Molekulare Analyse der Gene und Proteine der Cytochrom P450-haltigen Morpholin-Monooxygenase aus *Mycobacterium* sp. Stamm HE5

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Inhalt

Abkürzungen

CYP	Cytochrom P450
DNA	Desoxyribonukleinsäure
EPR	*Electron paramagnetic resonance*
FAD	Flavinadenindinukleotid
Fd	Ferredoxin
FdR	Ferredoxin-Reduktase
FMN	Flavinadeninmononukleotid
His-Tag	Zusätzliche Aminosäuresequenz mit 6 Histidin-Resten in rekombinanten Proteinen
HPLC	*High Performance Liquid Chromatography*
K_m	Michaelis-Menten-Konstante
NAD(P)H	Nikotinsäureamidadenindinukleotid(phosphat)
NBT	Nitroblautetrazoliumchlorid
P450	Cytochrom P450
PCR	*Polymerase chain reaction*

1 Einleitung

Der Heterozyklus Morpholin (1,4-Dioxazan) findet auf Grund seiner chemischen Eigenschaften breite Anwendung als Gummizusatz, Korrosionsschutz und Lösungsmittel. Außerdem wird es zur Synthese von optischen Aufhellern, pharmazeutischen Produkten, Pestiziden und Farbstoffen verwendet. Die weltweite Produktion von Morpholin wurde auf 25000 t pro Jahr geschätzt (WHO, 1995). Wegen seiner vielfältigen Anwendung und seiner guten Wasserlöslichkeit findet sich Morpholin sowohl in der Umwelt als auch in Nahrungsmitteln wieder. Da Morpholin durch biologische oder chemische N-Nitrosierung zum kanzerogenen N-Nitrosomorpholin umgewandelt werden kann (Enzmann *et al.*, 1995), ist der mikrobiologische Abbau dieser xenobiotischen Verbindung von großer Bedeutung.

Der Abbau der strukturanalogen Verbindungen Piperidin und Pyrrolidin durch *Pseudomonas fluorescens* wurde schon 1959 durch Jacoby & Fredericks gezeigt. Erst 1982 wurde der erste Mikroorganismus isoliert, der in der Lage ist, Morpholin abzubauen (Knapp, 1982). Dieses Bakterium wurde wie auch alle weiteren Mikroorganismen, die auf Grund dieser Eigenschaft isolierten wurden, als *Mycobacterium* identifiziert (Cech *et al.*, 1988; Combourieu *et al.*, 1998; Knapp & Brown, 1988; Knapp & Whytell, 1990; Mazure & Truffaut, 1994; Poupin *et al.*, 1999a; Poupin *et al.*, 1999b; Poupin *et al.*, 1998; Schuffenhauer *et al.*, 1999; Swain *et al.*, 1991). Dass anscheinend nur Mykobakterien Morpholin als Substrat nutzen können, mag mit den besonderen Eigenschaften dieser Familie zusammenhängen. So ist die Zellwand von Mykobakterien durch eingebaute Wachse und langkettige Mykolsäuren gekennzeichnet, wobei letztere diesen Bakterien ihren Namen verliehen. Hinsichtlich ihres Wachstums werden die Mykobakterien in schnell und langsam wachsende Arten unterteilt. Die Morpholin-abbauenden Mykobakterien gehören der ersten Gruppe an, während die zweite Gruppe pathogene Arten wie *Mycobacterium tuberculosis*, *Mycobacterium avium* oder *Mycobacterium leprae* umfasst (Madigan *et al.*, 2003). *Mycobacterium tuberculosis* ist der Verursacher der Tuberkulose, die jährlich rund zwei Millionen Todesopfer fordert (Dye *et al.*, 1999).

Mykobakterien gehören zur Ordnung *Actinomycetales*, deren Vertreter Gram positiv sind und einen hohen GC-Gehalt der DNA aufweisen (Stackebrandt *et al.*, 1997). Zahlreiche *Actinomycetales* sind für ihre Abbauleistungen bekannt, wie z.B. für den Abbau von Herbiziden durch *Streptomyces griseolus* und *Rhodococcus erythropolis*, von Phenolen durch *Rhodococcus rhodochrous* oder von Tetrahydrofuran durch *Pseudonocardia* sp. Stamm K1 (Eltis *et al.*, 1993; Nagy *et al.*, 1995; Omer *et al.*, 1990; Thiemer *et al.*, 2003). Außerdem sind Streptomyceten für die Synthese zahlreicher Antibiotika bekannt, wie z.B. Narbomycin, Oleandomycin oder Rapamycin (Molnar *et al.*, 1996; Rodriguez *et al.*, 1995; Xue *et al.*, 1998).

Über den Abbauweg von Morpholin war lange Zeit wenig bekannt. In Anlehnung an den Abbau von Pyrrolidin in *P. fluorescens* wurde ein Weg für den Abbau von Morpholin durch *Mycobacterium chelonae* morG postuliert (Swain *et al.*, 1991). Dieser wurde dann durch den Nachweis der Intermediate 2-(2-Aminoethoxy)acetat, Diglykolsäure und Glykolat beim Abbau von Morpholin durch *Mycobacterium aurum* MO1 und *Mycobacterium* sp. Stamm RP1 teilweise bestätigt (Combourieu *et al.*, 1998; Combourieu *et al.*, 2000; Poupin *et al.*, 1998). Daher ist anzunehmen, dass Morpholin über Diglykolsäure in die C_2-Verbindungen Glykolat und Glyoxylat gespalten wird (Abb. 1), die dann in den Zentralmetabolismus eingeschleust werden könnten (Fuchs, 1999).

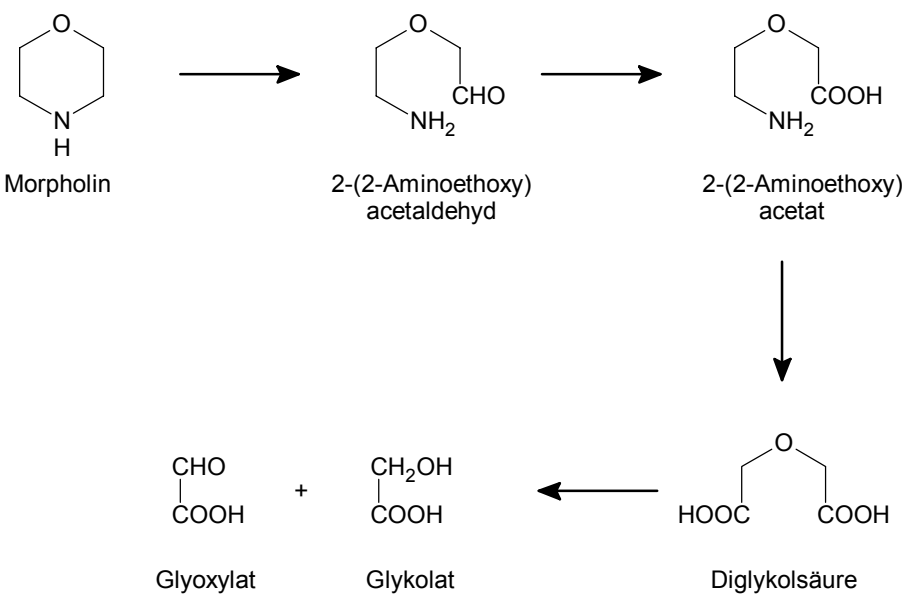

Abb. 1 Abbau von Morpholin durch Mykobakterien.

Die Detektion eines Cytochrom P450, das beim Wachstum dieser beiden Stämme auf Morpholin, Piperidin und Pyrrolidin induziert wird, ergab einen Hinweis auf den ersten Schritt beim Abbau dieser Verbindungen. Es wurde angenommen, dass dieses Cytochrom P450 als Teil einer Monooxygenase für die Hydoxylierung von Morpholin verantwortlich ist, was zur anschließenden spontanen Ringspaltung an der C-N-Bindung führen sollte (Poupin *et al.*, 1998). In *Mycobacterium smegmatis* mc^2155 konnte das Gen *pipA* isoliert werden, dessen Produkt ein Cytochrom P450 ist. Dieses Cytochrom P450 ist sehr wahrscheinlich am Abbau von Piperidin und Pyrrolidin beteiligt (Poupin *et al.*, 1999b).

Der in der vorliegenden Arbeit untersuchte *Mycobacterium* sp. Stamm HE5 wurde auf Grund seiner Eigenschaft isoliert, auf Morpholin, Piperidin und Pyrrolidin als alleiniger C-, N- und Energiequelle zu wachsen (Schuffenhauer *et al.*, 1999). *Mycobacterium* sp. Stamm HE5 zeichnet sich gegenüber den vorher isolierten Stämmen durch eine höhere Toleranz gegenüber Morpholin sowie ein schnelleres Wachstum auf diesem Substrat aus (Schräder *et al.*, 2000). Diese Eigenschaften machen *Mycobacterium* sp. Stamm HE5 auch für potenzielle biotechnologische Anwendungen interessant. Die ermittelte 16S-rDNA Sequenz zeigte die höchste Identität zu der von *Mycobacterium gilvum*, von dem er sich aber in einigen physiologischen Eigenschaften unterscheidet (Schräder *et al.*, 2000). Hohe Identitäten wurden auch zu der 16S-rDNA von *Mycobacterium fortuitum* und *Mycobacterium chlorophenolicum* gefunden. Beim Wachstum von *Mycobacterium* sp. Stamm HE5 auf Morpholin konnte ebenfalls ein spezifisch induziertes Cytochrom P450 detektiert werden (Schräder *et al.*, 2000). Dies war ein Hinweis darauf, dass in den bisher isolierten Mykobakterien ein ähnliches Enzym am Morpholin-Abbau beteiligt ist. Die Vermutung, dass dieses Cytochrom P450 die Hydroxylierung von Morpholin katalysiert, wurde indirekt dadurch bestätigt, dass beim Wachstum von *Mycobacterium* sp. Stamm HE5 auf dem wahrscheinlichen Intermediat Diglykolsäure kein Cytochrom P450 induziert wird (Debbab, 2003).

P450 Cytochrome sind Cytochrome vom b-Typ. Das heißt, sie besitzen ein Protoporphyrin IX als Häm-Kofaktor, der fest - aber nicht kovalent - am Enzym gebunden ist (Li, 2001). Diese Bindung am Protein erfolgt im Cytochrom P450 über

einen absolut konservierten Cystein-Rest, woraus sich charakteristische, spektroskopische Eigenschaften ergeben. So zeigt das reduzierte, CO-gebundene Enzym im Absorptionsspektrum eine Soret-Bande bei 450 nm, was diesen Enzymen ihren Namen verliehen hat. P450 Cytochrome finden sich in nahezu allen Organismen und katalysieren dort vielfältige Reaktionen, hauptsächlich jedoch Hydroxylierungen (Urlacher *et al.*, 2004).

Für die Aktivierung des molekularen Sauerstoffes am Häm-Kofaktor des Cytochrom P450 werden Elektronen benötigt, die durch die Oxidation von NAD(P)H durch eine Oxidoreduktase bereitgestellt werden. Das Cytochrom P450 wird entweder direkt durch eine FAD- und FMN-haltige Reduktase reduziert (Klasse II) oder die Elektronen werden von einer FAD-haltigen Ferredoxin-Reduktase über ein Fe-S-Protein (Ferredoxin) auf das Cytochrom P450 übertragen (Klasse I) (Munro & Lindsay, 1996). Alle eukaryotischen P450 Cytochrome gehören der Klasse II an, wobei das Cytochrom P450 meistens am Endoplasmatischen Retikulum gebunden ist. Prokaryotische P450 Cytochrome gehören in der Regel der Klasse I an und alle ihre Komponenten sind im Cytoplasma lokalisiert. Interessanterweise gehören die P450 Cytochrome aus Mitochondrien der Klasse I an (Abb. 2). Allerdings gibt es auch Ausnahmen wie die Fettsäuren-Monooxygenase $P450_{BM3}$ aus *Bacillus megaterium*, die in ihrem Aufbau der Klasse II ähnelt und als Besonderheit die FAD- und FMN-haltige Reduktase sowie das Cytochrom P450 auf einem Polypeptid vereint (Narhi & Fulco, 1986). P450 Cytochrome der Klasse III benötigen keinen Elektronendonator, da sie Peroxyverbindungen umsetzen, die bereits aktivierten Sauerstoff enthalten (Haurand & Ullrich, 1985). Diese P450 Cytochrome fungieren aber nicht als Monooxygenasen, genauso wenig wie die NO-Reduktase ($P450_{nor}$) aus *Fusarium oxysporum*, die NAD(P)H direkt oxidieren kann (Nakahara *et al.*, 1994).

Auf Grund der hohen Anzahl von P450-Sequenzen wird eine Nomenklatur verwendet, die neue P450 Cytochrome fortlaufend nummeriert (Nelson *et al.*, 1996). Die bakteriellen P450 Cytochrome beginnen mit der zuerst entdeckten Campher-Monooxygenase ($P450_{cam}$) aus *Pseudomonas putida* (Katagiri *et al.*, 1968), die als CYP101 (**Cy**tochrom **P**450 101) bezeichnet wird. Im Allgemeinen werden Proteine mit einer Identität von mehr als 40 % ihrer Aminosäuresequenz zu einer Familie

zusammengefasst. Bei Identitäten von mehr als 55 % werden diese Proteine einer Subfamilie zugeordnet, die durch einen angehängten Buchstaben angezeigt wird (z.B. CYP102A1). Im Januar 2005 waren 4504 Sequenzen von P450 Cytochromen bekannt, wovon ein großer Teil aus pflanzlichen Genomen stammt. So wurden allein im Genom von *Oryza sativa* 454 Gene, die für P450 Cytochrome kodieren, identifiziert (Nelson *et al.*, 2004). Die 472 Sequenzen von bakteriellen P450 Cytochromen wurden bislang in 154 Familien und 267 Subfamilien eingeteilt (http://drnelson.utmem.edu/cytochromeP450.html).

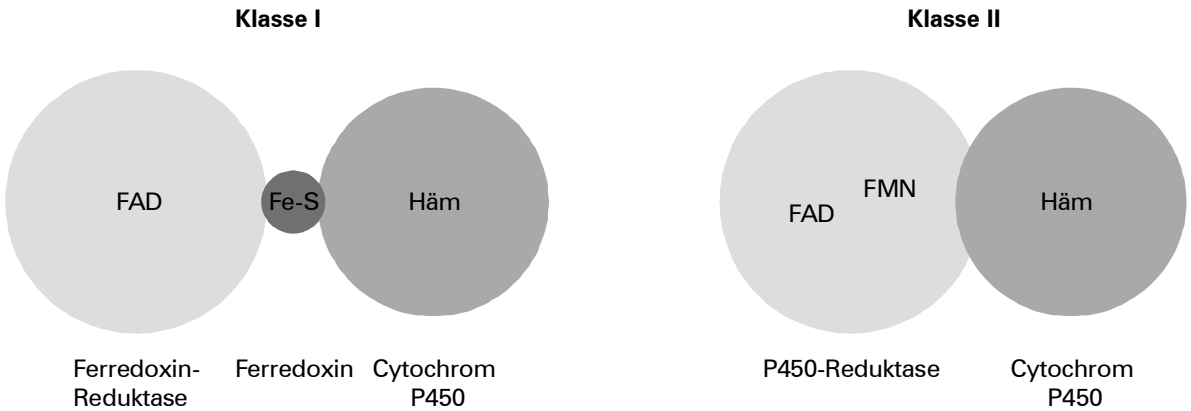

Abb. 2 Schematische Darstellung des Aufbaus von P450-haltigen Monooxgenasen.

In Säugetieren metabolisieren P450 Cytochrome zahlreiche Chemikalien, was zur Entgiftung, aber auch zur Aktivierung dieser Xenobiotika führen kann (Guengerich, 2001). Außerdem sind sie an den Synthesen wichtiger Steroide wie z.B. Cholesterin, Cortison und Aldosteron beteiligt (Bernhardt, 1996). Bakterielle P450 Cytochrome sind häufig in den Abbau diverser xenobiotischer Verbindungen involviert, wobei sie meistens die einleitende Hydroxylierung katalysieren. Ein Großteil der funktionell charakterisierten P450 Cytochrome wurde dabei aus *Actinomycetales* isoliert (Tab. 1). Intensiv untersucht wurden die Campher-Monooxgenase $P450_{cam}$ aus *P. putida* (Schlichting *et al.*, 2000) und die Fettsäuren-Monooxygenase $P450_{BM-3}$ aus *Bacillus megaterium* (Li & Poulos, 1997). Weitere bekannte Vertreter sind z.B. $P450_{terp}$ aus *Pseudomonas* spec. (Peterson *et al.*, 1992) oder $P450_{soy}$ aus *Streptomyces griseus* (Taylor *et al.*, 1999). Eine weitaus größere Zahl an P450 Cytochromen ist an der Synthese von Sekundärstoffen beteiligt und diese wurden fast ausschließlich in

Streptomyceten identifiziert (Tab. 2). Allerdings ergibt sich diese funktionelle Zuordnung meistens nur aus der Lokalisation der P450-kodierenden Gene in entsprechenden Biosynthese-Clustern. Die Bestätigung durch enzymologische Studien steht in den meisten Fällen noch aus.

Tab. 1 Am bakteriellen Abbau von xenobiotischen Verbindungen beteiligte P450 Cytochrome.

Cytochrom P450	Substrat	Organismus	Literatur
CYP101A1 ($P450_{cam}$)[a]	Campher	*Pseudomonas putida*	Koga *et al.*, 1985
CYP102A1 ($P450_{BM3}$)[a]	Fettsäuren	*Bacillus megaterium*	Narhi & Fulco, 1986
CYP105A3 ($P450_{sca}$)[a]	Compactin	*Streptomyces carbophilus*	Matsuoka *et al.*, 1989
CYP105A1 ($P450_{SU1}$)[a] CYP105B1 ($P450_{SU2}$)[a]	Sulfonylharnstoff-Herbizide	*Streptomyces griseolus*	Omer *et al.*, 1990
CYP105D1 ($P450_{soy}$)[a]	diverse Xenobiotika	*Streptomyces griseus*	Taylor *et al.*, 1999
CYP108A1 ($P450_{terp}$)[a]	Terpineol	*Pseudomonas spec.*	Peterson *et al.*, 1992
CYP111A1 ($P450_{lin}$)[a]	Linalool	*Pseudomonas putida*	Ropp *et al.*, 1993
CYP116A1	EPTC (Herbizid)	*Rhodococcus erythropolis*	Nagy *et al.*, 1995
CYP151A1	Piperidin, Pyrrolidin	*Mycobacterium smegmatis*	Poupin *et al.*, 1999b
CYP153A1	n-Alkane	*Acinetobacter* sp. EB104	Maier *et al.*, 2001
CYP176A1 ($P450_{cin}$)[a]	Cineol	*Citrobacter braakii*	Hawkes *et al.*, 2002
CYP177A1	RDX[b]	*Rhodococcus rhodochrous*	Seth-Smith *et al.*, 2002
CYP226A3	Resinsäure	*Pseudomonas diterpeniphila*	Morgan & Wyndham, 2002
CYP239A2	Isopropylamin	*Pseudomonas* sp. KIE171	de Azevedo Wasch *et al.*, 2002
CYP249A1	Ethyl-*tert*-butylether	*Rhodococcus ruber*	Chauvaux *et al.*, 2001
$P450_{RR1}$[c]	*ortho*-subst. Phenole	*Rhodococcus rhodochrous*	Eltis *et al.*, 1993
$P450_{EP1A}$[c]	2-Ethoxyphenol	*Corynebacterium* sp. EP1	Kawahara *et al.*, 1999
$P450_{dit}$[c]	Diterpenoide	*Pseudomonas abietaniphila*	Smith *et al.*, 2004
$P450_{PB\text{-}1}$[c]	Phenoxybutyrat-Herbizide	*Rhodococcus erythropolis*	Sträuber *et al.*, 2003
P450[d]	Veratrol	*Streptomyces setonii*	Sutherland, 1986
P450[d]	Phenantren	*Mycobacterium* sp. S1	Tongpim & Pickard, 1999
P450[d]	Ethyl-*tert*-butylether	*Comamonas testosteroni*	Kharoune *et al.*, 2001

a) In Klammern ist der Trivialname des Cytochrom P450 angegeben.
b) Hexahydro-1,3,5-Trinitro-1,3,5-Triazin.
c) Keine systematische Bezeichnung, da die Aminosäuresequenz nicht vollständig bekannt ist.
d) Keine Trivialnamen, da diese P450 Cytochrome noch nicht isoliert wurden.

Tab. 2 An der Synthese von Sekundärstoffen beteiligte bakterielle P450 Cytochrome.

Anwendung	Sekundärstoff	Cytochrom P450	Organismus[a)]	Literatur
Antibakteriell	Oleandomycin	CYP107D1, CYP235A1	*S. antibioticus*	Rodriguez *et al.*, 1995
	Tylosin	CYP113B1, CYP105L1	*S. fradiae*	Fouces *et al.*, 1999
	Rapamycin	CYP122A2, CYP107G1	*S. hygroscopicus*	Molnar *et al.*, 1996
	Geldanamycin	CYP105U1	*S. hygroscopicus*	Rascher *et al.*, 2003
	Clavulinsäure	CYP105M1	*S. clavuligerus*	Li *et al.*, 2000
	Novobiocin	CYP163A1	*S. speroides*	Chen & Walsh, 2001
	Clorobiocin	CYP163A2	*S. roseochromogenes*	Chen & Walsh, 2001
	Pikromycin	CYP107L1	*S. venezulae*	Xue *et al.*, 1998
	Nanchagmycin	CYP124B2	*S. nanchangnensis*	Sun *et al.*, 2003
	Erythromycin	CYP107A1, CYP107B1	*Saccharopolyspora erythrea*	Weber *et al.*, 1991
	Vancomycin	CYP165A3, CYP165B3, CYP165C4	*Amycolatopsis orientalis*	van Wageningen *et al.*, 1998
Fungizid	Simocyclinon	CYP163A3	*S. antibioticus*	Galm *et al.*, 2002
	Filipin	CYP105P1, CYP105D6	*S. avermitilis*	Ikeda *et al.*, 1999
	Pimaricin	CYP161A2, CYP105H3	*S. natalensis*	Aparicio *et al.*, 2000
	Amphotericin	CYP161A3, CYP105H4	*S. nodusus*	Caffrey *et al.*, 2001
	Nystatin	CYP161A1, CYP105H1	*S. nuorsei*	Brautaset *et al.*, 2000
	Monensin	CYP124B1	*S. cinnamonensis*	Oliynyk *et al.*, 2003
Insektizid	Nikkomycin	CYP162A1, CYP105H1	*S. tendae*	Zeng *et al.*, 2002
Antiparasitär	Avermectin	CYP171A1	*S. avermitilis*	Ikeda *et al.*, 1999
Phytotoxisch	Thaxtomin	CYP246A1	*S. acidscabies*	Healy *et al.*, 2002
Antitumor	Neocarzinostatin	CYP154J1, CYP208A2	*S. carzinostaticus*	Sthapit *et al.*, 2004
	Doxorubicin	CYP131A1, CYP129A2	*S. peucetius*	Lomovskaya *et al.*, 1999
	Daunorubicin	CYP131A2, CYP129A1	*S.* sp. Stamm C5	Dickens *et al.*, 1997
	Antibiotikum C-1027	CYP211A1	*S. globisporus*	Liu *et al.*, 2002
Anti-HIV	MitomycinC	CYP107C1, CYP160A1, CYP105F1	*S. lavendulae*	Mao *et al.*, 1999
	Complestatin	CYP165B5, CYP165E1	*S. lavendulae*	Chiu *et al.*, 2001
Vitamin	Biotin	CYP107H1	*Bacillus subtilis*	Green *et al.*, 2001

a) Die Abkürzung *S.* steht für *Streptomyces*.

Die Sequenzierung des Genoms von *Mycobacterium tuberculosis* führte zur Identifizierung der damals erstaunlich hohen Zahl von 22 Genen, die für P450 Cytochrome kodieren (Cole *et al.*, 1998). Mittlerweile wurde auch in anderen *Actinomycetales* eine hohe Anzahl an P450-Genen gefunden: 18 in *Streptomyces coelicolor* (Lamb *et al.*, 2002b), 33 in *Streptomyces avermitilis* (Lamb *et al.*, 2003) und sogar 40 in *Mycobacterium smegmatis* mc^2155 (Jackson *et al.*, 2003), was die bislang größte Zahl für ein Bakterium ist. Einige dieser Proteine wurden schon eingehend charakterisiert bis hin zur Aufklärung der Kristallstruktur, jedoch ohne dadurch mehr über ihre natürliche Funktion zu erfahren (Tab. 3).

Tab. 3 Charakterisierte bakterielle P450 Cytochrome mit unbekannter physiologischer Funktion.

Cytochrom P450	Bemerkung	Organismus	Literatur
CYP51[a)]	Homolog zu CYP51 aus Säugetieren[b)]	*Mycobacterium tuberculosis*	Podust *et al.*, 2001
CYP51	Homolog zu CYP51 aus Säugetieren[b)]	*Mycobacterium smegmatis*	Jackson *et al.*, 2003
CYP51	Homolog zu CYP51 aus Säugetieren[b)]	*Streptomyces coelicolor*	Lamb *et al.*, 2002a
CYP51FX	CYP51-Ferredoxin-Fusionsprotein[b)]	*Methylococcus capsulatus*	Jackson *et al.*, 2002
CYP106A2	-	*Bacillus megaterium*	Rauschenbach *et al.*, 1993
CYP119[a)]	Thermostabil	*Sulfolobus solfataricus*	Yano *et al.*, 2000
CYP121[a)]	-	*Mycobacterium tuberculosis*	Leys *et al.*, 2003
CYP154A1[a)]	Bindet Narbomycin	*Streptomyces coelicolor*	Podust *et al.*, 2003
CYP154C1[a)]	Hydroxyliert Narbomycin	*Streptomyces coelicolor*	Podust *et al.*, 2003
CYP175[a)]	Thermostabil	*Thermal thermophilus*	Yano *et al.*, 2003

a) Für diese P450 Cytochrome ist die Kristallstruktur aufgeklärt worden.
b) CYP51 ist eine α-Lanosterol-Demethylase, die an der Synthese von Cholesterin beteiligt ist.

Die Anzahl der Gene, die für die entsprechenden Ferredoxin-Reduktasen und Ferredoxine kodieren, ist weitaus geringer ist als die jeweilige Zahl der Gene, die für P450 Cytochrome kodieren. Zum Beispiel wurden im Genom von *S. avermitilis* zwar 33 P450-kodierende Gene identifiziert, aber nur sechs Gene, die für Ferredoxin-Reduktasen kodieren, und neun Gene, die für Ferredoxine kodieren (Lamb *et al.*, 2003). Bislang wurden nur wenige spezifische Ferredoxin-Reduktasen und Ferredoxine von P450-Systemen gereinigt und charakterisiert (Tab. 4). Intensiv

untersucht wurden Putidaredoxin-Reduktase und Putidaredoxin des $P450_{cam}$-Systems (Sevrioukova *et al.*, 2003; Sevrioukova *et al.*, 2004). Diese Proteine ähneln Adrenodoxin-Reduktase und Adrenodoxin, die verschiedene mitochondriale P450 Cytochrome reduzieren (Schiffler & Bernhardt, 2003). Einige Reduktasen oder Ferredoxine bilden mit dem jeweiligen Cytochrom P450 Fusionsproteine und sind daher als Ausnahmen anzusehen. Für die P450 Cytochrome der Klasse I ergibt sich eine weitere Differenzierung hinsichtlich der Art des genutzten Ferredoxins (Degtyarenko & Kulikova, 2001). Dieses trägt entweder einen Fe_2S_2-Cluster (Adrenodoxin-Typ) oder einen Fe_4S_4- bzw. Fe_3S_4-Cluster (bakterieller Typ). An Stelle von Ferredoxinen könnten Genanalysen zufolge auch Flavodoxine agieren, die anstatt eines FeS-Clusters einen FMN-Kofaktor tragen (Hawkes *et al.*, 2002).

Tab. 4 Charakterisierte Redoxpartner [a] **von bakteriellen P450 Cytochromen.**

Proteine	Kofaktoren	P450	Organismus	Literatur
Putidaredoxin-Reduktase Putidaredoxin	FAD Fe_2S_2	$P450_{cam}$	*Pseudomonas putida*	Roome *et al.*, 1983 Peterson *et al.*, 1990
Reduktase-Domäne von $P450_{BM3}$	FAD + FMN	$P450_{BM3}$	*Bacillus megaterium*	Narhi & Fulco, 1987
Terpredoxin	Fe_2S_2	$P450_{terp}$	*Pseudomonas spec.*	Mo *et al.*, 1999
Fd-1 Fd-2	Fe_3S_4 Fe_3S_4	$P450_{SU1}$ $P450_{SU2}$	*Streptomyces griseolus*	O'Keefe *et al.*, 1991
Fd_{soy}	Fe_3S_4	$P450_{soy}$	*Streptomyces griseus*	Trower *et al.*, 1992
Ferredoxin	Fe_3S_4	CYP51 [b]	*Mycobacterium tuberculosis*	Bellamine *et al.*, 1999
Reduktase-Domäne von P450RhF	FMN + Fe_2S_2	P450RhF	*Rhodococcus sp.*	Roberts *et al.*, 2002
Fer Protein	Fe_4S_4	P450 Biol [b]	*Bacillus subtilis*	Green *et al.*, 2003
Ferredoxin-Domäne von CYP51FX	Fe_3S_4	CYP51FX	*Methylococcus capsulatus*	Jackson *et al.*, 2002

a) Es sind nur Proteine aufgelistet, von denen auch die Aminosäuresequenz bekannt ist.
b) Als unspezifischer Redoxpartner für dieses Cytochrom P450 eingesetzt.

Vor allem in *Actinomycetales* konnten für das jeweilige Cytochrom P450 spezifische Ferredoxin-Reduktasen oftmals nicht nachgewiesen werden. Daher wurde vermutet, dass konstitutiv vorliegende Proteine deren Funktion übernehmen (O'Keefe & Harder, 1991). Transkriptionsanalysen für *S. coelicolor* haben gezeigt, dass für die Aktivität der 18 P450 Cytochrome in diesem Organismus drei Ferredoxin-

Reduktasen und sechs Ferredoxine ausreichend sind (Lei *et al.*, 2004). In den meisten Fällen wurden zur Messung der Monooxygenase-Aktivität in Ermangelung bekannter spezifischer Proteine heterologe Redoxpartner aus anderen Organismen, wie z.B. aus Spinat, eingesetzt (Green *et al.*, 2001; Kawahara *et al.*, 1999; Matsuoka *et al.*, 1989; Taylor *et al.*, 1999; Trower *et al.*, 1989). In einigen Fällen war auch eine direkte Aktivierung des Cytochrom P450 durch Hydroperoxyverbindungen erfolgreich (Coon *et al.*, 1996; Naqui *et al.*, 1986). Die einzige homologe bakterielle P450-haltige Monooxygenase, von der alle drei Proteine gereinigt und charakterisiert wurden, ist das schon erwähnte $P450_{cam}$-System aus *P. putida* (Tab. 4).

Nach der Identifizierung eines spezifisch Morpholin-induzierten Cytochrom P450 in *Mycobacterium* sp. Stamm HE5 (Schräder *et al.*, 2000) war das Ziel dieser Arbeit, die einzelnen Proteine dieses Systems molekularbiologisch sowie biochemisch zu charakterisieren und anschließend das gesamte System enzymologisch zu untersuchen. Im Rahmen der vorliegenden Dissertation wurden das Cytochrom P450 sowie sein Redoxpartner, ein Fe_3S_4-Ferredoxin, gereinigt, charakterisiert und partiell sequenziert. Ausgehend von den bestimmten Peptidsequenzen wurden die entsprechenden Gene kloniert und sequenziert. Dabei wurde auch das Gen identifiziert, das die Ferredoxin-Reduktase dieses P450-Systems kodiert. Die gereinigte rekombinante Ferredoxin-Reduktase wurde charakterisiert und mit verschiedenen rekombinanten Ferredoxin-Varianten enzymkinetisch untersucht. Das Cytochrom P450 wurde ebenfalls als rekombinantes Protein produziert und zu Bindungsstudien eingesetzt. Schließlich wurden alle drei rekombinanten Proteine zu einer aktiven Morpholin-Monooxygenase rekonstituiert. Die erhaltenen Ergebnisse sind in drei Publikationen detailliert beschrieben und werden im folgenden Kapitel zusammengefasst dargestellt.

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2 Zusammenfassung

2.1 Reinigung und Charakterisierung der Morpholin-induzierten Proteine Cytochrom P450 und Ferredoxin aus *Mycobacterium* sp. Stamm HE5

(Sielaff *et al.*, 2001)

Beim Wachstum von *Mycobacterium* sp. Stamm HE5 auf Morpholin wurde ein spezifisch durch diesen N-Heterozyklus induziertes Cytochrom P450 nachgewiesen. Auf Grund anderer Arbeiten (Kap. 1) wurde davon ausgegangen, dass dieses Cytochrom P450 als Teil einer Monooxygenase für die Hydroxylierung des Morpholins verantwortlich ist. Diese P450-haltige Monooxygenase sollte nun isoliert werden. Nach dem Aufschluss von Zellen, die auf Morpholin gewachsen waren, konnte jedoch keine Morpholin-abhängige Aktivität dieser Monooxygenase detektiert werden. Daher mussten Verfahren zum Nachweis der einzelnen Proteinkomponenten angewandt werden, um diese zunächst separat zu isolieren.

Die Identifizierung von P450 Cytochromen erfolgt im Allgemeinen durch das charakteristische Absorptionsspektrum ihres reduzierten CO-gebundenen Zustandes, welches ein Maximum bei 450 nm aufweist. Mit Hilfe dieses Nachweises wurde das Cytochrom P450 durch ein vierstufiges Reinigungsschema bis zur Homogenität isoliert. Die ermittelte molekulare Masse und das Absorptionsspektrum des gereinigten Proteins $P450_{mor}$ waren typisch für P450 Cytochrome. Für $P450_{mor}$ konnte im reduzierten CO-gebundenen Zustand nur noch ein Maximum bei 425 nm bestimmt werden, was eine Konversion des aktiven Cytochrom P450 zu seiner inaktiven Form (P420) anzeigt. Diese Konversion erfolgte im reduzierten Zustand von $P450_{mor}$ sehr schnell, wie verschiedene Messungen zeigten. Somit konnte $P450_{mor}$ nur in seiner inaktiven Form gereinigt werden. Das schien auch eine Erklärung dafür zu sein, dass eine Bindung der Substrate Morpholin, Piperidin oder Pyrrolidin an $P450_{mor}$ spektroskopisch nicht zu detektieren war.

Da $P450_{mor}$ N-terminal blockiert war, wurde nach proteolytischer Behandlung ein internes Peptid sequenziert. Diese Sequenz zeigte hohe Identitäten zu verschiedenen P450 Cytochromen, vor allem aus anderen *Actinomycetales*. Eine 100-prozentige

Identität zeigte sich zu der internen Sequenz eines Cytochrom P450 von *Mycobacterium smegmatis* mc^{2}155, das wahrscheinlich am Abbau von Piperidin und Pyrrolidin beteiligt ist. Es wurde davon ausgegangen, dass es sich bei dem aus *Mycobacterium* sp. Stamm HE5 isolierten Cytochrom P450 um ein ähnliches Enzym handelt, das sehr wahrscheinlich am Abbau der strukturanalogen Verbindung Morpholin beteiligt ist.

Gewöhnlich bestehen bakterielle P450-haltige Monooxygenasen aus drei Proteinen: einer FAD-haltigen Ferredoxin-Reduktase, die NADH oxidiert und die Elektronen über ein Ferredoxin auf den Häm-Kofaktor des Cytochrom P450 übertragt. Zur Identifizierung eines durch Morpholin induzierten Ferredoxins wurde der Nachweis des säurelabilen Schwefels genutzt. Im chromatographisch aufgetrennten zellfreien Extrakt von Morpholin-gewachsenen Zellen wurde ein Fe-S-Protein nachgewiesen, das sich nicht in dem von Succinat-gewachsenen Zellen bestimmen ließ. Dieses Ferredoxin wurde durch vier Säulenchromatographien zur Homogenität gereinigt und als Fd_{mor} bezeichnet. Die bestimmte molekulare Masse und vor allem das Absorptionsspektrum waren typisch für das von Fe_4S_4- oder Fe_3S_4-Ferredoxinen.

Die ermittelte N-terminale Sequenz von Fd_{mor} zeigte hohe Identitäten zu verschiedenen P450-assozierten Fe_3S_4-Ferredoxinen, die sich von den Fe_4S_4-Ferredoxinen durch das Fehlen eines Cystein-Restes unterscheiden. Die höchste Identität wurde zu dem Produkt des Gens *orf1* aus *M. smegmatis* mc^{2}155 gefunden, welches interessanterweise abwärts des Gen *pipA* liegt, das für das schon erwähnte Cytochrom P450 aus diesem Organismus kodiert. Die Ähnlichkeit zwischen den Ferredoxinen aus *Mycobacterium* sp. Stamm HE5 und *M. smegmatis* mc^{2}155 wurde als weiteres Indiz dafür gewertet, dass es sich hierbei um Monooxygenasen ähnlicher Funktion handeln sollte.

Nach der Reinigung von $P450_{mor}$ und Fd_{mor} sollte nun auch eine spezifisch durch Morpholin induzierte Ferredoxin-Reduktase isoliert werden. Zum Nachweis dieses Enzyms wurden zellfreie Extrakte von Morpholin- und Succinat-gewachsenen Zellen chromatographisch aufgetrennt. Anschließend wurde die NAD(P)H-abhängige Reduktion von verschiedenen künstlichen Elektronenakzeptoren in den einzelnen Fraktionen ermittelt. Hierbei konnte aber kein Unterschied zwischen den beiden

Extrakten festgestellt werden. Somit wurde auch kein Indiz für eine spezifisch Morpholin-induzierte Ferredoxin-Reduktase gefunden. Interessanterweise war in *M. smegmatis* mc^2155 neben den Genen *pipA* und *orf1*, die für das Cytochrom P450 bzw. das Ferredoxin kodieren, kein Gen identifiziert worden, das für eine Ferredoxin-Reduktase kodieren könnte. Ähnliche Ergebnisse wurden auch für andere P450-Systeme verschiedener *Actinomycetales* berichtet. Diese Genanalysen standen im Einklang mit den Ergebnissen dieser Arbeit, so dass zu diesem Zeitpunkt vermutet wurde, dass die für die Aktivität der Morpholin-Monooxygenase erforderliche Ferredoxin-Reduktase in *Mycobacterium* sp. Stamm HE5 ein konstitutiv gebildetes Protein ist.

Da $P450_{mor}$ nur in seiner inaktiven Form isoliert werden konnte und zudem keine spezifische Ferredoxin-Reduktase detektiert werden konnte, war es nicht möglich, die Aktivität der Morpholin-Monooxygenase zu messen. Dennoch war es das erste Mal, dass am Abbau von Morpholin beteiligte Enzyme gereinigt wurden. Die erstaunlich hohe Zahl von 22 P450-kodierenden Genen im Genom von *Mycobacterium tuberculosis* war ein Hinweis auf die Bedeutung dieser Enzyme für Mykobakterien. Daher war die Reinigung von $P450_{mor}$ ein wichtiger Schritt zum weiteren Verständnis der vielfältigen Funktionen von bakteriellen P450 Cytochromen. $P450_{mor}$ war erst das zweite Cytochrom P450, das aus einem *Mycobacterium* isoliert werden konnte.

Ebenso interessant war die Reinigung eines P450-assoziierten Fe_3S_4-Ferredoxins, da bislang nur zwei ähnliche Proteine aus *Streptomyces griseolus* isoliert und charakterisiert wurden. Alle anderen Informationen über diesen Typ von Ferredoxinen beruhen auf Sequenzdaten, die aus Genanalysen abgeleitet wurden. Dagegen gibt es sehr detaillierte Untersuchungen von Fe_2S_2-Ferredoxinen und den entsprechenden P450-Systemen (Kap. 1). Aus diesen Gründen stellte diese Arbeit einen viel versprechenden Ausgangspunkt für die weitere Untersuchung einer in vielfacher Hinsicht interessanten bakteriellen P450-haltigen Monooxygenase dar.

2.2 Klonierung und Sequenzierung des *mor* Operons und Charakterisierung der rekombinanten NADH:Ferredoxin-Reduktase des $P450_{mor}$-Systems

(Sielaff & Andreesen, 2005a)

Die ermittelten Aminosäuresequenzen der Peptide von $P450_{mor}$ und Fd_{mor} eröffneten nun die Möglichkeit, die genetische Basis dieser P450-haltigen Monooxygenase zu ermitteln. Durch verschiedene Amplifizierungs- und Klonierungsstrategien wurden mehrere spezifische DNA-Fragmente aus *Mycobacterium* sp. Stamm HE5 erhalten. Nach Sequenzierung und Analyse dieser Fragmente konnte ein Operon identifiziert werden, der sechs offene Leserahmen beinhaltete, von denen zwei unvollständig waren (Abb. 3). Das Gen *morA* und das stromabwärts von *morA* liegende Gen *morB* kodierten für die Proteine $P450_{mor}$ und Fd_{mor}, wie aus dem Vergleich der Genprodukte mit den zuvor ermittelten Proteindaten hervorging. Stromabwärts von *morB* wurde das Gen *morC* identifiziert, dessen Produkt signifikante Identitäten zu mehreren Ferredoxin-Reduktasen aus verschiedenen *Actinomycetales* zeigte. Diese wurden jedoch alle durch Genomanalysen identifiziert und noch nicht als Proteine charakterisiert. Stromabwärts von *morC* wurde ein unvollständiger offener Leserahmen (*orf1´*) identifiziert, der für eine Glutamin-Synthetase kodieren könnte.

Stromaufwärts von *morA* wurde ein unvollständiger offener Leserahmen (*´morR*) identifiziert, dessen abgeleitete Aminosäuresequenz eine hohe Identität zu einem regulatorischen Protein aus *M. smegmatis* mc^2155 zeigte. Das entsprechende Gen *pipR* liegt stromaufwärts von *pipA*, das für CYP151 kodiert. Zwischen *pipR* und *pipA* wurde ein Insertionselement identifiziert, welches zwischen *´morR* und *morA* nicht vorhanden war (Abb. 3). Stattdessen wurde in diesem Bereich ein offener Leserahmen (*orfX*) identifiziert, dessen Produkt aber wahrscheinlich nicht funktional ist. Ein ähnlicher offener Leserahmen konnte in dieser Arbeit auch in den entsprechenden Bereichen von *Mycobacterium* sp. Stamm RP1 und *M. smegm*atis mc^2155 identifiziert werden. In *M. smegm*atis mc^2155 wird dieser mögliche offene Leserahmen allerdings durch das Insertionselement unterbrochen (Abb. 3).

Die Gene *morA*, *morB* und *morC* waren identisch mit den entsprechenden Genen aus *Mycobacterium* sp. Stamm RP1, die kurz zuvor in einer anderen Arbeit bestimmt wurden. Es wurde hierbei auch berichtet, dass rekombinant produziertes MorA in

einem heterologen System mit Ferredoxin-Reduktase und Ferredoxin aus Spinat die Heterozyklen Morpholin, Piperidin und Pyrrolidin umsetzen kann. Das analysierte Fragment aus *Mycobacterium* sp. Stamm RP1 war über den gesamten Bereich identisch mit den entsprechenden Sequenzen aus *Mycobacterium* sp. Stamm HE5, einschließlich des unvollständigen offenen Leserahmens *orf1´* (Abb. 3).

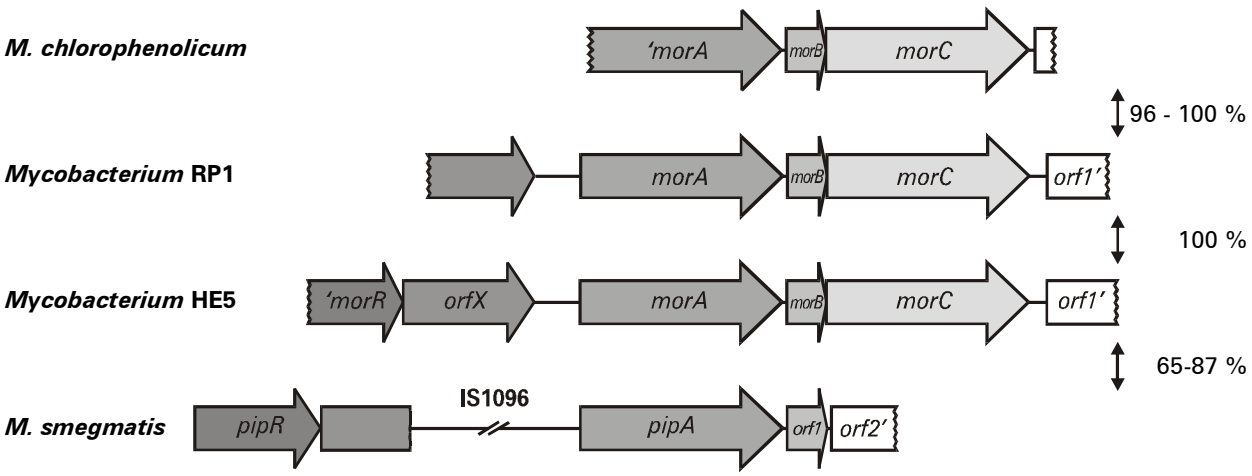

Abb. 3 Vergleich des *mor* Operons aus *Mycobacterium* sp. Stamm HE5 mit den entsprechenden Genbereichen anderer Mykobakterien. Die relativen Identitäten der entsprechenden Gene miteinander sind rechts angegeben.

Das Vorkommen völlig identischer P450-haltiger Monooxygenasen in *Mycobacterium* sp. Stamm HE5 und *Mycobacterium* sp. Stamm RP1 war erstaunlich, da die Homologie der entsprechenden Genbereiche damit höher lag als die ihrer 16s-rDNA. Ein weiterer Verwandter dieser Stämme ist *Mycobacterium chlorophenolicum* PCP-1, für den in unserem Labor gezeigt werden konnte, dass er auch Piperidin, Pyrrolidin und Morpholin als Substrat nutzen kann und dass beim Wachstum auf Morpholin ebenfalls ein Cytochrom P450 induziert wird. In dieser Arbeit wurden nun spezifische DNA-Fragmente aus *M. chlorophenolicum* PCP-1 amplifiziert. Die sequenzierten PCR-Produkte enthielten die nahezu identischen Gene *morA*, *morB* und *morC*. Größere Unterschiede wurden dann aber - beginnend mit einer veränderten Nukleinsäure im Stop-Codon von *morC* - stromabwärts von *morC* festgestellt. Die Region zwischen *morC* und *orf1´* war in *Mycobacterium* sp. Stamm HE5 etwas länger. Der folgende Sequenzabschnitt war dann wieder nahezu identisch zu dem aus *M. chlorophenolicum* PCP-1, allerdings in einem geringeren Maße. Dies ließe den Schluss zu, dass allein die *mor* Gene erst kürzlich in diese Genregion integriert wurden. Im Gegensatz dazu steht aber die völlige Identität der

gesamten analysierten Genbereiche in *Mycobacterium* sp. Stamm HE5 und *Mycobacterium* sp. Stamm RP1. Dies deutet eher daraufhin, dass die *mor* Gene zusammen mit den stromabwärts liegenden Sequenzen zwischen diesen Mykobakterien transferiert wurden und letztere erst später durch Deletionen oder Insertionen verändert wurden. Dies könnte auch bedeuten, dass die durch *orf1'* kodierte mögliche Glutamin-Synthetase nicht am Morpholin-Abbau beteiligt ist, obwohl eine Funktion dieses Proteins bei der Entsorgung des Stickstoffes vorstellbar wäre. Ein Plasmid könnte für den offensichtlichen horizontalen Gentransfer zwischen Morpholin-abbauenden Mykobakterien verantwortlich sein. In dieser Arbeit wurde aber kein Plasmid in *Mycobacterium* sp. Stamm HE5 detektiert.

Die Identifizierung von *morC* war ein wichtiges Ergebnis, da das entsprechende Protein zuvor nicht detektiert werden konnte (Kap. 2.1). *MorC* wurde nun als C-terminales His-Tag-Fusionsprotein exprimiert und das gereinigte Enzym FdR_{mor} konnte als NADH-abhängige, FAD-enthaltende Ferredoxin-Reduktase charakterisiert werden. FdR_{mor} war in der Lage, die künstlichen Elektronenakzeptoren Ferricyanid und Cytochrom c zu reduzieren. Die Ermittlung der kinetischen Parameter für die Reduktion dieser Elektronenakzeptoren ergab eine höhere katalytische Effizienz von FdR_{mor} mit Ferricyanid. Alle Aktivitäten waren strikt NADH-abhängig, da keine Reaktionen mit NADPH als Elektronendonator zu beobachten waren. Die Reduktion des vermuteten Redoxpartners Fd_{mor} durch FdR_{mor} wurde spektroskopisch direkt nachgewiesen. Die Zugabe von Fd_{mor} hatte keinen Effekt auf die FdR_{mor}-abhängige Reduktion von Ferricyanid. Jedoch wurde die Aktivität von FdR_{mor} gegenüber Cytochrom c durch die Gegenwart von Fd_{mor} um das fünffache erhöht. Weiterhin ermöglichte die Präsenz von Fd_{mor} die Reduktion von NBT durch FdR_{mor}, was FdR_{mor} alleine nicht möglich war. Durch diese Ergebnisse konnte eindeutig belegt werden, das FdR_{mor} in der Lage ist, Fd_{mor} zu reduzieren, welches seinerseits als Elektronenüberträger zu verschiedenen künstlichen Elektronenakzeptoren fungiert.

Um mehr über die Spezifität der Redoxpartner FdR_{mor} und Fd_{mor} zu erfahren, wurde untersucht, ob diese auch Aktivität mit den Proteinen Ferredoxin I aus Spinat, Adrenodoxin oder Adrenodoxin-Reduktase zeigen. Die letztgenannten Proteine dienen als Redoxpartner für verschiedene mitochondriale P450 Cytochrome und ähneln den Redoxpartnern von $P450_{cam}$: Putidaredoxin-Reduktase und

Putidaredoxin. Ferredoxin I, Putidaredoxin und Adrenodoxin sind als Fe_2S_2-Ferredoxine bekannt, während Fd_{mor} eindeutig einen Fe_3S_4-Cluster als Kofaktor trägt (Kap. 2.3). Zwar sind Aktivitäten von verschiedenen Ferredoxin-Reduktasen aus P450-Systemen auch mit anderen Ferredoxinen bekannt, jedoch wurden diese in keinem Fall als die natürlichen Redoxpartner dieser Reduktasen identifiziert.

FdR_{mor} war nicht in der Lage, Ferredoxin I zur Reduktion von Cytochrom c oder NBT zu nutzen. Adrenodoxin konnte aber die Reduktion von NBT durch FdR_{mor} vermitteln. Allerdings waren hierzu mikromolare Konzentrationen an Adrenodoxin notwendig, wohingegen im Fall von Fd_{mor} nanomolare Konzentrationen ausreichend waren. Dies deutete auf eine hohe Spezifität von FdR_{mor} für Fd_{mor} hin. Noch interessanter war aber, dass Adrenodoxin-Reduktase keinerlei Aktivität mit Fd_{mor} zeigte. Diese Ergebnisse deuten an, dass FdR_{mor} zwar in der Lage ist, verschiedene Ferredoxine zu nutzen, dass aber für die Reduktion des Fe_3S_4-Ferredoxins Fd_{mor} die spezifische NADH:Ferredoxin-Reduktase FdR_{mor} des $P450_{mor}$-Systems notwendig ist. Auf Grund der hier gezeigten Spezifität von Fd_{mor} für FdR_{mor} war die Darstellung von FdR_{mor} als katalytisch aktives Enzym ein wichtiger Schritt auf dem Weg zur Rekonstitution einer homologen P450-haltigen Morpholin-Monooxygenase.

2.3 Enzymatische Untersuchungen der Morpholin-Monooxygenase mit den gereinigten rekombinanten Proteinen FdR_{mor}, Fd_{mor} und $P450_{mor}$

(Sielaff & Andreesen, 2005b)

Nachdem die spezifische Ferredoxin-Reduktase des $P450_{mor}$-Systems identifiziert und auch als aktives Enzym dargestellt werden konnte, sollten nun die Interaktionen mit dem Redoxpartner Fd_{mor} kinetisch untersucht werden. Um ausreichende Mengen an Fd_{mor} zur Verfügung zu haben, sollte dieses auch als rekombinantes Protein produziert werden. Des Weiteren sollte auch $P450_{mor}$ rekombinant dargestellt werden, um durch ein vereinfachtes Reinigungsschema möglicherweise aktives Enzym zu erhalten, da die konventionelle Reinigung von $P450_{mor}$ aus *Mycobacterium* sp. Stamm HE5 immer zur inaktiven P420-Form geführt hatte (Kap. 2.1). Anschließend sollten alle drei rekombinanten Proteine zu einer katalytisch aktiven Morpholin-Monooxygenase rekonstituiert werden.

Rekombinantes Fd_{mor} wurde in drei Varianten dargestellt und gereinigt: als Wild-Typ-Protein sowie als C-terminales oder als N-terminales His-Tag-Fusionsprotein. Durch EPR-Spektroskopie wurde endgültig belegt, dass es sich bei Fd_{mor} um ein Fe_3S_4-Ferredoxin handelt. Für die Reduktion von NBT durch das Redoxpaar FdR_{mor}/Fd_{mor} konnte das pH-Optimum bestimmt werden und es wurde gezeigt, dass die Zugabe von Chloridionen die Aktivität herabsenkt. Dieser Einfluss der Ionenstärke ist ein Hinweis darauf, dass ionische Wechselwirkungen zwischen beiden Proteinen von Bedeutung sein könnten.

Für die Reduktion der künstlichen Elektronenakzeptoren Cytochrom c und NBT durch FdR_{mor}/Fd_{mor} wurden die kinetischen Parameter von FdR_{mor} für Fd_{mor} bestimmt. Die ermittelten katalytischen Effizienzen ergaben eine Präferenz des Redoxpaares FdR_{mor}/Fd_{mor} für NBT. Der sehr niedrige K_m-Wert von FdR_{mor} für Fd_{mor} mit NBT als Elektronenakzeptor war ein weiterer Hinweis auf eine hohe Spezifität. Alle diese Messungen wurden mit Wild-Typ-Fd_{mor} durchgeführt. Um festzustellen, ob die zusätzliche Aminosäuresequenz der His-Tag-Fusionsproteine einen Einfluss auf die Aktivität des Redoxpaares FdR_{mor}/Fd_{mor} hat, wurden die kinetischen Parameter auch mit diesen Fd_{mor}-Varianten bestimmt. Diese Ferredoxine zeigten allerdings schon ohne FdR_{mor} unspezifische Aktivitäten mit NADH und Cytochrom c. Dadurch waren die kinetischen Parameter nicht zu ermitteln und es konnten nur die katalytischen Effizienzen abgeschätzt werden, die ungefähr fünfmal niedriger lagen als die für Wild-Typ-Fd_{mor}. Mit NBT wurden ähnliche Hintergrund-Aktivitäten gemessen. Diese waren jedoch nicht so hoch wie die mit Cytochrom c gemessenen, so dass mit einer modifizierten Michaelis-Menten-Gleichung die kinetischen Parameter ermittelt werden konnten. Diese lagen im selben Bereich wie die für Wild-Typ-Fd_{mor} bestimmten Werte. Das ist ein Hinweis darauf, dass die zusätzlichen Aminosäuresequenzen in den His-Tag-Fusionsproteinen keinen Einfluss auf den Elektronentransfer zwischen FdR_{mor} und Fd_{mor} haben.

$P450_{mor}$ wurde mit einem N-terminalen His-Tag rekombinant dargestellt und konnte dadurch über eine einzige Affinitätschromatographie gereinigt werden. Im CO-Differenzspektrum des reduzierten Proteins wurden keine P420-Spezies detektiert (Kap. 2.1), so dass $P450_{mor}$ offenbar in seiner aktiven Form isoliert wurde. Jedoch führte - wie schon mit gereinigtem $P450_{mor}$ aus *Mycobacterium* sp. Stamm HE5

beobachtet (Kap. 2.1) - die Zugabe von Morpholin, Piperidin oder Pyrrolidin zu rekombinanten $P450_{mor}$ unter verschiedenen Bedingungen zu keiner Änderung des Spektrums, welche eine Substratbindung anzeigen würde. Die Änderung im Spektrum von P450 Cytochromen nach Bindung eines Substrats wird sehr wahrscheinlich durch die Verdrängung eines Häm-koordinierten Wassermoleküls durch das Substrat verursacht. Dies wird wahrscheinlich durch die hydrophobe Natur aller bis jetzt bekannten Substrate von P450 Cytochromen begünstigt. Dagegen sind die in dieser Arbeit untersuchten N-Heterozyklen polare und hydrophile Verbindungen, die möglicherweise deswegen keine Änderung im Spektrum von $P450_{mor}$ hervorrufen.

Um Näheres über den Zugang von Molekülen in das aktive Zentrum von $P450_{mor}$ zu erfahren, wurde die Bindung von verschiedenen Azol-Verbindungen an den Häm-Kofaktor von $P450_{mor}$ untersucht. Diese Moleküle generieren Bindungsspektren, die aus der Besetzung der sechsten Koordinationsstelle am Häm-Eisen durch den Azol-Stickstoff herrühren. Die mittels Titration bestimmten Bindungskonstanten für verschiedene Azole ergaben einen Hinweis darauf, dass $P450_{mor}$ möglicherweise größere und/oder hydrophobere Substrate bevorzugen könnte.

Allerdings schloss dies nicht aus, dass Morpholin trotzdem ein natürliches Substrat von $P450_{mor}$ ist. Mittels HPLC-Analyse wurde gezeigt, dass die aus den rekombinanten Proteinen FdR_{mor}, Fd_{mor} und $P450_{mor}$ rekonstituierte Monooxygenase Morpholin umsetzt. Damit war der direkte Beweis erbracht, dass es sich bei dem $P450_{mor}$-System um eine Morpholin-Monooxygenase handelt (Abb. 4). Daraufhin wurde die optimale Stöichiometrie der eingesetzten Proteine für einen maximalen Morpholin-Umsatz ermittelt und anschließend wurde das $P450_{mor}$-System mit den verschiedenen rekombinanten Fd_{mor}-Varianten rekonstituiert und der Umsatz an Morpholin verglichen. Der Umsatz war am höchsten, wenn Wild-Typ-Fd_{mor} eingesetzt wurde, während mit den C-terminalen oder N-terminalen His-Tag-Fusionsproteinen nur etwa die Hälfte dieses Umsatzes erreicht wurde. Da ein Einfluss der zusätzlichen Aminosäuresequenz in diesen Proteinen auf das Zusammenwirken zwischen Ferredoxin-Reduktase und Ferredoxin bereits ausgeschlossen wurde, scheint es jedoch möglich, dass Interaktionen zwischen Ferredoxin und Cytochrom P450 davon beeinflusst sein könnten.

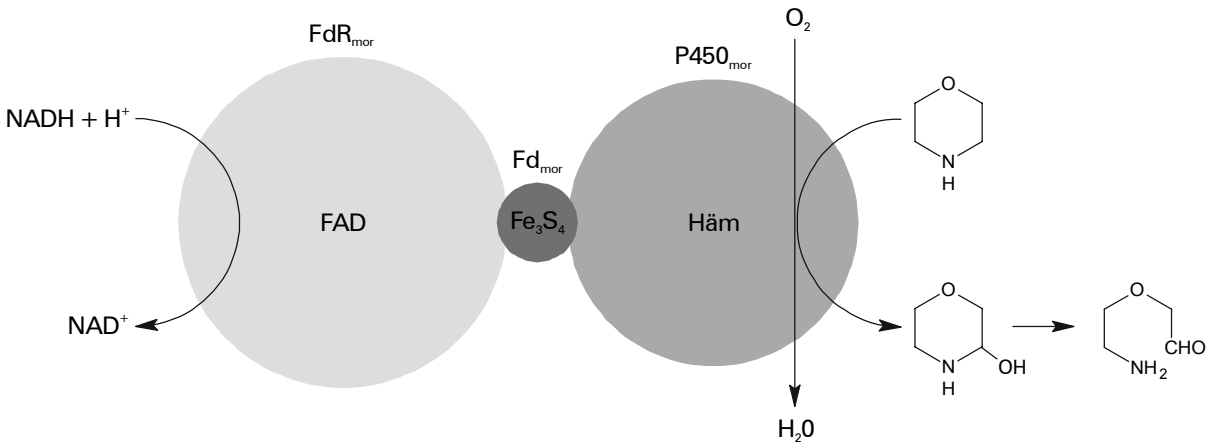

Abb. 4 Schematische Darstellung der Morpholin-Monooxygenase und der von ihr katalysierten Reaktion.

Wie schon in Kap. 1 erwähnt, wurden zur Messung der Aktivität von P450 Cytochromen in den meisten Arbeiten heterologe Redoxpartner aus anderen Organismen eingesetzt. Die Identifizierung und Sequenzierung des *mor* Operons und die anschließende, erfolgreiche Darstellung und Reinigung der rekombinanten Ferredoxin-Reduktase FdR_{mor}, des Ferredoxins Fd_{mor} und der Monooxygenase $P450_{mor}$ ermöglichten die erste homologe Rekonstitution eines katalytisch aktiven P450-Systems aus einem *Actinomycetales*. Außerdem waren die kinetischen Untersuchungen des Redoxpaares FdR_{mor}/Fd_{mor} die ersten, die mit einem Fe_3S_4-Ferredoxin durchgeführt wurden. Damit stellen die Ergebnisse der vorliegenden Dissertation eine solide Grundlage dar, um weitere detaillierte Untersuchungen der P450-haltigen Morpholin-Monooxygenase vorzunehmen, die sich durch die Nutzung eines Fe_3S_4-Ferredoxins und den Umsatz wasserlöslicher Substrate auszeichnet.

3 Publikationen

A cytochrome P450 and a ferredoxin isolated from *Mycobacterium* sp. strain HE5 after growth on morpholine (2001)
Bernhard Sielaff, Jan R. Andreesen and Thomas Schräder
Appl Microbiol Biotech **56**, 458-464

Analysis of the nearly identical morpholine monooxygenase-encoding *mor* genes from different *Mycobacterium* strains and characterization of the specific NADH:ferredoxin oxidoreductase of this cytochrome P450 system (2005a)
Bernhard Sielaff and Jan R. Andreesen
Eingereicht.

Kinetic and binding studies with purified recombinant proteins ferredoxin reductase, ferredoxin and cytochrome P450 comprising the morpholine mono-oxygenase from *Mycobacterium* sp. strain HE5 (2005b)
Bernhard Sielaff and Jan R. Andreesen
FEBS J **272**, 1148-1159

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ORIGINAL PAPER

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A cytochrome P450 and a ferredoxin isolated from *Mycobacterium* sp. strain HE5 after growth on morpholine

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Abstract A cytochrome P450 and an iron-sulfur protein, whose expression was specifically induced during growth of *Mycobacterium* sp. strain HE5 on morpholine as the sole source of carbon, nitrogen, and energy were purified to apparent homogeneity. Due to the lack of enzymatic activity, carbon monoxide difference spectra and determination of the acid-labile sulfur, respectively, were used to detect the proteins during purification. The cytochrome P450, designated $P450_{mor}$, was characterized as a monomer with an apparent molecular mass of 44.7 kDa. The amino acid sequence of an internal peptide comprising 19 amino acids was identical to the sequence derived from a gene encoding a cytochrome P450 from *Mycobacterium smegmatis* mc^2155 suggested to be involved in the utilization of piperidine and pyrrolidine. The iron-sulfur protein was characterized as a ferredoxin exhibiting a molecular mass of 6.8 kDa and named Fd_{mor}. An identity of 48–77% was obtained for the 30 N-terminal amino acids of Fd_{mor} and the corresponding sequences of different 3Fe-4S-ferredoxins known to be involved in P450-dependent reactions. From these data we concluded that growth of *Mycobacterium* sp. strain HE5 on morpholine led to the expression of a cytochrome P450-dependent monooxygenase system composed of at least two different proteins.

Introduction

Due to its chemical nature as a cyclic ether and secondary amine, morpholine is used for various industrial purposes, e.g. as a solvent, rubber additive or anticorrosive agent (Mijos 1978). Its release into the environment and subsequent conversion by chemical and biological N-nitrosation can lead to the formation of the carcinogenic compound *N*-nitrosomorpholine (Enzmann et al. 1995). Hypothetical pathways for the degradation of morpholine have been proposed by various authors (Swain et al. 1991; Mazure and Truffaut 1994; Combourieu et al. 1998, 2000). Recently 2-(2-aminoethoxy)acetate and diglycolic acid have been detected as intermediates during morpholine degradation by *Mycobacterium aurum* MO1 and an environmental *Mycobacterium* strain (Poupin et al. 1998; Combourieu et al. 1998, 2000). From these data it seems obvious that the initial ring-cleavage occurred at the C–N bond. Biochemical studies suggest that a cytochrome P450 may be involved in this initial step (Scheme 1) (Poupin et al. 1998; Poupin et al. 1999b). However, no morpholine-dependent enzymatic activity has ever been detected in bacteria grown on this substrate and thus nothing is known about the properties of the cytochrome P450. During our investigations on the degradation of cyclic amines by *Mycobacterium* sp. strain HE5, a specifically induced expression of a cytochrome P450 was observed during growth on morpholine, piperidine, and pyrrolidine (Schräder et al. 2000), strengthening the results obtained by Poupin et al. (1998, 1999b).

In most cases bacterial P450-dependent monooxygenases are composed of three components and electrons are transferred from NAD(P)H via an FAD-containing reductase and a small iron-sulfur protein to the cytochrome P450 where catalysis of the monooxygenase reaction takes place (for a review see Degtyarenko 1995; Munro and Lindsay 1996). Many P450-dependent monooxygenases are involved in the biosynthesis of secondary metabolites (e.g. antibiotics). Thus, these proteins often found in actinomycetes are of special interest for the biotechnological production of fine chemicals.

We report here the purification and characterization of a cytochrome P450 and a ferredoxin, whose expression was specifically induced during growth of *Mycobacterium* sp. HE5 on morpholine. Our data furthermore indicated that a very labile cytochrome P450-dependent monooxygenase is involved in the breakdown of morpholine by strain HE5.

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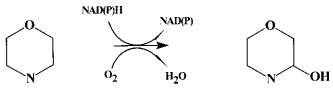

Morpholine 2-Hydroxymorpholine

Scheme 1 Reaction catalyzed by the proposed $P450_{mor}$-dependent morpholine monooxygenase

Materials and methods

Growth and preparation of cell extract

Mycobacterium sp. HE5 (DSM 44238) was grown on morpholine (30 mM) as the sole source of carbon, nitrogen, and energy as described by Schuffenhauer et al. (1999). Cell extracts were prepared from wet cells in accordance with Schuffenhauer et al. (1999).

Assays

Carbon monoxide difference spectra were used for the qualitative and quantitative determination of $P450_{mor}$. Spectra were recorded using samples containing 1–3 mg protein ml^{-1}. The samples were reduced by the addition of dithionite (2 mM) and carbon monoxide was carefully bubbled through the cuvette for 30 s. Reduced samples without carbon monoxide were used as a reference. The specificity of the formation of cytochrome $P450_{mor}$ was analyzed in cell extracts by recording the same spectra from succinate-grown cells. Quantification of the cytochrome P450 was achieved by using an extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm (Omura and Sato 1964). A second extinction coefficient was used for determination of the amount of cytochrome P450 already converted to cytochrome P420. This coefficient estimated with 176.5 mM^{-1} cm^{-1} at 420 nm was calculated after the complete conversion of defined amounts of cytochrome P450 to the corresponding cytochrome P420.

The iron-sulfur protein was detected by determination of the acid-labile sulfur present in the corresponding samples. The assay was carried out as described by Beinert (1983), estimating the methylene blue formation by the change in absorbance at 670 nm. An extinction coefficient of 43 $mM^{-1}cm^{-1}$ was calculated from a calibration curve obtained by using different concentrations of sulfide as a standard.

The assay for detection of a specifically formed reductase activity was performed in 50 mM potassium phosphate buffer, pH 7.2, containing NAD(P)H (0.1 mM) as electron donor and FAD and/or FMN (0.1 mM) as cofactor. The electron acceptors used in the assay were: different tetrazolium salts (0.2 mM), cytochrome *c* (0.05 mM), 2,6-dichlorophenol-indophenol (0.2 mM) and potassium hexacyanoferrate (1 mM). Activity measurements were carried out with crude extract (10–1,000 µg of protein) at different pH values (5.0–10.0) and temperatures (20–40°C) with or without the addition of phenazine methosulfate or phenazine ethosulfate (each 0.1 mM).

The activity of a putative morpholine monooxygenase was measured with crude extracts from morpholine-grown cells (10–1,000 µg of protein) or homogeneous proteins (1–10 µg of protein) at different pH values (5.0–10.0) and temperatures (20–40°C) after addition of NAD(P)H (0.1 mM) and FAD or FMN (0.1 mM) and/or dithiothreitol (1 mM) and/or Fe^{2+} (50 µM) to the assay mixtures containing 0.1–20 mM morpholine. Cell extracts were prepared in the presence or absence of stabilizing agents such as glycerol (20%) and/or dithiothreitol (1 mM) and/or Fe^{2+} (50 µM). Initial rates were derived from NAD(P)H oxidation following the decrease in absorbance at 340 nm (ε=6.3 mM^{-1} cm^{-1}). In addition the morpholine-dependent oxygen consumption was determined polarographically using a Clark electrode (Rank Brothers) under the conditions described above. Alternatively the morpholine-dependent activity was estimated by the consumption of H_2O_2. Peroxide (0.2 mM) was added to the morpholine-containing reaction mixture and after 10 min the remaining peroxide was determined by catalase-dependent (13 U) formation of oxygen estimated at an oxygen electrode.

Protein concentrations were determined according to the method described by Bradford (1976), using bovine serum albumin as a standard.

Protein purification

All purification steps, unless stated otherwise, were performed at 4°C. The first step in purification of $P450_{mor}$ was a chromatography of cell extract on a Sepharose Q column (17 ml) as described by Schuffenhauer et al. (1999), except that the potassium phosphate buffer was replaced by 50 mM Tris/HCl, pH 8.5. Since no morpholine-dependent enzyme activity could be determined in cell extracts using many varying conditions, a control for a specific induction of $P450_{mor}$ expression was prepared by separation of extracts from succinate-grown cells under the same conditions. The pooled fractions containing the cytochrome P450 were brought to a final concentration of 1.5 M $(NH_4)_2SO_4$ and precipitated protein was removed by centrifugation at 33,000 *g*. The supernatant obtained was subsequently loaded on a phenyl-Sepharose column (5 ml) previously equilibrated with 50 mM Tris/HCl, pH 7.2 (buffer A) containing 1.5 M $(NH_4)_2SO_4$ using a flow rate of 1 ml min^{-1}. Bound protein was eluted by a linear gradient from 1.5 M to 0 M $(NH_4)_2SO_4$ in buffer A, followed by an isocratic elution with 10 mM Tris/HCl, pH 7.2. The fractions containing the cytochrome P450 were pooled and concentrated by ultrafiltration using Centriprep tubes (Amicon). Subsequently the pool was applied at a flow rate of 0.5 ml min^{-1} to a Superdex 200 column (50 ml) equilibrated with buffer A. After identification of the fractions containing the cytochrome P450 they were pooled and supplied to a Mono Q column (1 ml) equilibrated with buffer A using a flow rate of 1 ml min^{-1}. Elution of the bound protein was achieved by a linear gradient from 0 to 1 M KCl in buffer A. Each fraction was analyzed by SDS-PAGE (12%) for homogeneity. Homogeneous fractions were pooled, dialyzed for 20 h against buffer A and stored at –20°C.

Chromatographies on Sepharose Q and phenyl-Sepharose as described for the cytochrome P450 were also used for purification of the iron-sulfur protein. However, the iron-sulfur protein eluted from the phenyl-Sepharose column during the washing step with 1.5 M $(NH_4)_2SO_4$ in buffer A. The resulting pool was dialyzed against 50 mM Tris/HCl, pH 8.5, and loaded on a Mono Q column equilibrated with the same buffer. After elution of the bound protein by a linear gradient from 0 to 1 M KCl in 50 mM Tris/HCl, pH 8.5, the fractions containing the iron-sulfur protein were pooled and concentrated by ultrafiltration. Chromatography on a Superdex 200 column equilibrated with buffer A was used as final purification step. Fractions were examined by SDS-PAGE (15%) and homogeneous fractions were pooled and stored at –20°C.

Analytical procedures

Fractions collected during the various chromatographies and samples of the purified proteins were analyzed under denaturing conditions by SDS-PAGE according to Laemmli (1970) using 12–15% polyacrylamide gels. Electrophoresis was carried out at constant current of 25 mA and maximal voltage. Gels were stained either by Coomassie Brilliant Blue G 250 or by silver stain.

The molecular masses of the subunits were either determined by SDS-PAGE (12–15%) or alternatively by mass spectrometry. The cytochrome P450 was analyzed by MALDI-mass spectrometry on a reflectron type time-of-flight mass spectrometer (Reflex; Bruker-Franzen Analytik). A VG BIO-Q (Fisons Instruments) which consists of an electrospray ion source followed by a triple quadrupole mass analyzer, was used for estimation of the molecular mass of the iron-sulfur protein. Both systems were run accord-

ing to the instruction manuals. The native molecular masses of both proteins were determined by gel filtration analysis on a Superdex 200 column.

Iron contents were determined by using *o*-phenanthroline and protein samples denatured by nitric acid (50%). After neutralization with NaOH, five volumes of denatured protein were adjusted to pH 4.5 by the addition of one volume of sodium acetate–acetic acid buffer (0.1 M). Subsequently hydroxylamine (0.1 volume, 20%) and *o*-phenanthroline (0.2 volume, 0.5%) were added after incubation for 15 min in the dark; the extinction was measured at 492 nm.

Absorption spectra of the purified proteins and carbon monoxide difference spectra of the cytochrome P450 were recorded on a Uvicon 930 spectrophotometer (Kontron Instruments) in cells of 1 cm path length at 25°C.

N-terminal sequence analysis and peptide mapping were carried out as previously described (Becker et al. 1997). The sequences were determined on an Applied Biosystems 476A Protein Sequencer.

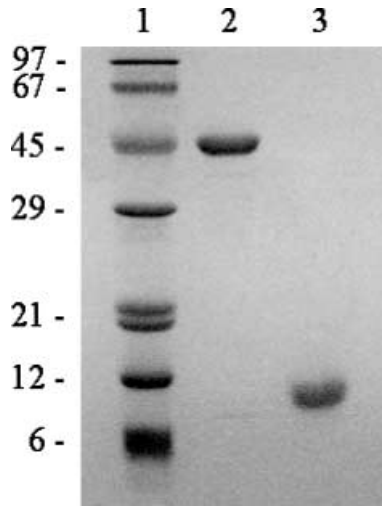

Fig. 1 SDS-PAGE of the purified cytochrome $P450_{mor}$ and Fd_{mor}. *Lane 1* Marker proteins (kDa); *lane 2* purified cytochrome $P450_{mor}$ after chromatography on the Mono Q column; *lane 3* purified Fd_{mor}

Results

Purification and characterization of $P450_{mor}$

As in previous studies, we were unsuccessful in detecting any substrate-dependent monooxygenase activity in extracts of *Mycobacterium* sp. HE5 grown on any of the three cyclic amines (Schuffenhauer et al. 1999; Schräder et al. 2000) even though the parameters used during the preparation of cell-free extracts were varied (see Materials and methods section). No monooxygenase reaction was obtained by using the peroxide shunt reported for P450-dependent enzymes (Joo et al. 1999). Thus, an alternative strategy was developed to detect enzymes induced by this substrate during purification. Comparison of carbon monoxide difference spectra from cell extracts obtained from morpholine- or succinate-grown cells were used for determination of cytochrome P450. These analyses led to the identification of a cytochrome P450 which was only observed if the strain was grown on morpholine (Schräder et al. 2000). Therefore, this cytochrome was designated cytochrome $P450_{mor}$. After ultracentrifugation, cytochrome $P450_{mor}$ was exclusively recovered in the supernatant using carbon monoxide difference spectra, indicating that it was a soluble protein. An average of 0.12 nmol cytochrome P450 (mg protein)$^{-1}$ was determined in extracts from morpholine-grown cells using carbon monoxide difference spectra. We developed a four-step purification scheme by which $P450_{mor}$ was obtained in an apparent homogeneous form. The results of a typical purification protocol are given in Table 1. Minor contaminating bands were removed by a final chromatography on a Mono Q column. Due to the loss of native protein, the amount of cytochrome P450 was not determined by carbon monoxide difference spectra after that final purification step. However, the cytochrome P450 could be simply identified by its color and the characteristic spectrum. The preparation of $P450_{mor}$ obtained was obviously homogeneous, as revealed by SDS-PAGE and silver-stain analyses (Fig. 1).

The subunit molecular mass of $P450_{mor}$ was determined to be 45 kDa by SDS-PAGE and 44.7 kDa by mass spectrometry. The native molecular mass was 65 kDa as estimated by gel filtration on a Superdex 200 column. Although this molecular mass did not exactly match to a monomeric native structure, it could nevertheless be suggested that $P450_{mor}$ is a monomer, as found for most other cytochromes P450.

Cytochrome $P450_{mor}$ was N-terminally blocked, thus, it was proteolytically digested using trypsin as protease and an internal peptide comprising 19 amino acids was sequenced by Edman degradation. A comparison of the obtained sequence with the SWISS–PROT database revealed a significant similarity to the sequences of different cytochromes P450 located between residues 90 and 120 (Fig. 2). This short sequence was identical to the sequence of a cytochrome P450 from *M. smegmatis* mc^{2}155 which is probably involved in the utilization of the two cyclic amines, piperidine and pyrrolidine (Fig. 2;

Table 1 Purification of cytochrome $P450_{mor}$ from *Mycobacterium* sp. strain HE5. The cytochrome P450 was quantified by carbon monoxide difference spectra

Purification step	Volume (ml)	Total protein (mg)	Total P450 (nmol)	Specific P450 (nmol mg^{-1})	Yield (%)	Purification (-fold)
Cell extract	35	432	23.7	0.06	100	1
Sepharose Q	18	67	12.7	0.2	53	3.5
Phenyl–Sepharose	5.5	5.5	6.5	1.2	27	21.3
Superdex 200	5.5	0.35	2.8	7.9	12	143

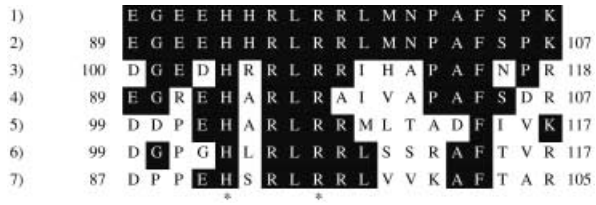

Fig. 2 Alignment of the internal peptide obtained after trypsin digestion of the cytochrome $P450_{mor}$ from *Mycobacterium* sp. HE5 with sequences of different cytochromes P450. The depicted sequences were obtained from the following sources: *1* cytochrome $P450_{mor}$ from *Mycobacterium* sp. HE5 (this study); *2* putative cytochrome P450 (PipA) involved in piperidine and pyrrolidine utilization by *M. smegmatis* mc^2155 (Poupin et al. 1999a); *3* P450 involved in daunomycin biosynthesis by *Streptomyces* sp. C5 (Dickens and Strohl 1996); *4* anaerobically expressed P450 from *B. japonicum* (Tully and Keister 1993); *5* involved in herbicide degradation P450 from *S. griseolus* (Omer et al. 1990); *6* P450 involved in carbomycin biosynthesis by *S. thermotolerans* (Arisawa et al. 1995); *7* putative P450 involved in biosynthesis of mycinamine by *M. griseorubida* (Inouye et al. 1994). The highly conserved residues proposed to be involved in binding of the porphyrin ring (Hasemann et al. 1994) are indicated by an *asterisk* (*) and residues identical to $P450_{mor}$ are *shaded*

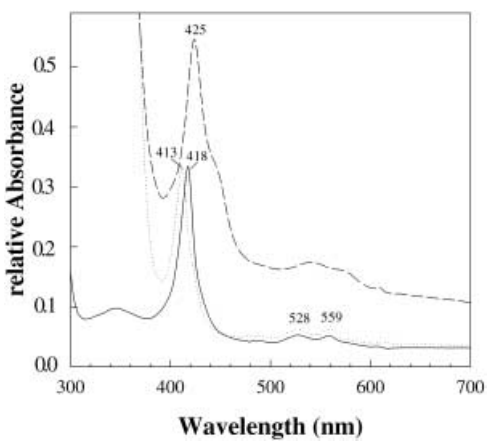

Fig. 3 Absorption spectra of cytochrome $P450_{mor}$. Spectra were recorded in 50 mM Tris/HCl, pH 7.2, at 25°C, for the oxidized (–), dithionite-reduced (···) and dithionite-reduced enzyme (0.1 mg ml^{-1}) after addition of carbon monoxide (- -)

Poupin et al. 1999a). An identity of 58% was estimated for the corresponding sequence of a cytochrome P450 from *Streptomyces* sp. strain C5, which is involved in daunomycin metabolism, and a cytochrome P450 with unknown function found in *Bradyrhizobium japonicum* (Tully and Keister 1993; Dickens and Strohl 1996; Walczak et al. 1999). The highly conserved amino acids His110 and Arg114 ($P450_{terp}$ numbering), shown to be involved in binding of the propionate-moiety of the porphyrin ring D (Hasemann et al. 1994), were also present in the sequence of cytochrome $P450_{mor}$ (Fig. 2).

Spectra recorded for the homogeneous protein showed absorption maxima at 418, 528, and 559 nm, respectively, for the oxidized enzyme (Fig. 3), indicating that it exists in the iron low-spin state. The addition of morpholine, piperidine, or pyrrolidine (0.1–10 mM) did not influence the absorption spectrum of cytochrome $P450_{mor}$. Maxima at 413, 528, and 559 nm were obtained for the dithionite-reduced protein. The addition of CO to the dithionite-reduced cytochrome $P450_{mor}$ resulted in a major absorption peak at 425 nm with a shoulder at 450 nm (Fig. 3). This unusual feature was due to the very fast conversion of the reduced cytochrome $P450_{mor}$ to its corresponding P420 form, as observed in reduced crude extracts at room temperature. Within 30 min the absorbance maximum at 450 nm was completely shifted to 420 nm (data not shown). The conversion of cytochrome $P450_{mor}$ to its P420 form was slower but also occurred in extracts kept oxidized.

Purification and characterization of Fd_{mor}

The characteristic features determined for the cytochrome $P450_{mor}$ indicated that it belongs to the class of cytochromes P450 associated with further proteins acting as an electron transport chain. In analogy to other bacterial P450-dependent monooxygenases, one of these proteins should be a small ferredoxin-like iron-sulfur protein (Degtyarenko 1995; Munro and Lindsay 1996). Thus, we analyzed cell-free extracts from morpholine- and succinate-grown cells, respectively, for the presence of an iron-sulfur protein by determination of the acid-labile sulfur. A concentration of 0.95 nmol acid-labile sulfur (mg of protein)$^{-1}$ was estimated in extracts from morpholine-grown cells. Only a relatively low amount of acid-labile sulfur [0.05 nmol (mg of protein)$^{-1}$] was detected in corresponding fractions from succinate-grown cells. After chromatography of the extracts from morpholine-grown cells on Sepharose Q, the protein containing the acid-labile sulfur eluted as a single dominant peak at a salt concentration of about 0.4 M KCl (data not shown). These data indicated that the expression of a single dominant, soluble iron-sulfur protein was in-

Table 2 Purification of the Fd_{mor} from *Mycobacterium* sp. strain HE5

Purification step	Volume (ml)	Total protein (mg)	Total acid labile sulfur (nmol)	Specific acid labile sulfur (nmol mg^{-1})	Yield (%)	Purification (-fold)
Cell extract	100	1,600	1,520	0.95	100	1
Sepharose Q	62	341	905	2.7	60	2.8
Phenyl–Sepharose	100	4.9	271	76	25	80
Mono Q	1.5	1.8	136	104	12	110
Superdex 200	5.5	0.4	89	265	7	279

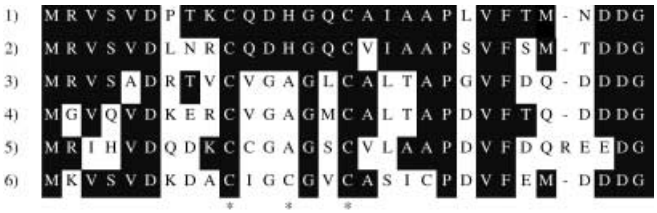

Fig. 4 Alignment of the N-terminal amino acid sequence of Fd_{mor} from *Mycobacterium* sp. HE5 with the corresponding sequences of different 3Fe-4S-ferredoxins. The sequences were obtained from: *1* Fd_{mor} from *Mycobacterium* sp. HE5 (this study); *2* putative 3Fe-4S-ferredoxin from *M. smegmatis* mc^2155 (Poupin et al. 1999a); *3* 3Fe-4S-ferredoxin (Fd-1) from *S. griseolus* (O'Keefe et al. 1991); *4* 3Fe-4S-ferrodoxin (fdx_{soy}) from *S. griseus* (Trower et al. 1992); *5* 3Fe-4S-ferredoxin (Fd-2) from *S. griseolus* (O'Keefe et al. 1991); and *6* 4Fe-4S-ferredoxin from *Thermococcus litoralis* (Busse et al. 1992). The cysteine residues involved in the formation of the iron-sulfur cluster (O'Keefe et al. 1991) are indicated by an *asterisk* (*) and residues identical to Fd_{mor} are *shaded*

duced during growth of *Mycobacterium* sp. strain HE5 on morpholine. The data obtained during purification of this protein are summarized in Table 2 and an SDS-PAGE analysis of the purified protein is shown in Fig. 1.

A molecular mass of 6.8 kDa was determined for the purified iron-sulfur protein using mass spectrometry. Gel filtration chromatography on Superdex 200 revealed a molecular mass of 14 kDa pointing to a dimeric native structure.

The absorption spectrum of the homogeneous oxidized iron-sulfur protein was typical for 4Fe-4S- or 3Fe-4S-ferredoxins showing an absorption maximum at 411 nm and a shoulder at about 560 nm (data not shown). After reduction of the protein by addition of dithionite, the absorption in the visible region of the spectrum decreased significantly, exhibiting a shoulder at 410 and 560 nm, respectively (data not shown).

The N-terminal amino acid sequence of the iron-sulfur protein that comprised 30 residues revealed the highest identity (77%) to the derived sequence of a putative 3Fe-4S-ferredoxin from *M. smegmatis* mc^2155 located downstream from the cytochrome P450 gene already described (Poupin et al. 1999a) (Fig. 4). Other 3Fe-4S-ferredoxins associated with cytochromes P450 showed an identity of up to 57% to the corresponding part of the iron-sulfur protein from *Mycobacterium* sp. HE5 (Fig. 4). An identity of 53% was obtained for a 4Fe-4S-ferredoxin from *Thermococcus litoralis*, which is easily converted to the corresponding 3Fe-4S-ferredoxin. The cysteine residue at position 13 is substituted by a histidine residue in the case of the iron-sulfur protein from *Mycobacterium* sp. strain HE5, and therefore it could be concluded that this protein is a 3Fe-4S-ferredoxin. Due to the involvement in morpholine metabolism, it was designated Fd_{mor}.

The data presented in Table 2 for the homogeneous iron-sulfur protein after the final chromatography on Superdex 200 indicated that it had lost about 60% of the four acid-labile sulfur atoms expected for the native enzyme. A loss of the iron-sulfur cluster was confirmed by determination of the iron present in the purified protein. An amount of 0.96 mol of iron per mol of protein was estimated, corresponding to a loss of at least 68% of the expected iron.

NAD(P)H-dependent reductase activities

After purification of both $P450_{mor}$ and Fd_{mor}, we assumed that there might be a third protein involved in the morpholine–monooxygenase reaction. This protein should be a flavin-containing reductase transferring electrons from NAD(P)H to the ferredoxin (Degtyarenko 1995; Munro and Lindsay 1996). The strategy for an identification of such a protein was the same as described for $P450_{mor}$ and the Fd_{mor}, separating crude extracts from morpholine- or succinate-grown cells on Sepharose Q. Subsequently, the reductase-activity was determined in each fraction using a variety of different electron acceptors (see Materials and methods section). However, the reductase activities, if obtained, were always about the same in both extracts (data not shown).

Discussion

We report here the first direct purification of a cytochrome P450 and a 3Fe-4S-ferredoxin from a *Mycobacterium* species and show that the expression of both proteins was specifically induced during growth of *Mycobacterium* sp. HE5 on morpholine. Although bacterial P450 systems are not that ubiquitous as they are in eukaryotes (Degtyarenko 1995; Munro and Lindsay 1996), 22 genes encoding putative cytochromes P450 have recently been identified in the genome of *Mycobacterium tuberculosis* (Cole et al. 1998). However, nothing is known about the function of the corresponding proteins. In general, there exist only a few reports about properties and functions of cytochromes P450 in mycobacteria (Uotila et al. 1992; Poupin et al. 1998, 1999a). To our knowledge, the only cytochrome P450 from a *Mycobacterium* which has been investigated is the CYP51-like protein from *M. tuberculosis* which has been isolated after heterologous expression of the corresponding gene in *Escherichia coli* (Aoyama et al. 1998).

The data obtained during the present study indicated that the isolated cytochrome $P450_{mor}$ and Fd_{mor} are components of a P450-dependent monooxygenase which catalyzes the initial oxidation of morpholine. Previously, the formation of 2-hydroxymorpholine by a cytochrome P450-dependent monooxygenase was suggested as the initial step in morpholine degradation by an environmental *Mycobacterium* strain designated RP1 (Poupin et al. 1998). A cytochrome P450 has been detected in crude extracts using carbon monoxide difference spectra. However, no data were given about the characteristic properties of the cytochrome P450 from strain RP1. So far, no enzyme assay has been be established in crude extracts, thus, the activity of the P450-dependent morpholine-con-

verting monooxygenases seems to be generally rather unstable. In the case of *Mycobacterium* sp. HE5, this instability was also demonstrated by comparing morpholine degradation rates of resting and permeabilized cells, respectively (Schräder et al. 2000). The low stability of the enzyme activity might be due to different factors. The monooxygenase system is composed of different components; thus, a disruption of the native structure could be one reason for inactivation of the enzyme. In addition the observed conversion of the cytochrome $P450_{mor}$ to the corresponding $P420_{mor}$ species occurred quite rapidly in case of the reduced enzyme and was also experienced to a lesser extent for the oxidized protein. This transition is a common feature of cytochromes P450, and the P420 form is known to be enzymatically inactive (Martinis et al. 1996 and references therein). The spontaneous conversion of $P450_{mor}$ to the corresponding $P420_{mor}$ seems to be an unusual feature, since this was usually only observed after chemical or physical treatment of the protein (Martinis et al. 1996). Thus, the fast conversion of cytochrome $P450_{mor}$ to its inactive P420 might be a further reason for our inability to detect a morpholine-dependent enzyme activity in crude extracts of *Mycobacterium* sp. HE5.

Cytochromes P450 usually shift by the addition of a substrate from the low- to the high-spin state, resulting in a shift of the Soret absorption peak to a lower wavelength (Jefcoate 1978; Poulos et al. 1986; Eltis et al. 1993). The addition of morpholine, pyrrolidine, or piperidine to partially purified or homogeneous cytochrome $P450_{mor}$ did not lead to spectral changes, indicating that the structure of the active site might have already been changed in such a way that the enzyme was unable to bind its substrate and thus was inactive.

Recently Poupin et al. (1999a) have identified a gene in *M. smegmatis* mc^2155, designated *pipA*, encoding a cytochrome P450. It is probable that PipA is involved in the degradation of the cyclic amines pyrrolidine and piperidine, which are structurally related to morpholine. The derived amino acid sequence of PipA was identical to the 19 amino acids comprising the peptide obtained from $P450_{mor}$ of *Mycobacterium* sp. HE5. Interestingly *M. smegmatis* mc^2155 is not able to grow on morpholine (Poupin et al. 1999a) indicating that this substrate might not be converted by PipA (e.g., due to differences between PipA and cytochrome $P450_{mor}$ in other regions of the sequence). These differences in the primary structures of both proteins were confirmed by preliminary studies on the gene encoding $P450_{mor}$ (unpublished results). An identification of the substrate-specific active site of these enzymes may be possible by a comparison of the complete amino acid sequences. PipA was classified as a member of the new family CYP151 (Poupin et al. 1999a) and our data indicated that $P450_{mor}$ also belongs to this family.

The N-terminal amino acid sequence of the morpholine-induced Fd_{mor} from *Mycobacterium* sp. HE5 that we obtained has the highest identity to a sequence derived from a gene of *M. smegmatis* mc^2155 encoding a putative 3Fe-4S-ferredoxin. Interestingly, this gene is located just downstream of the *pipA* gene, suggesting that both were cotranscribed and functionally connected (Poupin et al. 1999a). This suggestion is supported on the protein level by our data and thus we assume that Fd_{mor} is the second component of a monooxygenase responsible for the initial hydroxylation of morpholine. No gene encoding a reductase was detected adjacent to *pipA* and the putative ferredoxin gene (Poupin et al. 1999a) and this is in agreement with our observation that *Mycobacterium* sp. HE5 did not form a specific NAD(P)H-dependent reductase during growth on morpholine. Thus, the cytochrome P450-dependent monooxygenases from mycobacteria involved in the degradation of cyclic amines seem to be similar to the corresponding enzymes from streptomycetes, which also belong to the branch of Gram-positive bacteria with a high GC-content. The derived sequences of cytochrome P450-dependent monooxygenases from, for example, *Steptomyces griseolus* and *S. griseus* showed that there was only a ferredoxin-encoding gene adjacent to the cytochrome P450-encoding gene, but no reductase-encoding gene (O'Keefe et al. 1991; Trower et al. 1992). Thus, the reductase required for the respective monooxygenase activity seems to be a constitutively formed protein, perhaps involved in different cytochrome P450 systems.

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Analysis of the nearly identical morpholine monooxygenase-encoding *mor* genes from different *Mycobacterium* strains and characterization of the specific NADH:ferredoxin oxidoreductase of this cytochrome P450 system

Running title: *Mor* operon encoding mycobacterial $P450_{mor}$ system

Subject category: Biochemistry and Molecular Biology

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Abbreviations: AdR, adrenodoxin reductase; Adx, adrenodoxin; Fd, ferredoxin; FdI, spinach ferredoxin I; FdR, ferredoxin reductase; NBT, nitroblue tetrazolium; P450, cytochrome P450 monooxygenase.

The GenBank accession numbers for the nucleotide sequences reported here from *Mycobacterium* sp. strain HE5 and *M. chlorophenolicum* PCP-1 are AY816211 and AY960119, respectively.

SUMMARY

Cloning and sequencing of the *morABC* operon region revealed the genes encoding the three components of a cytochrome P450 monooxygenase, which is required for the degradation of the N-heterocycle morpholine by *Mycobacterium* sp. strain HE5. The cytochrome P450 ($P450_{mor}$) and the Fe_3S_4 ferredoxin (Fd_{mor}) encoded by *morA* and *morB*, respectively, have been characterized previously, whereas no evidence was obtained for a specific reductase, which is required to support activity of the $P450_{mor}$ system. Analysis of the *mor* operon now revealed the gene *morC* encoding the ferredoxin reductase of this morpholine monooxygenase. The genes *morA*, *morB* and *morC* were identical to those genes from *Mycobacterium* sp. strain RP1. We now identified almost identical *mor* genes in *Mycobacterium chlorophenolicum* PCP-1 in addition to an inducible cytochrome P450 pointing to a horizontal gene transfer. No evidence for a circular or linear plasmid was found in *Mycobacterium* sp. strain HE5. Analysis of the downstream sequences of *morC* revealed differences in this gene region between *Mycobacterium* sp. strain HE5 and *Mycobacterium* sp. strain RP1 on the one hand and *M. chlorophenolicum* on the other hand, indicating insertions or deletions after recombination. In all studied strains the gene *orf1´* was identified downstream of *morC*. Thus, the encoded putative glutamine synthetase is probably not related to morpholine degradation. The gene *morC* of *Mycobacterium* sp. strain HE5 was heterologously expressed, due to its presence in the *mor* operon. The purified recombinant protein FdR_{mor} was characterized as monomeric 44 kDa protein being a strictly NADH-dependent, FAD-containing reductase. The K_m values of FdR_{mor} for the substrate NADH (37·7 ± 4·1 µM) and the artificial electron acceptors potassium ferricyanide (14·2 ± 1·1 µM) and cytochrome c (28·0 ± 3·6 µM) were measured. FdR_{mor} was shown to functionally interact with its natural redox partner, the Fe_3S_4 protein Fd_{mor}, and with the Fe_2S_2 protein adrenodoxin, albeit with a much lower efficiency, but not with spinach ferredoxin. In contrast, adrenodoxin reductase, the natural redox partner of adrenodoxin, could not use Fd_{mor} in activity assays. These results indicated that FdR_{mor} might be able to utilize different ferredoxins, but that Fd_{mor} requires the specific NADH:ferredoxin oxidoreductase FdR_{mor} from the $P450_{mor}$ system for efficient catalytic function.

INTRODUCTION

The degradation of the secondary cyclic amines morpholine, piperidine and pyrrolidine has been reported for different mycobacteria (Cech *et al.*, 1988; Knapp & Brown, 1988; Poupin *et al.*, 1999a; Poupin *et al.*, 1998). The detection of intermediates during morpholine degradation in *Mycobacterium aurum* MO1 and in an environmental *Mycobacterium* strain strongly indicated that the initial ring cleavage occurs at the C-N bond (Combourieu *et al.*, 1998b; Combourieu *et al.*, 2000; Poupin *et al.*, 1998). Studies suggested that a cytochrome P450 may catalyse the hydroxylation of the carbon atom of this bond (Combourieu *et al.*, 1998a; Poupin *et al.*, 1999b; Poupin *et al.*, 1998).

An environmental bacterium was isolated in our laboratory and identified as new *Mycobacterium* sp. strain HE5, which was able to utilize morpholine, piperidine and pyrrolidine as sole source of carbon, nitrogen, and energy. A specifically induced expression of a cytochrome P450 was observed during degradation of these N-heterocycles (Schräder *et al.*, 2000) which supported the above mentioned reports. As observed for other mycobacterial strains, no enzymatic hydroxylation activity was detected in cell-free extracts of *Mycobacterium* sp. strain HE5. To tackle this problem, the cytochrome P450, designated $P450_{mor}$, and its proposed redox partner, a Fe_3S_4 ferredoxin (Fd_{mor}) were purified separately to homogeneity (Sielaff *et al.*, 2001). Thus, for the first time proteins involved in morpholine degradation could be isolated.

Cytochromes P450 are able to catalyse a wide range of reactions, mainly hydroxylations (Urlacher *et al.*, 2004). The activation of molecular oxygen species at the heme cofactor of these enzymes requires electrons, which are derived from the oxidation of NAD(P)H by an oxidoreductase. The cytochrome P450 can be reduced either directly by an FAD and FMN containing reductase (class II system) or electrons are transferred from an FAD containing reductase to the cytochrome P450 via a small iron-sulphur protein (class I system). Most bacterial P450 systems belong to the latter class (Munro & Lindsay, 1996). However, a specifically induced reductase could not be detected in cell-free extracts of *Mycobacterium* sp. strain HE5 (Sielaff *et al.*, 2001) or of other mycobacterial strains (Combourieu *et al.*, 1998a; Poupin *et al.*, 1998; Trigui *et al.*, 2004). The determined internal peptide of $P450_{mor}$ was identical to the translated sequence of the gene *pipA*, encoding a P450 (CYP151) from *Mycobacterium*

smegmatis mc^2155. PipA is involved in piperidine and pyrrolidine metabolism, but the *pip* operon lacked a gene encoding a reductase (Poupin *et al.*, 1999b). These results led to the assumption that the reductase might be a constitutively expressed protein (Sielaff *et al.*, 2001), as it was supposed earlier for other P450 systems from different *Actinomycetales* (O'Keefe & Harder, 1991). Transcription studies demonstrated that in *Streptomyces coelicolor* three ferredoxin reductases are sufficient to support activity of 18 P450 cytochromes (Lei *et al*., 2004) which was in agreement with this hypothesis.

The determined amino acid sequences of $P450_{mor}$ and Fd_{mor} provided now the opportunity to determine the genetic basis of the $P450_{mor}$ monooxygenase. Of special interest was the possibility to identify the ferredoxin reductase of the $P450_{mor}$ system. We report here the cloning of the operon encoding all structural genes of the $P450_{mor}$ monooxgenase. Sequence determination of this operon region revealed a gene encoding a ferredoxin reductase, which was expressed as enzymatically active recombinant protein. This is the first report of the characterization of a native NADH-dependent ferredoxin reductase from a P450 system, which is specifically required for enzymatic function with the Fe_3S_4 protein Fd_{mor}.

METHODS

Materials. All chemicals, NADH and spinach ferredoxin (FdI) were purchased from Sigma-Aldrich and Fluka. For molecular biological work all biochemicals and enzymes other than restriction endonucleases were provided by Roche Molecular Biochemicals. Restriction endonucleases were from Fermentas and New England Biolabs based on availability. Oligonucleotides were provided by Metabion. The Lambda ZAPII system was obtained from Stratagene. Cloning vectors were from Fermentas. Expression vectors and Ni-NTA affinity column material was from Novagen. All other column materials were obtained from Pharmacia. Purified adrenodoxin reductase (AdR) and adrenodoxin (Adx) were a kind gift from Prof. Rita Bernhardt and Dr. Frank Hannemann (Universität des Saarlandes).

Bacterial strains. *Mycobacterium* sp. strain HE5 (DSM 44238) was from our laboratory collection. *Mycobacterium chlorophenolicum* PCP-1 (DSM 43826^T) was kindly provided by Timo Nieminen (University of Oulo, Finland). *Escherichia coli* XL1 blue MRF´ and *E. coli* Rosetta(DE3) were purchased from Stratagene and Novagen, respectively.

Preparation of whole cell DNA. *Mycobacterium* sp. strain HE5 or *M. chlorophenolicum* PCP-1 were grown on 20 mM morpholine to an OD_{600} of ~ 1·0 and harvested as described previously (Schuffenhauer *et al.*, 1999). 400 µg cells were resuspended in 400 µl TENS-buffer (50 mM Tris/HCl, pH 8·0, 100 mM EDTA, 100 mM NaCl, 0·3 % SDS), passed in a 2 ml tube containing 1·6 g of glass beads (0·25 µm diameter) and these were shaken in a bead beater (Retsch) at maximum power for 10 min. After removing the cell debris and glass beads by centrifugation at 20 000 *g*, the supernatant was collected and incubated with RNase A (200 µg ml^{-1}) at 37 °C for 30 min. Subsequently, proteinase K was added (200 µg ml^{-1}) and the solution incubated at 55 °C for 1 h. The following steps were standard procedures and were performed in sequence: extraction with phenol and phenol/chloroform (60:40), ethanol-precipitation and resuspension of precipitated DNA in 10 mM Tris/HCl, pH 8·0, containing 1 mM EDTA.

DNA techniques. Molecular procedures were either standard techniques (Sambrook, 1989) or those recommended by the respective manufacturers. All PCRs and sequencing reactions were performed on a Mastercycler (Eppendorf). Nucleotide sequences were determined using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems) and analyzed using an ABI PRISM 377 DNA Sequencer. Attempts to isolate a potential circular plasmid were performed according to Larsen (2000). Plugs for usage in pulse field gel electrophoresis (PFGE) were prepared as described by Hughes et al. (2001). PFGE was performed on a CHEF-DR II system (Bio-Rad) at 13 °C and 170 V and the pulse time was raised linearly over 24 h from 20 to 80 s.

Amplification and cloning of DNA fragments. A specific DNA fragment was amplified from whole cell DNA using a degenerate primer (5´-CAC CAC CGS YTS CGS CGS YTS ATG AAC CC-3´) designed based upon the 19 aa internal fragment of $P450_{mor}$ (Sielaff *et al.*, 2001) and a primer (5´-GGC AGT GTG TTG GGT CCG GTG TTG C-3´) derived from the C-terminal part (1154 – 1171) of *pipA* from *M. smegmatis* mc^2 155 (Poupin *et al.*, 1999b). PCR was performed according to following standard protocol: 94 °C for 4 min; (94 °C for 15 s, 55 °C for 20 s, 72 °C for 1 min) for 10 cycles; (94 °C for 15 s, 55 °C for 20 s, 72 °C for 1 min plus 5 s at each cycle) for 20 cycles. This standard protocol was used for all PCRs, except that annealing temperatures and extension times were changed if necessary. The product P450-F1 was ligated into the pGem-T vector (Promega), transformed into *E. coli* XL1 blue MRF´ and of a positive clone the plasmid was sequenced using M13 forward and reverse primers. From the sequence of

P450-F1 two specific primers mor6 (5′-AAA CTC ATC GGC TCG CTC GTA CC-3′) and mor7 (5′-ACT CGC TGT ATA GGT GGA CGG TG-3′) were derived and used to amplify from whole cell DNA a 630 bp PCR product (annealing at 65 °C) which was directly sequenced and subsequently labeled using DIG High-Prime, yielding the probe P450m. This probe was used in Southern analysis of whole cell DNA digests using a bank of restriction enzymes (*Pst*I, *Xho*I, *Pvu*I, *Sma*I, and *Eco*RI). After separation by electrophoresis on a 0·8 % (w/v) agarose gel, DNA was transferred to a nylon membrane by vacuum blotting and probed with P450m. Chemiluminescence detection revealed a band of suitable size (4·5 kb) in the *Eco*RI digest. After repetition of digest and separation, bands of appropriate size were recovered by gel extraction (Qiagen Gel Extraction Kit) and ligated into the *Eco*RI site of the Lambda ZAPII vector. Recombinant clones were packaged in vitro and after infection of *E. coli* XL1 blue MRF′ the resulting phage particles were screened by plaque hybridization with P450m. The phagemids of positive plaques were in vivo excised and resulting clones were screened by colony hybridization and as a control by colony PCR. Because no positive clone could be detected, the recombinant clones were used as template in PCR with the primers mor6 and RPRN (modified M13 reverse primer: 5′-CAA TTT CAC ACA GGA AAC AGC TAT G-3′), which yielded the new fragment P450-F2 (annealing at 65 °C and extension for 1 min 30 s). P450-F2 was gel extracted and used as template for another PCR in order to obtain enough DNA for direct sequencing. Based upon the new sequence information of P450-F2, the primers mor8 (5′-GCG TAT CCG TAG ATC CCA CG-3′) and mor9 (5′-GCG GTT ATA AGG CAG GTG TC-3′) were designed for the new probe Fdfrm. PCR (annealing at 52 °C and extension for 30 s) with whole cell DNA yielded a 318 bp product which was labeled as described for P450m.

After restriction site analysis of the fragment P450-F2, digests of whole cell DNA were performed using the enzymes *Bst*XI, *Nru*I, *Pvu*II, *Sac*I, *Pae*I, and *Xma*III. Digested DNA was separated by electrophoresis, transferred to a nylon membrane and hybridization was performed one time with P450m and, after stripping the membrane, a second time with Fdfrm. Bands of suitable size were detected in the *Nru*I (2·0 kb), *Sfi*I (2·2 kb), and *Pae*I (2·0 kb) digests. Recovered DNA fragments were ligated into *Nru*I-digested pUC57 or into *Pae*I-digested pUC18, respectively, and transformed individually into *E. coli* XL1 blue MRF′. Colonies were transferred with a sterile stamp on two new agar plates, of which one was swapped off with 3 ml 10 mM Tris/HCl, pH 8·0, and 1 µl of this suspension was used as template for PCR with the primer pairs mor6/mor7 or mor8/mor9. For positive pools the corresponding plates were

screened by colony lifts and subsequent Southern hybridization. There was one positive clone detected each in the *Nru*I library (2500 clones tested) and the *Pae*I library (1500 clones tested). The plasmids of these positive clones were isolated (Qiagen Plasmid Purification Kit) and designated pMN21 and pMP10, respectively.

PCR with whole cell DNA of *M. chlorophenolicum* PCP-1 as template and the primers 5′-CGC TGA TCC GTC GTT CTC CAT-3′ with mor7 (annealing at 48 °C), mor6 with mor9 (annealing at 52 °C), 5′-GTC GTA GGC GGC TCA CTG-3′ with 5′-CCT CGT TGT TGT TTG GAC-3′ (annealing at 48 °C), and 5′-CTA TGG ATC ACC TGC TCT G-3′ with 5′-ATC GCT TGG AAA TAA ACG-3′ (annealing at 45 °C and extension for 30 s) yielded the products MC-F1, MC-F2, MC-F3, and MC-F4, respectively. These amplified DNA fragments were directly sequenced.

Cloning of *morC*. Primers were designed to either end of *morC* containing suitable restriction sites flanked by „spacer“ nucleotides at the 5′ end to facilitate efficient digestion. A *Pag*I site was incorporated in the N-terminal primer 5′-GAACTA TCATGA CCACCC CGCGG CACGTC-3′ to allow for an in frame ligation in the *Nco*I treated vector pET28b(+) to express *morC* as C-terminal His-Tag fusion protein. In the C-terminal primer 5′-CTAGAC AAGCTT TGCGGG CAGCTG GACGGC GG-3′ a *Hind*III site was incorporated (restriction sites underlined). PCR was performed using whole cell DNA as template according to the standard protocol (see above) with the annealing temperature set to 67 °C. The major 1·2 kb product was cut with *Pag*I and *Hind*III, extracted from the gel and ligated in the *Nco*I/ *Hind*III digested vector pET28b(+). This plasmid was transformed into *E. coli* XL1 blue MRF′ cells. Resulting recombinant cells were screened by PCR and plasmids of positive clones were purified and sequenced to confirm that no PCR errors were incorporated. A plasmid containing the correct insert was designated pMRC28 and transformed into cells of *E. coli* Rosetta(DE3). Glycerol stocks were prepared by adding 200 µl 40 % (v/v) glycerol to 800 µl of a cell culture previously grown to an OD_{600} of 1·0 and stored at –80 °C.

Production and purification of recombinant FdR_{mor}. 4 ml Luria Bertani medium with 30 µg kanamycin ml^{-1} were inoculated with 5 µl of a glycerol stock of *E. coli* Rosetta(DE3) containing pMRC28 and cultured overnight at 30 °C. This culture was used to inoculate four 2 l Erlenmeyer flasks containing each 500 ml Terrific Broth with 30 µg kanamycin ml^{-1}. The flasks were incubated at 37 °C until an OD_{600} of 0·8 was attained (~ 5 h). The cells were then induced

with 1 mM IPTG and incubated at 25 °C for 18 to 20 h. Cells were harvested by centrifugation (7500 *g*, 20 min, 4 °C) and stored at –20 °C. After resuspension in 20 ml buffer A (50 mM NaH_2PO_4, pH 8·0, 300 mM NaCl, 20 % (v/v) glycerol) containing 10 mM imidazole, 0·1 mM PMSF and 5 µl Benzonase, cells were disrupted by two passages through a 20 K French press cell (SLM-Amicon) at 120 MPa and the lysate was centrifuged at 18 000 *g* for 30 min (4 °C) to remove cell debris. The supernatant was loaded onto a 0·5 ml Ni-NTA His-Bind Resin flow through column, previously equilibrated with 3 ml buffer A containing 10 mM imidazole. After washing with 5 ml buffer A containing 20 mM imidazole, FdR_{mor} was eluted by stepwise addition of 0·25 ml buffer A containing 200 mM imidazole. Fractions containing FdR_{mor}, monitored by the flavin specific absorption at 452 nm, were pooled and concentrated in an ultrafiltration device (Vivascience). The second purification step was a gel filtration on Sephadex 75, run with buffer B (50 mM Tris/HCl, pH 7·5, 20 % (v/v) glycerol). Fractions were collected, concentrated and stored in aliquots at –20 °C.

Purification of Fd_{mor}. Growth of *Mycobacterium* sp. strain HE5 cells and preparation of crude extracts was performed as described previously (Sielaff *et al.*, 2001). The purification protocol for Fd_{mor} was modified. After eluting Fd_{mor} from Q-Sepharose fast flow in a lineaer gradient of 0 – 1 M KCl in buffer B, fractions containing Fd were identified by their brownish colour and collected according to their A_{280}/A_{425} value. The collected fractions were concentrated in an ultrafiltration device and proteins were then separated on a Sephadex 75 gel filtration column using buffer B. Subsequently Fd_{mor} was applied to a MonoQ column. After elution in a linear gradient of 0 – 1·5 M KCl in buffer B, the protein was desalted using a PD10 column run with buffer B. Fd_{mor} purified by this procedure was >95 % pure, as judged by SDS-PAGE analysis.

Molecular characterization methods. SDS/PAGE was carried out as described previously (Sielaff *et al.*, 2001). Analytical gel filtration analysis was performed on a FPLC system equipped with a Superdex 75 column (Pharmacia Biotech) run with buffer B. UV-Vis spectra were recorded on an Uvikon 930 spectrophotometer (Kontron). The reduction of FdR_{mor} with NADH was performed in a glove box (Coy) under nitrogen atmosphere at 4 °C. Buffers and solutions were made anaerobic prior to usage by several cycles of degassing and gassing with nitrogen using the sluice of the glove box. The quartz cuvette was sealed with a rubber cap. The extinction coefficient of the protein-bound flavin was determined spectrophotometrically quantitating the FAD released from the holoprotein following SDS treatment (Aliverti *et al.*,

1999). The identity of the enzyme bound flavin was assessed fluorometrically. After thermal denaturation of 10 µM holoenzyme at 100 °C for 15 min the released flavin was treated with 3 mU phosphodiesterase I (Aliverti *et al.*, 1999). Emission spectra (480 nm to 600 nm) were recorded in a fluorescence cuvette of 1 cm path length on a FluoroMax2 (Jobin Yvon-Spex) at 20 °C, using an excitation wavelength of 450 nm and a slit width of 5 nm. Visible CD spectra (320 nm to 600 nm) were recorded at 20 °C using a JASCO J-810 spectropolarimeter with a quartz cell of 1 cm pathlength (scan speed 50 nm min^{-1}). Spectra were recorded five times and averaged.

Activity assays. The activities of FdR_{mor} towards the artificial electron acceptors potassium ferricyanide, cytochrome c, and nitroblue tetrazolium (NBT) were determined spectrophotometrically using an Uvikon 930 spectrophotometer (Kontron). Potassium ferricyanide reduction was monitored at 420 nm (ε_{420} = 1020 M^{-1} cm^{-1}), cytochrome c reduction at 550 nm (ε_{550} = 21 100 M^{-1} cm^{-1}), and NBT reduction at 535 nm (ε_{535} = 18 300 M^{-1} cm^{-1}). Reactions were performed with 10 nM FdR_{mor} in 50 mM Tris/HCl, pH 8·5 at 30 °C, if not stated otherwise. For measurements of ferricyanide reducing activities at different pH values, a buffer was used composed of 25 mM Tris and 25 mM glycine, which was adjusted to the appropriate pH with either NaOH or HCl. Activity assays of FdR_{mor} with Fd_{mor} were performed in 50 mM glycine-buffer, pH 8·5. AdR/Adx activity was measured according to Uhlmann *et al.* (1994). Steady-state kinetic parameters were determined by varying the concentrations of the substrates in the standard assay. Initial velocities (v) were fitted to a hyperbolic function to obtain the kinetic parameters K_m and V_{max}.

RESULTS

Cloning and sequencing of the genes encoding the $P450_{mor}$ system

Degenerate primers were designed on basis of an internal 19 aa fragment of $P450_{mor}$ and the N-terminal 30 aa fragment of Fd_{mor} (Sielaff *et al.*, 2001), as it could be expected that these proteins are coded by adjacent genes. Primers were chosen according to codon usage in mycobacteria in order to minimize their inherent degeneracy. However, no specific fragment could be amplified by PCR with genomic DNA from *Mycobacterium* sp. strain HE5. A different approach was finally successful: The 19 aa $P450_{mor}$ fragment is identical to the corresponding translated

sequence of *pipA*, a gene coding for a P450 being involved in piperidine and pyrrolidine metabolism in *M. smegmatis* mc^2155 (Poupin *et al.*, 1999b). The combination of a new primer designed from the 3´ end of *pipA* and a degenerate primer derived from $P450_{mor}$ produced an 895 bp fragment (P450-F1), of which the internal sequence proved to encode the $P450_{mor}$ fragment. Based on this sequence, internal primers were designed to produce a probe which was then used in Southern hybridization experiments with different restriction enzyme digests. This led to the isolation of a 4·5 kb *Eco*RI fragment which was ligated into the Lambda ZAPII vector. After in vivo excision of the phagemids of plaques reacting positive in Southern hybridization experiments, no positive clone could be detected. However, using the ligated *Eco*RI fragment as template in PCR with an internal primer and a vector encoded primer, yielded a specific 1373 bp fragment (P450-F2) comprising 1272 bp from the 3´ terminal part of the cloned fragment (Fig 1).

Based on the new sequence of P450-F2, a second probe Fdfrm was amplified, of which the sequence was located downstream of probably suitable restriction sites. A restriction site map of the $P450_{mor}$ gene region (data not shown) was obtained by Southern analysis of several DNA digests with P450m, which was located upstream of these sites, or with Fdfrm. This allowed now cloning of specific fragments regarding their location and the expected extent of new sequence information. A 2·0 kb *Nru*I fragment was cloned, which was about 1·4 kb shorter at its 5´ terminal site than the *Eco*RI fragment. A positive clone was detected from this library and the plasmid pMN21 was isolated. Sequencing of the internal fragment revealed the upstream region of the $P450_{mor}$ operon. Cloning of a 2·0 kb *Pae*I fragment led to the isolation of the plasmid pMP10, which contained the downstream region of this operon. Summarizing, 4782 bp of the $P450_{mor}$ operon could finally be sequenced.

Analysis of the genes encoding the $P450_{mor}$ system

Sequencing of DNA fragments revealed a putative operon consisting of six open reading frames, of which two were truncated (Fig. 1). *MorA* encoded a protein of 400 aa, which contained the sequence identical to the previously determined internal 19 aa peptide of $P450_{mor}$ (Sielaff *et al.*, 2001). Thus, we concluded that *morA* encodes for the cytochrome $P450_{mor}$. There was a difference between the predicted molecular mass of 44 603 Da from *morA* and the molecular mass of 44 769 Da determined by mass spectrometry of $P450_{mor}$ (Sielaff *et al.*, 2001). $P450_{mor}$ proved to be N-terminally blocked in Edmann degradation, suggesting an N-terminal acylation,

which could account for this difference. *MorA* as well as the following genes *morB*, *morC* and *orf1´* were found to be identical to corresponding genes from *Mycobacterium* sp. strain RP1 (Trigui *et al.*, 2004). This point will be dealt with later.

The two ORFs downstream of *morA* appeared to encode the potential redox partners for a catalytically functional P450 system. *MorB* encodes the ferredoxin Fd_{mor} (62 aa) as confirmed by comparison of the translated sequence with the N-terminal 30 aa sequence determined for the previously purified protein Fd_{mor} from *Mycobacterium* sp. strain HE5. In addition, the predicted molecular mass of 6793 Da was in good accordance with the mass of 6795 Da determined for Fd_{mor} (Sielaff *et al.*, 2001). The following ORF *morC* encoded a 403 aa protein with a predicted molecular mass of 42 376 Da, which was, as mentioned above, identical to MorC from *Mycobacterium* sp. strain RP1 and exhibited identities to several ferredoxin reductases, all identified from genome sequences: 39 % in different overlaps to FprC from *Streptomyces avermitilis* MA-4680 (Ikeda *et al.*, 2003), and Rv0688 from *Mycobacterium tuberculosis* H37Rv (Cole *et al.*, 1998), and 37 % to FprA from *S. avermitilis* MA-4680 (Ikeda *et al.*, 2003). *FprC* as well as *fprA* were identified adjacent to genes encoding Fe_3S_4 ferredoxins and the P450s CYP105Q1 and CYP147B1, respectively (Lamb *et al.*, 2003). The identification of MorC as being actually a ferredoxin oxidoreductase was confirmed in this work by heterologous expression of *morC* and analysis of the protein. All previously purified P450 coupled reductases were reported to belong to the glutathione reductase family, of which all contain an FAD binding consensus sequence (GxGxxG) in the N-terminal region (Dym and Eisenberg, 2001). In FdR_{mor} this motif is changed (GGSLAG), whereas a second consensus sequence (GxGxxGxE) was found to be conserved. Sequence analysis of FprC and FprA from *S. avermitilis* revealed that they also contain the changed motif. However, despite such local differences, the overall homology of FdR_{mor} to putidaredoxin reductase (28 % identity) from the $P450_{cam}$ system from *Pseudomonas putida* (Sevrioukova *et al.*, 2004), indicated that these proteins are although related to the glutathione reductase family.

The *orf1´* downstream of *morC* was truncated and the derived amino acid sequence (141 aa) was identical to the deduced 74 aa of the truncated *orf1´* from *Mycobacterium* sp. strain RP1 and showed 85 % identity to the determined 130 aa, encoded by the also truncated *orf2´* from *M. smegmatis* mc^2155. Both sequences exhibit significant identities to N-terminal sequences of putative glutamine synthetases (Poupin *et al.*, 1999b; Trigui *et al.*, 2004).

Identical P450 genes in distinct mycobacterial strains

Quite recently, the genes encoding a cytochrome P450 system involved in secondary amine utilization in *Mycobacterium* sp. strain RP1 became known (Trigui *et al.*, 2004). The analyzed *Pst*I fragment, exhibiting the ORFs *morA*, *morB*, *morC* and *orf1´*, is identical to the corresponding sequence of *Mycobacterium* sp. strain HE5. The fact of a totally identical cytochrome P450 system was surprising, because the homology of this gene region is higher than that of their 16S rDNA (98·0 % identity). *M. chlorophenolicum* PCP-1 is another relative of these strains according to its 16S rDNA (98·4 % identity to *Mycobacterium* sp. strain HE5, 97·3 % to *Mycobacterium* sp. strain RP1) and known to be capable of degrading polychlorinated phenols (Apajalahti & Salkinoja-Salonen, 1987; Häggblom *et al.*, 1994). It was now shown in our laboratory, that *M. chlorophenolicum* PCP-1 is also able to use morpholine, piperidine and pyrrolidine as sole carbon, nitrogen and energy source and that a cytochrome P450 is induced during growth on morpholine, but not on the suggestive intermediate diglycolic acid (Debbab, 2003). The specific DNA fragments MC-F1, MC-F2, MC-F3, and MC-F4 (Fig. 1) could be amplified, using primers derived from the *mor* operon and whole cell DNA isolated from *M. chlorophenolicum* PCP-1 as template in PCR. Sequencing 2707 bp of these fragments revealed the nearly identical genes *morA*, *morB* and *morC* (only one nucleotide was different in *morB*). But a pronounced difference was detected downstream of *morC* beginning with a changed nucleotide in the stop codon of *morC* (Fig. 2). The intergenic region between *morC* and *orf1´* was 66 base pairs longer in *Mycobacterium* sp. strain HE5. The sequence following this stretch is again quite identical to that of *M. chlorophenolicum* PCP-1, although to a lower extent (95·5 %) compared to the identity of the *mor* genes. From these results it seemed obvious that these mycobacterial strains might have exchanged DNA. However, no plasmid could be detected in *Mycobacterium* sp. strain HE5, neither by standard procedures for the isolation of circular plasmids or by pulse field gel electrophoresis for the detection of linear plasmids.

Analysis of the upstream region of *morA*

The 3·9 kb *Pst*I fragment from *Mycobacterium* sp. strain RP1 (see restriction sites in Fig. 1) contained a 914 bp sequence upstream of *morA* which was previously identified as being noncoding (Trigui *et al.*, 2004). In this work, a larger extent of this region was sequenced. This enabled the identification of a stop codon upstream of *morA* at position 560 of the sequenced

mor operon (Fig. 1), which terminated a truncated ORF, designated ´*morR*. The translated 186 aa showed identities of 80 % to a putative regulatory protein, encoded by the gene *pipR* from *M. smegmatis* mc^{2}155 (Poupin *et al.*, 1999b), and of 39 % (in 177 bp overlap) to SAV1742, a putative GntR-family regulator from *S. avermitilis* MA-4680 (Ikeda *et al.*, 2003). PipR has been shown to be involved in the regulation of piperidine and pyrrolidine metabolism, which involves the cytochrome P450 CYP151, encoded by the gene *pipA* (87 % identity to *morA*) found downstream of *pipR*. Between *pipR* and *pipA*, an insertion element (IS*1096*) has been identified (Poupin *et al.*, 1999b), which was not present between ´*morR* and *morA* in *Mycobacterium* sp. strain HE5. Instead an ORF was identified, where the start codon overlapped with the stop codon of ´*morR*. This ORF, designated *orfX*, encoded a polypeptide of 260 aa, which showed identities of 31 % (in a 158 aa overlap) to the hypothetical proteins SAV1740 and SAV1124 from *S. avermitilis* MA-4680 (Omura *et al.*, 2001) and to a low extent to chemotactic transducers from different bacteria. No function can be assigned to the hypothetical protein (260 aa) encoded by *orfX*, as well as to SAV1740 (265 aa) and SAV1124 (278 aa), as they lack e.g. the C-terminal part of chemotactic transducers, which are composed in general of 600 to 700 aa. Interestingly, SAV1740 was found 39 bp downstream of SAV1742, which showed significant identities to the polypeptide encoded by ´*morR*. A possible ORF homologous to *orfX* could also be identified at the same position in the given sequence from *M. smegmatis* mc^{2}155, but two additional nucleotides are present here at position 1111 (position 273 of *orfX*), which were not detected in the corresponding sequences of *Mycobacterium* sp. strain HE5 and of *Mycobacterium* sp. strain RP1. If these two nucleotides would be deleted in case of *M. smegmatis* mc^{2}155, the predicted polypeptide (125 aa) would exhibit an identity of 65 % to that of *orfX*. However, in *M. smegmatis* mc^{2}155 this possible ORF was disrupted after 375 bp by the IS element.

Production and purification of *morC*

MorA and *morB* have now been shown to encode the previously isolated proteins $P450_{mor}$ and Fd_{mor}, thus, establishing them as being part of the morpholine-hydroxylating P450 system. A specific, morpholine-induced reductase could not be detected in *Mycobacterium* sp. strain HE5 by the methods employed (Sielaff *et al.*, 2001), but we now identified *morC* encoding the ferredoxin reductase FdR_{mor} of the $P450_{mor}$ system. The direct proof for this notion has been given in a separate publication by reconstitution of all three isolated proteins to an enzymatically

active morpholine monooxygenase (Sielaff & Andreesen, 2005). *MorC* was now expressed as a C-terminal His-Tag fusion protein to study its characteristics as ferredoxin reductase and to enable a comparison to previously purified ferredoxin reductases from other bacterial P450 systems.

An additional protein band was clearly visible in SDS-PAGE after growth of *E. coli* Rosetta(DE3) harboring pMRC28 at 37 °C and induction with 1 mM IPTG. But nearly all of the protein was found to form inclusion bodies. Lowering the growth temperature after induction significantly increased the amount of soluble protein which was found to be highest when cells were grown at 25 °C for 18 to 20 h. This protein, which from now on is called $FdR_{mor,}$ was isolated by chromatography on a Ni^{2+} affinity column and a subsequent gel filtration on Sephadex 75. The purified protein was judged to be about 90 % homogenous in SDS-PAGE (Fig. 3). Attempts to further purify FdR_{mor} by anion exchange chromatography on MonoQ resulted in the loss of the flavin cofactor and therefore in its activity. The cofactor could not be reconstituted neither by the addition of FAD nor of FMN. The amount of purified FdR_{mor} was calculated to be about 30 nmol (≅ 1·2 mg/L culture), using the estimated extinction coefficient for FdR_{mor} (see below).

Molecular properties of FdR_{mor}

FdR_{mor} showed a M_r of about 50 000 in denaturing PAGE (Fig. 2) which appeared to be in the same range as the calculated mass of 44 183 Da (42 736 calculated from the sequence of *morC* and 1147 from the linker sequence). The M_r of native FdRmor was determined by gel filtration to be 50 000 indicating that the protein was a monomer under these conditions. The pure FdR_{mor} enzyme exhibited in its oxidized state spectral features typical of flavin-containing enzymes, with spectral maxima at 273 nm, 378 nm and 452 nm. Shoulders were observed at 422 nm and 473 nm (data not shown). A value of 10·0 was calculated for the ratio of protein to flavin-specific absorption (A_{273}/A_{452}). Addition of excess sodium dithionite or NADH under anaerobic conditions led to full reduction of the flavin. No spectral signals attributable to flavin semiquinone species could be detected (data not shown). The non-covalently bound flavin in FdR_{mor} was identified as FAD. The fluorescence of the released flavin increased about ten-fold after addition of phosphodiesterase, as expected for the conversion from FAD to FMN. The extinction coefficient of FdR_{mor} at 452 nm was calculated to be ε_{452} = 11 070 M^{-1} cm^{-1} from the

amount of FAD released after protein denaturation by SDS. The visible CD spectrum of FdR_{mor} (Fig. 4) is mainly related to the chiral signal from the FAD cofactor. Minima are located at 450 nm (close to the electronic absorption maximum at 452 nm) and 478 nm. The overall shape of the visible CD spectrum is similar to that reported for the AdR-homologue FprA from *M. tuberculosis* (McLean *et al.*, 2003).

Catalytic properties of FdR_{mor}

FdR_{mor} was capable of oxidizing NADH and reducing the electron acceptors potassium ferricyanide and cytochrome c. The reduction of these acceptors by FdR_{mor} was strictly dependent on NADH, whereas no activity was obtained using NADPH as substrate. The addition of FAD to the assay had no enhancing effect on the activity of FdR_{mor}. The pH optimum for the NADH-dependent reduction of potassium ferricyanide by FdR_{mor} was determined to be at 9·4. At pH 7·5, activity of the enzyme declined to about 50 %. The optimal temperature for this reaction was found to be around 30 °C (data not shown). No NADH oxidase activity of FdR_{mor} could be observed under these conditions. The steady-state kinetic parameters of FdR_{mor} (Table 2) for the substrate NADH was determined with saturating concentrations of ferricyanide (1 mM) and those for the artificial electron acceptors ferricyanide and cytochrome c were determined with saturating concentrations of NADH (250 µM). The K_m values measured for these substrates were all found to be in the same range. The lowest K_m was obtained for ferricyanide, whereas those determined for cytochrome c and NADH were around two- and three-fold higher, respectively. The efficiency (k_{cat} / K_m) of NADH-dependent ferricyanide reduction by FdR_{mor} was about 25-fold higher, compared to cytochrome c reduction. This was mainly due to an about 14-fold higher k_{cat} for the reduction of ferricyanide.

Analysis of the *mor* operon in this study revealed that the $P450_{mor}$ monooxygenase is a class I system, composed of three components: The NADH-oxidizing ferredoxin reductase FdR_{mor}, the ferredoxin Fd_{mor} as electron-transfer protein and the cytochrome $P450_{mor}$, which acts as monooxygenase. Thus, FdR_{mor} should be able to interact catalytically with its proposed natural redox partner Fd_{mor}. The reduction of Fd_{mor} by FdR_{mor} was directly monitored by the decrease of the ferredoxin peak at 412 nm in the spectrum of Fd_{mor} after addition of NADH and a catalytic amount of FdR_{mor} (Fig. 5). Subsequently, the ability of Fd_{mor} to mediate the FdR_{mor} catalysed reduction of different electron acceptors was studied. The addition of Fd_{mor} had no effect on the

FdR_{mor}-dependent reduction of ferricyanide, whereas Fd_{mor} enhanced the reaction of FdR_{mor} towards cytochrome c up to five-fold. Furthermore, the presence of Fd_{mor} enabled the reduction of NBT by FdR_{mor}, which could not be catalysed by FdR_{mor} on its own. The results obtained now proved that FdR_{mor} reduces Fd_{mor}, which acts as an electron shuttle to different artificial electron acceptors. Thus, the systematic name for FdR_{mor} should be NADH:ferredoxin oxidoreductase.

The natural redox partners of previously characterized ferredoxin reductases have been always Fe_2S_2 ferredoxins. In contrast, it has been clearly demonstrated that Fd_{mor} contains a Fe_3S_4 cluster (Sielaff & Andreesen, 2005). To elucidate whether FdR_{mor} and Fd_{mor} specifically require each other for efficient catalysis, the cross reactivity of these proteins with AdR and Adx was examined. AdR and Adx serve as electron transport system of the mitochondrial P450 cytochromes and are the mammalian counterparts of putidaredoxin reductase and putidaredoxin (Schiffler & Bernhardt, 2003). The AdR-homologue FprA from *M. tuberculosis* was reported to interact functionally with Adx and spinach FdI (Fischer *et al.*, 2002). Therefore, the ability of FdR_{mor} was examined to utilize FdI in the reduction of NBT or cytochrome c, but no reactivity between these proteins was observed. In contrast, Adx could replace Fd_{mor} functionally in the NBT standard assay, although concentrations in the micromolar range were necessary (indicating a K_m value of FdR_{mor} for Adx of about 2 μM), compared to nanomolar concentrations in the case of Fd_{mor}. The reduction of cytochrome c by FdR_{mor} was not enhanced by the addition of Adx. More interestingly, AdR was not able to use Fd_{mor} neither in the reduction of cytochrome c nor in the reduction of NBT. These results suggest that FdR_{mor} might be able to interact with different ferredoxins, but that the reduction of the Fe_3S_4 ferredoxin Fd_{mor} requires the specific NADH:ferredoxin oxidoreductase FdR_{mor} from the $P450_{mor}$ system.

DISCUSSION

Cloning and sequencing of the *mor* operon from *Mycobacterium* sp. strain HE5 revealed six open reading frames of which three were found to encode the components of the $P450_{mor}$ system: *morA* encoding the cytochrome $P450_{mor}$, *morB* the Fe_3S_4 ferredoxin Fd_{mor} and *morC* the NADH:ferredoxin reductase FdR_{mor}. These genes were found to be identical to the corresponding genes from *Mycobacterium* sp. strain RP1. Only the gene *morA* from *Mycobacterium* sp. strain RP1 encoding CYP151A2 has been recently expressed and the protein could convert the heterocycles piperidine, pyrrolidine and morpholine in a heterologous system

with the alternate ferredoxin $NADP^+$ oxidoreductase and ferredoxin from spinach (Trigui *et al.*, 2004). MorA exhibits high identities to PipA, a cytochrome P450 (CYP151), which was shown to be involved in piperidine and pyrrolidine metabolism of *M. smegmatis* mc^2155 (Poupin *et al.*, 1999b).

In this study the almost identical genes *morA*, *morB*, and *morC* were also identified in *M. chlorophenolicum* PCP-1. Differences were detected downstream of these genes, where a shorter intergenic region is present only in *M. chlorophenolicum* PCP-1. The following gene *orf1´* is also highly conserved, but the lower identity compared to the *mor* genes correlates much better to the lower identity of their 16s rDNA. This indicated that only the *mor* genes might have been integrated recently into this gene region. In contrast, no differences between *Mycobacterium* sp. strain HE5 and *Mycobacterium* sp. strain RP1 were observed downstream of *morC*, suggesting that the *mor* genes were exchanged together with downstream sequences including *orf1´*. Subsequently, this gene region might have undergone deletions or insertions, thus, indicating a less importance of the putative glutamine synthetase, encoded by *orf1´*, for the degradation of morpholine. Transcription studies should indicate if *orf1´* is functionally related to the *mor* operon, e.g. by scavenging the nitrogen. The identification of identical *mor* genes in different mycobacterial strains suggested that this P450 system might be more widely distributed within this genus. In fact, a number of different mycobacteria, all able to degrade morpholine, piperidine and pyrrolidine, have been shown to specifically express a cytochrome P450 during growth on these heterocycles (Poupin *et al.*, 1999a). It seems very likely, that these enzyme systems should also be identical or at least should exhibit high identities to the $P450_{mor}$ system. Interestingly, the distribution of this P450 system seems not to follow the degree of relationship between mycobacterial strains. *Mycobacterium gilvum*, which was identified as the closest relative of *Mycobacterium* sp. strain HE5, was not able to grow on any of the heterocycles metabolized by the $P450_{mor}$ system (Schräder *et al.*, 2000). Similar results have been obtained for five distinct haloalkane-utilizing *Rhodococcus* strains, which all share the completely conserved gene *dhaA* encoding a haloalkane dehalogenase. The highly conserved gene region was detected on the chromosome as well as on plasmids in all these strains (Poelarends *et al.*, 2000). It was suggested that an ancestral plasmid was transferred and, subsequently, was integrated into the chromosome. A plasmid could also meet the requirements for horizontal gene transfer in morpholine-degrading mycobacterial strains, but no evidence for any sort of plasmid was found in *Mycobacterium* sp. strain HE5.

The main discrepancy between the P450 systems in *Mycobacterium* sp. strain HE5, *Mycobacterium* sp. strain RP1, *M. chlorophenolicum* PCP-1, and *M. smegmatis* mc^2155 on the genomic level is the lack of a ferredoxin reductase-encoding gene in the latter strain. In the *pip* operon of *M. smegmatis* mc^2155 the gene *orf1*, encoding the ferredoxin, is immediately followed by the gene *orf2´*, encoding a putative glutamine synthetase (Poupin *et al.*, 1999b). Sequencing the genome of *M. tuberculosis* had revealed 22 genes encoding P450 cytochromes (Cole *et al.*, 1998) and the genome of *M. smegmatis* mc^2155 was shown to exhibit even 40 P450 genes (Jackson *et al.*, 2003). This is the highest number found in a bacterium so far, but large sets of CYP genes were also identified in genomes of other actinobacteria. Many of these CYP genes are isolated in the genome, while a lower number is neighboured by genes encoding ferredoxins. Only few CYP genes are organized in operons including genes encoding for both reductase and ferredoxin. For instance, in the genome of *S. avermitilis* 33 CYP genes were identified, but only two were linked to ferredoxin and ferredoxin reductase genes (Lamb *et al.*, 2003). Interestingly, both these reductases showed significant homologies to FdR_{mor}. Gene expression studies with *S. coelicolor* revealed that only three reductases and six ferredoxins seemed to be sufficient to support activity of the 18 P450 cytochromes of this organism, which are all expressed during life cycle (Lei *et al.*, 2004). Thus, it seems likely that in *M. smegmatis* mc^2155 the missing reductase is functionally replaced by another ferredoxin reductase. The organization of the $P450_{mor}$ system recruiting a specific ferredoxin reductase is consistent with that of the classical bacterial $P450_{cam}$ system (Koga *et al.*, 1985). Other biodegradative P450s like $P450_{terp}$ (Peterson *et al.*, 1992), $P450_{cin}$ (Hawkes *et al.*, 2002), and $P450_{RRI}$ (Nagy *et al.*, 1995) are also organized into operons with their electron transfer proteins. This might imply that xenobiotic-metabolizing P450s generally utilize specific redox partners to ensure efficient functionality.

The amino acid sequence of FdR_{mor} showed identities to different ferredoxin reductases from different *Streptomyces* and *Mycobacterium* strains. So far, all of these have only been derived from nucleotide sequences and have not been characterized on the protein level. *MorC* was now expressed as C-terminal His-Tag fusion protein and the recombinant enzyme FdR_{mor} was characterized as NADH-dependent, FAD-containing ferredoxin reductase and shown to interact functionally with the Fe_3S_4 ferredoxin Fd_{mor}. FdR_{mor} showed some instability, which might explain that no specifically induced ferredoxin reductase could previously be identified in crude extracts of *Mycobacterium* sp. strain HE5, which at that time led us to propose that the reductase might be constitutively formed (Sielaff *et al*., 2001). Instability as well as low level of expression

might also be a reason for the only few reports on purified reductases of bacterial P450 systems. One of the best-known examples is the putidaredoxin reductase from *Pseudomonadas putida*, which uses putidaredoxin as electron transfer protein to reduce $P450_{cam}$ (Koga *et al*., 1985). This redox system is similar to mammalian AdR and Adx reducing mitochondrial P450 cytochromes (Schiffler & Bernhardt, 2003). FprA from *M. tuberculosis* was identified as an AdR-homologue and the heterologously expressed flavoprotein was able to reduce Adx, the Fe_2S_2 protein FdI from spinach, and a 7Fe ferredoxin from *M. smegmatis* (Fischer *et al*., 2002). While AdR is clearly a NADPH-dependent ferredoxin reductase, FprA also oxidizes NADH, although with a lower efficiency compared to NADPH. This distinguishes these proteins from FdR_{mor}, which is strictly NADH-dependent and cannot use NADPH as reductant. A soybean flour-induced NADH-dependent ferredoxin reductase was purified from *Streptomyces griseus* and shown to couple electron transfer to cytochrome $P450_{soy}$ in the presence of a 7Fe ferredoxin from *S. griseus* (Ramachandra *et al*., 1991). This 7Fe ferredoxin and those used in the studies of FprA were not the natural redox partners of these reductases. So far, only the specific redox partners of AdR and putidaredoxin reductase have been purified and characterized. Both proteins Adx and putidaredoxin contain Fe_2S_2 cluster, distinguishing them from Fd_{mor}, which was clearly identified as Fe_3S_4 ferredoxin (Sielaff & Andreesen, 2005). In contrast to FprA, FdR_{mor} was not able to interact functionally with FdI. FdR_{mor} was able to utilize Adx in NBT reduction, but the low catalytic efficiency of this reaction indicated a high specificity of FdR_{mor} for its natural redox partner Fd_{mor}. This is supported by the meanwhile measured low K_m value (5.6 nM) of FdR_{mor} for Fd_{mor} in the reduction of NBT (Sielaff & Andreesen, 2005). Interestingly, the reduction of cytochrome c by FdR_{mor} was not enhanced by the addition of Adx, although this electron acceptor is widely used to investigate AdR-Adx interactions (Grinberg *et al.*, 2000). Furthermore, Fd_{mor} could not replace Adx enzymatically in AdR/Adx activity assays with cytochrome c. Similarly, putidaredoxin and Adx could not substitute each other in activity assays of their respective reductases, although both ferredoxins share 37 % identity in their sequences (Geren *et al.*, 1986). In summary, we conclude from these data that Fd_{mor} requires for higher enzymatic efficiency the specific NADH:ferredoxin reductase FdR_{mor}, thus, reflecting the genomic organization of this P450 system, in which all genes were found adjacent in the same operon.

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Table 1. Steady-state kinetic parameters for NADH-dependent ferricyanide and cytochrome c reducing actvities of FdR_{mor}. Measurements were performed in triplicate in 50 mM Tris/HCl, pH 8·5, with 10 nM FdR_{mor}. Kinetic parameters were obtained by varying substrate concentrations in the standard assay. Standard errors from the fits of experimental data are included.

Substrate	k_{cat}	K_m	k_{cat} / K_m
	s^{-1}	µM	$s^{-1}µM^{-1}$
ferricyanide	60·2 ± 0·9	14·2 ± 1·1	4·24
cytochrome c	4·5 ± 0·2	28·0 ± 3·6	0·16
NADH	-	37·7 ± 4·1	-

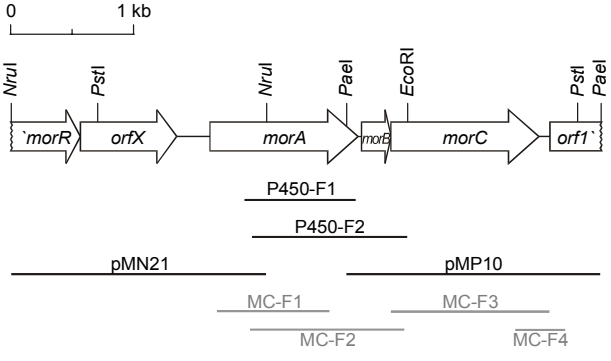

Fig. 1. Genetic organization of the *mor* operon region. Arrows indicate genes deduced from the nucleotide sequence, which was derived from PCR products P450-F1 and P450-F2 and from plasmids pMN21 and pMP10 (*Mycobacterium* sp. strain HE5). The PCR products MC-F1, MC-F2, MC-F3, and MC-F4 (*M. chlorophenolicum* PCP-1) are also shown. Only selected restriction sites of importance are indicated. Detailed information is given in the text.

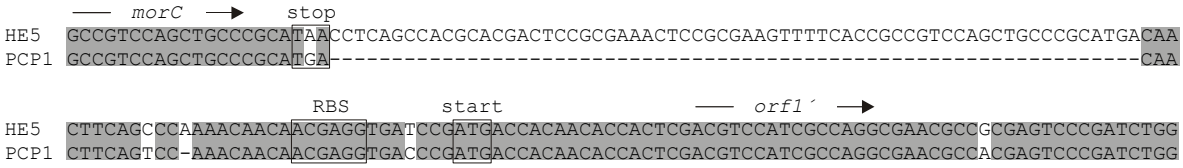

Fig. 2. Alignment of the gene regions downstream of *morC* from *Mycobacterium* HE5 (HE5) and *M. chlorophenolicum* PCP-1 (PCP1). Start codon, stop codon, and a putative ribosome binding site (RBS) are framed. Identical nucleotides are shaded.

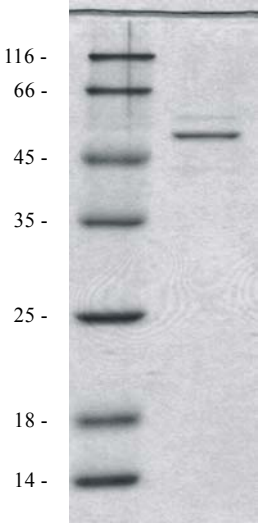

Fig. 3. SDS-PAGE (12·5 %) of purified C-terminal His-tagged FdR_{mor} (~ 2 µg). The molecular masses of the marker proteins are indicated in kDa.

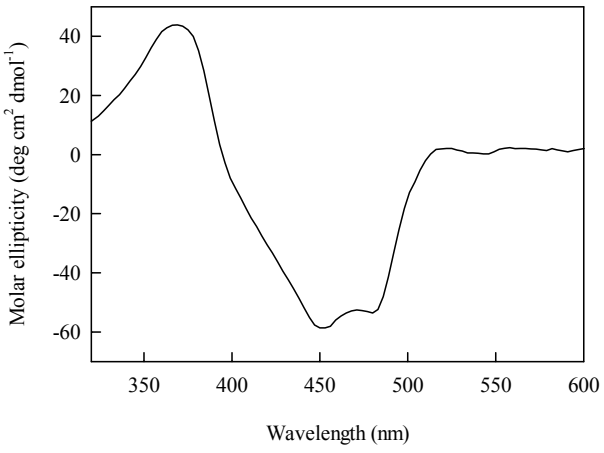

Fig. 4. Visible CD spectrum of FdR_{mor} (28·6 μM) in 50 mM Tris/HCl, pH 7·5, 10 % glycerol.

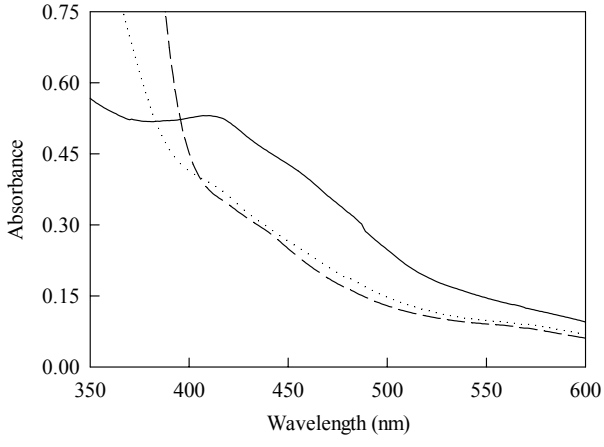

Fig. 5. Spectra of oxidized and reduced Fd_{mor}. Reduction of 50 μM Fd_{mor} (full line) was achieved by the addition of 1 nM FdR_{mor} and 0·25 mM NADH (dotted line) or the addition of a few grains of sodium dithionite (dashed line). Spectra were recorded ~ 20 s after addition of reductants to Fd_{mor} in 50 mM Tris/HCl, pH 7·5.

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Kinetic and binding studies with purified recombinant proteins ferredoxin reductase, ferredoxin and cytochrome P450 comprising the morpholine mono-oxygenase from *Mycobacterium* sp. strain HE5

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Keywords
cytochrome P450; ferredoxin; ferredoxin reductase; morpholine mono-oxygenase; *Mycobacterium*

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The $P450_{mor}$ system from *Mycobacterium* sp. strain HE5, supposed to catalyse the hydroxylation of different N-heterocycles, is composed of three components: ferredoxin reductase (FdR_{mor}), Fe_3S_4 ferredoxin (Fd_{mor}) and cytochrome P450 ($P450_{mor}$). In this study, we purified Fd_{mor} and $P450_{mor}$ as recombinant proteins as well as FdR_{mor}, which has been isolated previously. Kinetic investigations of the redox couple FdR_{mor}/Fd_{mor} revealed a 30-fold preference for the NADH-dependent reduction of nitroblue tetrazolium (NBT) and an absolute requirement for Fd_{mor} in this reaction, compared with the NADH-dependent reduction of cytochrome *c*. The quite low K_m (5.3 ± 0.3 nM) of FdR_{mor} for Fd_{mor}, measured with NBT as the electron acceptor, indicated high specificity. The addition of sequences providing His-tags to the N- or C-terminus of Fd_{mor} did not significantly alter kinetic parameters, but led to competitive background activities of these fusion proteins. Production of $P450_{mor}$ as an N-terminal His-tag fusion protein enabled the purification of this protein in its spectral active form, which has previously not been possible for wild-type $P450_{mor}$. The proposed substrates morpholine, piperidine or pyrrolidine failed to produce substrate-binding spectra of $P450_{mor}$ under any conditions. Pyridine, metyrapone and different azole compounds generated type II binding spectra and the K_d values determined for these substances suggested that $P450_{mor}$ might have a preference for more bulky and/or hydrophobic molecules. The purified recombinant proteins FdR_{mor}, Fd_{mor} and $P450_{mor}$ were used to reconstitute the homologous P450-containing mono-oxygenase, which was shown to convert morpholine.

P450 cytochromes are well known for their involvement in the synthesis of various antibiotics in different *Streptomyces* species [1–4]. But they also account for many of the various degradative abilities on xenobiotic compounds, which have been reported for other *Actinomycetales* [5–9]. The involvement of a cytochrome P450 in the degradation of the secondary cyclic amines morpholine, piperidine and pyrrolidine has been shown for different *Mycobacterium* species [10–14]. A P450-containing mono-oxygenase was supposed to catalyse the initial hydroxylation of these compounds [10,11], but enzymatic activity could not be recovered in cell-free extracts [15]. The cytochrome P450 ($P450_{mor}$) and its proposed redox partner, a Fe_3S_4 ferredoxin (Fd_{mor}), were purified for the first time from *Mycobacterium* sp. strain HE5 [15]. Nucleotide sequence determination of

Abbreviations
CHis-, C-terminal His-tag; Fd, ferredoxin; FdR, ferredoxin reductase; NBT, nitroblue tetrazolium; NHis-, N-terminal His-tag; P450, cytochrome P450 mono-oxygenase; wt, wild type.

the encoding operon revealed also the gene encoding the specific reductase, which is required for activity of the $P450_{mor}$ system (B. Sielaff & J. R. Andreesen, unpublished data).

Thus, the $P450_{mor}$ mono-oxygenase is a typical bacterial P450 system [16], composed of three components: NADH-oxidizing ferredoxin reductase (FdR_{mor}), ferredoxin (Fd_{mor}) as an electron-transfer protein and $P450_{mor}$, which acts as a mono-oxygenase. FdR_{mor} has already been cloned, purified and characterized as a NADH-dependent, FAD-containing protein and shown to be structurally distinct from previously purified P450 reductases (B. Sielaff & J. R. Andreesen, unpublished data), the latter of which all belong to the glutathione reductase-like family. An activity of just the cytochrome P450 component has recently been shown for the seemingly identical, recombinant CYP151A2 from *Mycobacterium* sp. strain RP1 using a heterologous system with both NADPH-dependent ferredoxin reductase and ferredoxin from spinach [17]. In most reports on bacterial P450 cytochromes activity has been reconstituted with heterologous redox partners [5,9,18–21]. For biotechnological purposes, strong oxidants like hydrogen peroxide have been used in a few cases for direct involvement of the P450 [22]. However, less attention has been paid, to date, to the homologous redox partners of P450s.

The aim of this study was to start a detailed examination of a complete bacterial P450 system distinct from other purified bacterial P450 systems which either utilize a Fe_2S_2 ferredoxin-like $P450_{cam}$ [23] or belong to the microsomal type of P450s like $P450_{BM3}$ [24] and are reduced by a diflavin reductase. This is the first report on the heterologous expression and purification of all components of a P450 system from an actinobacterium. Kinetic investigations were performed on the redox couple FdR_{mor}/Fd_{mor} and morpholine-converting activity could be demonstrated for the reconstituted, homologous $P450_{mor}$ mono-oxygenase.

Results

Production and purification of Fd_{mor} variants

morB, encoding Fd_{mor}, was expressed in *Escherichia coli* Rosetta(DE3)pLysS as wild-type protein wt-Fd_{mor}, as N-terminal His-tag fusion protein NHis-Fd_{mor} and as C-terminal His-tag fusion protein CHis-Fd_{mor}. All proteins were soluble and no inclusion bodies were formed as confirmed by SDS/PAGE analysis. The ferredoxins were purified as described in Experimental procedures. In the SDS gel (Fig. 1), the purified recombinant proteins appeared larger than expected

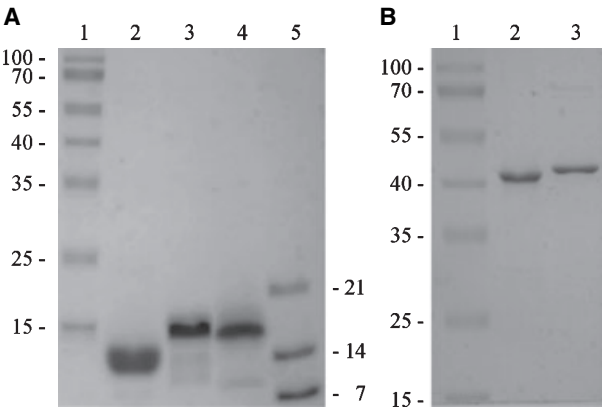

Fig. 1. SDS/PAGE of the purified recombinant Fd_{mor} variants (A) and purified recombinant $P450_{mor}$ (B). (A) Lane 1, marker proteins; lane 2, wt-Fd_{mor}; lane 3, NHis-Fd_{mor}; lane 4, CHis-Fd_{mor}; lane 5, marker proteins. (B) Lane 1, marker proteins; lane 2, $P450_{mor}$ purified from *Mycobacterium* sp. strain HE5; lane 3, NHis-$P450_{mor}$. Molecular masses of the marker proteins are indicated in kDa. Approximately 2 μg of each protein was applied to SDS/PAGE.

from their calculated masses, which was similar to findings for the wild-type protein Fd_{mor} isolated from *Mycobacterium* sp. strain HE5 [15]. However, the molecular masses determined by MS were in good agreement with those predicted from the sequences (Table 1). Absorption spectra were the same for all three recombinant proteins, containing only a single peak at 412 nm, and the protein peak at 280 nm. This is a typical feature of Fe_3S_4 proteins [25] and was found also for wild-type Fd_{mor} isolated from *Mycobacterium* sp. strain HE5 [15]. The obtained ratios of the absorbance of the Fe_3S_4 cluster to the protein-specific absorbance (A_{280}/A_{412}) differed between the recombinant proteins (Table 1). The lowest ratio was found for CHis-Fd_{mor}, indicating a high Fe_3S_4 cluster content. Higher ratios were found for NHis-Fd_{mor} and wt-Fd_{mor}, suggesting that the Fe_3S_4 cluster was not incorporated into these proteins to the same extent. In the case of wt-Fd_{mor}, this could be attributed to the

Table 1. Expression of the different recombinant Fd_{mor} variants. The amount of purified ferredoxin was determined spectrophotometrically using the absorption coefficient $\varepsilon_{412} = 9.8\ mM^{-1} \cdot cm^{-1}$. The absorbance ratio A_{280}/A_{412} indicates the amount of incorporated Fe-S cluster. Molecular masses were determined by ESI-MS.

Fd_{mor} variant	wt-Fd_{mor}	NHis-Fd_{mor}	CHis-Fd_{mor}
Purified ferredoxin (nmol·L^{-1} culture)	60	140	210
A_{280}/A_{412}	1.79	2.35	1.62
Predicted mass (Da)	6793	8820	8313
Estimated mass (Da)	6795	8824	8314

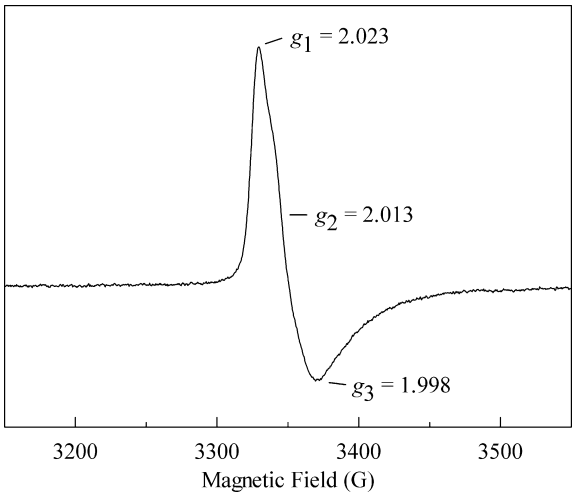

Fig. 2. EPR spectrum of oxidized wt-Fd_{mor}. Temperature, 10 K; microwave power, 0.2 mW; modulation amplitude, 2.8 Gauss. Sample concentration was 150 μM in 50 mM Tris/HCl, pH 7.5, 20% glycerol. The *g* factors are indicated in the figure.

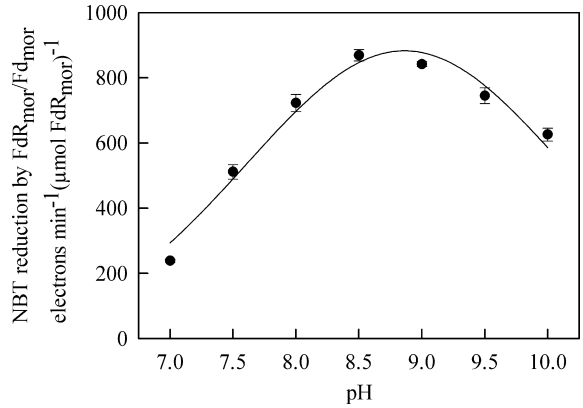

Fig. 3. NBT reduction by the FdR_{mor}/Fd_{mor} couple showing dependence on pH. Measured activities of the FdR_{mor}/Fd_{mor} couple (●) were fitted to a Gaussian curve (solid line). Error bars indicate the standard deviations of three independent measurements. Initial velocities were measured in a buffer composed of both 25 mM Tris and 25 mM glycine with 200 μM NADH, 5 nM FdR_{mor}, 50 nM wt-Fd_{mor} and 200 μM NBT.

different purification protocol, which might have led to some loss of cofactor. The highest ratio was found for NHis-Fd_{mor}, which might indicate less efficient incorporation of the Fe_3S_4 cluster and/or lower stability of the cofactor, compared with CHis-Fd_{mor} and wt-Fd_{mor}.

EPR-spectroscopy of oxidized wt-Fd_{mor} revealed a single signal with an average *g*-value of 2.01 which is characteristic of $[3Fe\text{-}4S]^{+}$, $S = 1/2$ oxidized three-iron cluster (Fig. 2). After recording spectra of different Fd_{mor} variants and determining the iron content of these Fd_{mor} solutions by atom absorption spectroscopy, an absorption coefficient for Fd_{mor} of $\varepsilon_{412} = 9.8\ mM^{-1}{\cdot}cm^{-1}$ could be calculated. The amount of purified recombinant ferredoxin was estimated using this absorption coefficient. The highest amount was obtained for CHis-Fd_{mor}, whereas wt-Fd_{mor} gave the lowest amount (Table 1), which might again be attributed to the purification procedure.

Catalytic properties of the recombinant FdR_{mor}/Fd_{mor} couple

Fd_{mor} was able to stimulate the NADH-dependent reduction of cytochrome *c* by FdR_{mor} approximately fivefold (B. Sielaff & J. R. Andreesen, unpublished data). Screening for other suitable electron acceptors revealed that the further addition of Fd_{mor} enabled reduction of nitroblue tetrazolium (NBT) by FdR_{mor}. There was an absolute requirement for Fd_{mor}, as no reduction was observed with NADH and FdR_{mor} alone.

The influence of the pH on the NADH-dependent reduction of NBT by the FdR_{mor}/Fd_{mor} couple was examined with wt-Fd_{mor} and revealed an optimum at ≈ pH 8.8 (Fig. 3). It has been shown previously that the activity of FdR_{mor} is dependent on the type of buffer used (B. Sielaff & J. R. Andreesen, unpublished data). In order to exclude this influence, measurements for the determination of the pH optimum were carried out in buffers composed of both 25 mM Tris and 25 mM glycine. Potassium chloride had an inhibitory effect on the NBT reducing activity of the FdR_{mor}/Fd_{mor} couple. The activity decreased more sharply if up to 50 mM potassium chloride was present. This inhibition declined between 50 and 800 mM potassium chloride, where ≈ 50% of the starting activity was reached (Fig. 4). Similar results were obtained when sodium chloride was added to the activity assays (data not shown). The ferricyanide-reducing activity of FdR_{mor} was not sensitive to ionic strength (data not shown), suggesting that the observed decrease in activity of the FdR_{mor}/Fd_{mor} couple was not caused by an inhibition of the FdR_{mor} activity.

Steady-state kinetic parameters of FdR_{mor} for wt-Fd_{mor} were determined at pH 8.6 with saturating concentrations of NADH (200 μM). With saturating concentrations of cytochrome *c* (150 μM), a Michaelis–Menten curve was obtained for the stimulation of the activity of FdR_{mor} towards cytochrome *c* by wt-Fd_{mor}, indicating an apparent V_{max} of 1534 ± 29 electrons·min^{-1} and an apparent K_m of FdR_{mor} for wt-Fd_{mor} of 316 ± 17 nM. Using NBT (200 μM) as the electron acceptor, an approximately twofold lower

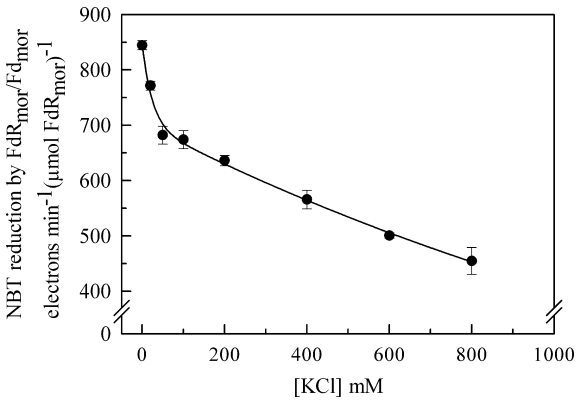

Fig. 4. NBT reduction by the FdR$_{mor}$/Fd$_{mor}$ couple showing dependence on the ionic strength. Activities were measured with 200 μM NADH, 5 nM FdR$_{mor}$, 50 nM wt-Fd$_{mor}$ and 200 μM NBT in 25 mM glycine-buffer, pH 8.6, adding varying concentrations of potassium chloride. Error bars indicate the standard deviations of three independent measurements.

V_{max} was obtained. Owing to a much lower K_m value of wt-Fd$_{mor}$ (Table 2), ≈ 60-fold with respect to the K_m measured with cytochrome *c*, the efficiency (V_{max}/K_m) of wt-Fd$_{mor}$ mediated NBT reduction was ≈ 30-fold higher compared with cytochrome *c* reduction ($V_{max}/K_m = 4.8$ electrons·min^{-1}·nM^{-1}). Thus, the FdR$_{mor}$/Fd$_{mor}$ couple seemed to show a preference for the two-electron acceptor NBT over the one-electron acceptor cytochrome *c*.

In order to check whether the added sequence providing the His-tag to the recombinant ferredoxins had an influence on the activity of the FdR$_{mor}$/Fd$_{mor}$ couple, kinetic parameters were determined with NHis-Fd$_{mor}$ and CHis-Fd$_{mor}$. Using cytochrome *c* as the electron acceptor, activities with a saturating concentration of NHis-Fd$_{mor}$ or CHis-Fd$_{mor}$ could not be determined correctly, as these recombinant ferredoxins showed unspecific activities with NADH and cytochrome *c* without any addition of FdR$_{mor}$. These background activities were negligible at low ferredoxin concentrations, but measurements at apparent saturating concentrations of ferredoxin yielded such high activities that it was not possible to measure initial velocities over a reasonable period. Thus, K_m and V_{max} values could not be determined under these conditions. However, from the slope of the initial linear range of the kinetic plot, the constants V_{max}/K_m of 1.1 electrons·min^{-1}·nM^{-1} for NHis-Fd$_{mor}$ and V_{max}/K_m of 0.9 electrons·min^{-1}·nM^{-1} for CHis-Fd$_{mor}$ could be estimated as approximate figure. These were approximately fivefold lower than the V_{max}/K_m determined with wt-Fd$_{mor}$.

NHis-Fd$_{mor}$ and CHis-Fd$_{mor}$ showed reducing activities towards NBT, similar to those seen in cytochrome *c* assays. In comparison with cytochrome *c* activities, there was a lower reduction of NBT by the FdR$_{mor}$/Fd$_{mor}$ couple as well as by His-tagged Fd$_{mor}$ on its own. Therefore, initial velocities could be measured with saturating concentrations of ferredoxin. However, kinetic plots did not show a typical Michaelis–Menten curve. Instead of reaching a plateau, velocities continued to increase in a linear dependence on the ferredoxin concentration (Fig. 5), which could be attributed to the unspecific background activities of His-tagged ferredoxins. Therefore, the data were fitted to a modified Michaelis–Menten equation (Experimental procedures) where a linear term was added to describe the FdR$_{mor}$-independent NBT reduction by the ferredoxin. This method revealed the kinetic parameters of FdR$_{mor}$ for NHis-Fd$_{mor}$ or CHis-Fd$_{mor}$, which

Table 2. Steady-state kinetic parameters for NBT reduction by FdR$_{mor}$ with the different Fd$_{mor}$ variants. Measurements were performed in 50 mM glycine-buffer, pH 8.6, with 200 μM NADH, 5 nM FdR$_{mor}$, and saturating concentrations of NBT (200 μM). Apparent kinetic parameters were determined by varying concentrations of each ferredoxin.

Fd$_{mor}$ variant	V_{max} (electrons·min^{-1})	K_m (nM)	V_{max}/K_m (electrons·min^{-1}·nM^{-1})
wt-Fd$_{mor}$	887 ± 9	5.3 ± 0.3	167
NHis-Fd$_{mor}$	952 ± 60[a]	10.5 ± 1.9[a]	91
CHis-Fd$_{mor}$	807 ± 26[a]	3.7 ± 0.5[a]	218

[a] Values obtained by fitting data to a modified Michaelis–Menten equation (Experimental procedures).

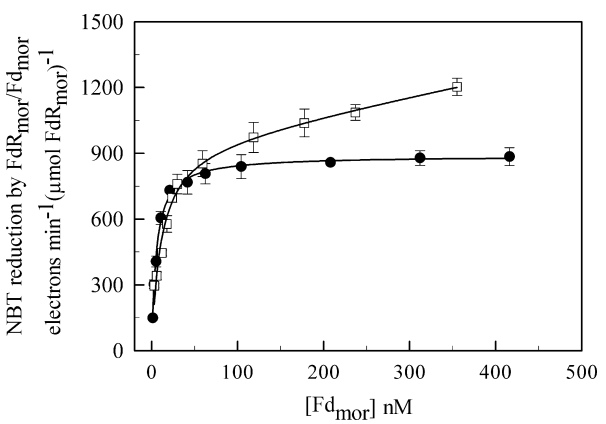

Fig. 5. Plot of NBT reducing activities of FdR$_{mor}$ with increasing concentrations of wt-Fd$_{mor}$ (●) or NHis-Fd$_{mor}$ (□). Activities were measured with 200 μM NADH, 5 nM FdR$_{mor}$ and 200 μM NBT in 25 mM glycine-buffer, pH 8.6. Initial velocities were plotted against the concentration of Fd$_{mor}$ and fitted to a hyberbolic function for wt-Fd$_{mor}$ or a modified Michaelis–Menten equation (Experimental procedures) for NHis-Fd$_{mor}$ to obtain the apparent kinetic parameters.

were found to be in the same range as those determined for wt-Fd_{mor} (Table 2).

Production and purification of recombinant $P450_{mor}$

morA, encoding $P450_{mor}$, was expressed as fusion protein with an N-terminal His-tag in *E. coli* Rosetta(DE3)pLysS cells. The reduced CO difference spectra of cytosolic extracts showed a characteristic maximum absorbance peak at 450 nm. Supplementation of the growth medium with the heme precursor δ-aminolevulinic acid increased the expression level of $P450_{mor}$ up to fivefold, suggesting that heme was limiting during the heterologous expression conditions. SDS/PAGE analysis revealed that apparently no inclusion bodies were formed. The protein was isolated by a single chromatography step on a Ni^{2+} affinity column and was judged to be homogenous by SDS/PAGE analysis. NHis-$P450_{mor}$ showed a molecular mass of 46 000 Da in SDS/PAGE, appearing larger than the wild-type $P450_{mor}$ (Fig. 1), as expected as a result from the added sequence. MS revealed a molecular mass of 46 705 Da which was in good agreement with the calculated mass of 46 700 Da for NHis-$P450_{mor}$.

The UV-Vis spectrum of NHis-$P450_{mor}$ was identical to that of wild-type $P450_{mor}$, isolated previously from *Mycobacterium* sp. strain HE5 [15]. In contrast to wild-type $P450_{mor}$, which could be purified only in the inactive P420 form, CO difference spectra of NHis-$P450_{mor}$ showed no peak at 425 nm, indicating that the protein was purified in its active form which was stable at −20 °C for over 6 months. Even multiple freeze–thaw cycles did not affect the integrity of the protein, as judged by its spectral properties.

The amount of purified protein was calculated to be ≈ 200 nmol·L^{-1} culture, using the extinction coefficient for oxidized $P450_{mor}$ of $\varepsilon_{418} = 181$ mM^{-1}·cm^{-1}, as calculated by determination of the protoheme content of NHis-$P450_{mor}$ as pyridine hemochromogen.

Binding studies with $P450_{mor}$

In the absence of substrates, most P450 enzymes are low-spin. Substrate addition usually shifts the heme to the high-spin state, which leads to a peak at 390 nm and a trough at 420 nm in the substrate-induced difference spectrum. Imidazole, which was used to elute NHis-$P450_{mor}$ from the Ni-NTA column, was bound to the heme group of NHis-$P450_{mor}$ (see below) during purification. Therefore, NHis-$P450_{mor}$ was dialysed prior to use in binding studies or activity assays to remove imidazole. Removal of imidazole was confirmed by spectral analysis of NHis-$P450_{mor}$. First and second deviations of spectra were calculated to ensure that no imidazole-bound species were left.

No significant spectral change could be observed upon addition of morpholine, piperidine or pyrrolidine (up to 50 mM each) to NHis-$P450_{mor}$. As it has been reported that the ionic strength can have an effect on the binding of substrates to some P450s [6,26], different NaCl concentrations (0–500 mM) were used in substrate-binding assays, but no significant perturbation of the low-spin spectrum of NHis-$P450_{mor}$ could be observed. The recombinant wt-Fd_{mor} was added to NHis-$P450_{mor}$ binding assays, as adrenodoxin facilitates the binding of cholesterol to CYP11A1 [27]. But wt-Fd_{mor} had no effect on the spin-state of NHis-$P450_{mor}$ in the presence or absence of any of the tested N-heterocycles.

In order to obtain more information about the binding properties of the active site of $P450_{mor}$ and the permitted access of molecules to it, the binding of different azole compounds to the heme group of NHis-$P450_{mor}$ was investigated. These molecules produce type II binding spectra as a result of the displacement of a water molecule by an azole nitrogen to the sixth coordination position of the heme iron [28]. The type II binding spectrum is characterized by a peak at 432 nm and a trough at 413 nm in the difference spectrum (Fig. 6). The P450–azole complex can be titrated leading to an estimation of the binding constant K_d (Fig. 6). The lowest affinity was determined for the

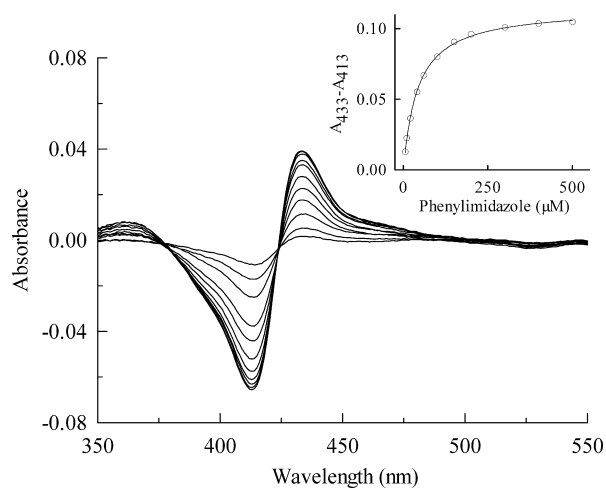

Fig. 6. UV-Vis spectra of $P450_{mor}$ titrated with phenylimidazole (5–500 μM) versus $P450_{mor}$ alone. The concentration of $P450_{mor}$ was 2.5 μM in 50 mM Tris/HCl, pH 7.5, 10% glycerol. The mean of three data sets were used to calculate a K_d for the enzyme–azole complex by plotting the absorbance difference against the phenylimidazole concentration (see inset).

binding of imidazole ($K_d = 1.23 \pm 0.02$ mM), whereas the affinity of NHis-$P450_{mor}$ to phenylimidazole was $\approx$ 25-fold higher ($K_d = 48.1 \pm 2.0$ μM). Binding of the azole antifungal drugs clotrimazole, econazole and miconazole to NHis-$P450_{mor}$ was too tight to analyse accurately. In case of these three azoles, the optical change observed upon azole addition occurred linearly with increasing azole concentrations, reaching a plateau at a concentration range similar to that of NHis-$P450_{mor}$ in these assays. These results were indicative of stoichiometric binding to NHis-$P450_{mor}$ and did not allow the determination of K_d values. It seems that binding to the heme of NHis-$P450_{mor}$ is favoured by the increasing number of hydrophobic phenyl groups of the azole compounds.

Pyridine, which is the analogous aromatic molecule of the potential substrate piperidine, and its derivate metyrapone (1,2-di-(3-pyridyl)-2-methyl-1-propanon) were also used in binding studies. These molecules also induce type II spectra with a peak at 428 nm and a trough at 411 nm in difference spectra. The binding of metyrapone showed an $\approx$ 300-fold higher affinity ($K_d = 24.6 \pm 1.6$ μM) than pyridine ($K_d = 7.99 \pm 0.72$ mM), which is an even larger difference than that between the binding of imidazole and phenylimidazole.

For CYP121, it had been reported that the addition of lanosterol increases the affinity to the azole antifungal ketoconazole [29]. No significant effect was observed upon the presence of up to 20 mM morpholine, piperidine or pyrrolidine on the binding of pyridine, metyrapone or the different azoles (see above) tested in this study.

Reconstitution of the catalytically active $P450_{mor}$ system

Assays with the reconstituted $P450_{mor}$ system were restricted to the substrate morpholine, which was also used for selective enrichment of this strain [15]. Using HPLC and UV detection, morpholine could be analysed directly from the assay buffer, without any need for derivatization or extraction.

In preliminary experiments we determined the optimal concentration of ferredoxin in the assay. First FdR_{mor} and NHis-$P450_{mor}$ were kept constant at 0.1 μM, whereas different concentrations of NHis-Fd_{mor}, ranging from 0.1 to 1 μM, were used in assays. Highest turnover [16.9 ± 2.8 nmol morpholine^{-1}·min^{-1}·(nmol P450)$^{-1}$] was observed using the enzymes in a ratio of 1 : 5 : 1 (FdRmor/Fdmor/P450). A further increase of the ferredoxin concentration did not lead to a significant enhancement of the reaction, indicating that the system was saturated by a fivefold excess of ferredoxin over the NADH-dependent reductase and the P450, respectively. Likewise, a higher concentration of FdR_{mor} did not increase the turnover of morpholine.

The activity of the $P450_{mor}$ system reconstituted with CHis-Fd_{mor} was determined to be 14.5 ± 3.4 nmol morpholine^{-1}·min^{-1}·(nmol P450)$^{-1}$, which is nearly the same as measured with NHis-Fd_{mor}. Using wt-Fd_{mor} as the electron transfer protein the conversion of morpholine by the $P450_{mor}$ system was 28.6 ± 3.0 nmol morpholine^{-1}·min^{-1}·(nmol P450)$^{-1}$, aproximately twofold higher than the activities obtained with NHis-Fd_{mor} and CHis-Fd_{mor}.

Discussion

The gene *morB* was heterologously expressed and the purified recombinant protein Fd_{mor} was confirmed by EPR spectroscopy to contain a Fe_3S_4 cluster, as predicted from the amino acid sequence and UV-Vis spectra [15]. Thus, Fd_{mor} can be classified as a bacterial-type ferredoxin, which distinguishes it from the adrenodoxin-type Fe_2S_2 ferredoxins. A well-studied example of the latter type is putidaredoxin, which serves as an electron transfer protein in the $P450_{cam}$ system [30]. In contrast, there are few reports on P450-associated bacterial-type ferredoxins. Two purified Fe_3S_4 ferredoxins have been spectroscopically characterized from *Streptomyces griseolus* and used to reconstitute $P450_{SUI}$ activity [25]. A recombinant Fe_4S_4 ferredoxin from *Bacillus subtilis* was shown to support activity of the cytochrome P450 BioI [31]. A heterologously expressed Fe_3S_4 ferredoxin from *Mycobacterium tuberculosis* was used in CYP51 activity assays [28]. However, the latter two ferredoxins were not specific for the respective P450 and no specific reductase was identified for any of these ferredoxins. The specific reductase of the $P450_{mor}$ system has been recently identified and the recombinant protein FdR_{mor} has been characterized (B. Sielaff & J. R. Andreesen, unpublished data). This enabled kinetic investigations on the FdR_{mor}/Fd_{mor} redox couple, which represent the first using a Fe_3S_4 ferredoxin.

An absolute requirement for ferredoxin in cytochrome *c* reduction has been shown for several P450 reductases [32–34]. FdR_{mor} was capable of reducing cytochrome *c* on its own, although Fd_{mor} enhanced the reaction significantly. Similar results were obtained for flavodoxin reductase from *E. coli* [35] and ferredoxin reductase from *Streptomyces griseus* [36]. In contrast to the latter and to putidaredoxin reductase [32], the two-electron reduction of NBT by FdR_{mor} was strictly dependent on Fd_{mor}. This allowed the direct measure-

ment of the K_m of FdR_{mor} for Fd_{mor}, which was found to be in the same range as that of the adrenodoxin reductase homolog FprA from *Mycobacterium tuberculosis* for a 7Fe ferredoxin from *Mycobacterium smegmatis* [33]. Investigations of other bacterial redox systems exhibited much lower affinities between reductases and their respective redoxins [35,37], although these might be attributed to the specificity of electron acceptors used. For instance, in this study a 60-fold higher K_m of FdR_{mor} for Fd_{mor} was measured with cytochrome *c* as the electron acceptor, compared with NBT reduction. However, the low K_m value of FdR_{mor} for Fd_{mor} in NBT reduction indicates a high specificity, possibly reflecting the genomic organization of this P450 system, in which all genes were found adjacent in the same operon (B. Sielaff & J. R. Andreesen, unpublished data). Increasing concentrations of potassium chloride retarded the reduction rates for Fd_{mor}, indicating that the association and electron-transfer reactions between FdR_{mor} and Fd_{mor} depend on the ionic strength and that electrostatic interactions contribute to the association. This has been shown to be similar for the reaction between putidaredoxin reductase and putidaredoxin [38]. In this study, a suitable activity test was established for further kinetic investigations of the FdR_{mor}/Fd_{mor} couple. These have to be restricted to the wild-type Fd_{mor} because the His-tagged variants showed unspecific background activities, competing with the FdR_{mor} catalysed redox reaction. These background activities might result from an acquired unspecificity of the His-tagged ferredoxins towards NADH, as they were observed with both electron acceptors cytochrome *c* and NBT. Electron transfer from FdR_{mor} to Fd_{mor} seemed not to be affected, as the K_m values of FdR_{mor} for the different recombinant Fd_{mor} variants did not show significant discrepancies.

The gene *morA* encoding $P450_{mor}$ was heterologously expressed as an N-terminal His-tag fusion protein and the amount of purified $P450_{mor}$ was in the range reported for N-terminal His-tagged CYP151A2 from *Mycobacterium* sp. strain RP1 [17], the amino acid sequence of which is identical to that of $P450_{mor}$ (B. Sielaff & J. R. Andreesen, unpublished data). However, the reported period of induction was much higher at 48 h, compared with 3 h for the expression system used in this study. The addition of an N-terminal His-tag to $P450_{mor}$ was an important improvement, as wild-type $P450_{mor}$ could not previously be purified in an active form [15]. NHis-$P450_{mor}$ could now be purified in a stable form without detectable formation of the inactive P420 species.

The binding of substrates to cytochromes P450 usually induces transition of the heme from the low-spin state to the high-spin state, which results in a shift of the heme Soret band, generating typical binding spectra. This is very likely caused by replacement of a heme-coordinated H_2O or OH^- molecule, which is accompanied by a rearrangement of the water structure in the active site [39]. This is very likely favoured by the hydrophobic nature of most cytochrome P450 substrates like, e.g. fatty acids [20], *n*-alkanes [40], camphor [41], terpineol [26] or cineole [21]. In streptomycetes, P450s are often involved in the biosynthesis of macrolide antibiotics such as pikromycin [1], oleandomycin [2], rapamycin [3] or nikkomycin [4], which are large, hydrophobic molecules. Morpholine, piperidine and pyrrolidine did not induce any observable change in the spectrum of $P450_{mor}$. This may be due to the polarity and hydrophilicity of these compounds in contrast to all other known substrates of P450 cytochromes. For $P450_{cam}$ it has been shown that the binding of substrate is a prerequisite for the beginning of the catalytic cycle [42]. But it has also been shown that binding of norcamphor to $P450_{cam}$ induced only $\approx 50\%$ high-spin species compared with the binding of camphor [43]. One should also note that binding of obtusifoliol to CYP51 resulted in only a minor change in the absorption spectra [28]. The binding of deoxycorticosterone to CYP106A2 resulted in no shift of the Soret band at all, although this substrate is converted by P450. However, binding of deoxycorticosterone to CYP106A2 was shown by infrared spectroscopy measurements [44]. It seems likely that binding of the proposed substrates to $P450_{mor}$ might not be detectable using the methods applied here. The crystal structure of progesterone-bound P450 3A4 revealed an initial binding site for the substrate. Access of the substrate to the heme would require a conformational movement, which was suggested to possibly arise from interactions with the cytochrome b_5, the reductase or even the membrane [45]. Similarly, adrenodoxin facilitates the binding of cholesterol to CYP11A1 [27]. Detectable binding of substrates to $P450_{mor}$ might also require binding of Fd_{mor}, but no evidence for this possibility was found in this study. The determination of binding constants of $P450_{mor}$ for different azoles revealed a higher affinity of $P450_{mor}$ for the more hydrophobic compounds, which coincides with a larger volume of these molecules. Similar results were found for the P450 BioI from *B. subtilis*, which hydroxylates fatty acids [20], and CYP121 from *M. tuberculosis* for which the substrate has yet to be elucidated [29]. The higher affinity of $P450_{mor}$ for metyrapone compared with pyridine might be explained by possible interactions of the second pyridinyl group with hydrophobic residues in the active site. At least, binding studies point to a preference of $P450_{mor}$ for

more bulky and/or hydrophobic compounds. However, it could not be excluded that morpholine is a natural substrate and, thus, converted by $P450_{mor}$. Therefore, activity assays were set up with the $P450_{mor}$ system.

As mentioned previously, in most cases, P450 activity was measured using heterologous redox partners from different sources [5,9,17–19]. The expression and purification of the ferredoxin reductase FdR_{mor}, the ferredoxin Fd_{mor} and the mono-oxygenase $P450_{mor}$ enabled now the first successful homologous reconstitution of a bacterial P450 system from an actinobacterium. Conversion of morpholine by the homologous $P450_{mor}$ system was highest if wt-Fd_{mor} was used as an electron transfer protein, whereas lower turnover was measured using the His-tagged ferredoxins. The additional His-tag sequence of recombinant ferredoxins seemed to have no effect on the electron transfer between FdR_{mor} and Fd_{mor} as concluded from our studies. Thus, lower activities of the $P450_{mor}$ system reconstituted with NHis-Fd_{mor} or CHis-Fd_{mor} might be explained by a less-efficient electron transfer to $P450_{mor}$ by these His-tagged ferredoxins. Quite recently, the conversion of morpholine was independently shown for the recombinant CYP151A2 from *Mycobacterium* sp. strain RP1 using $NADP^+$ ferredoxin reductase and ferredoxin from spinach as the electron donor system [17]. The reported apparent V_{max} value for conversion of morpholine by CYP151A2 was obviously just derived from the extrapolation of kinetic data and is therefore hard to compare with the turnover measured here. One also has to keep in mind that, in both cases, the assay conditions did not allow the measurement of initial velocities, which means that a maximum turnover was not measured. Therefore, time course analysis of morpholine conversion by the $P450_{mor}$ system should be performed next to settle this question.

So far, mycobacteria contain the largest variety of P450 cytochromes [46,47] and might therefore be suited best for morpholine degradation, as it coincides with their selective enrichments on this substrate [13,14,48]. This report is a basis to study an NADH- and Fe_3S_4 ferredoxin-dependent P450 system converting water soluble substrates.

Experimental procedures

Materials

All chemicals and NADH were purchased from Sigma-Aldrich (Taufkirchen, Germany). For molecular biological work, all biochemicals and enzymes other than restriction endonucleases were provided by Roche Diagnostics (Mannheim, Germany). Restriction endonucleases were from Fermentas and New England Biolabs (Beverly, MA, USA) based on availability. Oligonucleotides were provided by Metabion (Martinsried, Germany). Vectors and Ni-NTA affinity column material were from Novagen (Madison, WI, USA). Other column materials were from Pharmacia (Uppsala, Sweden). FdR_{mor} was prepared as described previously (B. Sielaff & J. R. Andreesen, unpublished data).

Cloning of the Fd_{mor} variants

Primers were designed to either end of *morB* containing suitable restriction sites flanked by 'spacer' nucleotides at the 5′-end to facilitate efficient digestion. A *Nde*I site was incorporated in the N-terminal primer 5′-GTCAGACTCATATG CGCGTATCCGTAGATC-3′ and an *Eco*RI site was incorporated in the C-terminal primer 5′-GTAGAATTCTCAAT CCTCGATGAAGATGG-3′ (restriction sites underlined). PCR was performed with whole-cell DNA as the template according to the following parameters: 94 °C for 4 min; 10 cycles of 94 °C for 15 s, 52 °C for 30 s, 72 °C for 30 s; 20 cycles of 94 °C for 15 s, 52 °C for 30 s, 72 °C for 30 s plus 5 s at each cycle. The obtained 200 bp product was digested with *Nde*I and *Eco*RI, extracted from the gel (Qiagen Gel Extraction Kit, Hilden, Germany) and ligated into the vector pET28b(+), treated in the same way. The ligated fragment was transformed into *Escherichia coli* XL1 blue MRF′ cells (Stratagene, La Jolla, CA, USA). Resulting recombinant cells were screened by PCR and plasmids of positive clones were purified and sequenced to confirm that no PCR errors were incorporated. A plasmid containing the correct insert was designated pMFN28 and used for the expression of *morB* as N-terminal His-tag fusion protein. In order to obtain Fd_{mor} as wild-type protein the *Nde*I/*Eco*RI digested fragment was ligated into the *Nde*I/*Eco*RI treated vector pET26b(+) to give pMF26.

For the expression of *morB* as C-terminal His-tag fusion protein the new C-terminal primer 5′-CGTAGCAA GCTTATCCTCGATGAAGATGGCC-3′, incorporating a *Hind*III site, was designed and used in PCR (conditions as above) in combination with the same N-terminal primer as described above. The obtained 200 bp product was cut with *Nde*I and *Hin*dIII, extracted from the gel and ligated into the *Nde*I/*Hin*dIII treated vector pET26b(+) to yield the plasmid pMFC26. All plasmids were finally transformed into *E. coli* Rosetta(DE3)pLysS cells (Novagen). Glycerol stocks were prepared by adding 200 μL 40% glycerol to 800 μL of a cell culture previously grown to D_{600} of 1.0 and stored at −80 °C.

Production and purification of Fd_{mor} variants

Four millilitres of Luria–Bertani medium with 30 $\mu g \cdot mL^{-1}$ kanamycin were inoculated with 5 μL of a glycerol stock of *E. coli* Rosetta(DE3)pLysS harbouring one of the expression plasmids pMFN28, pMFC26 or pMF26 and cultured

overnight at 30 °C. This culture was used to inoculate four 2 L Erlenmeyer flasks each containing 500 mL Terrific Broth with 30 $\mu g \cdot mL^{-1}$ kanamycin. The flasks were incubated at 37 °C until D_{600} of 1.0 was obtained ($\approx$ 5 h). The cells were then induced with 1 mM isopropyl thio-β-D-galactoside and incubated for another 3 h. Cells were harvested via centrifugation (7500 ***g***, 20 min, 4 °C) and stored at −20 °C.

For purification of the His-tagged ferredoxins, cells were resuspended in 20 mL buffer A [50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20% (v/v) glycerol] containing 10 mM imidazole, 0.1 mM phenylmethylsulfoxide and 5 μL Benzonase. Although *E. coli* Rosetta(DE3)pLysS cells lyse upon thawing, the suspension was passed once through a 20 K French press cell (Amicon, Urbana, IL, USA) at 120 MPa to complete cell lysis. After centrifugation (33 000 ***g***, 30 min, 4 °C), the supernatant was loaded onto a 1 mL Ni-NTA His-Bind Resin flow-through column, equilibrated with 5 mL buffer A containing 10 mM imidazole. After washing with 10 mL buffer A containing 20 mM imidazole, recombinant Fd_{mor} was eluted by stepwise addition of 0.5 mL buffer A containing 200 mM imidazole. Fractions (0.5 mL) containing Fd_{mor}, were identified by their brownish colour and pooled according to their A_{280}/A_{412} value. After concentration in an ultrafiltration device (Vivascience, Hannover, Germany), the protein solution was applied to gel filtration on Sephadex 75 run with buffer B (50 mM Tris/HCl, pH 7.5, 20% glycerol). Fractions were pooled, concentrated and stored in aliquots at −20 °C.

For the purification of wild-type Fd_{mor}, cells were resuspended in 1 $mL \cdot g^{-1}$ buffer B containing 0.1 mM phenylmethylsulfoxide and 0.25 $\mu L \cdot mL^{-1}$ Benzonase. The crude extract was prepared as described above and loaded on a Q-Sepharose fast-flow column, equilibrated with buffer B. After washing with buffer B, Fd_{mor} was eluted by a linear gradient from 0 to 1 M KCl in buffer B (flow rate 1 $mL \cdot min^{-1}$). Pooled fractions were desalted using a PD 10 column with buffer B and then concentrated by loading it onto a MonoQ column which was run under the same conditions as described for Q-Sepharose fast flow. Pooled fractions were then applied to gel filtration on a Sephadex 75 column using buffer B. The finally pure wt-Fd_{mor} was stored in aliquots at −20 °C.

Molecular characterization methods

SDS/PAGE was carried out as described previously [15]. Prior to MS, proteins were desalted by RP-HPLC on a Pronoril 300-5-C4 column (125 × 3 mm, Knauer, Berlin, Germany) using a HPLC system (Merck Hitachi, Tokyo, Japan). Proteins were eluted in a linear gradient from 5% acetonitrile, 0.05% trifluoroacetic acid (v/v/v) to 40% acetonitrile, 0.04% trifluoroacetic acid (v/v/v) over 35 min at a flow rate of 1 $mL \cdot min^{-1}$. ESI-MS was performed as described previously [15]. The iron content of the ferredoxin Fd_{mor} was determined by atom absorption spectroscopy on an AAnalyst 800 (Perkin–Elmer, Boston, MA, USA) using electrothermal atomization in the graphite furnace. The detection wavelength was set to $\lambda = 252.29$ nm and calibration was performed with dilution series (10–100 $\mu g \cdot L^{-1}$) of a $FeCl_3$ standard solution (Sigma-Aldrich). EPR spectra of recombinant wt-Fd_{mor} were recorded on an ESR-Spectrometer ESP 380e (Bruker, Leipzig, Germany) equipped with a Kryostat ESR-900 (Oxford, Instruments, Wiesbaden, Germany).

Activity assays

The activities of the FdR_{mor}/Fd_{mor} couple towards the artificial electron acceptors NBT and cytochrome *c* were determined spectrophotometrically using an Uvikon 930 spectrophotometer (Kontron, Milton Keynes, UK). NBT reduction was measured at 535 nm ($\varepsilon_{535} = 18\ 300\ M^{-1} \cdot cm^{-1}$) and cytochrome *c* reduction at 550 nm ($\varepsilon_{550} = 21\ 100\ M^{-1} \cdot cm^{-1}$). Reactions were performed in 50 mM glycine buffer, pH 8.6 at 30 °C, if not stated otherwise. For measurements at different pH values buffers were composed of 25 mM Tris and 25 mM glycine which were then adjusted either with NaOH or with HCl. Measurements were performed in triplicate. Initial velocities (v) were fitted to a hyperbolic function to derive the steady state kinetic parameters K_m and V_{max}. To obtain the apparent kinetic parameters of FdR_{mor} for the His-tagged ferredoxins data were fitted to following modified Michaelis–Menten equation:

$$v = \frac{V_{max}[Fd]}{K_m + [Fd]} + k[Fd]$$

The additional linear term k [Fd] describes the background activities, which were dependent on the concentration of the His-tagged ferredoxins.

Cloning of $P450_{mor}$

A *Spe*I site was incorporated in the N-terminal primer 5′-TATGTGACTAGTTCCCTCGCCCTCGGGCCTGTC-3′ to allow for an in-frame ligation in the *Nhe*I treated vector pET28b(+) to express *morA* as a N-terminal His-tag fusion protein. In the C-terminal primer 5′-GATTACGAATTCAGCGCGCCGGAGTGAAACCG-3′ an *Eco*RI site was incorporated (restriction sites underlined). PCR conditions were the same as above except that annealing temperature was 65 °C and the extension time was 1 min 30 s. The single 1.2 kb product was cut with the appropriate restriction enzymes, gel extracted and ligated in *Nhe*I/*Eco*RI digested pET28b(+) to yield the plasmid pMCN28. Other procedures were as described above.

Production and purification of $P450_{mor}$

Cell growth was performed as described above for the expression of Fd_{mor} except that, after induction, 0.75 mM

δ-aminolevulinic acid was added to the medium. Crude extract from 1 L cell culture was prepared as described above for the His-tagged ferredoxins. Ni-NTA affinity chromatography was performed as described for His-tagged ferredoxins. Fractions (0.5 mL) containing $P450_{mor}$ were identified by their reddish colour and pooled according to their A_{280}/A_{418} value. $P450_{mor}$ was finally desalted by gel filtration using a PD 10 column with 50 mM Tris/HCl, pH 7.5, 20% (v/v) glycerol and stored in aliquots at −20 °C.

Spectral analysis

UV-Vis absorption spectra were recorded on an Uvikon 930 spectrophotometer (Kontron) using quartz cells with 1 cm path length. The protoheme content of $P450_{mor}$ as pyridine hemochromogen was determined according to Hawkes *et al*. [21]. CO difference spectra were recorded as described previously [15]. P450 inhibitors econazole, miconazole, clotrimazole and phenylimidazole were prepared as stock solutions in dimethylsulfoxide. Imidazole, pyridine and metyrapone were made up in 50 mM Tris/HCl, pH 7.5. Spectral binding assays were performed using 1–3 μM $P450_{mor}$ in 50 mM Tris/HCl, pH 7.5, 10% glycerol divided between sample and reference cuvette. After recording the baseline between 350 and 650 nm, dissolved substrate was added to the sample cuvette and the same volume of solvent was added to the reference cuvette. Solutions were mixed by carefully pipetting up and down and difference spectra were recorded after each addition of substrate. The maximal absorbance changes calculated from each difference spectrum were plotted against the concentration of inhibitor. Data points were then fitted to a hyperbolic function to generate the K_d value. All values presented here were determined using the mean of three independent titration experiments.

HPLC analysis of morpholine conversion

Reactions were performed in a final volume of 500 μL 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM morpholine, 50 pmol FdR_{mor}, 250 pmol of one of the Fd_{mor} variants and 50 pmol $P450_{mor}$. Reactions were set up in triplicate and initiated by addition of 1 mM NADH. Immediately after mixing, 250 μL were removed and treated with 1 μL 20% (v/v) H_2SO_4 in order to terminate the reaction. This sample was used as a reference in HPLC analysis. The remaining reaction mixture was incubated for 30 min at 30 °C and then terminated in the same way. Precipitated proteins were removed by centrifugation.

The content of morpholine was determined according to Meister & Wechsler [49] on a HPLC apparatus (Varian) using a Hypersil column (5 μm, 150 mm × 4.6 mm, Phenomenex). Samples (50 μL) were injected and chromatography was performed at 50 °C with a mixture of 52% acetonitrile and 48% 10 mM potassium phosphate buffer (pH 6.7) at a flow rate of 1 mL·min^{-1}. Morpholine eluted at 7.3 min and was detected by UV absorption at 192 nm. The detection limit was found to be 10 nmol. Activities were calculated from the differences between the amount of morpholine in the reference samples and in the samples taken after 30 min.

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4 Anhang

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10/1991 – 09/1992	Anstellungen bei der Kaufhof AG, Bonn und der Bundeszentrale für politische Bildung, Bonn
07/1989 – 09/1990	Zivildienst beim Arbeiter-Samariter-Bund, Bonn

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Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die aus fremden Quellen entnommenen Gedanken sind als solche kenntlich gemacht. Die vorliegende Arbeit wurde bisher noch keiner anderen Prüfungsbehörde vorgelegt.

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