Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues

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The class II PHA (polyhydroxyalkanoate) synthases [PHA_{MCL} synthases (medium-chain-length PHA synthases)] are mainly found in pseudomonads and catalyse synthesis of PHA_{MCL}s using CoA thioesters of medium-chain-length 3-hydroxyfatty acids (C_6–C_{14}) as a substrate. Only recently PHA_{MCL} synthases from *Pseudomonas oleovorans* and *Pseudomonas aeruginosa* were purified and *in vitro* activity was achieved. A threading model of the *P. aeruginosa* PHA_{MCL} synthase PhaC1 was developed based on the homology to the epoxide hydrolase (1ek1) from mouse which belongs to the α/β-hydrolase superfamily. The putative catalytic residues Cys-296, Asp-452, His-453 and His-480 were replaced by site-specific mutagenesis. In contrast to class I and III PHA synthases, the replacement of His-480, which aligns with the conserved base catalyst of the α/β-hydrolases, with Gln did not affect *in vivo* enzyme activity and only slightly *in vitro* enzyme activity. The second conserved histidine His-453 was then replaced by Gln, and the modified enzyme showed only 24 % of wild-type *in vivo* activity, which indicated that His-453 might functionally replace His-480 in class II PHA synthases. Replacement of the postulated catalytic nucleophile Cys-296 by Ser only reduced *in vivo* enzyme activity to 30 % of wild-type enzyme activity and drastically changed substrate specificity. Moreover, the C296S mutation turned the enzyme sensitive towards PMSF inhibition. The replacement of Asp-452 by Asn, which is supposed to be required as general base catalyst for elongation reaction, did abolish enzyme activity as was found for the respective amino acid residue of class I and III enzymes. In the threading model residues Cys-296, Asp-452, His-453 and His-480 reside in the core structure with the putative catalytic nucleophile Cys-296 localized at the highly conserved γ-turns of the α/β-hydrolases. Inhibitor studies indicated that catalytic histidines reside in the active site. The conserved residue Trp-398 was replaced by Phe and Ala, respectively, which caused inactivation of the enzyme indicating an essential role of this residue. In the threading model this residue was found to be surface-exposed. No evidence for post-translational modification by 4-phosphopantetheine was obtained. Overall, these data suggested that in class II PHA synthases the conserved histidine which was found as general base catalyst in the catalytic triad of enzymes related to the α/β-hydrolase superfamily, was functionally replaced by His-453 which is conserved among all PHA synthases.

Key words: catalytic mechanism, class II polyhydroxyalkanoate synthase (class II PHA synthase), polyhydroxyalkanoate (PHA), *Pseudomonas aeruginosa*.

INTRODUCTION

PHA (polyhydroxyalkanoate) synthases are the key enzymes of PHA biosynthesis and catalyse the conversion of 3-hydroxyacyl-CoA substrates into PHAs with the concomitant release of CoA (Scheme 1). Meanwhile, more than 50 different PHA synthases were cloned and assigned [1,2]. The multiple alignment of the primary structures of these PHA synthases showed an overall identity of 21–88 % with only eight strictly conserved amino acid residues. The PHA_{MCL} synthases (medium-chain-length PHA synthases) from *Pseudomonas aeruginosa* share approx. 37 % identity with the class I PHA synthase from *Ralstonia eutropha*. PHA synthases have been assigned to three classes based on their substrate specificity and subunit composition. The class I PHA synthases, with *Ralstonia eutropha* synthase as prototype, are composed of a single type of polypeptide chain and use mainly (*R*)-3-hydroxybutyryl-CoA, (*R*)-3-hydoxyvaleryl-CoA and other short-carbon-chain-length hydroxyalkanoic acid CoA thioesters as substrates. The class III PHA synthases, as represented by the *Allochromatium vinosum* enzyme, are composed of two different subunits, each of approx. 40 kDa [3]. The substrate specificity is similar to that of class I synthases, although some medium-chain-length 3-hydroxyfatty acids are incorporated. Both types of PHA synthases were purified and *in vitro* activity has already been achieved [4–6]. The catalytic reaction mechanism of these enzymes was studied intensively. Most of the site-specific mutations considering putative catalytic residues have been performed with the class I PHA synthase from *R. eutropha* [6–9]. Experimental evidence was obtained that Cys-319, Asp-480 and His-508, which align with the corresponding residues of the *A. vinosum* PHA synthase, are directly involved in covalent catalysis [8]. The highly conserved Trp-425 was replaced by alanine, which reduced *in vivo* activity to 19 % and *in vitro* activity to 0.003 % of wild-type activity. This Trp-425 has been postulated to play an important role in protein–protein interaction, i.e. in the dimerization of the PhaC subunit, by generating a hydrophobic surface [8]. The post-translational modification of a conserved serine residue with 4-phosphopantetheine described for class I PHA synthase from *R. eutropha*, which was supposed to provide a catalytically active second SH group [6,10], was not

Abbreviations used: PHA, polyhydroxyalkanoate; PHA_{MCL} synthase, medium-chain-length PHA synthase; LB, Luria–Bertani; DEPC, diethylpyrocarbonate.

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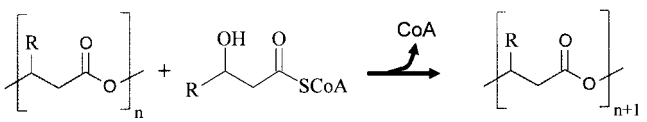
Table 1 Strains and plasmids used in this study

Strain/plasmid	Relevant characteristic	Reference
Strains		
Escherichia coli		
XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_K^-,m_K^+), *supE44*, *relA1*, λ^-, *lac* [*F*, *proAB*, *lacI*^{q}, *lacZ*Δ*M15*, *Tn10*(*Tc*^{r})]	[23]
LS1298	*el4*$^-$(*mcrA*$^-$), *supE44*, *thi-1*, *thr-1*, *leuB6*, *lacY1*, *tonA21*, *fadB*::*Kan*^{r}	[24]
BMH71-18 *mutS*	*thi*, *supE*, Δ(*lac-proAB*), [*mutS*:*Tn10*] [*F*′, *proAB*; *lacI*^{q} *lacZ* Δ*M15*]	Invitrogen
SJ16	*F*$^-$ *panD2 zad-220*::*Tn10* λ^- *216 relA1 spoT1 metB1* λ^r *Tc*^{r} *10*	[25]
Plasmids		
pBBR1MCS	*Cm*^{r}, *mob*$^+$, *tra*$^-$, *lacPOZ*′	[26]
pAA11	pBluescript SK$^-$ harbouring N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa* with mutated *phaC1* gene containing the mutation D452N	This study
pAA12	pBluescript SK$^-$ harbouring N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa* with mutated *phaC1* gene containing the mutation H480Q	This study
pAA13	pBluescript SK$^-$ harbouring N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa* with mutated *phaC1* gene containing the mutation W398A	This study
pAA14	pBluescript SK$^-$ harbouring N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa* with mutated *phaC1* gene containing the mutation W398F	This study
pAA15	pBluescript SK$^-$ harbouring N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa* with mutated *phaC1* gene containing the mutation C296A	This study
pAA16	pBluescript SK$^-$ harbouring N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa* with mutated *phaC1* gene containing the mutation C296S	This study
pAA17	pBluescript SK$^-$ harbouring N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa* with mutated *phaC1* gene containing the mutation H453Q	This study
pMCS69	pBBR1MCS containing the *phbA-phbB* genes downstream of *lac* promoter	This study
pBHR80	pBluescript SK$^-$ containing N-terminal His_6 tag *phaC1*$_{Pa}$ (*Bam*HI, *Eco*RI) from *P. aeruginosa*	[22]



Scheme 1 Reaction catalysed by class II PHA synthase

R = alkyl chain (3–11 carbon atoms).

supported by studies of the enzyme in the natural host [7]. The current model of active class I and II PHA synthase involves two subunits forming a homodimer, and forming a multimeric heterodimer (PhaC and PhaE) in case of class III PHA synthases. Accordingly, class I, II and III PHA synthases possess two thiol groups provided by the conserved cysteine residue of the PhaC subunit considering at least two subunits of PhaC in the active PHA synthase. Mutagenesis studies with the class III *A. vinosum* PHA synthase revealed that His-331 is the general base catalyst that activates the nucleophile Cys-149 for covalent catalysis. Asp-302 is assumed to function as a general base catalyst by deprotonating the 3-hydroxy group of 3-hydroxybutyryl-CoA or that of the bound 3-hydroxybutyrate to render possible the nucleophilic attack on the covalently linked thiol ester intermediate [11].

The class II enzymes which, as class I PHA synthases, are composed of only one type of subunit, are only found in pseudomonads such as, e.g. *P. aeruginosa*. One major difference between class II and both class I and III PHA synthases is the substrate specificity. Class II PHA synthases incorporate preferentially 3-hydroxyfatty acids of medium chain length (C_6–C_{14}) into PHA, and the resulting product is a latex-like polymer [12–14]. *In vivo* these substrates are mainly derived from intermediates of fatty acid β-oxidation [15,16] or from fatty acid *de novo* biosynthesis [17–20], provided fatty acids or simple non-related carbon sources, such as e.g. carbohydrates, were added, respectively. Only recently class II PHA synthases were purified by immobilized metal-chelate affinity chromatography and *in vitro* activity was achieved [21]. The purification of this enzyme was further improved by matrix-assisted refolding, which recently enabled a first biochemical characterization of this class II PHA synthase [22]. Since class I and III enzymes were already intensively studied with respect to catalytic residues and reaction mechanism, we investigated the role of conserved amino acid residues in class II enzymes.

Table 2 Primers used in this study

Primer name	Sequence (5′ → 3′)
PHAC1MC296A	AACCTCCTCGGCGCCGCCTCCGGCGGATCACCA
PHAC1MH453Q	GGTCTGAACGACCAGATCACCCCCTGGG
PHAC1MC296S	CCTCCTCGGCGCCTCCTCCGGCGGGATCAC
PHAC1MW398A	CGACATCCTCTACGCCAACAACGACACCAC
PHAC1MW398F	CGACATCCTCTACTTCAACAACGACACCAC
PHAC1MD452N	CGCCGGTCTGAACAACCACATCACCCCCTG
PHAC1MH480Q	CTCCAACAGCGGTCAGATCCAGAGCATCCT

EXPERIMENTAL

Bacterial strains, plasmids and growth of bacteria

Strains and plasmids used in this study were summarized in Table 1. *Escherichia coli* was grown at 37 °C in LB (Luria–Bertani) medium. Different carbon sources were added as indicated.

Isolation, analysis and manipulation of DNA

All genetic procedures and manipulations of DNA were conducted as described by Sambrook et al. [27]. DNA sequencing was carried out by the dideoxy chain-termination method with single-stranded or double-stranded alkali-denatured plasmid DNA, but with 7-deazaguanosine 5′-triphosphate instead of dGTP. Oligonucleotides used for site-specific mutagensis were summarized in Table 2. Site-specific mutagenesis was conducted using the kit GeneEditor™ (Promega GmbH, Mannheim, Germany). Instructions were followed as provided in the manufacturer's protocol. All site-specific mutations generated in this study were confirmed by DNA sequencing.

SDS/PAGE and Western immunoblotting

SDS/PAGE was performed according to Sambrook et al. [27]. Proteins were separated in SDS/12.5 %-polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. Western blotting was performed using the Semidry Fastblot (Biometra, Goettingen, Germany). On Western blots [28] using nitrocellulose membranes

the *P. aeruginosa* PHA_{MCL} synthase PhaC1 was detected applying mono-specific, polyclonal anti-PhaC1 antiserum and an alkaline-phosphatase-antibody conjugate as a secondary antibody. Bound antibodies were detected using Nitro Blue Tetrazolium chloride and the toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate.

Analysis of 4-phosphopantetheinylated proteins

The procedure of Rusnak et al. [29] was followed. *E. coli* was cultivated in Dex-E-B1-met medium containing 0.5 % (w/v) glucose, 1 mM thiamine and 0.002 % (w/v) methionine. Media contained the appropriate antibiotic, 1 mM isopropyl β-D-thiogalactoside and 20 mM [U-^{14}C]β-alanine (220 mCi/mmol). Cells were cultivated for 24 h. Crude extracts were prepared, and proteins were separated by SDS/PAGE. Autoradiography was performed to visualize 4-phosphopantetheinylated proteins. Immunoblotting was conducted to identify the class II PHA synthase. Cells were also analysed with respect to PHA accumulation to obtain evidence for *in vivo* activity of class II PHA synthases.

Class II PHA synthase activity *in vivo*

PHA synthase activity was confirmed by expression of the respective PHA synthase gene in various metabolic backgrounds favouring PHA_{MCL} synthesis, e.g. *E. coli* RS3097 (only in the presence of β-oxidation inhibitor acrylic acid) and *E. coli* LS1298 [12,14]. Recombinant bacteria harbouring the respective plasmid were cultivated in the presence of 0.25 % (w/v) decanoate. PHA accumulation was determined by GC/MS analysis of lyophilized cells and indicated *in vivo* PHA synthase activity.

GC/MS of polyester in cells

PHA was qualitatively and quantitatively analysed by GC/MS. Liquid cultures were centrifuged at 10 000 ***g*** for 15 min, then the cells were washed twice in saline and lyophilized overnight. Lyophilized cell material (8–10 mg) was subjected to methanolysis in the presence of 15 % (v/v) H_2SO_4. The resulting methyl esters of the constituent 3-hydroxyalkanoic acids were assayed by GC/MS as described in [30]. GC analysis was performed by injecting 3 μl of sample into a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (Palo Alto, CA, U.S.A.). The same column as for the GC analysis was applied using a temperature profile as described previously [30].

Class II *in vivo* substrate specificity

The *in vivo* substrate specificity of the class II PHA synthase and the respective mutants was determined, transferring the respective plasmid into *E. coli* LS1298 (fadB) which provides medium-chain-length (*R*)-3-hydroxyacyl-CoAs, when cultivated in LB medium plus 0.5 % (w/v) decanoate [13,31]. Cells harbouring the respective plasmid were cultivated for 48 h and PHA content and composition were analysed by GC/MS analysis. The PHA was indicative of the relative substrate specificity of the modified PHA synthases compared with the wild-type PHA synthase.

Analysis of (*R*,*S*)-3-hydroxydecanoyl-CoA

The substrate (*R*,*S*)-3-hydroxydecanoyl-CoA was synthesized as described previously [17,19]. (*R*,*S*)-3-Hydroxydecanoyl-CoA was purified using Sep-Pak cartridges (reversed-phase C_{18} column; Waters, Milford, MA, U.S.A.) and eluting the CoA thioester with 0.01 M NaOH in 20 % (v/v) methanol. The purified (*R*,*S*)-3-hydroxydecanoyl-CoA was analysed by HPLC, and its concentration was determined by hydroxylamine treatment, which causes the release of bound CoA. The concentration of free CoA before and after hydroxylamine treatment [32] was analysed by the Ellman method [33].

Class II PHA synthase assay *in vitro*

The *in vitro* activity of class II PHA synthase using (*R*,*S*)-3-hydroxydecanoyl-CoA as a substrate was determined as described previously [22].

RESULTS

Development of a threading model of the *P. aeruginosa* class II PHA synthase 1

The conserved domain homology search strongly suggested that PhaC1 contains the α/β-hydrolase domain. The conserved domain alignment revealed that the region of amino acid residues 249–492 exerted 30 % similarity and 17 % identity to the conserved α/β-hydrolase domain (Figure 1). The conserved and proposed

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PhaC1:  249  QTFIVSWRNPTKSQREWGLTTYIEALKEAIEVVLSITGSKDLNLLGACSGGITTATLVGH  308
α/β:    2    DVILFDLRGFGQSSPSDLAEYRFDDLAEDLEALLDALGLDKVILVGHSMGGAIAAAYAAK  61

PhaC1:  309  YVASGEKKVNAFTQLVSVLDFELNTQVALFADEKTLEAAKRRSYQSGVLEGKDMAKVFAW  368
α/β:    62   YPE----RVKALVLVSAPHPALL----------------SSRLFPRN-LFGLLLANFRNR  100

PhaC1:  369  M-RPNDLIWNYWVNNYLLGNQPPAFDILYWNNDTTRLPAALHGEFVELFKSNPLNRPGAL  427
α/β:    101  LLRSVEALLGRALKQFFLLGRPLVSDFLKQFELSSLIRFGEDDGGDGLL----WVALGKL  156

PhaC1:  428  EVSGTPIDLKQVTCDFYCVAGLNDHITPWESCYKSARLLGGKCEFILSNSGHIQSILNPP  487
α/β:    157  LQWDVSADLKRIKVPTLVIWGDDDPLVPPDASEKLSALFPNAEVVVIDDAGHLAQLEKPE  216

PhaC1:  488  GNPKA  492
α/β:    217  EVAEL  221
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Figure 1 Alignment of the *P. aeruginosa* PHA synthase PhaC1 with the conserved α/β-hydrolase fold region (α/β)

The alignment was performed using PSI-BLAST and the conserved domain search, respectively. Conservative replacements and identical residues are highlighted with light-grey backgrounds. The catalytic residues of α/β-hydrolases are highlighted with a dark-grey background. Mutagenized residues are labelled with stars.

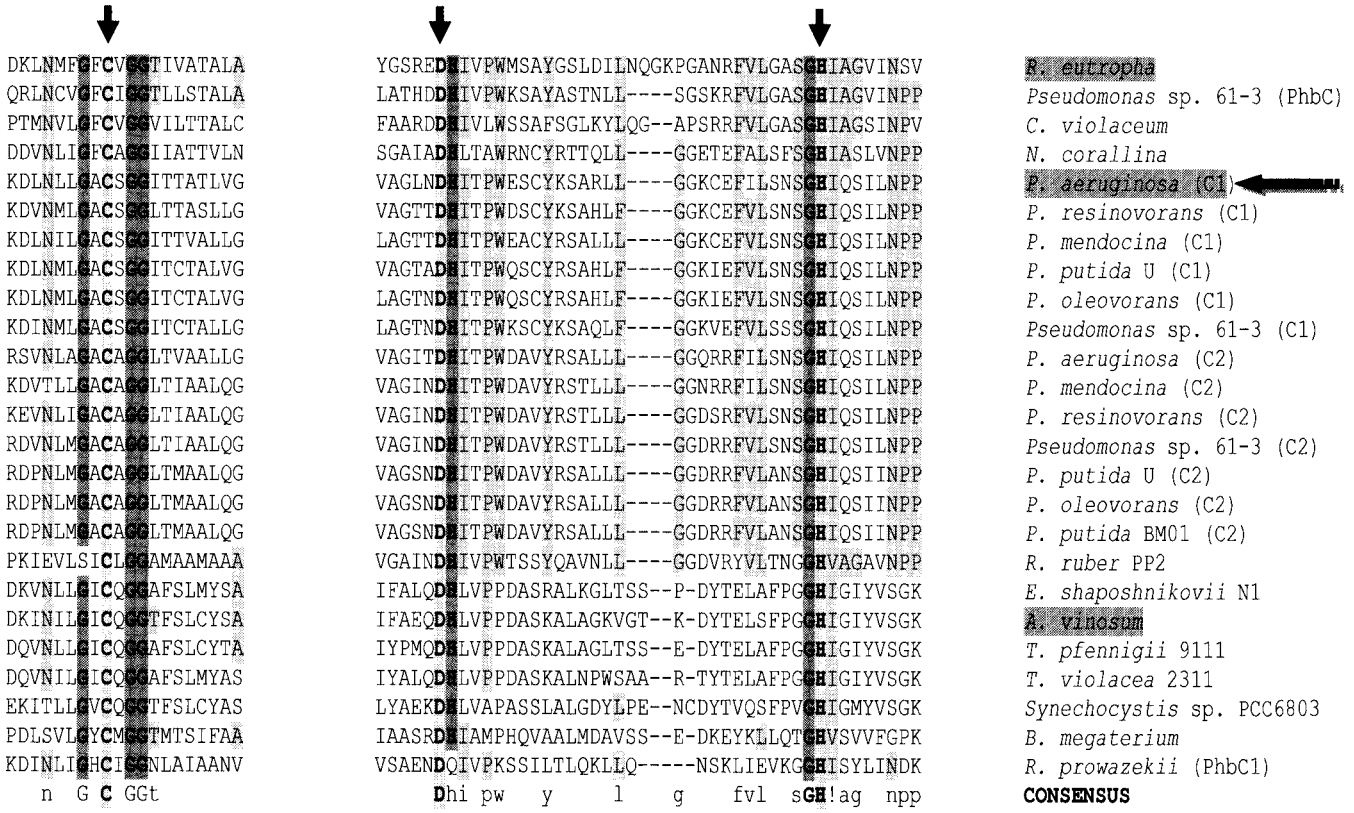

Figure 2 Multiple alignment of the proposed catalytic residues of PHA synthases

The proposed catalytic residues are indicated by arrows. See the Figure 1 legend for details of the shading of the sequences. On the Consensus line bold capital letters indicate residues that are conserved in all PHA synthases (! represents **I**); other capital letters represent highly conserved residues and lower-case letters represent moderate conservation. On the right, bacteria with PHA synthases that have been studied extensively are shown with a dark-grey background. Genus names (top to bottom): *R*, *Ralstonia*; *C*, *Chromobacterium*; *N*, *Nocardia*; *P*, *Pseudomonas*; *R. ruber*, *Rhodococcus ruber*; *E*, *Ectothiorhodospira*; *A*, *Allochromatium*; *T. pfennigii*, *Thiocapsa pfennigii*; *T. violacea*, *Thiocystis violacea*; *B*, *Bacillus*; *R. prowazekii*, *Rickettsia prowazekii*.

catalytic residues of the PhaC1 aligned with amino acid residues constituting the catalytic triad in enzymes belonging to α/β-hydrolases (Figures 1 and 2). A 3D-PSSM [34] similarity search resulted in an alignment showing approx. 55% similarity of PhaC1 with the epoxide hydrolase (1ek1) from mouse, which belongs to the α/β-hydrolase superfamily (Figure 3). This alignment in combination with the conserved domain alignment was used to generate a threading model of PhaC1 (Figure 4). The N-terminal region (amino acids 1–184) and five further regions (234–239, 302–306, 402–407, 434–443 and 455–459) were deleted in the PhaC1 used for the protein model. Deletions were introduced because no homology for these regions with structurally conserved regions was found and the loop search against a loop-fold library failed (HOMOLOGY software package from Molecular Simulations). Moreover, deletions were located exclusively in highly variable regions according to the multiple alignment of PHA synthases [1,2]. A threading model of PhaC1 was finally developed using software packages HOMOLOGY and DISCOVER (from Molecular Simulations; Figure 4). Energy minimization was performed employing the consistent valence force field (CVFF) implemented in DISCOVER. The stereochemistry of the model structure was evaluated with the program PROCHECK [35] and the residue environment was analysed with the VERIFY_3D program that implements the algorithm of Lüthy et al. [36]. The resulting model suggests that PhaC1 is a member of the protein family possessing an α/β-hydrolase fold, the core structure, as in the case of the epoxide hydrolase. Additional submission of the PhaC1 sequence to three other algorithms that search structural databases (SAM-T02 [37], 3D-PSSM [34] and the UCLA Foldserver [38]) resulted also in fits to other enzymes belonging to the α/β-hydrolase fold family with high confidence levels (results not shown). Inspection of the protein model of PhaC1 showed that the active-site Cys-296, the conserved His-480 and the Asp-452, presumably forming a catalytic triad, are adjacent to the core structure (Figure 4). These residues are conserved in all PHA synthases and are proposed to be required for catalytic activity [39]. The active-site Cys-296 was located at the nucleophile elbow, a sharp γ-turn containing the nucleophilic residue, positioned between a β-strand and an α-helix, which is one of the most conserved features of the α/β-hydrolase enzymes.

Site-specific mutagenesis of the class II PHA synthase from *P. aeruginosa*

The five amino acid residues Cys-296, Trp-398, Asp-452, His-453, and His-480, which are conserved among all classes of PHA synthases, were investigated by site-specific mutagenesis. The corresponding amino acid residues of the three amino acid residues Cys-296, Asp-452 and His-480 have been analysed in the class I PHA synthase from *R. eutropha* and the class III PHA synthase from *A. vinosum*, suggesting strongly that these residues are involved in covalent catalysis [6,8,11,40]. These residues

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           151                                                        200
PhaC1___PSS  HHHHHHHHHH HHHHHHHHCC CCCCCCCCCE EEECCCCCCC CCCE....EE
PhaC1___Seq  GGKSLLDGLG HLAKDLVNNG GMPSQVDMDA FEVGKNLATT EGAV....VF
-----------                                    --++-- +++V    V+
d1ek1a2_Seq  .......... .......... .......... ....LPVPCN PNDVSHGYVT
d1ek1a2_SS   .......... .......... .......... ....CCCCCC CCCCCCCCCE
CORE         .......... .......... .......... ....000030 0003000050

           201                                                        250
PhaC1___PSS  ECCCEEEEEE ECCCCCCCCC EEEEECCCCC CHHHHCCCCH HHHHHHHHHC
PhaC1___Seq  RNDVLELIQY RPITESVHER PLLVVPPQIN KFYVFDLSPD KSLARFCLRN
-----------  ++++++L ++ --++   ~++ +L-++++-- +++++-++      +++++++
d1ek1a2_Seq  VKPGIRL.HF VEMG...SGP ALCLCHGFPE SWFSWRYQ.. ...IPALAQA
d1ek1a2_SS   EECCEEC.CE EEEC...CCC CEEEECCCCC CCCCCCCC.. ...CHHHHHC
CORE         3000007.04 2050...000 3678901520 01002000.. ...7107400

           251                                                        300
PhaC1___PSS  CCCEEEECCC CCCCCHHCCC HHHHHHHHH. .HHHHHHHHH CCCCCEEEEE
PhaC1___Seq  GVQTFIVSWR NPTKSQREWG LTTYIEALK. .EAIEVVLSI TGSKDLNLLG
-----------  G+++-+++++ +++-S+++-- +++Y+--L+   E++-++-+- G++++-++G
d1ek1a2_Seq  GFRVLAIDMK GYGDSSSPPE IEEYAMELLC KEMVTFLDKL .GIPQAVFIG
d1ek1a2_SS   CCCEEEECCC CCCCCCCCCC CHHHCCCCCC CCCCHHHHHH .CCCCEEEEE
CORE         0109578040 0100100000 0000010011 0071064003 .010043865

           301                                                        350
PhaC1___PSS  ECHHHHHHHH HHHHHHHHCC CCCCEEEEEE EECCCCCCCC HHHHHHHH.H
PhaC1___Seq  ACSGGITTAT LVGHYVASGE KKVNAFTQLV SVLDFELNTQ VALFADEK.T
-----------  +-+-G+++     ++++--++ ++V+A+--L- +++-++++-- -++-+-++ +
d1ek1a2_Seq  HDWAGVMV.. ..WNMALFYP ERVRAVASLN TPFMPPDPDV SPMKVIRSIP
d1ek1a2_SS   EHHHCCCC.. ..CCHHHCCC CCEEEEEEEC CCCCCCCCCC CHHHHHCCCC
CORE         00042048.. ..20471000 0050185430 1120000000 0000000000

           351                                                        400
PhaC1___PSS  HHHHHHHHHC CCCCCH...H HHHHHH.... .......... .........H
PhaC1___Seq  LEAAKRRSYQ SGVLEG...K DMAKVF.... .......... .........A
-----------  +---++-+-- -GV-E+   K -M+++F                          -
d1ek1a2_Seq  VFNYQLYFQE PGVAEAELEK NMSRTFKSFF RASDETGFIA VHKATEIGGI
d1ek1a2_SS   CCCCCCHHHC CCCCCCCCCC CCCCHHHCCC CCCCCCCCCC CCCCCCCCCC
CORE         1001001600 0004001900 0100220251 0000000043 1004001017

           401                                                        450
PhaC1___PSS  CCC.HHHHHH HHHHHHHHCC CCHHHHHHHH HHHCCCCCCH HHHHHHHHHH
PhaC1___Seq  WMR.PNDLIW NYWVNNYLLG NQPPAFDILY WNNDTTRLPA ALHGEFVELF
-----------  +-+ P+D-+- ++      --+ +++++F+I++ -----+R-P- -+++
d1ek1a2_Seq  LVNTPEDPNL SK.....ITT EEEIEFYIQQ FKKTGFRGPL NWYR......
d1ek1a2_SS   CCCCCCCCCC CC.....CCC HHHHHHHHHH HHHHCCCHHH HHCC......
CORE         4001000000 00.....000 0001000000 2000040015 0110......

           451                                                        500
PhaC1___PSS  HHCCCCCCCC CEECCEECCH H.HCCCCEEE EEECCCCCCC HHHHHHHHHH
PhaC1___Seq  KSNPLNRPGA LEVSGTPIDL K.QVTCDFYC VAGLNDHITP WESCYKSARL
-----------     +--R+-+ +++-G    L + ++-+++++ V-+++D-+-- -E++++++++
d1ek1a2_Seq  ...NTERNWK WSCKG....L GRKILVPALM VTAEKDIVLR PEMSKNMEKW
d1ek1a2_SS   ...CCCCCCC CCCCC....C CCCCCCCEEE EEECCCCCCC HHHHCCHHHC
CORE         ...0000000 00000....0 0003050532 2010001110 0001001000

           501                                                        550
PhaC1___PSS  CCCCCEEECC CCCCEEEEEC CCCCCEE.EE ECCCCCCCCH HHHHHCCEEC
PhaC1___Seq  LGGKCEFILS NSGHIQSILN PPGNPKA.RF MTNPELPAEP KAWLEQAGKH
-----------  ++--++-+++ ++GH+++I+- P-+++-+ -+ ++++E
d1ek1a2_Seq  IPFLKRGHIE DCGHWTQIEK PTEVNQILIK WLQTE..... ..........
d1ek1a2_SS   CCCCEEEEEC CCCCCCCCCC CCCCCCCCCC CCCCC..... ..........
CORE         2002001020 0000010000 0002002410 14000..... ..........

           551                                                  595
PhaC1___PSS  CCCCCHHHHH HHHHCCCCCC CCCCCCCCCC CCCCCCCCCC EEEEC
PhaC1___Seq  ADSWWLHWQQ WLAERSGKTR KAPASLGNKT YPAGEAAPGT YVHER
-----------
d1ek1a2_Seq  .......... .......... .......... .......... .....
d1ek1a2_SS   .......... .......... .......... .......... .....
CORE         .......... .......... .......... .......... .....
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Figure 3 Alignment of the *P. aeruginosa* PHA synthase PhaC1 with mouse epoxide hydrolase (1ek1)

The alignment was performed using the 3D-PSSM algorithm [34]. Mutagenized residues are shown with a grey background. PhaC1_PSS, secondary structure prediction of PhaC1; PhaC1_Seq, amino acid sequence of PhaC1; ----, showing no similarity (−), similarity (+) and amino acids in the proteins that are identical in that position; d1ek1a2_Seq, amino acid sequence of structurally resolved protein epoxide hydrolase; d1ek1a2_SS, secondary structure of protein epoxide hydrolase; Core, hydrophobic residues residing in the core structure of library structure (0 means least important and 9 means very important for hydrophobic interactions in the core structure).

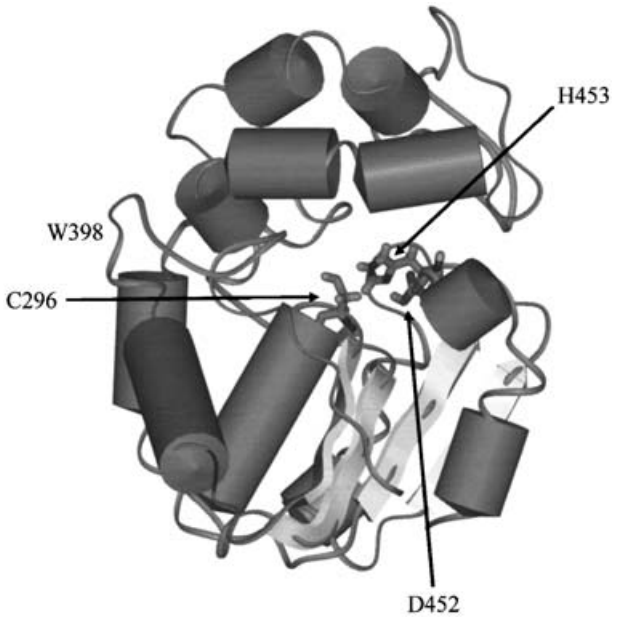

Figure 4 Threading model of PhaC1 from *P. aeruginosa*

The threading model was developed based on the alignment shown in Figure 3. The software packages HOMOLOGY and DISCOVER (Molecular Simulations) were applied to generate the model. Cylinders represent α-helical structures. Big arrows in dark grey represent β-strands. The putative catalytic residues are given as stick side chains and indicated by arrows.

Table 3 *In vivo* and *in vitro* enzyme activity of modified class II PHA synthases

Means ± S.D. from three independent experiments are shown. The *in vivo* activity of 100 % corresponds to PHA accumulation contributing to 18 % of CDW (cellular dry weight). The *in vitro* activity of 100 % corresponds to 30 m-units · mg^{-1}. ND, not detectable.

Mutation	PHA content (%, w/w of CDW)	*In vivo* activity (% of wild-type activity)	*In vitro* activity (% of wild-type activity)
wt	18 ± 2	100	100 ± 5
C296A	ND	ND	ND
C296S	6.3 ± 1	35	14 ± 3.5
D452N	ND	ND	ND
H480Q	18 ± 1	100	41 ± 3
H453Q	4.4 ± 0.5	24	7 ± 2
W398A	ND	ND	ND
W398F	ND	ND	ND

were considered to constitute the catalytic triad, which was found in enzymes belonging to the α/β-hydrolases, and were residing in the core structure of the developed PhaC1 model. The following mutations of PhaC1 were performed using site-specific mutagenesis: C296A, C296S, D452N, H453Q and H480Q (Figures 2 and 4). Mutational analysis of the class I PHA synthase from *R. eutropha* has suggested that the conserved Trp-425 is involved in protein–protein interaction, i.e. in dimerization, by generating a hydrophobic surface. Therefore the corresponding amino acid Trp-398 of the class II PHA synthase was analysed by site-specific mutagenesis generating the following mutants: W398A and W398F. In order to compare class II PHA synthases with class I and class III PHA synthases, the same respective amino-acid replacements were used. *In vivo* activity was achieved by expression of the respective mutated PHA synthase genes located on plasmid pBluescript SK^- co-linear to the *lac* promoter in *E. coli* LS1298 and by quantitative analysis of the accumulated PHA (Table 3). Crude extracts of the same cells were subjected to *in vitro* PHA synthase activity measurements using purified (*R*)-3-hydroxydecanoyl-CoA (Table 3). The expression levels of the

respective mutated PHA synthase genes were evaluated by immunoblotting analyses, which indicated that all PHA synthase genes were expressed equally (results not shown).

In vivo and *in vitro* activity of modified class II PHA synthases

Analysis of the *in vivo* activity of the respective modified PhaC1 showed that replacement of Cys-296 with Ala abolished enzyme activity, whereas the replacement with Ser resulted in an enzyme with 35 % of wild-type enzyme *in vivo* activity (Table 3). The *in vitro* activity was only 14 % of wild-type activity. The D452N mutation resulted in inactivation of PhaC1, whereas the H480Q mutation did not affect the *in vivo* activity. In contrast to the *in vivo* activity, the *in vitro* activity was reduced to 41 % of wild-type activity. Replacement of the second conserved His-453 with Gln resulted in strong reduction of *in vivo* and *in vitro* activity to 24 and 7 % of wild-type activity, respectively (Table 3).

Analysis of substrate specificity of the various class II PHA synthase mutants

In order to investigate whether or not the substrate specificity was changed by the respective mutation, we transferred the respective plasmid into either *E. coli* LS1298 or *E. coli* XL1-Blue-harbouring plasmid pMCS69. *E. coli* LS1298 provides medium-chain-length 3-hydroxyacyl-CoA thioesters as a substrate for PHA synthase when grown on fatty acids, and *E. coli* XL1-Blue (pMCS69) provides 3-hydroxybutyryl-CoA as a substrate independently of the carbon source used. Plasmid pMCS69, a derivative of pBBR1MCS which contains the β-ketothiolase gene (*phbA*) and acetoacetyl-CoA reductase (*phbB*) gene from *R. eutropha* co-linear to the *lac* promotor, was constructed to provide a plasmid that co-replicates with the pBluescript SK$^-$ derivatives mediating provision of (*R*)-3-hydroxybutyryl-CoA from acetyl-CoA (Table 1). None of the mutants were able to use 3-hydroxybutyryl-CoA as a substrate (Figure 5). Interestingly, the substrate specificity with respect to 3-hydroxyacyl-CoA thioesters, ranging from 6 to 12 carbon atoms, was strongly altered in the C296S and the H453Q mutants (Figure 5). Both mutants showed an approx. 2-fold increase in affinity towards incorporation of 3-hydroxyhexanoyl-CoA and 3-hydroxydodecanoyl-CoA into PHA (Figure 5).

Active-site residues

We probed the active site of *P. aeruginosa* PhaC1 and its permissive site-specific mutants by incubating the enzymes, respectively, with the hydroxyl-specific agent PMSF. Incubation of PhaC1 with PMSF caused only weak inhibition (Table 4). In contrast, incubation of the C296S mutant with PMSF caused strong inhibition (Table 4). Moreover, preincubation in the presence of the substrate 3-hydroxydecanoyl-CoA reduced the level of inhibition, indicating the likelihood that Ser-296 provides the nucleophilic hydroxyl at the active site (results not shown).

Furthermore, the histidine reagent DEPC (diethylpyrocarbonate) was used to study the role of histidines in enzyme activity. Incubation of PhaC1 and its mutants with DEPC, a histidine reagent, caused strong inhibition of enzymic activity, except for the C296S mutant, which was only weakly inhibited (Table 4). Interestingly, both histidine mutants (H453Q, H480Q) were inhibited strongly by DEPC. The inhibition of activity depends upon the concentration of DEPC. To determine whether the inactivation of PhaC1 results from modification of an active-site residue, we looked for substrate protection against DEPC inactivation. The substrate 3-hydroxydecanoyl-CoA protects PhaC1 from inactivation by DEPC (Table 4). Protection by 3-hydroxyacyl-CoA suggests that the modified residue accounting for the inactivation is in the *P. aeruginosa* PhaC1 active site or at least in the cleft which docks 3-hydroxyacyl-CoA.

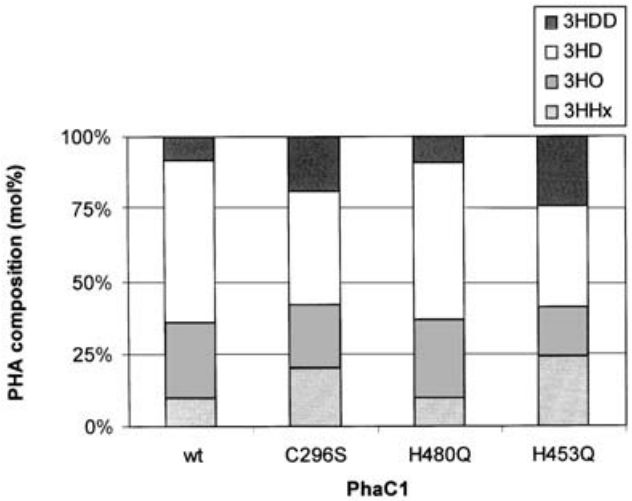

Figure 5 Analysis of *in vivo* substrate specificity of PhaC1 by determining the composition of the respective polyester

E. coli LS1298 harbouring the respective plasmids was used. LB medium containing 0.4 % (w/v) decanoate, 1 mM isopropyl β-D-thiogalactoside and antibiotics was used. Cultivations were conducted in 300-ml Erlenmeyer flasks in 50 ml of medium for 48 h at 37 °C. Whole cells were used to determine the PHA composition by GC/MS. Means from three independent measurements are presented. 3HDD, 3-hydroxydodecanoate; 3HD, 3-hydroxydecanoate; 3HO, 3-hydroxyoctanoate; 3HHx, 3-hydroxyhexanoate.

Table 4 Relative inhibition of wild-type class II PHA synthase and the respective modified PHA synthases by DEPC and PMSF

	In vitro activity (% of wild-type enzyme activity) of modified PhaC1			
Inhibitor (mM)	wt	H480Q†	C296S†	H453Q†
DEPC				
0	100 ± 5	41 ± 3	14 ± 3.5	7 ± 2
1*	53 ± 7	55 ± 5.2	90 ± 3	75 ± 5.2
0.1	65 ± 5.5	63 ± 3.3	92 ± 2.2	84 ± 1
1	40 ± 4	48 ± 4	92 ± 6	74 ± 4.2
2	34 ± 3	27 ± 12	92 ± 1.8	29 ± 11.7
PMSF				
0	100 ± 5	41 ± 3	14 ± 3.5	7 ± 2
1*	90 ± 5.5	80 ± 10	60 ± 1.8	90 ± 5
0.1	99 ± 6	96 ± 7	80 ± 3.2	93 ± 3.6
1	85 ± 4	84 ± 5	52 ± 4	88 ± 2.3
2	83 ± 7	79 ± 2.7	41 ± 4	84 ± 2.4

* Enzmyes were preincubated with 500 μM (*R*,*S*)-3-hydroxydecanoyl-CoA. The *in vitro* activity of 100 % corresponds to 30 m-units · mg^{-1}.

† The enzyme activity of modified PHA was set to 100 %, when calculating the relative enzyme activity of inhibitor-treated enzyme.

Post-translational modification of PhaC1 by 4-phosphopantetheine

Recently, evidence was provided that class I and class III PHA synthases are post-translationally modified by 4-phosphopantetheine [10]. In this study we investigated whether the class II

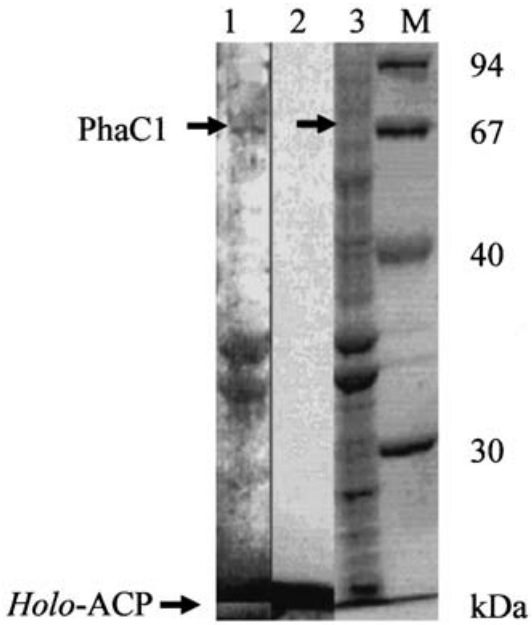

Figure 6 Analysis of 4-phosphopantetheinylation of the class II PHA synthase in recombinant *E. coli* SJ16

Lane 1, immunoblot analysis of lane 3 with mono-specific anti-PhaC1 antibodies; lane 2, autoradiograph of lane 3; lane 3, SDS/PAGE of crude extracts of recombinant *E. coli* SJ16 harbouring plasmid pBHR80, which was cultivated in the presence of [2-^{14}C]β-alanine; lane M, molecular-mass standards. The positions of PHA synthase PhaC1 and *holo*-ACP (*holo*-acyl carrier protein) are indicated.

PHA synthase is post-translationally modified by 4-phosphopantetheine. The *phaC1* gene was expressed in the β-alanine auxotrophic *E. coli* SJ16 (*panD*) in the presence [^{14}C]β-alanine, which serves as a precursor for CoA and 4-phosphopantetheine. Respective crude extracts were separated by SDS/PAGE and exposed to X-ray films. Crude extracts of the same cells, but grown in the presence of β-alanine, were subjected to SDS/PAGE analysis combined with immunoblot analysis. The *phaC1* gene was functionally expressed as confirmed by immunoblot analysis and measurement of *in vitro* enzyme activity. However, no labelling of PhaC1 by [^{14}C]4-phosphopantetheine could be detected (Figure 6). *Holo*-acyl carrier protein of *E. coli*, which is 4-phosphopantetheinylated, served as a positive control.

DISCUSSION

The multiple alignment of the primary structures of 54 PHA synthases showed the presence of six conserved blocks and eight conserved amino acid residues [2]. It has been noted that all PHA synthases contain a putative lipase box, Gly-Xaa-(Ser/Cys)-Xaa-Gly, in which the essential active-site serine of the lipase is replaced with a cysteine in the PHA synthase [9,10]. A conserved domain search strongly suggested that all classes of PHA synthases contain an α/β-hydrolase domain with the putative active-site cysteine residue aligning with the active-site residue (Ser, Cys, Asp) of enzymes belonging to the superfamily of α/β-hydrolases [2,9,10] and threading models of class III and class I PHA synthase have been developed [9,11]. Moreover, all three postulated catalytic residues constituting the catalytic triad were found to align with respective residues of α/β-hydrolases.

In this study, we developed the first threading model of a class II PHA synthase, the PHA synthase I from *P. aeruginosa*, based on the homology to the epoxide hydrolase (1ek1) from mouse, the structure of which has been determined by X-ray analysis [41]. Many of the features of the α/β-hydrolase fold are maintained in the protein model. The central β-sheet in the core of the molecule conforms to the α/β-hydrolase fold (Figure 4). Inspection of the proposed PhaC1 model showed that the proposed catalytic residues Cys-296, His-480 and Asp-452 are located adjacent to the core structure of PhaC1 protein model with the catalytic Cys-296 located at the nucleophile elbow (Figure 4). The active-site Cys-296 lies at the C-terminal end of a β-strand, corresponding to strand β5 of the lipases, in the strand-turn-helix motif.

Site-specific mutagenesis analysis of the class III *A. vinosum* PHA synthase has indicated that residues Cys-149–His-331 constitute an essential catalytic dyad in which the nucleophilic Cys-149 is involved in covalent catalysis and His-331 serves as a general base catalyst [11]. Studies with Asp-302 mutants suggest that Asp-302 functions as a general base catalyst in activation of the 3-hydroxyl of 3-hydroxybutyryl-CoA (or a hydroxybutyrate acyl enzyme) for nucleophilic attack on the covalently linked thiol ester intermediate [11]. Mutational studies with the class I *R. eutropha* PHA synthase indicated an essential catalytic dyad (Cys-319–His-508) in which the nucleophilic Cys-319 is involved in covalent catalysis and His-508 serves as a general base catalyst [8]. A conserved Asp-480 was shown not to be required for acylation of Cys-319 by sT-CoA [a terminally saturated trimer of (*R*)-3-hydroxybutyryl-CoA] and is proposed to function as a general base catalyst to activate the hydroxyl of (*R*)-3-hydroxybutyryl-CoA for ester formation [8]. In addition, the highly conserved Trp-425 has been replaced by alanine, which reduced *in vivo* activity to 19 % and *in vitro* activity to 0.003 % of wild-type activity. This Trp-425 has been postulated to play an important role in protein–protein interaction, i.e. in the dimerization of the PhaC subunit, by generating a hydrophobic surface [8,9]. Interestingly, this was consistent with our threading model for this class I PHA synthase in which Trp-425 was located at the surface. Furthermore, the site-specific mutations Y445F and L446K have resulted in reduced activity and since Tyr-445 and Leu-446 are hydrophobic residues and are located adjacent to Trp-425 at the surface of the protein model, these residues might exert a similar function in view of dimerization [9].

In this study, we generated for the first time site-specific mutants of a class II PHA synthase. The class II PHA synthase PhaC1 from *P. aeruginosa* was used as a representative enzyme. The highly conserved and proposed catalytic residues Cys-296, Asp-452 and His-480 were considered to constitute the catalytic triad, as was found for class I and class III PHA synthases (Figure 2).

To our surprise, replacement of the catalytic nucleophile Cys-296 with Ser only reduced *in vitro* enzyme activity to 14 % of wild-type enzyme activity, whereas the same replacement in class I and class III synthases strongly reduced enzyme activity to 0.001 % and 0.1 % of wild-type enzyme activity, respectively [8,11]. This finding suggested strongly that serine can functionally replace the Cys-296, presumably enabling the formation of an acyl oxoester enzyme intermediate. The oxoester intermediates are thermodynamically more stable than the original thioester intermediates, which might interfere with the elongation reaction that requires hydrolysis of acyl ester enzyme intermediates for chain elongation. This might explain the reduction in enzyme activity.

However, replacement of Asp-452 with Asn did abolish enzyme activity, which was consistent with the data obtained from analysis of class I and III PHA synthases, suggesting a function as a general base catalyst in activation of the 3-hydroxyl of hydroxy acyl enzyme for nucleophilic attack on the covalently linked thiol ester intermediate. Thus Asp-452 is assumed to function as a general base catalyst by deprotonating the 3-hydroxy group of

3-hydroxyacyl-CoA or that of the bound 3-hydroxyacyl to render possible the nucleophilic attack on the covalently linked ester intermediate.

In contrast to class I and III PHA synthases, replacement of the proposed general base catalyst His-480 with Gln did not affect *in vivo* enzyme activity and only reduced *in vitro* enzyme activity to 41 % of wild-type enzyme activity (Table 3). The replacement of the corresponding His residue in the class I *R. eutropha* PHA synthase and the class III *A. vinosum* PHA synthase resulted in *in vitro* enzyme activity relative to the wild-type enzyme of $< 5 \times 10^{-4}$ and 0.017 %, respectively.

Since a general base catalyst would be required for activation of the catalytic nucleophile for nucleophilic attack, the second conserved histidine His-453 was investigated by site-specific mutagenesis. The mutant H453Q was strongly impaired in enzyme *in vivo* and *in vitro* activity, exerting 24 and 7 % of wild-type activity, respectively (Table 3). These data suggested that His-453 might be the general base catalyst, but might be functionally replaced by His-480.

In order to further evaluate the role of histidine residues in covalent catalysis by class II PHA synthase, the histidine-specific inhibitor DEPC was used (Table 4), which showed that the wild-type enzyme and the histidine mutants are inhibited by this reagent. These data suggested that histidine residues play a role in catalysis (Table 4). Moreover, substrate preincubation studies which revealed that the substrate protects against inhibition by DEPC indicated that the respective histidine residues reside in the active site. Inhibitor studies using PMSF as serine-specific inhibitor indicated that in the mutant C296S, serine functions as a catalytic nucleophile, whereas serine plays no role in catalysis of the wild-type enzyme (Table 4).

The highly conserved Trp-425 in the *R. eutropha* class I PHA synthase was replaced by alanine, which reduced *in vivo* activity to 19 % and *in vitro* activity to 0.003 % of wild-type activity. Replacement of the corresponding Trp-398 with Ala abolished *in vivo* and *in vitro* enzyme activity, indicating the importance of this residue for enzyme activity presumably by enabling dimerization. Previously, we reported that dimerization of PhaC1 was induced by addition of substrate [22].

The discrepancies observed for *in vivo* and *in vitro* activities of the respective mutants, generated in this study, might be due to the fact that the enzymes were still capable of efficient acylation but catalysis of polymerization reaction was impaired [9,42].

Recently, Yuan et al. [10] reported that 4-phosphopantetheinylation might play a role for enzyme activity of class I and III PHA synthases. However, analysis of 4-phosphopantethenylation of the class II PHA synthase did not provide evidence for such a post-translational modification (Figure 6). Thus the second nucleophilic group might be provided through dimerization of PhaC1 [22].

Overall, a first threading model of a class II PHA synthase was developed, which basically is in agreement with various mutations generated in this study. This model served as a working hypothesis for our mutational approach. This is the first approach to investigate amino acid residues involved in catalysis of class II PHA synthases, which suggested a catalytic triad (Cys-296–Asp-452–His-453) with the originally proposed general base catalyst His-480 functionally replaced by His-453 (Figure 4). Moreover, replacement of the catalytic nucleophile Cys-296 with Ser kept the enzyme highly active, which was not the case in the respective class I and III PHA synthases. Interestingly, mutants C296S and H453Q showed a significantly changed substrate specificity, which has not been described for the respective class I and class III PHA synthases (Figure 5). This is an example where a single amino acid replacement exerted a strong impact on substrate specificity. A conclusion of this study is that besides differences in substrate specificity the class II PHA synthases differ strikingly from class I and III PHA synthases with respect to catalytic residues.

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