für die untersuchten heteroaromatischen Verbindungen nicht beobachtet.

Die Ergebnisse dieser Untersuchungen sollten sich für die Zukunft sowohl für die Verbesserung der periplasmischen Produktion von Antikörperfragmenten als auch für die Erarbeitung optimierter Rückfaltungsprotokolle für inclusion body-Material bei physiologischen pH-Werten als hilfreich erweisen.

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1. Introduction

1.1 Antibodies and antibody fragments

1.1.1 The immune system and immunoglobulins

The immune system of vertebrates is a complex system of molecules and cells designed to protect the body against viruses, micro-organisms, and parasites by distinguishing between "self" and "foreign". Antibodies or immunoglobulins (Ig) are part of the humoral immune response, are produced by mature B cells or plasma cells and can either inactivate the foreign particle, the antigen, or label it for further processing by the complement system or macrophages. Human immunoglobulins can be divided into 5 classes according to their function and physico-chemical properties: IgG, IgM, IgA, IgE and IgD (Janeway and Travers, 1997; Nilsson and Tolmachev, 2007; Sorensen, 2007)

1.1.2 Antibodies

IgG's are the most abundant class of antibodies present in human immunoglobulins. The IgG molecule consists of two identical light chains and two identical heavy chains, which have a considerable variation due to alternate splicing and somatic recombination. Each chain has a variable domain (V_H and V_L), which is important for antigen binding. Proteolytic digestion of entire IgG molecules with papain results in two antigen-binding fragments (Fab) and one crystalline fragment (Fc) (Fig. 1.1). These domains are structurally related and are called immunoglobulin domains. They consist of two β-sheets in a sandwich-like arrangement, each consisting of three or four antiparallel β-strands in the constant domains and four or five antiparallel β-strands in the variable domains (Fig. 1.2). The structure of the antiparallel β-strands is fixed by a disulfide bond between two essential cysteines and stabilised by hydrophobic interactions. In the variable domains, three loops consisting of the complementarity-determining regions (CDRs) contribute to specific antigen binding. The Fc region is responsible for the effector functions of the IgG.

Antibodies with binding specificity for a certain antigen can be obtained by immunisation of experimental animals. The hybridoma technique developed by Köhler and Milstein (1975) allows the production of monoclonal antibodies (mAb's) specific for only one epitope. Here splenic B-cells are isolated after immunisation and fused with tumour cells. By selection and proliferation of a single hybridoma cell expressing antibodies with the desired binding specificity, mAb's can be produced in theoretically unlimited amounts.

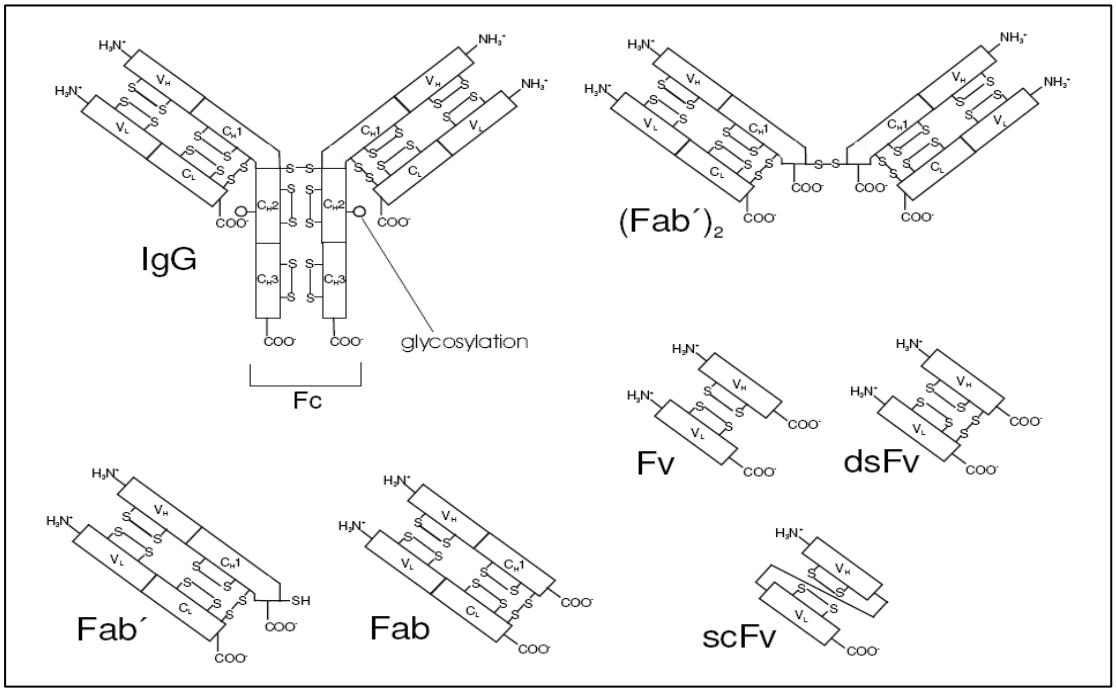


Fig. 1.1: Structure of an IgG and antibody fragments: $(Fab´)_2$ can be obtained by digestion with pepsin, Fab´ by subsequent reduction. Fab results from digestion with papain. Fv is usually expressed as a recombinant protein. DsFv and scFv are stabilised by a genetically introduced disulfide bond or a peptide linker, respectively.

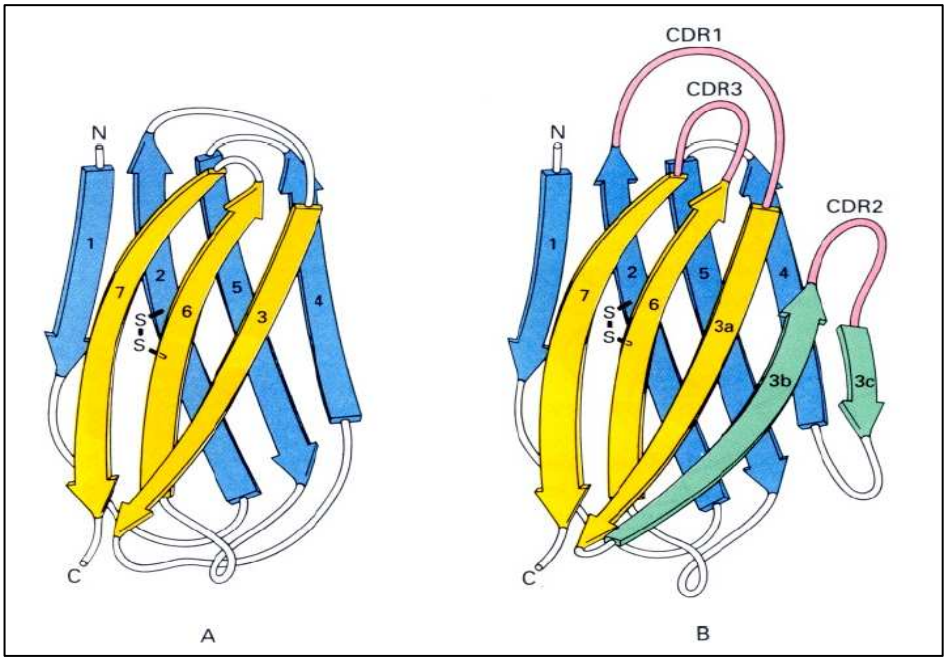


Fig. 1.2: Immunoglobulin folds of a constant domain (A) and a variable domain (B): Blue: N-terminal β-sheet, yellow: C-terminal β-sheet, green: extra β-strands in variable domains, red: complementarity determining regions.

1.1.3 Antibody Fragments

For many clinical and diagnostic applications such as ELISA or *in situ* labeling techniques, the antigen binding activity is of interest, but not the effector activities of the constant regions. Therefore, often, antibody fragments consisting only parts of the parental mAb which confer the characteristic binding activity are prepared (Fig. 1.1). Small antibody fragments have advantages compared to whole immunoglobulins for some clinical applications, such as good penetration of solid tumours, rapid clearance (Huston *et al.*, 1993, and 1996; Holliger and Hudson, 2005), easy derivatisation, and lack of assembling requirements. Additionally, they can be produced in various expression systems, such as plants (Fiedler and Conrad, 1998, Orecchia *et al.*, 2008), bacteria (Buchner and Rudolph, 1991; Sletta *et al.*, 2004), or yeasts (Edqvist *et al.*, 1991; Khatri and Hoffmann, 2006).

The smallest antibody fragment that retains an intact antigen-binding site is the variable fragment (Fv) that consists only of V_H and V_L (Fig. 1.1). However, this fragment is unstable, as these domains are free to dissociate. Two strategies have been adopted to overcome this. The first is to link the domains with a peptide to generate a single chain Fv (scFv). The second is to introduce cysteines at the interface between the V_H and the V_L domains, resulting in an Fv stabilised by disulfide bond formation (dsFv) (Verma *et al.*, 1998). The first approach is more commonly used because it also reduces the number of genes to be expressed to one and a linker consisting of $(Gly_4Ser)_3$ has been proven to work well (Pantoliano *et al.*, 1991). In addition, large numbers of scFv's can be screened for antigen binding by phage display techniques (McCafferty *et al.*, 1990; Schirrmann *et al.*, 2008).

1.1.4 Recombinant antibodies as modern bio-therapeutics

Engineered antibodies (mAb's or fragments) are now fairly well established as biopharmaceuticals, and as of 2005 itself, since 18 antibody products have reached the market and, more than 100 are in clinical trials Holliger and Hudson (2005). Analysts are predicting that by 2010, engineered antibodies are likely to account for over 30% of all revenues in the biotechnology market across the globe. Smaller recombinant antibody fragments (for *e.g.*, classical monovalent antibody fragments, Fab, scFv) (Fig. 1.1) and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies) are now emerging as feasible alternatives. These fragments retain the targeting specificity of whole mAb's but can be produced more economically and possess other unique and superior properties for a range of diagnostic and therapeutic applications. Antibody fragments have also been linked to therapeutic payloads (such as, radionuclides, toxins, enzymes, liposomes and viruses) to produce multivalent and multispecific reagents, and engineered for enhanced therapeutic efficacy (Holliger and Hudson, 2005).

Many of these products are now in clinical and preclinical trials. These include, a Fab fragment (chimeric) for cardiovascular disease, an scFv fragment (humanised) for coronary artery bypass, and an scFv fragment fused to poly ethylene glycol (PEG) for the treatment of breast cancer (Nellis *et al.*, 2005; Holliger and Hudson, 2005).

1.1.5 The scFv fragment against the hapten oxazolone

In this PhD work, the feasibility of producing an scFv fragment that recognises the hapten oxazolone (Berek and Milstein, 1988) was examined. This scFv was chosen because (1) of its well-defined antigen binding properties, which are similar to that of the parental mAb, (2) of the availability of sequence data (Fiedler and Conrad, 1999), (3) of its physiological neutrality in bacteria (Berek and Milstein, 1988), and (4) the assay for this scFv is inexpensive and without requirements for special regents (oxazolone is commercially available). The V_L and V_H domains of this antibody fragment are joined by a synthetic peptide linker $(Gly_4Ser)_3$ (Fiedler and Conrad, 1999).

1.2 Production of recombinant proteins in *Escherichia coli*

1.2.1 Recombinant proteins as bio-therapeutics

For a long time, extraction from native tissue had been the only option to obtain proteins, *e.g.*, peptide hormones. Drawbacks associated with this were poor yields leading to extremely expensive products and possible allergic reactions with therapeutic products extracted from animal tissues. For example, the amino acid sequence of bovine insulin differs from that of human insulin at three positions of the B chain, whereas porcine insulin differs from human insulin by one amino acid. As a result, both bovine and porcine insulin are allergenic or immunogenic when used for the treatment of type 1 diabetes (Peacock *et al.*, 1983). Moreover, even commercial recombinant human insulin that has an identical primary structure to natural insulin of humans has been found to be immunogenic in some cases (Fineberg *et al.*, 1983 and 2007). However, severe immunological complications occur rarely, and less severe events affect a small minority of patients.

During the last decades, there have been great improvements in technologies for the isolation, identification, characterisation and manipulation of genes. This enabled the cloning and amplification of DNA encoding heterologous proteins of interest. Recombinant protein technology has enabled the production of large amounts of high value proteins at affordable cost. Several recombinant products are now available for therapeutic applications, and some protein therapeutics have achieved great commercial significance. Examples include erythropoetin (Procrit, Johnson and Johnson, USA) which is used to treat anaemia associated with chronic kidney failure, HIV infection and cancer chemotherapy; erythropoetin (Epogen,

Amgen USA) for the treatment of anaemia and as a doping agent sporting competitions; and insulin (Novo Nordisk, Denmark) (Pavlou and Reichert, 2004).

A variety of host systems are available for the production of recombinant proteins for different purposes. This includes eukaryotic systems such as yeasts, filamentous fungi and cells from insects, plants, and mammals, as well as prokaryotes including bacteria belonging to *Escherichia, Bacillus* and *Staphylococcus*. Each cell type has its merits and demerits. The amount and quality of the recombinant product are influenced by gene copy number, transcription efficiency, presence of absence of introns, mRNA stability, the efficiency of nuceocytoplasmic transport of the transcript, translation efficiency, and stability and solubility of the proteins. In addition, post-translation modifications are many times critical for folding, stability, transport and function of proteins. Certain post-translational modifications of proteins like glycosylation, amidation, and methylation, can only be carried out by eukaryotic cells. For certain therapeutic applications of IgG's, glycosylation is essential (Trill *et al.*, 1995), as it plays a role in effector functions of the constant regions. Yeast cells can export proteins into the medium and can glycosylate proteins, but normally the glycosylation pattern is different from that in animal cells (Hamilton *et al.,* 2003). Recently, the authentic glycosylation pattern of humanised IgG's was obtained from engineered *Pichia pastoris* (Li *et al.,* 2006). However, for large proteins with complex glycolysation patterns and other post-translational modifications, animal cells are preferred. Unfortunately, sensitivity to contamination and need for expensive complex media are the limitations of mammalian cell system. Furthermore, less information is available about the interrelation between physiology and cultivation parameters for eukaryotic systems in comparison to prokaryotic systems.

Therefore, the production of recombinant proteins in microbial cells represents a convenient and cost-effective option. *E. coli* is genetically and physiologically well-characterised, and in addition, a fast growth rate and simple media requirements make it one of the dominant hosts for protein production. Nine of the 31 therapeutic proteins approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) in 2003 were produced in *E. coli* (Walsh, 2006).

1.2.2 Protein folding machinery and limiting factors in the production of recombinant proteins in *E. coli*

Some eukaryotic proteins, which need sophisticated post-translational modifications not achievable in the prokaryotic cell, simply cannot be functionally expressed in *E. coli*. However, for many non-glycosylated proteins, *E. coli* is a convenient expression system. Still, many factors at the level of transctiption, translation, translocation and protein folding can

impede the efficient expression of functional proteins. Additionally, the behaviour of the host cells under extreme conditions such as high cell density cultivation has to be taken into account. These issues are discussed in the following sections.

Transcription

Transcription is the transfer of the information encoded in the DNA sequence to mRNA, which in turn is decoded by the ribosomes, where the actual protein synthesis takes place. The number of transcripts that reach the ribosomes depends on the number of DNA molecules carrying the respective gene, the transcription rate and the stability of the mRNA. The use of stable high-copy plasmids encoding the gene of interest and the coupling of translation directly to transcription in prokaryotes reduces this problem to the transcription rate, which depends very much on the promoter system and the RNA polymerase (Dubendorff and Studier, 1991; Gräslund *et al*., 2008).

Translation

Translation is a highly complex process by which the nucleotide sequence encoded in the mRNA is translated into an amino acid sequence, which determines the structure and function of the protein. In concert with aminoacyl tRNAs and various translation factors, translation is performed by the ribosomes, which in prokaryotes consist of three different rRNA species and 55 different proteins. Translation is regulated by features upstream of the open reading frame encoding the protein. The start codon AUG coding for formylmethionine initiates translation in most cases. Ringquist *et al.* (1992) found that a distance of eight nucleotides between the Shine-Delgarno sequence and the start codon, combined with an AAA +2 codon led to the highest expression levels in *E. coli*. It has been assumed that a positive effect on gene expression by adenine at the first position of the +2 codon may be associated with the decoding tRNA and that evolution has favoured codons at the +2 position for high translation initiation (Stenstrom *et al.*, 2001b). The level of mRNA may also need to be regulated for optimum protein expression. If mRNA is present in excess, secondary structures, which influence translation initiation, may form (Arnold *et al.*, 2001). It is also well known that codon optimisation needs to be done for recombinant protein expression in prokaryotic system. In attempts to improve the heterologous gene expression in *E. coli*, helper vectors, *e.g.*, pUBS520 have been used (Brinkmann *et al.*, 1989). This vector carries the *dnaY* gene encoding the tRNA for the arginine codons AGA and AGG, which are rare in *E. coli* and thus often limit expression of genes containing these codons.

Protein folding

It is generally assumed that the native state of a protein is in most cases determined by its amino acid sequence and corresponds to the global minimum in its conformational energy landscape (Noelting, 1999). However, a protein can be trapped in a misfolded state corresponding to a local energy minimum. Such misfolding events are more likely to occur when a protein is taken out of its original environment, in which it folds correctly, to another environment with different chemical and physical properties, *e.g.*, pH value, viscosity or redox potential.

Some proteins need helper proteins for correct folding even in the original environment. Helper proteins do not change the energy landscape of a protein but help the protein in attaining its global energy minimum. They can be grouped into folding catalysts and molecular chaperones. The former such as, DsbA and DsbC increase the recovery yields of active proteins by accelerating rate-limiting steps along the folding pathway, such as disulfide bond formation and isomerisation. Prolyl *cis/trans* isomerase catalyses the *cis/trans* transition of propyl residues, which may be rate-limiting for folding of some proteins. Disulfide oxido-reductases can be used to facilitate formation of correct disulfide bonds. The chaperones like, DnaK and DnaJ favour on-pathway folding by transiently interacting with folding intermediates and suppressing their aggregation (Baneyx and Mujacic, 2004).

Heterologous proteins can be expressed in the periplasm or the cytoplasm in native and active form or alternatively, by production as inclusion bodies (IB's) followed by renaturation. Due to the different physico-chemical properties of the proteins in question and limitations of the protein folding machinery in these compartments of *E. coli*, the most favourable expression strategy is directly coupled to the protein folding problem (Duguay and Silhavy, 2004; Georgiou and Segatori, 2005).

Strategies for the improvement of protein production in vivo

It has been proposed that the yield of native protein depends on the respective rates of protein synthesis, folding and aggregation (Kiefhaber *et al.*, 1991; Rudolph, 1996; Gasser *et al.*, 2008). According to this model, a decrease in the rate of protein synthesis or an increase in the rate of folding would lead to increased amounts of correctly folded protein. Strategies like cultivation at low temperature, use of a weaker promoter, or partial induction can reduce or avoid the formation of IBs (Kopetzki *et al.*, 1989; Georgiou and Segatori, 2005; Mansell *et al.*, 2008; Gasser *et al.*, 2008). Altering cultivation parameters like pH value, temperature or medium composition can also result in higher concentration of soluble product (Kopetzki *et al.*, 1989; Georgiou and Segatori, 2005; Mansell *et al.*, 2008; Gasser *et al.*, 2008).

It was reported that during the production of proinsulin as fusion protein with DsbA, the highest product concentrations were obtained at 30°C, but the total amount of soluble protein was higher at 25°C (Winter *et al.,* 2001). Addition of 1% ethanol to the medium 30 min before induction resulted in enhanced protein expression (Winter *et al.,* 2001), probably by stimulating the host cells' chaperone machinery, which after induction served as safeguard to prevent aggregation of the newly synthesised protein. Similar to this, an up shift (40°C) or downshift (10°C) of temperature before induction was beneficial for active protein production (Kopetzki *et al.,* 1989).

In another study, it was found that the addition of low molecular weight additives like L-Arginine and reduced glutathione to the culture medium enhanced periplasmic production of r-tPA (recombinant tissue type plasminogen activator) by 10- and 37-fold, respectively (Schäffner *et al.,* 2001). This effect might be due to the small molecular size of L-Arginine and reduced glutathione, making it possible for them to passively enter the periplamsic space. L-Arginine might have helped in stabilisation of the native state of the protein and reduced glutathione might have played a role in providing appropriate redox potential for the correct folding of the newly secreted polypeptide.

1.3 Production with secretion into the periplasm

Periplasmic production of recombinant proteins has emerged as a possible alternative to overcome the drawbacks of *in vitro* protein renaturation techniques (Choi and Lee, 2004; Georgiou and Segatori, 2005). Various components their translocation pathways, and protein folding in the *E. coli* periplasm have been illustrated in Fig. 1.3. Advantages of the secretory production in periplasm are: (1) the oxidising environment of the periplasm allows for the formation of disulfide bonds, which does not occur in the reducing environment of the cytoplasm (Makrides, 1996, Nagradova, 2008); (2) the periplasm contains two foldases, disulfide oxidoreductase (DsbA) and disulfide isomerase (DsbC) that catalyse the formation and isomerisation of disulfide bonds; (3) the removal of N-terminal translation initiator Met by methionine amino peptidase, which may be crucial for the function and stability of proteins (Takano *et al.,* 1999; Georgiou and Segatori, 2005). In case of periplasmic production, the N-terminal amino acid residue of the secreted product can be identical to the natural protein after cleavage of the signal sequence by a specific signal peptidase; (4) since the periplasm contains fewer proteases, proteolysis is less than that in the cytoplasm (Baneyx and Mujacic, 2004; Choi and Lee, 2004); and (5) since the periplasm is the outermost compartment of a cell, it is possible to recover a protein of interest simply by means of disrupting the outer membrane by osmotic shock procedures. This avoids mixing with other cellular proteins.

Despite several advantages, there are still some drawbacks involved during periplasmic production of recombinant proteins. These include: (1) incomplete processing of signal sequences; (2) variable secretion efficiency depending on the characteristics of the proteins; (3) in some cases, low or undetectable amounts of recombinant protein; (4) cell toxicity; (5) formation of IB's; and (6) incorrect formation of disulfide bonds (Pritchard *et al*., 1997; Chung *et al*., 1998; Lucic *et al*., 1998; Jeong and Lee, 2000; Wong *et al*., 2003).

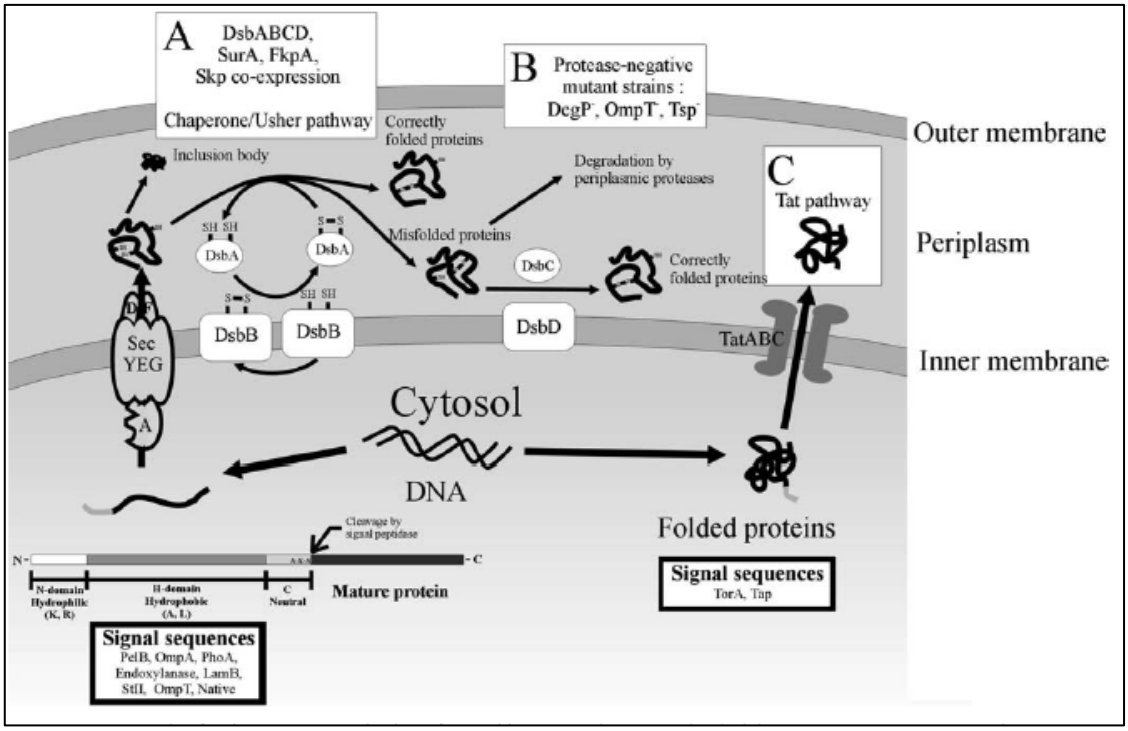


Fig. 1.3: Protein folding catalysts, translocation pathways, export machinery, and pathways of protein folding in *E. coli* periplasm (adapted from Choi and Lee, 2004 and the literature cited within). The Sec system, the twin-arginine translocation (TAT) system, and the strategies for enhancing secretory protein production using periplasmic chaperones and protease-negative mutants are shown. **A.** The co-expression of periplasmic chaperones, such as disulfide-bond formation (Dsb) family proteins, SurA, FkpA, and Skp, can improve the efficiencies of secretory production and protein folding. **B.** Protease-negative mutant strains can improve secretory production of recombinant proteins by reducing proteolysis. **C.** The TAT system can directly secrete folded proteins to the periplasm.

Co-secretion of foldases

Several chaperones and foldases play a role in folding proteins in the periplasm of *E. coli*. The major class belongs to the disulfide bond forming group ("Dsb"), commonly referred to as foldases. They are members of the thioredoxin family containing an active site with a Cys-X-X-Cys motif (Fabianek *et al*., 2000, Nagradova, 2008; Gleiter and Bardwell, 2008). At least six proteins, DsbA, DsbB, DsbC, DsbD, DsbF and DsbG have been reported to be involved in disulfide bond formation and isomerisation. Efficient folding of antibodies *in vivo* also involves molecular chaperones and foldases such as protein disulfide isomerase and

peptidyl-proline *cis-trans* isomerase (Knarr *et al.,* 1995). The functional yield of antibodies by *in vitro*-renaturation and by *E. coli* cell-free translation systems is also enhanced in the presence of molecular chaperones and foldases (Buchner *et al.,* 1992a; Lilie *et al.,* 1993; Ryabova *et al.,* 1997).

In bacteria, DsbA catalyses the oxidation of cysteine residues in the nascent polypeptide to form disulfide bonds into the periplasm. DsbA is the second most potent cysteine-oxidising enzyme known (next only to the membrane protein DsbB, which oxidises DsbA under physiological conditions) (Grauschopf *et al.,* 2003). Co-expression of DsbA has beneficial effects for the production of human leptin (Jeong *et al*., 2000), and insulin like growth factor-I (IGF-I) (Joly *et al*., 1998). DsbC, a protein disulfide isomerase, when co-expressed in the periplasm, enhances the yield of tissue type plasminogen activator (tPA), a complex protein containing 17 disulfide bridges (Qiu *et al*., 1998).

Knappik *et al.,* (1993) tested the effect of over-expression of DsbA and the proline *cis-trans* isomerase PPIase A (poly propyl peptidase A) of *E. coli* on the functional co-expression of Fv, Fab and scFv forms of the antibody McPC603, whose functional expression yield was limited by the periplasmic folding process. Over-expression of PPIase marginally increased functional expression in all instances, except for scFv in the V_L/V_H orientation (scFv-L), by 1.8 fold. No further increase of yield was observed when scFv-L was co-expressed with both PPIase and DsbA. Co-expressing the Fab with DsbA in a different expression format did not increase its functional expression. This suggests that aggregation, which precedes or is independent of isomerisation, might limit protein folding in this case. A lack of positive effect of DsbA on soluble yield was also observed for another scFv (Ryabova *et al.,* 1997) and may reflect its weak disulfide bond isomerisation activity. Being highly reactive, oxidation of substrate proteins by DsbA occurs very rapidly, and seems to be dictated by the proximity and chemical reactivity of individual cysteine residues in protein rather than by their actual pairing in the three-dimensional structure of the native protein (Berkmen *et al.,* 2005). This results in the formation of incorrect disulfide bonds in need of isomerisation, a function catalysed by DsbC and DsbG.

It was demonstrated that disulfide bond isomerisation but not thiol oxidation is the rate-limiting step in expression of recombinant protein in the periplasm (Ostermeier 1996; Maskos, 2003). In order to overcome the problem of disulfide bond isomerisation, rat protein disulfide isomerase (rPDI) was co-expressed in the bacterial periplasm (Ostermeier 1996). Co-expression of rPDI increased the yield of bovine pancreatic trypsin inhibitor several fold, an effect that was enhanced when reduced glutathione was added to the growth medium

(Humphreys *et al.,* 1996). Surprisingly, the results of this study showed that rPDI, when expressed in the bacterial periplasm, mostly acted as thiol-oxidase and not as disulfide isomerase (Ostermeier 1996). Eukaryotic PDI is a dimer of 55 kDa, which additionally adds to the burden of heterologous protein expression. Another alternative to achieve disulfide isomerisation was co-expression of the bacterial protein disulfide isomerase DsbC. DsbC is involved in reshuffling of incorrect disulfides in a human alkaline phosphatase mutant (Sone *et al.,* 1997) when over-expressed in the periplasm of *E. coli*. However, other attempts to over-express this protein resulted in cell death 3-4 h post-induction (Qui *et al.,* 1998).

These studies suggest that there is a need for protein disulfide isomerase of *E. coli* origin, the over-expression of which does not affect host physiology. *In vitro* studies showed that active site variants of DsbA (Wunderlich *et al.,* 1995), in which the active site cysteine (C33) was replaced by serine or arginine, mimicked disulfide isomerase. Therefore, the effect of co-expression of active site variants of DsbA on the periplasmic production of the model protein scFvOx was explored in this study.

1.3.1 Impact of promoter strength on export to the periplasm versus aggregation in the cytoplasm

If the translation rate is too high, the efficiency of secretion can decrease, depending on the protein. Therefore, the optimal translation rate has to be determined empirically for each heterologous protein. Promoters have been optimised for recombinant expression in *E. coli*. Usually, the objective is to obtain soluble, active protein, and for protein secretion, slower and more sustained production is preferred. The important parameter is probably the cell-specific rate of protein synthesis. Weak promoters allow production of heterologous protein at relatively small concentrations (10 - 20% of the total cell protein) (Banyex, 1999; Graumann and Premstaller, 2006). This slower rate of protein synthesis may allow cells to export and fold heterologous protein properly.

On the other hand, a very powerful expression system is the bacteriophage *T7* promoter in combination with the T7 RNA polymerase (Novagen, USA). Induction of this promoter system leads to the synthesis of large amounts of mRNA, and, quite often resulting in production of the desired protein at extremely high concentrations (40 - 50% of the total cell protein) (Banyex, 1999; Graumann and Premstaller, 2006). It is so efficient that mRNA is synthesised much faster than it can be translated by the typical set of ribosomes in *E. coli*. From a process integration point of view, this rapid and dynamic performance is very difficult to handle. High levels of mRNA can cause ribosome destruction and cell death (Dong *et al.,* 1997), and leaky expression of T7 RNA polymerase may result in plasmid or expression

instability (Banyex, 1999). An additional limitation of the *T7* and other strong promoter systems is that the target protein is often unable to reach the native conformation, and either partially or completely accumulates within IB's. Typically, the more rapid the intracellular product synthesis, the greater is the probability of product aggregation.

High translation levels might overwhelm the secretory apparatus for the translocation of proteins. For the 14 kDa protein NT3, higher translation rates were favourable, while for the 8 kDa protein RANTES, a lower translation level led to higher yields of secreted protein (Simmons and Yansura, 1996). Use of F-plasmid-based expression vectors that are stably maintained at 1-2 copies per cell (Jones and Keasling, 1998) might be a solution to this. Clearly, the total accumulation of expressed protein (β-galactosidase) is lower than with higher copy number vectors, even when the mRNA is stabilised with a 5′ hairpin secondary structure (Carrier *et al.,* 1998). But, if allowed for longer expression periods, this lower rate of expression may still provide significant product yield while avoiding aggregation and/or saturation of the secretory pathway.

The mechanism of translocation of proteins across the cell membrane in *E. coli* is mediated by leader sequences. Although leader sequences of genes such as *ompA* and *pelB* have been proven to be generally effective, this does not hold in all cases. Additionally, toxic effects and formation of periplasmic aggregates may counteract the high level expression of active protein in the periplasm (Choi and Lee, 2004; Graumann and Premstaller, 2006).

1.3.2 The influence of growth rate in fed-batch cultivations on product stability

The overall goals in recombinant protein production are to simultaneously reach a high specific production rate, a high cell density, and to ensure product stability. Only few strategies are able to achieve more than one of these at a time (Shokri *et al.,* 2003).

Productivity and protein stability are affected by the growth rate (Rozkov and Enfors, 2004). The specific growth rate is one of the most important process variables influencing the state of micro-organisms during fermentations, mainly because it often affects the biosynthesis of the recombinant products. For control of growth rate, two techniques are typically used: fed-batch and continuous cultivation, both controlled by the ingoing feed rate (Shokri and Larsson, 2004). As opposed to batch fermentation, in fed-batch cultures, it is possible to maintain the pre- and post-induction specific growth rate to provide a desirable metabolic condition to attain maximum productivity of the recombinant protein (Curless *et al.,* 1990; Lim and Jung, 1998; Sanden *et al.,* 2003). For *E. coli*, the specific growth rate should be kept below a certain threshold in order to avoid the accumulation of acetic acid (Jana and Deb,

2005). In order to keep the specific growth rate at a pre-determined value, the most common approach is to apply a feed-forward exponential feeding strategy, where the nutrients required by the culture for achieving the desired growth rate are pre-determined and supplied at any moment. However, it might be difficult to control desired specific growth rate under experimental conditions, due to too many process variables (Lubenova *et al.*, 2003).

High cell density also plays an important role in recombinant protein production. High cell density cultivations start with a growth phase to produce biomass, followed by a production phase. On the cellular level, there are several unfavourable factors associated with the metabolic stress of protein production, *e.g.*, increased cell lysis, accumulation of endotoxins, and increased membrane stiffness (conferring increased resistance to downstream mechanical treatment and osmotic permeabilisation). Fluctuations of oxygen and glucose concentrations during cultivation may affect the production of recombinant proteins because of the induction of the various related stress responses. All these have to be taken into consideration for the optimisation of cultivation conditions (Shokri *et al.*, 2002).

High cell density culture techniques for the periplasmic production of recombinant proteins are less well developed than those for cytosolic production (Choi and Lee, 2004). As a result, for periplasmic recombinant protein production, the relationship between specific growth rate and product formation is poorly understood. Very few studies have addressed the impact of growth or feeding rates specifically for periplasmic production. Early reports showed that secretion efficiencies can show a sharp maximum with respect to the pre-induction growth rate (Curless *et al*., 1994). Recently, it was demonstrated that growth rate influences the composition of the membranes and leakage of periplasmic proteins to the culture supernatant (Shokri *et al*., 2002; and 2003). This observation was in good agreement with a recent report showing that during batch cultivation, recombinant protein was found to migrate into the extra-cellular medium at high growth rates (Dresler *et al.*, 2006).

Influence of proteolysis

Proteolysis has a significant impact on production of recombinant proteins, and yet, little is its importance, little is known about the factors affecting the recognition of recombinant proteins as protease targets. The heat shock protein DnaK, *e.g.*, has been suggested to participate in protein degradation (Sherman and Goldberg, 1992), and its induction by low oxygen and high glucose may therefore increase the proteolysis of recombinant protein. In order to avoid or minimise the problem of proteolysis, proteolytically sensitive motives or structures may be removed by genetic engineering of the recombinant protein (Rozkov and Enfors, 2004).

Little is known about the influence of cultivation conditions on proteolysis of recombinant proteins in the fed-batch cultures. Fermentation scale-up was shown to have a significant impact on the production and proteolysis of the recombinant ZZT2 (Rozkov and Enfors, 2004). The final accumulation level of the product declined to 35 mg g^{-1} of biomass in the large-scale compared to 50 mg g^{-1} level in the laboratory-scale expression, which was explained by a combination of a higher proteolysis rate and declining synthesis rate (Rozkov and Enfors, 2004).

During periplasmic production of recombinant proteins in *E. coli*, there is additional potential for undesired proteolytic degradation. Several periplasmic and outer membrane proteases have been described in *E. coli*, including Prc (*prc*), DegP (*degP*), OmpT (*ompT*), and Protease III (*ptr3*) (Baneyx and Georgiou, 1991, 1992; Meerman and Georgiou, 1994). Genes that encode periplasmic proteases have been deleted to minimise proteolysis of recombinant proteins (Baneyx and Georgiou, 1991, 1992; Meerman and Georgiou, 1994). Analysis of strains cultured in high cell density fermentations showed that the combination of mutations in *degP*, *prc* and *spr* was necessary for the cells to produce high levels of the desired antibody fragment (Chen *et al*., 2004). However, a downside of this was that these mutations adversely affected cell physiology. Mutations in prc lead to cell envelope defects and render cells temperature-sensitive (Hara *et al.*, 1991 and 1996), and in fermentors, a sudden cessation of growth and lysis of prc strains was observed (Chen *et al.*, 2004).

1.3.3 Periplasmic production of antibody fragments

For medical applications, scFv's are needed in large amounts, and the ability to produce high yields in *E. coli* has gained considerable interest (Humphreys, 2003). The secretion of most scFv and Fab fragments leads to the formation of insoluble aggregates in the periplasm (Robert *et al.*, 2006). Functional periplasmic production of antibody fragments can also be limited by the sequence-dependent efficiency of periplasmic folding (Skerra and Plückthun, 1991; Knappik *et al.*, 1993; Knappik and Plückthun, 1995; Ulrich *et al.*, 1995; Forsberg *et al.*, 1997). In some instances the expression of antibody fragments is accompanied by cell lysis (Skerra and Plückthun, 1991; Knappik *et al.*, 1993; Sletta *et al.*, 2004). In other cases, neither translation efficiency nor stability but other unknown factors impeded periplasmic expression of antibody fragments (Oelschlaeger *et al.*, 2003). Periplasmic expression of scFv-phOx also leads to cell lysis (Sletta *et al.*, 2004), and controlled expression rates are essential to achieve high product yields. After process optimisation, a high volumetric yield (1.2 g L^{-1}) of this scFv was obtained in high cell density cultures of *E. coli*.

1.4 Production by means of renaturation of inclusion bodies

The over-expression of recombinant proteins in *E coli* frequently yields inactive protein, aggregated in the form of IB's (Fig. 1.4). These IB's are very dense amorphous particles (Bowden *et al.*, 1991) containing almost exclusively the over-expressed protein (Fig. 1.4) (Rudolph *et al.*, 1997; Villaverde and Carrió, 2003; Singh and Panda, 2005). Therefore, a first purification of the recombinant protein can be achieved by IB isolation. After isolation, IB's are generally solubilised by high concentrations of chaotropic agents such as guanidinium hydrochloride (GdnHCl) or urea. GdnHCl is usually preferred for two reasons. First, GdnHCl is a strong chaotroph, which solubilises even extremely sturdy IB's that are resilent to solubilisation by urea. Second, urea solutions may contain isocyanate leading to carbamylation of free amino groups of the polypeptide, especially upon long term incubation at alkaline pH values (Rudolph and Lilie, 1996). In addition to the solubilising agent, the presence of low molecular weight thiol reagents such as dithiothreitol (DTT) or 2-mercaptoethanol is generally required. These substances reduce non-native inter- and intra-molecular disulfide bonds possibly formed by air oxidation during cell disruption and will keep the cysteines in their reduced state (Rudolph *et al.*, 1997; De Bernardez Clark *et al.*, 1999; Jungbauer and Kaar, 2007). Finally, the pH must be reduced before the removal of the reducing agent from the solution containing the solubilised protein to prevent the formation of undesired disulfide bonds. IB isolates collected after maximum cell disruption usually are relatively homogeneous. Therefore, the proteins can be renatured directly after solubilisation without further purification of the solubilised denatured protein (Rudolph and Lilie, 1996).

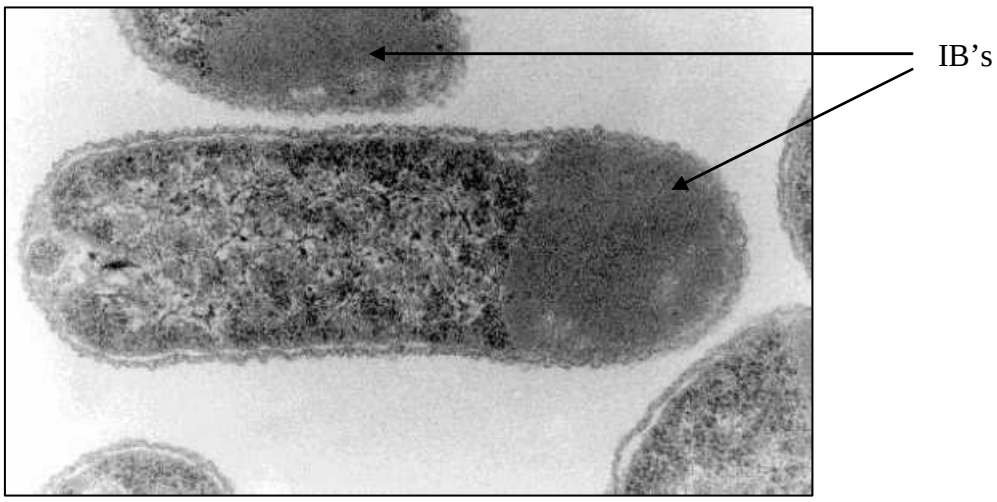


Fig. 1.4: *E. coli* cell filled with IB's (adopted from Marston, 1986).

Kinetic competition between folding and aggregation

Upon *in vitro* folding, misfolding as well as aggregation compete with correct folding. A kinetic model has been put forward to explain this (Kiefhaber *et al.*, 1991). As per this hypothesis, aggregation reactions are second- (or higher) order processes, whereas, correct folding is generally determined by first-order reactions. Consequently, an increase of the rate of unproductive aggregation is observed by increasing the concentration of unfolded polypeptide chains. Thus, aggregation predominates upon renaturation above a limiting concentration of denatured protein, and as result, renaturation must often be performed at an extremely high dilution (Rudolph and Lilie, 1996; Middelberg, 2002).

Renaturation followed by formation of mixed disulfide

Folding polypeptide chain experiences massive aggregation problems at an early stage of renaturation (Rudolph and Lilie, 1996). The propensity of the competing precipitation reactions can be greatly reduced by formation of mixed disulfide before renaturation, *e.g.*, glutathione. It is assumed that upon mixed disulfide formation, numerous charged residues are incorporated into the polypeptide chain that reduces extent of precipitation during the early stages of renaturation. Indeed, significant enhancement is observed in renaturation yield of recombinant tissue type plasminogen activator (r-tPA) produced as IB's in *E. coli* by formation of mixed disulfide with glutathione as a starting material (Rudolph *et al.*, 1985a and 1985b).

One of the major obstacles in maximising protein renaturation yields is the thermodynamic stability of a protein under given experimental conditions. Reduction of the two intra-chain disulfide bonds may lead to lack of stability of the native proteins, resulting in aggregation of scFv's (Martineau and Betton, 1999). This conclusion was drawn from the observation that it is not possible to refold most antibody fragments *in vitro* under reducing conditions, and that the thermodynamic stability of an oxidised scFv or V_L domain is comparable to the loss of free energy resulting from the reduction of two disulfide bonds in other protein models (Goto and Hamaguchi, 1979; Betz 1993; Martineau and Betton, 1999).

1.4.1 Techniques for protein renaturation

Direct dilution

The simplest renaturation procedure is to dilute the concentrated protein-denaturant solution into a renaturation buffer that allows the formation of the native structure of the protein. Most frequently, the final protein concentration after dilution is in the range of 1-100 μg mL^{-1} in order to favour the productive renaturation instead of the unproductive aggregation pathway. Though ideal at laboratory scale, this technique has serious drawbacks during scale-up, as

huge renaturation vessels and additional cost-intensive concentration steps are required after renaturation. A major improvement of this technique was the development of a method where the solubilised, denatured protein is added in pulses or continuously into the renaturation buffer (Rudolph and Fischer, 1990; Katoh and Katoh, 2000). By stepwise addition of the solubilised denatured protein at time intervals that are sufficiently large for the polypeptides to fold past the aggregation-prone early stages on the folding pathway, the concentration of unfolded protein and folding intermediates is kept sufficiently low at any given time during the renaturation process. This was demonstrated by increased renaturation yield of proinsulin upon stepwise dilution of denatured protein into the renaturation buffer (Winter *et al.*, 2002b). This technique retains the simplicity of the direct dilution method while considerably increasing the final concentration of the renatured protein.

Membrane controlled denaturant removal

Another technique to transform the solubilised and unfolded protein to native structure is the utilisation of dialysis and diafiltration systems for denaturant removal (Tsumoto *et al.*, 1998; Takemura *et al.*, 2000; Umetsu *et al.*, 2003). Stepwise removal of denaturant and controlled addition of oxidiant and L-Arginine were found to be the effective conditions to achieve almost complete recovery of functional monomeric scFv from IB's (Tsumoto *et al.*, 1998). However, often these techniques cause more aggregation during renaturation compared to the direct dilution method (Gu *et al.*, 2002).

Chromatographic methods for protein renaturation

The basic idea of matrix-assisted protein renaturation is to make use of the specific interaction of the protein that is to be renatured with a chromatography material in order to prevent non-specific aggregation. This method sometimes also referred as on column renaturation. To achieve this goal, three prerequisites must be fulfilled: (1) protein binding to the matrix must be possible under denaturing conditions; (2) the interaction between the protein and the matrix during the renaturation step must allow for the flexibility of the polypeptide chain that is required for proper structure formation; and (3) the interaction must be tight enough to prevent inter-chain bonding, which might result in aggregate formation. This can be achieved by ion exchange resins (Creighton 1985) or sometimes by hydrophobic interaction chromatography (West *et al.*, 1998; Vallejo and Rinas 2004). An interesting approach to matrix-assisted protein renaturation was described by Berdichevsky *et al.* (1999). In this case, the folding of an scFv fused to a cellulose binding domain (CBD) was analysed in the matrix-bound form and compared to standard renaturation in solution. Following solubilisation of the fusion protein IB's in urea, the CBD recovered its native structure while the Fv part was still

unfolded. After binding to crystalline cellulose, the antibody fragment could then be refolded while still bound to the matrix via its fusion partner, the native CBD.

Size exclusion chromatrography (SEC) can also be used for renaturation. Elution with the renaturation buffer results in a renatured protein with a considerably higher concentration compared to that reached by the simple dilution technique (Müller and Rinas, 1999; Batas and Chaudhuri, 1999; Fahey and Chaudhuri, 2000). Sometimes however, this technique may result in poor separation among different folding intermediates thereby boosting protein precipitation (Müller and Rinas, 1999).

Artificial chaperone-mediated renaturation

Cyclodextrins are known to have destabilising effect on the native state of proteins (Cooper, 1992). This can be explained by a shift in the equilibrium in favour of the unfolded state, which is caused by the direct interaction of the cyclodextrins with buried hydrophobic side chains of the protein. Detergent-based micellar systems mimic the two-step mechanism of chaperone-assisted protein folding. The capturing step is performed by diluting the denatured protein into a detergent solution, which prevents protein aggregation through the formation of mixed protein-detergent micelles (Rozema and Gellman, 1995; 1996a; and 1996b). The release of the folding-competent protein is subsequently initiated by the addition of cyclodextrins (Rozema and Gellman, 1995; 1996a; and 1996b).

Renaturation additives

In order to suppress aggregation, enhance the solubility of unfolded species, and for stabilisation of native species, additives are essential components of renaturation buffers. Additives can be classified depending on working optimum concentration. Surfactants and detergents are effective at very low concentrations, while salts, amino acids, and solvents (*e.g.* glycerol) are effective at higher concentrations, sometimes up to their solubility limit. L-Arg is nowadays the most commonly used renaturation-aiding agent (De Bernardez Clark *et. al.*, 1999; Vallejo and Rinas 2004; Lange and Rudolph, 2005). It impedes aggregate formation by enhancing the solubility of folding intermediates, presumably by shielding hydrophobic regions of partially folded chains (De Bernardez Clark *et. al.*, 1999; Vallejo and Rinas 2004; Lange and Rudolph, 2005). In addition, it has been shown that numerous other low molecular weight additives such as detergents, protein-stabilising agents such as glycerol or even low residual concentrations of denaturants improve renaturation yields by enhancing solubility of unfolded species, stabilisation of native species and suppressing aggregation (De Bernardez Clark *et. al.*, 1999; Vallejo and Rinas 2004; Lange and Rudolph, 2005). Because of the relevance to the work, this aspect is briefly reviewd in section 1.4.2.

Other methods for the enhancement of renaturation yield

In addition to these well-studied renaturation techniques, pressure-induced folding (Gorovits *et al.*, 1998) as well as a temperature leap technique (Xie and Wetlaufer, 1996) have also been reported. For bovine carbonic anhydrase II (bcaII) renaturation was optimal when first induced at 4°C for 120 min, followed by a final folding phase after warming the solution to 36°C. During the renaturation of bcaII, an early intermediate is formed that is still susceptible to aggregation. If renaturation is performed at elevated temperatures, this intermediate is largely converted to aggregates. If, however, the initial stage of renaturation is allowed to proceed at lower temperatures, the early intermediate rearranges to a late intermediate that is more resistant to aggregation and can then be renatured at a higher rate at higher temperatures (Xie and Wetlaufer, 1996).

1.4.2 Co-solvents and additives

Proteins are inherently unstable in aqueous solution and are degraded by a variety of routes, the most common of which is aggregation. Aggregation is the assembly of non-native protein conformations into multimeric states, often leading to phase separation and precipitation. It has been observed that by adding low molecular weight components, such as salts, sugars, or polyols, to protein solutions, the propensity of a protein to aggregate can often be affected significantly (Wang, 1999; Lange and Rudolph, 2005). Unfortunately, because proteins are diverse in chemistry and structure, additives that work well for a particular protein may not work universally.

Timasheff and co-workers have extensively characterised the interaction of co-solvents with proteins by using a 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 the determination of preferential interaction, utilising high-precision densitometry and differential refractometry. The co-solvent concentration inside and outside the dialysis bag is determined. This allows performing affinity measurements of one particular co-solvent in relation to a protein. In those cases where co-solvent becomes more concentrated inside the dialysis bag, the effect is called preferential binding. In cases where the concentration is reduced inside the dialysis bag, the effect is called preferential hydration, which is equivalent to preferential exclusion of the co-solvent (Fig. 1.5 and 1.6).

Preferential exclusion of co-solvent increases the free energy of the whole system. According to Le Chatelier's principle, the system tries to move towards the lower free energy (Fig. 1.5). In this case, this means that the equilibrium is driven towards a smaller exclusion volume for

the native protein. This explains why co-solvents, which experimentally show preferential exclusion, stabilise the native state and suppress unfolding of the protein. The salting-out effect of some compounds on proteins works in a similar way. The salted-out precipitates of protein are excluded from water.

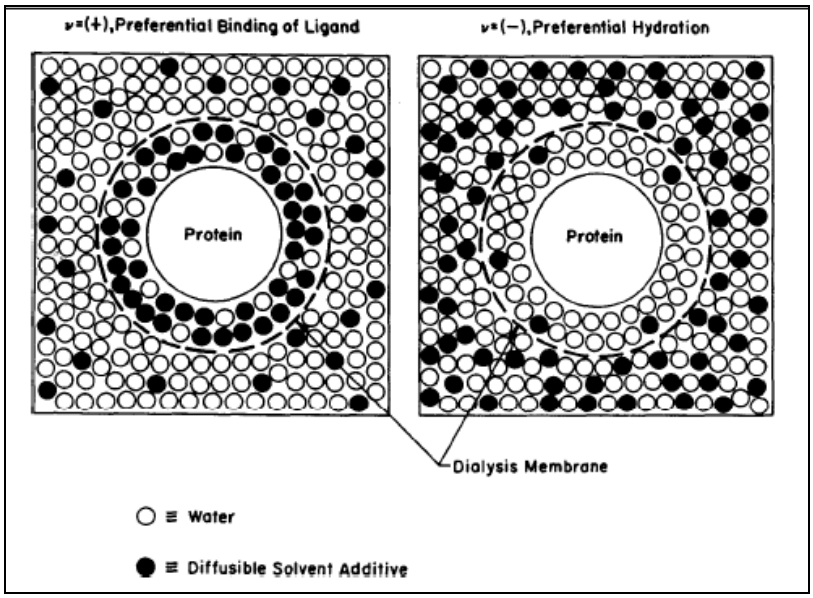


Fig. 1.5: Schematic representation of preferential binding and preferential hydration (adopted from Timasheff, 1992a).

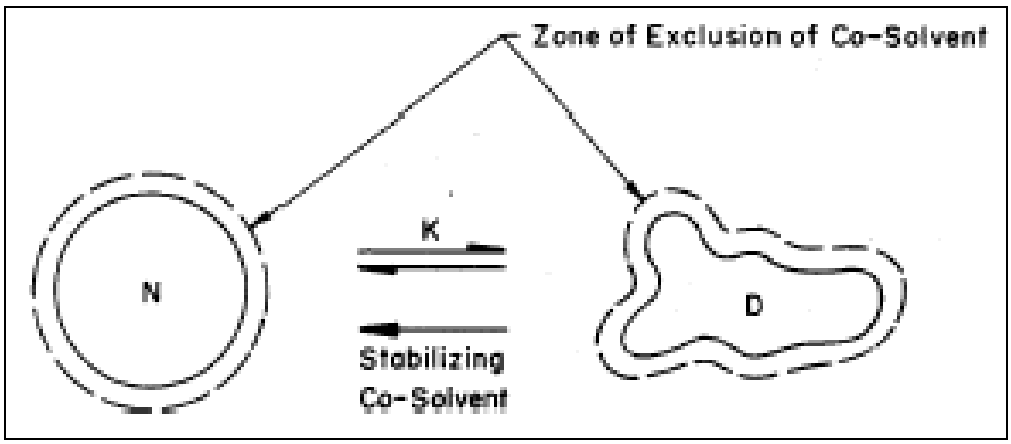


Fig. 1.6: Visualisation of the zone of exclusion, which becomes bigger when the protein is, unfolded (adopted from Timasheff, 1992a).

Two basic types of mechanisms contribute to co-solvent exclusion: (1) Interactions are determined by solvent properties. In this case, a protein remains inert towards the additive and only presents a surface. Interactions between the protein and co-solvent are not affected by changes in the pH or changes of the concentration of co-solvent. This type of interaction is caused by steric exclusion of water from the protein surface or through effects caused by increased surface tension; (2) The chemical nature of the protein surface determines the

interactions, attractive as well as repulsive. The effects of most co-solvents cannot be exclusively assigned to either mechanism, but show characteristics of both.

Some salts, which are well known for their ability to salt-out proteins, have the effect of stabilising proteins in solution. Through combinations of different ions and counter ions it has been determined that each ion contributes separately. The measure of strength of salts was documented long back and is commonly referred to as "Hofmeister series" (Hofmeister, 1888). It describes the capability of cations and anions to salt-out proteins. 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 stabilisation of the native protein structure can be well explained by the above-mentioned effects, it is not possible to predict the effect on denatured proteins, since they are not accessible to characterisation without addition of co-solvents (denaturants). If a co-solvent 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, stabilising the protein. It is not possible to make a reliable prediction on how a given co-solvent will affect the equilibrium between native and denatured protein. Direct interactions between protein and co-solvents may occur, which may be stronger than the above-mentioned effects.

Ionic Liquids as renaturation additives

Ionic liquids (IL's) are organic salts that are liquid at temperatures below 100°C (Huddleston *et al.*, 2001). In biotechnology, they have been utilised as mixtures with water especially in the enzymology of lipases (Kragl *et al.*, 2000; Schöfer *et al.*, 2001; Wehofsky *et al.*, 2008). Lipases are stable and active in these solvents and the hydrophobic substrates of lipases are soluble in IL's. Besides lipases, different proteases have also been shown to be active in IL's (Kragl *et al.*, 2000), even if IL's were used as pure solvents. These salts generally consist of combinations of organic cations, namely derivatives of N, N' substituted imidazolium, N-substituted pyridinium, tetraalkyl ammonium, and tetraalkyl phosphonium, and either organic or inorganic anions. Their solution properties are amenable to be engineered by changes in substitution patterns. A recent report described a new family of biocompatible IL's based on the dihydrogen phosphate anion (Fujita *et al.*, 2005); these IL's were able to dissolve substantial quantities of cytochrome c and the protein was shown to retain its structure and activity. One feature of these solutions was the fact that the protein retained its structure to much higher temperatures than in aqueous solution.

Besides the above-mentioned applications, IL's have also been demonstrated to be useful as additives for protein renaturation (Summers and Flowers, 2000, Rudolph *et al.,* 2005). Tetraethyl and tetrabutyl ammonium nitrate enhance renaturation yields of hen egg lysozyme by suppressing aggregation when used as co-solvents, whereas the pure IL's were found to denature the protein (Summers and Flowers, 2000). r-tPA could be renatured with increasing yields in solutions containing the 1-butyl-4-methyl-pyridinium tetrafluoroborate (Rudolph *et al.,* 2005).

Preferentially excluded co-solvents tend to stabilise the native state of proteins while promoting aggregation ("salting out"). IL's containing the phosphate anion belong to this category (Fujita *et al.,* 2005). Preferentially bound co-solvents, in turn favour protein denaturation and solubility ("salting in"). IL's that act as suppressors of aggregation, *e.g.,* ethyl ammonium nitrate, fall into this second category.

1.4.3 Redox shuffling system: effect of aromatic thiols

Cysteine residues of cytosolic proteins are usually reduced, while extra-cellular proteins usually have cysteine residues that are oxidised to form intra-molecular disulfide bridges (Collet *et al.,* 2002). Most therapeutically relevant proteins require correctly formed disulfide bonds to be active. The majority of inclusion body protein is completely misfolded. Therefore, suitable measures need to be taken during renaturation for correct folding.

Metal ion-catalysed 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 (Jacob *et al.,* 2006). A more commonly used strategy is the use of oxido shuffling systems, which promote oxidation of free sulfhydryl groups as well as disulfide rearrangement for the formation of correct disulfide bonds.

Oxido shuffling systems consist of an oxidising and a reducing sulfhydryl component. Mixtures of reduced (GSH) and oxidised (GSSG) glutathione are used most 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 oxidised 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 and Lilie, 1996; Lilie *et al.,* 1998).

The thiolate anion is the active species in thiol-disulfide exchange. Thus, the pH of renaturation buffers must lie in the range of the pK_a of the thiol group (*i.e.*, between pH 7.5 and pH 9.5). This might be a potential problem for proteins for which such slight to moderate alkaline conditions are unfavourable. Glutathione, mercaptoethanol, and almost all other aliphatic thiols have thiol pK_a values above 7.0. In comparison, aromatic thiols have thiol pK_a values between 4.0 and 7.0 and are more reactive than aliphatic thiols of similar pK_a values towards aromatic disulfides.

Over the past few years, several low-molecular-weight thiol compounds have been designed with the aim of improving thiol-disulfide exchange during the oxidative renaturation of proteins. For example, the synthetic dithiol (G)-trans-1,2-bis(2-mercaptoacetamido) cyclohexane (Vectrase P), which has a pK_a value of 8.3 and an $E^{0'}$ value of -0.24 V, was designed to mimic the properties of the active site of protein disulfide isomerases (Woycechowsky *et al.*, 1999). This compound improved the activation of scrambled RNAse A more efficiently than comparable monothiols. The synthetic dithiol compound was also reported to improve the renaturation yield of human proinsulin (Winter *et al.*, 2002). Recently, various aromatic monothiols, characterised by low thiol pK_a values, were tested for their effect on rate and yield of the oxidative renaturation of scrambled RNAse A (Gough *et al.*, 2002 and 2003). The rate of RNAse A renaturation was increased with decreasing thiol pK_a value.

1.4.4 Production of antibody fragments by *in vitro*-renaturation

The advantages and disadvantages of periplasmic and cytoplasmic expression of recombinant proteins in general and of antibody fragments in *E. coli* have been extensively discussed (Wulfing and Pluckthün, 1994, Martineau *et al.*, 1998; Schirrmann *et al.*, 2008). Functional expression in the cytoplasm of *E. coli* has been improved by using mutant genes coding for thioredoxin reductase and gluthathione oxidoreductase (Jurado *et al.*, 2002). These mutant cells create an oxidising cytoplasm capable of forming disulfide bridges in proteins. As an alternative, the functional production yields of antibody fragments in the cytoplasm can also be significantly improved by co-expression with chaperones and foldases or by a fusion protein strategy. In some instances, where antibody fragments are sufficiently stable without the conserved disulfide bonds (Proba *et al.*, 1997; 1998) and, thus, their folding is possible under the redox conditions of the cytoplasm, functional expression can be achieved without resorting to *in vitro*-renaturation. Such disulfide-free antibody fragments are also important tools for the intrabody technology where antibody fragments fold in the reducing environment of the cytoplasm (Worn and Pluckthün, 1998; Ewert *et al.*, 2004).

The expression of antibody fragments as cytoplasmic IB's, however, still continues to represent the most straightforward and generally applicable methodology. Renaturation of scFv's can be achieved by gradual removal of denaturant by stepwise dialysis (Tsumoto *et al.,* 1998, Takemura *et al.,* 2000; Umetsu *et al.,* 2003). However, these protocols have not been found to be applicable to other tested scFv constructs, where severe aggregation problems were observed (Gu *et al.,* 2002; Sinacola and Robinson, 2002). Successful matrix-assisted renaturation (Stempfer *et al.,* 1996a, and 1996b) of scFv fragments was reported recently (Gu *et al.,* 2002, Yang *et al.,* 2005; Guo *et al.,* 2006). For renaturation by dialysis as well as for matrix-assisted renaturation, process scale-up may present problems due to sample volume limitations. For these reasons, dilution protocols for the *in vitro*-renaturation of antibody fragments remain in widespread use.

Production of recombinant scFvOx as IB's and its renaturation offers potential advantages over periplasmic expression due to relatively higher protein synthesis levels. Increasingly higher demands of recombinant therapeutic proteins have clearly indicated the need for systematic optimisation of efficient renaturation. In the present work, in efforts to maximise the yield of recombinant protein, the effects of renaturation additives like Ionic Liquids and redox buffers such as aromatic thiols were explored.

Successes and challenges of antibody fragment production from inclusion bodies

The cytoplasmic approach benefits from a high expression level of antibodies using a strong promoter (*e.g., T7* promoter). An advantage of the cytoplasmic expression approach is that following lysis of bacterial cells, IB's can easily be separated from other cellular components because of their large size and high density. Moreover, this approach is useful for producing antibody-based fusion proteins such as immunotoxins that might be toxic for bacterial cells (Buchner *et al.,* 1992b) or antibody fragments that are unstable due to intracellular degradation when expressed in a soluble or secreted form. However, correct *in vitro*-renaturation and purification of functional product is a complex and time-consuming process, requiring expertise and involving many steps. Problems and limitations commonly encountered with this approach, for antibodies in particular, are (1) difficulties in predicting the tendency of different sequences to form IB's and their susceptibility to proteases (Hoogenboom *et al.,* 1992; Maynard and Georgiou, 2000), (2) renaturation efficiency which is highly variable depending on the specific antibody fragment with yields varying from 10-40% for Fab and Fv fragments (Buchner *et al.,* 1991; Duenas *et al.,* 1995; Umetsu *et al.,* 2003), and (3) the need for separation of correctly and incorrectly folded fractions of the protein (Huston *et al.,* 1991; Fernandez, 2004).

1.5 Objectives of the work

In this thesis, different production strategies for obtaining a single chain variable fragment recognising the hapten oxazolone, scFvOx, in biologically active form were explored.

Periplasmic expression of recombinant proteins in *E. coli* offers the opportunity to obtain proteins in functional form, albeit at a lower expression rate. Therefore one of the objectives of this thesis was to establish a strategy for functional, periplasmic expression of the recombinant protein. In addition, the effect of the co-expression of the helper protein DsbA and its two active site variants, as well as of different expression vectors on the functional expression was explored. It was planned to implement this approach at the bioreactor scale and study the effect of cultivation parameters on productivity and product stability.

The second approach aimed at the improved production of scFvOx by *in vitro*-renaturation after expression into cytosolic IB's. A new renaturation strategy using IL's as renaturation additives was explored. The effect of N'-alkyl N-methyl imidazolium and N'-(ω-hydroxyalkyl) N-methyl imidazolium chlorides as well as pyridinium and ammonium-derived compounds was systematically studied.

In order to establish optimal protocol for *in vitro*-renaturation of scFvOx, a systematic investigation of thermodynamic and pH dependent stability, and the effect of pH value on renaturation of scFvOx was performed. Low molecular weight aromatic thiols were explored as redox reagents for protein renaturation. Simultaneously, the effect of pH on renatutation in the presence of aromatic thiols was investigated. In order to understand the mechanism of how aromatic thiols effect protein renaturation, scFvOx renaturation kinetics were studied. Based on the information from previous renaturation experiments, protein characterisation, stability studies and on the effect of pH on renaturation, an improved renaturation strategy for scFvOx was proposed.

2. Materials and Methods

2.1 Materials

Instruments

Instruments	Manufacturer	Origin
Äkta FPLC	Amersham Biosciences	Uppsala, Sweden
Avanti J-20 and Avanti J-25 preparative centrifuges	Beckman	Fullerton, CA, USA
BIAcore	Biacore AB	Uppsala, Sweden
Bioreactor process control system MFCSwin	B. Braun Biotech International	Melsungen, Germany
Bioreactor, BIOSTAT C	B. Braun Biotech International	Melsungen, Germany
EM 900 transmission electron microscope	Zeiss	Jena, Germany
Filtron Minisette, cross-flow device	PallGelman	Ann Arbor, MI, USA
Fluorescence spectrometer	HORIBA Jobin Yvon	Munich, Germany
French press, homogeniser	Gaulin	Unna, Germany
Gel electrophoresis apparatus	Pharmacia Biotech	Uppasala, Sweden
Gene Pulser electroporation apparatus	BioRad	Munich, Germany
Gyncotek Analytical HPLC-System	Dionex	Germering, Germany
J-710 CD spectrometer	Jasco	Gross Umstadt, Germany
LiCor 4000 DNA sequencer	LiCor	Lincoln, NE, USA
Peristaltic pump (type 101U/R)	Watson Marlow Ltd	Rommerskirchen, Germany
pH-Meter	WTW	Weilheim, Germany
Semidry transfer cell	BioRad	Munich, Germany
Shaking incubator, Certomat BS-T	Sartorius	Goettingen, Germany

Sunrise microtiter plate reader	Tecan	Crailsheim, Germany
Thermocycler	Eppendorf	Duesseldorf, Germany
Trans-illuminator table	KAISER Fototechnik	Buchen, Germany
Ultra-Turmax T25	Janke and Kunkel, IKA Labortechnik	Staufen, Germany
UV/VIS spectrophotometer	Pharmacia Biotech	Uppsala, Sweden

Various materials

Equipments	Provider	Origin
1 mL and 5 mL pre-packed SP Sepharose FF Äkta columns	Amersham Biosciences	Uppsala, Sweden
Amicon	Millipore	Billerica, MA, USA
Cassette or screen for Western blot	Roche Diagnostics	Penzberg, Germany
CD and fluorescence cuvettes	Hellma	Darmstadt, Germany
Developer and fixer solutions	Sigma Aldrich	Steinheim, Germany
Dialysis bags and clamps	Spectrum	Houston, TX, USA
Electroporation cuvettes, Gene Pulser II	Biorad	Munich, Germany
ELISA plates	NUNC	Roskilde, Denmark
Horizontal mini electrophoresis chamber	Roth	Karlsruhe, Germany
Lumi film (chemiluminescent detection film)	Roche Diagnostics	Penzberg, Germany
Plastic foil (Barrier food wrap)	Saran (Dow)	Hamburg, Germany
C4, C8, C18, di-phenyl RP-HPLC columns	Vydac	Hesperia, CA, USA
Slide-A-Lyzer, Mini dialysis unit	Roth	Karlsruhe, Germany

TSK Gel Alpha column, size exclusion chromatography	TOSOH	Tokyo, Japan

Chemicals and kits

Chemical	Provider	Origin
1 kb DNA ladder	New England Biolabs	Frankfurt, Germany
ABTS substrate	Roche Diagnostics	Penzberg, Germany
Acetonitrile	Roth	Karlsruhe, Germany
Aromatic thiols	TCI	Tokyo, Japan
Benzonase	Merck	Darmstadt, Germany
Bradford-reagent	BioRad	Munich, Germany
Blocking reagent	Roche Diagnostics	Penzberg, Germany
Bromphenol blue	Roth	Karlsruhe, Germany
BSA	Merck	Darmstadt, Germany
Dithiotheitrol (DTT)	ICN Biomedicals	Irvine, USA
Gluthathione, oxidised (GSSG)	Boehringer Mannheim	Mannheim, Germany
Gluthathione, reduced (GSH)	Boehringer Mannheim	Mannheim, Germany
Guanidinium hydrochloride (GdnHCl)	Nigu Chemie	Waldkraiburg, Germany
Ionic Liquids	As indicated in section 2.1.2	-
L-Arginine hydrochloride	Ajinomoto	Tokyo, Japan
Lysozyme	Merck	Darmstadt, Germany
Mutagenesis primers	Metabion	Martinsried, Germany
Oxazolone	Sigma Aldrich	Steinheim, Germany
PEG, 50%	Roth	Karlsruhe, Germany
Primary antibody, anti-*c-myc* tag, from mouse	Roche Diagnostics	Penzberg, Germany
QIA molecular biological kits	Qiagen	Hilden, Germany
Quick change kit	Stratagene	La Jolla, CA, USA

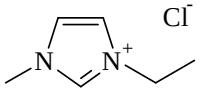
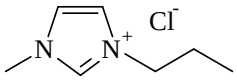
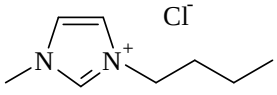
SDS-PAGE LMW	Amersham Biosciences	Uppsala, Sweden
Secondary antibody	Chemicon	Temecula, CA, USA
SequiTherm EXCEL II DNA sequencing kit	Epicentre	Madison, WI, USA
T4-DNA-Ligase, *Pfu*-DNA-Polymerase, restriction enzymes	New England Biolabs	Frankfurt, Germany
Urea	ICN Biomedicals	Irvine, CA, USA

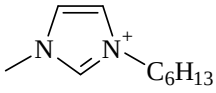
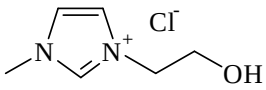
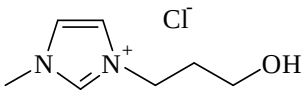
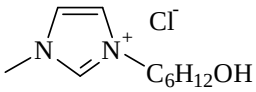
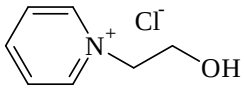
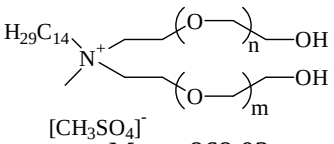
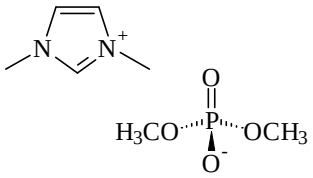
All reagents were of analytical grade or of higher purity, and Millipore water was used for buffer and media preparations.

2.1.1 Ionic Liquids

The IL's employed in this study were obtained from various sources, as indicated in table 2.1, and used without further purification. In all cases they were more than 98% pure, as per product specifications.

Table: 2.1

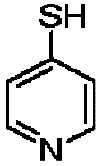
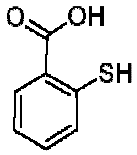
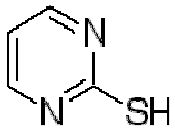
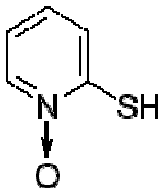
Compound	Formula	Source
N-Ethyl-N'-methyl-imidazolium chloride (EMIM Cl)	 M_r = 146.62	TCI, Tokyo, Japan
N-Propyl-N'-methyl-imidazolium chloride (PMIM Cl)	 M_r = 160.64	Solvent Innovation, Cologne, Germany
N-Butyl-N'-methyl-imidazolium chloride (BMIM Cl)	 M_r = 174.67	DEGUSSA, Trostberg, Germany

Compound	Formula	Source
N-Hexyl-N‘-methyl-imidazolium chloride (HMIM Cl)	 M_r = 202.72	Solvent Innovation, Cologne, Germany
N-(2-Hydroxyethyl)-N‘-methyl-imidazolium chloride (OH-EMIM Cl)	 M_r = 162.62	DEGUSSA, Trostberg, Germany
N-(3-Hydroxypropyl)-N‘-methyl-imidazolium chloride (OH-PMIM Cl)	 M_r = 176.64	DEGUSSA, Trostberg, Germany
N-(6-Hydroxyhexyl)-N‘-methyl-imidazolium chloride (OH-HMIM Cl)	 M_r = 218.72	Solvent Innovation, Cologne, Germany
N-(2-Hydroxyethyl)-pyridinium chloride (OH-EPY Cl)	 M_r = 159.61	DEGUSSA, Trostberg, Germany
cocosalkyl penta-ethoxy-methyl ammonium methyl-sulfate (ECOENG™-500)	 $<M_r>$ = 868.03	Solvent Innovation, Cologne, Germany
N,N‘-Dimethyl-imidazolium dimethylphosphate (ECOENG™-1111P)	 Mr =222.18	Solvent Innovation, Cologne, Germany

2.1.2 Aromatic thiols

The aromatic thiol compounds used in this study were procured from TCI (Tokyo, Japan) as indicated in table 2.2 and were used without further purification. Stock solutions of the thiol compounds were prepared in 5% methanol at the concentration of 10 mM.

Table: 2.2

Compound	Formula	Molecular weight	Thiol p*Ka*[a]	Provider
4-Mercaptopyridine (4-MPYN)		111.16	2.21 (11.91)	TCI, Tokyo, Japan
Thiosalicylic acid (ThioSA)		154.19	(3.5) 8.2 [(b)]	TCI, Tokyo, Japan
2-Mercaptopyrimidine (2-MPYMN)		112.15	1.65 (8.14)	TCI, Tokyo, Japan
2-Mercaptopyridine N-oxide (2-MPYN-Ox)		127.16	-0.48 (5.44)	TCI, Tokyo, Japan

(a) pK_a values are given for the thiol proton; values in brackets correspond to the pK_a values of other protonable groups. All pK_a's were calculated using Advanced Chemistry Development Software V8.14 for Solaris (ACD/Labs, Toronto, Canada) in SciFinder Scholar v.2006, except (b) Gough *et al.*, 2006.

2.1.3 Strains and plasmids

For periplasmic production: *E. coli* strains used in this work are shown in Table 2.3. The expression construct pHEN1scFvOx, containing the coding DNA sequence of recombinant scFvOx fused to sequences coding for an N-terminal *pelB* signal sequence and a C-terminal *c-myc* tag, was used for this work (Fiedler and Conrad, 1995) (Table 2.4). This vector expresses the desired protein under control of the *lac* promoter, and carries a resistance against ampicillin. In case of the plasmid pET20(+)scFvOx, the *scFvOx* gene along with an N-

terminal *pelB* signal sequence was cloned under the control of a *T7* promoter; the vector carries resistance against ampicillin (Table 2.4) (courtesy of Dr. Brigitte Söhling). During these studies, an scFvOx protein standard obtained from plant seeds served as positive control (courtesy of Dr. Udo Conrad). This protein was additionally fused with an endoplasmic reticulum retention signal sequence (Fiedler *et al.*, 1997).

These vectors were generally co-expressed with the helper plasmid pUBS520 (Brinkmann *et al.*, 1989), which bears the p15A replication origin and a kanamycin resistance. This vector also carries the *dnaY* gene encoding the tRNA for the arginine codons AGA and AGG, which are rare in *E. coli* and thus often limit expression of genes with these codons (Brinkmann *et al.*, 1989).

For cytosolic production: The gene for scFvOx was fused at the N- and C-termini to sequences coding for hexa-histidine and *c-myc* tags, respectively. This sequence was inserted between the unique *Nde*I and *Xho*I restriction sites of the bacterial expression vector pET15b(+) (Table 2.4) (courtesy of Dr. Claudia Humbeck). This vector carries resistance against ampicillin and expresses the desired protein under control of the *T7* promoter. ScFvOx protein was expressed in *E. coli* BL21 (DE3) cells that had been co-transformed with the helper plasmid pUBS520.

Table 2.3: E. coli strains used in this work

Strains	Genotype and/ or description	Source
BL 21(DE3)	*E. coli B F⁻ dcm ompT hsdS*(r_B^- m_B^-) *gal λ*(DE3)	Stratagene, USA
MC4100	*araD139 Δ*(*argF⁻ lac*)*U169 rpsL150 relA1 flbduB5301 deoC1 ptsF25 rbsR*	Prof. F. Baneyx, University of Washington, USA

Table 2.4: Plasmids used in this work

Plasmids	Description	Source
pHEN1scFvOx	scFvOx gene cloned between the *SfiI* and *NotI* restriction sites of vector, *pelB* signal	Fiedler and Conrad, 1995

	sequence, *lac* promoter, Amp^R	
pUBS520 (Appendix A)	*dnaY* gene, *trc* promoter, Kan^R	Brinkmann *et al.*,1989
pET15b(+)scFvOx (Appendix B)	scFvOx gene cloned between *NdeI* and *BamHI* restriction sites of vector, *T7* promoter, Amp^R	Dr. Claudia Humbeck
pET20b(+)scFvOx (pScPelB) (Appendix C)	scFvOx cloned between *NdeI* and *Hin*dIII restriction sites of vector, *pelB* signal sequence, *T7* promoter, Amp^R	Dr. Brigitte Söhling
pUBS520DsbA(pDsbA3) (Appendix A)	*dsbA* gene was inserted in *Bam*HI restriction site of vector, *trc* promoter, Kan^R	Dr. Jeannette Winter
pUBS520DsbAC33S and pUBS520DsbAC33A (pDsbA3) (Appendix A)	The C33S, C33A mutants of *dsbA*, *trc* promoter, Kan^R	Generated in this work

2.2 Molecular biological methods

2.2.1 Plasmid isolation

Plasmid DNA was isolated from 5 mL cultures of the respective *E. coli* strains grown overnight in LB media at 30°C. Plasmid preparations were performed using the QIAprep Spin Miniprep kit according to the protocol recommended by the manufacturer.

2.2.2 Agarose gel electrophoresis

The test for the presence of plasmids as well as the separation of DNA fragments was carried out on 1% (w/v) agarose gels in TAE buffer (Tris/acetate, pH 8.0, containing 1 mM ethylene diamine tetraacetic acid (EDTA)). DNA samples were mixed with loading buffer (40% (w/v) glycerol, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.1 M EDTA, 0.2% (w/w) bromophenol blue) and loaded on the gel. The gels were run in a horizontal mini electrophoresis chamber under voltages between 50 V and 80 V. The 1 kb DNA ladder (New England Biolabs, Frankfurt, Germany) served as standard. The gel was stained for 20 min with an ethidium bromide solution (5 μg mL^{-1}), and the DNA was visualised on a UV trans-illuminator.

2.2.3 Site directed mutagenesis

The plasmid pUBS520DsbA (courtesy of Dr. J. Winter) had been constructed by inserting the *dsbA* gene with its own signal sequence from pDsbA3 (courtesy of Prof. R. Glockshuber) into the unique *Bam*HI restriction site of pUBS520. Cysteine 33 in the active site of DsbA (codon TGC) was changed to serine (codon AGC) or alanine (codon GCC) by site directed mutagenesis at position 154 using the Quick Change kit (primers are described in Appendix A). The amplified recombinant DNA was transformed into *E. coli* XL1 blue. Plasmids containing the mutant forms of the gene were isolated from overnight cultures and sequenced to confirm the mutation.

2.2.4 DNA sequencing

Sanger's di-deoxynucleotide chain termination method, where single-stranded DNA molecules that differ in length by a single nucleotide can be separated on polyacrylamide gels, was used for sequencing (Sanger *et al.*, 1977). The SequiTherm EXCELTM II kit was used for the sequencing reaction and the reaction mixtures were analysed on a Li-COR sequencer DNA sequencing system.

2.2.5 Transformation of *E. coli* cells

Preparation of competent cells

LB medium was inoculated with 1% (v/v) of an overnight pre-culture of *E. coli* cells. The culture was incubated at 37°C until the OD_{600} reached about 0.5-0.8. After this, the culture was chilled on ice for 10-20 min, and then centrifuged at 10,000 rpm at 4°C for 10 min. The resulting cell pellet was washed 3 times with ice-cold 10% (v/v) glycerol, and then re-suspended in 0.5 volumes of the same solution. Aliquots of 40 µL were transferred into sterile Eppendorf tubes, shock frozen in liquid nitrogen and stored at -80°C.

Transformation via electroporation

Electroporation was carried out in a Gene Pulser II apparatus. About 1-10 ng plasmid or 10-100 ng of ligation mix were mixed with a 40 µL aliquot of cells, and transferred into the previously chilled cuvette (0.2 cm gap). Electroporation was carried out at 25 µF capacitance, 200 Ω resistance and 2.5 kV voltage. After pulsation, the cells were transferred into 1 mL LB medium and were incubated at 37°C at 650 rpm on a rotary thermo-mixer for 30-60 min. One hundred µL of incubated cells were directly plated on an LB plate containing appropriate antibiotics. The remaining volume was centrifuged at 3000 rpm for 2 min and after removal of about 750 µL supernatant, the remaining cells were re-suspended and plated on a fresh LB agar plate containing the appropriate antibiotics.

2.3 Cultivation media and conditions

2.3.1 Media

Defined medium (Le Thanh, 2005)

Component	Batch medium	Feeding solution
Glucose[a]	5 or 10 g L^{-1}	875 g L^{-1}
$MgSO_4 *7 H_2O$	1.2 g L^{-1}	20 g L^{-1}
KH_2PO_4	13.3 g L^{-1}	-
$(NH_4)_2HPO_4$	4.0 g L^{-1}	-
Citric acid $*H_2O$	1.7 g L^{-1}	-
Ferric citrate	100.08 mg L^{-1}	40.0 mg L^{-1}
$CoCl_2 *6 H_2O$	2.5 mg L^{-1}	4.0 mg L^{-1}
$MnCl_2 *4 H_2O$	15.0 mg L^{-1}	23.5 mg L^{-1}
$CuCl_2 *2 H_2O$	1.5 mg L^{-1}	2.3 mg L^{-1}
H_3BO_3	3.0 mg L^{-1}	4.7 mg L^{-1}
$Na_2MoO_4 *2 H_2O$	2.1 mg L^{-1}	4.0 mg L^{-1}
$Zn(CH_3COO)_2 *2 H_2O$	33.8 mg L^{-1}	16.0 mg L^{-1}
EDTA	14.1 mg L^{-1}	13.0 mg L^{-1}

[a] The initial concentration of glucose for pre-culture and shake flask experiments was 20 g L^{-1}.

Trace element stock solutions (Le Thanh, 2005)

Component	Concentration g L^{-1}
Ferric citrate	12
$CoCl_2 *6 H_2O$	2.5
$MnCl_2 *4 H_2O$	15
$CuCl_2 *2 H_2O$	1.5
H_3BO_3	3.0
$Na_2MoO_4 *2 H_2O$	2.5
$Zn(CH_3COO)_2 *2 H_2O$	13.0
EDTA	8.4

The required quantities of trace element solutions were autoclaved separately and added to the media in the bioreactor separately after autoclaving to avoid precipitation. Antibiotics were sterilised by filtration and added aseptically to the media. The final concentrations of the antibiotics were 100 µg mL^{-1} for ampicillin and 70 µg mL^{-1} for kanamycin.

Complex medium

For preparation of modified Luria-Bertani (LB) medium, 10 g L^{-1} bacto tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl were dissolved in distilled water.

Agar plates

For the preparation of LB agar plates, LB medium was supplemented with 1.5% agar powder.

2.3.2 Cultivation conditions

For shake flask experiments, preculture and main cultures were grown in LB medium supplemented with the appropriate antibiotics. For the preculture, complex medium was inoculated with a single colony from an LB agar plate and incubated overnight on a rotary shaker at 30°C, 140 rpm. For the main culture, complex medium in a shake flask with baffles was inoculated with 1% (v/v) of the overnight preculture and was incubated at 30°C to an OD_{600} of about 0.5. After this, scFvOx synthesis was induced by 0.5 mM of isopropyl-β-D thiogalactopyranoside (IPTG), and the culture was transferred to 24°C. For shake flask experiments in defined medium, cultures were inoculated with 1% (v/v) of the precultures.

For cultivation in the bioreactor, the first preculture was grown in complex medium for 6-8 h. The second preculture was grown in defined medium under the same conditions overnight. The main cultivation was carried out in a 10 L BIOSTAT C bioreactor equipped with a digital measurement and control unit (DCU) to control agitation, pH value, temperature as well as dissolved oxygen tension, and connected to the process control system MFCSwin. The cultivation was started as batch culture with an initial volume of 8 L, and a glucose concentration of 5 or 10 g L^{-1} at a temperature of 30°C. Samples were drawn with a sterile syringe at indicated intervals of time. Airflow rates (4 to 16 L^{-1}) and stirrer speed (300 to 1200 rpm) was controlled with a sequential cascade program to maintain a constant dissolved oxygen tension above 40%. A cascade control system is a multiple-loop system where the primary variable is controlled by adjusting the set point of a related secondary variable controller. The secondary variable then affects the primary variable through the process.

The pH value was maintained at 6.8 by automatic addition of aqueous ammonia (25% (w/v)). Foam formation was suppressed by the addition of the antifoam agent polypropylene glycol. After depletion of glucose, the stirrer speed controlled by the DCU decreased, because the cells stopped to consume oxygen. The decrease of the stirrer speed was the signal for the process control system to activate the flow controller at the DCU. The DCU operated a peristaltic pump to infuse the feeding solution, which was placed on a balance connected to the DCU so the exact feeding rate could be feed-back controlled. The feeding solution was

placed on a balance connected to the DCU so the exact feeding rate could be feed-back controlled. A feeding rate that increases exponentially over time allows the specific growth rate to be kept constant. The feeding rate was calculated continuously throughout the cultivation according to equation (1).

$$F = \frac{1}{S_0} \cdot q_s \cdot X \cdot V = \frac{1}{S_0} \cdot \left(\frac{\mu_{set}}{Y_{X/S}} + m_E \right) \cdot X_f \cdot V_f \cdot exp\left[\mu_{set} \cdot \left(t - t_f \right) \right] \quad (1)$$

where,

F: feeding rate, (g h^{-1})

S_0: substrate concentration in the feeding solution, (g g^{-1})

q_s: specific substrate consumption rate, (g g^{-1} h^{-1})

X: cell density, (g L^{-1})

V: culture volume, (L)

μ_{set}: set point of the specific growth rate, (h^{-1})

Yx/s: yield coefficient for biomass per substrate utilised excl. maintenance, (g g^{-1})

m_E: maintenance coefficient, (g g^{-1} h^{-1})

X_f: dry cell weight at the time of feeding start, (g L^{-1})

V_f: volume at the time of feeding start, (L)

t: cultivation time, (h)

t_f: time of feeding start, (h)

2.4 Protein harvesting methods

2.4.1 Isolation of periplasmic fractions by osmotic shock

Periplasmic fractions were prepared by the osmotic shock method according to Kang and Yoon (1994). Cells were harvested at 13,000 rpm for 2 min. One hundred µL of resuspension buffer (0.3 M Tris/HCl, pH 8.0, containing 25% sucrose, 0.5 mM $MgCl_2$, and 1 mM EDTA) was added to the cell pellet for each mL of culture corresponding to OD 1.0. After re-suspension and 10 min incubation at room temperature, the suspension was centrifuged for 10 min at 5000 rpm. The cells were re-suspended in the same volume of ice-cold osmotic shock buffer (10 mM Tris/HCl, pH 8.0, containing 0.5 mM $MgCl_2$, and 1 mM EDTA) and incubated on ice for 10 min. The supernatant after 10 min centrifugation at 13,000 rpm and 4°C was collected as periplasmic fraction.

2.4.2 Inclusion body isolation and solubilisation

E. coli cells were harvested by centrifugation at 13,000 rpm for 10 min. The biomass was homogenised at 4°C in 5 mL of re-suspension buffer (0.1 M Tris/HCl, pH 7.0, containing 1

mM EDTA) per 1 g of *E. coli* wet cell weight in the presence of 0.3 mg mL^{-1} lysozyme, and incubated for 30 min at 4°C. The cells were mechanically broken open using a French press, and cellular DNA was digested for 30 min with 10 µg mL^{-1} Benzonase in the presence of 3 mM $MgCl_2$. The solution was mixed with 0.5 volumes of 60 mM EDTA, 6% (v/v) Triton X-100, 1.5 M NaCl, pH 7.0, and incubated for a further 30 min at 4°C. The IB's were pelleted by centrifugation at 12,000 rpm for 10 min at 4°C, washed with 40 mL of re-suspension buffer per each g wet cell weight, and harvested by a final centrifugation step.

IB's were solubilised in 1 mL of solubilisation buffer (0.1 M Tris/HCl, pH 8.0, containing 6 M GdnHCl, 0.1 M DTT and 1 mM EDTA) per 10 mg wet weight for 2 h at 25°C. The solubilisation mixture was acidified to pH 4.0 by the addition of HCl and cleared by centrifugation, dialysed twice against 100 volumes of 4 M GdnHCl, 10 mM HCl at room temperature and once against 200 volumes of 4 M GdnHCl at 4°C.

2.5 Protein renaturation and purification

2.5.1 Renaturation

Preparative renaturation of scFvOx was performed by dilution of scFvOx IB solubilisate to a final concentration of 140 µg mL^{-1} in degassed renaturation buffer (100 mM Tris/HCl, pH 8.5, containing 2.5 mM GSH, 2.5 mM GSSG, 1 mM EDTA and 1 M L-Arg/HCl). Renaturation was allowed to proceed for 96 h at 15°C.

2.5.2 Protein purification by ion exchange chromatography

Following renaturation, the mixture was concentrated using a Filtron Minisette cross-flow device. The resulting solution was dialysed against 10 volumes of 25 mM sodium phosphate pH 6.0, containing 10 mM NaCl and 10% glycerol at 4°C (3 changes), and clarified by centrifugation at 10,000 rpm in a JA-10 rotor for 15 min.

Subsequently, this protein was loaded onto a 1 mL HiTrap SP Sepharose HP column that had been pre-equilibrated with 20 column volumes (CV) of loading buffer (25 mM sodium phosphate, pH 6.0, containing 10 mM NaCl). The column was then washed with 10 CV of loading buffer, followed by elution with a linear gradient of 5 -40% elution buffer (25 mM sodium phosphate, pH 6.0, containing 2 M NaCl) in 15 CV. Fractions containing pure scFvOx were identified by SDS-PAGE, silver staining and ELISA, then pooled and dialysed against 50 mM sodium phosphate, pH 7.0, containing 50 mM NaCl and stored at -80°C.

2.6 Protein quantification

2.6.1 Bradford's method

The protein concentration in different samples was determined by Bradford's method (Bradford, 1976) using BioRad protein assay reagent, according to the manufacturer's instructions, with BSA as the standard. In order to determine protein concentrations in samples containing GdnHCl, the respective standard curve was measured in the same concentration of GdnHCl.

2.6.2 Molar extinction coefficient

For the determination of concentration of purified protein, a spectrum was measured between 240 to 340 nm against a reference from the respective buffer. The specific extinction coefficient of scFvOx at 280 nm (49,740 M^{-1} cm^{-1}, corresponding to 1.731 mL mg^{-1} cm^{-1}) was calculated from the amino acid composition by ProtParam (ExPASy proteomic tool) according to Gill and von Hippel (1989). The concentration of purified refolded scFvOx was determined using the calculated extinction coefficient.

2.6.3 Enzyme-linked immunosorbent assay

Preparation of the BSA-oxazolone conjugate for ELISA

BSA-oxazolone conjugates were prepared according to Alfthan *et al.*, (1995). Briefly, 1 g BSA was dissolved in 20 mL of 5% (v/w) $NaHCO_3$; to this 75 mg 4-ethoxy-methylene-2-phenyl-2-oxazolin-5-one (Oxazolone) were added. The mixture was incubated at 4°C for 24 h with gentle shaking. The undissolved material was removed by centrifuging the mixture for 40 min at 16,000 rpm in a JA-20 rotor. The supernatant was dialysed extensively against 150 mM NaCl. The coupling ratio was determined by measuring the optical density of oxazolone at 352 nm (ε_{352} = 32,000 M^{-1} cm^{-1}) and protein determination by Bradford's assay. The coupling ratio was found to be 64.5 moles of oxazolon per mole of BSA. For storage, 1 volume of glycerol was added, and aliquots of 1 mL were stored at -20°C.

ELISA procedure

Concentrations of active scFvOx were determined by ELISA. For this purpose, 96-well ELISA microtiter plates were coated overnight with 120 µL per well of 25 µg mL^{-1} BSA-oxazolone conjugate in 0.1 M sodium carbonate, pH 9.6. The coated plates were washed three times with blocking reagent (1.5% BSA, 0.05% Tween 80 in PBS), followed by blocking for 90 min at room temperature. Samples and standards were diluted in blocking reagent to a final volume of 100 µL per well, loaded onto the plates, incubated for 90 min, and unbound material was removed by three washes with blocking reagent. For the detection of bound scFvOx, the plates were incubated for 1 h with 100 µL per well of a 1:5000 dilution of mouse

anti-*c-myc* tag antibody in blocking reagent. After one washing with blocking reagent, the plates were incubated with horseradish peroxidase-coupled chicken anti-mouse-IgG (1:3000). The plates were washed with substrate buffer (3.25 mM sodium perborate, 40 mM sodium citrate, 60 mM sodium phosphate, pH 4.5) before applying 1 mg mL^{-1} 2,2'-azino-di-(3-ethylbenzthi-azoline-6-sulfonic acid) (ABTS) in substrate buffer. Colour development was followed at 405 nm in a Sunrise microplate reader. Bound scFvOx was determined from the increase in the absorption at 405 nm over 30 min. Purified protein obtained as above (cf. section 2.5) served as the reference, and used at concentrations of 250, 125, 65, 30, 15, 7, and 3 ng mL^{-1} to generate the standard curve

2.7 Analytical methods

2.7.1 Surface plasmon resonance analysis of periplasmic scFvOx

ScFvOx activity in crude periplasmic fractions was analysed by surface plasmon resonance spectroscopy using a BIACORE X instrument, with the help of Dr. Jan Oschmann. BSA-oxazlone conjugate was covalently immobilised on a CM5 sensor chip surface using the amine coupling method (Johnsson *et al.,* 1991) according to the manufacturer's instructions. Sixteen hours after induction, periplasmic fractions from MC4100:pHEN1scFvOx:pUBS520 were dialysed against 150 mM NaCl, and concentrated using centricon devices (Millipore, USA). Two different volumes of periplasmic fraction, 15 µL and 50 µL were applied for analysis. Periplasmic extraction buffer (10 mM Tris/HCl, pH 8.0, containing 0.5 mM $MgCl_2$ and 1 mM EDTA) was used as running buffer, samples were injected over the flow cell and the dissociation phase was followed by a regeneration step (10 mL, 100 mM HCl). Samples applied to CM-5 chip coated with glucagons-like peptide-1 served as negative control (Lopez de Maturana *et al.*, 2003). The experiments were performed at 25°C and the flow rate was set to 50 µL min^{-1}. All sensorgrams were corrected by subtracting the signal of the control reference surface.

2.7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Discontinuous SDS-PAGE was carried out using the Hoefer Mighty small II system, according to Laemmli (1970). Protein samples were dissolved in 100 µL loading buffer (125 mM Tris/HCl, pH 6.8, containing 20% (v/v) glycerol, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.02% (w/v) bromophenol blue), heated to 95°C for 10 min, and loaded on gels and run at 30 mA for 50-55 min. Running buffer was 25 mM Tris/HCl, 192 mM glycine, 0.1% (w/v) SDS. The low molecular weight proteins from 14.4 to 94 kDa served as molecular weight standard (Amersham Biosciences, Uppsala, Sweden).

Coomassie staining

Gels were stained for at least one hour with Coomassie Brilliant Blue solution (0.1% (w/v) Coomassie Brilliant Blue R250, 30% (v/v) methanol, 10% (v/v) acetic acid) and destained in 30% (v/v) methanol, 10% (v/v) acetic acid.

Silver staining

Gels were first fixed in 50% acetone solution containing 2.5% (v/v) tri-chloroacetic acid (TCA) and 0.04% (v/v) formaldehyde solution, for 5 min. Then they were rinsed in acetone solution for 5 min, then in 0.17% (v/v) aqueous sodium thiosulphate ($Na_2S_2O_3$) solution for 1 min, and later in aqueous solution containing 1.3% (v/v) silver nitrate ($AgNO_3$) and 1% (v/v) formaldehyde for 8 min. Before transfer from one solution to the next, gels were washed thoroughly with deionised water. The gels were developed in aqueous solution containing 2% (w/v) Na_2CO_3, 0.04% (v/v) $Na_2S_2O_3$ and 0.04% (v/v) formaldehyde. Transfer to 1% acetic acid stopped the development of the gels.

2.7.3 Western blot

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes using a semidry transfer cell. The stack of membranes and blotting paper was soaked in Tris buffered saline (TBS buffer, 0.1 M Tris/HCl, pH 7.4, containing 1.5 M NaCl) and a current of 15 V was applied for 45 min. The membrane was then air-dried and blocked in blocking buffer (1% casein in TBS buffer) overnight at 4°C. Three 10 min washing steps in TBS buffer followed this. The membrane was further incubated with mouse anti-*c-myc* tag antibody (1:500) diluted in blocking buffer. After this, the membrane was washed twice for 20 min in 0.3% (v/v) Tween 20 in blocking buffer, followed by three 10 min washes in TBS. Subsequently the membrane was incubated for one hour under gentle agitation with horseradish peroxidase-coupled chicken anti-mouse-IgG (1:1000) diluted in blocking buffer, followed by three 10 min washes in TBS. The membrane was then treated with enhanced chemiluminescent solutions (ECL, Sigma Aldrich, USA) ECL1 and ECL2 for around 5 min. After removal of excess ECL solutions, the membrane was wrapped with Saran wrap. The light from the horse raddish peroxidase (HRP) -catalysed reaction was visualised by exposure of the wrapped membrane to an X-ray film.

2.7.4 Size exclusion chromatography

Analytical size exclusion chromatography (SEC) was performed using a 7.8 mm × 30 cm TSK Gel Alpha column, at a flow rate of 1.0 mL min^{-1}. The column was equilibrated with 4-5 column volumes of 0.1 M of sodium phosphate, pH 7.0. Molecular weights were determined

by comparison with the standard low molecular weight calibration kit (Amersham Biosciences, Uppsala, Sweden).

2.7.5 Reversed phase high performance liquid chromatography

Reversed phase HPLC (RP-HPLC) was performed using a 4.6 mm x 250 mm diphenyl column (cf. material list). The protein was eluted at 60°C with a flow rate of 0.5 mL min^{-1} and a linear gradient from 20 to 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 40 min.

2.7.6 Mass spectroscopy

After separation with RP-HPLC peaks were manually collected and submitted for mass spectrometric analysis. These analyses were performed by Dr. Angelika Schierhorn, Max Plank Research Unit for Enzymology of Protein Folding, Halle.

2.7.7 Fluorescence spectroscopy

Fluorescence spectroscopy measurements were performed with a FluoroMax-3 spectrophotometer. Spectroscopic analyses were carried out at a temperature of 20°C in a 1 cm quartz cuvette. Slit widths for excitation and emission were set to 5 nm. The excitation wavelength was set to 280 nm, and emission spectra were recorded in 1 nm intervals from 290 to 440 nm.

2.7.8 Circular dichroism spectroscopy

CD spectroscopy was performed with a Jasco J710 CD spectrometer. Measurements were performed at 20°C in a 0.5 mm quartz cuvette. Far UV CD spectra were recorded between 190 and 260 nm in 1 nm intervals, and 10 recordings were averaged.

2.7.9 Analytical ultra-centrifugation

Analytical ultra-centrifugation experiments were performed by PD Dr. Hauke Lilie, Institute for Biotechnology, Halle. Sedimentation velocity scans were measured at 20°C in a Beckman XL-A ultra-centrifuge using double sector cells and an An Ti 50 rotor. The protein concentration was 0.15 mg mL^{-1}. The experiments were performed at 40,000 rpm, and optical scans were recorded every 10 minutes at 280 nm.

2.7.10 Immuno-labelling and determination of protein localisation by transmission electron microscopy (TEM)

E. coli cells were harvested 5 h after induction, and transferred onto a nitrocellulose capillary of 250 µm diameter. The capillary tube was incubated overnight in a fixative (phosphate buffer pH 7.2, containing 0.25% glutaraldehyde and 3% para-formaldehyde) on a rotator at

4°C. The capillaries were washed with the same buffer three times (10 min each) and then post fixed with 1% (w/v) osmium tetroxide (OsO_4) for 1 h at room temperature. After washing three times with PBS (50 mM potassium phosphate, pH 7.2, containing 150 mM sodium chloride,), samples were dehydrated through a series of solutions containing ethanol in ascending concentrations (30 min each, 10%, 30%, 50%, 90%, 2 x 100%) and three changes of 100% propylene oxide. After this, the samples were embedded in Epon812 (AGAR Scientific Ltd). Thin sections (below 100 nm) were made with a Reichert Ultracut-S microtome and post stained with 0.5% uranyl acetate. These samples were incubated with anti-*c-myc* tag primary antibody (dilution 1:1000) followed by the secondary anti-mouse IgG antibody coupled to gold nanoparticles (Roche, dilution 1:1000). The stained samples were observed using a TEM (EM 900 transmission electron microscope) at 120 kV. This experiment was performed with help of Dr. Gerd Hause, Biozentrum, Halle.

2.8 Protein renaturation

2.8.1 Renaturation in the presence of Ionic Liquids

For the renaturation screening, scFvOx IB solubilisate was diluted to a final concentration of 80 µg mL^{-1} in renaturation buffer (80 mM Tris/HCl, pH 8.7, containing 2.5 mM GSH, 2.5 mM GSSG and 1 mM EDTA) containing organic salts as indicated. All buffers were degassed before use. Renaturation was allowed to proceed for 96 h at 15°C. Renaturation yields were determined by antigen binding ELISA.

2.8.2 Preparation of the glutathione-modified mixed disulfide

Purified scFvOx was denatured and reduced as described in section 2.4.2. One mg mL^{-1} of reduced scFvOx was incubated overnight at 20°C in 6 M GdnHCl, buffered with 0.1 M Tris/HCl, pH 9.0, containing a mixture of 0.1 mM GSH and 250 mM GSSG. This preparation was dialysed against 10 mM HCl and cleared by centrifugation. Homogeneity of this preparation was assessed by SDS-PAGE and RP-HPLC.

2.8.3 Optimisation of GSH concentration for the renaturation of the mixed disulfide

The glutathione-modified mixed disulfide species was used as starting material for renaturation experiments at the concentration of 140 µg mL^{-1}. Protein renaturation was performed in 0.1 M Tris/HCl, pH 8.5, containing 1 mM EDTA. GSH was applied at 0.1 to 10 mM range. 1 M L-Arg/HCl was included as additive in all experiments. All buffers were degassed before use. Renaturation yields were analysed after 96 h at 15°C.

2.8.4 Effect of pH value on protein renaturation

Renaturation was performed in buffers containing 0.1 M acetic acid/NaOH for pH values 4.0-5.0, Bis-Tris/HCl for pH 6.0, Tris/HCl for pH values 7.0-8.5, and boric acid/NaOH for pH values 9.0-9.5, in the presence of 1 mM EDTA. 1 M L-Arg/HCl was included as additive. All buffers were degassed before use. Experiments were performed in a sample volume of 300 µL. Renaturation yield was analysed by ELISA after 96 h incubation at 15°C.

2.8.5 Renaturation in the presence of aromatic thiols

Protein renaturation was performed in 0.1 M acetic acid/NaOH for pH values 4.0-5.0, Bis-Tris/HCl for pH 6.0, Tris/HCl for pH values 7.0-8.5, and boric acid/NaOH for pH values 9.0-9.5, in the presence of 1 mM of EDTA. The glutathione-modified mixed disulfide species was used as starting material for renaturation experiments at a concentration of 140 µg mL^{-1}. Aromatic thiols or GSH were applied at 0.2 mM concentration. 1 M L-Arg/HCl was included as additive in all experiments. All buffers were degassed before use. Renaturation yields were analysed after 96 h at 15°C. In order to test the effect of aromatic thiols on renaturation kinetics, experiments were performed at pH 7.0. Samples were withdrawn at indicated time intervals. Determination of the apparent rate of renaturation was done by single exponential fit using Sigma Plot 8.0 software.

2.9 Protein stability

2.9.1 Denaturant-induced unfolding and refolding

The urea-dependent unfolding and refolding of scFvOx was monitored by changes in tryptophan fluorescence. For the equilibrium stability experiments, 7.5 µg mL^{-1} of native or denatured scFvOx were mixed with buffer containing either 0.1 M sodium phosphate, pH 7.0, or 0.1 M Tris/HCl, pH 8.5, and urea in the indicated concentrations. The samples were equilibrated for 18 h at 20°C before fluorescence measurements were performed as indicated above (cf. section 2.7.7). The fraction of unfolded protein was calculated from the change in fluorescence intensity at 360 nm.

2.9.2 pH-dependent stability

The pH-dependent stability analysis was performed by monitoring changes in intrinsic tryptophan fluorescence. The native protein concentration used for this experiment was 7.5 µg mL^{-1}. Buffers contained 0.1 M citric acid, 0.1 M phosphoric acid and 0.1 M boric acid, and were adjusted to the indicated pH values by addition of NaOH. The samples were incubated for 24 h at 20°C. Measurements were performed as indicated above (cf. section 2.7.7).

3. Results

Various strategies were investigated to achieve functional production of scFvOx. Previous studies suggest that expression of this class of proteins in *E. coli* leads to formation insoluble aggregates into the periplasm. In addition, scFv's are known to be prone to aggregation during *in vitro*-renaturation. Therefore, scFvOx served as a good model system for studying effect of various expression strategies into periplasm as well as screening of various new compounds for *in vitro*-renaturation.

3.1 Periplasmic production of scFvOx

3.1.1 Secretion of scFvOx into the periplasm

Recombinant scFvOx was produced in *E. coli* from the expression vector pHEN1scFvOx (Fiedler and Conrad, 1995). The protein was directed into the periplasm by a *pelB* leader sequence. Samples were withdrawn at 4 and 16 h after induction. The soluble periplasmic fraction and the cytosolic fraction were analysed by Western blot (Fig. 3.1), using scFvOx from a plant source as standard for this analysis (courtesy of Dr. Udo Conrad, IPK, Gatersleben).

Proteins from the periplasm were separated from the remainder, especially cytoplasmic and membranes which are labeled here "cytosolic", by extraction. No cell growth inhibition was observed after several hours of induction. No signals of scFvOx were detected in pre-induction and medium supernatant samples (data not shown). This indicates that scFvOx production was tightly controlled, and that there was no leakage of scFvOx to the medium.

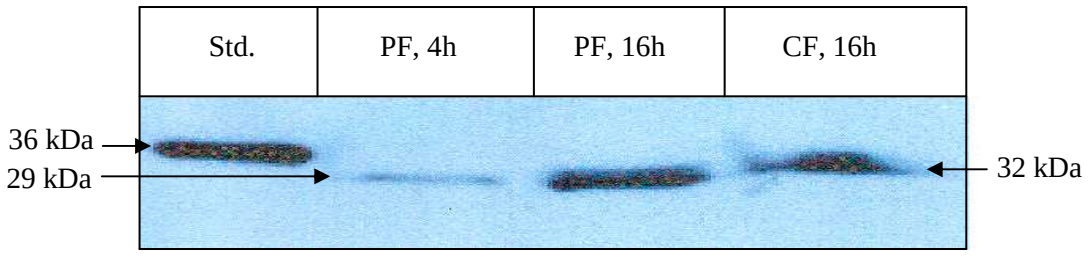


Fig. 3.1: Localisation of scFvOx: The *E. coli* MC4100:pHEN1scFvOx cells were grown at 30°C, induced with 1 mM IPTG, and further cultivated at 24°C. ScFvOx produced in plant seeds served as standard. PF, 4 h and PF, 16 h represent periplasmic fractions 4 h and 16 h after induction, CF, represents the cytosolic fraction 16 h after induction. For analysis, 20 µL of PF prepared out of 1 mL of culture corresponding to OD_{600} 1. (cf. section 2.4.1), sonicated cell pellet homogenate from 200 µL culture volume for CF, and 7 µL of the crude standard preparation were applied (Std.). Samples were separated by 12% SDS-PAGE, and Western blotted using anti-*c-myc* tag primary antibody and anti-mouse IgG coupled to HRP conjugate. Expected molecular weights of the proteins are indicated.

The product was found to be localised in the target compartment, *i.e.*, the periplasm. The level of scFvOx expression was higher after 16 h than after 4 h post induction (Fig. 3.1). Production was sustained for a long time but the rate of product synthesis was slow. A considerable amount of scFvOx was also found in the cytosolic fraction (Fig. 3.1). This might be due to export problems. As expected, the apparent size of scFvOx protein in the periplasmic fraction was lower than the standard protein extracted from plant seeds, which is additionally fused with an endoplasmic reticulum retention signal sequence (Fiedler *et al.*, 1997). A major fraction of the product (approx. 60%) was correctly translocated into the target compartment, *i.e.*, the periplasm. However, a significant fraction of the expressed scFvOx remained trapped in the cytosol. scFvOx located in the cytosolic fraction migrated more slowly than that from the periplasmic extract, suggesting that the cytosolic protein still carried the export leader sequence (Fig. 3.1). The size of the *pelB* signal sequence is 2457 Da. Thus, the relative positions of the bands were in agreement with what was expected, and this indicates that scFvOx could be expressed in the periplasm.

3.1.2 Sub-cellular localisation of scFvOx

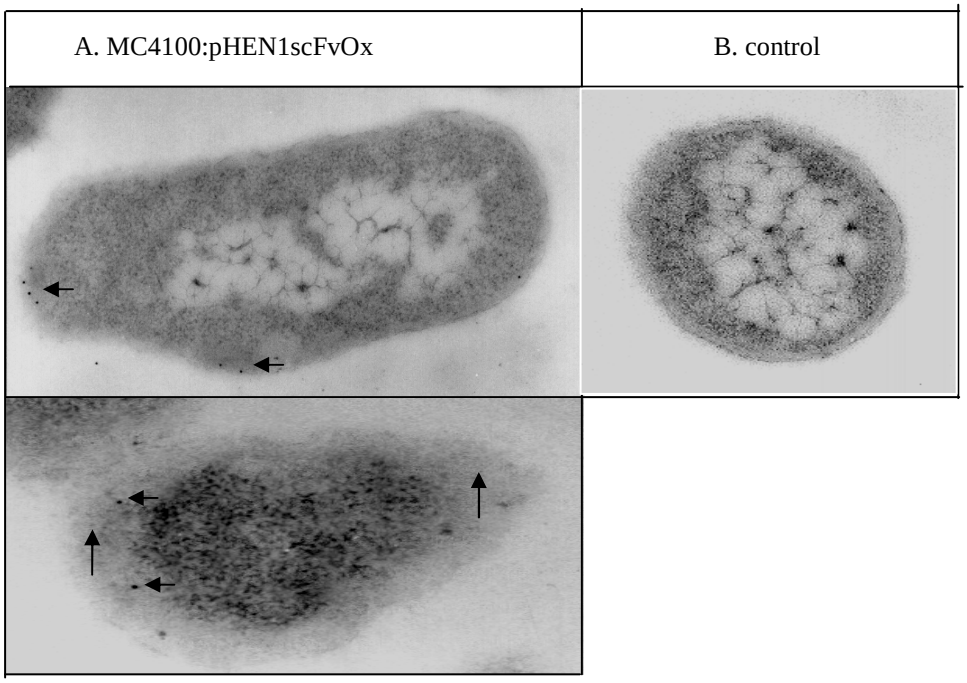


Fig. 3.2: Immunogold analysis of expression and sub-cellular localisation of scFvOx: A. *E. coli* MC4100:pHEN1scFvOx cells were grown at 30°C, induced with 1 mM IPTG, and further cultivated at 24°C for 5 h. B. MC4100 cells without expression plasmid. The samples in both A and B were incubated with anti-*c-myc* tag antibody (dilution 1:1000) followed by anti-mouse IgG coupled to gold (dilution 1:1000). Horizontal arrows indicate binding of scFvOx-specific antibodies and vertical arrows indicate bulging of the periplasm.

The morphology of cells expressing scFvOx and the sub-cellular localisation of the product were examined by transmission electron microscopy (TEM). This experiment was performed

with the help of Dr. Gerd Hause. No periplasmic IB's were observed. Immunogold staining was carried out in order to gain information about the sub-cellular localisation of periplasmically expressed scFvOx (Fig. 3.2). Black immunogold spots indicating the presence of the product was detected only in cells expressing scFvOx, and only in the periplasmic space (shown by horizontal arrows) (Fig. 3.2). However, the signal in the periplasm was not very strong. This might suggest that the general efficiency of the immunogold-labeling was quite low. In a number of cells, additional bulging of the periplasm was observed, which suggested an accumulation of the product in this compartment (Fig. 3.2, indicated by vertical arrows). No signals were obtained either in the cytoplasm or in control cells. This suggests that scFvOx was efficiently exported into the periplasm. However, this is in contradiction to the observations with Western blot analysis (cf. section 3.1.1), where significant amounts of scFvOx were detected in the cytoplasmic fraction. A potential reason for this observation could be accessibility problems for the cytoplasmic fraction.

3.1.3 ScFvOx activity analysis using surface plasmon resonance

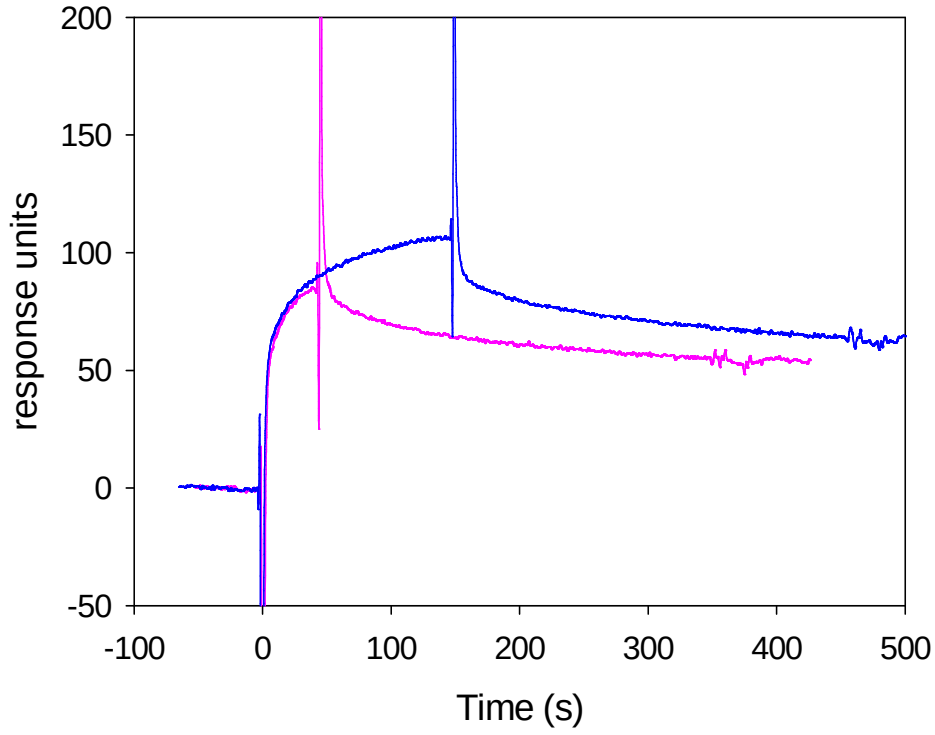


Fig. 3.3: Binding activity of periplasmic scFvOx to immobilised BSA-oxazolone conjugate: 300 RU of the oxazolone conjugate were covalently immobilised on the sensor chip surface. Periplasmic fraction from MC4100:pHEN1scFvOx:pUBS520 16 h post induction was dialysed against 150 mM NaCl, 15µL (—) and 50µL (—) were injected over the sensor surface. Periplasmic extraction buffer (10 mM Tris/HCl, pH 8.0, containing 0.5 mM $MgCl_2$, 1 mM EDTA) was used as running buffer. The flow rate was 50 µL min^{-1}.

The hapten binding activity of periplasmically produced scFvOx was assessed qualitatively by BIAcore analysis. Crude periplasmic fraction 16 h post induction was dialysed overnight and centrifuged thoroughly to remove aggregated protein before analysis. Total protein concentration in the sample was 45 µg mL^{-1}.

A binding of scFvOx to immobilised antigen-BSA conjugate was specific (Fig. 3.3) as periplasmic samples did not bind to a sensor chip coated with glucagon-like peptide-1 (Lopez de Maturana *et al.,* 2003) (data not shown). These observations strongly indicate that the periplasmic expression of scFvOx yielded biologically active protein capable of specifically binding its antigen. However, a reliable quantification of active scFvOx in the periplasm was not possible due to the lack of homogeneous purified scFvOx at this stage of work.

3.1.4 Periplasmic scFvOx production from a pET vector system

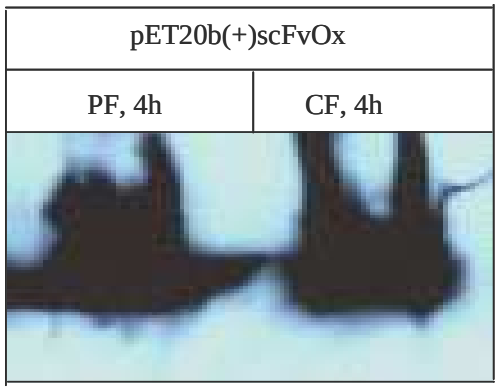


Fig. 3.4: Periplasmic expression of scFvOx under the control of *T7* promoter: *E. coli* BL21(DE3):pET20b(+)scFvOx cells were grown at 30°C, induced with 1 mM IPTG, and cells were further cultivated at 24°C. PF and CF represent periplasmic and cytosolic fractions respectively. Samples were taken 4 h after induction. For analysis, 20 µL of PF prepared out of 1 mL of culture corresponding to OD_{600} 1 (cf. section 2.4.1), and CF equivalent to sonicated cell pellet homogenate from 200 µL were applied. Samples were separated by 12% SDS-PAGE, and Western blotted using anti-*c-myc* tag antibody followed by anti-mouse IgG coupled to HRP conjugate.

Periplasmic expression of scFvOx under the control of a strong promoter, *i.e., T7* was evaluated. For this purpose, the *E. coli* host strain BL21(DE3):pET20b(+)scFvOx was used. In shake flask cultivation experiments, cell growth stopped 3-4 h post induction, at which time, strong expression of scFvOx was observed (Fig. 3.4). Despite the inhibition of cell growth, 10-12 fold higher amounts of scFvOx were detected (Fig. 3.4) in comparison to expression using MC4100:pHEN1scFvOx (Fig. 3.1). Approximately equal quantities of product were found in the periplasmic and cytosolic fractions, respectively (Fig. 3.4). Due to the overloading of gel, it was not possible to compare the size of scFvOx extracted from the periplasmic and cytosolic fractions. Therefore, information on processing of *pelB* leader sequence could not be interpreted.

Thus, high production levels were obtained with *T7* However, problems were observed with respect to uncontrolled expression levels and cessation of host cell growth.

3.1.5 Effect of co-expression of *dnaY,* DsbA and active site variants of DsbA

The effect of co-expression of the thiol-oxidase DsbA and its variants with presumed disulfide isomerase activity on the production of scFvOx was investigated (Fig. 3.5). Since the *E. coli* strain BL21(DE3) has an added advantage over MC4100 in being deficient in periplasmic proteases, this strain was used for subsequent expression studies. For co-expression experiments, the plasmids pHEN1scFvOx and pUBS520 were used with or without the gene for DsbA and its active site variants. *E. coli* BL21(DE3):pHEN1scFvOx without additional plasmids served as a control. Samples were analysed at two different time points (4 and 16 h) after induction. Biologically active scFvOx in the periplasmic fractions was quantified by ELISA using purified scFvOx as a standard (kindly provided by Dr. Claudia Humbeck).

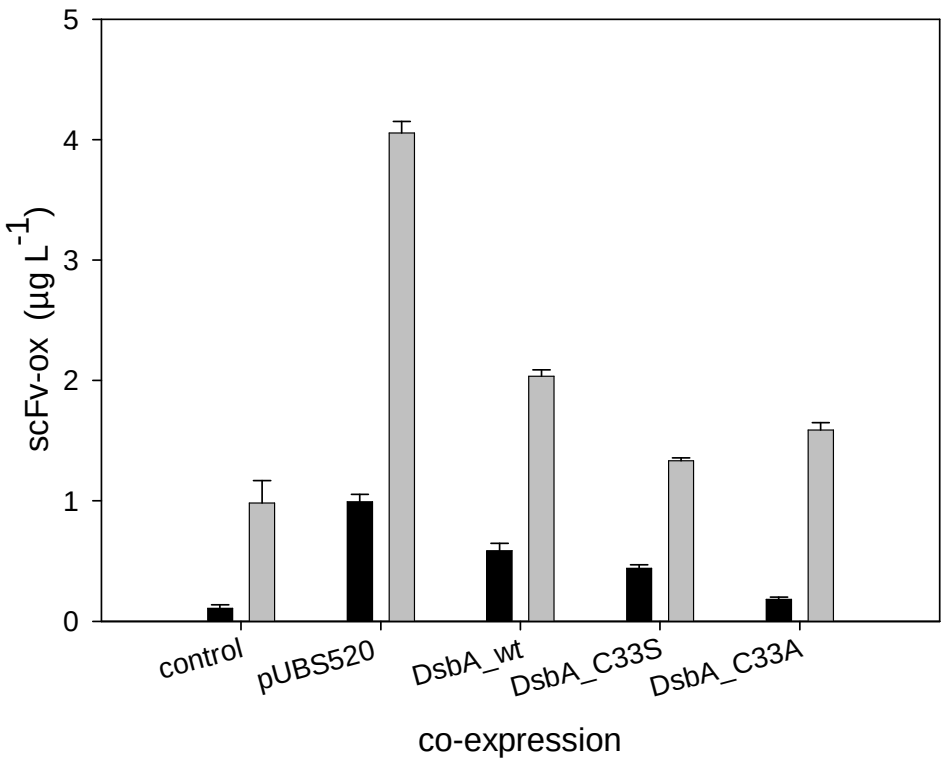


Fig. 3.5: Effect of co-expression of DsbA and active site mutants on the production of scFvOx: Different BL21(DE3) *E. coli* strains harbouring the expression vector pHEN1scFvOx and the indicated additional plasmids were were grown at 30°C, induced with 1 mM IPTG, and further cultivated at 24°C. Black and grey bars represent samples taken 4 h and 16 h post induction, respectively. scFvOx concentrations were determined by ELISA. Error bars indicate the standard deviations of three independent experiments.

No scFvOx was detected in the pre-induction samples. In all cases, the amount of scFvOx in samples taken 16 h post induction was higher than in the samples taken after 4 h (Fig. 3.5). The highest specific concentration of scFvOx, 4.15 μg L^{-1} was detected when scFvOx was co-expressed with *dnaY* (pUBS520), whereas additional co-expression of DsbA or its active site mutants was found to reduce the yield of periplasmic expression (Fig. 3.5). Co-expression of scFvOx with *dnaY* and wild type *dsbA* resulted in a yield of 2.1 μg L^{-1} of scFvOx, which was 2-fold higher than in the control without pUBS520, whereas no enhancement in the production of scFvOx was detected when active site variants of DsbA were co-expressed. In other words, disulfide isomerase activity, as assessed from the co-expression of DsbA or of its active site variants, failed to enhance the periplasmic production of scFvOx. These studies revealed the strain BL21(DE3):pHEN1scFvOx:pUBS520 as best system for the periplasmic production of scFvOx. Therefore, this strain was used for further process optimisation.

3.1.6 Bioreactor scale production of scFvOx

After identifying the optimal expression strain the next objective was to establish a periplasmic expression process under controlled conditions in a bioreactor. Fed-batch cultivations are commonly used to achieve far higher cell densities of recombinant *E. coli* than those that could be reached in batch cultivations. Another advantage of fed-batch cultivation strategies is that limiting feeding can closely control the growth rate. Fed-batch cultivations in this work were performed with an exponential increase in feeding rate over time to establish a constant growth rate μ_{set}.

Production of scFvOx, using the strain BL21(DE3):pUBS520:pHEN1scFvOx, was scaled up to 15 L. Cultures were initially grown at 30°C. After induction with 0.5 mM IPTG, the temperature was shifted to 24°C. A pH of 6.5 was maintained throughout the cultivation by controlled addition of aqueous ammonia. After inoculation, a 20-30 minute lag phase was observed, followed by the batch phase when the cells grew at their maximum rate (μ_{max}) of about 0.48 h^{-1}. After consumption of the supplied glucose, the programmed automatic feeding procedure initiated, from when it took 2 h for the culture to reach stable growth at the set rate. Cultivations were monitored only after induction of protein synthesis. Online data such as aeration, pH, feeding rate, stirrer speed, and CO_2 expulsion rate were recorded by the MFCSwin software, which was connected to the DCU system. The average specific growth rate was calculated from fit to the optical densities by single exponential function. Further details on cultivation parameters, process control and operational procedure are given in sections 2.3.1 and 2.3.2.

Effect of growth rate on scFvOx production

One of the objectives in recombinant protein production is to simultaneously reach a high specific recombinant protein production rate, a high cell density, and to ensure product stability. Both productivity and protein stability are affected by the growth rate (Rozkov and Enfors, 2004). The influence of the growth rate on the periplasmic production of scFvOx was investigated by comparing the bioreactor-scale fed-batch production of scFvOx with μ_{set} values of 0.12 h^{-1} and 0.06 h^{-1} respectively.

Cultivation using faster growth rate

For cultivation with a *set* growth rate of 0.12 h^{-1}, the induction of protein synthesis was carried out at an OD_{600} of 8.8, which was reached at 10 h. Upon induction, an increase of scFvOx activity in the periplasmic fractions was observed (Fig. 3.6, A). The rate of product synthesis was high. This trend continued up to 18 h, with a maximum of 4.9 µg L^{-1} specific activity of soluble antibody fragment. A similar trend was found for the volumetric activity. The maximum volumetric activity was 66 mg L^{-1} at 18 h. After this point, scFvOx activity declined. The active production phase lasted for 8 h.

The feeding profile was tightly maintained throughout the cultivation (Fig. 3.6, B). During the fed-batch phase, the feeding rate, which was calculated by the process control system based on the set specific growth rate μ_{set} = 0.12 h^{-1}, increased exponentially with time (Fig. 3.6, B). The optical cell densities continuously increased (Fig. 3.6, C). The actual average growth rate remained much lower, *i.e.* 0.045 h^{-1}, instead of μ_{set} = 0.12 h^{-1} (Fig. 3.6, C). These observed difficulties in controlling desired specific growth rate are in agreement with previous reports (Lubenova *et al.*, 2003). The inherent features of a feed-forward method limit the application of this feeding scheme, due to the likely occurrence of external perturbations or variations of culture parameters under experimental conditions (Lubenova *et al.*, 2003).

Cultivation at slower growth rate

In order to investigate the effect of slower growth on periplasmic production of scFvOx, a fed-batch cultivation with a set growth rate μ_{set} = 0.06 h^{-1} was performed. After 2 h of feeding, the OD_{600} of the culture was 36. At this point, *i.e.*, after 14 h of cultivation, the expression of scFvOx was induced. The concentration of scFvOx in the periplasmic fractions increased more gradually compared to cultivation with faster growth rate. This trend continued up to 38 h, with a maximum specific yield of 2.3 µg L^{-1} of active antibody fragments (Fig. 3.7, A). The maximum volumetric product concentration was 120 mg L^{-1} at 38 h.

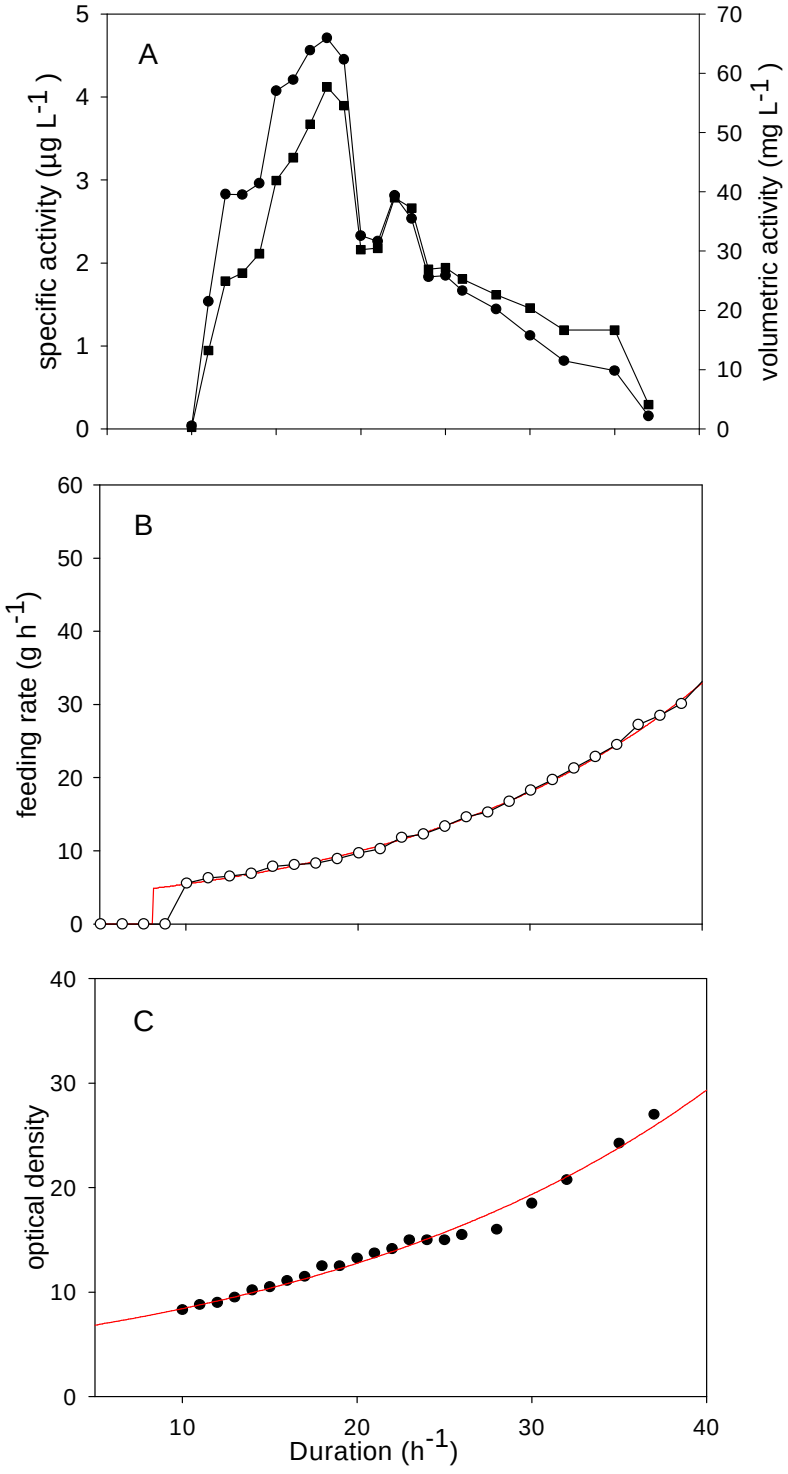


Fig. 3.6: Fed-batch cultivation for production of recombinant scFvOx at faster growth rate (μ_{set} 0.12 h^{-1}): *E. coli* BL21(DE3):pHEN1scFvOx:pUBS520 cells were cultivated in a bioreactor in fed-batch mode. At 10 h scFvOx production was induced with 0.5 mM IPTG. **A.** Specific scFvOx concentration (■) and volumetric concentration (●). **B.** Set point (—), and process value (-○-) of the glucose feeding rate and **C.** Optical density (●), and fit to the data (—) to calculate average specific growth rate are shown.

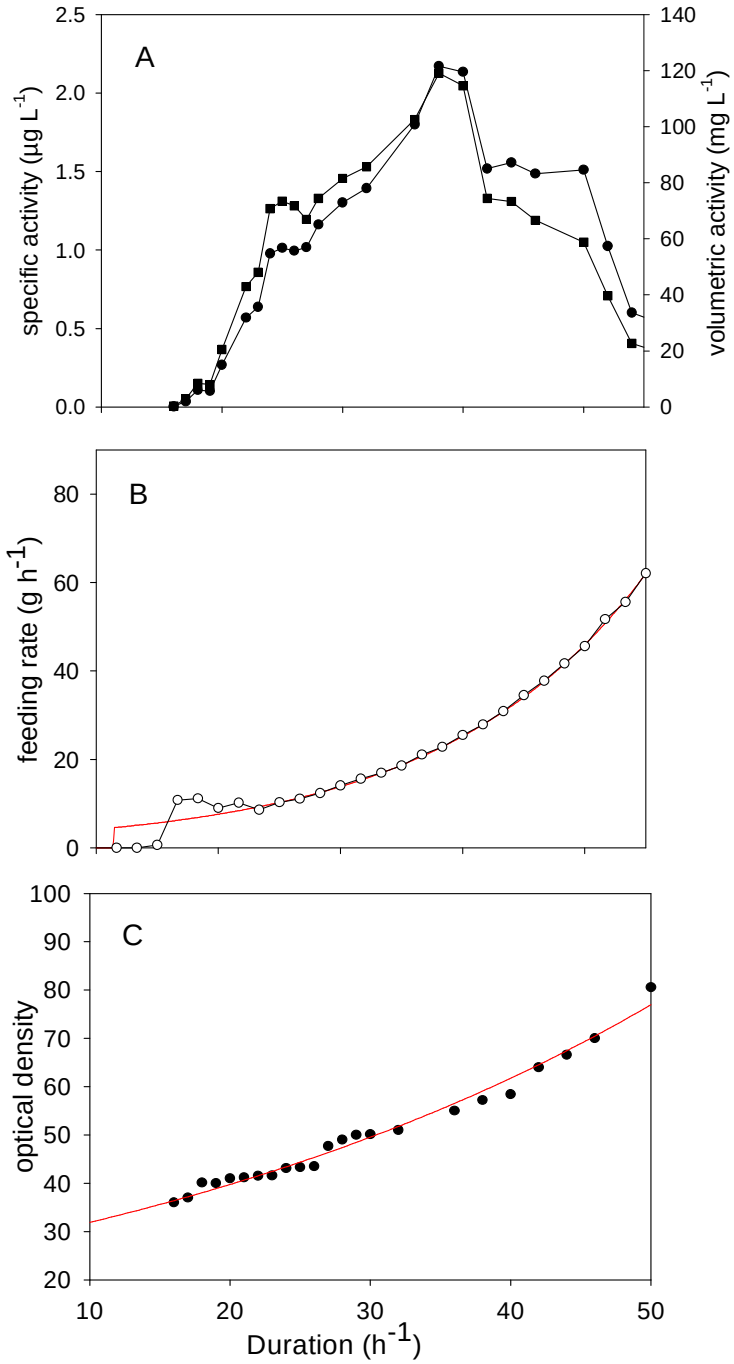


Fig. 3.7: Fed-batch cultivation for production of recombinant scFvOx at slower growth rate (μ_{set} 0.06 h^{-1}): *E. coli* BL21(DE3):pHEN1scFvOx:pUBS520 was cultivated in a bioreactor in fed-batch mode. At 14 h, scFvOx production was induced with 0.5 mM IPTG. **A.** Specific scFvOx concentration (■) and volumetric concentration (●). **B.** Set point (—), and process value (-○-) of the glucose feeding rate and **C.** Optical density (●), and fit to the data (—) to calculate average specific growth rate are shown.

In comparison with the cultivation at faster growth rate, scFvOx production profile was steadier and significantly more sustained. The specific product concentration obtained in this cultivation was approximately 2-fold higher than in the cultivation at higher growth rate.

scFvOx activity suddenly dropped after 38 h. In contrast to cultivation at a higher growth rate, the production phase was prolonged to 16 h. The actual average growth rate during the production phase, before product degradation set in, was found to be 0.022 h^{-1} (Fig. 3.7, C). This was considerably lower than the set point μ_{set} = 0.06 h^{-1}. Thus, the specific growth rate had a pronounced effect on the production of scFvOx, *i.e.*, 2-fold increase in activity and prolonged production phase as compared to cultivation at a higher growth rate were achieved.

Product localisation during fed-batch cultivation

ELISA detects only active and soluble material. For the quantification of total product including inactive conformations, immuno-detection of the produced protein on a Western blot is a suitable tool. It is specific for the product and highly sensitive. Periplasmic and cytoplasmic fraction samples at indicated time points during the cultivation were analysed by this method. These samples were from the batch with μ_{set} = 0.12 h^{-1}.

No scFvOx was detected in pre-induction samples. Increased amounts of scFvOx were observed with increasing time after induction (Fig. 3.8). It was found that the product concentration increased for up to 8 h, in both periplasmic and cytoplasmic fractions. Apparently, the amount of scFvOx in the cytosolic fraction was higher than in the periplasmic fractions. Possibly under fed-batch conditions, the stress on the cells was higher and translocation less efficient. After 8 h of cultivation the product concentration decreased, which was in agreement with results described above (Fig. 3.6 and 3.7). Possible reasons for this are proteolytic degradation of the product, cell death or growth of non-producing cells. Thus, scFvOx was obtained as a mixture of soluble active and aggregated material in cytosol during the periplasmic expression of the protein.

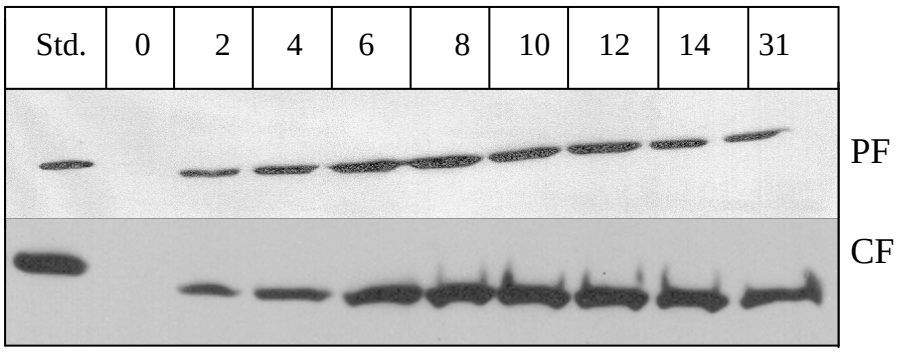


Fig. 3.8: Analysis of product during fed-batch cultivation. Periplasmic fractions and pellet taken at indicated intervals of time from fed-batch cultivation were examined. Renatured and purified protein from IB's served as standard. For analysis 20 µL of PF prepared out of 1 mL of culture corresponding to OD_{600} 1 (cf. section 2.4.1), and for CF, sonicated cell pellet homogenate from 200 µL culture volume were applied. Samples were separated by 12% SDS-PAGE and Western blotted using anti-*c-myc* tag antibodies.

During this study, periplasmic expression of biologically active scFvOx was achieved under the control of lac promoter. However, expression under the control of strong promoter resulted in a very high but un-regulated production. Co-expression of *dnaY* was found to be beneficial for the production of scFvOx. Co-expression of DsbA and its active site variants was not beneficial for the enhancement of periplasmic production of scFvOx. During bioreactor-scale cultivation studies at lower growth rate, an enhancement in the production of scFvOx and a prolonged production phase were observed.

In addition to the periplasmic expression of scFvOx, the namely production as cytoplasmic IB's and its *in vitro*-renaturatuion was also investigated. The objective behind these studies was to compare both systems and identify the best production protocol for antibody fragments.

3.2 Cytosolic production, preparative renaturation and purification of scFvOx

Production of scFvOx inclusion bodies

IB's predominantly contain the over-expressed protein, and therefore serve as a one of the rich source of recombinant product needed for academic and industrial applications. This section describes studies on isolation of scFvOx from IB's, investigation of protein stability and various strategies explored for improvement in renaturation yield.

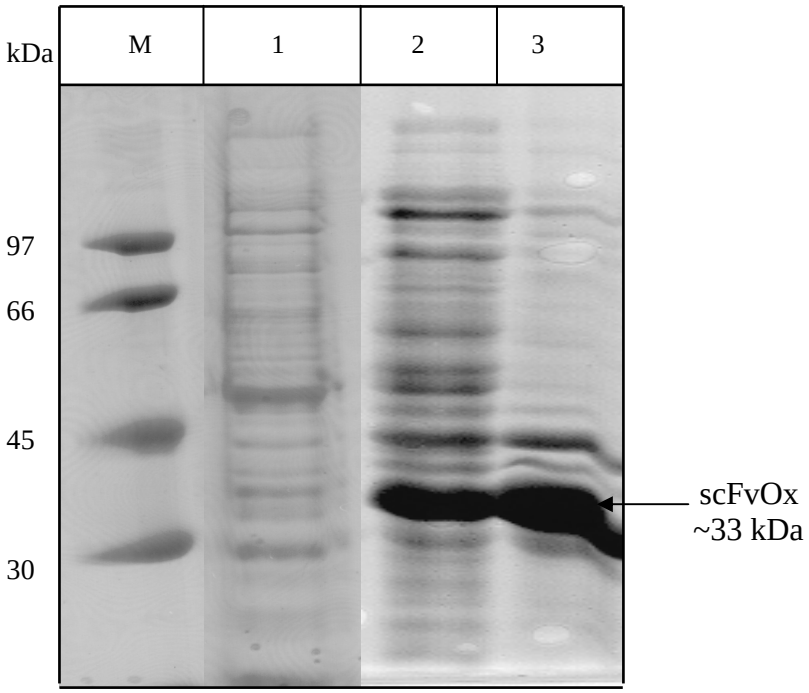
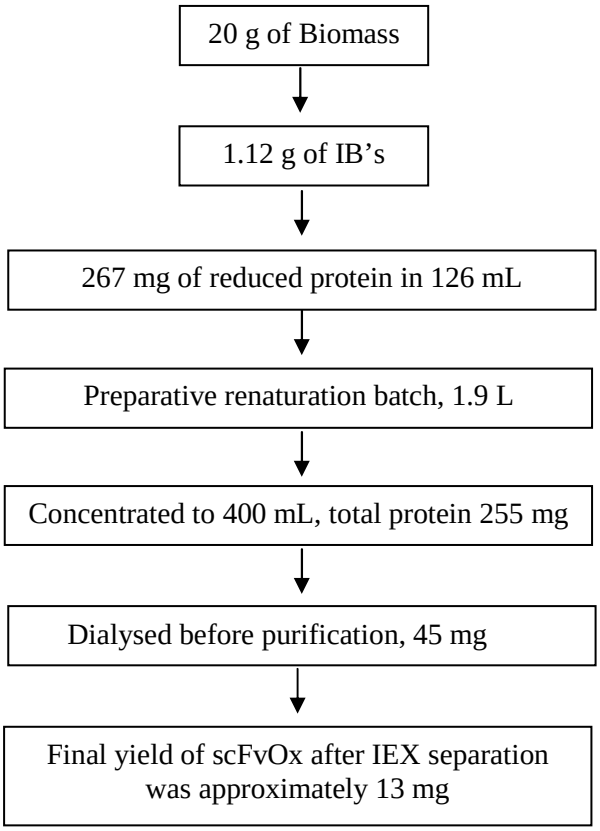


Fig. 3.9: Production and isolation of scFvOx IB's: IB's were expressed in *E. coli* BL21 (DE3):pUBS520:pET15b(+)scFvOx. Proteins were analysed by 12% SDS-PAGE and staining with Coomassie Blue. M, low molecular weight marker; lane 1, whole cell extract from un-induced cells; lane 2, whole cell extract from induced cells (4 h, post-induction); lane 3, IB preparation. For each lane the pellet of 200 µL culture volume was applied.

scFvOx was produced in *E. coli*. Cells were harvested 4 h post induction and IB's prepared according to section 2.4.2. Pre-induction, post-induction and IB samples were analysed by SDS-PAGE (Fig. 3.9). No scFvOx production was observed in the pre-induction sample (Fig. 3.9, lane, 1). Post-induction, the crude cell extract showed strong expression (Fig. 3.9, lane, 2), corresponding to ~30-50% of the total cellular protein. Apparently, the strong *T7* promoter drove very high protein production. scFvOx was produced as insoluble IB's, as described in section 2.4.2 and the preparation contained ~ 80% scFvOx polypeptide (Fig. 3.9, lane, 3).

Preparative renaturation of scFvOx from IB's



Scheme 3.1: Production of IB's, preparative renaturation and purification of scFvOx

Isolation of IB's was done from 20 g of cell biomass (scheme 3.1), resulting in 1.12 g of IB's. After reduction, denaturation and dialysis, 267 mg of IB solubilisate in a total volume of 126 mL was obtained (scheme 3.1). From this reduced protein, a 1.9 L renaturation experiment was performed (cf. section 2.5). After cross-flow concentration, 400 mL of renatured sample was obtained, corresponding to 255 mg of protein (scheme 3.1). This concentrated protein was dialysed and clarified by centrifugation to remove aggregates, resulting in 45 mg of protein (scheme 3.1). Purification by ion exchange chromatography (IEX) resulted in 13 mg of pure scFvOx, corresponding to 5% of the applied total IB protein (scheme 3.1).

Purification of scFvOx

After production, preparative isolation and renaturation of scFvOx, the protein was purified by IEC, using a Hi-Trap SP-Sepharose column. The renatured protein applied for purification was relatively pure (Fig. 3.10 B, lane 4). 45 mg of the protein was loaded on the column. No scFvOx was detected in the unbound and washing fractions (Fig. 10, B, lanes, 5 and 16).

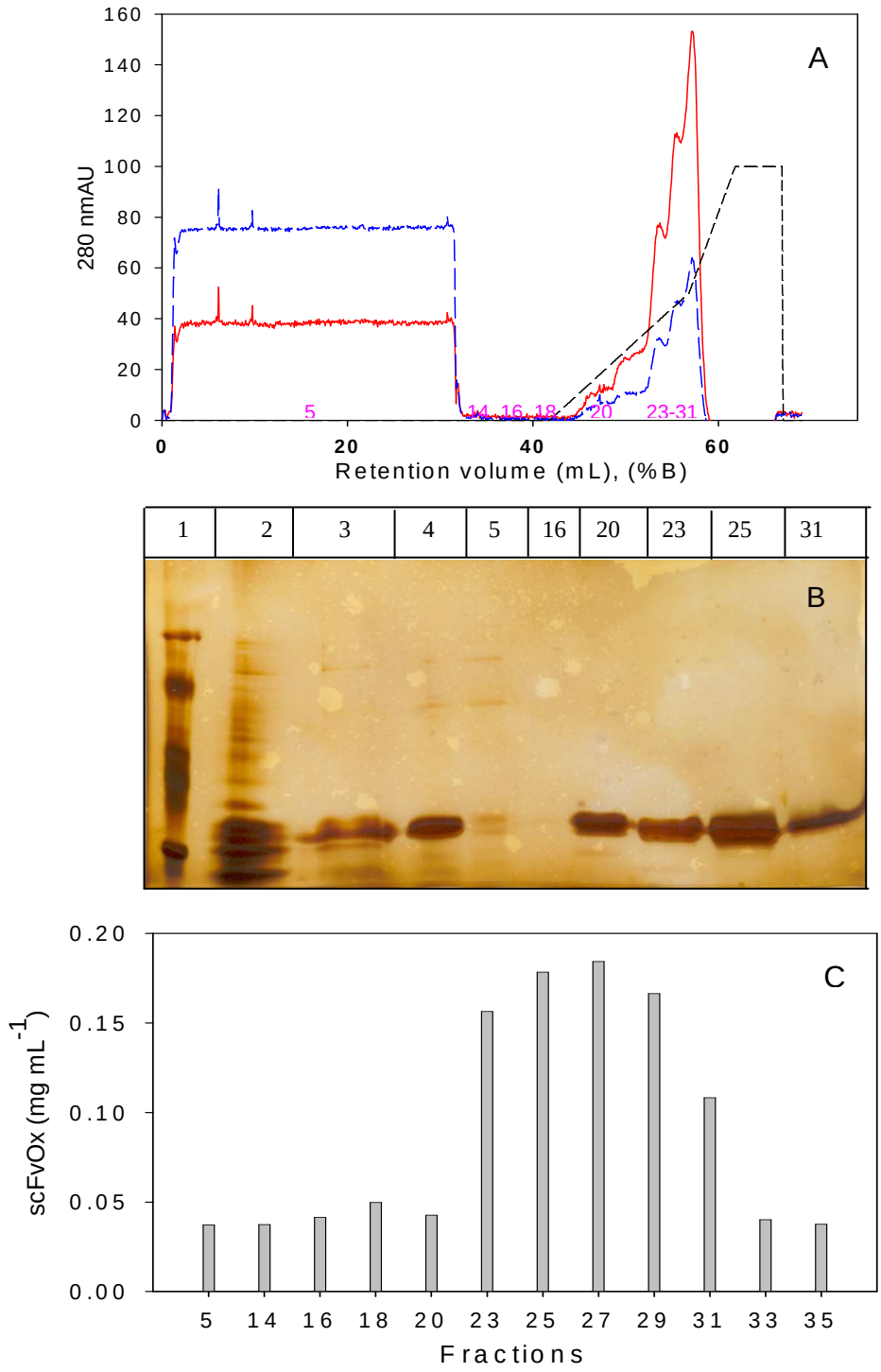


Fig. 3.10: Purification of scFvOx by ion exchange chromatography: Protein was applied to a Hi-Trap SP-Sepharose column (1 mL) and eluted with a gradient of 10 mM (0%) to 2 M (100%) NaCl in 25 mM of sodium phosphate, pH 6.0. **A. Chromatogram** - Absorption at 280 nm (---), and 260 nm (---), and salt gradient (---). **B. SDS-PAGE analysis** - 20 µL of respective samples were analysed by 12 % SDS-PAGE and silver staining. 1, LMW markers; 2, whole cell extract; 3, IB preparation; 4, protein applied to column (after dialysis); 5, flow-through, 16, wash fraction, 20, early eluates and 23 -31, elution fractions. **C. scFvOx antigen binding activity** - Antigen binding activity was determined by ELISA.

Bound protein was eluted with a gradient of increasing NaCl concentration. Eluted scFvOx protein was found in the fractions 23-31 (Fig. 3.10, B) corresponding to NaCl concentrations

between 0.23 and 0.6 M. The protein eluted in three overlapping peaks. All peak fractions contained an apparently homogeneous pure protein with a relative molecular weight of 29 kDa as observed by silver stained polyacrylamide gel. The activity assay by ELISA revealed scFvOx antigen binding activity in all peak fractions (Fig. 3.10, C), with specific scFvOx activities between 0.16 to 0.19 mg mL^{-1}. Characterisation by CD spectroscopy, analytical SEC, RP-HPLC and mass spectroscopy (cf. section 3.3) failed to detect any differences between the protein in the different elution fractions. This indicates that the observation of three overlapping elution peaks was not due to heterogeneity of the purified protein. Instead, it is conceivable that scFvOx adopted different conformations with different elution properties upon binding to the ion exchange matrix, in agreement with conformational flexibility of antibody fragments reported in the literature (*e.g.*, Dumoulin *et. al.*, 2002).

3.3 Characterisation of purified scFvOx

3.3.1 Reversed-phase HPLC

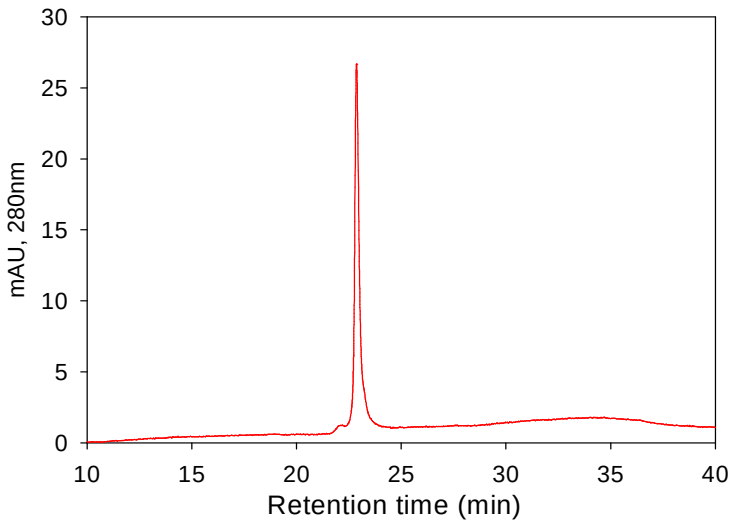


Fig. 3.11: Analytical RP-HPLC of scFvOx: Retention profile of scFvOx monitored by optical absorption at 280 nm (---). Separations were performed on a di-phenyl column (219TP54, VyDac) in a gradient from 20 to 60% acetonitrile in 0.1% TFA over 45 min. The flow rate was maintained at 0.5 mL min^{-1}. The experiment was performed at 60°C.

The purity and homogeneity of isolated purified protein was further assessed by RP-HPLC. Different RP-HPLC column materials (C18, C8 and C4) and buffer systems (acetonitrile, methanol and acetone) were tested, but the observed resolution and reproducibility were not satisfactory. A reversed phase di-phenyl solid phase column (219TP54, VyDac) was found to produce good resolution with acetonitrile / TFA buffer system, when the experiment was performed at 60°C (Fig. 3.11). Under these conditions, the separation of purified protein

samples gave rise to single sharp peaks, indicating that the protein had been purified to homogeneity.

3.3.2 Mass spectrometry

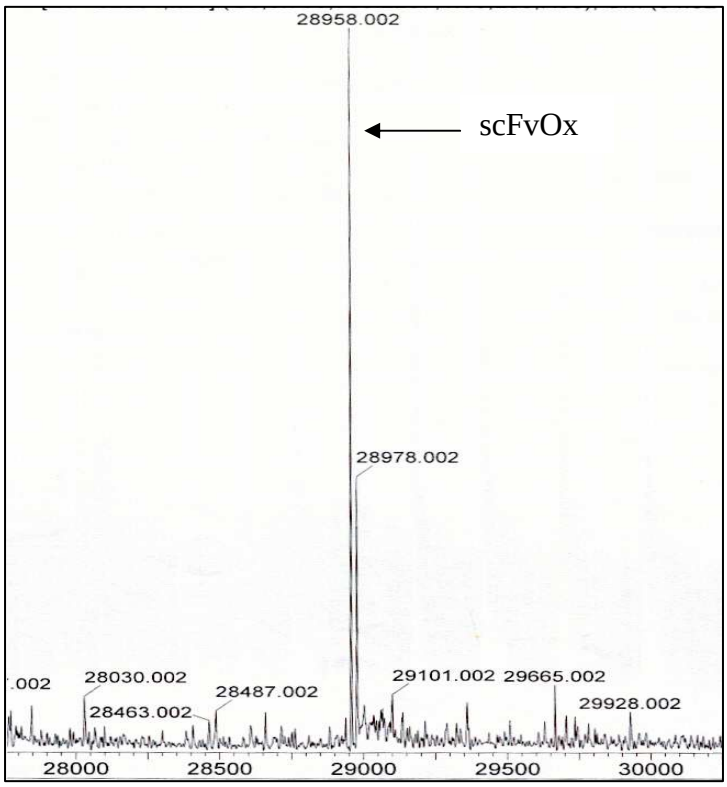


Fig. 3.12: ESI-TOF mass spectrum of scFvOx

The mass of the recombinantly expressed and purified scFvOx was determined by Electro Spray Ionisation-Time-of-Flight (ESI-TOF) mass spectrometric analysis of the collected peak fractions from the RP-HPLC runs described in section 3.3.1. The analysis was performed by Dr. Angelika Schierhorn (Max Plank Research Unit for Enzymology of Protein Folding, Biozentrum, Halle). The mass of the protein was found to be 28,958 Da (Fig. 3.12). This was in good agreement with the theoretically expected mass of scFvOx (28,960 Da).

3.3.3 Spectroscopic characterisation

Fluorescence spectroscopy

Fluorescence emission spectra of renatured-purified scFvOx were recorded in phosphate buffer, with or without GdnHCl (Fig. 3.13, A). When excited at 280 nm, the protein showed an emission maximum at 348 nm in the absence of denaturant. Upon exposure to 6 M GdnHCl, the emission maximum shifted to 359 nm, while the emission intensity at the maximum increased by almost three-fold (Fig. 3.13, A). These changes are in agreement with

an increased solvent exposure of aromatic amino acids upon unfolding, and therefore indicate that the renatured purified scFvOx assumes a defined folded structure in phosphate buffer.

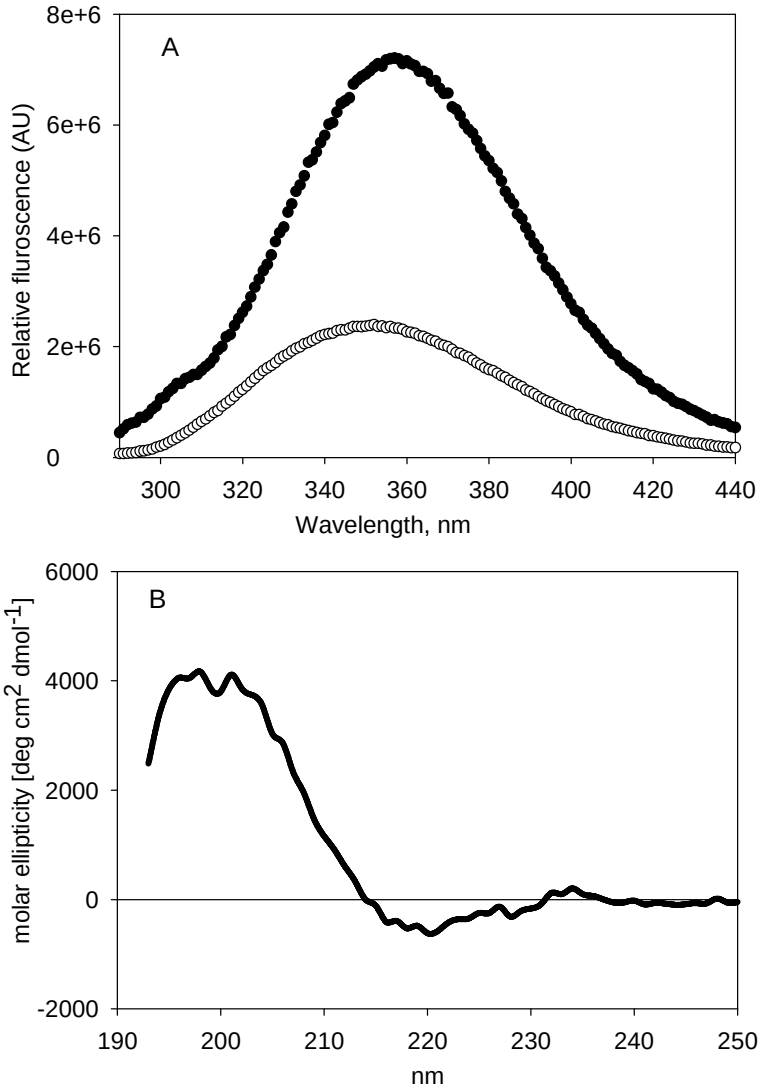


Fig. 3.13: Spectroscopic characterisation of scFvOx: A. Fluorescence spectra of renatured purified scFvOx (7.5 µg mL^{-1}) in 50 mM in sodium phosphate, pH 7.0, containing 50 mM NaCl (-○-), or 6 M GdnHCl (-●-) at 280 nm. **B. Far UV CD spectrum of native scFvOx** (0.15 mg mL^{-1}) in 25 mM phosphate, pH 7.0 (—), containing 50 mM NaCl.

Circular dichroism

Far-UV circular dichroism (CD) is generally used to estimate the secondary structure content of proteins. The far-UV CD spectrum of scFvOx in phosphate buffer was recorded between 190 and 260 nm (Fig. 3.13, B). An atypical CD spectrum with a relatively shallow minimum of the molar ellipticity at 218 nm (-530 deg cm^2 $dmol^{-1}$) and a pronounced maximum between 195 and 200 nm was obtained. Atypical CD spectra are a feature commonly observed with immunoglobulins and antibody fragments (*e.g.*, Tetin *et al.*, 2003). The large positive peak in

the CD signal at 200 nm supports the notion that the renatured purified scFvOx assumed a defined, natively folded structure.

Secondary structure analysis using algorithms SELCON3, CDSSTR and CONTIN contained in the CDpro package (Sreerama and Woody, 2000) predicted β-sheet, helix and turn-like elements of 18±5%, 0.7±0.1%, and 22±1%, respectively. This observation was consistent with literature that antibody fragments are β-sheet structure-containing proteins (Tetin *et al.*, 2003).

3.3.4 Analytical size exclusion chromatography

Analytical SEC on a TSK Gel 3000 HPLC column was employed to probe the oligomerisation state of purified scFvOx (Fig. 3.14). No aggregates were detected (Fig. 3.14, A). The molecular weight of the protein, as estimated by comparison with a calibration curve obtained with a low molecular weight gel filtration calibration kit (Amersham Biosciences) (Fig. 3.14, B), was found to be 37.6 kDa (log Mw 1.57), which was in reasonable agreement with the expected 28.7 kDa (log Mw 1.45). The value expected for a dimer would be 57.4 kDa (log Mw 1.75), so the data suggests mainly monomeric scFvOx. Pronounced peak tailing was observed, indicating interaction of the protein with the silica based material of the column, which makes the size determination questionable (Fig. 3.14, A). Therefore, in order to determine the in-solution status of scFvOx, analytical ultra-centrifugation was performed as discussed in section 3.3.5.

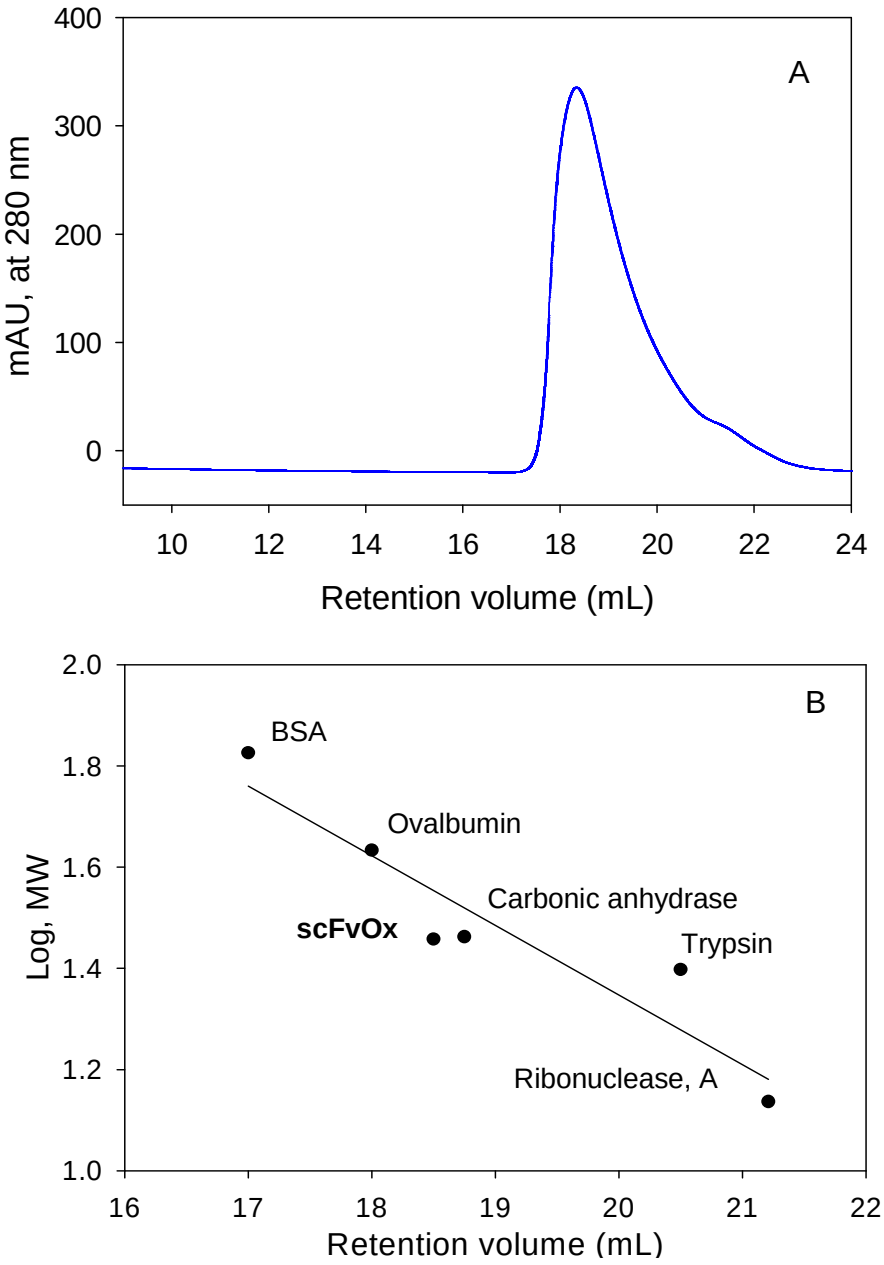


Fig. 3.14: Analytical SEC of scFvOx: A. Retention profile of scFvOx – The experiment was performed using a TSK Gel 3000 column, in 25 mM sodium phosphate, pH 7.0, containing 50 mM NaCl. Ten µg of protein were applied for analysis. Retention profile of scFvOx was monitored by optical absorption at 280 nm (---). **B. Calibration curve using low molecular weight gel filtration calibration markers** – For scFvOx, the expected molecular weight is plotted against the experimentally observed retention volume.

3.3.5 Analytical ultra-centrifugation analysis of scFvOx

In an analytical ultra-centrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption. This allows the operator to observe the evolution of the sample concentration profile versus the axis of rotation as a result of the applied centrifugal field. This experiment was performed with the help of PD Dr. Lilie. Since the earlier experiments described in section 3.3.4 could not give exact information

about the status of scFvOx in solution, analytical ultra-centrifugation analysis was performed. Under the conditions of the measurement, scFvOx was not found to form aggregates (Fig. 3.15, A).

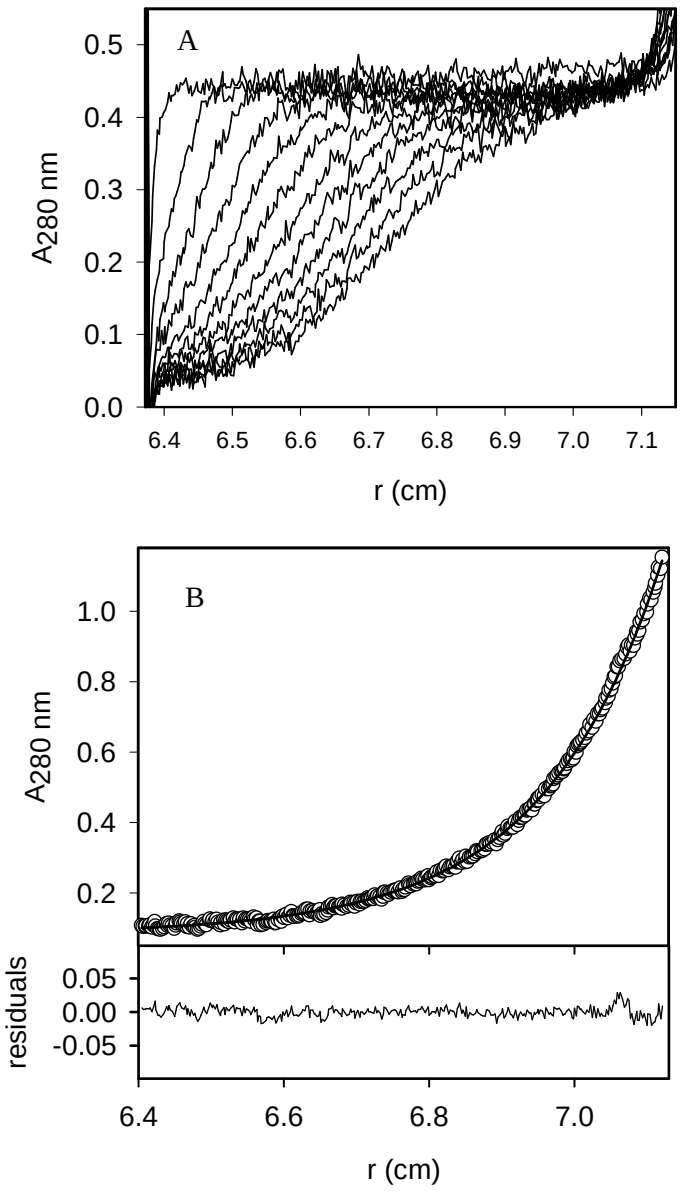


Fig.3.15: Analytical ultra-centrifugation: A. Sedimentation velocity experiment - The sedimentation velocity run was carried out at 40,000 rpm (---). Scans were taken every 10 minutes. **B. Equilibrium sedimentation experiment** - The sedimentation equilibrium was established by centrifugation at 16,000 rpm for 45 h. The equilibrium distribution of a monomeric species was fit to the data (-○-). Lower panel: Residuals of the fit. The analysis was performed in 25 mM sodium phosphate, pH 7.0, containing 50 mM NaCl, at 20°C. The protein concentration was 110 µg mL^{-1}.

The sedimentation velocity of the native protein corresponded to a sedimentation coefficient *s*(app) of 2.16 *S*. This value was consistent with monomeric scFvOx. The evaluation of the equilibrium sedimentation experiment (Fig. 3.15, B) gave a molecular mass of 27.4 kDa, in good agreement with the expected value of 29 kDa for the monomeric protein.

3.3.6 Urea-induced unfolding and refolding

Urea-induced unfolding/refolding transitions at pH 7.0 and 8.5 were compared (Fig. 3.16) to evaluate the effect of pH on the *in vitro*-refolding of scFvOx. For unfolding, the protein was incubated in a series of buffers containing the indicated urea concentrations for 24 h. For refolding, the protein was denatured in 8 M urea for 6 h, diluted into the urea buffer series and then incubated for 24 h. Tertiary structure was monitored by measuring the intrinsic fluorescence at 360 nm (Fig. 3.16).

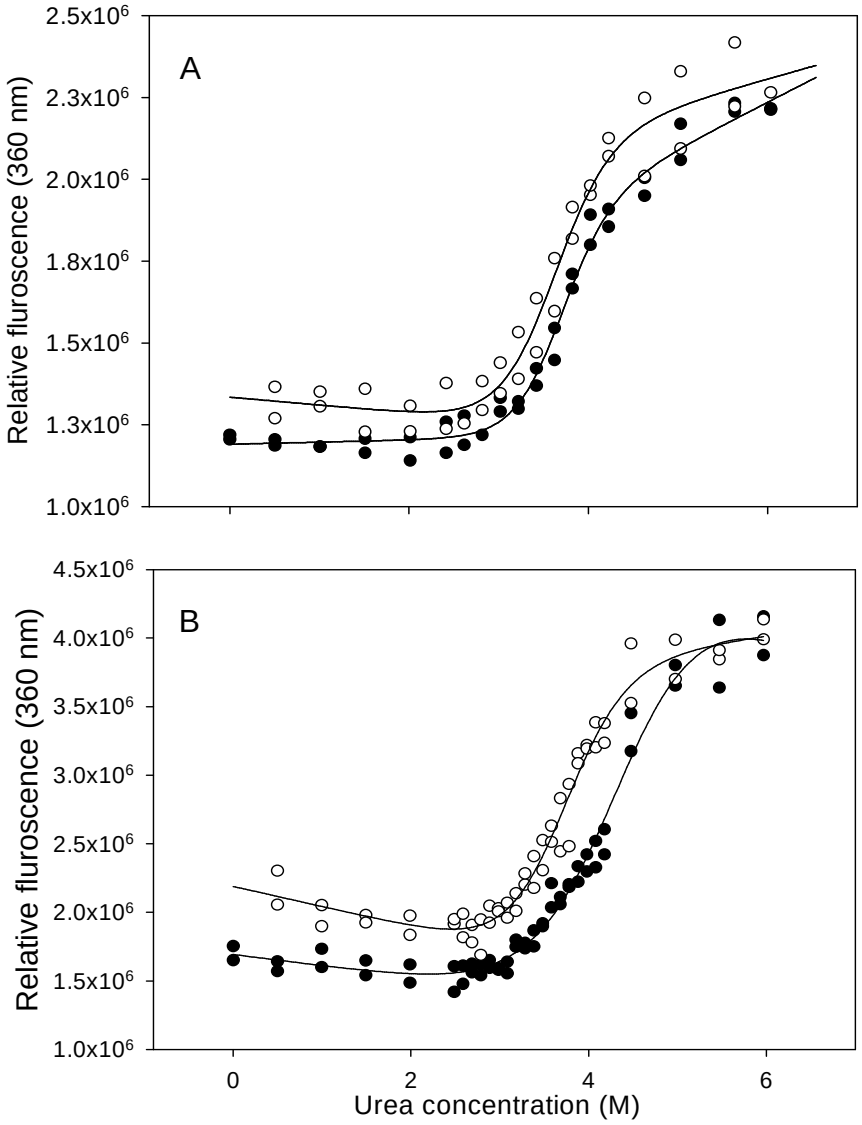


Fig. 3.16: Urea-induced unfolding and refolding transitions: A. Unfolding (-●-) and refolding (-○-) of scFvOx in 0.1 M sodium phosphate, pH 7.0, at 20°C. **B.** Unfolding (-●-) and refolding (-○-) of scFvOx in 0.1 M Tris/HCl, pH 8.5, at 20°C. The protein concentration in the experiments was 7.5 µg mL^{-1}. The fluorescence emission at 360 nm was monitored. The excitation wavelength was set to 280 nm. A two-state equilibrium folding / unfolding transition (see text) was fit to the data (—).

In each case, *i.e.*, for unfolding or refolding at either pH, sigmoid curves were obtained. At pH 7.0, the midpoints of the unfolding and refolding transitions were 3.7 M and 3.6 M, respectively. At pH 8.5, a considerable difference between the transition midpoints was noticed (4.35 M and 4.0 M, respectively). The unfolding and refolding baselines at pH 7.0 were more coinciding than at pH 8.5 (Fig. 3.16). This observation suggests that the degree of refolding obtained at pH 7.0 was higher than that at pH 8.5 (Fig. 3.16, B). At pH 7.0, 80% of denaturation of the protein was apparently reversible, as judged by visual inspection of the unfolding and refolding baselines (Fig. 3.16), whereas approx. 60% of denaturation was reversible at pH 8.5. These results indicate that pH 7.0 is more suitable than 8.5 for refolding of scFvOx. The effect of pH on the apparent stability of scFvOx was further investigated over a wider range of pH values as described in section 3.3.7.

Using the data presented in Fig. 3.16, the thermodynamic parameters of scFvOx unfolding were calculated at both pH values, assuming two-state unfolding transitions. Due to the observed lack of reversibility, the values (Table 3.2) are only estimates. The estimates for the free energy of unfolding ΔGUo values fall into the range reported for other single chain antibody fragments.

Experiment	ΔG_{Uo} (kJ mol^{-1})	*m* (kJ mol^{-1} M^{-1})	IC_{50} (M)
pH 7.0	33 ± 4	-8.9 ± 0.8	3.6 ± 0.5
pH 8.5	22 ± 3	-5.0 ± 0.5	4.4 ± 0.3

Table: 3.2: Fit parameters of urea-induced unfolding of scFvOx.

3.3.7 pH dependent stability of scFvOx

The influence of pH on the structural stability of scFvOx was investigated at pH between 2.0 and 10.0 (Fig. 3.17, A). For this purpose, purified scFvOx samples were incubated in a combined buffer system at the desired pH values for 24 h. In order to assess pH-induced structural changes, the fluorescence emission of the aromatic residues was measured at 350 nm (Fig. 3.17, A).

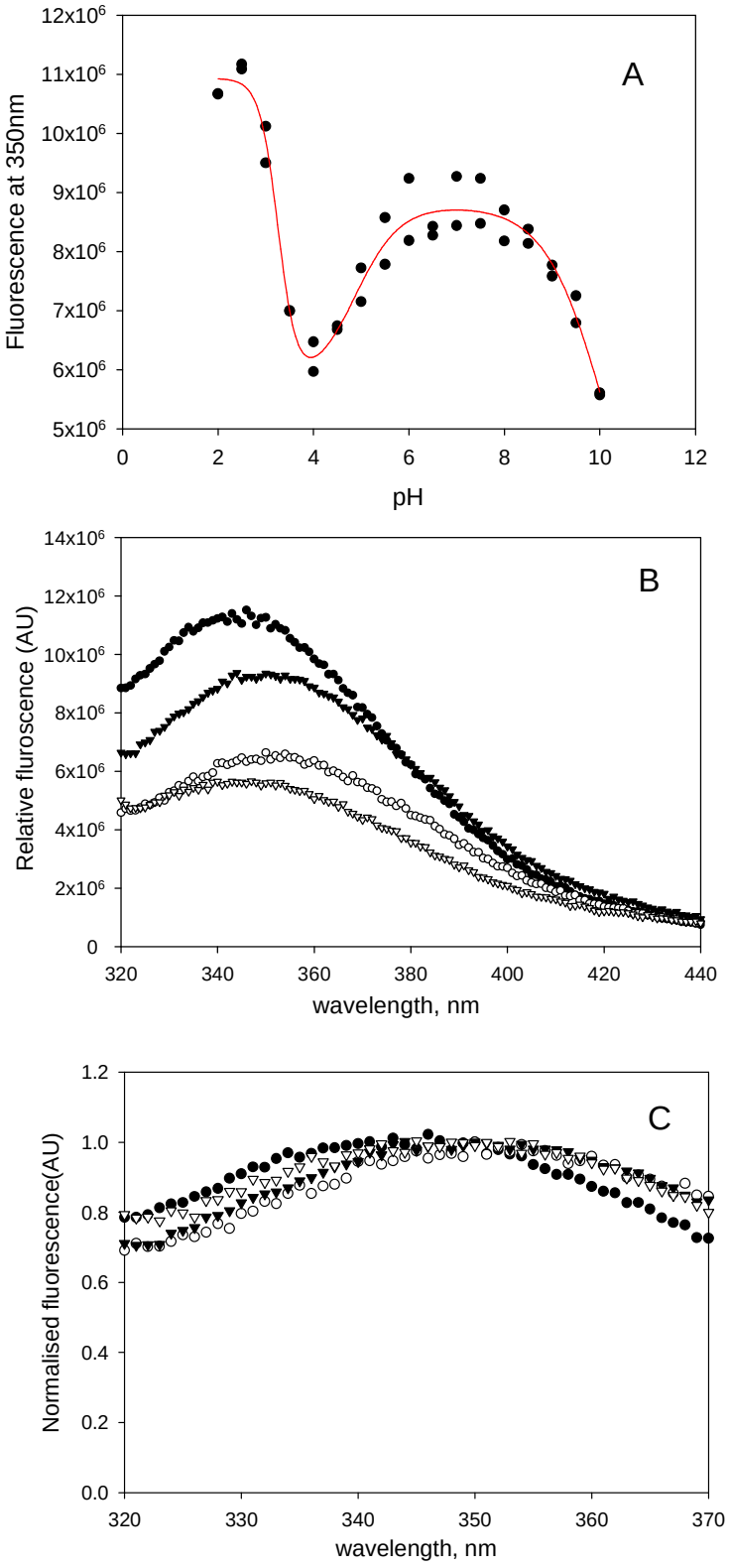


Fig. 3.17: pH dependent stability of scFvOx: A. pH transition of scFvOx - For analysis, 7.5 µg mL^{-1} protein was incubated at 20°C in a buffer containing 0.1 M citric acid, 0.1 M phosphoric acid and 0.1 M boric acid adjusted to the indicated pH values. Excitation was done at a wavelength 280 nm. Fluorescence emission at 350 nm was monitored, (-●-). The solid line is meant to guide the eye. **B. Fluorescence emission spectra of scFvOx at different pH values -** at pH 2.0 (-●-), at pH 4.0 (-○-), at pH 7.0 (-▼-), and at pH 9.5 (-▽-) respectively. **C. Normalised fluorescence data -** from figure B.

Protein was found to be stable in between pH 6.0 to 8.0. Apparently, acid and base-induced denaturation occurred below pH 5.0 and above pH 9.0, respectively (Fig. 3.17, A). Below pH 4.0, an apparent second acid-induced transition caused a strong increase in the fluorescence signal at 350 nm (Fig. 3.17, A). The maxima of the fluorescence emission spectra of scFvOx at pH 4.0 and pH 9.5, respectively, show a red shift, similar to the spectrum of GdnHCl-denatured protein at pH 7.0 (cf. section, 3.3.3, Fig. 3.13, A and Fig. 3.17, B and C).

The overall fluorescence intensity of the acid- and base-denatured protein was decreased compared to that of the native state, while chemical denaturation (cf. section, 3.3.3, Fig. 3.13, A and Fig. 3.17, B and C) had led to a strong increase in maximum fluorescence intensity. In contrast, the fluorescence emission spectrum of scFvOx at pH 2.0 showed a significant blue shift of the emission maximum to 341 nm (Fig. 3.17, B and C). The maximum fluorescence emission intensity was approximately 30% higher than that of the native protein at pH 7.0 (Fig. 3.17, B and C). The data have been normalised in Fig. 3.17, C.

These observations suggest the sequestration of the aromatic amino acids within the interior of the protein and therefore some degree of structure formation of scFvOx polypeptide at pH below 3.0. Acid-induced alternatively folded or molten globule-like conformational states have been described for a series of proteins, including immunoglobulins and antibody fragments (Buchner *et al.*, 1991 and 2001).

3.3.8 Characterisation of the acid-induced state

GdnHCl-induced unfolding of the acid-induced state

The putative acid-induced state of scFvOx was further characterised. For this purpose, the fluorescence spectra of scFvOx were recorded at pH 2.0 in the presence or absence of the denaturant GdnHCl and compared to the spectra of native and denatured protein at pH 7.0 (cf. section 3.3.7) (Fig. 3.17, A and B). As described above, scFvOx exhibited an increase in fluorescence emission intensity and a blue shift of the emission maximum to 343 nm (Fig. 3.18, A, B) at pH 2.0. This blue shifted fluorescence spectrum was clearly distinguishable from that of the native as well as from that of the GdnHCl-denatured spectrum at pH 7.0.

After addition of 6 M GdnHCl at pH 2.0, a red shift in the fluorescence emission maximum of scFvOx to 359 nm was observed (Fig. 3.18 B), this corresponds to fluorescence maximum of the GdnHCl-denatured protein at pH 7.0. These findings suggest that the acid-induced conformation at pH 2.0 and the GdnHCl-induced conformations represent distinct protein states.

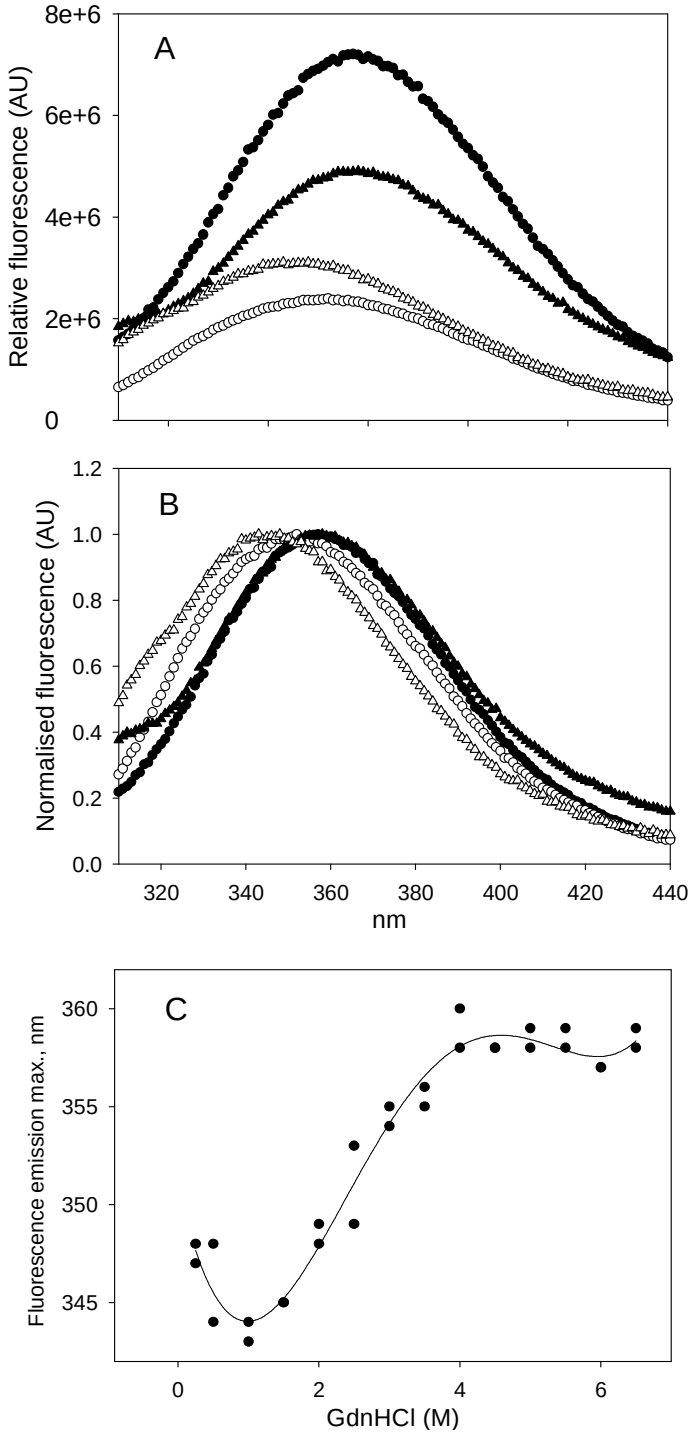


Fig. 3.18: Spectroscopic characterisation of acid-induced state: A. Fluorescence spectra and **B. Normalised data of the fluorescence spectra A -** For analysis native 7.5 µg mL^{-1} of protein was incubated in 50 mM sodium phosphate, pH 7.0, containing 50 mM NaCl (-○-), or 6 M GdnHCl (-●-). 50 mM sodium phosphate, pH 2.0, containing 50 mM NaCl (-△-), or 6 M GdnHCl (-▲-). **C. Denaturant-induced unfolding transition -** For analysis 7.5 µg mL^{-1} of protein was incubated in 50 mM sodium phosphate, pH 2.0, containing the indicated concentrations of GdnHCl. After incubation for 16 h at 20°C, fluorescence emission spectra were measured after excitation at 280 nm. The solid line is meant to guide the eye.

In order to investigate the conformational shift from the acid-induced state to the GdnHCl-induced state at pH 2.0, scFvOx was incubated for 6 h at pH 2.0 and subsequently transferred to buffers containing increasing concentrations of GdnHCl at pH 2.0. After additional incubation for 24 h, fluorescence spectra of the protein samples were measured. Formation of aggregates at pH 2.0 precluded a quantitative evaluation of the transition between acid-induced and GdnHCl-induced state (cf. section 3.3.9). However, when the maxima of the fluorescence emission spectra were plotted against the concentration of denaturant, a co-operative transition with a midpoint at approx. 2.6 M GdnHCl was observed (Fig. 3.18, C). This suggests that the acid-induced state contains tertiary contacts that have to be disrupted simultaneously upon unfolding, and defines an alternative folded state of the protein.

Circular dichroism

The far-UV CD spectrum of the acid-induced state of scFvOx was recorded at pH 2.0 (Fig. 3.19). Interestingly, the spectrum at 220 nm showed significantly higher amplitude than that of the native protein and indicated a significant content of ordered secondary structure elements. The content of β-sheet, helix and turn was 22.07% ±7.2, 1.9% (± 0.018) and 23.21% (± 0.019), respectively. These observations support the idea that the acid-induced state of scFvOx assumes an alternative, ordered conformation with a high content of secondary structure elements.

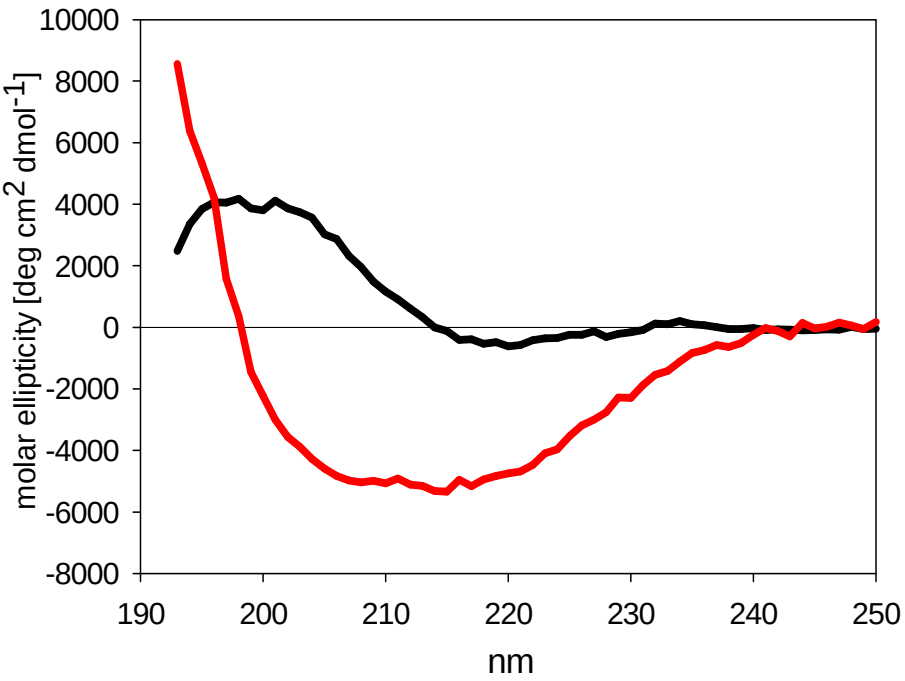


Fig. 3.19: CD spectra of scFvOx: Recorded in 25 mM sodium phosphate, containing 50 mM NaCl, adjusted to pH 2.0 (—) , or pH 7.0 (—) respectively.

Ultra-centrifugation analysis of the acid-induced state

During the GdnHCl-induced unfolding of scFvOx at pH 2.0 (cf. sub-section above), a quantitative analysis of the results was precluded by loss of signal during the incubation of the samples, possibly due to protein aggregation. In order to evaluate oligomerisation of aggregation status, scFvOx was subjected to a sedimentation velocity ultra-centrifugation at pH 2.0 (Fig. 3.20). Unlike the native protein at pH 7.0 (Fig. 3.15, A), the protein rapidly settled down in the measurement cell, clearly indicating aggregation. A quantitative determination of sedimentation velocities for the acid-induced state of scFvOx at pH 2.0 was therefore not possible.

The very limited solubility of the acid-induced conformation of scFvOx also precluded its thorough biophysical characterisation. Although hallmarks of a proper alternatively folded state could be observed spectroscopically, definite proof for the existence of a distinct thermodynamic state remained elusive.

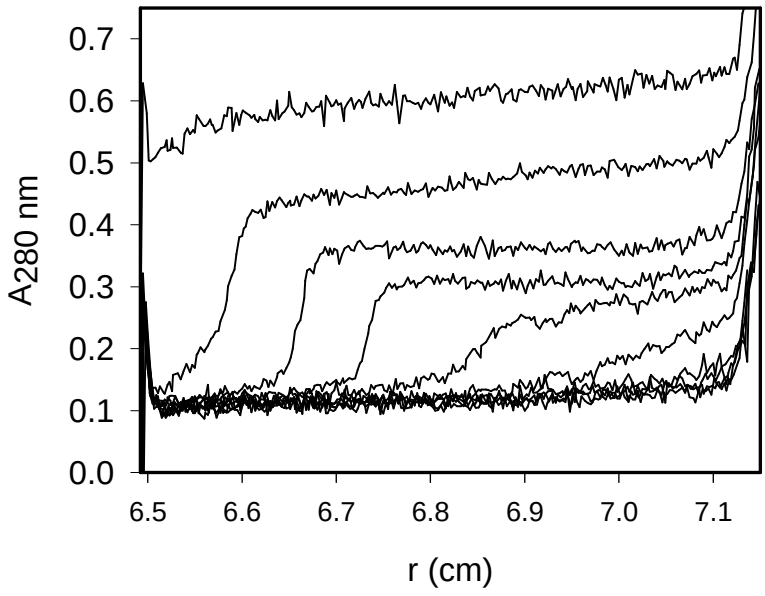


Fig. 3.20: Analytical ultra-centrifugation of scFvOx at pH 2.0: The analysis was performed in 25 mM sodium phosphate, pH 2.0, containing 50 mM NaCl, (---). Protein concentration was 110 µg mL^{-1}. The sedimentation velocity run was carried out at 40,000 rpm and 20°C. Scans were taken every 10 minutes.

3.4 Ionic Liquids as renaturation additives

Renaturation of proteins is often limited by a kinetic competition between productive folding and irreversible off-pathway reactions such as aggregation. In case of scFv's, the formation of two disulfide bonds is required for correct folding. Furthermore, scFv's are vulnerable to aggregation. In the absence of additives, renaturation yields are typically close to the detection limit. Although functional scFvOx could be successfully renatured *in vitro* in the

presence of L-arginine, an improvement of renaturation yields would be desirable. In addition, testing novel additives for the further improvement in renaturation yield remains one of the areas of interest in research. In order to study the renaturation of scFvOx, a series of N'-alkyl and N'-(ω-hydroxyalkyl) N-methylimidazolium chlorides, with alkyl chain lengths varying between two and six carbon atoms, were investigated.

3.4.1 Influence of N'-alkyl N-methyl imidazolium chlorides on renaturation of scFvOx

The inclusion body solubilisate of scFvOx as described in the section 2.4.2 served as starting material for these renaturation experiments. The oxidative renaturation of scFvOx was first performed at concentrations of IL's ranging from 0.5 M to 4.0 M. Renaturation in the presence of L-Arg/HCl served as reference for this experiment. Renaturation yields were determined by the antigen binding activity by ELISA.

The results are represented in Fig. 3.21. An L-Arg/HCl concentration 1.0 M resulted in 3.5% renaturation yield (Fig. 3.21). Significant enhancement in renaturation yield was observed in the presence of EMIM-Cl (Fig. 3.21) - up to 4.5% at 0.5 M EMIM-Cl (Fig. 3.22). This was an increase of 28.6% in the renaturation yield in comparison to L-Arg/HCl. Compounds OH-EMIM-Cl and OH-PMIM-Cl were also found to promote renaturation of scFvOx, but only slightly compared to that with L-Arg/HCl. Up to 2.2% and 2.3% renaturation yields were obtained in the presence of 1 M OH-EMIM-Cl and 0.5 M OH-PMIM-Cl, respectively (Fig. 3.21, B). For the IL's that did promote renaturation, lower concentrations were also evaluated (Fig. 3.22). Maximum renaturation yields were observed in the presence of 0.5 M EMIM-Cl (Fig. 3.22, A). The combination of least concentration and highest renaturation yield were 0.8 M and 0.3 M and 2.5% and 2.4% in case of OH-EMIM-Cl and OH-PMIM-Cl, respectively (Fig. 3.22, B and C). Concentration of the IL's higher than 1 M was counterproductive to the yield of renaturation. The salts carrying longer alkyl side chains at the substituted methylimidazolium cations did not act as effective renaturation enhancers for scFvOx, and the detrimental effect at higher concentrations was more pronounced with such compounds. In the presence of compounds PMIM-Cl, BMIM-Cl, and OH-HMIM-Cl, up to 0.8%, 0.7% and 1.2% recovery, respectively, of the denatured protein was seen in the presence of 0.5 M salt concentration (Fig. 3.22, A), and this was close to the detection limit of the assay. No scFvOx was recovered in the case of HMIM-Cl.

Apart from these imidazolium chlorides, other IL's with varying cations and anions were tested as renaturation additives, which are described below (section 3.4.2).

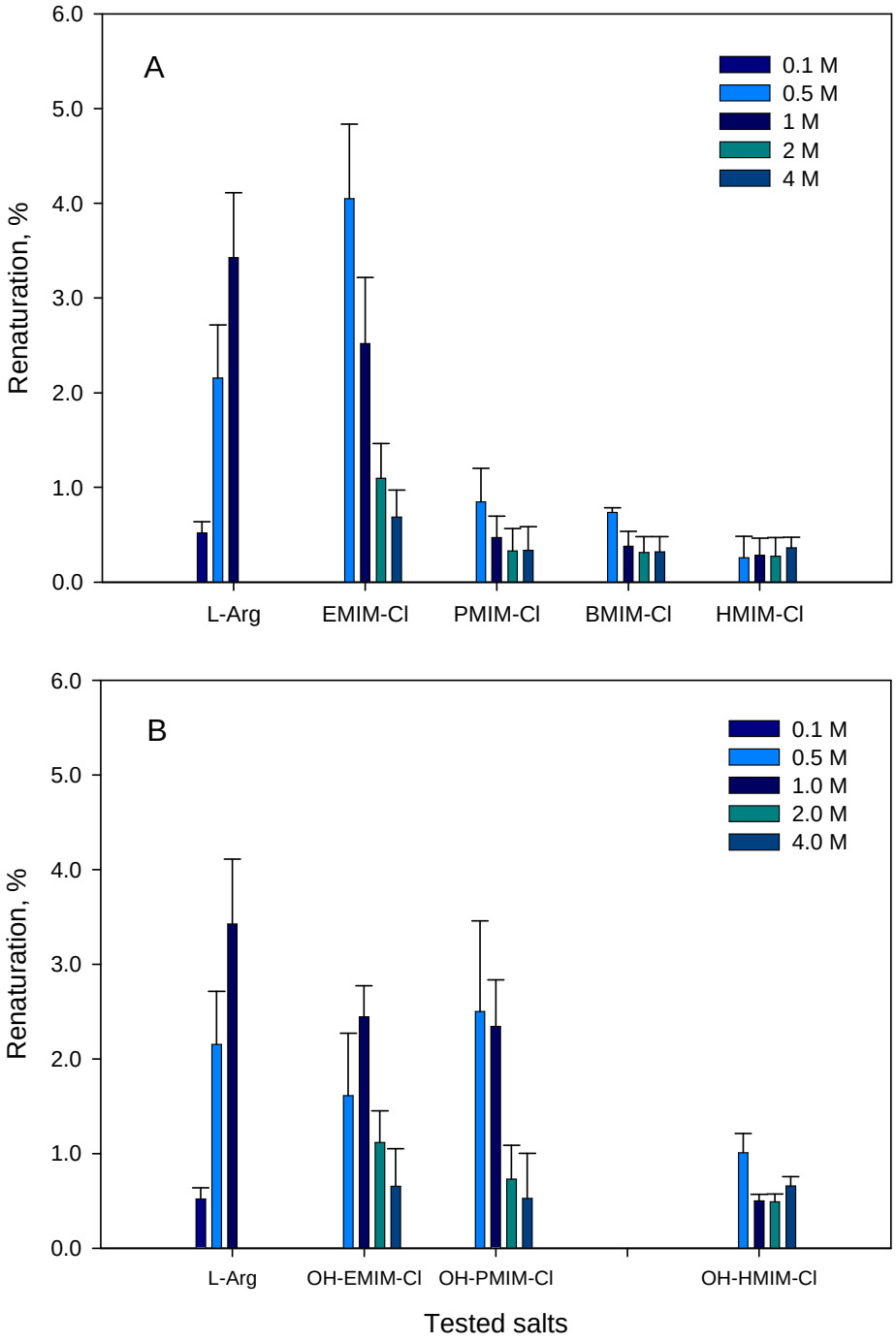


Fig. 3.21: Renaturation of scFvOx in the presence of A. N'-alkyl N-methylimidazolium chlorides and B. N'-(ω-hydroxyalkyl) N-methylimidazolium chlorides: Reduced scFvOx at 80 µg mL^{-1} was diluted into renaturation buffer (80 mM Tris/HCl, pH 8.7, containing 2.5 mM GSH, 2.5 mM GSSG, 1 mM EDTA) containing organic salts as indicated. Renaturation was allowed to proceed for 96 h at 15°C. Renaturation in presence of L-Arg/HCl was included as a reference. Renaturation yields were determined by ELISA. Bars are colour-coded and marked with the molar concentrations of the respective organic salts. Error bars indicate the standard deviations of four independent experiments.

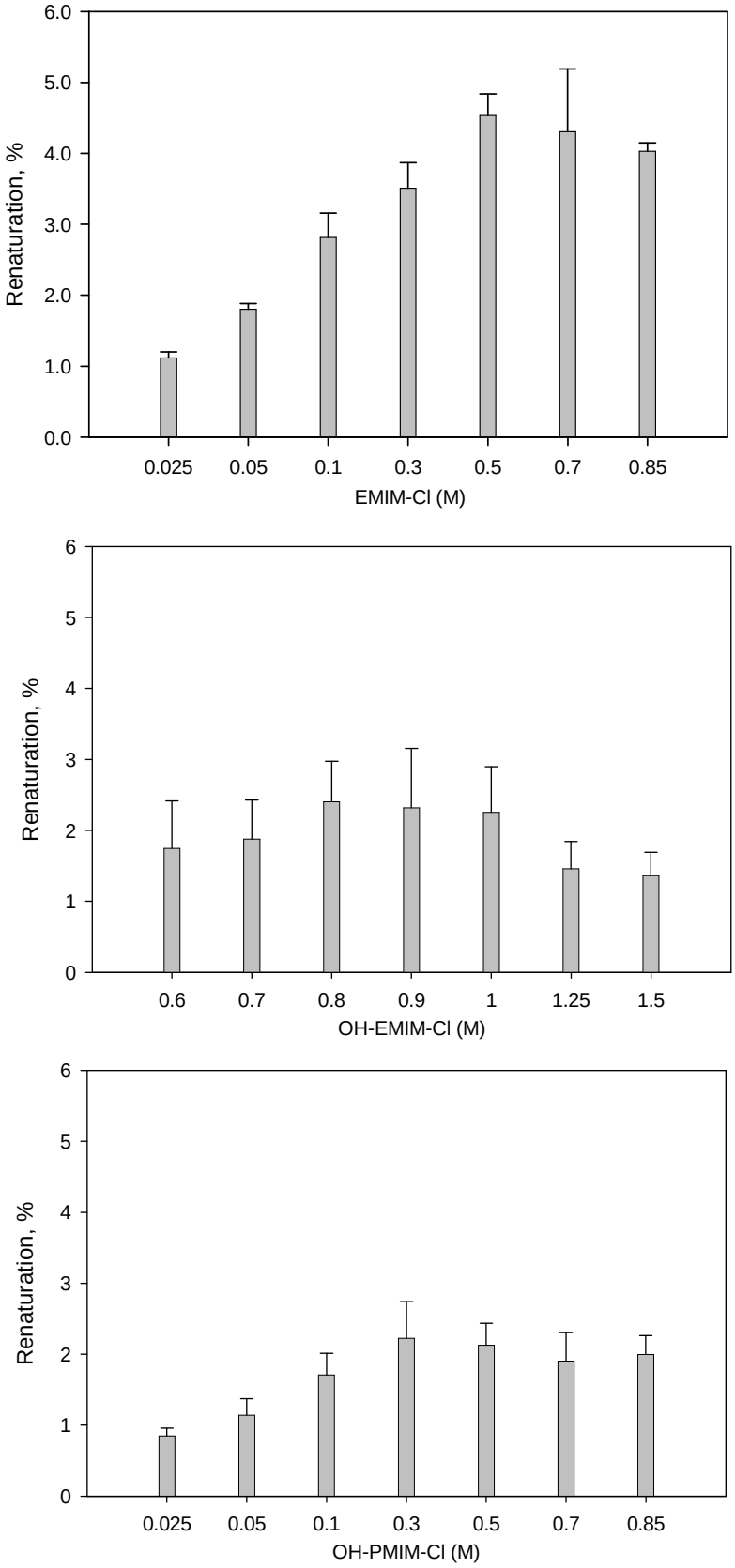


Fig. 3.22: Renaturation of scFvOx at lower concentration of IL's that showed yield enhancement: Renaturation was performed as described in the legend of Fig. 3.21. Renaturation yields were determined by ELISA. Error bars indicate the standard deviations of four independent experiments.

3.4.2 Effect of other Ionic Liquids

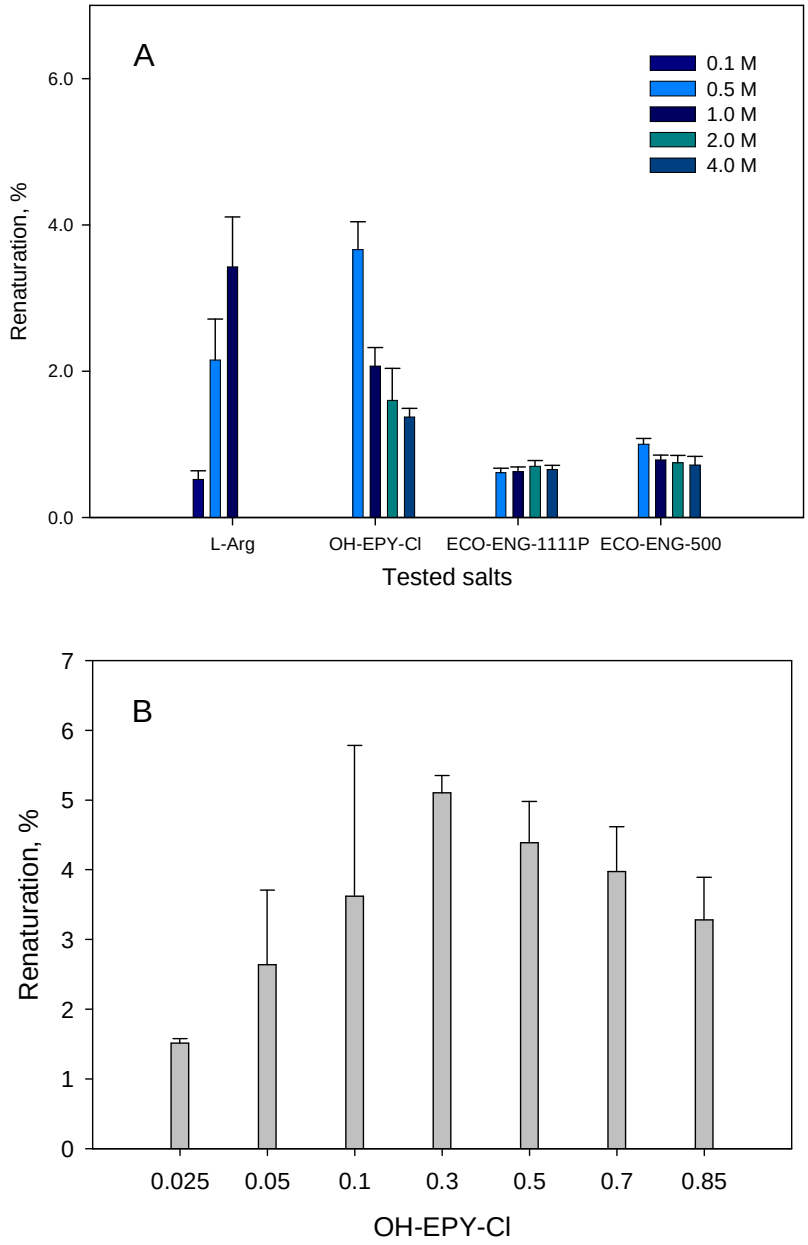


Fig. 3.23: Effect of IL's with varying cations on the renaturation of scFvOx: A. Renaturation in the presence of various IL's and **B. Screening of lower concentration of OH-EPY-Cl -** Renaturation was performed as described in the legend of Fig. 3.21. Renaturation yields were determined by ELISA. Renaturation in presence of L-Arg/HCl was included as a reference. Bars are marked with the molar concentrations of the respective organic salts. Error bars indicate the standard deviations of four independent experiments.

Renaturation screening experiments in this set included N-(2-hydroxyethyl) pyridinium chloride (OH-EPY-Cl), N,N'-dimethylimidazolium dimethylphosphate (ECO-ENG-1111P) and cocoasalkyl pentaethoxymethyl ammonium methyl sulfate (ECO-ENG-500). The remaining conditions for the renaturation experiments were the same as described above in section 3.4.1. OH-EPY-Cl was found to enhance the renaturation yield of scFvOx compared

to L-Arg/HCl. Up to 3.8% renaturation yield was obtained at a salt concentration of 0.5 M (Fig. 3.23, A), and the compound was most effective at 0.3M, when > 5.2% of the protein was recovered (Fig. 3.23, B). The highest renaturation was an increase of about 50% as compared to renaturation obtained in the presence of L-Arg/HCl. However, as observed in case of other IL's (cf. section 3.4.3), increased concentrations of OH-EPY-Cl were not helpful for the renaturation of scFvOx. The other two compounds, N, N'-dimethyl imidazolium dimethylphosphate (ECO-ENG-1111P) and the quaternary ammonium methylsulphate (ECO-ENG-500) were not beneficial for the renaturation of scFvOx (Fig. 3.23, A).

In conclusion, IL's EMIM-Cl, OH-EMIM-Cl, OH-PMIM-Cl and OH-EPY-Cl promoted *in vitro*-renaturation of scFvOx, and their effects were slightly higher or equivalent to that of the widely used additive L-Arg/HCl. In certain cases, an increase of 28-50% renaturation yield over that of L-Arg/HCl was observed. Compound OH-EPY-Cl was the most effective.

3.5 Renaturation from the mixed disulfide of scFvOx and GSH

The applicability of the mixed disulfide of scFvOx with glutathione as starting material for *in vitro*-renaturation was explored in order to establish a protocol for the screening of aromatic thiol compounds. The mixed disulfide was prepared by reacting the reduced-denatured protein with an excess of glutathione, as described in section 2.9.2. Renaturation was performed in the presence of GSH concentrations ranging from 0 to 10 mM.

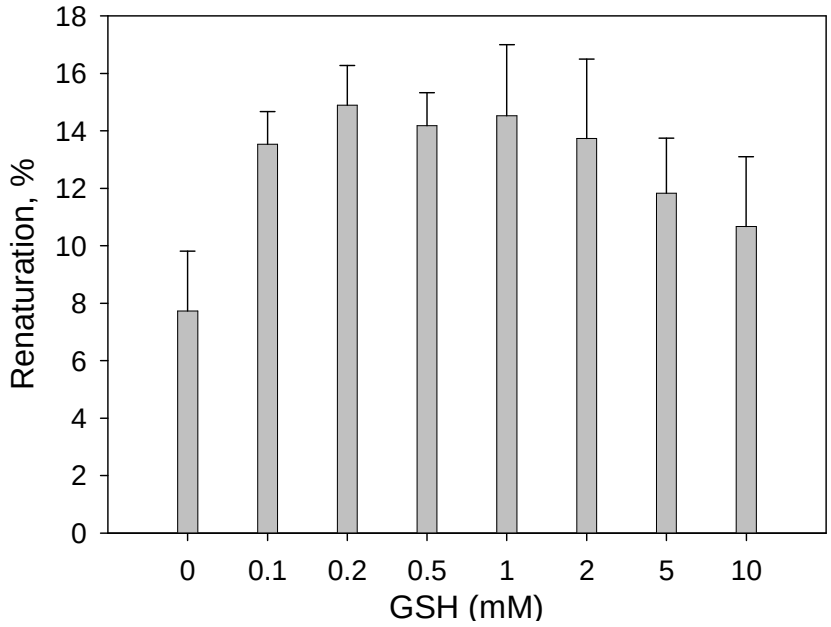


Fig. 3.24: Optimisation of GSH concentration for the renaturation of glutathione modified mixed disulfide species: One hundred forty µg mL^{-1} glutathione modified mixed disulfide species were diluted into the renaturation buffer containing 80 mM Tris/HCl, 1 mM EDTA, pH 8.7. 1 M L-Arg/HCl was included as additive in all experiments. Renaturation yields were analysed by ELISA after 96 h of renaturation at 15°C. Error bars indicate the standard deviations of three independent experiments.

The optimum concentration of GSH for renaturation of the glutathione modified mixed disulfide of scFvOx was 0.2 mM (Fig. 3.24). However, a broad GSH concentration optimum, between 0.2 mM and 1.0 mM was observed. A further increase in concentration was not helpful for *in vitro*-renaturation. Therefore, GSH or aromatic thiols were used at 0.2 mM in further experiments.

3.6 pH-dependence of scFvOx renaturation and GSH

Thermodynamic factors like pH and temperature have a considerable influence on protein stability and therefore on the *in vitro*-renaturation of proteins. The pH dependence of the *in vitro*-renaturation of scFvOx was investigated using purified reduced-denatured scFvOx as well as its mixed disulfide with glutathione as starting material. This experiment was performed in the presence of L-Arg/HCl as additive, while GSH and GSSG were used as redox buffer components.

The renaturation of scFvOx was highly dependent on the pH (Fig. 3.25). In case of the reduced-denatured-protein (Fig. 3.25, black bars), an optimum renaturation yield of 11% was obtained at pH 7.0. At pH 8.5, the renaturation dropped to below 8%. Thus, renaturation yield obtained at pH 7.0 was up to 37.5% higher than at pH 8.5. At pH 6.0, only 7.3% renaturation yield was obtained. Below pH 5.0 and above pH 8.5, the amount of active protein was close to the detection limit of the assay. Incidentally, the renaturation pH optimum correlated with the stability of scFvOx observed in the pH-dependent denaturation experiment, as discussed in section 3.3.7.

In a parallel set of experiments using mixed disulfide with glutathione (Fig. 3.25, gray bars), reduced glutathione was used at a concentration of 0.2 mM to catalyse the formation of disulfide bonds. The pH optimum was lower compared to that for reduced-denatured-protein. The highest renaturation yield of 12% was observed at pH 6.0. At pH 8.5, the renaturation yield was below 7% (Fig. 3.25). Thus, renaturation yield obtained at pH 7.0 was 70% higher than at pH 8.5. Below pH 5.0 and above pH 8.5, renaturation yields were close to the detection limit of the assay. Importantly, since the highest renaturation yield was at pH 7.0, well below the thiol pK_a of pH 8.7 for glutathione, these results indicate the stability of scFvOx.

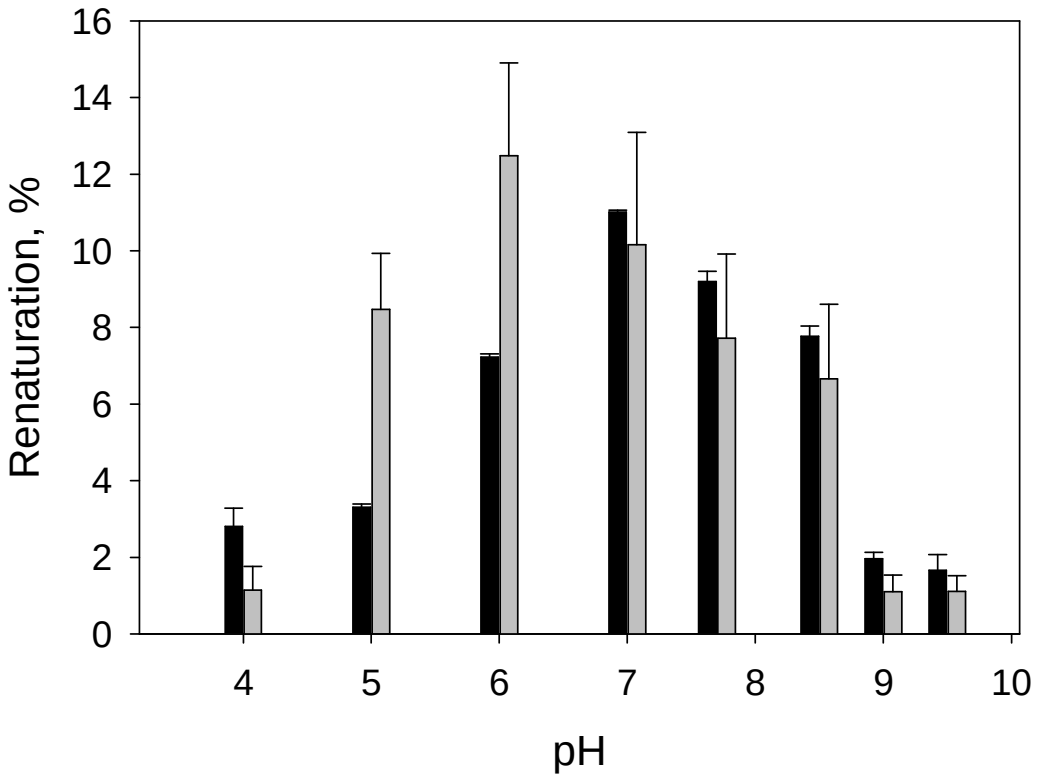


Fig. 3.25: Effect of pH on the renaturation of IB solubilisate and of the mixed disulfide with GSH: Renaturation was performed in buffers containing 0.1 M acetic acid/NaOH for pH values 4.0-5.0, Bis-Tris/HCl for pH 6.0, Tris/HCl for pH values 7.0-8.5 and boric acid/NaOH for pH values 9.0-9.5, in the presence of 1 mM of EDTA. Experiments were performed at concentrations of 140 µg mL^{-1} starting material. For renaturation of reduced protein (black bars), GSH and GSSG were applied at a concentration of 2.5 mM. The mixed disulfide of scFvOx with glutathione was refolded in presence of 0.2 mM GSH, (gray bars). 1 M L-Arg/HCl was included as additive in all experiments. Renaturation yield was analysed by ELISA after 96 h of renaturation at 15°C. Error bars indicate the standard deviations of four independent experiments.

These observations revealed two important points: (1) earlier renaturation experiments (cf. section, 3.2.1 and 3.2.2) were performed under sub-optimal conditions, and (2) an alternative redox buffer system which functions more effectively at lower pH is desirable. In addition to information concerning the pH optima for the renaturation of scFvOx, the results described here also indicated that renaturation using the mixed disulfide is a suitable system for studying the effect of various thiol compounds on the *in vitro*-renaturation of scFvOx.

3.7 Aromatic thiols as redox buffers

3.7.1 Effect of aromatic thiols on the renaturation of the mixed disulfide of scFvOx with glutathione

The effects of four aromatic thiol compounds, thiosalicylic acid (ThioSA), 4-mercaptopyridine (4-MPYN), 2-mercaptopyrimidine (2-MPYMN), and 2-mercaptopyridine N-oxide (2-MPYN-ox) (cf. section, 2.1.2) as redox buffers on the *in vitro*-renaturation of the

mixed disulfide of scFvOx with glutathione were tested in comparison to GSH over the pH range of 4.0 to 8.5 (Fig. 3.26). The thiol compounds were used at a total concentration of 0.2 mM. Therefore, the relative concentrations of the protonated and the active thiolate form, respectively, were not fixed but varied as a function of thiol pK_a and solution pH. These experiments were performed in the presence of L-Arg/HCl as additive.

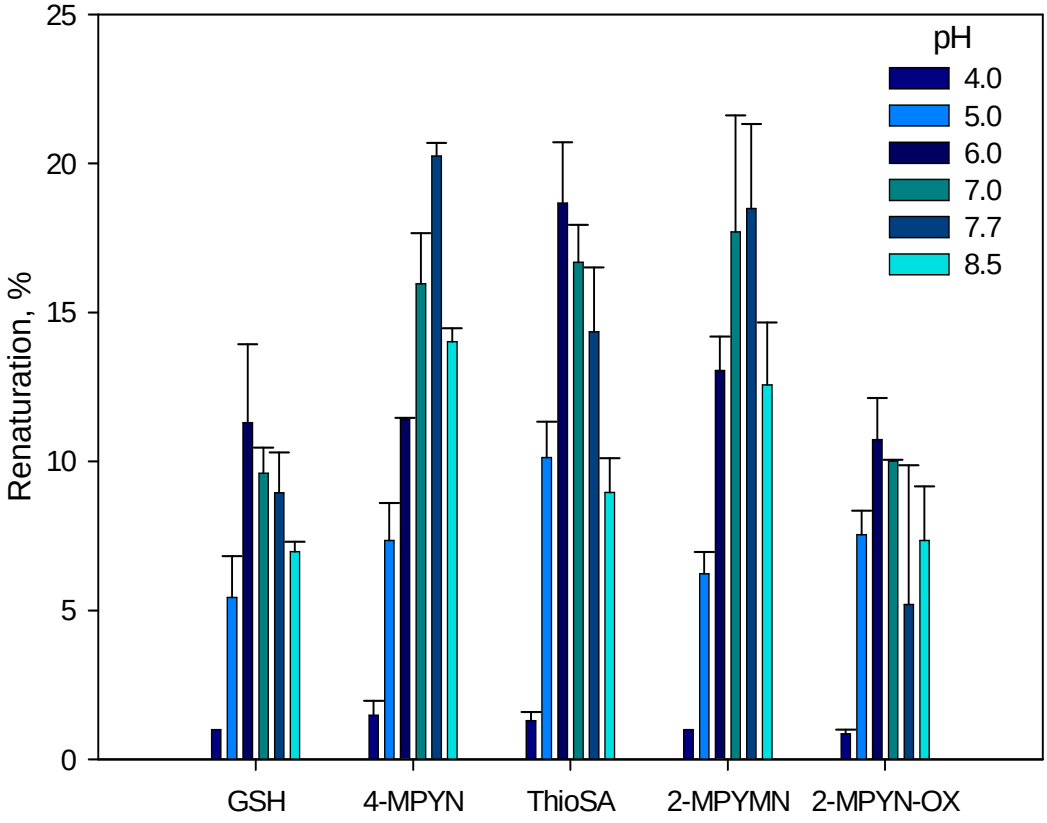


Fig. 3.26: Renaturation of the glutathione-modified mixed disulfide of scFvOx in the presence of aromatic thiols: Renaturation was performed in buffers containing 0.1 M acetic acid/NaOH for pH 4.0-5.0, Bis-Tris/HCl for pH 6.0, Tris/HCl for pH 7.0-8.5 and boric acid/NaOH for pH 9.0-9.5 in the presence of 1 mM of EDTA. Glutathione modified mixed disulfide was used as starting material at 140 µg mL^{-1}, and aromatic thiols or GSH were applied at 0.2 mM. 1 M L-Arg/HCl was included as additive in all experiments. Renaturation yields were analysed after 96 h at 15°C. Error bars indicate the standard deviations of six independent experiments.

The tested aromatic thiols were found to have significant effects on the renaturation of scFvOx (Fig. 3.26), with similar pH dependence in all cases. The pH optima ranged from 6.0 for TihoSa to 7.7 for 4-MPYN. Presence of GSH resulted in 12% renaturation yield. In comparison, for three of the four tested compounds, significantly enhanced renaturation yields were observed at least at one pH value, while the yield was significantly reduced above pH 6.0 with 2-MPYN-ox (Fig. 3.26).

Enhanced renaturation yield was observed in case of 4-MPYN, with up to 21% at pH 7.7, an increase of up to 75% compared to renaturation in the presence of GSH. Inclusion of ThioSA resulted in 18% yield, with the renaturation optimum shifted to pH 6.0. A pH optimum of 7.0-7.7 was observed in the case of 2-MPYMN, with renaturation yield around 18%, a 50% increase over that in the presence of GSH. 2-MPYN-ox did not enhance renaturation of scFvOx, as 11% renaturation yield was observed at pH 6.0, which was comparable to that in the presence of GSH.

Importantly, the overall effect of all three compounds on the final renaturation yield was not marked, as in all three cases renaturation maximum was 18-21%, an increase 50-75% over ranaturation in the presence of GSH. However, the most important finding was that, all these compounds showed renaturation pH optima lower than 8.5. At pH 5.0, some recovery of scFvOx was observed in all cases, with up to 10% renaturation yield obtained in the presence of ThioSA. At pH 4.0, no recovery of scFvOx could be detected above the background signal of the ELISA analysis.

In conclusion, three of the four tested compounds significantly enhanced renaturation yields of scFvOx in comparison to glutathione. The compound 2-mercaptopyridine N-oxide did not enhance the renaturation of scFvOx.

3.7.2 Effect of aromatic thiol compounds on renaturation kinetics of the mixed disulfide of scFvOx with glutathione

For proteins containing disulfide bonds, the renaturation rate is usually limited by thiol-disulfide exchange reactions. These reactions occur within the protein itself or between the protein and the redox buffer, which consists of a mixture of a thiol and the corresponding disulfide. Therefore, the reactions were allowed to take place between the mixed disulfide of scFvOx on the one hand, and glutathione or aromatic thiols on the other. Renaturation kinetics in the presence of the aromatic thiols were compared with that of the reaction in the presence of GSH, under identical experimental conditions. After starting the assay, samples were withdrawn at defined time intervals and analysed by ELISA (Fig. 3.27). Although it may be assumed that renaturation follows complex kinetics, it was not possible to distinguish kinetic phases within the uncertainty of this analytical method. Therefore, *in vitro*-renaturation of scFvOx from its mixed disulfide was described with a single apparent time constant, k_{app}, when reasonable fits of data could be obtained (Fig. 3.27, A).

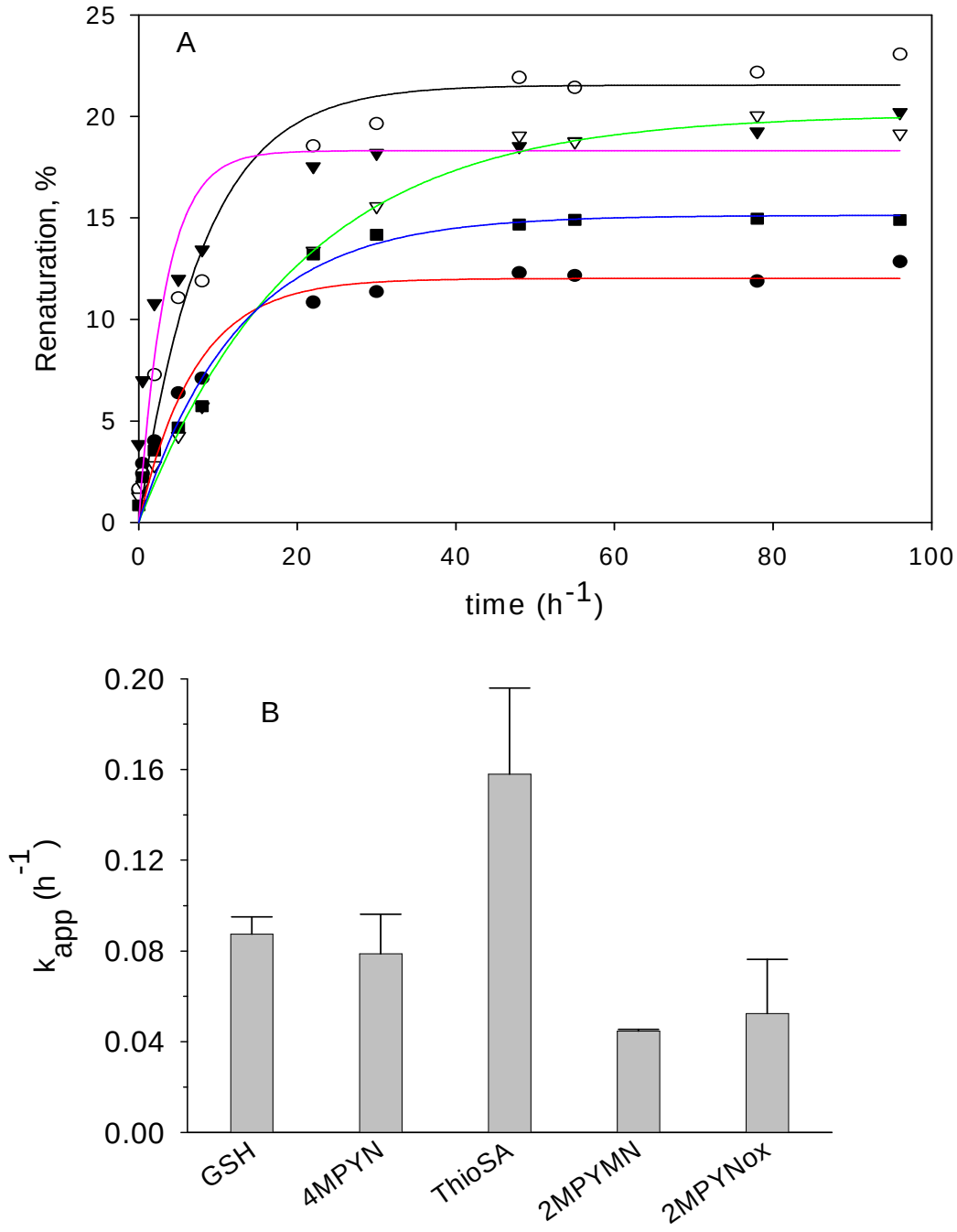


Fig. 3.27: Effect of aromatic thiols on renaturation kinetics: A. Renaturation kinetics at pH 7.0 - Renaturation was performed in 0.1 M Tris/HCl, pH 7.0, containing 1 mM EDTA. Mixed disulfide of scFvOx with glutathione was used as starting material at 140 µg mL^{-1} and renaturation was performed in the presence of 0.2 mM of GSH (-●-), 4-MPYN (-○-), ThioSA (-▼-), 2-MPYMN (-▽-) and 2-MPYN-Ox (-■-), respectively. 1 M L-Arg/HCl was included as additive in all experiments. Renaturation yields were analysed by ELISA. Solid lines represent single-exponential fits to the data. **B. Apparent rate constants -** Apparent first-order rate constants k_{app} for the renaturation of the mixed disulfide of scFvOx. Error bars indicate the standard deviations of three independent experiments.

Although the adopted method of analysis was relatively crude, considerable differences in the kinetics of renaturation could be resolved in the presence of the tested thiol compounds (Fig. 3.27). The apparent rate of renaturation in the presence of 4-MPYN was the same as that in

the presence of GSH. The compounds 2-MPYMN and 2-MPYN-OX significantly affected the apparent rate of the renaturation reaction. Highest apparent renaturation of scFvOx was observed with thiosalicylic acid: k_{app} = 0.16 h^{-1} (Fig. 3.27, B), a rate approximately twice that in the case of glutathione.

In conclusion, a 2.5-fold enhancement in the recovery of scFvOx was achieved in the presence of aromatic thiols by performing renaturation reaction at the pH where protein was most stable. In addition, renaturation kinetics studies revealed that time required for the completion of reaction could be reduced from 96 h to 48 h.

4. Discussion

The bacterial expression of antibody fragments has been a primary driving force behind the rapid expansion and major successes of antibody engineering in the past two decades. Antibody library screening by phage display, which is now a relatively routine procedure for the *de novo* isolation of mAb fragments and for improving antibody properties by evolutionary approaches, is contingent upon expression of properly folded. Antibody engineering and bacterial expression provide a convenient means of generating antigen binding fragments for evaluation, isolation, and production of antibodies, alleviating any concerns about the use of animals for such purposes (Huston *et al.*, 1988, 1991, 1993 and 1996; Holliger and Hudson, 2005; Schirrmann *et al.*, 2008).

As applications for mAb's and their derivatives continue to increase, it is likely that bacterial expression will continue to play a role in their manufacture. However, it is unlikely that bacterial expression of whole antibodies will become practical because of the difficulties in expressing such large molecules in bacteria and the requirements for glycosylation. For full length mAb's mammalian cell and sometimes yeast are the host of choice, but for antibody fragments, *E. coli* remains one of the most attractive host organisms. Although some information is available about the factors influencing antibody yield in *E. coli*, it is not possible to identify general conditions that will give good expression of a particular antibody format. Studies have shown that antibody sequence remains the key factor in determining yield and various engineering and expression strategies may have to be investigated for each individual molecule in order to achieve acceptable product yield (Huston *et al.*, 1988, 1991, 1993 and 1996; Holliger and Hudson, 2005; Schirrmann *et al.*, 2008). Antibody expression is influenced by intrinsic factors such as transcription and translation efficiencies, toxicity to *E. coli*, amino acid sequence, stability and susceptibility to proteolytic degradation, and spontaneous protein folding. Extrinsic or physiological factors such as translocation inside the cell, processing, protein degradation, assisted protein folding, aggregation and limited renaturation yields have so far been shown to affect production of antibody fragments (Wulfing and Plückthun, 1994; Worn and Plückthun, 2001; Oelschlaeger *et al.*, 2003). In line with some of these established facts, production of antibody fragment by means of secretion into the periplasm and recovery by means of renaturation of IB's were investigated in this work.

4.1 Periplasmic production of scFvOx

The periplasmic strategy imitates the natural folding process and secretion of immunoglobulins in eukaryotes. In bacteria, the secretory machinery directs proteins carrying specific signal sequences such as *pel*B, *pho*A and *omp*A to the periplasmic space (Pugsley, 1993; Georgiou and Segatori, 2005). The periplasmic space is a more oxidising environment than the cytoplasm and is equipped with a number of proteins (DsbA and DsbC) which catalyse disulfide bond formation and rearrangement, and periplasmic chaperones such as Skp or FkpA (Bothmann and Plückthun, 2000 and 2004), which are important for folding and assembly of recombinant proteins. However, the periplasmic production of recombinant proteins in native, active form still presents a challenge today (Choi and Lee, 2004; Georgiou and Segatori, 2005).

Results of scFvOx expression studies here showed that a significant fraction of scFvOx was trapped inside the cytosol, although higher product levels were obtained in periplasm (Fig. 3.1). This suggests that although the *pelB* signal sequence was successful in translocating scFvOx across the inner membrane, possible pre-export aggregation led to a fraction of the protein being retained in the "cytosolic" cell fraction (Fig. 3.1). On the other hand, no inclusion body formation was observed in the periplasm (Fig. 3.2), similar to the observation with scFv for E-selectin (Casalvilla *et al.,* 1999), and with fragment of antibody for hepatitis B surface antigen (Ayala *et al.,* 1995). Further, periplasmically produced scFvOx was biologically active (Fig. 3.3).

The position of protein bands during Western blot suggested that scFvOx was properly processed (Fig. 3.1) by the periplasmic export machinery. Regrettably, the amount of available product was not high enough for a successful purification of periplasmically expressed scFvOx. Thus, an N-terminal sequencing that could have definitely confirmed the correct processing of the periplasmic export sequence (*pelB* leader) was not carried out.

When scFvOx was expressed under the control of the strong promoter *T7*, there was very high production of scFvOx (Fig. 3.4), but this led to inhibition of cell growth 2-3 h after induction and to increased protein accumulation in the cytosol. Overload of the cellular export machinery by high expression levels might hinder proper protein processing, as has been observed with the periplasmic production of penicillin acylase (PAC) under the control of *T7* promoter (Xu *et al.,* 2006). Upon induction with IPTG, the production of PAC was limited by the accumulation of PAC precursors as IB's, growth inhibition and cell lysis. Interestingly, when arabinose induction was used, the production of PAC was significantly enhanced (Xu *et al.,* 2006), reflecting the weak induction efficiency of this system compared to that of IPTG.

The vector pUBS520 (Brinkmann *et al.,* 1989) carries the *dnaY* gene encoding the tRNA for the arginine codons AGA and AGG. These are rare codons in *E. coli* and thus often limit gene expression. Co-expression of pUBS520 enhanced the periplasmic production of scFvOx (Fig. 3.5). On the other hand, neither co-expression of DsbA nor of its active site mutants C33A of C33S were found to be beneficial for periplasmic expression of scFvOx (Fig. 3.5). By contrast, co-expression of DsbA enhances the yield of T-cell receptor fragments (Wulfing *et al.,* 1994), IGF-I (Joly *et al.,* 1998) and human leptin (Jeong *et al.,* 2000), and has been used successfully as a solubilising partner in protein fusions (Zhang *et al.,* 1998; Winter *et al.,* 2001). Recently, Sandee *et al.,* (2005) reported that co-expression of *dsbABCD* moderately enhanced the periplasmic production of an antibody fragment against hepatocellular carcinoma. In order to significantly improve the productivity, the addition of 0.5 M sorbitol to the culture medium was necessary. Sorbitol is known to stabilise the native state of the protein. DsbA and its active site variants were reported to mimic protein disulfide isomerases under i*n vitro* conditions (Wunderlich and Glockshuber, 1993). Recently, it was demonstrated for other scFv fragments as well that the oxidative folding in the periplasm is independent of the Dsb system. Neither periplasmic and inner membrane-bound Dsb enzymes (*i.e.* DsbC, DsbG, DsbB and DsbD) nor cytoplasmic thioredoxins (TrxA and TrxC) were required for the oxidation of the scFv fragment HlyA (Fernandez and de Lorenzo, 2001).

Cultivation at elevated growth rate enabled rapid synthesis of scFvOx, but was followed by a sudden drop in the level of already formed product 12 h after induction (Fig. 3.6). As depicted in figure 3.7, a slower specific growth rate allowed a sustainable synthesis of scFvOx. The slower growth prolonged the active recombinant protein production phase by 8-10 h compared to cultivation at faster growth rate. Hence, the specific growth rate played a very important role in improving yield of scFvOx. The specific growth rate has been shown to be one of the most important process variables characterising the state of micro-organisms during fermentations. The biosynthesis of many products of interest is often related to this parameter, and both pre- and post-induction specific growth rates should be closely controlled in order to achieve maximum productivities for the desired recombinant protein. For example, an increase in the yields of recombinant *trp1* and consensus interferon were observed in parallel to an increase in pre-induction specific growth rate (Siegel and Ryu, 1985; Curless *et al.,* 1990). Such direct correlation between the efficiency of production of recombinant fusion proteins and growth rate was found to be associated with changes in cellular ribosomal content (Shin *et al.,* 1998). At the same time, several groups (Zabriskie *et al.,* 1987; Bech-Jensen and Carlsen, 1990) have observed no correlation between the specific growth rate and specific recombinant product formation. For example, a reduction in yield of recombinant β-lactamase was observed when the growth rate was increased by a change in medium

composition in chemo-stat cultures (Seo and Bailey, 1985). Riesenberg *et al.,* (1990) reported that the cellular level of human interferon α1 was higher at low growth rates in glucose limited chemo-stat studies. Taken together, it appears that the characteristics of the recombinant system, *e.g.,* the promoter strength, host/vector interaction and toxicity of the product dictate the relationship between recombinant protein production and growth rate. Nevertheless, during the fed-batch process, the growth rate is determined by the feeding rate (Shin *et al.,* 1998), and high cell densities and high volumetric product activity can be simultaneously achieved.

In all cultivations for the periplasmic production of scFvOx at bioreactor scale, a sudden loss in the cells' ability to form product was noticed at a certain time point (Fig. 3.6 and 3.7). This tendency might be due to proteolytic degradation of scFvOx (Fig. 3.8), as highlighted when proteolytically stable or the unstable protein was studied in high cell density cultures (Schmidt *et al.,* 1999). It was shown that the inhibition of growth of cells synthesising the proteolytically unstable protein was connected to an increased dissimilation of the carbon substrate, resulting in a concomitant reduction of the growth rate and the biomass yield coefficient with respect to the carbon source. In order to overcome the problem of proteolytic degradation, protease deficient strains have been developed (Chen *et al.,* 2004). The use of mutants deficient in all major cell envelope proteases (*degP, prc, ptr, ompT*) resulted in a drastic decrease in proteolysis of a secreted recombinant polypeptide (Baneyx and Georgiou, 1991). However, the growth of various protease-deficient strains is somewhat impaired relative to wild-type *E. coli* (Hara *et al.,* 1991 and 1996). In addition, the *prc*-deficient strain was found to be vulnerable to cell lysis in the fermentor, and mutations in *prc* lead to cell envelope defects, rendering cells temperature sensitive for growth in low salt culture medium (Hara *et al.,* 1991).

Taking into account the observations from this work and the published literature, an optimisation of cultivation conditions offers substantial potential to improve the production of recombinant single chain antibody fragments in the periplasm of *E. coli.*

4.2 Stability of scFvOx

The relative stabilities of the native protein, on-pathway folding intermediates, and misfolded conformations during *in vitro*-renaturation of proteins depends on the amino acid sequence of the individual protein and on the renaturation environment (De Bernardez Clark, 2001). One objective of this work was to identify factors which contribute to the optimisation of the *in vitro*-renaturation and stability of scFvOx.

The results presented in this work gave some indications that the formation of the native conformation of scFvOx at slightly alkaline pH was limited by the stability of the protein (Fig. 3.16). Alkaline conditions were destabilising for the protein and pH 8.5 was the upper limit for the stability of scFvOx (Fig. 3.17). Therefore, the original protocol involving renaturation of scFvOx at pH 8.7 was sub-optimal.

Under the conditions of the folding / unfolding experiments performed in this study, equilibrium transitions were not obtained since the refolding process was not fully reversible, which could potentially be due to the tendency of scFvOx to aggregate. A similar situation was observed by Martineu and Betton (1999) during studies on the thermodynamic stability of scFv's. The authors demonstrated the presence of a fraction of the protein that had high tendency to aggregate during renaturation, whereas neither native nor denatured protein was susceptible to aggregation. However, the estimated ΔGUo for scFvOx at pH 7.0 and pH 8.5 are in good agreement with those reported by Dumoulin *et al.,* (2002) for a series of other scFv fragments, which were estimated by GdnHCl, urea, temperature and pressure induced unfolding.

The acid-induced state of scFvOx

Acid-induced alternatively folded state of scFv fragments, as observed for scFvOx for pH below 3.0 in this study (Fig. 3.17, 3.18 and 3.19), has not been previously reported. This state was characterised by a blue shift in the fluorescence emission maximum, apparent co-operative folding and a pronounced tendency to aggregate (Fig. 3.17, 3.18 and 3.19). CD spectroscopic observations suggested that the acid-induced state had higher content of β-sheet secondary structures than native scFvOx. This co-operative unfolding indicates an ordered conformation with tertiary contacts, which suggests the formation of an alternatively folded state of scFvOx.

Incubation of proteins at low pH often leads to the loss of their native structure. The product of this denaturation process is often not a random coil but an assembly of conformations termed molten globule (Kuwajima, 1989; Ptitsyn, 1996; Arai and Kuwajima, 2000). The molten globule, originally characterised for α-lactalbumin (Dolgikh *et al.,* 1981; Ptitsyn and Uversky, 1994; Schulman *et al.,* 1997; Redfield, 1999), exhibits a significant amount of secondary structure but only few stable tertiary contacts (Kuwajima, 1989; Ptitsyn, 1996). The stability of the molten globule is only marginal and unfolding is not a co-operative process.

In the case of antibodies and antibody fragments (Fab, C(H)3 domain) however, incubation of the protein at pH 2.0 appears to lead to a specific non-native structure and not a molten globule (Vlasov *et al.,* 1996; Welfle *et al.,* 1999; Buchner *et al.,* 2001). This conformation, termed the alternatively folded state, is characterised by co-operative unfolding transition indicating a stable tertiary fold. A similar conformation was described for a Fab fragment (Lilie and Buchner, 1995) and a dimeric C(H)3 domain (Thies *et al.,* 2001). Domain interactions between the two-polypeptide chains of the Fab fragment were found to stabilise the alternatively folded state (Lilie and Buchner, 1995). Thus, incubation of scFvOx at acidic pH leads to the formation of an apparent alternatively folded state of scFvOx. This phenomenon could be a common feature of the immunoglobulin fold.

4.3 *In vitro*-renaturation of scFvOx

In order to achieve maximum renaturation yield, systematic optimisation of a series of parameters such as the denatured protein concentration, pH, temperature, time, redox buffers and effect of different additives is required (De Bernardez Clark, 2001; Middelberg, 2002; Lange and Rudolph, 2005). Certain additives such as L-Arg (for suppression of aggregation), sucrose and glycerol (for stabilsation of the native state) have been widely used to enhance renaturation yields of the proteins. Also reduced and oxidised glutathiones and small molecular weight thiols are commonly used as redox buffers for disulfide shuffling (De Bernardez Clark, 2001; Middelberg, 2002; Lange and Rudolph, 2005). In order to select the right low-molecular weight compounds as co-solvents for renaturation reactions, it is crucial to maintain the balance between several factors such as preservation of the stability of the native state of a protein and stabilisation of denatured polypeptides and intermediates.

The results of the present work clearly showed that the IL's from the series of N´-substituted N-methylimidazolium chlorides (Fig. 3.21 and Fig. 3.22) and N-(2-hydroxyethyl) -pyridinium chloride promote the renaturation of scFvOx. On the other hand, higher concentrations of these compounds exert a destabilising effect on the native state of the protein, as loss in the renaturation yield of scFvOx was observed. This effect was more pronounced in case of compounds with longer alkyl side chains. In good agreement with present findings, significant enhancement in renaturation yield of lysozyme compared to arginine hydrochloride and Triton X-100 was reported by use of N-methylimidazolium cations with a short N'-alkyl chain, such as an N'-ethyl or N'-butyl chain (Yamaguchi *et al.,* 2008). Importantly, kinetic analyses performed in this report to illustrate mechanism how these IL's function revealed that the effective cations selectively decreased the aggregation rate constant. This means that IL's act as aggregation suppressing agents in a manner similar to conventional renaturation additives. Similar enhancement in renaturation yields of lysozyme

were reported with the aid of ethylammonium nitrate (Summers and Flowers, 2000). In the same study, butylammonium nitrate and ammonium nitrate only marginally increased renaturation yield.

The results obtained with the studied IL's were compared to the amino acid salt L-Arg/HCl, one of the most widely used additives for protein renaturation. The efficacy of N´-substituted N-methylimidazolium and N-(2-hydroxyethyl) -pyridinium chloride was found to be comparable to, and in some instances even better than, that of L-Arg/HCl for renaturation of scFvOx. L-Arg/HCl has also been shown to suppress the aggregation of unfolded proteins without destabilising the native state of proteins (Arakawa and Tsumoto, 2003; Reddy K *et al.*, 2005). The destabilising effect of IL's as well as their ability to suppress aggregation in this study was clearly correlated with the hydrophobicity of the substituted imidazolium cations and anions, similar to recent report (Yamaguchi *et al.*, 2008).

Stabilisation of protein activity and structure by other IL's has been reported (Lozano *et al.*, 2001; Baker *et al.*, 2004; de Diego *et al.*, 2004). IL's with dihydrogen phosphate anion stabilise cytochrome c (Fujita *et al.*, 2005 and 2006), but dimethyl imidazolium dimethyl phosphate did not lead to an enhancement of the renaturation yield of scFvOx (Fig. 3.23). By contrast the negligible enhancement of renaturation of scFvOx by cocoasalkyl pentaethoxymethyl ammonium methyl sulphate was in agreement with low to marginal renaturation of active lysozyme in the presence of ethylammonium phosphate and bis-ethylammonium sulphate, respectively (Summers and Flowers, 2000). This could possibly due to these compounds not imparting a stabilising effect on the native state or lack the ability to suppress aggregation.

The *in vitro*-renaturation of antibody fragments has been less intensely studied than that of other disulfide containing proteins such as RNAse A, lysozyme or bovine pancreatic trypsin inhibitor. Despite few reports on the systematic optimisation of renaturation of antibody fragments (Buchner and Rudolph, 1991; Gu *et al.*, 2002; Umetsu *et al.*, 2003), studies testing systematic effects of small molecule thiols as redox buffers do not exist.

Aliphatic thiols are expected to be more effective redox buffers for protein renaturation under alkaline (pH 8.0-8.5) than at neutral (pH 6.0-7.5) conditions. Aromatic thiols have recently been reported as more versatile redox buffers which can function at lower pH (DeCollo and Lees, 2001; Gough *et al.*, 2002, 2003 and 2006; Gough and Lees, 2005a and 2005b).

At a pH 8.5, scFvOx was at its limiting edge of stability (Fig. 3.17). The protein renaturation optimum was pH 7.0 for reduced-denatured protein and pH 6.0 for the mixed disulfide (Fig. 3.25). Therefore, compounds serving as redox buffers at pH lower than 8.0 were desirable. Aromatic thiols have been reported to significantly increase the folding rate and yield of a disulfide containing protein, *i.e.* scrambled RNAse A (DeCollo and Lees, 2001; Gough *et al.*, 2002, 2003, and 2006; Gough and Lees, 2005a and 2005b), by promoting the rate of renaturation kinetics. In agreement with these findings, aromatic thiols functioned as redox buffers and increased the renaturation yield of scFvOx in comparison to GSH (Fig. 3.26). Their effect as renaturation enhancers was highly pH-dependent.

Surprisingly, overall scFvOx renaturation yield did not vary dramatically with three aromatic thiols (Fig. 3.26). This observation was in agreement with renaturation of lysozyme, where, yield of active protein was similar for four of the five aromatic thiols and for glutathione at pH 7.0 as well as for glutathione at pH 8.2 (Gurbhele-Tupkar *et al.*, 2008). In addition, aromatic thiols eliminated the lag phase at low disulfide concentrations, increased the folding rate constant up to 11-fold, and improved the yield of active lysozyme relative to GSH (Gurbhele-Tupkar *et al.*, 2008). A similar enhancement in apparent renaturation rate was observed in this work.

Replacement of GSH with the heteroaromatic thiols improved renaturation yields but did not accelerate the apparent rate of ranaturation kinetics of scFvOx, in contrast to thiosalicylic acid, the aromatic compound with the least acidic thiol proton. The correlation between thiol proton pK_a and renaturation rate that had been observed by Lees and coworkers (2003 and 2005a) did not hold for the renaturation of scFvOx in the presence of heteroaromatic thiols.

In addition to the acidic thiol pK_a, the enhancement effect on renaturation yield could be due to the hidden cysteine residues in molecular confirmation being more accessible to the smaller size of the aromatic thiols tested. The demonstrated efficacies of aromatic thiols at slight acidic conditions might be helpful in establishing renaturation protocols at physiological or even lower pH conditions for other therapeutically relevant proteins. Thus, up to 28-48% increase in renaturation yield of scFvOx could be obtained in comparison to L-Arg/HCl by using IL's as renaturation additives. Information obtained from protein stability and pH-dependent renaturation experiments could be used to increase renaturation yield by 50-75% by selective use of aromatic thiols and by performing the reaction at lower pH.

5. Conclusions

New strategies were examined for the production of a single chain antibody fragment *in vivo* and *in vitro*. Table 5.1 summarises the production of scFvOx by the different methods explored in this work. Periplasmic production at shake flask level in the presence of pUBS520 co-expression yielded 4.15 μg L^{-1} of scFvOx (Table 5.1). Bioreactor scale resulted in 4.9 μg L^{-1} of scFvOx (Table 5.1). Renaturation in the presence of IL's resulted in 4.8 mg L^{-1} (Table 5.1). The preparative renaturation strategy provided 6.8 mg L^{-1} amount of the pure protein (Table 5.1). However, it should be noted that this experiment was performed in the presence of L-Arg/HCl. In addition, both these experiments were performed using reduced-denatured protein from IB and used GSH/GSSG as redox buffers, and at the sub-optimal pH 8.7. Based on findings from protein stability and pH dependency, renaturation of scFvOx was performed using glutathione modified mixed disulfide and new redox buffer components. Thus, renaturation in the presence of aromatic thiols was found to be a better method for the production of scFvOx, yielding 29.4 mg L^{-1} of the product in 48 h of renaturation time. In essence, periplasmic strategy provided both specific and volumetric concentrations whereas *in vitro*-renaturation produced specific concentration of scFvOx. Therefore, direct comparision of scFvOx production using different strategies was not possible.

Experiment	Quantity	Remark, quality of scFvOx
Shake flask level	4.15 μg L^{-1}	Not pure
Bioreactor scale	4.9 μg L^{-1}	Not pure
Preparative renaturation batch	6.8 mg L^{-1}	Pure
Renaturation in the presence of IL's	4.8 mg L^{-1}	Relatively pure
Renaturation in the presence of aromatic thiols	29.4 mg L^{-1}	Relatively pure

Table 5.1: Smmary of production of scFvOx by different strategies

Recovery of purified scFvOx might be easier by *in vitro*-renaturation from IB's than periplasmic expression, since relatively pure protein was subjected to renaturation reaction. On the other hand, *in vitro*-renaturation often involves prolonged reaction times (*i.e.* 48-96 h) and usage of relatively large reaction volumes during down-stream operations, whereas periplasmic production often results in a biologically active product in a single step. However, low yields and the risk of protein loss during purification are the drawbacks in this case. Therefore, an ideal protocol for scFvOx is to use a vector with a promoter to promote

sustainable periplasmic production over prolonged periods. At the same time, high cell density cultivations at bioreactor scale should add new dimensions to the yield. The inclusion of Ionic Liquids as aggregation suppressing agents during *in vitro*-renaturation should further enhance the renaturation yield of scFvOx. This class of compounds offers great potential to engineer a solvent to serve a given individual purpose.

Although successful renaturation can be achieved by using glutathione as a redox buffer, the working pH might limit the renaturation yields of proteins which are sensitive to slightly alkaline pH conditions. Aromatic thiols were found to be possible alternatives to establish protocols at physiological pH conditions. These compounds lead to higher recovery of scFvOx in reaction times. Other results also suggest that renaturation by means of glutathione modified mixed disulfide is a better protocol than renaturation via reduced protein. The renaturation yield could also be improved by performing renaturation at a pH at which the protein was apparently more stable. This finding emphasises the importance of thermodynamic stability of a protein in dictating the conditions for its *in vitro*-renaturation.

It should be noted that the screening experiments were performed at the test-tube scale. Expanding to a laboratory scale bioreactor, which allows homogenous mixing of renaturation reaction components, should allow stringent control of parameters like temperature, oxygen exclusion and pH, resulting in further improvement in moving up to industrial scales.

In conclusion, findings from this thesis may be helpful for the optimisation of the periplasmic production of antibody fragments as well as for establishing optimised renaturation protocol for IB material at physiological pH.

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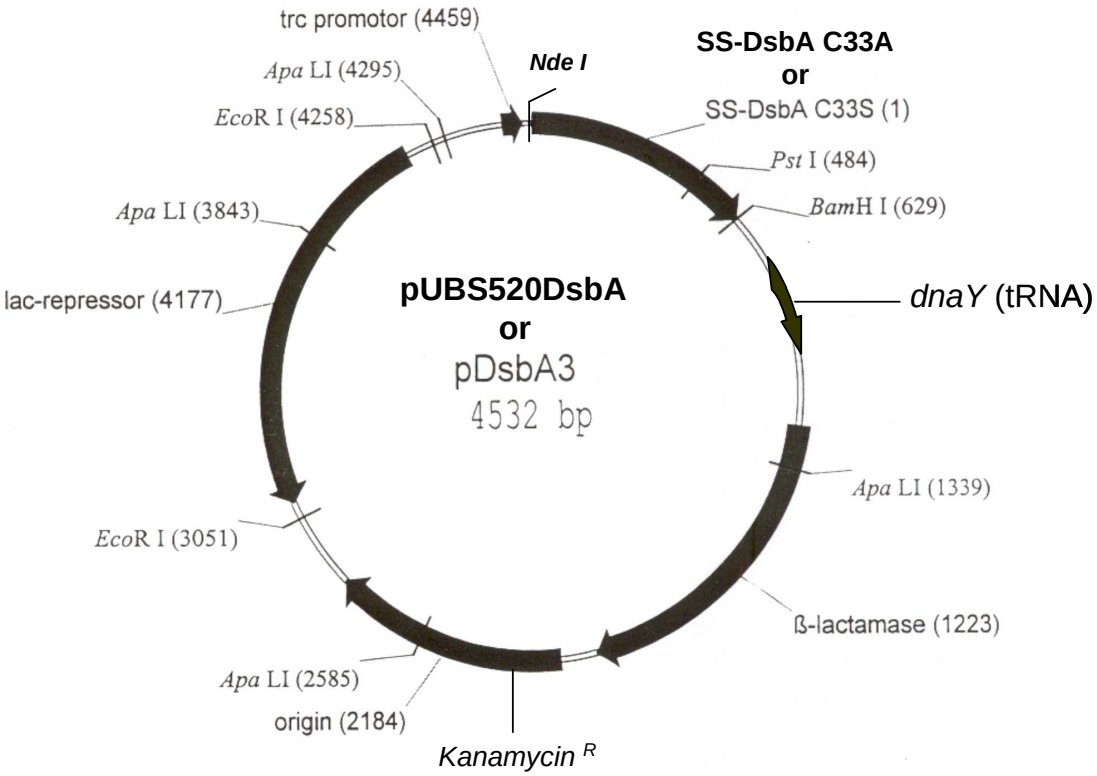
7. Appendix

A. Diagrammatic representation of pUBS520DsbA (or pDsbA3, Dr. Jeannette Winter, unpublished data) or Cysteine 33 Serine (C33S) or Cysteine 33 Alanine (C33A).

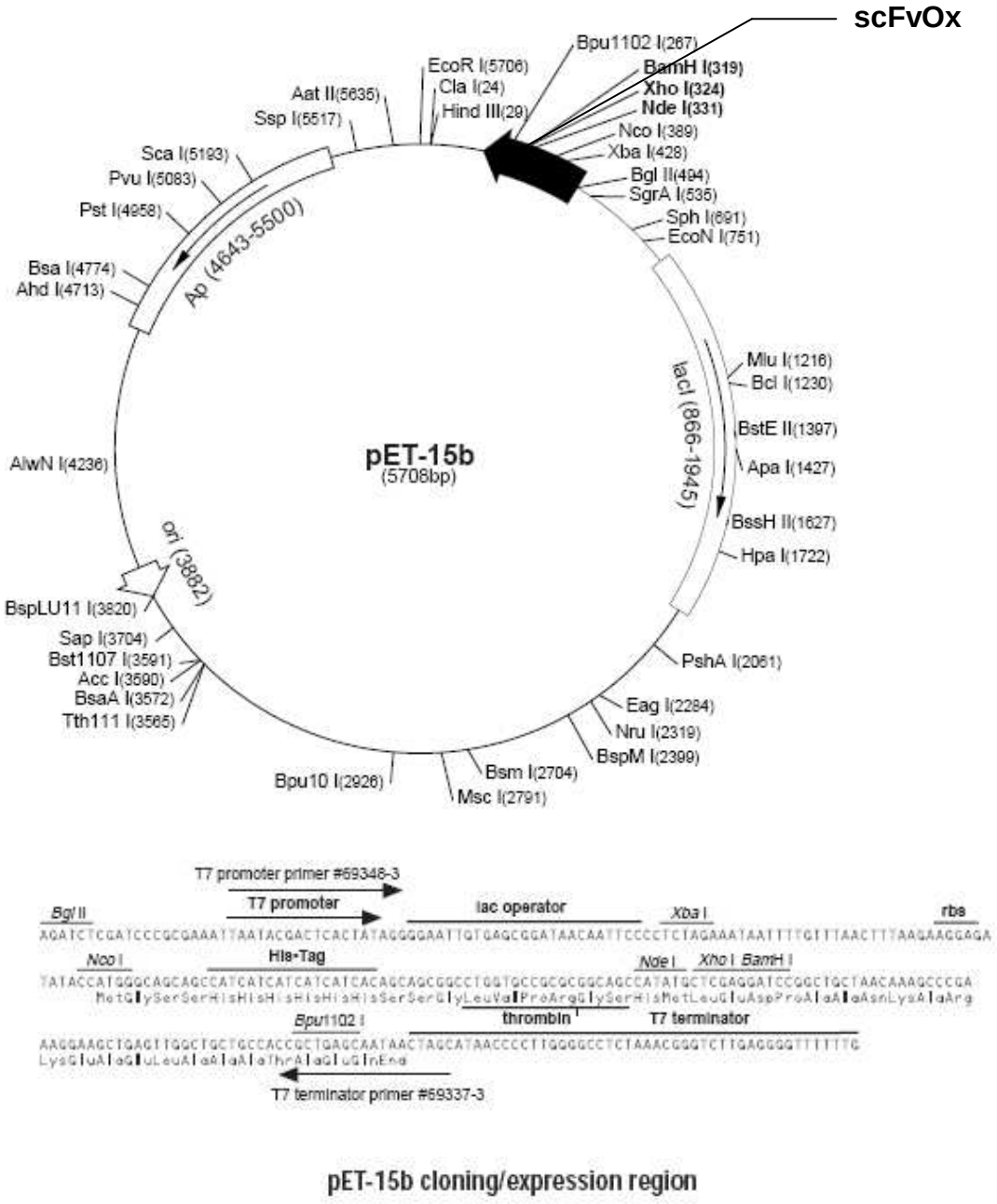
The *dsbA* gene was cloned between the *Nde* I and *Bam* HI restriction sites of pUBS520.



Oligonucleotides for mutagenesis and sequencing:

1. FwDsbAC33S: 5´CTTCTGCCCGCACAGCTATCAGTTTGAAGAAGTTCTGC-3´
2. RwDsbAC33S: 5´-GCAGAACTTCTTCAAACTGATAGCTGTGCGGGCAGAAG-3´
3. FwDsbAC33A: 5´-CTTCTGCCCGCACGCCTATCAGTTTGAAGAAGTTCTGC-3´
4. RvDsbAC33A: 5´-GCAGAACTTCTTCAAACTGATAGGCGTGCGGGCAGAAG-3´
5. DsbA_Seq_pri_fw: 5´-ATGAAAAAGATTTGGCTGGCGCTGGCTGG - 3´

B. Diagrammatic representation of pET15b(+)scFvOx (Dr. Claudia Humbeck, unpublished data).



pET-15b cloning/expression region

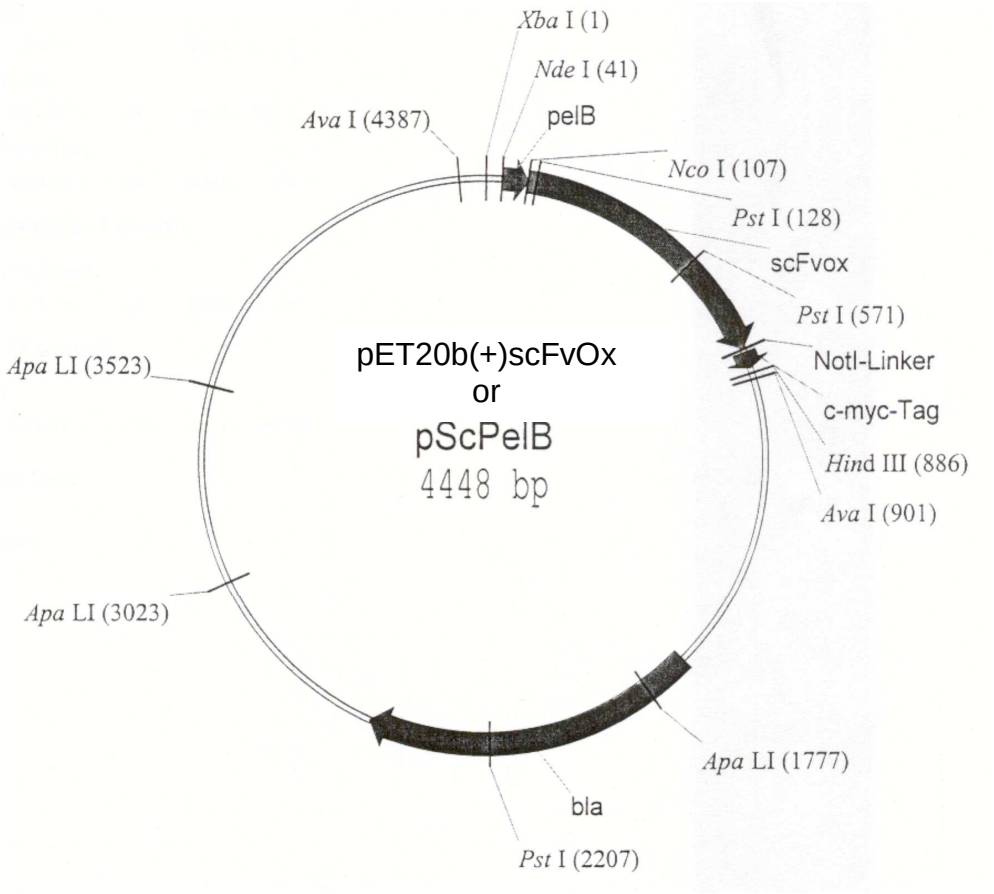
The coding sequence for scFvOx was cloned between the *Nde* I and *Xho* II restriction sites. This scFvOx gene contained an N-terminal hexahistidine, and a C-terminal *c-myc* tag.

His-Tag thrombin ndeI

GSSHHHHHHSSGLVPRGSHM**AEVKLQESGGGLVQPGGSRKLSCAASGFTFSSFGMHWVRQ**
APEKGLEWVAYISSGSSTIYYADTVKGRFTISRDNPKNTLFLQMTSLRSEDTAMYYCARD
YGAYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPAIMSASPGEKVTMTCSASSSVR
YMNWFQQKSGTSPKRWIYDTSKLSSGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQW
SSNPLTFGAGTKLELKRAAAEQKLISEEDLNGAA

c-myc tag xhoII

C. Diagrammatic representation of pET20b(+)scFvOx (or pScPelB, Dr. Brigitte Söhling, unpublished data).



D. Abbreviations

% (m/v)	mass percent
% (v/v)	volume percent
°C	degree centigrade
2-MPYN-Ox	2- mercaptopyridine N-oxide
2-MPYMN	2- mercaptopyrimidine
4-MPYN	4- mercaptopyridine
A	absorbance
Å	Ångström (=10^{-10} m)
Amp^R	ampicillin resistance
APS	ammonium persulfate
BSA	bovine serum albumin
CD	circular dichroism
Da	Dalton
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5´-triphosphate
DTT	dithiothreitrol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
Fab	antigen binding fragment
Fc	cristalline fragment
Fv	variable fragment
GdnHCl	guanidinium hydrochloride
GSH	glutathione, reduced
GSSG	glutathione, oxidised
h	hour
HEWL	hen egg-white lysozyme
HPLC	high-performance liquid chromatography
IB's	inclusion bodies
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactoside
L-Arg/HCl	L-arginine hydrochloride
LB	Luria-Bertani
LMW	low molecular weight
min	minute
OD_{600}	optical density = absorbance at 600 nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer with sodium chloride

PCR	polymerase chain reaction
PEG	polyethylenglycol
pI	isoelectric point (of a protein)
pO2	oxygen partial pressure
PVDF	polyvinylidene fluoride
Rf	retention factor
rpm	rotations per minute
RT	room temperature
r-tPA (rPA)	recombinant tissue type plasminogen activator
s	second
S	Svedbergs's constant
scFv	single-chain variable fragment
SDS	sodium dodecyl sulfate
TAE	Tris/acetate/EDTA buffer
TCA	trichloroacetic acid
TE	50 mM Tris/HCl, 1 mM EDTA, pH 8.0
TEMED	N,N,N´,N´-tetramethyl-ethylendiamine
TFA	trifluoroacetic acid
ThioSA	thiosalicylic acid
tPA	tissue type plasminogen activator
Tris	tris-(hydroxymethyl)-aminomethane
TS	Tris/sodium chloride buffer
UV	ultraviolet
UV/VIS	ultraviolet-visible
V	volume

Additionally, the nucleotides and amino acids were used with their standard codes.

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F. Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel verfasst habe. Die aus den anderen Werken wörtlich oder inhaltlich übernommenen Stellen sind als solche gekennzeichnet. Hiermit erkläre ich, dass ich mich mit der vorliegenden Arbeit erstmals um die Erlangung des Doktorgrades bewerbe.

Die Arbeit wurde noch keiner anderen Prüfungskommission vorgelegt.

Halle/Saale, den

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