

**Cloning, expression, purification and characterization
of a novel cytochrome P450 isolated from
Thermus thermophilus HB27.**

Von der Fakultät Chemie der Universität Stuttgart
zur Erlangung der Würde eines Doktors der
Naturwissenschaften (Dr. rer. nat.) genehmigte Abhandlung

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Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

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Abbreviations

A	absorbance
Å	Ångström (=10 ⁻¹⁰ m)
AdR/AdX	bovine adrenodoxin reductase/adrenodoxin
Amp ^R	ampicillin resistance
APS	ammonium persulfate
B p	base pairs
CIAP	calf intestinal alkaline phosphatase
Cm	chloramphenicol
CYP119	gene encoding the P450 from <i>S. solfataricus</i>
CYP175A1	gene encoding the P450 from <i>T. thermophilus</i>
Cys	cysteine
DC	direct current
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
Gly	glycine
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
IEX	ion exchange chromatography
IPTG	isopropyl-β-D-thiogalactoside
KAc	potassium acetate
kb	kilobases
k _d	dissociation constant
kDa	kilo Dalton
KPi	potassium phosphate buffer
LB	Luria-Bertani
LMW	low molecular weight
M	mole per liter
MD	molecular dynamics

mg	milligram
min	minute
ml	milliliter
mol	mole
NaAc	sodium acetate
NADPH	β -nicotinamidadeninucleotidephosphate
nm	nanometer
OD	optical density
ORF	open reading frame
ori	origin of replication
P450_Tth	P450 from <i>Thermus thermophilus</i>
PCR	polymerase chain reaction
PEG	polyethylenglycol
rms	root mean square
rpm	rotations per minute
r.t.	room temperature
R _z	$A_{418\text{nm}}/A_{280\text{nm}}$ (R _z 1.6 corresponds to electrophoretic pure protein preparation)
sec	second
SDS	sodium dodecyl sulfate
TAE	Tris/acetate/EDTA buffer
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylendiamine
TLC	thin layer chromatography
Tris	Tris-(hydroxymethyl)-aminomethane
TSS	transformation storage solution
UV	ultraviolet
VIS	visible
% (w/v)	weight percent
% (v/v)	volume percent
°C	degree Celsius
λ	wavelength
μg	microgram

μl microliter

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1. Zusammenfassung

Cytochrome sind Proteine, die in tierischen und menschlichen Organismen in der "Atmungskette", einem Teilprozeß der oxidativen Phosphorylierung, vorkommen. Das Suffix "450" erhalten bestimmte Cytochromsysteme, weil sie mit dem starken Inhibitor CO (Kohlenmonoxid) einen Komplex bilden, der Licht mit einer Wellenlänge von 450 nm absorbiert. Das Cytochrom P450 wurde zum ersten Mal von Klingenberg und Williams aus Rattenleber-Mikrosomen isoliert [Klingenberg und Williams, 1958] und dann von Sato und Omura [Sato und Omura, 1964] weiter charakterisiert, die auch die Methode zur quantitativen Bestimmung von Cytochrom P450 entwickelten [Omura und Sato, 1964a], [Omura und Sato, 1964b]. Nach internationaler Nomenklatur werden die einzelnen Cytochrome mit der Abkürzung CYP bezeichnet und sodann mit einer arabischen Zahl für die Familie, mit einem Großbuchstaben für die Unterfamilie und mit einer weiteren arabischen Zahl für das individuelle Enzym versehen (z.B. CYP1A1). Alle Familien zusammen bilden die Superfamilie Cytochrom P450. Die Mitglieder der CYP-Superfamilie führen aliphatische und aromatische Hydroxylierungen, Oxidationen, Epoxidierungen, Reduktionen sowie Dehalogenierungen aus. Durch die Hydroxylierung von $-X-CH_3$ ($X = O, S, NR$) sind die entsprechenden Dealkylierungen möglich. Die CYP-Enzyme sind bezüglich der Substratspezifität sehr variabel. Sie sorgen für die Metabolisierung endogener Substanzen wie auch für die Umwandlung xenobiotischer Substanzen.

Die Suche nach P450-Genen im Genom von thermophilen Mikroorganismen führte zur Identifikation einer Region im Genom von *Thermus thermophilus* (Abb. 1) mit einer Homologie zu P450 BM3 (Monooxygenase-Domäne) aus *Bacillus megaterium*. Obwohl die allgemeine Homologie, wie üblich bei P450-Proteinen, nicht besonders hoch war, zeigten die Aminosäuresequenzen in einigen Bereichen hohe Übereinstimmung (Abb. 1). Das P450_Tth wies die typischen Sequenzmerkmale auf. Die Helix K, die P450 „Signatur“ und die Häm-Bindungsregion. Die Häm-Bindungsstelle enthält die konservierte Phe-X-X-Gly-X-Arg-X-Cys-X-Gly Konsensussequenz, wobei die Aminosäure Cystein, die in allen Cytochromen P450 enthalten ist, als fünfter Ligand an das Häm bindet. Die Helix K enthält das konservierte Glu-X-X-Arg-Motiv, das wahrscheinlich an der Stabilisierung der

Struktur beteiligt ist. Schließlich ist die Konsensussequenz Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser zu erkennen, die als P450 Signatur bekannt ist.

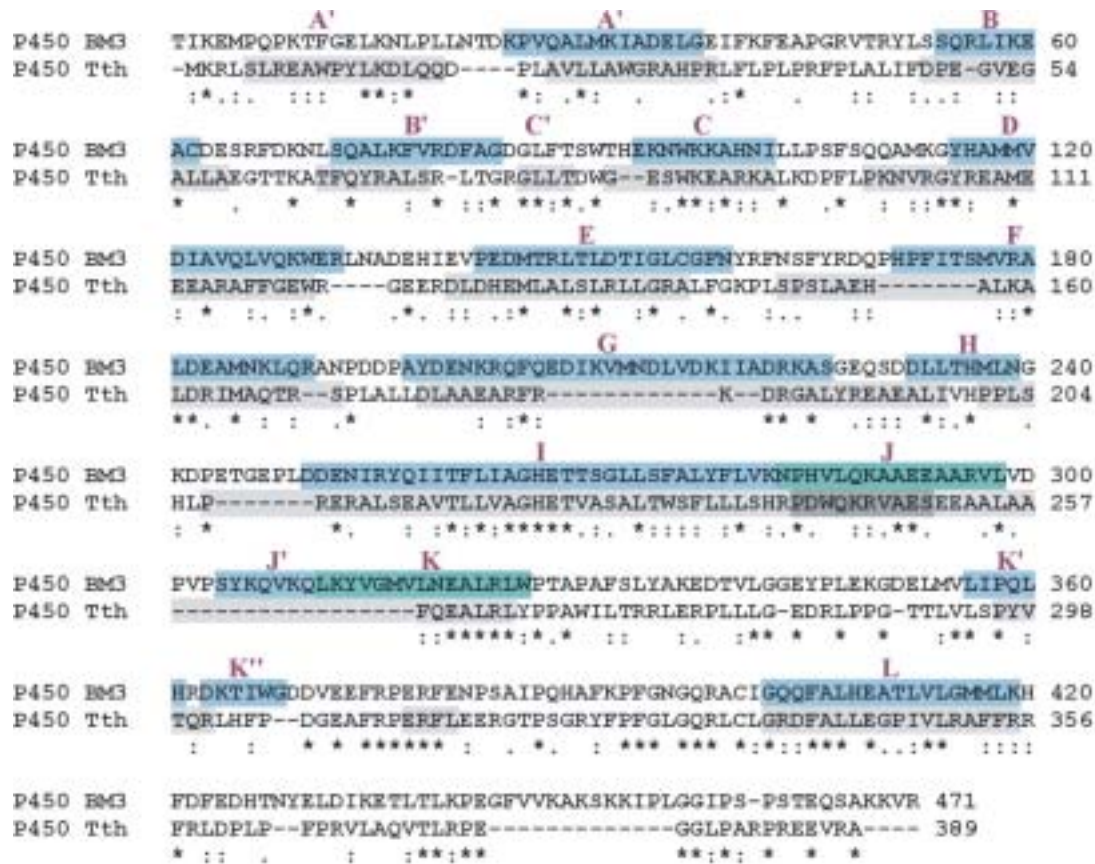


Abbildung 1: Sequenzvergleich zwischen P450_Tth und P450_BM3. Schattierte Regionen zeigen α -Helices in den beiden Proteinen. Mit (*) markierte Aminosäuren sind identisch, während (:) auf eine große und (.) auf eine geringe Ähnlichkeit hinweisen.

Thermus thermophilus HB27 ist ein aus heißen Wasserquellen isoliertes Gram-negatives Bakterium. Sein Genom wird zur Zeit am Institut für Mikrobiologie und Genetik der Universität Göttingen sequenziert. Der GC-Gehalt des Genoms beträgt ca. 69 %. Die optimale Kultivierungstemperatur für *Thermus* liegt zwischen 47 und 85 °C. Proteine aus *Thermus thermophilus* sind im allgemeinen thermostabil, nur 10 % der gesamten Proteine werden nach 5 minutiger Inkubation bei 110 °C denaturiert.

Diese Arbeit behandelt die Klonierung, Expression, Aufreinigung und Charakterisierung eines neuen P450-Enzyms aus *T. thermophilus* HB27. Das kodierende Gen der P450-Monooxygenase (Abb. 2) wurde mittels PCR amplifiziert und in einen passenden Expressionsvektor kloniert.

P450_Tth (CYP175A1)

ATGAAGCGCCTTTCCCTGAGGGAGGCCTGGCCCTACCTGAAAGACCTCCAGCAAGATCCC	60
CTCGCCGTCCTGCTGGCGTGGGGCCGGGCCCCACCCCGGCTCTTCCTTCCCTTGCCCCGC	120
TTCCCCCTGGCCCTGATCTTTGACCCCCGAGGGGGTGGAGGGGGCGCTCCTCGCCGAGGGG	180
ACCACCAAGGCCACCTTCCAGTACCGGGCCCTCTCCCCGCTCACGGGGAGGGGCCTCCTC	240
ACCGACTGGGGGAAAGCTGGAAGGAGGCGCGCAAGGCCCTCAAAGACCCCTTCCCTGCCG	300
AAGAACGTCCGCGGCTACCGGGAGGCCATGGAGGAGGAGGCCCGGGCCTTCTTCGGGGAG	360
TGGCGGGGGAGGAGCGGGACCTGGACCACGAGATGCTCGCCCTCTCCCTGCGCCTCCTC	420
GGGCGGGCCCTCTTCGGGAAGCCCTCTCCCAAGCCTCGCGGAGCACGCCCTTAAGGCC	480
CTGGACCGGATCATGGCCCAGACCAGGAGCCCCCTGGCCCTCCTGGACCTGGCCGCGGAA	540
GCCCCGCTTCGGAAGGACCGGGGGCCCTCTACCGCGAGGCGGAAGCCCTCATCGTCCAC	600
CCGCCCCCTCTCCACCTTCCCCGAGAGCGCGCCCTGAGCGAGGCCGTGACCCCTCCTGGTG	660
GCGGGCCACGAGACGGTGGCGAGCGCCCTCACCTGGTCTTTCTCCTCCTCTCCCACCGC	720
CCGGAAGTGGCAGAAGCGGGTGGCCGAGAGCGAGGAGCGGCCCTCGCCGCCTTCCAGGAG	780
GCCCTGAGGCTCTACCCCCCGCCTGGATCCTCACCCGGAGGCTGGAAGGCCCTCCTC	840
CTGGGAGAGGACCGGCTCCCCCGGCACCACCTGGTCTCTCCCCCTACGTGACCCAG	900
AGGCTCCACTTCCCCGATGGGGAGGCCTTCCGGCCCGAGCGCTTCTTGGAGGAAAGGGGG	960
ACCCCTTCGGGGCGCTACTTCCCTTTGGCTGGGGCAGAGGCTCTGCCTGGGGCGGGAC	1020
TTCCGCTCCTCGAGGGCCCCATCGTCTCAGGGCCTTCTTCCGCGCTTCCGCCTAGAC	1080
CCCCTCCCTTCCCCGGGTCTCGCCAGGTCACCTGAGGCCCGAAGGCGGGCTTCC	1140
GCGCGCCTAGGGAGGAGGTGCGGGCGTGA	1170

Abbildung 2: Nukleotidsequenz des offenen Leserahmens aus *T. thermophilus* mit Homologie zu P450 BM3. Der offene Leserahmen ist 1170 Basenpaare lang und das entsprechende Protein sollte 390 Aminosäuren umfassen, was einem berechneten Molekulargewicht von 44 kDa entspricht.

Für die rekombinante Expression wurde *E. coli* BL21 CodonPlus verwendet, denn die Seltenheit einiger Kodons schien der limitierende Faktor für die Expression in anderen *E. coli* Stämmen zu sein. Nach Optimierung der Expressionsbedingungen konnten 40 mg P450 in einem Liter Kulturmedium nachgewiesen werden. Abhängig von der für verschiedene Anwendungen benötigten Reinheit des P450 Proteins wurden zwei unterschiedliche Aufreinigungsprotokolle etabliert. Der erste Schritt in beiden Protokollen war eine Ammoniumsulfat-Fällung. Die sonst für thermostabile Proteine oft verwendete Hitze-Präzipitation konnte in diesem Fall nicht benutzt werden, da das P450 aus *T. thermophilus* anscheinend Membran-assoziiert war und durch Hitze-Behandlung mit den Membranbestandteilen der *E. coli*-Zellen ausfiel. Das erste Aufreinigungsprotokoll bestand aus zwei chromatographischen Trennungen. Zuerst wurde eine Ionaustauschchromatographie und dann eine Hydroxyapatit-chromatographie durchgeführt. Die gesamte Ausbeute war ca. 20 % und das gewonnene Protein war elektrophoretisch homogen mit R_z 1.6, wobei $R_z = A_{417\text{nm}}/A_{280\text{nm}}$ ein oft verwendetes Maß für die Reinheit von P450 Proteinen ist. Das nach diesem Protokoll gereinigte Protein wurde für Kristallisationsexperimente eingesetzt. Das zweite Protokoll bestand aus einer chromatographischen Trennung, die auf hydrophoben Wechselwirkungen basierte. Die Ausbeute dieses Schrittes war ca. 50 % und das gewonnene Protein mit einem R_z von 1.4 und wurde für die weitere

biochemische Charakterisierung verwendet. Das Enzym aus *Thermus* zeigte die typischen spektroskopischen Eigenschaften der P450-Monooxygenasen. Im UV-VIS Bereich besaß das Protein im oxidierten Zustand ein Absorptionsmaximum bei 417 nm (so genanntes Soret Band).

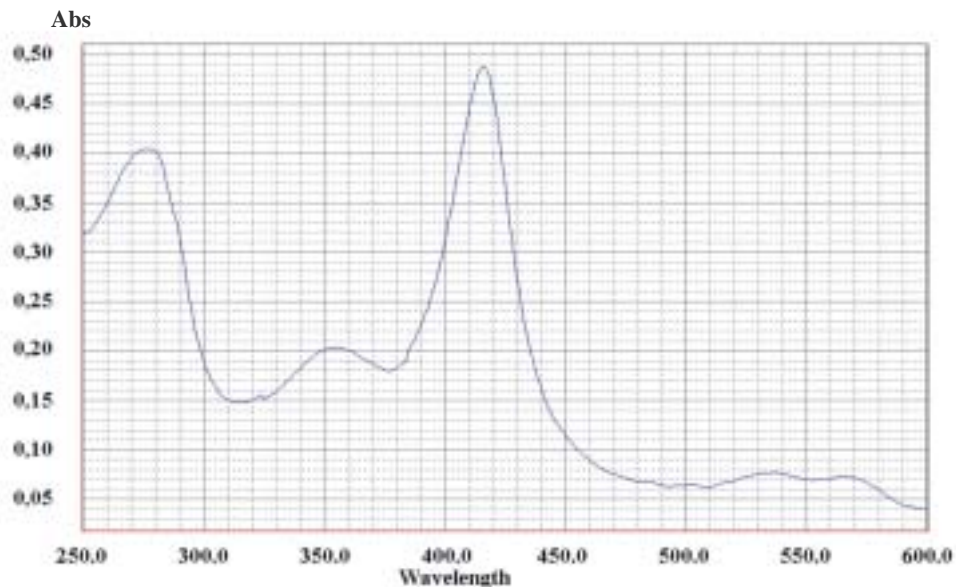


Abbildung 3: UV-VIS Spektrum von P450_Tth. Das Absorptionsmaximum bei 417 nm wird als Soret-Bande bezeichnet. Auch die beiden Banden bei 537 nm und 567 nm sind typisch für P450 Enzyme.

Der reduzierte CO-Komplex des Cytochroms-P450 zeigte das typische Absorptionsmaximum bei 450 nm (Abb. 4).

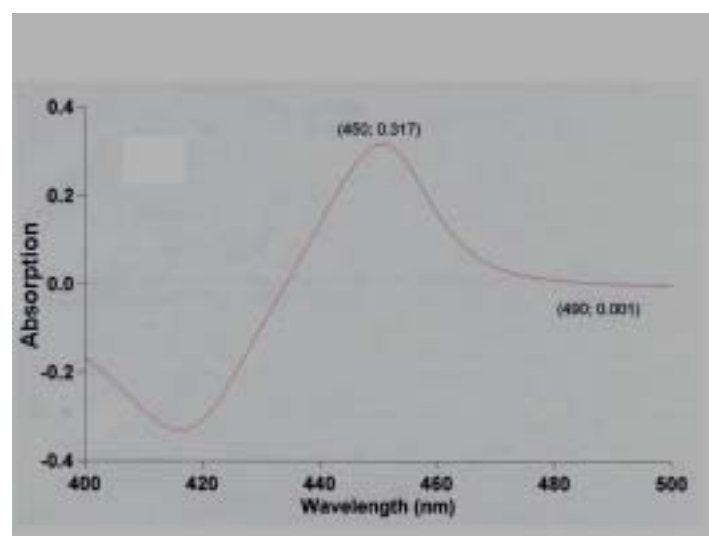


Abbildung 4: CO-Differenzspektrum von P450_Tth

Die g Werte des EPR Spektrums (Abb. 4) waren denen anderer P450-Monooxygenasen sehr ähnlich. Dies deutete an, dass die Orientierung und die Natur der Liganden des Häm-Eisens denen in anderen P450-Systemen weitgehend entspricht.

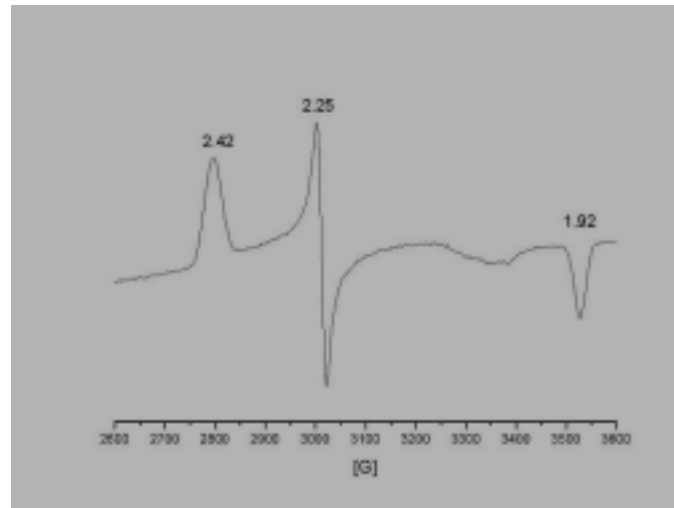


Abbildung 5: EPR-Spektrum von P450_Th. P450_Tth 214 μ M in KPi 20 mM pH 7.4. Temperatur der Probe: 10K. Frequenz, 9.47 GHz. Amplitudenmodulation: 5 G. Mikrowellenleistung 0.32 mW.

Untersuchungen bezüglich der Thermostabilität zeigten, dass der Schmelzpunkt des P450-Enzyms aus *T. thermophilus* bei 88 °C lag (Abb. 6).

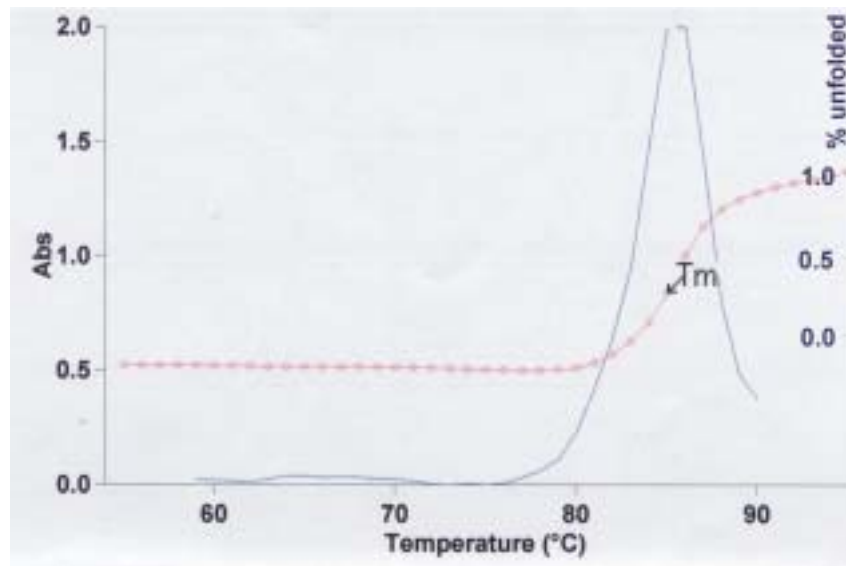


Abbildung 6: Schmelzkurve von P450 Tth. Der Anstieg der Absorption des Häms bei 418 nm wurde gemessen (blau). Der prozentuale Anteil an denaturiertem Protein (rot) wurde nach der Gleichung in 4.10.3 („Determination of melting temperatures“) berechnet. Die Schmelzkurve führte zu einem Schmelzpunkt von 88 °C.

Das P450-Protein aus *Thermus* ist das zweite Beispiel einer thermostabilen P450-Monooxygenase. Das erste ist CYP119 aus dem Archeon *Sulfolobus solfataricus* [McLean, Maves et al. 1998], dessen Schmelzpunkt bei 91 °C liegt. Die Kristallstruktur des P450 aus *T. thermophilus* wurde von T. Poulos innerhalb eines gemeinsamen Projekts aufgeklärt. Die Struktur zeigt die typische P450-Faltung mit 17 α -Helices und 11 β -Strängen, die 4 β -Faltblättern entsprechen.

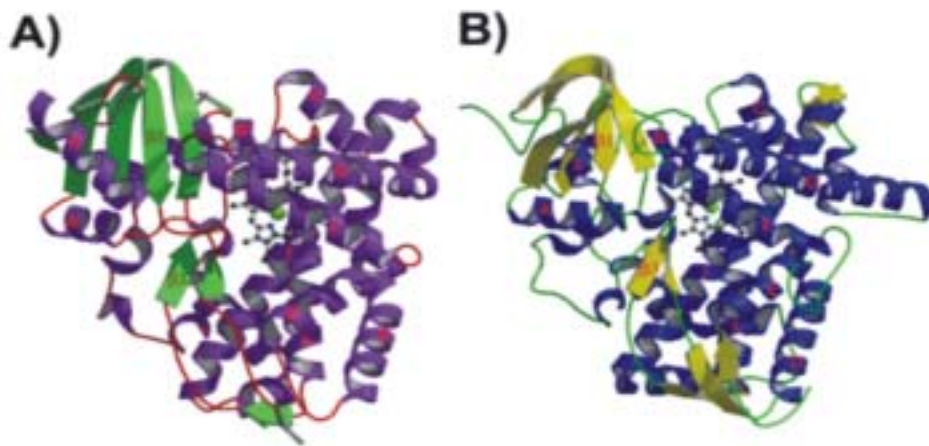


Abbildung 7: Darstellung der Sekundärstrukturelemente von A) CYP175A1 und B) P450 BM-3.

Das Sequenz ähnlichste bekannte P450 ist das Protein aus *Bacillus megaterium* (P450 BM3), dessen Kristallstruktur ebenfalls bekannt ist. Der Strukturvergleich (Abb. 7) zwischen den beiden Enzymen zeigt allgemeine strukturelle Ähnlichkeiten. Insbesondere im Bereich der Bindungstasche weisen die zwei Proteine große Ähnlichkeit auf. Nach der Etablierung von Expression und Aufreinigung für das P450 aus *Thermus thermophilus* wurden Experimente zur Evaluierung des Substratspektrums durchgeführt. Die Substratsuche bei P450-Systemen im allgemeinen, besonders jedoch bei dem in der vorliegenden Arbeit untersuchten Enzym, ist häufig schwierig. Da die P450-Enzyme von weiteren, Elektronenliefernden Enzymen abhängig sind, müssen artifizielle Systeme verwendet werden, die diese Aufgabe übernehmen können, falls der natürliche Redoxpartner unbekannt ist. Artifizielle Redoxsysteme sind in der Literatur beschrieben, sie sind jedoch nur

auf eine geringe Zahl von P450-Monooxygenasen anwendbar. Auch der „peroxide shunt“, der das Problem des Elektronentransports umgeht, ist bislang nur für wenige Beispiele beschrieben. Ein weiteres Problem war die Thermophilie des untersuchten Enzyms, das seine optimale Aktivität bei einer relativ hohen Temperatur zeigt. Bei diesen Temperaturen denaturieren potentielle Redoxpartner aus mesophilen Organismen, die in den beschriebenen künstlichen Redoxsystemen mesophiler P450-Systeme verwendet werden, während bei geringeren Temperaturen die Aktivität des P450_Tth möglicherweise nicht mehr nachweisbar ist. Desweiteren war das potentielle Substratspektrum des untersuchten Enzyms extrem groß, da die P450-Monooxygenasen eine enorme Substratvariabilität und eine Vielzahl verschiedener Reaktionstypen aufweisen. Aus den beschriebenen Problemen ergab sich eine experimentelle Situation, in der verschiedene Parameter gleichzeitig unbekannt waren. Zunächst wurde eine intensive Suche nach dem Redoxpartner im Genom von *Thermus thermophilus* auf der Basis von Sequenzhomologien bekannter Redoxpartner aus mesophilen Organismen durchgeführt, die nicht zum Erfolg führte. Die strukturelle Homologie der P450-Monooxygenasen aus *Thermus thermophilus* und *Bacillus megaterium* und die Sequenzidentität der zwei Proteine deutete an, dass endogene Substrate des P450 aus *Thermus* Fettsäuren sein könnten. Da die endogene Reduktase der P450-Monooxygenase aus *Thermus* unbekannt war, wurden alternative Möglichkeiten getestet, um die katalytische Aktivität des Proteins zu charakterisieren. Trotz mehrerer Versuche konnten weder Bindung an das P450 noch Umsetzung von Fettsäuren nachgewiesen werden. Aus diesem Ergebnis konnte nicht eindeutig geklärt werden, ob die getesteten Substrate bzw. die Reduktasesysteme passend waren. Die sorgfältige Analyse der Region des *Thermus* Genoms, in der sich das P450 Gen befindet, führte zur Entdeckung des kodierenden Gens für die Phytoin-Synthase (*crtB*). Die Herstellung von Carotinoiden [Brock 1984] sowie die Anordnung carotinogener Gene in einem Cluster [Tabata et al. 1994] wurde in *Thermus thermophilus* schon nachgewiesen. Zusätzlich scheinen P450-Enzyme an der Carotinoidbiosynthese beteiligt zu sein [Schoefs et al. 2001]. Aus diesen Informationen ließ sich vermuten, dass P450 aus *T. thermophilus* ein carotinogenes Enzym sein könnte. Die *in vivo* heterologe Komplementation ist eine häufig angewandte Methode für die Charakterisierung von carotinogenen Proteinen, die oft membrangebunden und deswegen *in vitro* schwierig zu charakterisieren sind. Mittels modifizierter *E. coli* Zellen, die Carotinoide bildeten, konnte die katalytische Aktivität

2. Summary

Enzymes produced by extremophiles are of considerable biotechnological interest. They show activity and stability at extreme temperatures, low water activity and high hydrostatic pressure. The growing knowledge about “thermozymes” from thermophilic organisms obtained during the recent years and the importance of P450 monooxygenases as potential biocatalysts for industrial applications has created high interest in P450 enzymes from thermophilic bacteria and archaea. Although the homology between P450s is generally low, a few domains and sequence signatures are conserved and can be identified by homology searches. Following this approach a homology search of recently sequenced genomes from thermophiles was performed to identify novel P450 enzymes of this origin. Indeed, a region showing homology to a known bacterial P450 (P450 BM3 from *Bacillus megaterium*) was found within the *Thermus thermophilus* HB27 genome, currently being sequenced in Göttingen. *Thermus thermophilus* is a non-sporulating Gram-negative bacterium isolated from hot springs. Its genome of 1.82 Mbp (excluding the mega-plasmid pTT27 of 0.24 Mbp) has a GC-content of approximately 69 %. The optimal temperature for growth is between 65 and 72 °C, the maximum being 85 °C and the minimum being 47 °C. Bulk protein extracted from this thermophile is much more stable to heat than mesophilic protein extracts; and only about 10 % of the total protein is denatured by heating up to 110 °C for 5 min.

Cytochrome P450 proteins, named for the absorption band at 450 nm of their carbon monoxide bound form, are a large superfamily of enzymes. P450s are ubiquitous enzymes essential for steroid biosynthesis, catabolism of drugs, utilization of carbon compounds as an energy source in bacteria, and in the production of various macrolide antibiotics. Sequence identity among P450s is often low (normally less than 20 %), but the structural fold has been conserved during evolution. P450s are heme thiolate proteins; the most conserved structural feature is a cysteine as fifth ligand to the heme iron. Normally P450s use electrons from NAD(P)H to catalyze activation of molecular oxygen, leading to regiospecific and stereospecific oxidative attack (often hydroxylation) of non-activated hydrocarbons at physiological temperatures. The substrates of P450s are extremely diverse. They are involved in the biosynthesis of hormones or antibiotics, as well as carcinogenesis and degradation of xenobiotics.

The selective oxidation of an unactivated C-H bond in a complex organic molecule to the alcohol functionality (C-OH) has wide applications in chemical synthesis.

The present work reports cloning, expression, purification, crystallization and characterization of CYP175A1, a cytochrome P450 from the thermophilic bacterium *Thermus thermophilus* HB27. For over expression the gene was amplified by PCR in the presence of specific primers from a DNA fragment kindly provided by Prof. H. J. Fritz (University of Göttingen). The PCR product was ligated into the polylinker region of pKK223-3 for expression under control of the tac promoter. The resulting clone was transformed in *E. coli* BL21(DE3)RP CodonPlus and selected for ampicillin and chloroamphenicol resistance. Expression was induced by IPTG and a yield of approx. 40 mg protein/liter culture was obtained. After cell disruption and ammonium sulfate precipitation P450_Tth was purified to homogeneity, following two different purification protocols. The fractions corresponding to the highest purity were used for crystallization, while the others were used for further biochemical characterization. The 44 kDa protein is soluble and displays an absorption spectrum in the reduced, oxidized, and carbonyl adduct analogous to those of other P450 enzymes. This enzyme exhibits thermostability with a melting temperature 30 °C higher than that of P450cam from *P. putida*. The crystal structure of CYP175A1 has been determined in co-operation with T. Poulos (University of California, Irvine). CYP175A1 exhibits the typical prism like P450-fold composed of 17 α -helices and 11 β -strands, corresponding to four beta sheets. The closest sequence homologue to CYP175A1 is P450 BM3 from *B. megaterium*. Based on the sequence identity and close structural position of regions associated with the active site, it seemed possible that CYP175A1 may act as a fatty acid hydroxylase. However, the characteristic low- to high-spin shift that accompanies the binding of substrates to P450s was not observed using a range of even chain, commercially available fatty acids (C10-C20) either at room temperature or temperatures up to 70 °C. The location of CYP175A1 in the *T. thermophilus* genome (close to *crtB* encoding a phytoene synthase), and the fact that genes for carotenoid biosynthesis occur as a cluster in *T. thermophilus* as well as in other carotenoid-producing strains, suggested that the gene may be involved in carotenoid biosynthesis. Heterologous complementation using *Escherichia coli* strains genetically engineered to produce β -carotene indicated that CYP175A1 was able to catalyze the conversion of β -carotene to zeaxanthin via cryptoxanthin.

3. Introduction

3.1 Cytochrome P450 monooxygenases

First discovered in 1955 in rat liver microsomes [Klingenberg, 1958], the P450 enzymes nowadays constitute a large superfamily of heme-thiolate proteins involved in the metabolism of a wide variety of both exogenous and endogenous compounds. [Degtyarenko et al., 2001]. They are characterized by an intensive absorption band at 450 nm in the presence of carbon monoxide [Omura et al., 1964a], [Omura et al., 1964b]. P450s use electrons from NAD(P)H to catalyze the activation of molecular oxygen, leading to the regiospecific and stereospecific oxidative attack of structurally diverse chemicals [Werck-Reichhart et al., 2000]. Usually acting as terminal oxidases in multicomponent electron-transfer chains, P450s are ubiquitous enzymes essential for steroid biosynthesis, catabolism of drugs, utilization of carbon compounds as an energy source in bacteria, and in the production of various macrolide antibiotics. P450s are found throughout the biosphere and various genome projects have revealed a remarkable and unexpectedly large number of P450s in all the three phylogenetic lines of organisms.

3.1.1 *Gene organization and evolutionary history*

P450 superfamily genes (called CYP) are classified according to the recommendation of a nomenclature committee [Nelson et al., 1996] on the basis of amino acid identity, phylogenetic criteria and gene organization. The enzymes are divided into families based on amino acid sequence similarities, and each family can be further separated into subfamilies, which are designated by capital letters following the family designation (e.g., CYP3A). Individual enzymes are subsequently indicated by Arabic numerals (e.g., CYP3A4). A P450 enzyme belongs to a specific family when the amino acid sequence reveals more than 40% sequence similarity to this family, enzymes sharing more than 55% homology form a subfamily. Sequences with more than 97% similarity are variants of one individual enzyme [Nelson, Koymans et al., 1996].

3.1.2 Structural features

P450-containing monooxygenase systems originally belonged to two major classes separated by their intracellular location: bacterial/mitochondrial (class I), and microsomal (class II) which differ in subunit organization as shown in Fig. 1.

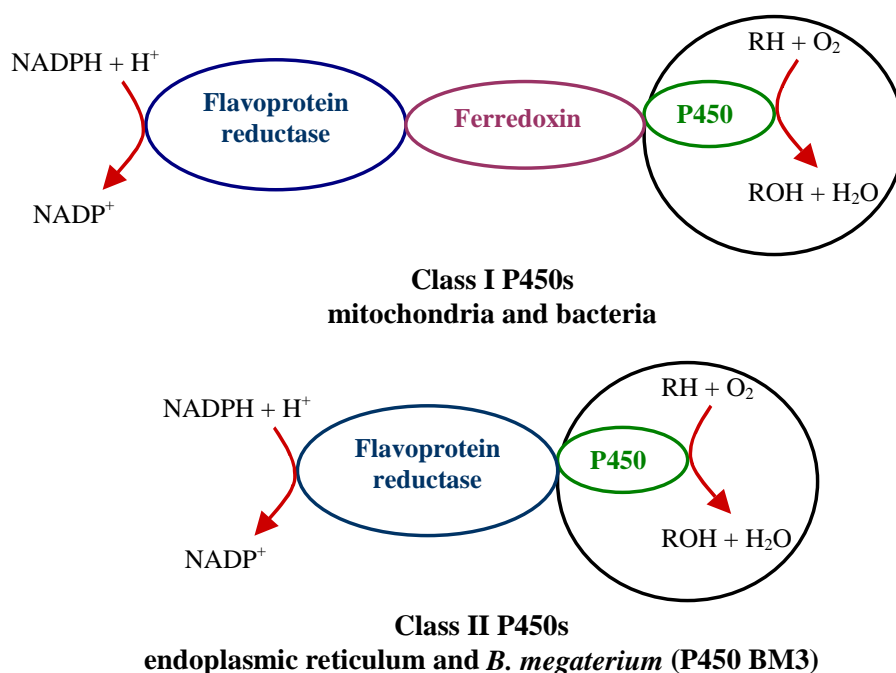


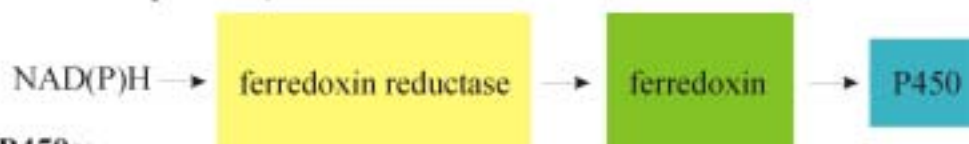
Fig. 1: Electron transfer systems for P450 monooxygenases

With growing knowledge about the variety of P450-containing systems they were classified according to the number of their protein components (Fig. 2): mitochondrial and most bacterial P450 systems (class I) have three components - an FAD-containing flavoprotein (NADPH or NADH-dependent reductase), an iron-sulfur protein, and the P450; the eukaryotic microsomal P450 systems (class II) contain two components - NADPH:P450 reductase (a flavoprotein containing both FAD and FMN) and P450; and a soluble monooxygenase P450BM-3 from *Bacillus megaterium* exists as a single polypeptide chain with two functional parts (the heme and flavin domains), and represents a unique bacterial one-component system. Sequence and functional comparisons show that these domains are more similar to P450 and the flavoprotein of the microsomal two component P450 monooxygenase system than to the proteins of the three component system. P450s from class III are self-sufficient and do not

require molecular oxygen or an external electron source. They catalyze the rearrangement or dehydration of alkylhydroperoxides or alkylperoxides generated by dioxygenase [Mansuy, 1998]. Only one P450 enzyme of class IV has been described to date. P450_{nor} (nitric oxide reductase) is a fungal P450 that receives electrons directly from NADH or NADPH [Mansuy, 1998].

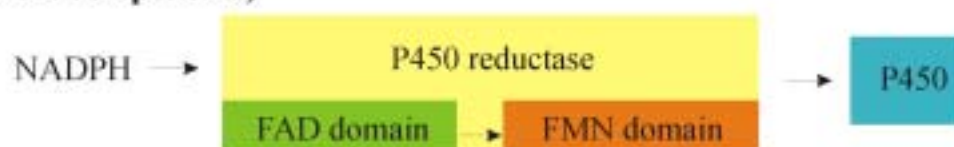
Class I P450s:

(three protein components)

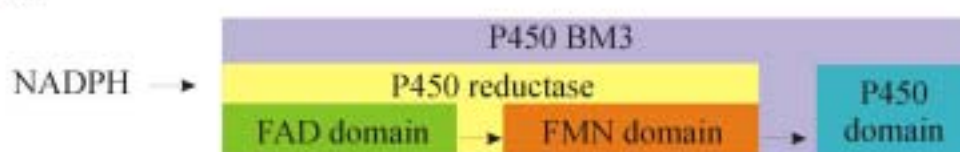


Class II P450s:

(two protein components)

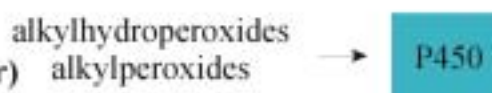


P450 BM3:



Class III P450s:

(one protein component, no cofactor)



Class IV P450s:

(one protein component)



Fig. 2: Examples of relevant electron transport chains within the different classes of P450 enzymes.

Sequence identity among P450 proteins is often less than 20 %, and there are only three absolutely conserved amino acids. Nevertheless their structural fold is highly conserved [Graham et al., 1999]. Highest structural conservation is found in the core of the protein around the heme and reflects a common mechanism of electron and proton transfer and oxygen activation. The conserved core is formed by a four-helix (D, E, I and L) bundle, the helices J and K, two sets of β sheets, and a coil called the meander (Fig. 3).

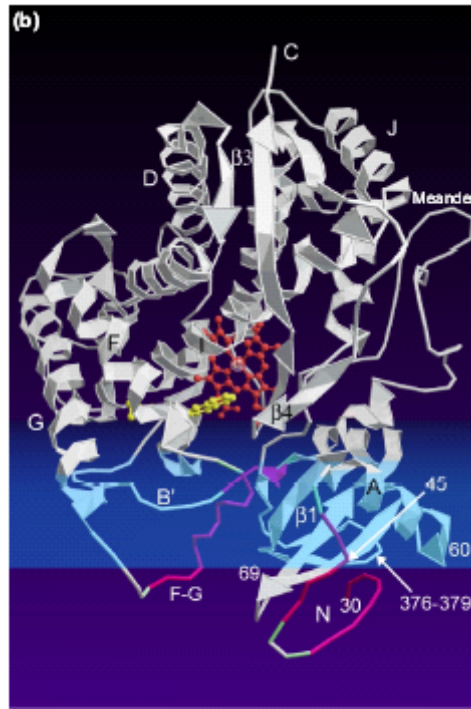


Fig. 3: P450 BM3 crystal structure

These regions comprise: a) the heme binding loop, containing the most characteristic P450 consensus sequence (Phe-X-X-Gly-X-Arg-X-Cys-X-Gly) with the absolutely conserved cysteine that serves as fifth ligand to the heme iron; b) the conserved Glu-X-X-Arg motif probably needed to stabilize the core structure; and c) the consensus sequence considered as P450 signature (Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser), which corresponds to the proton transfer groove on the distal side of the heme.

3.1.3 Functions in biological systems

Prokaryotic P450s are soluble proteins. They enable prokaryotes to catabolyze compounds used as carbon sources or to detoxify xenobiotics. Other functions described for prokaryotic P450s include fatty acid metabolism and biosynthesis of antibiotics. Eukaryotic class I (mitochondrial) enzymes catalyze several steps in the biosynthesis of steroid hormones and vitamin D₃ in mammals. Class II enzymes are the most common in eukaryotes. They are anchored on the outer face of the endoplasmic reticulum by amino-terminal hydrophobic anchors. Function of class II enzymes are extremely diverse. In fungi, they synthesize membrane sterols and mycotins, detoxify phytoalexins, and metabolize lipid carbon sources. In insects the

P450 enzymes fulfill many important tasks, from the synthesis and degradation of ecdysteroids and juvenile hormones to the metabolism of foreign chemicals of natural or synthetic origin [Feyereisen, 1999]. In plants class II P450s are involved in the biosynthesis or catabolism of all types of hormones, in the oxygenation of fatty acids, and in all the pathways of secondary metabolism. In animals P450s are involved in the biosynthesis and catabolism of signaling molecules, steroid hormones, retinoic acid and oxylipins [Hasler, 1999]. P450 enzymes are responsible for the metabolism and removal of exogenous compounds such as drugs and harmful compounds [Guengerich, 1995], [Hasler, 1999] but also contribute to carcinogenesis by activating chemicals to electrophilic metabolites that are capable of binding to DNA and causing gene mutation. The result of P450 catalysis is not always C-oxidation, but can also be a N-, or S-oxidation, dealkylation, deamination and dehalogenation (Tab. 1).

Reaction	Reaction scheme	Substrate
Aliphatic carbon hydroxylation	$\text{R-CH}_2\text{-CH}_3 \longrightarrow \begin{array}{c} \text{OH} \\ \\ \text{R-CH-CH}_3 \\ + \\ \text{R-CH}_2\text{-COOH} \end{array}$	Barbiturate
N-dealkylation	$\text{R}_1\text{-N} \begin{array}{l} \text{H} \\ \diagdown \\ \text{CH}_2\text{R}_2 \end{array} \longrightarrow \text{R}_1\text{-NH}_2 + \begin{array}{c} + \\ \text{R}_2\text{-C} \\ // \quad \backslash \\ \text{O} \quad \text{H} \end{array}$	Ephedrine, Methamphetamine
Deamination	$\text{R-CH}_2\text{-NH}_2 \longrightarrow \begin{array}{c} \text{O} \\ // \\ \text{R}_2\text{-C} \\ \backslash \\ \text{H} \end{array} + \text{NH}_3$	Histamine, Noradrenaline, Mescaline
O-dealkylation	$\text{R}_1\text{-CH}_2\text{-O} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \\ \text{---} \\ \text{---} \\ \text{---} \end{array} \text{R}_2 \longrightarrow \text{HO} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \\ \text{---} \\ \text{---} \\ \text{---} \end{array} \text{R}_2 + \text{R}_1\text{-CHO}$	Phenacetin, Codeine, Mescaline
Aromatic carbon hydroxylation	$\text{C}_6\text{H}_5\text{-R} \longrightarrow \text{HO-C}_6\text{H}_4\text{-R}$	Phenorbitale, Chlorpromazin
Oxidation of aromatic amines	$\text{C}_6\text{H}_5\text{-NH}_2 \longrightarrow \text{C}_6\text{H}_5\text{-NH-OH}$	Aniline derivatives
S-oxidation	$\begin{array}{c} \text{R}_1 \\ \diagdown \\ \text{S} \\ \diagup \\ \text{R}_2 \end{array} \longrightarrow \begin{array}{c} \text{R}_1 \\ \diagdown \\ \text{S} \\ \diagup \\ \text{R}_2 \end{array} \text{---} \text{O} \longrightarrow \begin{array}{c} \text{R}_1 \\ \diagdown \\ \text{S} \\ \diagup \\ \text{R}_2 \end{array} \begin{array}{l} \nearrow \text{O} \\ \searrow \text{O} \end{array}$	Phenothiazine
Dehalogenation	$\text{R}_1\text{-CH} \begin{array}{l} \text{X} \\ \end{array} \text{R}_2 \longrightarrow \begin{array}{c} \text{O} \\ // \\ \text{R}_1\text{-C} \\ \backslash \\ \text{R}_2 \end{array} + \text{H-X}$	Ethandibromide, Chloroform

Tab. 1: Examples of different P450 catalyzed reaction patterns

3.1.4 Catalytic cycle of cytochrome P450s dependent reactions

P450s are usually monooxygenases, catalyzing the insertion of one of the atoms of molecular oxygen into a substrate, whereas the second oxygen atom is reduced to water. In the proposed catalytic cycle (Fig. 4) [Wong, 1998], the ferric resting state of the enzyme (1) has a low-spin coordinated heme iron. Upon substrate binding (2) a low- to high-spin shift of the heme iron takes place and an increase in the reduction

potential is observed, by which cytochrome P450 can easily be reduced. One electron reduction (3) of the complex iron generates a ferrous form of the enzyme that rapidly binds dioxygen (4) to give the ferrous-oxy form which is further reduced (5). The oxyferryl species (6), resulting from the cleavage of the O-O bond (one oxygen atom leaves with two electrons and two protons as water) is supposed to be the C-H bond oxidizing species which inserts the oxygen atom into the substrate. The oxygenated product can be released from the enzyme (7), resulting in the initial state of cytochrome P450. The catalytic turnover of most microsomal/eukaryotic P450s is relatively low compared to those of prokaryotic P450s, typically between 0.1 and 10 min⁻¹. However, P450 BM3 with its covalently linked reductase has the highest known catalytic turnover of any P450 with 4600 min⁻¹ [Ruckpaul et al., 1984], [Archakov et al., 1990].

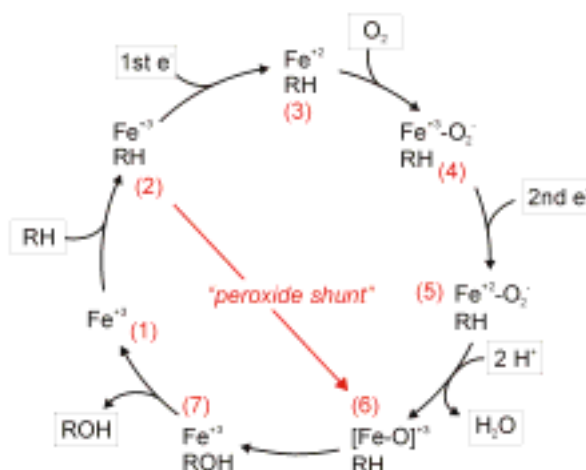


Fig. 4: Schematic view of cytochrome P450 reaction mechanism

Making use of the so-called “peroxide shunt” [Joo et al., 1999] the initial enzyme-substrate complex (2) can be directly converted into the reactive iron-oxygen intermediate (6). As a result of this peroxide pathway no electrons need to be supplied by the redox partner(s) and from NAD(P)H [Roberts, 1999].

3.1.5 P450 monooxygenases in fine chemical synthesis

In spite of the variety of chemical reactions catalyzed and the number of substrates attacked, the application of P450s in fine chemical synthesis is rather limited. Only a few *in vivo* processes are described, such as the production of dicarboxylic acids

[Mueller, 1990], steroids such as progesterone [Duport et al., 1998], subterminal hydroxylated fatty acids and polymers. There are two major limitations in the use of P450s as biocatalysts. The first problem is the low thermal stability of P450s. For example 90 % of human liver P450s are denatured after 6 hours at 25 °C [Yamazaki et al., 1997]. Bacterial P450s seem to be more thermostable than the eukaryotic counterparts, especially P450s from thermophilic microorganisms may be interesting enzymes for biotechnological applications [Sideso et al., 1997], [Sideso et al., 1998], [McLean et al., 1998]. A second major problem to be solved is that almost all known P450s require NAD(P)H as cofactor which has to be effectively recycled in a possible industrial process. Approaches for replacing NADPH by an electrochemical reduction using platinum-electrodes and different mediators are known. Taylor et al. described [Taylor et al., 2000] an enzyme-coupled recycling system for NADH regeneration for CYP105D1 from *Streptomyces griseus* using formate dehydrogenase. The recombinant enzyme expressed in *Escherichia coli* was functionally immobilized and the required cofactor could effectively be sustained by the recycling system. In other studies, NADPH was replaced by [cobalt(III) sepulchrate trichloride] for the electrocatalytical omega-hydroxylation of lauric acid [Faulkner et al., 1995], [Schwaneberg et al., 2000]. The use of hydrogen peroxide or other strong oxidants has been reported to support P450 monooxygenase activity through the “peroxide shunt” [Matsunaga et al., 2000], [Matsunaga et al., 1999]. Unfortunately, hydrogen peroxide interacts with the heme iron generating radicals that bleach the apoenzyme [Karuzina et al., 1994].

Other limitations to the application of P450s in fine chemical synthesis is that for class I and II proteins redox partners are required in order to catalyze the electron transfer from the cofactor to the heme. Reconstitution of the monooxygenase activity with separate enzymes [Shaw et al., 1997] or artificial fusion proteins [Deeni et al., 2001], [Gilep et al., 2001] have been described. Additionally, membrane-bound P450s are difficult to dissolve in water, a problem which can be partly solved by modification of the N-terminus [Scott et al., 2001], [Kempf et al., 1995].

3.2 *Thermus thermophilus*

Thermus thermophilus is a non-sporulating Gram-negative bacterium isolated from hot springs. Its genome of 1.82 Mbp (excluding the mega-plasmid pTT27 of 0.24

Mbp) has a GC-content of approximately 69% [Oshima, 1974]. 16S rDNA phylogeny indicates that *Thermus thermophilus* is associated to the green non-sulfur bacteria. The optimal temperature for growth is between 65 and 72 °C, the maximum is 85 °C and the minimum 47 °C. *Thermus* cells are yellow pigmented rods. The organism is strictly aerobic and sensitive to various antibiotics including those which are known to be rather ineffective against other Gram-negative bacteria. Bulk protein extracted from this thermophile is much more stable to heat than mesophile protein extracts; only about 10 % of the total protein is denatured by heating up to 110 °C for 5 min. One characteristic of the genus *Thermus* is that many strains produce carotenoids [Brock, 1984]. As carotenoids are hydrophobic substances associated with the cell membrane, they may play an important role in membrane stability in *Thermus* strains at high temperatures. Genes for carotenoid biosynthesis are arranged as a cluster on a large plasmid in *Thermus thermophilus* [Tabata et al., 1994], like in other carotenoid-synthesizing strains such as *Erwinia herbicola* [Armstrong et al., 1990], *Erwinia uredovora* [Misawa et al., 1990] and *Rhodobacter capsulatus* [Armstrong et al., 1989]. The main carotenoids of *T. thermophilus* are thermozeaxanthins and thermobiszeaxanthins, which are identified as carotenoid-(di)glucoside-branched fatty acid (di)esters [Yokoyama et al., 1995] (Fig. 5).

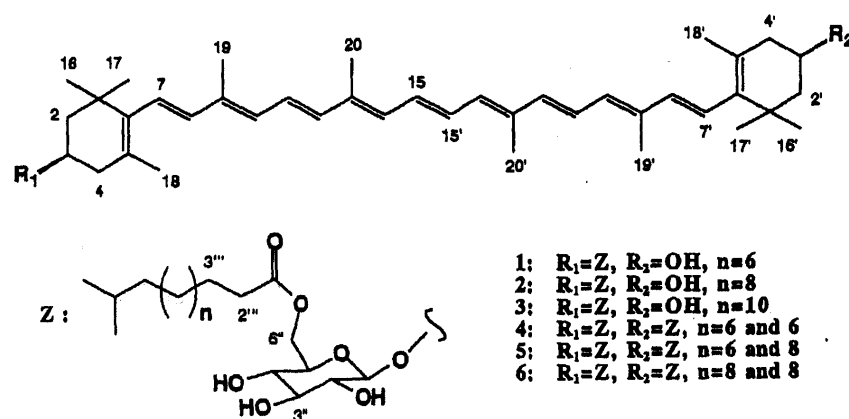
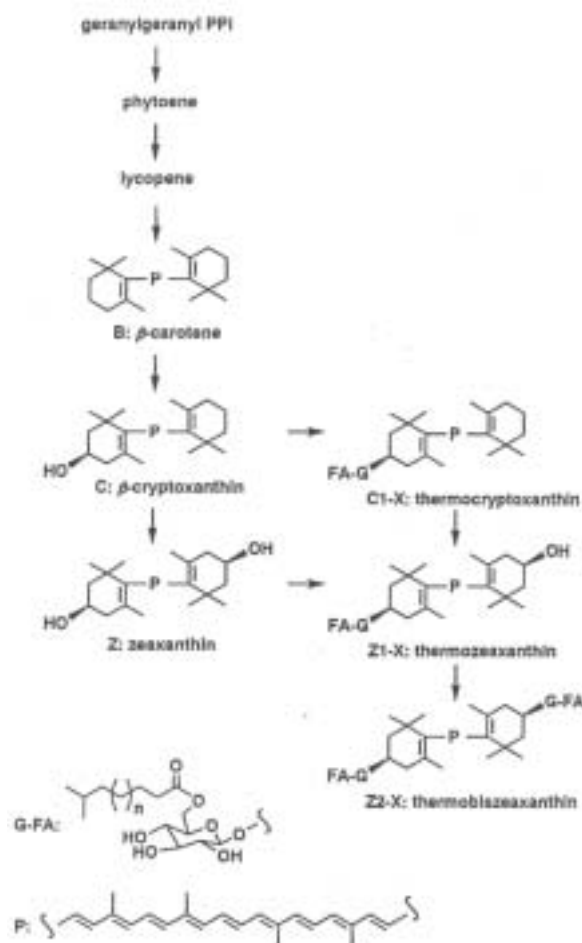


Fig. 5: Structure of thermoxanthins in *Thermus thermophilus* [Yokoyama et al., 1995]

The proposed biosynthetic pathway (Scheme 1) resembles to some extent that of *Erwinia*, in which non-esterified zeaxanthin diglucoside is the end product. However, *Thermus* carries out the esterification of the glucose moiety with fatty acids of various lengths as an additional step, leading to the synthesis of thermoxanthins with thermocryptoxanthin as intermediate. These new carotenoid glucoside esters are exclusively found in *Thermus* [Yokoyama et al., 1996].



Scheme 1: Carotenoid biosynthetic pathway in *Thermus thermophilus* [Yokoyama et al., 1996]

3.2.1 Biotechnologically relevant enzymes from *Thermus*

Enzymes produced by *T. thermophilus* are assumed to be thermostable [Oshima, 1974]. They are not only more thermostable but also more resistant to chemical agents than their mesophilic homologues, properties that make them of considerable biotechnological interest [Lasa et al., 1993]. The molecular mechanisms responsible for thermostability are different and vary from protein to protein. These mechanisms include hydrophobic interaction, packing efficiency, salt bridges, hydrogen bonds, loop stabilization etc. [Zeikus et al., 1998]. Pantazaki et al. have reviewed biotechnologically relevant enzymes from *T. thermophilus* that are functional under extreme conditions [Pantazaki et al., 2002]. Thermozyms involved in the conversion of starch to glucose, maltose and oligosaccharides have been described [Leuschner et al., 1995]. A fusion gene encoding the α -amylase from *Bacillus stearothermophilus* and the glucose isomerase from *T. thermophilus* has been constructed [Beaujean et al.,

2000] and integrated into the potato genome. The enzyme complex was active only at 65 °C, offering novel strategies for starch-producing industries. Thermostable xylose isomerases were purified from *T. thermophilus* and *T. caldophilus* and their structures were determined [Chang et al., 1999]. The *Thermus thermophilus* gene encoding xylose isomerase was cloned and expressed in *Saccharomyces cerevisiae*. The recombinant enzyme showed the highest activity at 85 °C in the production of ethanol during oxygen-limited xylose fermentation [Walfridsson et al., 1996]. α - and β -glucosidases and α - and β -galactosidases from *T. thermophilus* have been isolated and characterized. The application of thermophilic proteases as biocatalysts has been proposed as a method for enzyme-catalyzed synthesis of dipeptides [Monter et al., 1991]. Several phosphatases have been isolated from *T. thermophilus*. The need for thermostable alkaline phosphatase in clinical medicine and molecular biology makes this enzyme biotechnologically interesting. *T. thermophilus* pyrophosphatase is commercially available and is applied in several fields such as PCR, RT-PCR, stabilization of DNA and RNA at high temperatures [Tabor et al., 1990]. Thermostable DNA polymerases have facilitated the automation of the thermal cycling part of the PCR protocol. The first thermostable DNA polymerase applied in PCR was isolated from *Thermus aquaticus*. Another thermostable DNA polymerase was isolated from *T. thermophilus* which is able to catalyze both reverse transcription and polymerase chain reaction (RT-PCR), a valuable process for detection, quantitation, cloning and analysis of gene expression at RNA level. Several *Thermus thermophilus* ligases have been characterized [Takahashi et al., 1984], [Hjörleifsdottir et al., 1997]. Thermostable DNA ligases have the advantage that the reaction can be performed at temperatures near the melting point of the primers which enhances the specificity dramatically. Endonucleases Tth111I and Tth111II have been isolated from *T. thermophilus* and the recognition sequences have been identified [Shinomiya et al., 1980a], [Shinomiya et al., 1980b].

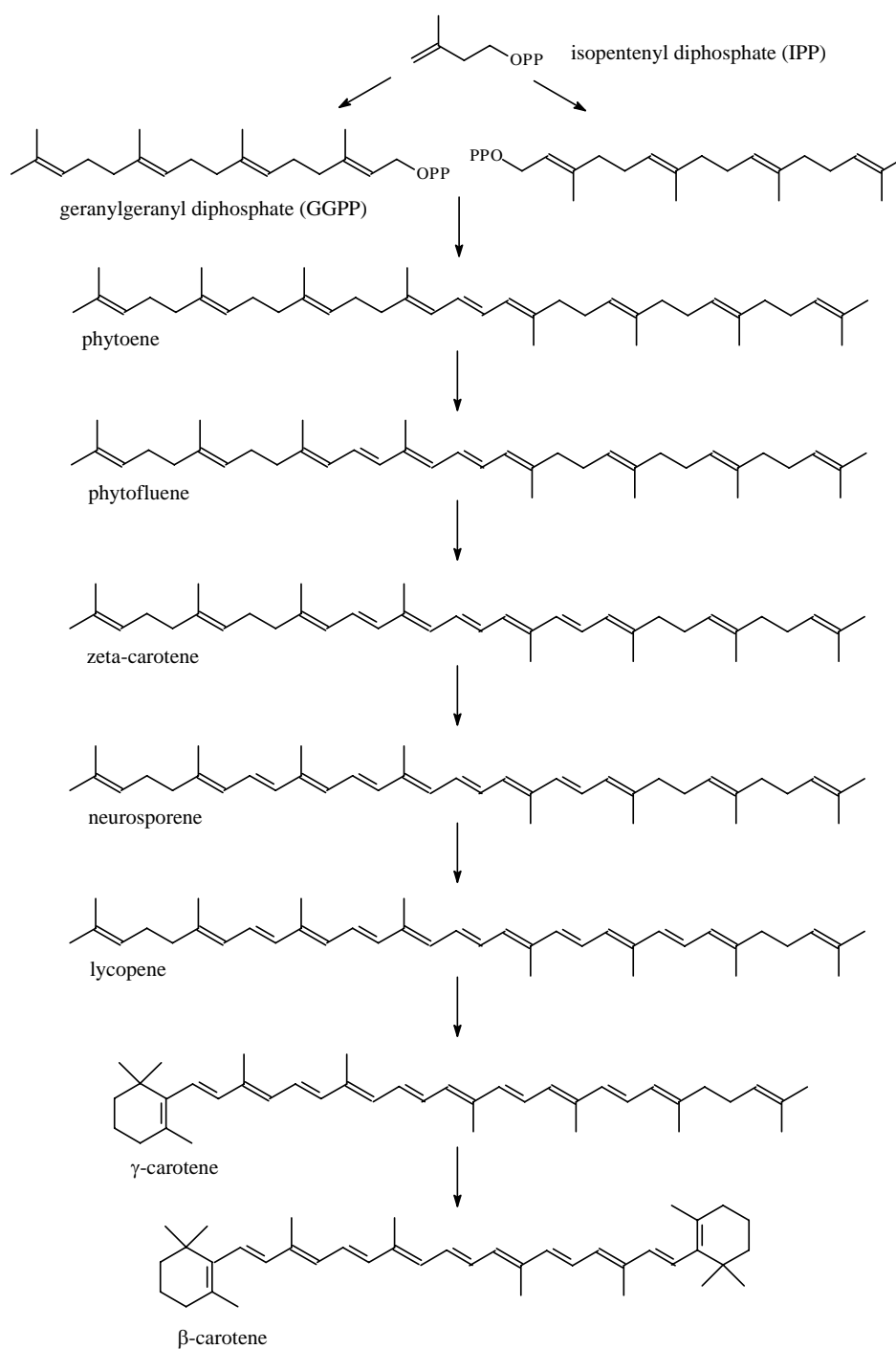
3.3 Carotenoids

Carotenoids are a class of natural fat-soluble pigments principally found in plants, algae, and photosynthetic bacteria, where they play a crucial role in the photosynthetic process. They also occur in some non-photosynthetic bacteria, yeasts, and molds,

where they may carry out a protective function against damage by light and oxygen. Although animals appear to be incapable of synthesizing carotenoids, many animals incorporate carotenoids from their diet. Within animals, carotenoids provide bright coloration, serve as antioxidants, and can be a source for vitamin A activity [Ong et al., 1992], [Britton, 1995b]. Carotenoids are responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colors of some birds, insects, fish, and crustaceans. Some familiar examples of carotenoid coloration are the orange of carrots and citrus fruits, the red of peppers and tomatoes, and the pink of flamingoes and salmon [Pfander, 1992]. Some 600 different carotenoids are known to occur naturally [Ong and Tee, 1992], and new carotenoids are continuously identified [Mercadante et al., 1999].

3.3.1 Carotenoid biosynthesis

Carotenoids, being terpenoids, are synthesized from the basic C₅-terpenoid precursor, isopentenyl diphosphate (IPP) (Scheme 2). This compound is converted to geranylgeranyl diphosphate (GGPP). The dimerization of GGPP leads to phytoene (7,8,11,12,7',8',11',12'-octahydro- γ,γ -carotene) and the stepwise dehydrogenation via phytofluene (15Z,7,8,11,12,7',8'-hexahydro- γ,γ -carotene, zeta-carotene (7,8,7',8'-tetrahydro- γ,γ -carotene), and neurosporene (7,8-dihydro- γ,γ -carotene) gives lycopene. Subsequent cyclizations, dehydrogenations, oxidations, etc., lead to hundreds of different natural carotenoids. Carotenoids are defined by their chemical structure. The hydrocarbon carotenoids are known as carotenes, while oxygenated derivatives of these hydrocarbons are known as xanthophylls. Beta-carotene, the principal carotenoid in carrots, is a familiar carotene, while lutein, the major yellow pigment of marigold petals, is a common xanthophyll (Scheme 2).



Scheme 2: Biosynthesis of β -carotene

3.3.2 Carotenoid functions

Besides their ubiquitous occurrence the various biological properties and functions of the carotenoids are the main reason for the importance of this class of compounds [Krinsky, 1993]. In photosynthesis the energy transfer involves the direct excitation of carotenoids by light to form the first excited singlet state, and the subsequent transfer

of this excitation energy to chlorophyll to initiate the process of photosynthesis. This type of process can effectively extend the wavelength of light available to an organism for photosynthesis. Carotenoids also play a major role in the photoprotection of cells and tissues. This ability is the result of energy transfer reactions in which the energy of triplet state sensitizers or singlet oxygen is transferred to carotenoid molecules in the ground state, forming triplet state carotenoid molecules. The energy acquired by the carotenoids is then lost as heat and the ground state carotenoid is regenerated to undergo another cycle of photoprotection.

In human beings, carotenoids can have several important functions. The most widely studied and well-understood nutritional role for carotenoids is their provitamin A activity. Deficiency of vitamin A is a major cause of premature death in developing nations, particularly among children. Vitamin A, which has many vital systemic functions in humans, can be produced within the body from certain carotenoids, notably beta-carotene [Britton, 1995b]. Dietary beta-carotene is obtained from a number of fruits and vegetables, such as carrots, spinach, peaches, apricots, and sweet potatoes [Mangels et al., 1993]. Other provitamin A carotenoids include alpha-carotene (found in carrots, pumpkin, and red and yellow peppers) and cryptoxanthin (from oranges, tangerines, peaches, nectarines, and papayas). Carotenoids also play an important potential role in human health by acting as biological antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Lycopene, the hydrocarbon carotenoid that gives tomatoes their red color, is particularly effective at quenching the destructive potential of singlet oxygen [Di Mascio et al., 1989]. Lutein and zeaxanthin, xanthophylls found in corn and in leafy greens such as kale and spinach, are assumed to function as protective antioxidants in the macular region of the human retina [Snodderly, 1995]. Astaxanthin, a xanthophyll found in salmon, shrimp, and other seafoods, is another naturally occurring xanthophyll with potent antioxidative properties [Di Mascio et al., 1991]. Other health benefits of carotenoids potentially related to their antioxidative potential include enhancement of immune system function [Bendich, 1989], protection from sunburn [Mathews-Roth, 1990], and inhibition of the development of certain types of cancer [Nishino, 1998].

3.3.3 Industrial aspects and synthesis

In recent years, carotenoids have gained outstanding importance in the nutraceutical and OTC fields as many studies confirm their anti-oxidative properties and associated effects on the health status of elder people. In many industrialized countries, the population is aging and with an increasing standard of living, health consciousness also increases, which drives the markets for health products. Carotenoids play an outstanding role in that sector. According to a study from Business Communications Co., Inc. (www.bccresearch.com) RGA-110 the worldwide carotenoid market has estimated to be worth \$786 million in 1999. With an estimated AAGR (average annual growth rate) of 2.9 %, this market is expected to reach \$935 million by 2005 (Tab. 2).

	1999	2005
Food	209	235
Feed	462	527
Pharma	115	173
Total	786	935

Tab. 2: Global market for carotenoids by application segments, 1999-2005 (\$ Millions)

The most important types of carotenoids are astaxanthin and β -carotene, accounting about 28% of total sales each. The third largest carotenoid is canthaxantin with 19% of total sales. All other carotenoids (Fig. 6) show sale volumes in the range of \$10 million to \$80 million. The production of carotenoids is still dominated by chemical synthesis, and although substantial efforts were undertaken to produce carotenoids by fermentation or extraction, the importance of these methods remains negligible.

With the application of molecular genetic techniques in combination with other biochemical and chemical approaches there are now exciting perspectives for rapid progress in this field. The benefits of this approach are not purely academic. The industrial production of natural carotenoids through microbial biotechnology is already established and expanding, mainly through the exploitation of some microalgae (particularly *Dunaliella*) which can synthesize large amounts of carotenoids.

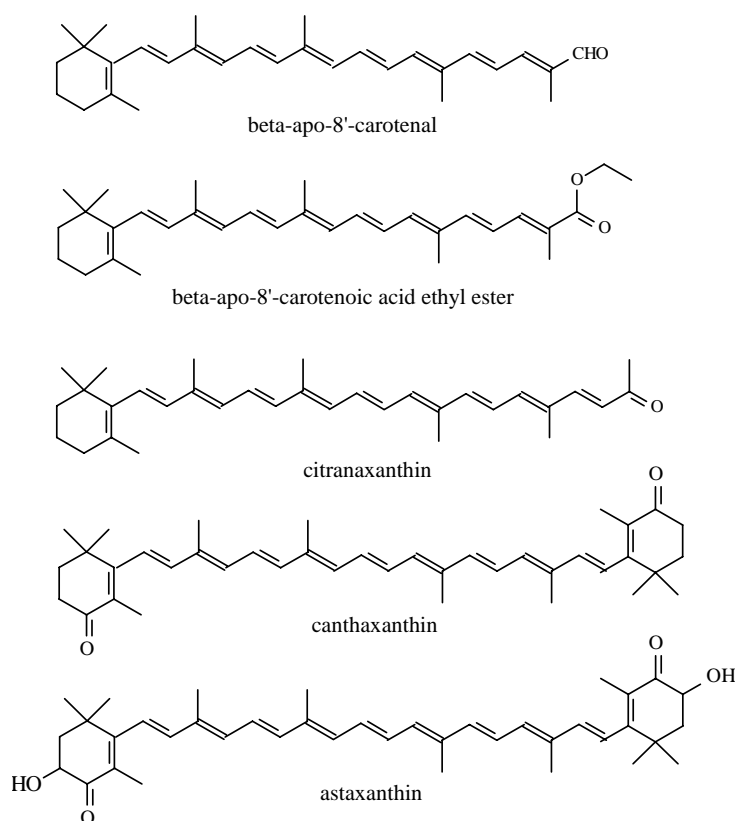
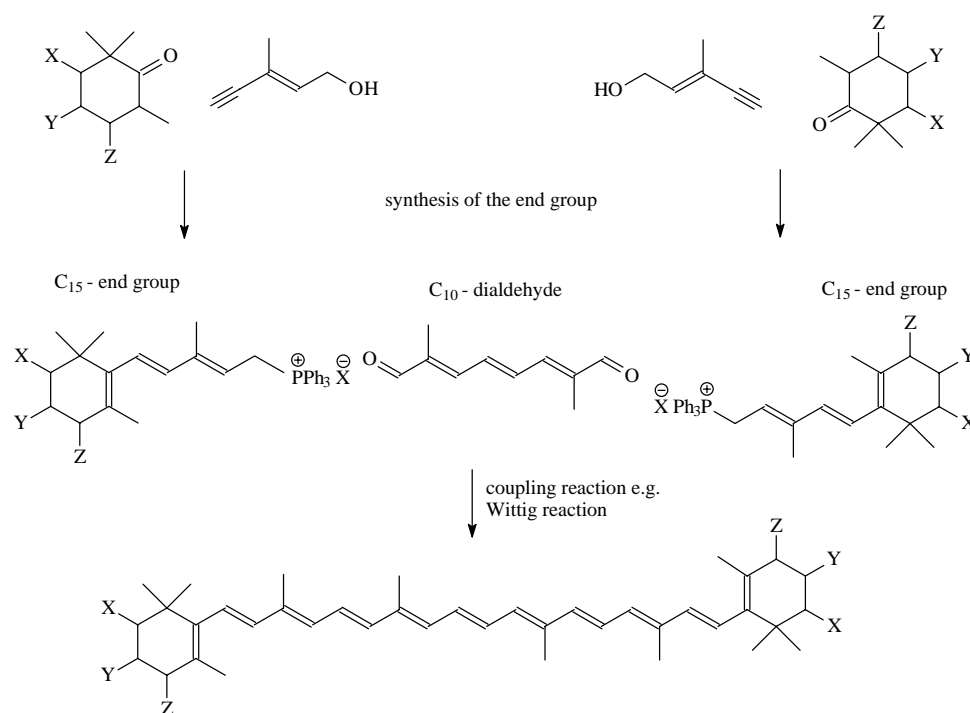


Fig. 6: Commercially important carotenoids

The first synthesis of beta-carotene was reported independently by Karrer, Inhoffen and Milas [Milas et al., 1950]. The Inhoffen synthesis was later developed into an industrial process and since 1954 beta-carotene has been produced commercially. Modern synthetic organic chemistry would permit the preparation of carotenoids by an almost countless number of strategies. However, in reality the few methods and strategies that became established early last century have been used repeatedly and only a few of these have been developed to the scale of industrial syntheses. Due to the great structural diversity of carotenoids, it is not possible to develop a general concept. Thus, an appropriate strategy is essential for a total synthesis of each individual target carotenoid. Since industry tends to evaluate systematic approaches to the major carotenoids, there has been a tendency to unify the strategies and base them on just a few general building blocks which, after slight modification, allow the preparation of various available key starting materials such as 6-oxoisophorone (Roche) or trimethylcyclohexanone (BASF).

The general strategy for the synthesis of a symmetrical C₄₀-carotenoid with identical cyclic end groups is presented in Scheme 3:



Scheme 3: General strategy for the synthesis of C₄₀-carotenoid

These approaches form the basis of the procedures developed in the early 1960s and of the present day industrial syntheses of such carotenoids as astaxanthin and zeaxanthin [Widmer et al., 1981], [Widmer et al., 1990].

3.3.4 Biotechnological approaches to carotenoids

Carotenoids have been successfully synthesized in non-carotenogenic fungi. The first attempts were made with *Saccharomyces cerevisiae* which was transformed with the carotenogenic genes from *Erwinia uredovora* for the production of lycopene [Yamano et al., 1994]. Since then, *Candida utilis* has been developed systematically as a production host for lycopene, β -carotene and astaxanthin. The engineered strain yielded 7.8 mg lycopene per g dry weight [Misawa et al., 1997]. *E. coli* is another convenient host for heterologous carotenoid production [Sandmann et al., 1999]. Most of the carotenogenic genes from bacteria, fungi and plants can be functionally expressed in this bacterium. With the development of new and powerful molecular biology methods, e.g. plasmids belonging to different incompatibility groups with different antibiotic resistance markers, one can not only increase the production of rare carotenoids in novel hosts, but also evolve new activities in existing and recombined pathways. In fact novel carotenoids have been obtained by combination

of genes from different organisms that follow different branches of the pathway. For example, this combinatorial approach yielded 1-hydroxylated acyclic structures with up to 13 conjugated double-bonds [Albrecht et al., 2000]. Another way for the synthesis of novel molecules is molecular pathway breeding, including mixing genes and modifying catalytic functions by *in vitro* evolution. With this method two phytoene desaturases from two *Erwinia* species were shuffled [Schmidt-Dannert et al., 2000] generating a recombined gene and obtaining an enzyme which introduced six instead of four double bonds.

Industry utilizes *Dunaliella bardawil* to produce β -carotene on algal farms with more than 10% of the dry weight in the form of β -carotene [Ben-Amotz et al., 1990]. *Dunaliella salina* has been the subject of extensive biochemical analyses in the laboratory, and became the likely organism to find wide application in mass culture and production in photobioreactors as commercially viable sources of lutein and zeaxanthin. The production of zeaxanthin by bacterial species of the genus *Flavobacterium* has been described and disclosed in several patents (U.S. Pat. No. 3,891,504, U.S. Pat. Nos. 3,841,967; 3,951,742; and 3,951,743, U.S. Pat. No. 4,026,949). The optimized process (U.S. Pat. No. 5,308,759) provides, dependent on the strain and on the medium used, up to 500 mg zeaxanthin per l culture, at lower costs and more rapidly than known methods and microorganisms.

Commercial production of astaxanthin from the microalga *Haematococcus pluvialis* is a growing business worldwide, primarily due to the rapid growth of this microorganism and its high astaxanthin content. Other commercial ventures for natural astaxanthin production utilize fermentation of the pink yeast *Xanthophyllomyces dendrorhous* or extraction of the pigment from by-products of crustacea such as the Antarctic krill (*Euphausia superba*). A fermentative process using the yeast *Phaffia rhodozyma* has also been established for astaxanthin production, yielding 10.4 mg astaxanthin per l culture.

3.4 Aim of the work

The focus of this work was the isolation, cloning, expression and biochemical characterization of a novel P450 monooxygenase from *Thermus thermophilus* HB27. Enzymes from thermophilic bacteria are interesting for basic as well as for applied research. In fact these molecules may contribute to a better understanding of the thermostability, and are also suitable for biotechnological processes. To date only one thermostable P450 (CYP119) has been characterized [McLean, 1998] and the crystal structure determined [Yano et al., 2000]. A comparison of the CYP119 structure with mesophilic P450s indicated that a ladder of aromatic residues may partly account for the enhanced thermostability of CYP119. The discovery that *T. thermophilus* also produces a P450 provides a second example of a thermostable P450. A detailed characterization of CYP175A1 is important for understanding P450 thermostability if the two thermostable proteins share common structural features which can be associated to such feature. No information concerning the natural substrates and the function of this novel P450 are to date available, therefore one of the targets of this work was the screening for substrates, which may be of industrial interest. Additionally the application of a P450 protein as a biocatalyst in a bioreactor is also a challenging goal, since to date it has not effectively been attained.

4. Materials and methods

4.1 Instruments

Instrument	Characteristics	Company
Agarose gel electrophoresis	DNA Sub Cell™ Mini Sub™ DNA Cell Mini Sub™ Cell GT Video Copy Processor P66E BWM 9X Monitor UV-lamp table	BioRad Mitsubishi Javelin Electronics MWG-Biotech
Balances	Basic, MCI Research RC 210 D Precision Advanced	Sartorius OHAU®
Centrifuges	Eppendorf Centrifuge 5417 C Eppendorf Centrifuge 5417 R Universal 30F KR 22 i (Rotor: AK 500-11, 155 mm) G412 Sorvall RC – 5B (Rotor: SA 600)	Eppendorf Hettich Jouan Jouan Du Pont Instruments
Electroporation	Gene Pulser®, Pulse Controller	BioRad
Incubators	WTE HT-incubator Certomat R Incubator UM 500	Binder Infors AG Braun Memmert
Microwave oven	Micro-Chef FM A935	Moulinex
Power supplies	Power Pac 3000 Power Pac 300 Model 200/2.0 Power supply	BioRad
PCR-Cycler	Master Cycler Gradient Robocycler® Gradient 40	Eppendorf Stratagene®
pH-Meter	Digital pH Meter pH525	WTW
Polyacrylamide gel	Minigel-Twin G42	Biometra®

electrophoresis	Model 583 Gel Dryer	BioRad
Rotavapor	Rotavapor R-134	Büchi
Sonifier	Sonifier 250	Branson
	Sonorex Super RK 514 H	Brandelin
Spectrophotometer UV/VIS	Ultrospec 3000	Pharmacia Biotech
	Cary 3E UV-VIS	
Vacuum concentrator	SpeedVac Concentrator 5301	Eppendorf
Vortex	Vortex Genie 2	Scientific Industries
Thermomixer	Thermomixer 5436	Eppendorf

4.2 Materials

Supplier	Material
ARK scientific GmbH, Darmstadt	Oligonucleotides
Applichem, Darmstadt	IPTG
BIO-RAD Laboratories, Richmond, USA	LMW protein standard
Boehringer Mannheim, Mannheim	Restriction endonucleases, T4-DNA ligase, RNase (DNase-free), CIAP (calf intestinal alkaline phosphatase)
Calbiochem, Darmstadt, Germany	Hydroxylapatite, fast flow
DIFCO-laboratories, Detroit, USA	Yeast extract, Tryptone, Bacto-tryptone, Bacto-yeast extract
Fluka Chemie, Buchs, Schweiz	Agar, Ampicillin (Na-salt), Chloroamphenicol, Tetracycline, Bromphenolblue, Coomassie Brilliant Blue, EDTA, Ethidium bromide, Lysozyme, NaCl, PEG 6000, SDS, Tris-(hydroxymethyl)-aminomethane, Triton [®] X-100, Na ₂ HPO ₄ , NaH ₂ PO ₄ , K ₂ HPO ₄ , KH ₂ PO ₄ , NaOH, KAc, Acetic acid, β-carotene, Sodium dithionite

Gibco BRL GmbH, Eggenstein	1 kb-Ladder (DNA standard)
MBI Fermentas, St. Leon-Rot	Restriction endonucleases, T4- DNA ligase, <i>Taq</i> -polymerase
Merck, Darmstadt	Glacial acetic acid, TLC plates (0.2 mm, Silica 60 F ₂₅₄), HPLC water
MWG, Ebersberg	DNA sequencing
Pharmacia LKB, Freiburg	Phenyl sepharose, fast flow (low substrate)
Qiagen GmbH, Hilden	Midi Plasmid Kit, Spin Mini Plasmid Kit, QIAquick Gel Extraction Kit, RT-PCR Kit, RNEasy Kit
Riedel-de-Haën, Seelze, Germany	Acetone, Methanol, Diethylether, Dichloromethane, DMSO, Ethylacetate, Glycine
Schleicher & Schuell, Dassel	Paper filters
Serva Feinbiochemika GmbH, Heidelberg	Agarose
Sigma Chemie GmbH, Deisenhofen	Ammoniumpersulfate, DMF, PMSF
Stratagene, Heidelberg, Germany	<i>Pfu</i> -polymerase, <i>Pfu</i> turbo-polymerase
Whatman international Ltd., Maidstone, England	DE52 (anion exchange chromatography matrix)

4.3 Buffers and commonly used solutions

4.3.1 Common buffers

<i>TE buffer:</i>	Tris	10	mM
	EDTA	1	mM
			pH 8.0
<i>TSS buffer (Chung et al., 1989):</i>	PEG 6000	10	% (w/v)
	DMSO	5	% (w/v)
	MgCl ₂	5	% (w/v)
	LB medium up to 100 ml		pH 6.5

<i>Potassium Phosphate Buffer:</i> (0.1 M, pH 7.4)	K ₂ HPO ₄ 1M	80.2	ml
	KH ₂ PO ₄ 1M	19.8	ml
	H ₂ O	900	ml

4.3.2 Buffers for mini preparation of plasmid DNA

<i>Resuspension buffer:</i>	Tris/HCl (pH 7.5)	100	mM
	EDTA	10	mM
	RNase I	250	µg/ml
<i>Lysis buffer:</i>	NaOH	200	mM
	SDS	1	% (w/v)
<i>Neutralization buffer:</i>	potassium acetate	29.4	g
	acetic acid	11.5	ml
	H ₂ O	28.5	ml

4.3.3 Buffers and solutions for agarose gel electrophoresis

<i>1 % agarose gel:</i>	Agarose	4.0	g
	1 x TAE (Tris-acetate)	400	ml
<i>50 x TAE buffer:</i>	Tris base	242	g/l
	glacial acetic acid	57	ml
	EDTA (0.5 M, pH 8.0)	100	ml
<i>6 x DNA loading buffer:</i>	Glycerol	30	% (w/v)
	bromphenol blue	0.2	% (w/v)
	EDTA (pH 7.5)	5	mM

4.3.4 Buffers and solutions for polyacrylamide gel electrophoresis

<i>4 x Lower Tris (200 ml):</i>	Tris base	36.46	g
	SDS	0.8	g
		pH 8.8	
<i>4 x Upper Tris (200 ml):</i>	Tris base	12.11	g

	SDS	0.8	g
		pH 6.8	
<i>Resolving gel (12.5 %)</i>	acrylamide bisacrylamide 37.5:1 (30 %)	3.33	ml
	Lower Tris 4 x	2.0	ml
	H ₂ O _{bidest.}	2.67	ml
	TEMED	4.0	μl
	ammonium persulfate (10 % (w/v))	40	μl
<i>Stacking gel (3.9 %)</i>	acrylamide bisacrylamide 37.5:1 (30 %)	0.52	ml
	upper Tris 4 x	1.0	ml
	H ₂ O _{bidest.}	2.47	ml
	TEMED	4.0	μl
	ammonium persulfate (10 % (w/v))	40	μl
<i>5 x Tris glycine buffer</i>	Tris	15	g/l
	glycine	72	g/l
	SDS	10	% (w/v)
		pH 8.3	
<i>6 x loading buffer</i>	Tris/HCl (pH 6.8)	320	mM
	glycerol	50	% (v/v)
	SDS	10	% (m/v)
	β-mercaptoethanol	25	% (m/v)
	bromophenol blue	0.1	% (m/w)
Staining with Coomassie Brilliant Blue:			
<i>staining solution:</i>	Coomassie Brilliant Blue	0.1	% (m/v)
	R-250		
	methanol	30	% (v/v)

<i>destaining solution:</i>	glacial acetic acid	10	% (v/v)
	methanol	30	% (v/v)
	glacial acetic acid	10	% (v/v)

4.3.5 Antibiotics and others

Compound	Stock solution	Solvent	Working concentration
Ampicillin (Amp)	100 mg/ml	H ₂ O _{bidest}	100 µg/ml
Chloramphenicol (Cm)	50 mg/ml	Ethanol	50 µg/ml
IPTG	1 M	H ₂ O _{bidest}	1 mM

4.4 Bacterial strains

Microorganism	Strain	Genotype
<i>Escherichia coli</i>	DH5α	<i>Sup E44</i> <i>ΔlacU169 (Φ80lacZΔM15)</i> <i>hsdR17 recA1 endA1 gyrA96</i> <i>relA1</i>
<i>Escherichia coli</i>	JM109	<i>E14-(McrA-) recA1 endA1</i> <i>gyrA96 thi-1 hsdR17(rk- mk+)</i> <i>Sup E44 relA1 Δ(lac-proAB)</i> <i>[F' traD36 proAB lacI^fZ ΔM15]</i>
<i>Escherichia coli</i>	BL21(DE ₃)	<i>E. coli B F- dcm+ Hte ompT</i> <i>hsdS(r_B- m_B-) gal l (DE3) endA</i> <i>Tet^r</i>
<i>Escherichia coli</i>	BL21-CodonPlus(DE ₃)-RP	<i>F- ompT hsdS(r_B- m_B-) dcm+</i> <i>Tet^r gal l (DE3) endA Hte [argU</i> <i>proL Cam^r]</i>
<i>Thermus thermophilus</i>	DSM 674	

4.5 Vectors

Vector	Size [kb]	Properties
pUC18	2.7	Amp ^r , LacZ [Vieira et al., 1982]
pKK223-3	4.6	Amp ^r , P _{tac} [Nock et al., 1995]
pCYTEXP1	5.0	cIts857, Amp ^r , P _{RP_L} [Belev et al., 1991]

4.6 Oligos used for the PCR reactions

Oligonucleotides were dissolved in TE Buffer to a final concentration of 100 pmole/ μ l.

Name	Sequence 5' to 3'
Tt_NdeI_F:	CGA AGC TCA TAT GAA GCG CCT TTC CCT GAG
Tt_EcoRI_R:	GCG AAT TCA CGC CCG CAC CTC CTC CCT AGG
Tt_EcoRI_F:	CCG GAA ATT CAT GAA GCG CCT TTC CCT GAG G
Tt_PstI_R:	CCA TGC ATT GGT TCT GCA GTC ACG CCC GCA CCT CCT CCC TA
pKK223_F:	GTT GAC AAT TAA TCA TCG GCT CGT ATA ATG
pKK223_R:	CAG ACC GCT TCT GCG TTC TGA TTT AAT CTG
qc_gly_F:	CAG AGG CTC GGA CTG GGG CGG GAC TTC
qc_gly_R:	GAA GTC CCG CCC CAG TCC GAG CCT CTG
Tt_16S_F:	TTG TAG TGG GCA TTG TAG CAC G
Tt_16S_R:	GGA AGA GGG GGA CAA CCC
Tt_CrtB_F:	GAA AAT GCC CGC AAG TAT GGA GC
Tt_CrtB_R:	TCA GGG GCT TCC CTC CGG
Tt_RNA_F:	GCG CCT TTC CCT GAG GGA GG
Tt_RNA_R:	ACC TCC TCC CTA GGC CGC GC
Tt_DNA_F:	GCC CCC TGG TGC GCC GTT AA
Tt_DNA_R:	AGG GGC GGG AGG AGG AAG AC

4.7 Molecular biological methods

4.7.1 Isolation of plasmid DNA from *E. coli* with Mini/Midi-Prep kit (Qiagen)

Highly pure plasmid DNA was obtained by means of QIAprep[®] kit. This procedure is based on alkaline lysis of bacterial cells [Birnboim et al., 1979], followed by purification of the DNA by ion-exchange chromatography.

Procedure:

After harvesting the cells from an overnight culture by centrifugation, the plasmid isolation was carried out according to the instructions of the manufacturer. Depending on the amount of DNA, either QIAprep Spin Miniprep kit or QIAGEN Midi Plasmid Purification kit were used.

4.7.2 Mini preparation of plasmid DNA for fast tests

Plasmid DNA obtained by this method was normally used to screen transformants for cloned products. After isolation plasmid DNA could be properly digested in order to verify the presence of the desired gene. The procedure was also based on alkaline lysis of bacterial cells.

Procedure:

1.5 ml from an overnight culture were centrifuged (1 min, 5000 rpm, r.t.). The supernatant was removed and the cell pellet was resuspended in 200 µl resuspension buffer. The cells were then lysed by addition of 200 µl lysis buffer. After neutralization (200 µl neutralization buffer) proteins and genomic DNA were precipitated and separated by means of centrifugation (5 min, 14000 rpm, r.t.). The supernatant was removed and plasmid DNA precipitated with isopropanol (420 µl) for 10 min at room temperature. The mixture was centrifuged (20 min, 14000 rpm, 4 °C) and the DNA pellet separated and washed with 200 µl 70 % ethanol. After centrifugation and solvent removal the pellet was dried in vacuum, resuspended in 20 µl TE buffer and stored at -20°C.

4.7.3 Purification/Concentration of DNA with ethanol and isopropanol

The most widely used method for concentrating DNA is precipitation with alcohols. The precipitated DNA, which is formed in the presence of monovalent cations, is recovered by centrifugation and resuspended in an appropriate buffer.

Isopropanol precipitation can be used together with ethanol precipitation in order to reduce the volume of dilute solutions to the point where the DNA can easily be recovered by precipitation with ethanol.

Isopropanol precipitation

Procedure:

1 vol. of DNA solution was mixed with 0.7 vol. isopropanol. The mixture was kept for 15 min at r.t. and then centrifuged (15 min, 14000 rpm, r.t.). The supernatant was removed, the pellet washed with 200 μ l 70% ethanol, and recovered by centrifugation. Finally the DNA was dried and resuspended in water or TE buffer.

Ethanol precipitation

Procedure:

To 1 vol. of DNA solution 1/10 vol. 3M NaAc, pH 4.8 and 2.5 vol. 100 % ethanol (-20 °C) were added. The mixture was kept for 30 min at -20 °C, then the DNA was recovered by centrifugation (30 min, 14000 rpm, 4 °C). The supernatant was removed, the pellet washed with 200 μ l 70% ethanol and recovered by centrifugation. Finally the DNA was dried and resuspended in water or TE buffer.

4.7.4 DNA digestion with restriction endonucleases

DNA digestions were carried out according to the instructions of the manufacturer. Normally 0.1-10 μ g DNA was restricted by 1-8 U of enzyme endonuclease. Specific enzyme buffers provided with the enzymes were used to achieve the best results, and finally water was added so that the salt concentration met the specifications. Double digestions were performed if the enzymes (optimal buffer and temperature) were compatible. Restriction digestions were performed in 10-50 μ l scale. Incubation times and temperatures were depended on the enzymes used, typically 2-16 hours at 37 °C. After incubation the restriction endonucleases were denatured by incubating the mixture at 72 °C for 10 minutes. The digested DNA was analyzed by agarose gel electrophoresis and isolated by gel extraction.

4.7.5 Dephosphorylation of DNA

In cloning experiments one of the most common factors decreasing cloning efficiency is the recircularization of the vector during ligation without insertion of foreign DNA. One way to prevent religation is to treat the cleaved vector with a phosphatase which removes the 5' phosphate group from the vector. Since ligation reactions absolutely

need a 5' phosphate group, this treatment effectively blocks the religation of the vector as the required phosphate group can only come from the DNA insert. The terminal 5' phosphates can be removed from DNA by treatment with calf intestinal alkaline phosphatase (CIAP), which can afterwards be completely heat inactivated.

Procedure:

CIAP (1 U) was added to the digestion mixture and incubated 1 hour at 37 °C and finally denatured by treatment at 72 °C for 15 minutes. Purification of the DNA after dephosphorylation was performed by means of gel extraction.

4.7.6 *Ligation of DNA*

Ligation of a stretch of DNA to a linearized plasmid vector involves the formation of new bonds between phosphate residues located at the 5' termini of double-stranded DNA and adjacent 3'-hydroxyl moieties. When both strands of the plasmid vector carry 5'-phosphate residues, four new phosphodiester bonds are generated. The formation of phosphodiester bonds between adjacent 5'-phosphate and 3'-hydroxyl residues can be catalyzed *in vitro* by DNA ligases.

Procedure:

A 3-5 fold molar excess of foreign DNA was mixed with the plasmid after digestion. Ligase buffer (5 µl), T4 ligase (1 U) and finally water up to 50 µl were added to the mixture, which was incubated 1 hour at room temperature.

4.7.7 *Agarose gel electrophoresis*

Agarose gel electrophoresis is an effective method for analytical as well as preparative separation of DNA molecules depending on their size. Due to the negative charge of DNA molecules, they migrate in an electrical field towards the anode. The migration rate depends on the size of the molecule itself. Larger molecules migrate more slowly than smaller molecules because of greater frictional drag. The rate of migration also depends on other parameters such as the size of the pores and the conformation of the DNA. Ethidium bromide, a fluorescent dye, is used in order to

detect DNA in agarose gel. It intercalates between stacked base pairs and upon ultraviolet illumination makes DNA visible.

Preparation:

Agarose powder was added to 1 x TAE buffer to a final concentration of 1 % (w/v). The slurry was heated in a microwave oven until the agarose was dissolved. The agarose solution was cooled to 60 °C and then ethidium bromide was added (1 µl 1 % solution to 10 ml agarose). The warm agarose was poured into the mold and the comb was properly positioned. After the gel was completely set, it was transferred into the electrophoresis tank and covered with 1 x TAE buffer. DNA samples were mixed with DNA loading buffer (5 vol. DNA solution + 1 vol. DNA loading buffer) and loaded into the slots of the gel. As a size standard 1 kb-ladder was used. Electrophoresis was performed at 120 V for 30 min. The gel was examined by ultraviolet light and documented.

4.7.8 *Isolation of DNA fragments from agarose gel*

After PCR or digestion and agarose gel electrophoresis, the DNA was isolated from the agarose by means of gel extraction. The band corresponding to the desired DNA fragment was cut using a scalpel, then the agarose piece was treated as described in the QIAquick Gel Extraction kit.

4.7.9 *Isolation of total RNA from Thermus thermophilus HB27*

For the isolation of high quality total RNA the RNeasy Mini kit (Qiagen) was used. After lysozyme digestion of the bacterial cell wall and lysis, RNA is bound to a silica-gel-based membrane, while the contaminants are efficiently washed away.

Procedure:

An overnight culture of *Thermus thermophilus* was used to inoculate fresh appropriate medium. The cells were grown at 65 °C to OD 0.6. 2 ml of culture was harvested and the pellet treated according to the instructions of the manufacturer. After elution the RNA was incubated for 30 min at 37 °C with RNase free DNase (1 U) in the specific

buffer. The DNase was heat inactivated (15 min at 65 °C) and finally the RNA was purified by means of RNeasy column.

4.7.10 Quantitation of DNA and RNA

A simple and accurate method for quantitating the DNA concentration is based on the spectrophotometric measurement of the absorption in the ultraviolet range. Readings should be taken at 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA and 40 µg/ml for single-stranded DNA and RNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure DNA and RNA preparations have OD_{260}/OD_{280} values between 0.8 and 2.0.

4.7.11 In vitro amplification of DNA by the polymerase chain reaction (PCR)

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of known sequence. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalysed by a DNA polymerase. The template DNA is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotides to anneal to their target sequence, then the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is then repeated many times. Because the products of one round of amplification serve as template for the next, each successive cycle essentially doubles the amount of the desired DNA product.

Procedure:

Plasmid DNA (Spin Mini Prep) was used as DNA template. Polymerase specific buffer was used. The components of the mixture were pipetted together according to the following general scheme:

Component	Volume [μ l]	Final concentration
10 x polymerase buffer	10	1 x
DMSO	10	10 % (v/v)
dNTP mix (containing 10 mM of each dNTP)	4	0.1 mM
sense primer	2	0.2 μ M
antisense primer	2	0.2 μ M
DNA template	variable	ca. 100 ng
DNA polymerase	1	2.5 U
H ₂ O	variable	-
Total volume	100	

PCRs were performed in a thermocycler (Eppendorf) using the following general program:

Cycle	Denaturation	Annealing	Extension
Initial denaturation	95 °C, 1 min		
PCR cycles (30)	95 °C, 1 min	55 °C, 1 min 30 sec	72 °C, 1 min 30 sec
Final extension			72 °C, 4 min

4.7.12 Quick change kit for site-directed mutagenesis

In vitro site-directed mutagenesis was carried out with the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). The basic procedure utilizes a circular, double stranded DNA vector with an insert of interest and two synthetic complementary oligonucleotide primers containing the desired mutation. By elongation of the annealed oligonucleotide primers a mutated plasmid is generated using temperature cycling and *Pfu* DNA polymerase. Following the temperature cycling the product is treated with *DpnI*. This endonuclease, specific for methylated and hemimethylated DNA, is used to digest template DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore, in contrast to the newly synthesized strands, susceptible to *DpnI* digestion.

Procedure:

Plasmid DNA (Spin Mini Prep) was used as DNA template. The components of the mixture were pipetted together according to the following general scheme:

Component	Volume	Final concentration
ddH ₂ O	variable	-
10 x <i>Pfu</i> buffer	5.0 µl	1x
DMSO	5.0 µl	10 % (v/v)
dNTP Mix (containing 10 mM of each dNTP)	1.0 µl	200 µM of each dNTP
Primer A	1.1 µl	0.022 µM
Primer B	1.1 µl	0.022 µM
<i>Pfu</i> DNA polymerase	1.0 µl	2.5 U/µl
Template DNA	variable	5 – 50 ng
Total volume	50 µl	-

PCRs were performed in a thermocycler (Eppendorf) using the following general program:

Cycle	Denaturation	Annealing	Extension
Initial denaturation	95 °C, 1 min		
PCR cycles (30)	95 °C, 30 sec	65 °C, 1 min	68 °C, 2 min/1 kb
Final extension			68 °C, 4 min

4.7.13 RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) consists of two parts: synthesis of cDNA from RNA by reverse transcription (RT) and amplification of a specific cDNA by polymerase chain reaction (PCR).

RT-PCR is a method of RNA detection and can also be used for cDNA library construction, probe synthesis, differential display, and signal amplification in *in situ* hybridizations.

Procedure:

A master mix was generated by mixing the components according to the following scheme :

Component	Volume	Final concentration
Master mix		
RNase-free water	Variable	-
5x QIAGEN OneStep RT-PCR buffer	10 μ l	1x
dNTP Mix (containing 10 mM of each dNTP)	2.0 μ l	400 μ M of each dNTP
Primer A	3.0 μ l	0.6 μ M
Primer B	3.0 μ l	0.6 μ M
QIAGEN OneStep RT-PCR enzyme Mix	2.0 μ l	-
Template RNA		
Template RNA	Variable	1 pg – 2 μ g/reaction
Total volume	50 μl	-

The RT-PCR program was carried out according with the following scheme:

Reverse Transcription:	30 min	50 °C
Initial PCR activation:	15 min	95 °C
3-step cycling		
Denaturation:	1 min	94 °C
Annealing:	90 sec	55 °C
Extension:	90 sec	72 °C
Number of cycles:	30	
Final extension:	10 min	72 °C

Control reactions were carried out without reverse-transcriptase (the mixture was kept on ice until the cycler had reached 95 °C for the HotStarTaq DNA Polymerase). Alternatively, control reactions were set up adding the RNA during the HotStarTaq DNA Polymerase activation step.

4.8 Microbiological methods

4.8.1 Media

The quantities are referred to 1 l H₂O. For solid media 16 g agar were added.

<u>LB(Luria-Bertani)-medium:</u>	tryptone	10.0	g
	yeast extract	5.0	g
	NaCl	5.0	g
		pH 7.5	
<u>2 x YT medium:</u>	tryptone	16.0	g
	yeast extract	10.0	g
	NaCl	5.0	g
<u>SOC medium:</u>	tryptone	20.0	g
	yeast extract	5.0	g
	NaCl	10.0	mM
	MgCl ₂	10.0	mM
	MgSO ₄	10.0	mM
	KCl	2.0	mM
<u>Terrific broth:</u>	bacto-tryptone	12	g
	bacto-yeast extract	22	g
	glycerol	4	ml
	KH ₂ PO ₄	2.31	g
	K ₂ HPO ₄	12.54	g
		in 100 ml H ₂ O	
<u>Thermus medium:</u>	yeast extract	4.0	g
	tryptone	1.0	g
	NaCl	1.0	g

4.8.2 Transformation of *E. coli* strains by heat shock

Transformation is the alteration of the genotype of a cell by the direct introduction of foreign DNA into the cell. The cells which are capable of taking up DNA are called competent. The following method of transformation is very fast and easy and the cells can be readily frozen for later use. As in most methods of transformation the exact mechanism by which the procedure works is unclear. However, DMSO is a well known agent for permeabilizing many cell types, so its utility in aiding transformation is perhaps not surprising.

Preparation of *E. coli* competent cells by TSS method:

A fresh overnight culture of bacteria was diluted 1:100 into prewarmed LB broth and the cells incubated at 37°C with shaking (225 rpm) to an OD₆₀₀ 0.4-0.6. The cells were spinned down at 6000 rpm for 10 min in a centrifuge. They were resuspended in 1/25 volume of ice-cold TSS buffer and mixed gently. For long term storage, cells were aliquoted (200 µl), frozen immediately in liquid nitrogen and stored at -70°C.

Transformation:

1 µl DNA solution or 20 µl ligation mixture were pipetted into a 200 µl aliquot of cells and mixed gently. The mixture was incubated on ice for 30 min, and then transferred for 40 sec in a water bath that had been preheated to 42 °C. 800 µl LB medium were added, and the cells were incubated at 37°C with shaking (200 rpm) for 1 hour to allow expression of the antibiotic-resistance gene. The mixture was centrifuged for 1 min at 6000 rpm and the supernatant discarded. The pellet was resuspended in the remaining supernatant and spread on LB agar plates containing the appropriate antibiotic.

4.8.3 Transformation of *E. coli* by electroporation

Electroporation involves introduction of DNA into cells with a transient pulse of high voltage. The DC pulse is thought both to disrupt temporarily the membrane and to electrophorese DNA into cells. The efficiency of this technique is 10 to 20 times higher than that obtained with competent cells prepared by chemical methods. Transformation efficiencies of 10⁹-10¹⁰ transformants/µg of DNA have been achieved

by optimizing various parameters, including the strength of the electrical field, the length of the electrical pulse, and the concentration of the DNA [Dower et al., 1988]. The plasmid size seems not to have any effect on the transformation efficiency.

Preparation of competent *E. coli* cells for electroporation:

500 μ l of an overnight culture were used to inoculate 50 ml LB medium. Bacteria were grown to mid-log phase (OD_{578} 0.3-0.5), chilled (30 min on ice), harvested by centrifugation (15 min, 6000 rpm, 4°C) and then washed extensively with sterile ice cold 10% glycerol to reduce the ionic strength of the cell suspension. Cells could be used immediately or could be frozen in 0.2 ml aliquots in microcentrifuge tubes using a dry ice-ethanol bath. Frozen cells were stored at -70°C .

Electroporation:

1 μ l of the DNA was added to an aliquot of electrocompetent cells and mixed. 50 μ l of the cell-DNA mixture were transferred into a chilled cuvette. The cuvette was placed in the electroporation chamber and the electrical pulse was started. The sample was inoculated in 1 ml LB medium and the mixture incubated for 1 hour at 37°C. Finally the cells were harvested by centrifugation (2 min, 6000 rpm) and plated on SOC medium containing the appropriate antibiotic.

Standard conditions for <i>E. coli</i> electroporation	
Voltage	2.5 kV
Electric field strength	25 μ F
Resistance	200 Ohm

4.9 Storage and cultivation of bacteria

4.9.1 Stab culture

1 ml LB agar was autoclaved in 1.5 ml Eppendorf tube. A single colony was picked from an agar plate and stabbed by means of a sterile inoculation needle in LB agar. The vial was stored at room temperature in the dark.

4.9.2 Storage of cells

Long-term storage of cells was performed as glycerol stocks. To 1 ml of overnight culture 1 ml sterile 87 % glycerol was added. The mixture was vortexed to ensure that the glycerol was evenly dispersed and then stored at – 80 °C. To recover the bacteria, the surface of the frozen culture was scraped with a sterile inoculation needle, and then streaked onto the surface of an LB agar plate containing the appropriate antibiotics.

4.9.3 Cultivation in Erlenmayer flasks

A single colony was picked from an agar plate and inoculated in fresh liquid medium with the appropriate selection antibiotics. An overnight culture was used to inoculate 1/100 fresh medium with selection antibiotics. Liquid cultures were normally incubated at 37 °C and 65 °C for *E. coli* and *T. thermophilus* respectively. For experiments related to carotenoid production, the cultures were incubated at 30 °C.

4.9.4 Heterologous expression in *E. coli*.

Over-expression was carried out in *E. coli* BL21-CodonPlus(DE3)-RP cells and was controlled by the strong *tac* promoter. Induction was performed by addition of 1mM IPTG.

An overnight culture was used to inoculate (1:100) 2xYT medium with ampicillin (100 µg/ml). The cells were grown at 37 °C and 200 rpm to OD≈1 and then IPTG to a final concentration of 1 mM was added. After induction the culture was incubated at 30 °C and 150 rpm for 48 hours. Finally the culture was centrifuged (6000 rpm, 30 min, 4 °C) and the pellet stored at –20°C.

4.10 Protein methods

4.10.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique is used to assess the purity and estimate the molecular weight of a protein. Proteins migrate to the anode of the gel when an electric current is applied. The rate at

which the proteins move in the gel is affected by their size (i.e. smaller proteins move faster in the gel).

Preparation:

After assembling the glass plates properly, the resolving gel was prepared and poured into the gap between the glass plates and allowed to polymerize after overlaying gently with iso-propanol. After the separating gel had polymerized, the overlay was decanted, the stacking gel was prepared and poured. A comb was inserted into the stacking gel solution. After the stacking gel had polymerized, the comb was removed and the gel mounted in the electrophoresis chamber. The samples were prepared by heating them at 95°C for 5 min in SDS gel-loading buffer. Finally they were loaded up into the wells of the gel together with the LMW standard. For each gel the electrophoresis was carried out at 10 mA for 10 min and 25 mA for approximately 50 min. After electrophoresis proteins were fixed with the staining solution. The excess of dye was then allowed to diffuse from the gel during a prolonged period of destaining. After destaining, gels were dried at 80 °C under vacuum between filter paper and cellophane foil in order to be stored.

LMW standard (BIO-RAD):

Protein	Molecular weight [kDa]
Phosphorylase B	97.4
Serum albumine	66.2
Ovalbumine	45.0
Carbonic anhydrase	31.0
Trypsin inhibitor	21.5
Lysozyme	14.0

4.10.2 Cell lysis and purification

1. Cultures were harvested by centrifugation (Sorvall RC – 5B, rotor AK 500-11) and cells lysed by sonication (10 min, 40 % intensity) in lysis buffer. Cell debris was removed by centrifugation.

Lysis buffer:

Component	Concentration
KPi pH 7.5	50 mM
Emulgen (Japan)	0.1%
Phenylmethylsulfonyl fluoride (PMSF)	1 mM
EDTA	20 mM
MgCl ₂	25 mM
Lysozyme	1 mg/ml

2. Heat precipitation

Heat precipitation is a common method to roughly separate mesophilic proteins from thermophilic ones. Taking advantage of the thermal stability of proteins from thermophilic bacteria, the cell lysate of *E. coli* can be heated in order to denature the homologous protein, which precipitate, retaining the thermostable fraction in the supernatant.

Procedure:

After lysis, cell debris was removed by centrifugation at 20000 rpm (Sorvall RC-5B, rotor: AK 50-22) for 20 min. The supernatant was heated at 60-75 °C for 15 min and then the denatured proteins were removed by centrifugation at 15000 rpm (Sorvall RC-5B, rotor: AK 50-22) for 30 min.

3. Ammonium sulfate precipitation

The purpose of this procedure is to obtain the desired protein in a bulk form that can be used as the starting material for a fine fractionation. The solubility of protein depends, among other things, on the salt concentration in the solution. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule. However, as the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is decreasing amount of water available to solubilize the proteins. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules.

Procedure:

Cell free extract was brought to 35% ammonium sulfate concentration by adding solid ammonium sulfate with constant stirring at 5 °C. Precipitated proteins were removed by centrifugation and the supernatant was brought to a concentration of 50% ammonium sulfate. The resulting precipitate was centrifuged, resuspended in minimal volume, and dialyzed against 50 mM KPi, pH 7.5 overnight (3 times buffer exchange).

4. Ionic Exchange Chromatography (IEX)

IEX separates proteins with differences in charge. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic matrix. Proteins bind as they are loaded onto a column. Elution is usually performed by an increase in salt concentration or changes in pH.

Procedure:

The matrix of choice was DE52 (diethylaminoethyl cellulose, Whatman). The column with the dimensions of 2.5 cm x 20 cm was equilibrated with 50 mM KPi buffer pH 7.5. Flow rate was approximately 1 ml/min. Elution was carried out with a salt (NaCl) gradient. The dialyzed supernatant resulting from the ammonium sulphate cut, was passed over a DE52 column to help remove contaminants since CYP175A1 has limited affinity for DE52. In order to remove the little amount of P450 which weakly bound to DE52, a salt gradient was performed. 50 mM NaCl was added to the elution buffer and fractions with an absorbance ratio ($A_{418\text{nm}}/A_{280\text{nm}}$) > 1 were pooled, concentrated and dialyzed against KPi 50 mM pH 7.4. Purity and concentration of P450 were determined spectrophotometrically.

5. Hydroxylapatite Chromatography

Hydroxylapatite, a form of calcium phosphate, can be used as a matrix for the chromatography of proteins. Protein binding to hydroxylapatite is mediated through interactions between the amino and the carboxyl group of the protein and the Ca^{2+} and PO_4^{3-} groups of the matrix. Basic proteins are thought to bind via electrostatic interaction between their amino group and the surface of PO_4^{3-} groups, while acidic and neutral proteins bind to Ca^{2+} sites via their carboxyl groups. Molecular separation is not completely dependent on any single property of the molecule such as molecular

weight, size, or isoelectric point. As a general principle, proteins can be eluted by increasing the concentration of the phosphate buffer. Other elution procedures include adding Mg^{2+} or Ca^{2+} ions or NaCl or changing pH.

Procedure:

The matrix of choice was hydroxylapatite supplied by Bio Rad. The column with the dimensions of 2.5 cm x 20.0 cm was equilibrated with KPi buffer 50 mM pH 7.4. Flow rate was approximately 0.5 ml/min by gravity. Elution was carried out with a linear salt (KPi) gradient.

The protein was loaded onto the hydroxylapatite column and washed with ca. 5-6 column volumes of 50 mM KPi pH 7.4. Elution was carried out with a linear gradient of KPi pH 7.4 from 50-300 mM over 10 column volumes. Fractions with an absorbance ratio (A_{418nm}/A_{280nm}) above 1.55 were pooled, concentrated, and exchanged into 50 mM Tris pH 7.4 on a PD10 column (Pharmacia). The protein was then concentrated to 40 mg/ml and stored at -20 °C.

6. Hydrophobic Interaction Chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The separation is based on the reversible interaction between a protein and the hydrophobic surface of a chromatographic matrix. This interaction is enhanced by high ionic strength buffer which makes HIC an ideal “next step” after precipitation with ammonium sulfate. Samples in high ionic strength solution bind as they are loaded onto a column. Elution is usually performed by a decrease in salt concentration. Other elution procedures include reducing eluent polarity, adding chaotropic species or detergents, changing pH or temperature.

Procedure:

The matrix of choice was phenyl sepharose (low substrate) supplied by Amersham Pharmacia. The column with the dimensions of 5 cm x 6 cm was equilibrated with buffer A. Flow rate was 5 ml/min. Elution was carried out with a decreasing ammonium sulfate gradient and with the addition of sodium cholate.

The precipitate obtained by ammonium sulfate precipitation was resuspended in a minimal volume of KPi 20 mM and loaded onto a phenyl sepharose column. The high affinity of the desired protein for the resin allows the selective removal of the most

contaminants by washing with buffer A (ca. 5 column volumes) and with buffer B (ca. 3 column volumes) respectively. Elution was performed in buffer C (ca. 1,5 column volumes) and with buffer D (ca. 5 column volumes) respectively.

Buffer A: 20 mM KPi pH 7.4 + ammonium sulfate 20% (w/v)

Buffer B: 20 mM KPi pH 7.4

Buffer C: 20 mM KPi pH 7.4 + sodium cholate 1% (w/v)

Buffer D: 20 mM KPi pH 7.4 + sodium cholate 1.5% (w/v)

4.10.3 Spectroscopic determinations

1. Determination of P450 concentration and purity

Carbon monoxide can bind ferrous P450, inducing a shift of the maximum absorbance of the heme to 450 nm. Differential spectrophotometry can be used to calculate P450 concentration in solution [Omura and Sato, 1964a], [Omura and Sato, 1964b].

$$[\text{P450}] = (A_{450} - A_{490}) \times \text{dil.} \times 1000/\epsilon$$

Procedure:

The sample of P450 containing solution was added of a few crystals of sodiumdithionite and divided in two cuvettes. One of the aliquots was treated with CO. The absorption of the sample in the region between 400 and 500 nm was measured using as a reference the aliquot that was not treated with CO.

A visible extinction coefficient of $\epsilon_{418\text{nm}} = 104 \text{ mM}^{-1}$ was determined by extracting and measuring the total amount of heme present in a pure sample using the pyridine hemochromagen assay [Sato, 1964] and compared to the spectra obtained for the intact protein. Purity was measured by the appearance of a single band on a Coomassie blue stained SDS-PAGE and corresponded to an absorbance ratio ($A_{418\text{nm}}/A_{280\text{nm}}$) of 1.60.

2. Monitoring of substrate binding

The UV spectral changes which accompany substrate interactions with P450s have been used to indicate certain binding characteristics, or so called types of ligand binding. There are three categories: type I, type II and reverse type I. These changes,

which occur in the UV spectra following ligand-P450 complexation reactions, represent a method for classifying different varieties of P450 active site interactions [Schenkman et al., 1981]. Specifically type I spectral change entails a reduction in the Soret absorption at 420 nm and a concomitant increase in the 390 nm absorption, which may be regarded as a shift in the hemoprotein equilibrium from low- to high-spin ferric P450. Substrates which exhibit a type II spectral change appear to act primarily as inhibitors of the enzyme by ligating the heme iron, forming relatively stable, tightly bound complexes. The type II spectral change is characterized by a decrease in absorption at around 390-405 nm accompanied by an increase at 425-435 nm. The third variety of spectral change is essentially a mirror image of the type I case. Consequently, in reverse type I there is an increased absorption at 420 nm and a decrease at 390 nm. Substrates that displace the six-coordinated solvent in the resting P450 usually induce a shift to the blue which reflects a low- to high-spin transition of the iron. Differential spectrophotometry is used to monitor binding of substrates.

Procedure:

Potential substrates and purified enzyme were pipetted together according to the following general scheme:

Component	Working concentration
P450:	2.5 μ M
Substrate (*):	2.5 mM
KPi	20 mM, pH 7.4
Total volume	1 ml

(*) α -ionone and β -ionone, fatty acids (C-10 up to C-20), naphthalene, indole, styrene were added as DMSO solutions.

The absorption maximum of the Soret peak (417.5 nm) was observed for 30 min at 60 °C.

3. Determination of melting temperatures

The thermal melting temperatures were measured by recording the absorbance of the heme peak at a linear temperature rate of 1 °C/minute on a Cary 3E UV – visible spectrophotometer equipped with a temperature controller. All P450s were

irreversibly denatured. P450BM-3 consists of the heme domain only (residues 1-455). The absorbance increase at the heme peak was followed for CYP119 and CYP175A1, while the decrease in absorbance at the heme peak was followed for all other P450s. All proteins were pure to homogeneity. All measurements were carried out in 1 ml solution, in the absence of substrates in 50 mM Tris pH 7.4, except CYP175A1, which was carried out in 50 mM KPi pH 7.4. Protein concentrations ranged from about 6 to 12 μ M and corresponded to an absorbance at the heme peak of 0.8 to 1.0. The heme peak absorbance was 418 nm for P450BM3, P450eryF, P450cam and P450_Tth, while the P450 from *S. solfataricus* heme peak absorbance was 414 nm. The percentage of denatured protein was calculated based on the disappearance of the heme peak. The following equation was used :

$$\text{denaturation (\%)} = 1 - (\text{Absorbance}_{418 \text{ nm, rt}} - \text{Absorbance}_{418 \text{ nm, xt}})$$

Absorbance_{418 nm, rt} represents the absorbance at 418 nm of the protein at room temperature, while absorbance_{418 nm, xt} represents the absorbance at 418 nm measured at various temperatures.

4. Electron Paramagnetic Resonance (EPR) spectroscopy

EPR spectroscopy is a useful technique in the study of systems containing unpaired electrons. The theory of EPR as applied to hemoprotein investigations has been summarized by Palmer [Palmer, 1983], and the use of the technique in the study of P450 systems has been reviewed by Lewis [Lewis, 1986] and Weiner [Weiner, 1986]. In low-spin ferric-hemes, EPR spectra exhibit three resonant absorptions corresponding to the effect of the three non-equivalent x, y and z components of the g tensor, which is a factor governing the interaction between unpaired electron spins and the external magnetic field. This so called anisotropy in the g-factor is due to the tetragonal (axial) and rhombic distortion splitting the degeneracy of the t_{2g} orbitals under the influence of the non-symmetric ligand field experienced by the low-spin ferric iron in the heme environment.

One important feature of the EPR technique, as applied to P450s or other heme proteins, is the fact that it is possible to study transition low- to high- spin Fe^{3+} , as there are significant differences between the EPR signals which correspond to these two ferric iron spin states.

The orientations of the ligands as well as the nature of the heme axial ligands was investigated by means of EPR. The *g*-values are sensitive to such parameters and thus can provide with important information about the iron coordination sphere. The EPR spectra were recorded using Bruker EMX X-band EPR spectrometer Bruker equipped with an Oxford continuous flow helium cryostat ESR 900. Data were obtained under the following instrumental conditions: modulation amplitude, 5.00 Gauss; microwave power, 0.32 mW; frequency 9.47 GHz; modulation frequency, 100 kHz. Protein preparation used to record EPR spectra was pure to homogeneity and contained 214 μ M P450_Tth in 50 mM KPi buffer pH 7.4.

4.10.4 Heterologous complementation

The practical difficulties associated with reproducing carotenoid conversion *in vitro* have been documented [Bramley, 1985]. The carotenogenic enzymes are all membrane-associated or integrated into the membranes. Furthermore, the water solubility of carotenoids is so low that it represents a limiting factor in the characterization of the enzymes *in vitro*. Heterologous complementation using *E. coli* strains genetically engineered to produce carotenoids represents an interesting alternative [Sandmann et al., 1999] [Sandmann, 2002]. Several factors determine the potential of this host system for heterologous carotenoid production. Most of the carotenogenic genes can be functionally expressed in this bacterium. Furthermore, plasmids belonging to different incompatibility groups with different antibiotic resistance markers are available. They can all be introduced simultaneously in *E. coli* for carotenoid synthesis allowing combinations of individual genes. A combination of genes from different organisms which follow different branches of a pathway makes it possible to synthesize novel compounds. The structurally diverse carotenoids may be useful to study the relationship between structural features and antioxidative activities as well as pharmaceutical properties.

Procedure:

Host for heterologous complementation were *E. coli* JM109 cells. Plasmids (pACYC_Y and Tth-pKK) were introduced one at a time by electroporation. Cultures of *E. coli* JM109 harboring the different plasmids were grown in 50 ml LB medium at

30 °C for 48 hours. Ampicillin, chloramphenicol and IPTG (0.5 mM) were added as required.

4.10.5 Carotenoid extraction and analysis

Because of the long conjugated double bond system the carotenoids are generally sensitive to several factors, e.g., light and oxygen. Carotenoid samples should always be stored *in vacuo* or in a completely inert atmosphere.

Procedure:

Cultures were harvested by centrifugation (Eppendorf Centrifuge 5417 C) and wet cells extracted with acetone (200 µl/ml culture) and then reextracted with an equal volume of hexane after addition of 1/4 volume water.

The two layers were separated and the organic one used for analysis. TLC and HPLC were the methods of choice to identify carotenoids present in a mixture. β-Cryptoxanthin and zeaxanthin standard were provided by Roche vitamins and BASF respectively. For the separation of β-carotene from oxygenated derivatives, thin-layer chromatography (TLC) was generally carried out in acetone/hexane (1:2). Due to the intense color of the products to examine, the spots on the TLCs were visually detected. For HPLC-MS analysis a Dionex device was used. Carotenoid extracts were applied to a DXSIL C8 (3µm, 120A, 2.1 x 100mm) column and eluted with water-acetonitrile (5:95) at a flow rate of 0.35 ml/min. UV-VIS detection was also performed (453nm).

4.10.6 Activity tests

1. Peroxide shunt.

Oxidizing agents such as H₂O₂ and organic peroxides (ROOH) bypass the reduction steps of the NAD(P)H pathway and form the ferryl ion by donating one oxygen atom to the ferric enzyme. This ferryl ion species of cytochrome P450 decomposes with the liberation of hydroxylated product and regenerated ferric cytochrome P450.

Procedure:

Potential substrates, hydrogen peroxide and purified enzyme were pipetted together according to the following general scheme:

Component	Working concentration
P450:	10 – 30 μ M
Substrate (*):	7.5 – 10 mM
H ₂ O ₂ (**):	5 mM

(*) styrene, naphthalene, β -ionone and α -ionone, fatty acids (C-10 up to C-16) were added as DMSO solutions. (**) Cumene hydroperoxide was also used as oxidizing agent.

Reactions were performed in 20 mM KPi pH 7.4 at 30 °C – 40 °C for 3 - 5 hours. Control reactions were carried out without P450 and without H₂O₂. The reaction mixtures (1 ml) were finally extracted with CH₂Cl₂ (2 x 500 μ l) and the organic phases analyzed by GC.

2. Coupling CYP175A1 with bovine adrenodoxin and adrenodoxin reductase.

The adrenal redox proteins AdX and AdR are known to support bacterial cytochrome P450 activity. The two proteins were used as P450 reductase system in the conversion of fatty acids by CYP175A1.

Procedure:

Fatty acids and enzyme solution were incubated for 15 – 30 min in KPi 20 mM pH 7.4 at 60 °C. After incubation AdX/AdR and NADPH were added to the mixture according to the following general scheme:

Component	Working concentration
P450:	0.5 μ M
Substrate (*):	50 μ M
AdR:	0.2 μ M
AdX:	2 μ M
NADPH:	0.13 μ M

(*) fatty acids (C-10 up to C-18) were added as DMSO solutions

Activity was estimated following the rate of NADPH consumption at 340 nm for 5 min at room temperature. Control reactions were carried out without redox proteins. The reaction mixtures (1 ml) were finally extracted with CH₂Cl₂ (2 x 500 µl) and the organic phases analyzed by GC.

4.11 *In silico* methods

4.11.1 Docking of β-carotene in the active site of CYP175A1

In order to generate a model of the complex CYP175A1 β-carotene AutoDock 3.0 [Morris et al., 1998] was the program of choice. AutoDock predicts the bound conformations of a small, flexible ligand to a nonflexible macromolecular target of known structure [Morris et al., 1996]. The technique combines simulated annealing for conformation searching with a rapid grid-based method of energy evaluation based on the AMBER force field. AutoDock actually consists of three separate programs: AutoDock performs the docking of the ligand to a set of grids describing the target protein; AutoGrid pre-calculates these grids; and AutoTors sets up which bonds will be treated as rotatable in the ligand.

Procedure:

Prior to investigation ethylene glycol molecules as well as tartrate and water (with the exception of the molecule coordinating the heme iron) were deleted from the crystal structure. Bonds and atom types were adjusted, hydrogen atoms were added, charges of the iron and the nitrogens in the heme group were modified according to the ones calculated for a model system (porphyrin ring bound to a cysteine) by means of MOPAC ("MOPAC 2002", J. J. P. Stewart, Fujitsu Limited, Tokyo, Japan (1999) (Fig.7).

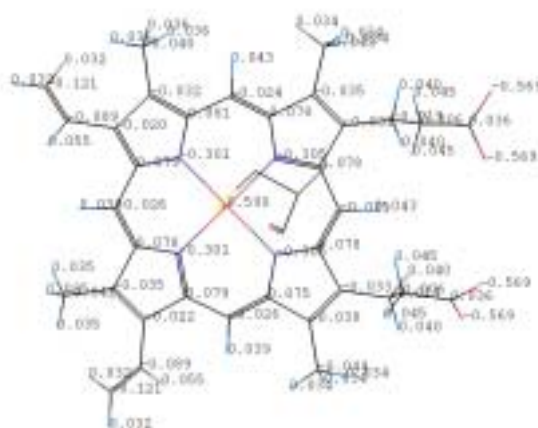


Fig. 7: Charges of the heme modified by MOPAC and used for docking and MD simulations.

The starting structure was minimized (1000 iterations) by means of the minimization tool of Sybyl 6.8 (Sybyl 6.8, Tripos Inc., 1699 South Hanley Road, St Louis, Missouri, 63144, USA.). A channel was identified as potential binding site, connecting the outer surface of the protein with the region close to the heme. This channel showed a rather large pocket at the bottom, in proximity of the heme and a narrow access channel. The nature of the residues forming the channel walls was mostly hydrophobic with very few exceptions. The structure of β -carotene was built by means of the Build/Edit tool of Sybyl and minimized. The ligand was docked in the identified potential binding site by means of AutoDock 3.0.

4.11.2 Screening for potential substrates by means of DockSearch

The program DockSearch (*Skvortsov V.S., Molecular docking in investigation of ligand-protein complexes, Ph.D. thesis, Moscow, 1998*) utilizes the procedure of geometric docking of two molecules, adapted for screening of databases containing three-dimensional coordinates of small molecules. DockSearch is based on an iterative process. Each iteration is the rigid geometric docking of mobile ligand (novel molecule for each screening iteration) and fixed target molecule (constant for all screening iterations). The coordinates of ligand are undertaken from the database containing 3D structures of small molecules. The description of target molecule must contain its coordinates as well as coordinates of known ligand and/or marks for atoms,

declared as binding site. The docking procedure forms a great number of trial mutual dispositions of molecules by moving and twisting of ligand. For each trial conformation the value of molecules' penetration (space overlapping) is examined. It should not exceed the value specified by the user. On a final stage, for an elimination of closely located ligand positions, clusterization by standard deviation is carried out. For each cluster only one ligand position with the best parameters is undertaken and written as output result.

Procedure:

A set of approximately 600 test compounds was selected from the available chemicals database depending on molecular weight, number of cycles, heteroatoms content, rotatable bonds etc. The conformers for flexible molecules were generated by using random search Sybyl procedure (not more than 10 for each molecule). By means of DockSearch the variants were generated, which could fit in the active site. The docking was optimized by Leap Frog (Sybyl 6.8, Tripos Inc.) and eight solutions were selected, according to both energetic and structural criteria, to perform short time MD simulations (first step was 100 iterations of minimization, second – 1 ps of molecular dynamics in NEV ensemble conditions (constant energy simulation) with 1 fs step, third - 500 iterations of minimization).

5. Results

5.1 From gene isolation to protein purification

5.1.1 Homology search

The amino acid sequence of the P450BM3 heme domain (residues 1- 471) was used as template for a homology search within bacterial genome databases with a special focus on thermophilic microorganisms to find thermostable P450 enzymes. The homology search yielded a homologous region within the genome of *Thermus thermophilus* HB27 which is currently sequenced at the Institute of Microbiology and Genetics in Göttingen. The open reading frame (ORF) with a length of 1170 basepairs (Fig. 8) was not yet annotated and no informations regarding the potential function or activity were available.

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P450_Tth (CYP175A1)

ATGAAGCGCCTTTCCCTGAGGGAGGCCTGGCCCTACCTGAAAGACCTCCAGCAAGATCCC      60
CTCGCCGTCCTGCTGGCGTGGGGCCGGGCCACCCCCGGCTCTTCCTTCCCCCTGCCCCGC      120
TTCCCCCTGGCCCTGATCTTTGACCCCGAGGGGGTGGAGGGGGCGCTCCTCGCCGAGGGG      180
ACCACCAAGGCCACCTTCCAGTACCGGGCCCTCTCCCGCCTCACGGGGAGGGGCCTCCTC      240
ACCGACTGGGGGGAAAGCTGGAAGGAGGCGCGCAAGGCCCTCAAAGACCCCTTCCTGCCG      300
AAGAACGTCCGCGGCTACCGGGAGGCCATGGAGGAGGAGGCCCGGGCCTTCTTCGGGGAG      360
TGGCGGGGGGAGGAGCGGGACCTGGACCACGAGATGCTCGCCCTCTCCCTGCGCCTCCTC      420
GGGCGGGCCCTCTTCGGGAAGCCCTCTCCCCAAGCCTCGCGGAGCACGCCCTTAAGGCC      480
CTGGACCCGGATCATGGCCCAGACCAGGAGCCCCCTGGCCCTCCTGGACCTGGCCGCGGAA      540
GCCCCCTTCGGGAAGGACCGGGGCCCTTACC CGAGGCGGAAGCCCTCATCGTCCAC      600
CCGCCCCCTCTCCACCTTCCCCGAGAGCGCGCCCTGAGCGAGGCCGTGACCCCTCCTGGTG      660
GCGGGCCACGAGACGGTGGCGAGCGCCCTCACCTGGTCTTTCTCCTCCTCTCCACCGC      720
CCGGACTGGCAGAAGCGGGTGGCCGAGAGCGAGGAGCGGGCCCTCGCCGCCTTCCAGGAG      780
GCCCTGAGGCTTACCCCCCGCCTGGATCCTCACCCGGAGGCTGGAAAGGCCCTCCTC      840
CTGGGAGAGGACCGGCTCCCCCGGGCACCACCCTGGTCTCTCCCCCTACGTGACCCAG      900
AGGCTCCACTTCCCCGATGGGGAGGCCTTCCGGCCCGAGCGCTTCTGGAGGAAAGGGGG      960
ACCCCTTCGGGGCGCTACTTCCCTTTGGCCTGGGGCAGAGGCTCTGCCTGGGGCGGGAC      1020
TTCGCCCTCCTCGAGGGCCCCATCGTCTCAGGGCCTTCTTCCGCGCTTCCGCCTAGAC      1080
CCCCCTCCCTTCCCCCGGGTCTCGCCAGGTACCCCTGAGGCCCGAAGGCGGGCTTCCC      1140
GCGCGGCCTAGGGAGGAGGTGCGGGCGTGA      1170

```

Fig. 8: Nucleotide sequence corresponding to the ORF in *T. thermophilus* which showed homology to P450 BM3. The ORF is 1170 basepairs long and the corresponding protein is expected to have 390 amino acids and a calculated molecular weight of approx. 44 kDa.

The sequence alignment of the amino acid sequence of the P450 BM3 heme domain and the translated ORF from *T. thermophilus* (Fig. 9) showed a relatively low overall homology of approx. 28 %. With 389 residues CYP175A1 is comparable in size to

CYP119 from *Sulfolobus solfataricus* (378 residues), but considerably smaller than other known soluble bacterial P450s such as P450BM3 (heme domain) with 471 residues.

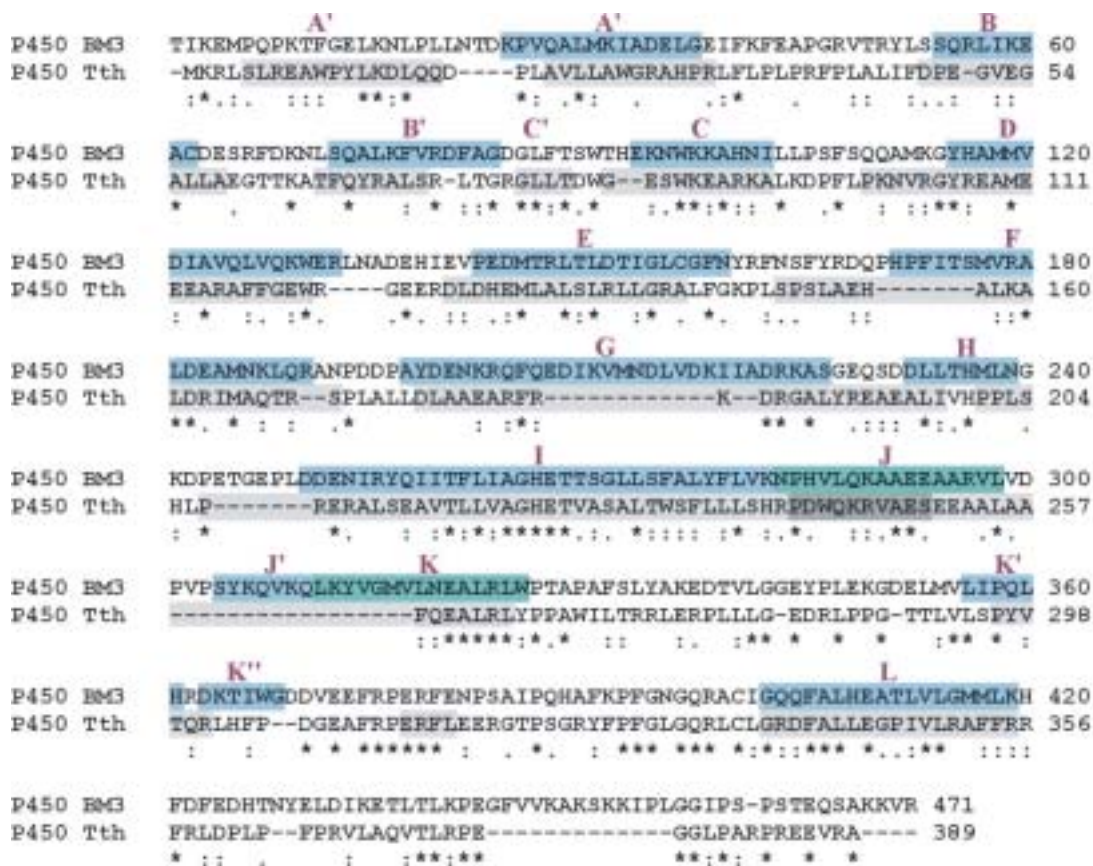


Fig. 9: Sequence alignment of P450 BM3 heme domain and P450_Tth and comparison of secondary structural elements. Shaded regions indicate the α -helices in the two proteins. Residues marked with (*) are identical, while (:) indicate strong similarity and (.) weak similarity respectively.

From the sequence alignment between P450BM3 and P450_Tth one can see that most of the differences between the two P450s are located in the loops connecting the helices. P450BM3 has a seven residue insertion in the loop connecting helices E and F (residues 159–172 in P450 BM3, residues 146–152 in CYP175A1). Another difference includes the β -5 region in P450BM-3 (residues 239–250) compared with the loop found in CYP175A1 (residues 202–205), a difference of eight residues. This region forms a tight loop between the H and I helices, and resembles what was previously found in CYP119 and P450nor [Park et al., 1997]. In contrast, all other soluble bacterial P450 structures show this ill-defined β -hairpin similar to P450BM3. Interestingly, the crystal structure of a complex formed between the heme domain of P450BM3 and the FMN domain has shown that this hairpin region makes direct

contact with the FMN domain [Sevrioukova et al., 1999]. The most conserved regions are located in the I and L helices. The I helix contains the highly conserved Thr (Thr-225) residue, which is hydrogen bonded to the peptide backbone. This region has been postulated to be involved in a proton shuttle network considered to be important for delivering solvent protons required for the activation of O₂ during the catalytic cycle [Poulos et al., 1992], [Schlichting et al., 2000]. The L helix contains the Cys-336 serving as the fifth ligand to the heme iron.

5.1.2 Cloning of *CYP175A1* in *pCYTEXP1* and expression

The plasmid tthb66, kindly provided by Prof. H.-J. Fritz (Institute of Microbiology and Genetics, University of Göttingen) contained a fragment of *Thermus* genomic DNA which overlaps with the ORF homologous to the heme domain of P450 BM3. This plasmid was used as a template for the specific amplification of the putative P450_Tth gene (Fig. 10) by PCR.

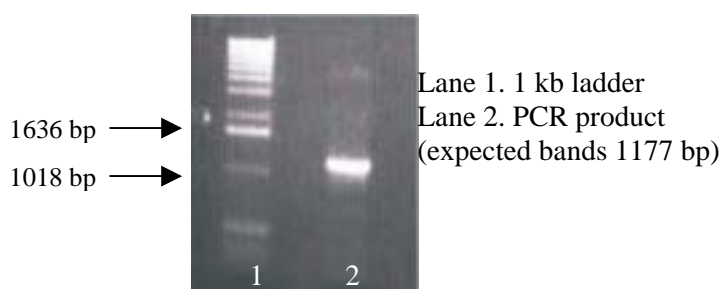
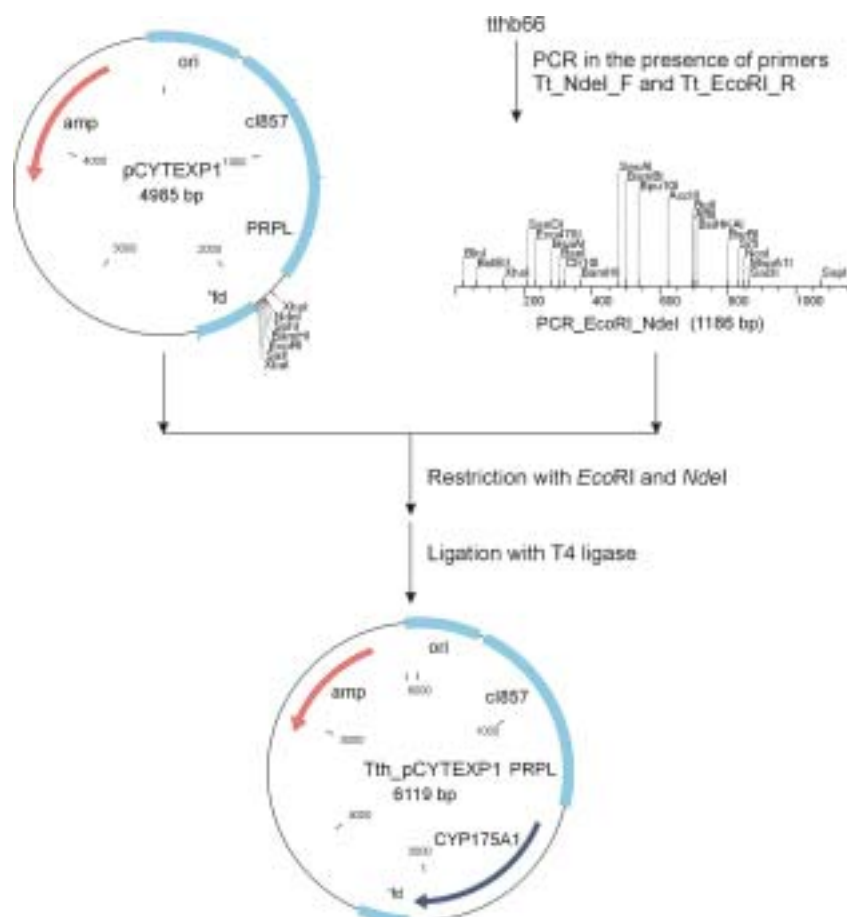


Fig. 10: Amplification of the P450_Tth gene by PCR.

NdeI and *EcoRI* restriction sites were introduced by specific primers to clone the gene into the multiple cloning site of *pCYTEXP1* (Scheme 4). This vector was chosen because of the strong, temperature-inducible lambda P_LP_R promoter. High levels of heterologous expression in *E. coli* are usually achieved with *pCYTEXP1*, overcoming the use of expensive inducers like IPTG.



Scheme 4: Cloning of CYP175A1 in pCYTEXP1.

After transformation and the isolation of the plasmid DNA, positive clones were identified by analytical restriction analysis using *EcoRI* and *NdeI* (Fig. 11). The insert of positive clones was sequenced, in order to exclude the presence of undesired mutations. One clone (Fig. 11, lane 4) was selected for further experiments and named Tth-pCYTEXP1.

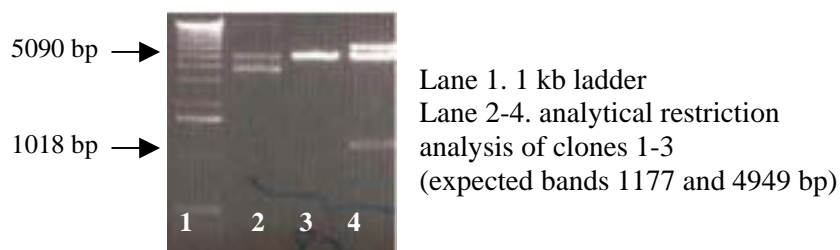


Fig. 11: Analytical restriction analysis of plasmid DNA isolated after ligation of *EcoRI* and *NdeI* restricted PCR product and vector. Clone nr. 3 is likely to be positive, since it contains an insert of the right size.

For protein expression, Tth-pCYTEXP1 was transformed into *E. coli* DH5 α . The transformants were grown in 50 ml LB medium and induced at OD = 0.7 by

temperature shift (37 °C to 42 °C). Aliquots (1 ml) of the cultures were sampled before induction and then every hour after induction for SDS-PAGE. Twenty four hours after induction the cells were harvested. No over-expression could be detected after SDS-PAGE.

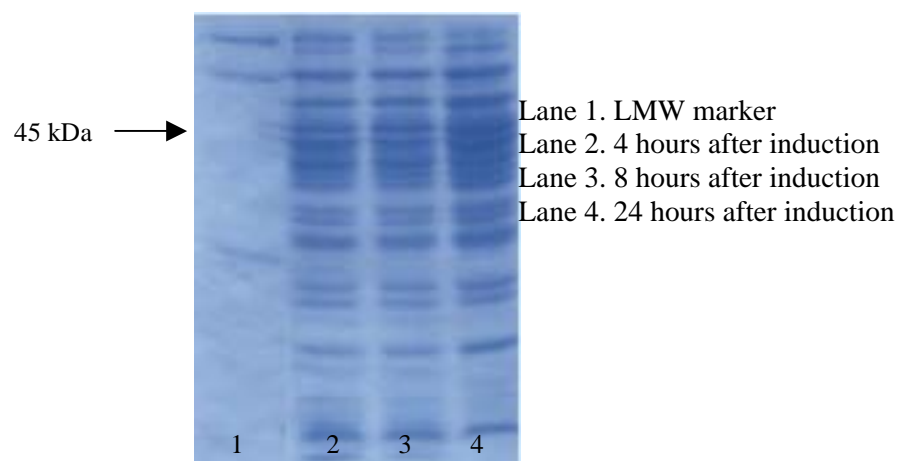


Fig. 12: SDS PAGE of the expression of P450_Tth in *E. coli* DH5 α . *E. coli* DH5 α harboring Tth_pCYTEXP1 were cultivated in 50 ml LB medium with ampicillin at 37 °C before induction and 42 °C after induction. No over expression of the protein is detected (calculated molecular weight 44 kDa).

In addition cell extracts were analyzed by CO difference spectroscopy showing very little P450 expression (Fig. 13).

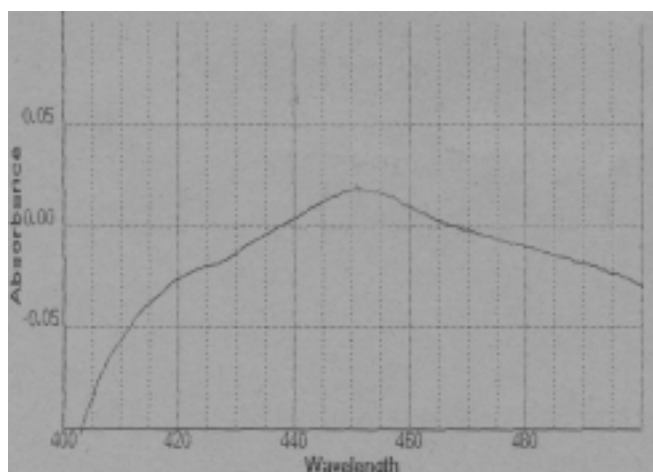


Fig. 13: CO-difference spectrum of *E. coli* cell expressing P450_Tth.

5.1.3 Cloning of CYP175A1 in pKK223-3 and expression

Assuming that the vector pCYTEXP1 was not suitable for expression, the P450 gene was cloned into another vector. The one of choice was pKK223-3. This vector is

generally used for over-expression of heterologous proteins in *E. coli* under the control of the strong *tac* promoter and was tested here as an example for an IPTG inducible system. PCR was used to add *EcoRI* and *PstI* restriction sites into the CYP175A1 gene (Fig. 14).

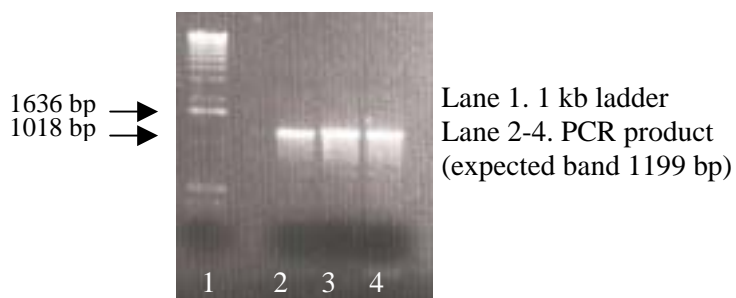
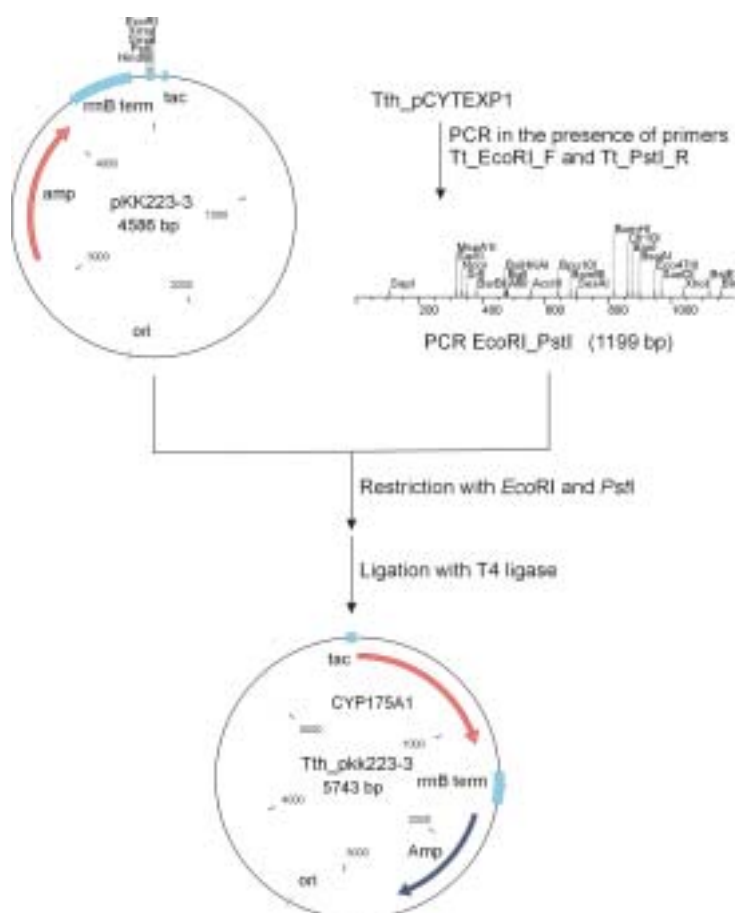


Fig. 14: Amplification of the P450_Tth gene by PCR.

After restriction digest of the PCR product the CYP175A1 gene was cloned into the *EcoRI* and *PstI* sites of the pKK233-3 vector (Scheme 5).



Scheme 5: Cloning of CYP175A1 in pKK233-3.

The screening of the transformants was carried out by analytical restriction analysis with *EcoRI* and *PstI* (Fig. 15).

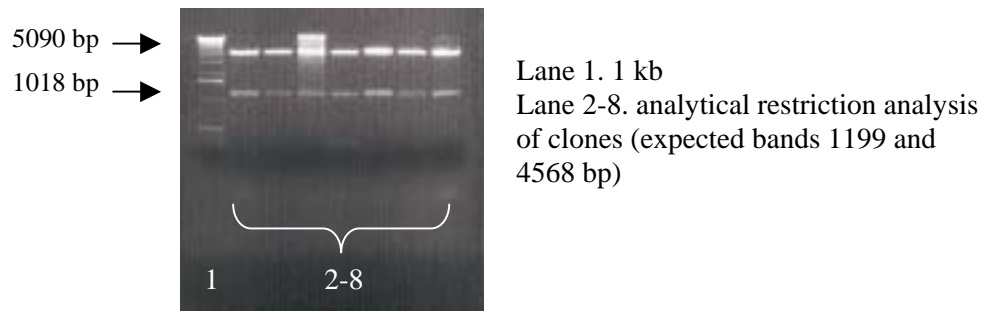


Fig. 15: Test digest of plasmid DNA isolated after ligation of *EcoRI* and *PstI* restricted PCR product and vector. All the clones seem to contain an insert of the right size.

The inserts of positive clones was sequenced (Fig. 16) after plasmid DNA isolation, in order to exclude the presence of undesired mutations.

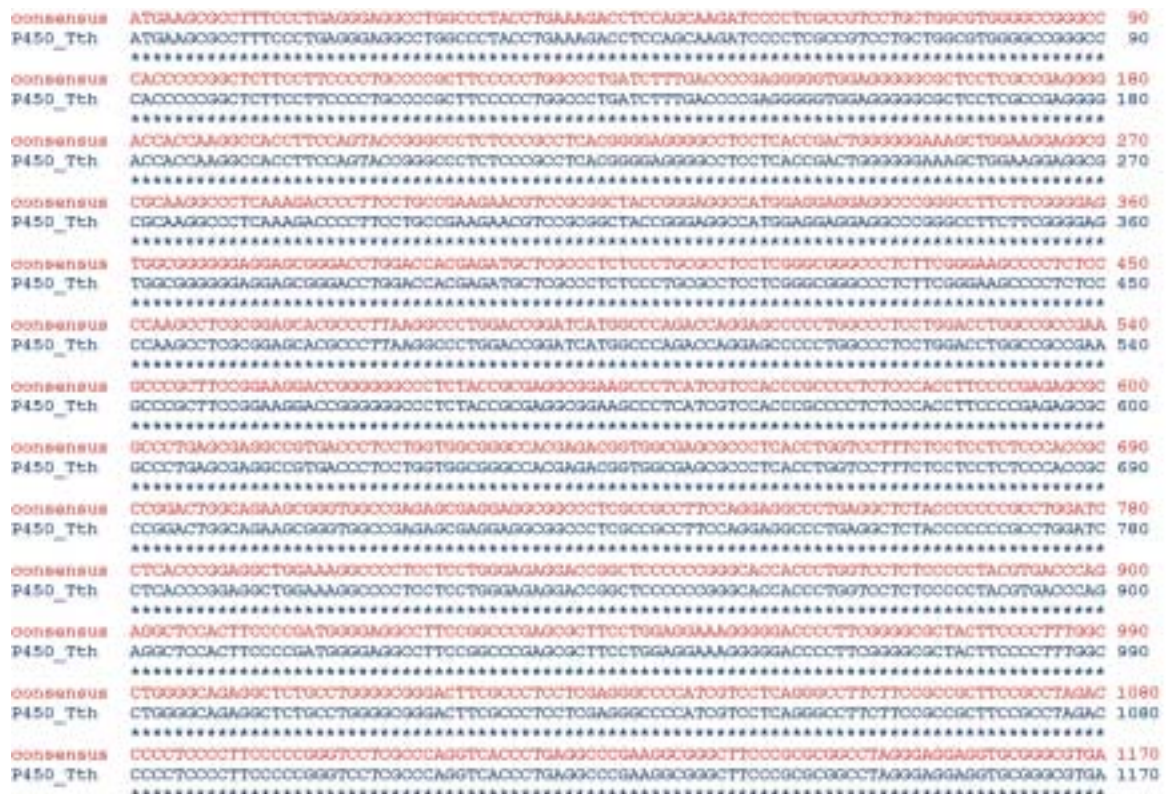


Fig. 16: Sequence alignment between the consensus sequence of the sequencing reads and the nucleotide sequence of P450_Tth. The sequences are 100% identical.

For P450 expression, Tth-pKK was transformed into *E. coli* DH5 α . The cells were grown in 50 ml LB medium until OD = 0.7 and then induced by 1 mM IPTG. The

vector pKK223-3 contains an IPTG inducible *tac* promoter. The cultures were sampled (1 ml) before induction and then every hour after. Twenty four hours after induction the cells were harvested. No over-expression could be detected on the SDS PAGE. Cell extract was analysed by means of CO difference spectroscopy showing very little expression (<1 mg protein / 1 culture). The use of *E. coli* BL21(DE3) as host strain for over expression yielded analogous results.

5.1.4 Optimization of the expression level

In order to optimize the expression level, the influence of parameters such as host cells, medium, expression time, temperature and shaker speed was investigated. An important parameter was the choice of the host cells. The candidates were first *E. coli* DH5 α and *E. coli* BL21, but with both strains the expression level was very low. Then the codon usage of *T. thermophilus* was analyzed and compared with the one of *E. coli*. The result is summarized in Tab. 3.

UUU	22.3	7.3 (7.7)	UCU	8.5	0.7 (0)	UAU	16.2	1.4 (0)	UGU	5.2	0.2 (0)
UUC	16.6	29.2 (46.2)	UCC	8.6	16.1 (20.5)	UAC	12.2	27.4 (17.9)	UGC	6.5	3.7 (2.6)
UUA	13.9	0.8 (0)	UCA	7.2	0.3 (0)	UAA	2.0	0.8 (0)	UGA	0.9	1.3 (2.6)
UUG	13.7	8.5 (0)	UCG	8.9	3.9 (2.6)	UAG	0.2	0.9 (0)	UGG	15.2	12.1 (20.5)
CUU	11.0	13.7 (12.8)	CCU	7.0	3.9 (5.1)	CAU	12.9	0.9 (0)	CGU	20.9	1.6 (0)
CUC	11.1	60.6 (103)	CCC	5.5	45.0 (66.7)	CAC	9.7	18.4 (20.5)	CGC	22.0	30.1 (38.5)
CUA	3.9	2.5 (2.6)	CCA	8.4	1.4 (2.6)	CAA	15.3	3.0 (2.6)	CGA	3.6	1.2 (2.6)
CUG	52.6	37.9 (61.5)	CCG	23.2	11.5 (10.3)	CAG	28.8	24.2 (20.5)	CGG	5.4	33.4 (48.7)
AUU	30.3	4.4 (0)	ACU	9.0	0.5 (0)	AAU	17.7	0.5 (0)	AGU	8.8	0.3 (0)
AUC	25.1	30.9 (12.8)	ACC	23.4	30.1 (33.3)	AAC	21.7	18.6 (2.6)	AGC	16.1	13.0 (15.4)
AUA	4.4	1.5 (0)	ACA	7.1	0.5 (0)	AAA	33.6	3.0 (5.1)	AGA	2.1	1.1 (0)
AUG	27.9	16.6 (10.3)	ACG	14.4	10.4 (5.1)	AAG	10.3	42.4 (23.1)	AGG	1.2	14.7 (30.8)
GUU	18.3	2.4 (0)	GCU	15.3	2.8 (0)	GAU	32.1	2.0 (5.1)	GGU	24.7	2.6 (41.0)
GUC	15.3	26.7 (17.9)	GCC	25.5	78.5 (89.7)	GAC	19.1	39.1 (33.3)	GGC	29.6	38.0 (16.0)
GUA	10.9	1.4 (0)	GCA	20.1	1.4 (0)	GAA	39.4	10.0 (15.4)	GGA	8.0	4.5 (2.6)
GUG	26.4	50.1 (17.9)	GCG	33.6	24.8 (25.6)	GAG	17.8	81.5 (74.4)	GGG	11.1	41.7 (16.0)

fields: [triplet] [frequency]

Tab. 3: Comparison of the codon usage frequency of *E. coli* (blue) and *T. thermophilus* (purple). In parenthesis the codon usage referred to the P450_{Tth} gene is shown. Shaded fields correspond to the triplets for proline (grey) and arginine (bright blue) respectively. Frequencies are referred to thousand triplets.

The codon usage differs in at least two amino acids (proline and arginine) which showed the largest differences in the codon frequency. Proline residues are mainly encoded by the triplet CCG in *E. coli* while in *T. thermophilus* the preferred codon for this amino acid is CCC. To evaluate the influence of the rarity of certain tRNAs which frequently limits heterologous expression in *E. coli*, BL21-CodonPlus[®] cells were used for expression experiments. BL21-CodonPlus[®] strains are genetically

engineered to contain additional copies of genes encoding the tRNAs that most frequently limit translation of heterologous protein in *E. coli*. BL21-CodonPlus(DE3)-RP cells contain extra copies of the *argU* and *proL* genes. These genes encode tRNA that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively. The BL21-CodonPlus(DE3)-RP strain was designed to provide the tRNAs that restrict translation of heterologous proteins of organisms that have GC-rich genomes like *Thermus thermophilus*.

The use of *E. coli* BL21-CodonPlus(DE3)-RP in LB medium increased the expression level to approx. 15 mg protein/l culture. Furthermore, parameters such as shaking speed and ratio volume medium / volume Erlenmeyer flask were adjusted in order to control the oxygen content of the medium. The temperature after induction was set up at 30 °C, whereas before the cells were grown at 37 °C. Additionally, relatively late induction during the logarithmic growth phase of the host organism ($OD \approx 1$) had a positive effect on the amount of expressed P450 as well. After optimization (Tab. 4) the expression was increased to approx. 40 mg protein/l culture.

Medium:	2xYT
Temperature (before induction):	37 °C
Shaker speed (before induction):	200 rpm
Induction ($OD \approx 1$):	1 mM IPTG
Temperature (after induction):	30 °C
Shaker speed (after induction):	150 rpm
<i>E. coli</i> strain	BL21 CodonPlus
Expression time:	48 hours

Tab. 4: Optimized expression conditions for the expression of P450_Tth in *E. coli*.

However, as indicated by the absence of a band corresponding to 44 kDa in Fig. 17 no clear over expression could be detected after SDS PAGE.

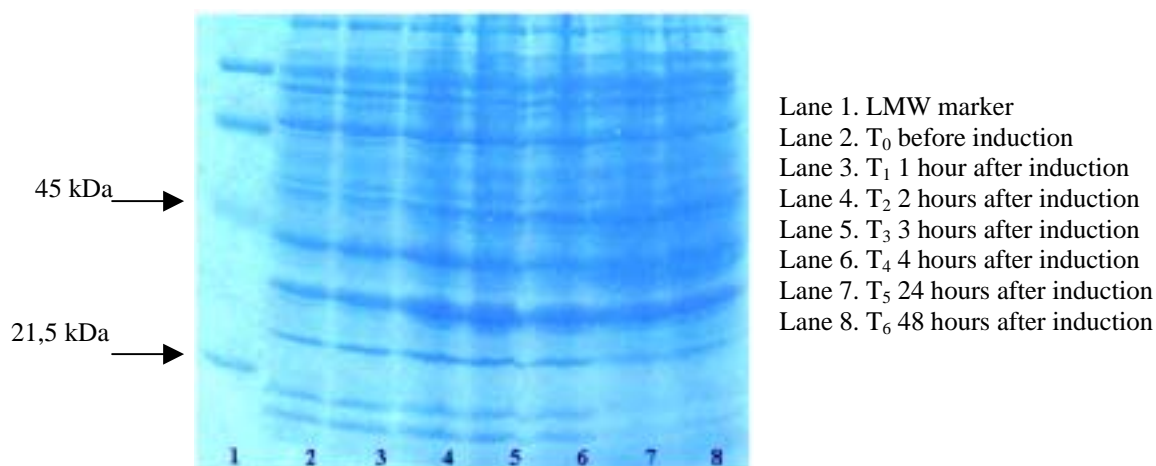


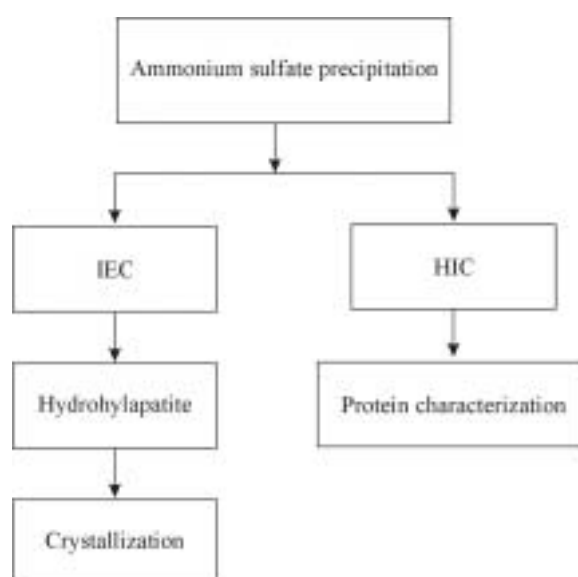
Fig. 17: SDS PAGE of the expression of P450_Tth in *E. coli* BL21-CodonPlus(DE3)-RP. No over expression of the protein is detected (calculated molecular weight 44 kDa). The band observed at approx. 25 kDa is due to the chloramphenicol acetyl transferase, the protein that provides chloramphenicol resistance.

5.1.5 Purification

Heat precipitation

After cell lysis and removal of cell debris by centrifugation at 20000 rpm for 30 min at 5 °C, the supernatant was heated at 60-75 °C for 15 min. The denatured proteins were separated by centrifugation. Significant loss of P450 was observed by P450 concentration measurement in the supernatant. Therefore this step was not used later on for preparative purposes.

The purification protocols of P450_Tth are summarized in Scheme 6.



Scheme 6: Representation of the purification of P450_Tth.

Ammonium sulfate precipitation

A purification/concentration step was performed using a fractionated ammonium sulfate precipitation. The fractions between 35 and 50% were pooled, dialysed against the appropriate buffer and used for further purification.

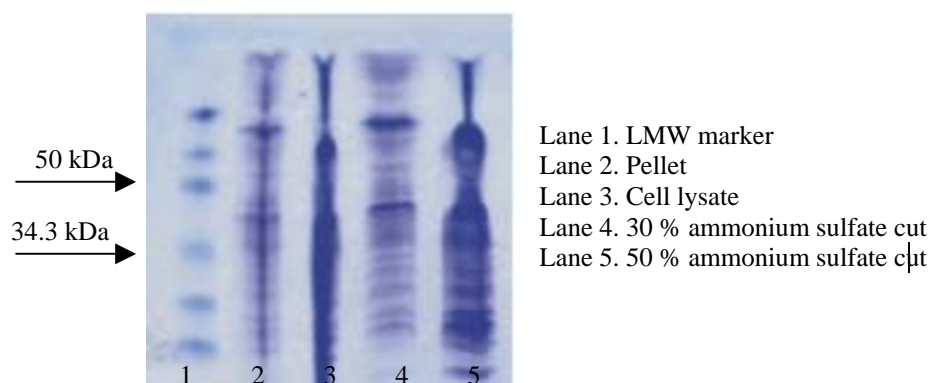


Fig. 18: SDS PAGE of the ammonium sulfate precipitation.

Two different purification protocols were established for further purification of the precipitated P450 fractions:

Protocol 1:

Ion exchange chromatography followed by hydroxylapatite chromatography.

This protocol was used to purify the protein later on used for crystallization experiments to homogeneity. It consisted of the two following purification steps with a general overall yield of approx. 20%, based on CO-difference spectroscopy.

Due to the low affinity of the protein for the DE52 matrix, the desired protein was recovered from the flow through of the ion exchange chromatography in this first purification step. However, the most *E. coli* proteins bound to the matrix and the eluted P450 showed $R_z \approx 1$ (Fig. 19 and 20). The recovery rate of this first step was approx. 50%. The little amount of P450 which weakly bound to DE52 was eluted with 50 mM NaCl in the elution buffer (KPi buffer 50 mM pH 7.5).

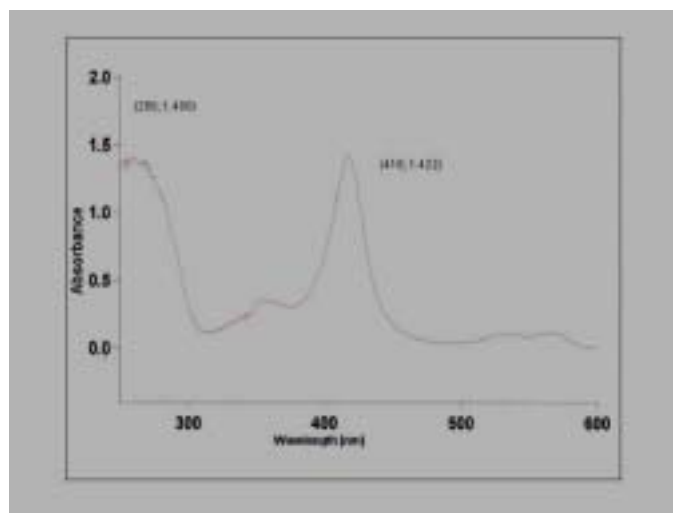


Fig. 19: Flow through from DE52. P450_Tth has limited affinity for DE52 and can be collected directly from the flow through.

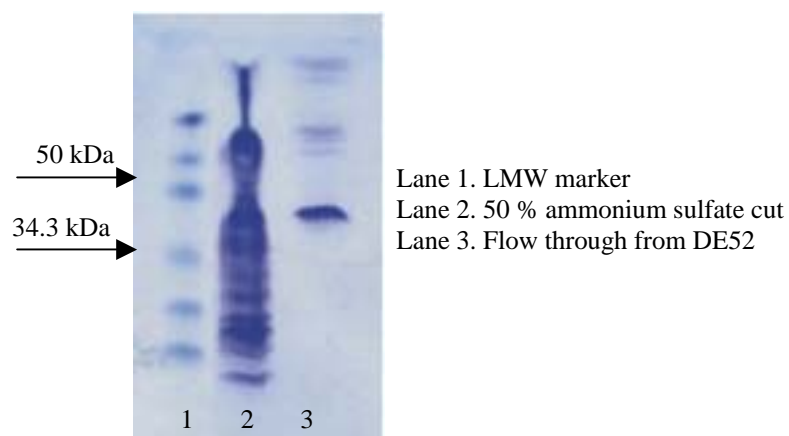


Fig. 20: SDS PAGE after ammonium sulfate precipitation and ion exchange chromatography.

The protein obtained from the first step was then further purified to electrophoretic homogeneity using a hydroxylapatite column. The protein preparation had $R_z > 1.6$ (Fig. 21) and the recovery rate was approx. 20%. After buffer exchange and concentration the enzyme was used for crystallization experiments.

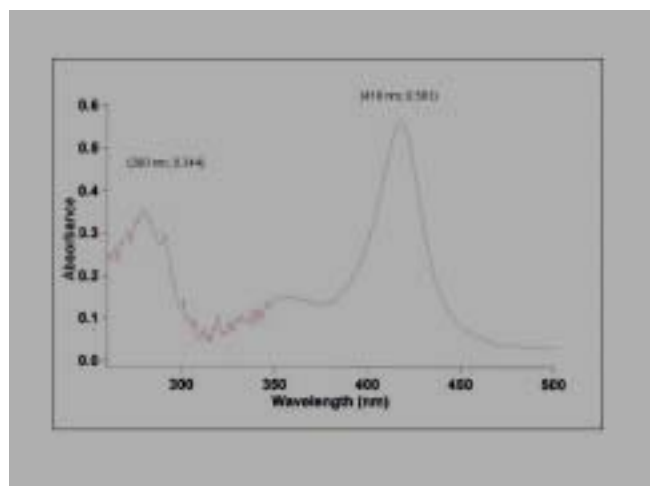


Fig. 21: Eluate from hydroxylapatite. Elution was carried out with a linear gradient of KPi pH 7.4 from 50-300 mM.

The purity of the fractions was controlled by SDS PAGE (Fig. 22).

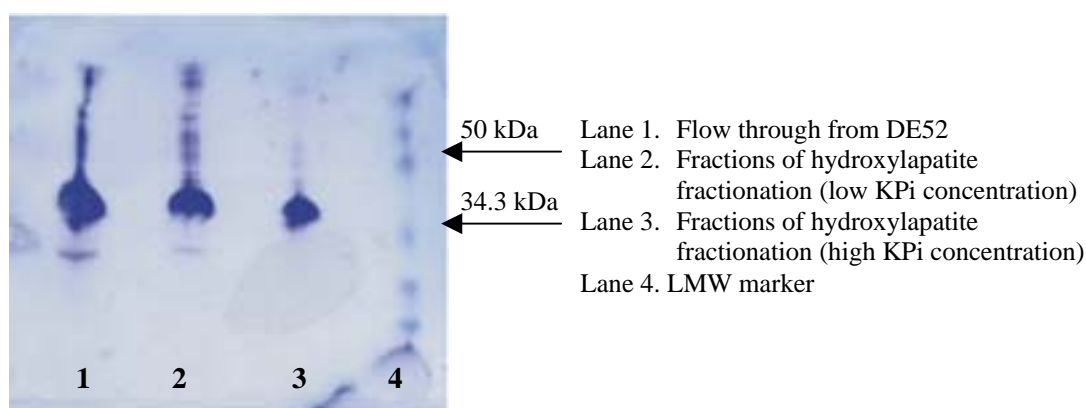


Fig. 22: SDS PAGE of the eluate from hydroxylapatite.

Protocol 2:

This protocol was used to generate nearly pure Tth_P450 to be used for the characterization. It consisted of the one purification step with a general overall yield of approx. 50%.

Hydrophobic interaction chromatography.

The pellet obtained from the ammonium sulfate precipitation was further purified on a Phenyl Sepharose matrix. This resulted in a protein preparation with $R_z \approx 1.5$ (Fig. 23), and a recovery rate of approx. 50 %, based on CO-difference spectroscopy measurements.

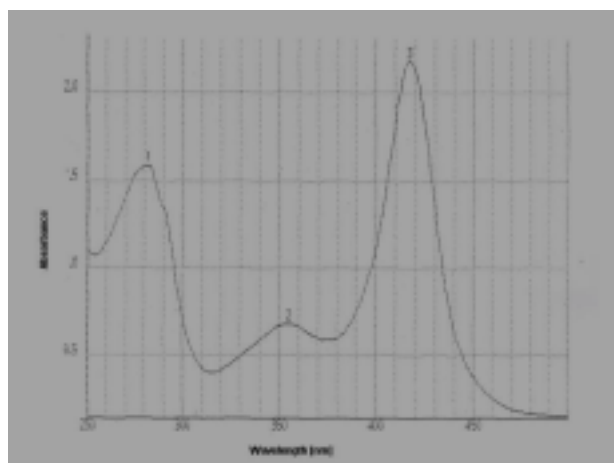


Fig. 23: Eluate from Phenyl Sepharose. Elution was carried out with a decreasing ammonium sulfate gradient and with the addition of sodium cholate.

The purity of the fractions was also controlled by SDS PAGE (Fig. 24).

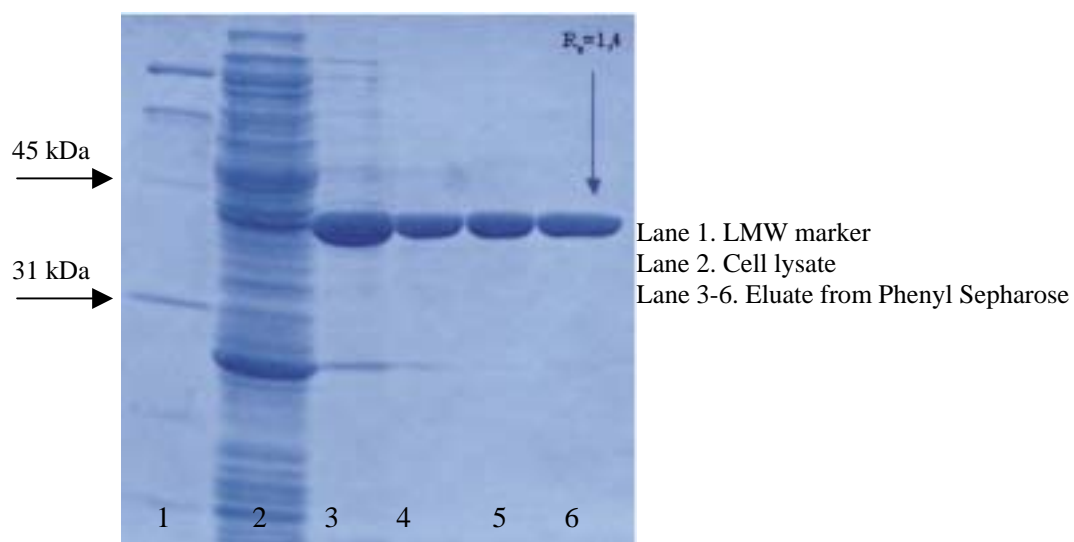


Fig. 24: SDS PAGE of the eluate from Phenyl Sepharose.

5.2 Protein characterization

5.2.1 Spectroscopic characterization

P450_Tth displayed absorption spectra analogous to those of other P450 enzymes. The ferric form of P450_Tth showed a Soret maximum at 418 nm and two visible bands between 500 and 600 nm (Fig. 25).

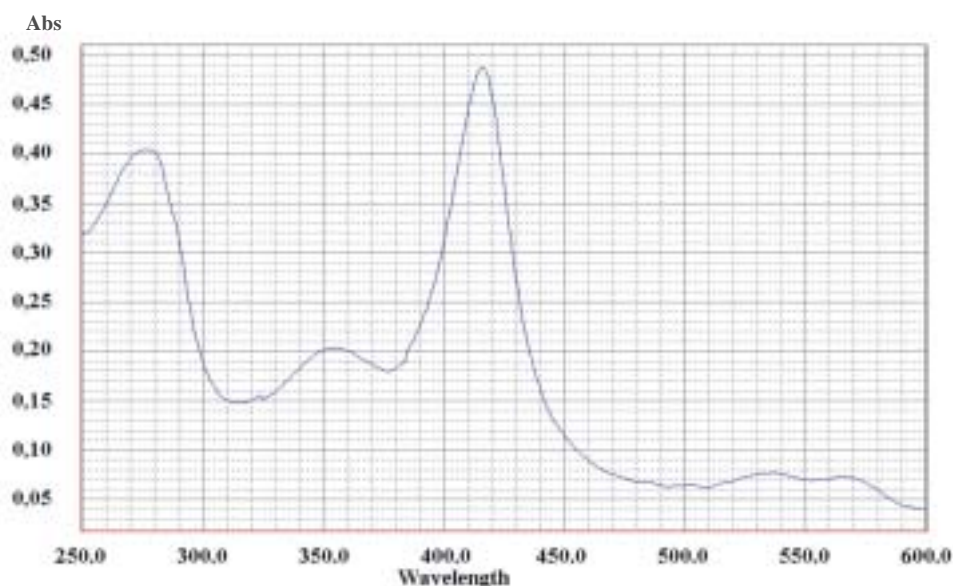


Fig. 25: UV-VIS spectrum of P450_Tth. The substrate-free ferric form of P450_Tth shows a Soret maximum at 417 nm and two visible bands at 537 nm and 567 nm.

The reduced CO bound form revealed the characteristic maximum at 450 nm (Fig. 26). The absence of the peak at 420 nm which is typical for disrupted P450 enzymes confirmed that the protein was not denatured during purification.

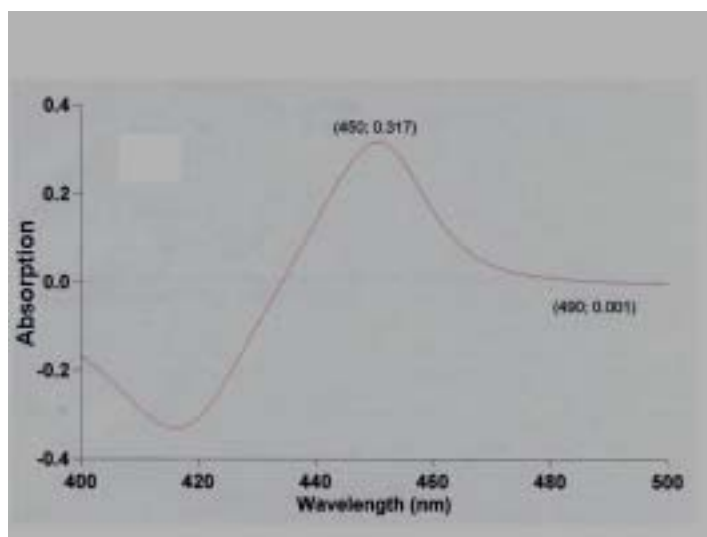


Fig. 26: CO difference spectrum of P450_Tth

The X-band EPR spectrum of the low-spin ferric P450_Tth shows signals at g 2.42, 2.25, and 1.92 (Fig. 27).

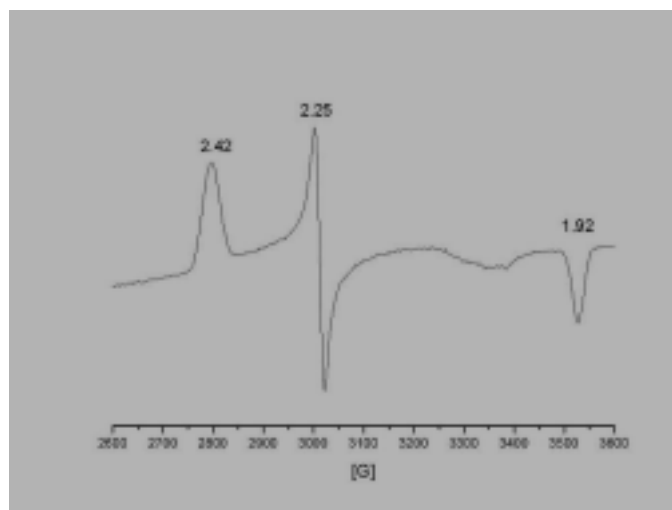


Fig. 27: EPR spectrum of P450Tt. P450_Tth 214 μ M in KPi 20 mM pH 7.4. Temperature of sample: 10K. Frequency, 9.47 GHz. Modulation amplitude 5 G. Microwave power 0.32 mW.

These g -values fit to the ranges reported for other P450s (Tab. 5). The g -values refer to the nature of the heme axial ligands and to perturbations such as the orientations of the ligands. The results show that the heme ligation in P450_Tth is similar to that of P450 cam and P450 BM-3.

P450 (low spin)	g Values
P450 cam	2.45, 2.26, 1.91 [Lipscomb, 1980]
P450 BM3	2.42, 2.26, 1.92 [Miles et al., 1992]
Rat liver microsomes	2,43, 2,25, 1,92 [Chevion et al., 1977]
P450_Tth	2.42, 2.25, 1.92

Tab. 5: g values of known P450s

5.2.2 Thermal Stability

The melting point of P450_Tth was spectrophotometrically calculated. As expected, P450_Tth resulted to be thermostable, the melting point being 88 $^{\circ}$ C (Fig. 28).

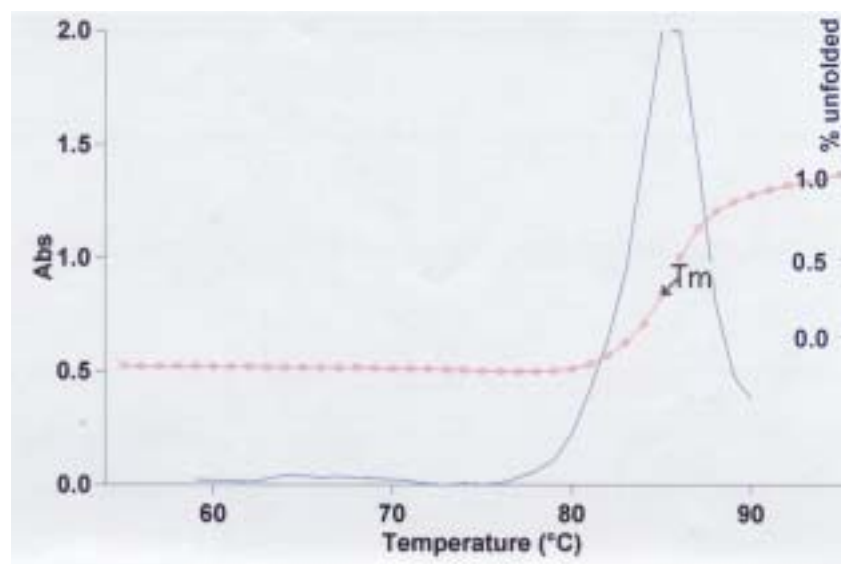


Fig. 28: Melting curve of P450_Tth. The absorbance increase at the heme peak at 418 nm was measured (blue curve). The percentage of denatured protein (red curve) was calculated according to the equation reported in 4.10.3 (“*Determination of melting temperatures*”). The melting curve indicates a melting point of 88 °C.

Melting curves for the two known thermophilic P450s, CYP175A1 and CYP119, and three mesophilic P450s were compared (Fig. 29). The three mesophilic P450s exhibit melting temperatures (T_m) values ranging from 47°C to 61°C while CYP175A1 and CYP119 have T_m values of 88°C and 91°C, respectively.

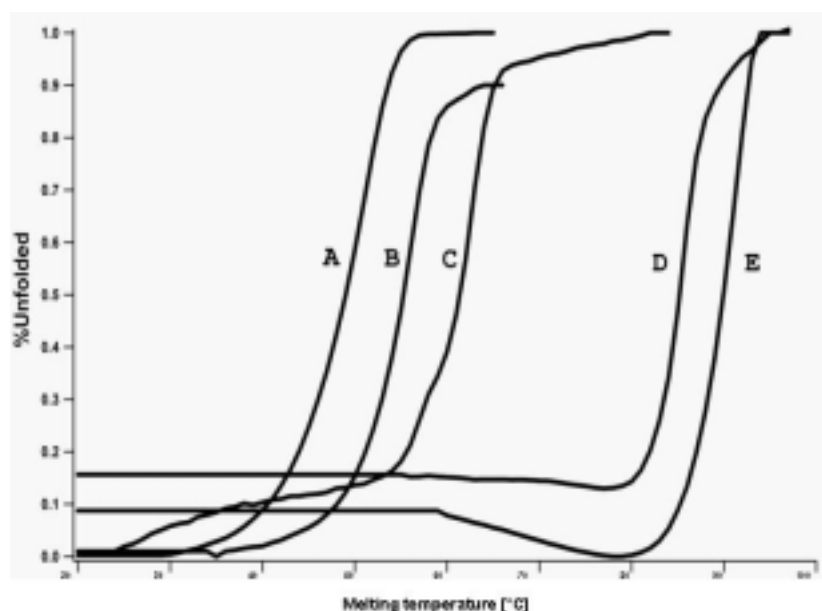


Fig. 29: Thermal melts of P450s. A) P450 BM3 heme domain, B) P450eryF, C) P450cam, D) P450_Tth, E) P450 from *S. solfataricus*. The absorbance increase at the heme peak was followed for CYP119 and CYP175A1, while the decrease in absorbance at the heme peak was followed for the all other P450s.

These values were in agreement to those obtained by differential scanning calorimetry. In these experiments, the T_m for CYP119 was found to be 91°C while that for P450cam was 54°C [Maves et al., 2001], [McLean et al., 1998].

5.2.3 *Crystal structure*

The 3-dimensional structure of P450_Tth was solved as a molecular replacement structure with P450 BM3 as a model. The X-Ray crystallography was performed by the group of T. Poulos (University of California, Irvine) in the frame of a cooperation. The structure is here described because it was used during this study for modeling experiments.

CYP175A1 exhibits the typical prism like P450-fold (Fig. 30) composed of 17 α -helices and 11 β -strands, corresponding to four beta sheets. At the core of the molecule is a four-helix bundle composed of helices D, E, I and L and two α -helices (J and K) [Graham and Peterson, 1999]. The heme prosthetic group is embedded between the distal I and proximal L helices, with Cys-336 serving as the fifth axial thiolate ligand to the heme iron. The propionate side chains of the heme are tethered to the protein through interactions with Trp-87, Arg-273 and Arg-334. The I helix spans the length of the entire molecule and contains the highly conserved Thr (Thr-225) residue, which is hydrogen bonded to the peptide backbone.

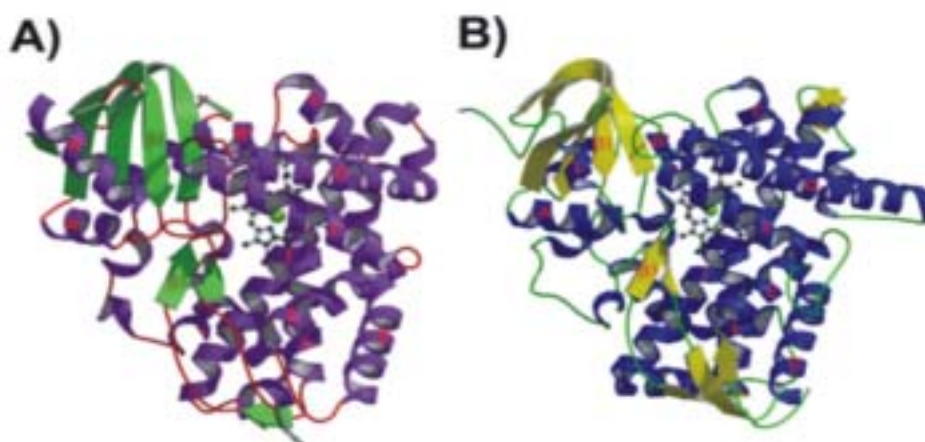


Fig. 30: Ribbon diagram of A) CYP175A1 and B) P450 BM3.

Since the closest homologue to CYP175A1 is P450 BM3, the structure were compared. The rms backbone deviation between CYP175A1 and palmitoleic acid bound P450 BM3 (PDB accession number 1FAG) [Li et al., 1997] is 1.64 Å for 304 C α atoms. Most of the differences between CYP175A1 and P450 BM3 are located in the loops connecting the helices. Another interesting region is the substrate binding environment, formed by the F and G helices, the B' helix and neighboring loops. The major portions of the F-G region and the B' helix which are known to be important in substrate binding [Gotoh, 1992] are in nearly identical conformations in CYP175A1 and P450 BM3 (Fig. 31). The B' helix is thought to form a portion of the lid to the substrate access channel in P450 BM3 and makes intimate contact with the G-helix. The average rms deviation for the B' helix is 1.23 Å, reflecting the general overlap of this structural element. The F-helix in CYP175A1 (residues 150-170) is nearly identical in both size and location to the F-helix in P450 BM3 (residues 171-190). The core region of the F-helix (residues 157-167 in CYP175A1) has an average rms deviation of 1.66 Å, despite being shifted by about half a turn.



Fig. 31: Schematic drawing of superimposed substrate binding regions from P450_Tth and P450 BM3. P450_Tth is depicted in *dark gray* while P450BM-3 is depicted in *light gray*.

The G helix is about two turns shorter in CYP175A1 (residues 176-198 in CYP175A1 compared to residues 197-226 in P450 BM3). Although the G helix is shorter, residues 176-195 in CYP175A1 align very well with residues 198-217 in P450BM3 with an average rms deviation of 1.14 Å.

5.3 *In vitro* protein characterization

5.3.1 *Conversion of fatty acids and other substrates*

P450_Tth likely belongs to the class I of P450s, which require electron transfer proteins to transfer electrons from NAD(P)H to heme iron (Fig. 2). So far the homologous electron transfer proteins have not been identified and for the characterization of the catalytic activity of P450_Tth alternative redox partners were used. The peroxide shunt pathway (Fig. 4) as well as adrenal redox proteins adrenodoxin and adrenodoxin reductase were tested. Adrenodoxin and adrenodoxin reductase are electron transfer proteins, which transfer electrons from NADPH to P450, making them an irreplaceable component of the steroid hormone biosynthesis in the adrenal mitochondria of vertebrates [Grinberg et al., 2000]. Adrenodoxin reductase is an FAD containing flavoenzyme, and adrenodoxin is [2Fe-2S] ferredoxin type iron-sulfur protein. FAD of adrenodoxin reductase accepts two electrons from NADPH, and these are transferred one at a time to adrenodoxin, which is one-electron carrier [Ziegler et al., 1999]. In these respects the mitochondrial P450 electron transfer system is similar to that of some bacterial P450s such as P450cam from *Pseudomonas putida* that includes a ferredoxin reductase and a ferredoxin (putidaredoxin). Whereas the mitochondrial P450s are tightly bound to the membrane, the electron transfer proteins are soluble in the matrix. Furthermore the electron transfer proteins are not specific for individual P450s and serve as electron donors for different cytochrome P450s in different tissues. Functional interaction between adrenal redox proteins and a bacterial P450 has been already reported [Simgen et al., 2000] as well as the one between mammalian P450 and bacterial electron transfer proteins [Jenkins et al., 1994].

Based on the sequence identity and close structural position of regions associated with the active site, it seemed possible that CYP175A1 may act as a fatty acid hydroxylase. In order to limit the number of chemicals to be tested as potential substrates *in vitro*, a preliminary spectroscopic investigation was carried out. The effect of the potential substrates on the Soret band of the protein was analyzed, looking for a shift of the absorption maximum. This would imply the displacement of the water, the sixth ligand of the heme iron, by another molecule likely to be a substrate. Even chain fatty acids (C-10 to C-20) and aromatics (styrene, naphthalene and indole) were tested also

in large molar excess at temperatures ranging between 25 and 70 °C. However, the characteristic low- to high-spin shift that accompanies the binding of substrates to P450s was not observed.

Hydrogen peroxide and cumene hydroperoxide were used to bypass the need of redox partners which provide P450_Tth with the electrons necessary for the catalytic activity. In fact by peroxide shunt the heme iron can be directly oxidized to the ferryl intermediate capable to hydroxylate the substrate. GC analysis of the reaction mixtures showed no detectable hydroxylations of even chain fatty acids (C10-C16), naphthalene, styrene and ionones.

Bovine adrenodoxin/adrenodoxin reductase were also tested as redox partners of P450_Tth. After incubation of the mixture containing substrate and P450_Tth at 60 °C in the presence of fatty acids (C10-C16), the adrenal redox proteins AdX/AdR were added together with NADPH. No hydroxylation products were detected by GC analysis of the reaction mixtures.

5.3.2 Conversion of ionones

The fact that P450 BM3 catalyzes the conversion of α - and β -ionone to the corresponding hydroxyl derivatives (Urlacher, unpublished results) together with the knowledge that in *Thermus* the β -ionone ring of β -carotene is hydroxylated at the C-3 (see introduction), indicated that P450_Tth might catalyze the hydroxylation of ionones. Thus it was expected that β -ionone is being hydroxylated to 3-hydroxy- β -ionone (Fig. 32), which might be used as building block for zeaxanthin and astaxanthin. This hypothesis was additionally corroborated by the fact that the addition of α - and β -ionone to a solution containing P450_Tth induced a shift in the absorption maximum of the Soret peak (417.5 nm to 416.5 nm and to 414.8 nm respectively). Thus ionones seemed to bind in the active site of P450_Tth so that the water molecule as the sixth ligand of the heme iron was displaced.

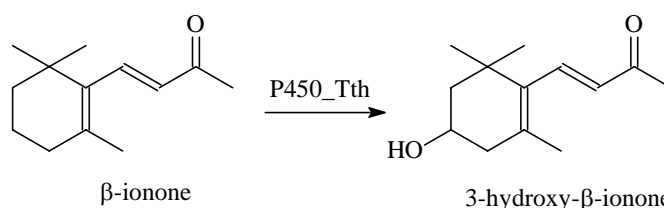


Fig. 32: Expected hydroxylation of β -ionone catalyzed by P450_Tth.

The attempts to hydroxylate β -ionone in the presence of the purified enzyme and using H_2O_2 as oxidizing reagent yielded very little amount (5 %) of 4-hydroxy- β -ionone. Anyway control reactions performed without enzyme, confirmed that the observed conversion was not catalyzed by P450_Tth. In fact it was a chemical hydroxylation taking place at the activated C-atom.

5.4 *In vivo* characterization

5.4.1 *Heterologous complementation*

In order to examine the catalytic activity of CYP175A1, *E. coli* strains were used which had been genetically engineered to produce carotenoids. Heterologous complementation experiments indicated that CYP175A1 was able to catalyze the conversion of β -carotene to zeaxanthin via β -cryptoxanthin. Transformation of plasmid pACYC_Y (Fig. 33) which contained the *crtE*, *crtB*, *crtI* and *crtY* genes from *E. uredovora* in *E. coli* resulted in the accumulation of β -carotene which confers a yellow pigmentation on *E. coli*.

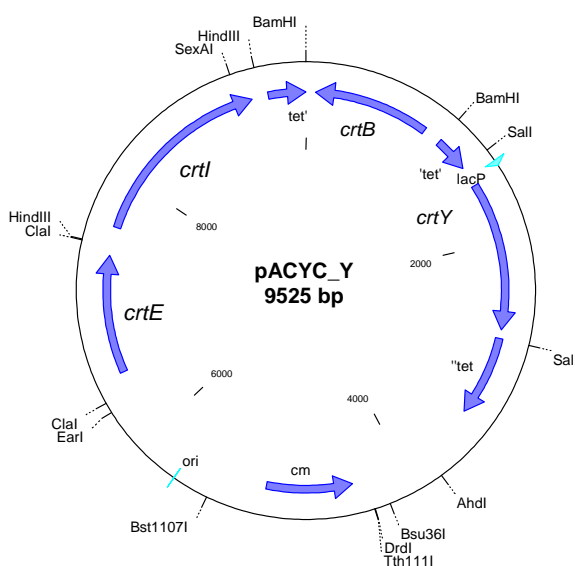


Fig. 33: Plasmid map of pACYC_Y, carrying the carotenogenic genes from *E. uredovora*. The *CrtE* gene codes for phenyl transferase to synthesize GGPP from farnesyl diphosphate. Two molecules of GGPP are condensed to phytoene by *crtB*. The next enzyme encoded by *crtI* is needed for the conversion of phytoene to lycopene. Finally the *crtY* gene product cyclizes lycopene to β -carotene.

After co-transformation of plasmid pACYC_Y and Tth_pkk223-3 containing the P450 from *T. thermophilus*, the corresponding *E. coli* transformants were shown to produce β -cryptoxanthin and zeaxanthin (Fig. 34).

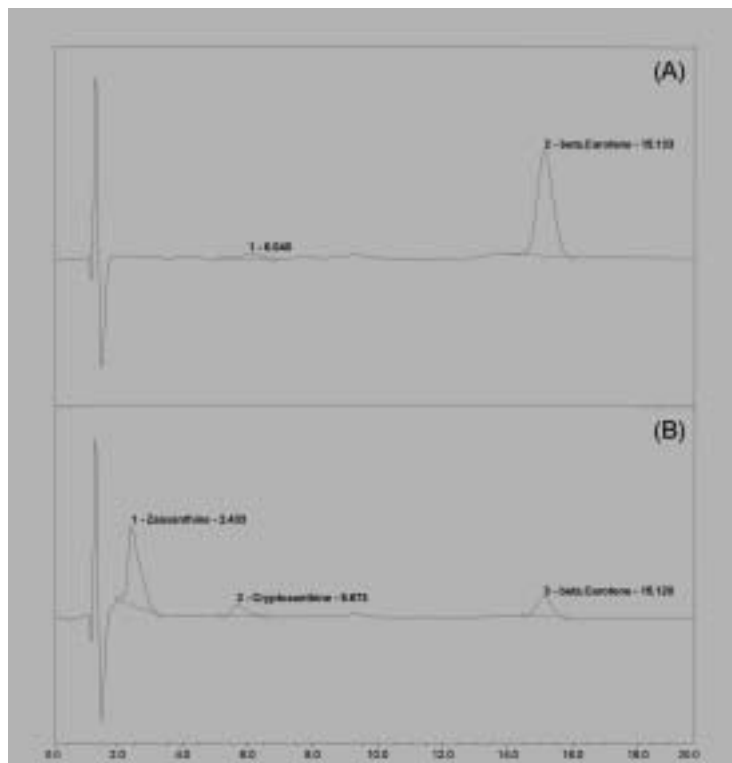


Fig. 34: HPLC separation of carotenoid pigments extracted from *E. coli* cells carrying either the plasmid pACYC_Y only (A) or the plasmid pACYC_Y together with Tth_pkk223-3 (B). Cultivation was carried out in 50 ml LB medium for 48 hours at 30°C.

As negative controls *E. coli* cells carrying the plasmid pACYC_Y (Kauffmann, PhD Thesis, University of Stuttgart, 2002) were co-transformed with empty pKK223-3. Additionally, an inactive mutant of Tth_pkk223-3 (C400G) was generated by site directed mutagenesis and this plasmid was transformed into the β -carotene producing *E. coli* strain. As a result the corresponding *E. coli* transformants were shown to produce β -carotene and no detectable hydroxylated products were detected in the extract (Fig. 35).

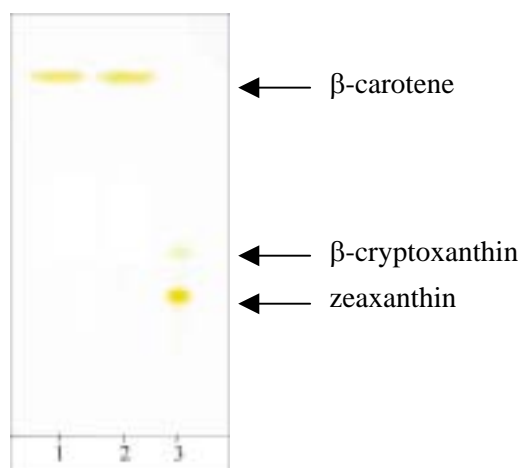


Fig. 35: TLC separation of carotenoid pigments extracted from *E. coli* cells carrying either the plasmid pACYC_Y only (lane 1) or the plasmid pACYC_Y together with Tth_C400G_pkk223-3 (lane 2) and Tth pkk223-3 (lane 3) respectively. Cultivation was carried out in 5 ml LB medium with chloramphenicol (30 $\mu\text{g/ml}$), ampicillin (100 $\mu\text{g/ml}$) and IPTG (0.5 mM) for 48 hours at 30°C.

5.4.2 In vivo biotransformation of β -ionone

T. thermophilus cells were cultivated in *Thermus* specific medium at 65 °C. After 24 hours β -ionone (0.01 % w/v) was added to the culture. At several time points the culture was sampled and the extract analyzed by GC. After 5 days the cultivation was terminated. 4-hydroxy- β -ionone could be isolated from the reaction mixture, but the hydroxylation was found to be an artifact due to the cultivation conditions. In fact a control, β -ionone incubated in medium under the same experimental conditions showed also hydroxylation at the C-4. This position is chemically activated and at 65 °C (temperature needed for the growth of *Thermus thermophilus*) a spontaneous hydroxylation took place.

5.4.3 Screening for substrates and /or inducers

RT-PCR indicated that P450_Tth was not expressed under normal cultivation conditions (Fig. 36). Control reactions showed that also *crtB* (phytoene synthase) was not expressed under the same cultivation conditions. A negative control was performed, using P450_Tth specific primers but inhibiting the reverse transcriptase by incubation on ice. As positive control 16S RNA specific primers were used leading to the successful amplification of a 1000 bp fragment of the 16S rDNA.

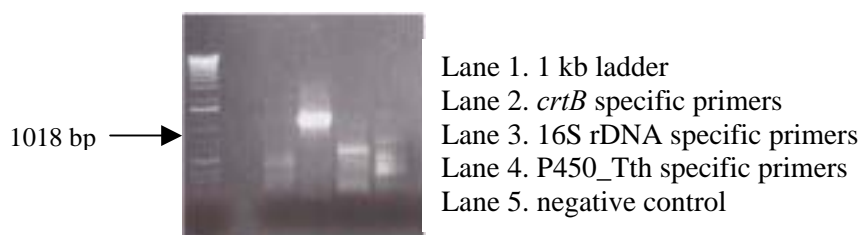


Fig. 36: RT-PCR in the presence of different primer pairs. A positive control was performed with 16S rDNA specific primers, whereas a negative control was carried out with P450_Tth specific primers but without RT.

The influence of the addition of potential P450 substrates/inductors (10 μ M – 10 mM) (Tab. 6) to an overnight culture of *T. thermophilus* was investigated. The culture was screened for P450 up to five hours after the addition. Neither the SDS PAGE nor the CO-difference spectroscopy detected any expression.

Class	Compound
Alkanes	Octane
	Decane
	Octadecane
Aromatics	Naphataline
	Toluene
	Benzene
Fatty acids	Palmitic acid
	Lauric acid
	Decanoic acid
Heteroaromatics	Quinolin
	Hydroxycumarin
	Indole

Tab. 6: Chemical compounds screened as potential *in vivo* inductors of P450_Tth.

5.5 *In silico* characterization

5.5.1 Docking of β -carotene in the active site of CYP175A1

The active site of CYP175A1 was identified as an up-side-down funnel shaped surface (Fig. 37). A narrow channel leads from the surface of the protein to a region

above the heme where a pocket is located. With very few exceptions the active site is formed by hydrophobic amino acids.

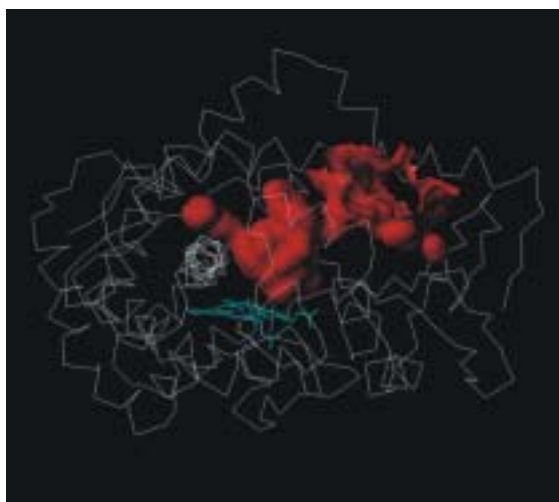


Fig. 37: CYP175A1 active site.

The model of the complex β -carotene-CYP175A1 (Fig. 38) demonstrates how well the hydrophobic environment suited the substrate molecule. The substrate fitted excellently into the binding pocket, accommodating the long aliphatic chain in the narrow channel and the ring in the pocket. The position of β -carotene within the complex model showed the C-3 of the β -ionone ring as the closest atom of the ligand to the heme iron, confirming that the observed regioselectivity of the β -carotene hydroxylation was catalyzed by CYP175A1. In fact only hydroxylation at C-3 was observed experimentally.

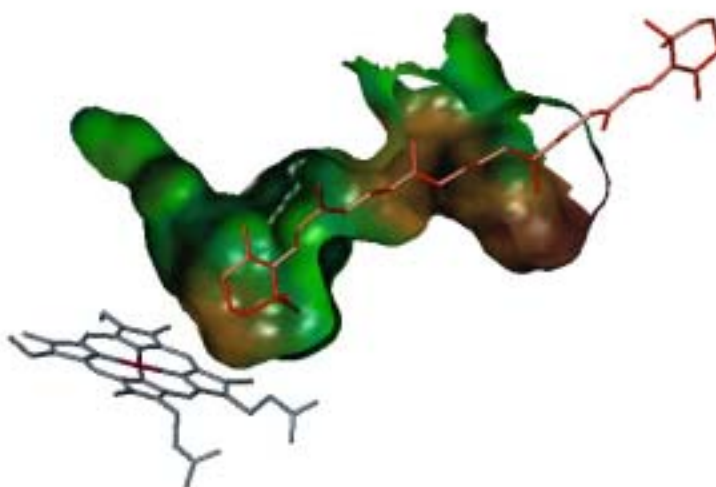


Fig. 38: Model of the complex β -carotene-CYP175A1. The surface of the active site was colored by molecular lipophilicity potential [Heiden et al., 1993]. Green and brown surfaces indicate hydrophobic residues, blue surface indicates hydrophilic ones.

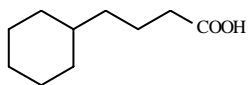
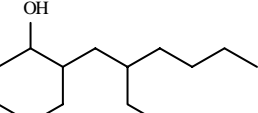
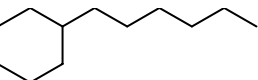
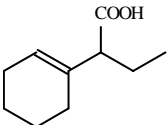
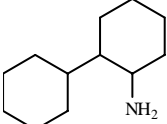
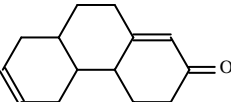
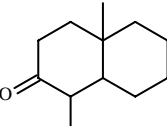
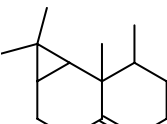
A very similar binding modus was identified for canthaxanthin. After minimization followed by short-time MD simulation (first step was 100 iterations of minimization, second - 5 ps of molecular dynamics in NEV (constant energy simulation) ensemble conditions with 1 fs step, third - 2000 iterations of minimization) the two final complexes were compared and were found very similar, indicating canthaxanthin as potential substrate for CYP175A1. K_d values for the complexes of CYP175A1 with β -carotene and canthaxanthin, respectively, were predicted according to different scoring functions (Tab. 7) [Krepets et al., 2000].

Substrate	K_d [nm]	K_d' [nm]
β -carotene	264,9	91,4
canthaxanthin	206,3	97,2

Tab. 7: K_d predicted for the complexes CYP175A1- β -carotene and CYP175A1-canthaxanthin, according to two different scoring functions [Krepets et al., 2000].

5.5.2 In silico screening for new potential substrates

A set of approximately 600 compounds from the available chemical database was screened as potential substrates depending on molecular weight, number of cycles, heteroatom content, rotatable bonds etc. The variants were generated, which could fit in the active site and then eight solutions were selected, according to both energetic and structural criteria, to perform short time MD simulations. The final result was that five of the eight compounds moved away from the heme, suggesting that they were not likely to bind CYP175A1. The other three did not move significantly. The K_d values for the complexes were predicted (Tab. 8) [Krepets et al., 2000], showing very large differences in comparison with the K_d of the β -carotene – CYP175A1 complex. Apparently, the binding of the test compounds is far less tight than the binding of β -carotene itself.

Ligand	K_d [nm]	K_d '[nm]
β-carotene	264,9	91,4
	17754565,2	20504,1
	1068452,8	2078,4
	7389627,0	4030,5
	25943625,9	20246,0
	5979905,0	7994,0
	1136843,9	3929,4
	4512541,7	5289,7
	1582475,1	3113,0

Tab. 8: K_d predicted for CYP175A1 complexes.

6. Discussion

6.1 From gene isolation to protein purification

The growing knowledge about “thermozymes” from thermophilic organisms obtained during the recent years and the importance of P450 monooxygenases as potential biocatalysts for industrial applications has created high interest in P450 enzymes from thermophilic bacteria and archaea. Different examples from literature [Koo et al., 2000] have demonstrated a broad potential application spectrum of thermostable P450s. Although homology between P450s is generally low, a few domains and sequence signatures are conserved and can be identified by homology search. Following this approach a homology search of recently sequenced genomes from thermophiles was performed to identify novel P450 enzymes of this origin. Indeed, a region showing homology to a known bacterial P450 (P450BM3) was found within the *Thermus thermophilus* HB27 genome, currently sequenced at the Institute of Molecular Microbiology and Genetics in Göttingen. Although the overall homology to known P450s did not exceed 28% some structural features could be inferred from the primary structure which indicated a P450 enzyme. The ORF was successfully amplified by PCR and cloned in common *E. coli* expression vectors.

Unfortunately, first expression experiments demonstrated that the correspondent protein was either not expressed at all or very poorly expressed in *E. coli*. Careful analysis of the gene indicated different reasons for this observation: first of all, the gene product could be toxic or a metabolic burden for the host cells. This can be avoided by employing inducible expression system. However, most of the commonly used promoters in *E. coli* are negatively and not tightly regulated [Wilms et al., 2001] and the basal expression from the start of cultivation can lead to plasmid instability and growth inhibition by the gene products. If the protein is toxic expression should not be induced until high cell densities are reached and tight regulation is necessary. However, the gene product could be cloned into different expression vectors without these problems indicating that the P450 monooxygenase from *Thermus thermophilus* is not toxic for *E. coli*.

Secondly, the high GC content could be responsible for the poor expression in *E. coli*. The high GC-content of the *Thermus* gene was reflected by differences in the codon

usages of *T. thermophilus* and *E. coli*, which could also lead to poor expression in *E. coli*. To circumvent this problem and to enhance the expression level several alternative ways can be used. An artificial gene modifying *T. thermophilus* codon usage and adapting it to *E. coli* could be synthesized. The target gene should be “*de novo*” designed, so that the rare codons could be replaced by more common ones without introducing amino acid mutations. Different approaches to synthesize artificial genes are described in literature [Khorana, 1979]. However, all of these approaches are relatively expensive and time-consuming. An alternative approach was the one developed by Stratagene with *E. coli* BL21-CodonPlus[®] competent cells. These cells enable efficient high-level expression of GC-rich heterologous proteins in *E. coli*. They are engineered to contain extra copies of genes that encode the tRNAs that most frequently limit translation of heterologous proteins in *E. coli*. From the comparison of the codon usages the main differences were observed for proline and in smaller extent also for arginine. The *E. coli* BL21-CodonPlus(DE3)-RP strain contains extra copies of *argU* (AGA, AGG) and *proL* (CCC), two of the tRNA genes which could limit translation. P450_Tth contained 33 proline and 26 of them were encoded by CCC, while 12 of the 47 arginine residues were encoded by AGG. Both CCC and AGG are rare codons for *E. coli* (Tab. 3). The use of *E. coli* BL21-CodonPlus[®] yielded a much higher (approx. 40 folds) expression level than conventional *E. coli* BL21. This showed that the rarity of some tRNAs was one of the reasons for poor expression of P450_Tth in *E. coli*.

Thirdly, it might be possible that strong mRNA secondary structures were also a limiting factor for the overexpression [Kozak, 1986]. Especially GC-rich genes are known for strong secondary structures which influence not only heterologous expression but also the sequencing or amplification of the genes. The translation initiation sequence is of crucial importance for the expression of GC-rich genes and is therefore a possible target for optimization of the expression [Tang et al., 1999]. Another practical method for the overexpression of GC-rich genes in *E. coli* by modification of the initial sequence was found in literature [Ishida et al., 1997]. It was reported that the *leuB* gene (encoding for 3-isopropylmalate dehydrogenase) and the *pfk1* gene (encoding for phosphofructokinase) of *T. thermophilus* were scarcely expressed in *E. coli*, unless a leader sequence was added to the N-terminal sequence of the gene. The first 15 base pairs were identical to those coded by *E. coli lacZ*. The authors found an increased expression level and demonstrated that the expression was

limited by secondary structure(s) in the translational initiation region of the mRNA. This inhibitory effect was relieved by the introduction of a leader open reading frame [Ishida et al., 1994].

The observation that low shaking speed and high ratio culture volume/Erlenmeyer flask volume yielded a better expression level indicated that the oxygen content in the medium could be an important factor influencing the expression. A possible explanation for this observation is the oxidative inactivation of cytochrome P450 [Karuzina and Archakov, 1994]. Moreover, it was reported that the first step in heme degradation is thought to be the cytochrome reductase-, NADPH-, and O₂- dependent α -meso-hydroxylation of heme by heme oxygenase [Liu et al., 1997]. The effect of the temperature decrease after induction and of the induction point have no straightforward explanations but have been observed in other cases as well (Yano, pers. comm.).

Unlike other thermostable proteins, the heat precipitation of *E. coli* proteins after cell disruption was not applicable for P450_Tth. Although the protein was not membrane-bound it seemed to be membrane-associated. This assumption is confirmed by the fact that P450_Tth precipitates together with the membrane fraction and the *E. coli* proteins during heat precipitation. However the purified enzyme is stable up to 88°C. Instead of heat precipitation an ammonium sulfate precipitation was used as a first purification/concentration step.

For further purification several matrices were screened according to the capability to bind P450_Tth. Cation exchange chromatography matrices did not bind P450_Tth under the experimental conditions and hydrophobic interaction chromatography (HIC) matrices such as butyl sepharose bound the protein so strongly that the elution of the P450 fraction was impossible under non-denaturing conditions. Two protocols were developed for the purification of P450_Tth, depending on the purity of the protein preparation needed. The first protocol was a two step chromatographic fractionation using anion exchange and hydroxylapatite chromatography. The second protocol was a one step chromatographic purification via HIC. For practical reasons both the purification procedures were carried out at room temperature. Due to the thermal stability of P450_Tth no inactivation during the purification process at room temperature was observed.

6.2 Protein characterization

P450 monooxygenases are good candidates for spectroscopic characterization. They display typical absorption spectra, which make the identification convenient. Additionally, the spectroscopic features provide insights into the coordination state as well as the oxidation state of the heme iron. P450_Tth displayed UV-VIS absorption spectra analogous to those of other P450 enzymes. Thus, the isolated enzyme was identified as a P450 although the overall sequence homology is relatively low. Furthermore, the heme iron was present as low spin Fe(III). From the absence of any absorption signal at 420 nm, it could also be excluded that inactivation occurred during purification. The X-band EPR spectrum showed that the coordination of the heme iron in P450_Tth is analogous to the one of other known P450s (iron octahedral coordinated with the nitrogens of the porphyrin ring, a cysteine and water as axial ligands) confirming the results from the UV-VIS spectrum.

The melting point of the protein was determined spectroscopically and calorimetrically. It was reported [McLean et al., 1998], [Maves and Sligar, 2001] that the results of differential scanning calorimetry (DSC), which is normally the technique of choice for such measurements, do not differ significantly from the ones predicted by spectroscopic determinations. The results obtained for both methods confirmed this observation. P450_Tth melting point was determined to be 88 °C, which makes it interesting for biotechnological applications.

6.3 Crystal structure

The crystal structure of P450_Tth was solved by T. Poulos' group in the frame of a co-operation. Recently, this group solved the X-ray structure of the first thermally stable P450 (CYP119 from *S. solfataricus*). A comparison of the CYP119 structure with mesophilic P450s indicated that a large aromatic network extending ~ 39 Å (C-alpha – C-alpha) down one side of the molecule might in part account for the enhanced thermal stability of CYP119. The discovery that *T. thermophilus* also produces a P450 provides a second example of a thermostable P450. A detailed characterization of P450_Tth is important for understanding if P450_Tth and CYP119 share common structural features related to thermal stability.

The primary difference between the mesophilic and thermophilic P450s is the investment of charged residues into salt-linked networks at the expense of single charge-charge interactions. Additional factors involved in the thermal stability are a decrease in the overall size, especially shortening of loops and connecting regions, and a decrease in the number of residues such as Asn, Gln and Cys. After comparison of CYP175A1 with CYP119 and P450BM3 it appears that the two thermophilic P450s share some common structural features expected to contribute to enhanced thermal stability. Both are smaller than mesophilic P450s, which is especially notable in CYP175A1. CYP175A1 closely resembles P450BM3 without the loops. In addition, both thermophilic proteins contain a significant increase in salt bridged networks compared to mesophilic P450s. The two thermostable P450s sharply differ in the extensive aromatic network found in CYP119 which is not present in CYP175A1 or any other P450 for which a structure is available.

The comparison between the structure of CYP175A1 and the one of P450BM3, showed a rms backbone deviation of 1.64 Å for 304 C α atoms. Most of the differences between CYP175A1 and P450BM3 are located in the loops connecting the helices. Moreover, the major portions of the F-G region and the B' helix which are known to be important in substrate binding are in nearly identical conformations in CYP175A1 and P450BM3.

6.4 *In vitro* characterization

A common problem with new enzymes of unknown activity obtained from genome sequences is to screen for substrates. The situation is even more complicated if the enzyme also requires support from other unknown proteins or cofactors to carry out a given reaction. In the case of the catalytic activity of P450_Tth neither the substrate nor the corresponding reductase system was known. Furthermore, this thermophilic enzyme probably exhibits the highest activity at elevated temperatures which required reductases which are stable at this temperature as well. Despite the information obtained from the preliminary characterization and from the X-ray, no insights concerning the natural substrates of P450_Tth were obtained. Moreover, a homology search within the *T. thermophilus* genome did not identify any flavodoxin/flavodoxin reductase like proteins, which are generally required in P450 catalyzed reactions.

Thus, the characterization of the P450_Tth had to deal with two unknown parameters at the same time. Different approaches have been tested to identify the substrates and a suitable reductase system.

Even though it was reported [Simgen et al., 2000] that the Soret band is not always an appropriate spectroscopic probe to monitor substrate binding, this method was used as a pre-screening to limit the number of substrates for the *in vivo* experiments. This spectroscopic method was used to identify compounds which bound to CYP175A1. A variety of potential substrates were screened with a large molar excess of the test compounds but a shift from the low-spin to the high-spin form could only be detected in two cases: α and β -ionone.

The second approach was based on the so called “peroxide shunt” to overcome the lack of homologous redox partners. Hydrogen peroxide was reported to substitute both cofactor and electron transfer proteins, generating directly the oxyferryl oxidizing species [Joo et al., 1999]. Compounds belonging to different chemical classes (alkanes, aromatics, fatty acids, heteroaromatics etc.) were tested as substrates but no conversion was detected. Based on these results it was tried to reconstitute the P450 activity using bovine adrenodoxin/adrenodoxin reductase as electron transfer system as successfully applied for other bacterial P450 enzymes [Simgen et al., 2000]. The NADPH consumption (decrease of the absorption at 340 nm) was determined and the reaction mixture was analyzed by GC. The results as well as the conclusions were identical as described above. However, it cannot be excluded that the tested compounds were substrates, since it could not be investigated whether the peroxide shunt or the bovine reductase system was applicable for the P450_Tth.

6.5 *In vivo* characterization

From the analysis of the region flanking CYP175A1 in the genome of *T. thermophilus*, it was possible to identify the vicinal ORFs of the gene. Interestingly, one ORF had been identified as phytoene synthase (*crtB*) [Hoshino et al., 1993]. Carotenogenic genes in *Thermus* like in other organisms are usually arranged in a biosynthetic cluster [Tabata et al., 1994]. This was the first hint to assume that

CYP175A1 might be involved in the carotenoids biosynthesis acting as a β -carotene hydroxylase. These enzymes generally share high sequence homologies as demonstrated in the alignment of the amino acid sequences of the known bacterial β -carotene hydroxylases (*crtZ*) (Fig. 38).

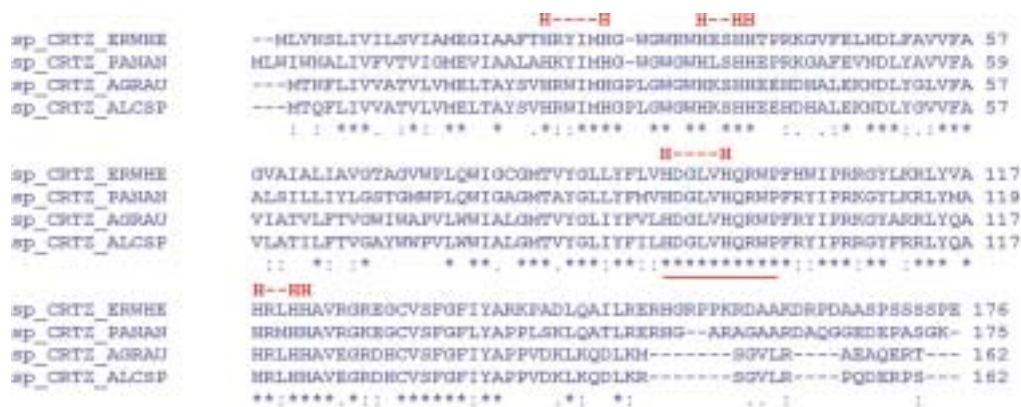


Fig. 38: Amino acid sequence alignment of the known bacterial β -carotene hydroxylases. Amino acid residues which were either well or perfectly conserved in all the sequences are indicated by (.) and (*) below the alignment, respectively. Conserved histidine motifs are shown above the sequences. The highly conserved motif 1 is underlined. GenBank accession numbers: CRTZ_ERWHE (*Erwinia herbicola*) M87280; CRTZ_PANAN (*Erwinia uredovora*) D90087; CRTZ_AGRAU (*Agrobacterium aurianticum*) D58420; CRTZ_ALCSP (*Alcaligenes* sp.) D58422.

There are very few gaps in the alignment, and a number of highly conserved regions are evident, the most notable being the underlined so called motif 1 (amino acids HDGLVHXRXP; [Linden, 1999], [Sun et al., 1996]). The identity between the sequences is relatively high (>40 %). Additionally, four characteristic histidine motifs can be recognized. They were proposed to be involved in iron binding and their importance was proven by site directed mutagenesis [Bouvier et al., 1998]. CYP175A1 did not show overall amino acid sequence similarity to the known β -carotene hydroxylases and neither of the above mentioned motifs could be recognized in the sequence (Fig. 39).

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sp_CRTE_AGRAU      -----MTNFLIVVAT-----VLVMEL 16
sp_CRTE_ALCSP     -----MTQFLIVVAT-----VLVMEL 16
sp_CRTE_ERWHE     -----MLVNSLIVILS-----VIAMEG 17
sp_CRTE_PANAN     -----MLNIWNGALIVFVT-----VIGMEV 19
thermus            MKRLSLREANPYLKDLDQDD PLAVLLANWGRAHPRLFLPLPRFFLALIPDFEFGVREGALLAEG 60
                                     : + : : *

      H-----H           H--HH
sp_CRTE_AGRAU     TAYSVHERN---IMHGP---LGNWGHKSHHEERDHALEK----- 48
sp_CRTE_ALCSP     TAYSVHERN---IMHGP---LGNWGHKSHHEERDHALEK----- 48
sp_CRTE_ERWHE     IAAFTHERY---IMHG---WGNWGHKSHHTPRKGVFEL----- 48
sp_CRTE_PANAN     IAALAHKY---IMHG---WGNWGHKSHHEPRKGAFEV----- 50
thermus            TTGATFQYRALSRLTGRGLLDWGESWKEARKALKDFPLPQSVRGYREAMEERARAFFGE 120
      : . . : : *      * * : : : . . :

sp_CRTE_AGRAU     -----NDLYGLVFAVIATVLF-----TVGMINAPVLNWIALG----- 80
sp_CRTE_ALCSP     -----NDLYGVVFAVLATILF-----TVGAYNWPVLNWIALG----- 80
sp_CRTE_ERWHE     -----NDLFAVVFAGVAIALI-----AVGTAGVWPLQWIGCG----- 80
sp_CRTE_PANAN     -----NDLYAVVFAALSILLI-----YLGSTGRWPLQWIGAG----- 82
thermus            WRGRRERDLHEMLALSRLGRALPGKPLSPSLAEHALKALDRINAGQTRSPALLDLAAE 180
      : : : : : * : : : : * *

      H-----H
sp_CRTE_AGRAU     ---MTVYGLIY---FVLEDGLVBQR-----WPFRIYPRK 108
sp_CRTE_ALCSP     ---MTVYGLIY---FILEDGLVBQR-----WPFRIYPRR 108
sp_CRTE_ERWHE     ---MTVYGLLY---FLVHDGLVBQR-----WPFHWIYPR 108
sp_CRTE_PANAN     ---MTAYGLLY---FMVHDGLVBQR-----WPFRIYPRK 110
thermus            ARPKRDRGALYREARALIVHPFLSHLPRERALSEAVTLLVAGHETVASALTWSEFLLSHR 240
      . * : * : : : * * * * * * * * * * * * * * * * * * * * * * * *

sp_CRTE_AGRAU     GYARR-----LYQAH 118
sp_CRTE_ALCSP     GYFRR-----LYQAH 118
sp_CRTE_ERWHE     GYLKR-----LYVAH 118
sp_CRTE_PANAN     GYLKR-----LYMAH 120
thermus            POWQKRVASSEEAAALAAFQEARLRYPPAWILTRRLERPLLLGEDRLPPGTTLVLSPIVTQ 300
      : : * : :

      --HH
sp_CRTE_AGRAU     RLHHAVE-----GRDHCVSFGFIYAPPVDKLEQDLKM---- 150
sp_CRTE_ALCSP     RLHHAVE-----GRDHCVSFGFIYAPPVDKLEQDLKR---- 150
sp_CRTE_ERWHE     RLHHA VR-----GRGCVSFGFIYARKPADLQAILRER--H 152
sp_CRTE_PANAN     RMHHA VR-----GRGCVSFGFIYAPPLSKLQATLRER--H 154
thermus            RLHFDDGEAFRPERFLEEROTPSGRYFFPFGLOQLCLORDFALLEGPIVLRRAFFRRFLD 360
      * : * . . * : * : * . . * * : :

sp_CRTE_AGRAU     -----SGVLR---AEAGERT----- 162
sp_CRTE_ALCSP     -----SGVLR---PQDERPS----- 162
sp_CRTE_ERWHE     GRPPKRDAAKDRPDAASPSSSSPE----- 176
sp_CRTE_PANAN     G--ARAGAARDAQGGEDFPASGK----- 175
thermus            PLFFFRVLAQVTLRPEGGLPARPREEVRA 389
      : :

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Fig. 39: Amino acid sequence alignment of CYP175A1 with the known bacterial β -carotene hydroxylases.

However, the β -carotene hydroxylase from *Synechocystis sp.* PCC6803, active in functional complementation experiments, showed very low sequence similarity to the known bacterial β -carotene hydroxylases as well [Masamoto et al., 1998]. The alignment between CYP175A1 and the enzyme isolated from *Synechocystis sp.* revealed also no overall homology. Beside that it was reported that in *Haematococcus pluviialis* a cytochrome P450 dependent enzyme might be involved in the bioconversion of β -carotene to astaxanthin [Schoefs et al., 2001]. Furthermore, neither *crtW* nor *crtB* from *H. pluviialis* show homology to P450s in general and more in detail to P450_Tth.

To evaluate this hypothesis heterologous complementation experiments with *E. coli* cells engineered to produce β -carotene were performed. These experiments showed that CYP175A1 was able to catalyze the hydroxylation of β -carotene to zeaxanthin via β -cryptoxanthin. A surprising feature of P450_Tth is the finding that the catalytic activity of this thermophilic enzyme was functionally supported by a redox partner from *E. coli*, a mesophilic bacterium. The flavodoxin/flavodoxin reductase (Fpr-Fld) system from *E. coli* may function as redox partner and supply P450_Tth with electrons needed for the catalytic activity. The system Fpr-Fld fulfills in *E. coli* several tasks. Specifically, Fpr primarily provides reduced flavodoxin which activates, directly or indirectly, different enzymes like cobalamin dependent methionine synthase, pyruvate-formate lyase, anaerobic ribonucleotide reductase, and biotin synthase. This system had been described to support bovine cytochrome P450c17 hydroxylase activity [Jenkins and Waterman, 1994] and had been fully characterized [Jenkins et al., 1998]. If the assumption is correct it is surprising that the soluble Fpr-Fld system could replace a P450 reductase in supporting microsomal cytochrome P450 activity, since *E. coli* does not contain endogenous P450 enzymes or immunologically detectable P450 reductase [Porter et al., 1987].

6.6 *In silico* characterization

In theory, there should be some form of structural explanation for the various phenomena associated with P450-mediated reactions, and modeling represents a possible way of achieving such molecular structural rationalizations for many aspects of the P450 system. Furthermore, with increasing knowledge about structure-function relationships of P450 systems molecular modeling might help to explain whether there are common 3-D structural characteristics which could facilitate the prediction of P450-mediated metabolism using computational techniques.

The model of the β -carotene-CYP175A1 complex was generated using AutoDock 3.0. The hydrophobic nature of the ligand fitted well into the binding site of CYP175A1 which contained mostly hydrophobic or neutral residues. The position of the ligand in the active site of the enzyme showed that the C-3 of the β -ionone ring is the closest atom to the heme iron confirming the experimentally observed regioselectivity. The

binding site formed a narrow channel, where the long C-chain of β -carotene was accommodated, and a broad pocket corresponding to the region above the heme, where the cyclohexenyl ring was placed. The second ring was located outside of the protein. CYP175A1 symmetrically introduced OH groups to β -carotene, as shown by the traces of β -cryptoxanthin experimentally isolated. Thus β -cryptoxanthin is also a substrate of CYP175A1. Apparently, β -cryptoxanthin diffused out of the enzyme and then bound again to an enzyme molecule with the non hydroxylated side. The OH group which is already introduced into the molecule is assumed to influence the re-association of the enzyme-substrate complex because it changes the polarity and the steric volume of the hydroxylated endgroup. This would explain the release of symmetrically hydroxylated zeaxanthin which was experimentally observed.

A model of the complex canthaxanthin-CYP175A1 was generated and investigated. The model looked very similar to the β -carotene-CYP175A1 complex. Despite of the keto group on the cyclohexenyl ring, the position of canthaxanthin in the active site did not differ significantly from the one of β -carotene. K_d values of the two complexes were predicted according to two different scoring functions. However, no prediction could be made since the observed differences were in the same order of magnitude like the method's error. Apparently, none of the scoring functions were suitable to describe the binding of CYP175A1 with either β -carotene and canthaxanthin.

A virtual screening for new substrates was carried out by means of Docksearch. A database of approximately 600 compounds was screened. Finally, a set of six compounds was selected, according to both energetic and structural criteria. K_d values were predicted and differed significantly from that predicted for the *in vivo* substrate β -carotene. The size of these compounds might be responsible for the difference in K_d because β -carotene is indeed at least twice as large as the virtual test compounds, thus involving larger protein-ligand interactions and better stabilization of the complex. However, these results could not exclude *a priori* binding but suggested that for a substrate binding larger interactions with the protein were needed to make the binding tighter.

7. References

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8. Curriculum vitae

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1977-1985 Elementary school, Augusta (SR), Italy
 1985-1990 Gymnasium, Augusta (SR), Italy (High school equivalent)
 1990-1995 Chemistry studies (comparable to M. Sc.), Catania, Italy (110/110 magna cum laude)
 Topic: „Kinetic resolution of *Rac*-Suprofen by Lipase catalyzed enantioselective esterification“
 1995-1997 Specialization School in Synthetic Organic Chemistry, Milano, Italy (100/100 magna cum laude)
 Topic: „Asymmetric synthesis of fluoroalkylated amino acids“
 2000 to date PhD studies, Stuttgart (Germany)
 Topic: “Cloning, expression, purification and characterization of a novel cytochrome P450 isolated from *Thermus thermophilus* HB27”
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Publications:

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