Appl Microbiol Biotechnol (2004) 64: 671–674
DOI 10.1007/s00253-003-1529-7

F. Blasco · I. Kauffmann · R. D. Schmid

CYP175A1 from *Thermus thermophilus* HB27, the first β-carotene hydroxylase of the P450 superfamily

Received: 26 August 2003 / Revised: 13 November 2003 / Accepted: 21 November 2003 / Published online: 16 January 2004

Abstract The biological function of thermostable P450 monooxygenase CYP175A1 from *Thermus thermophilus* HB27 was studied by functional complementation in *Escherichia coli*. The gene product of CYP175A1 added hydroxyl groups to both β rings of β-carotene to form zeaxanthin (β,β-carotene-3,3′-diol) in *E. coli*, which produces β-carotene due to the *Erwinia uredovora* carotenoid biosynthesis genes. In addition, spectroscopic methods revealed that *E. coli* carrying CYP175A1 and the cDNA of the *Haematococcus pluvialis* carotene ketolase was able to synthesise hydroxyechinenone. The predicted amino acid sequence of the enzyme from *T. thermophilus* does not show substantial similarity with other known β-carotene hydroxylases, but 41% with the cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP102A1, P450 BM3). It is concluded that CYP175 A1 represents a new type of β-carotene hydroxylase of the P450 superfamily.

Introduction

P450 monooxygenases are widespread enzymes, and various genome projects have identified a remarkably high number of P450s in bacteria, plants and animals. In the presence of suitable redox partners, P450 monooxygenases use electrons from NAD(P)H to catalyse the activation of molecular oxygen, leading to the regio- and stereospecific oxidative attack of various structurally different chemicals (Werck-Reichhart and Feyereisen 2000). In bacteria, P450s play an essential role in steroid biosynthesis, drug catabolism, and the utilisation of carbon compounds as an energy source. They are also involved in the biosynthesis of various macrolide antibiotics. Recently, they were invoked in the transformation of β-carotene to astaxanthin in the green microalgae *Haematococcus pluvialis* (Schoefs et al. 2001).

Thermus thermophilus is a Gram-negative, aerobic microorganism that grows at temperatures above 75°C. Many species of the genus *Thermus* produce carotenoids (Brock 1984). The carotenoids of *T. thermophilus* include thermozeaxanthins and thermobiszeaxanthins, which are carotenoid-(di)glucoside-branched fatty acid (di)esters (Yokoyama et al. 1995). Currently, the only known carotenogenic gene from *T. thermophilus* encodes phytoene synthase (*crtB*) (Hoshino et al. 1993). Genes encoding β-carotene hydroxylases (*crtZ*) have been described for several bacteria (Misawa et al. 1995; Masamoto et al. 1998; Linden 1999) but not for *T. thermophilus*.

Recently, we have isolated a thermostable P450 monooxygenase from *T. thermophilus* and analysed its crystal structure (Yano et al. 2003). Screening of the *T. thermophilus* genome for ORFs revealed that the P450 monooxygenase CYP175A1 gene is located close to an ORF previously identified as phytoene synthase (*crtB*) (Hoshino et al. 1993). Like in carotenoid-synthesising bacteria such as *Erwinia herbicola* (Hundle et al. 1994), *Erwinia uredovora* (Misawa et al. 1990) and *Rhodobacter capsulatus* (Armstrong et al. 1989), the genes required for carotenoid biosynthesis in *T. thermophilus* (Tabata et al. 1994) also occur as a cluster on a large plasmid. This led us to believe that CYP175A1 might be involved in carotenoid biosynthesis by acting as a β-carotene hydroxylase. In the present paper, we characterise the catalytic activity of CYP175A1 by functional complementation of *Escherichia coli* strains transformed with the *Erwinia uredovora* carotenoid biosynthesis genes that synthesise β-carotene. In addition, *E. coli* strains transformed with the β-carotene ketolase from *H. pluvialis* were used to study the capability of CYP175A1 to utilise oxygenated compounds as substrates.

F. Blasco · I. Kauffmann · R. D. Schmid (✉)
Institute of Technical Biochemistry, University of Stuttgart,
Allmandring 31,
70569 Stuttgart, Germany
e-mail: Rolf.D.Schmid@rus.uni-stuttgart.de
Tel.: +49-711-6853192
Fax: +49-711-6853196

Materials and methods

E. coli strains, plasmids and growth conditions

E. coli JM109 strains containing the various plasmids mentioned below were used for complementation experiments. Cultures were grown in LB medium at 30°C for 2 days and ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), tetracycline (10 μg/ml) and isopropyl-β-thio-galactopyranoside (IPTG) (0.5 mM) were added as required.

The pUCbkt plasmid harbouring the β-carotene ketolase gene (*bkt*) from *H. pluvialis*, and pRK404 were kindly provided by G. Sandmann (University of Frankfurt, Germany). Plasmids pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ carrying the carotenogenic genes *crtE*, *crtB*, *crtI* and *crtY* from *E. uredovora*, and pKK_CYP carrying the CYP175A1 gene of *T. thermophilus* are described elsewhere (Kaufmann 2002; Yano et al. 2003).

Plasmid construction

Polymerase chain reaction (PCR) was applied to introduce *Eco*RI and *Asp*718 restriction sites flanking the CYP175A1 gene by using the oligonucleotide primers 5′-CCGGAATTCATGAAGCGCCTTTCCCTGAGG-3′ and 5′-GGCGGTACCTCACGCCCGCACCTCCTC-3′ (*Eco*RI and *Asp*718 restriction sites underlined). The reaction mixture consisted of 100 ng template DNA (pKK_CYP), 2.5 U *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), 5 μl 10× reaction buffer, 5 μl DMSO, 0.4 μm of each oligonucleotide, and 400 μm dNTPs (final volume 50 μl). PCR cycling parameters were: 95°C 1 min, followed by 95°C 1 min, 55°C 30 s, 72°C 2 min 30 s for 30 cycles, and a final extension at 72°C for 5 min. The CYP175A1 gene sequence was confirmed by DNA sequencing. Subsequently, the CYP175A1 gene was cloned into the *Eco*RI/*Asp*718 sites of pBluescript II SK (Stratagene) to yield pBS_CYP. The insert of pUCbkt was isolated by restriction with *Bam*HI/*Hin*dIII and cloned into the *Bam*HI/*Hin*dIII sites of pRK404 to yield pRK_bkt.

Carotenoid extraction and identification

For the isolation of carotenoids from *E. coli*, 2 ml culture was harvested and the pellet extracted with acetone (200 μl) and re-extracted with one volume of hexane after the addition of one-fifth volume water. Pigments were analysed by silica gel (60F$_{254}$) thin-layer chromatography (TLC) developed with acetone:hexane (1:2). A Shimazu device, equipped with a photodiode array detector was used for HPLC analysis of the carotenoids. Separations were carried out on a nucleosil 100-5 C18 column (Macherey-Nagel, Düren, Germany). UV-VIS detection was performed at 440 nm. Separation of keto carotenoids was performed according to the method described by Linden et al (1999). To separate β-carotene and hydroxylated derivatives, acetonitrile/methanol/2-propanol (85:10:5, v/v/v) was applied as eluent for 17 min followed by acetonitrile/2-propanol (80:20, v/v) for 20 min. For mass spectrometry, the carotenoids were separated by silica gel column chromatography developed with a step-wise gradient of hexane to hexane/acetone (2:1). Purified carotenoids were identified using spectroscopic methods. Absorption spectra were measured with a Pharmacia Biotech Ultrospec 3000, UV-VIS spectrophotometer (Pharmacia, Freiburg, Germany). FAB mass spectral data were obtained with a Finnigan-Mat mass spectrometer model MAT 95. 500 MHz ^{1}H-NMR spectra were run in $CDCl_3$ on a Bruker ARX 500 spectrometer. Standards for analysis such as β-carotene and astaxanthin were purchased from Sigma (Steinheim, Germany). Synthetic samples of zeaxanthin and canthaxanthin were provided by BASF (Ludwigshafen, Germany) and β-cryptoxanthin by Roche Vitamins (Basel, Switzerland).

Results

DNA and amino acid sequence of the β-carotene hydroxylase from *T. thermophilus*

The predicted amino acid sequence of the protein shows similarities to known bacterial P450 monooxygenases, but not to any other β-carotene hydroxylases.

Functional complementation in *E. coli*

Transformation of plasmid pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ in *E. coli* led to an accumulation of β-carotene and yellow pigmentation of the cells. After co-transformation of plasmids pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ and pKK_CYP, the corresponding *E. coli* transformants were a slightly different colour. Carotenoid pigments were identified by TLC and HPLC (Fig. 1) as β-cryptoxanthin, zeaxanthin and β-carotene. This was confirmed by NMR. A control strain transformed with pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ and the cloning vector pKK223-3 contained only β-carotene (data not shown).

Carotenoid pigments were also extracted from *E. coli* JM109 transformed with the three plasmids pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$, pBS_CYP (containing CYP175A1) and pRKbkt (containing the β-ketolase from *H. pluvialis*). Several carotenoids were detected by TLC and HPLC (Fig. 2). Canthaxanthin and zeaxanthin were identified by comparison to authentic standards. MS (M^+ 566) and comparison with other reports (Yokoyama et al. 1995) led

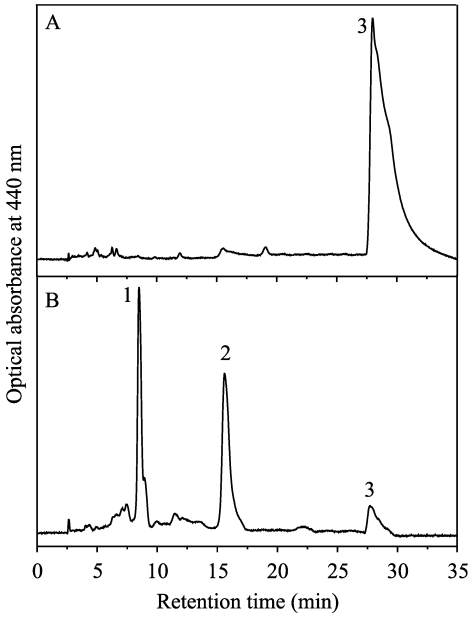

Fig. 1 HPLC separation of carotenoid pigments extracted from *Escherichia coli* cells carrying either plasmid pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ alone (**A**) or plasmid pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ along with pKK_CYP (**B**). Carotenoids identified by their retention times in comparison to authentic standards: *1* zeaxanthin, *2* β-cryptoxanthin, *3* β-carotene

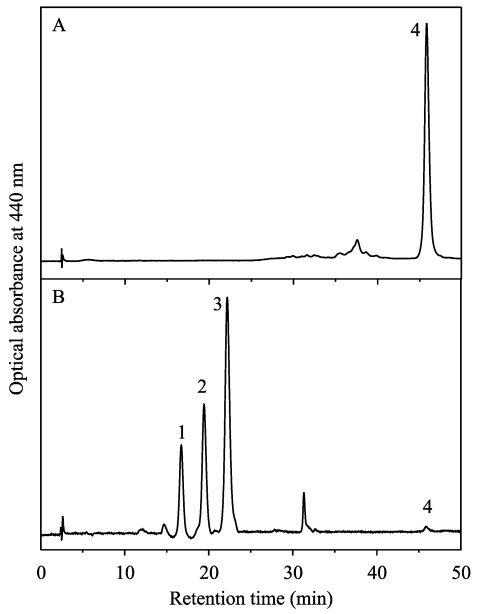

Fig. 2 HPLC separation of carotenoid pigments extracted from *E. coli* cells carrying plasmid pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ alone (**A**) or plasmid pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ along with pBS_CYP and pRKbkt (**B**). Carotenoids identified by their retention times in comparison to authentic standards: *1* zeaxanthin, *3* canthaxanthin, *4* β-carotene. 3′-Hydroxyechinenone was identified by MS and by comparison with other reports

to the identification of a third product: 3′-hydroxyechinenone.

Discussion

In the present paper we characterised the catalytic activity of P450 monooxygenase from *T. thermophilus* (CYP175A1). As practical difficulties are often associated with carotenoid conversions in vitro (Bramley 1985), we decided to use heterologous complementation with genetically engineered *E. coli* strains that are able to synthesise carotenoids (Sandmann 2002; Sandmann et al. 1999). Zeaxanthin and canthaxanthin were isolated from the transformants containing the plasmids pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ and pKK_CYP, whereas only β-carotene could be isolated from the transformants harbouring pAC-crtE$_{EU}$-crtB$_{EU}$-I14-Y$_{EU}$ and the empty cloning vector pKK223-3. We therefore are led to assume that CYP175A1 encodes a β-carotene hydroxylase that is capable of introducing OH groups into the β-ionone rings of β-carotene, producing zeaxanthin via β-cryptoxanthin (Fig. 3).

Surprisingly, the catalytic activity of the thermophilic CYP175A1 is functionally supported by an unknown redox partner from *E. coli*, a mesophilic bacterium that does not contain endogenous P450 enzymes. We assume that the soluble flavodoxin/flavodoxin reductase (Fpr-Fld) system from *E. coli* might replace P450 reductase in supporting cytochrome P450 activity. An analogous situation has already been observed for a microsomal P450 (P450c17) (Jenkins and Waterman 1994).

β-Carotene hydroxylases share a high level of sequence similarity, with the sequence identity usually exceeding 40%. Sequence comparison of CYP175A1 with *crtZ* genes encoding β-carotene hydroxylases did not, however, show any similarity in amino acid sequence. Thus, histidine-rich iron-binding motifs that are typical for β-carotene hydroxylase and ketolases were not found in CYP175A1, suggesting a different catalytic mechanism. Masamoto et al. (1998) reported a β-carotene hydroxylase from *Synechocystis* sp. PCC6803 without sequence similarity to other known *crtZ* genes and suggested different evolutionary origins of bacterial and plant enzymes to account for this finding. The alignment between CYP175A1 and the enzyme isolated from *Synechocystis* sp. did not reveal any significant sequence similarity. Thus, CYP175A1 might be the first example of a P450 enzyme involved in carotenoid biosynthesis. Recently, a cytochrome P450-dependent enzyme was invoked in the bioconversion of β-carotene to astaxanthin in *H. pluvialis* (Schoefs et al. 2001); however, neither *crtW* nor *crtZ* from *H. pluvialis* exhibit substantial sequence similarity to P450s in general or to CYP175A1.

CYP175A1 substrate specificity was also tested in canthaxanthin-producing *E. coli* strains containing β-carotene ketolase of *H. pluvialis*, an enzyme that converts β-carotene into canthaxanthin via echinenone. It is already known that hydroxylated carotenoids such as zeaxanthin and β-cryptoxanthin are poor substrates for the ketolase (Breitenbach et al. 1996). Since we isolated hydroxyechinenone along with zeaxanthin and canthaxanthin only from the *E. coli* strain transformed by three expression vectors for β-carotene synthesis, β-carotene ketolase and CYP175A1, we tend to assume that CYP175A1 is a β-carotene-specific enzyme and does not accept canthaxanthin as a substrate. Computer models of the enzyme-substrate interaction suggest that the keto group of the β-ionone ring might interfere with the catalytic activity of this hydroxylase from *T. thermophilus*.

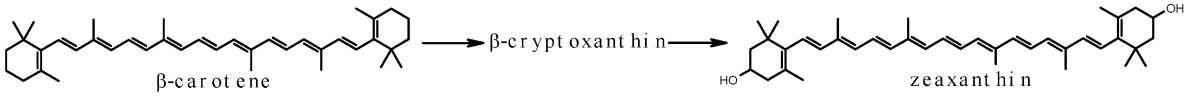

Fig. 3 Hydroxylation of β-carotene catalysed by CYP175A1 in transformants of *E. coli* containing plasmids pAC-crtE$_{EU}$-crtB$_{EU}$-114-Y$_{EU}$ and pKK_CYP

Acknowledgements We would like to thank Prof. G. Sandmann, University of Frankfurt, Germany, for providing the plasmid pUCbkt and the vector pRK404. Thanks are due to BASF AG, Ludwigshafen, and to Roche AG, Basel, for providing authentic carotenoid standards, and to BASF AG for financial support. Helpful discussions with Dr. Vlada Urlacher and Dr. Kyoko Momoi are gratefully acknowledged.

References

Armstrong G, Alberti M, Leach F, Hearst J (1989) Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. Mol Gen Genet 216:254–268

Bramley P (1985) In vitro carotenoid biosynthesis. Adv Lipid Res 21:243–279

Breitenbach J, Misawa N, Kajiwara S, Sandmann G (1996) Expression in *Escherichia coli* and properties of the carotene ketolase from *Haematococcus pluvialis*. FEMS Microbiol Lett 140:41–246

Brock T (1984) Genus thermus, Brock and Freeze. Williams & Wilkins, Baltimore, Md.

Hoshino T, Fujii R, Nakahara T (1993) Molecular cloning and sequence analysis of the crtB gene of *Thermus thermophilus* HB27, an extreme thermophile producing carotenoid pigments. Appl Environ Microbiol 59:3150–3153

Hundle B, Alberti M, Nievelstein V, Beyer P, Kleinig H, Armstrong GA, Burke DH, Hearst JE (1994) Functional assignment of *Erwinia herbicola* Eho10 carotenoid genes expressed in *Escherichia coli*. Mol Gen Genet 245:406–416

Jenkins C, Waterman M (1994) Flavodoxin and NADPH-flavodoxin reductase from *Escherichia coli* support bovine cytochrome P450c17 hydroxylase activities. J Biol Chem 269:27401–27408

Kaufmann I (2002) PhD thesis, Stuttgart University, Stuttgart, Germany

Linden H (1999) Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. Biochim Biophys Acta 1446:203–212

Masamoto K, Misawa N, Kaneko T, Kikuno R, Toh H (1998) Beta-carotene hydroxylase gene from the cyanobacterium *Synechocystis* sp. PCC6803. Plant Cell Physiol 39:560–564

Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izawa Y, Nakamura K, Harashima K (1990) Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. J Bacteriol 172:6704–6712

Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiwara S, Saito T, Ohtani T, Miki W (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. J Bacteriol 177:6575–6584

Sandmann G (2002) Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. Chembiochem 3:629–635

Sandmann G, Albrecht M, Schnurr G, Knorzer O, Boger P (1999) The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli*. Trends Biotechnol 17:233–237

Schoefs B, Rmiki N, Rachadi J, Lemoine Y (2001) Astaxanthin accumulation in *Haematococcus* requires a cytochrome P450 hydroxylase and an active synthesis of fatty acids. FEBS Lett 500:125–128

Tabata K, Ishida S, Nakahara T, Hoshino T (1994) A carotenogenic gene cluster exists on a large plasmid in *Thermus thermophilus*. FEBS Lett 341:251–255

Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genome Biol 1:3003.1–3003.9

Yano JK, Blasco F, Li H, Schmid RD, Henne A, Poulos TL (2003) Preliminary characterization and crystal structure of a thermostable cytochrome P450 from *Thermus thermophilus*. J Biol Chem 278:608–616

Yokoyama A, Sandmann G, Hoshino T, Adachi K, Sakai M, Shizuri Y (1995) Thermozeaxanthins, new carotenoid-glycoside-esters from thermophilic eubacterium *Thermus thermophilus*. Tetrahedron Lett 36:4901–4904