The crystal structure of a novel bacterial adenyltransferase reveals half of sites reactivity

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Phosphopantetheine adenyltransferase (PPAT) is an essential enzyme in bacteria that catalyses a rate-limiting step in coenzyme A (CoA) biosynthesis, by transferring an adenyl group from ATP to 4'-phosphopantetheine, yielding dephospho-CoA (dPCoA). Each phosphopantetheine adenyltransferase (PPAT) subunit displays a dinucleotide-binding fold that is structurally similar to that in class I aminoacyl-tRNA synthetases. Superposition of bound adenyl moieties from dPCoA in PPAT and ATP in aminoacyl-tRNA synthetases suggests nucleophilic attack by the 4'-phosphopantetheine on the α -phosphate of ATP. The proposed catalytic mechanism implicates transition state stabilization by PPAT without involving functional groups of the enzyme in a chemical sense in the reaction. The crystal structure of the enzyme from *Escherichia coli* in complex with dPCoA shows that binding at one site causes a vice-like movement of active site residues lining the active site surface. The mode of enzyme product formation is highly concerted, with only one trimer of the PPAT hexamer showing evidence of dPCoA binding. The homologous active site attachment of ATP and the structural distribution of predicted sequence-binding motifs in PPAT classify the enzyme as belonging to the nucleotidyltransferase superfamily.

Keywords: coenzyme A/crystal/half site reactivity/nucleotidyltransferase superfamily/phosphopantetheine adenyltransferase

Introduction

Coenzyme A (CoA), the principal acyl group carrier in all living cells, is required for numerous synthetic and degradative reactions in intermediary metabolism (Robinshaw and Neely, 1985). It is invariably synthesized from pantothenate (vitamin B₅), cysteine and ATP in five steps. The penultimate step is the transfer of an adenyl group from ATP to 4'-phosphopantetheine, catalysed by phosphopantetheine adenyltransferase (PPAT), yielding dephospho-CoA (dPCoA) and pyrophosphate (Figure 1). Subsequent phosphorylation at the 3'-hydroxyl of the ribose ring by dephospho-CoA kinase (dPCoAK) produces the acyl group carrier, CoA.

CoA metabolic intermediates in *Escherichia coli*, including 4'-phosphopantetheine, are present in significant

amounts (Jackowski and Rock, 1984), suggesting that PPAT catalyses a rate-limiting step in the pathway. PPAT is thus a plausible target for inhibition, aiming at reducing the intracellular levels of CoA and preventing bacterial growth. In mammalian systems, PPAT and dPCoAK occur as a bifunctional enzyme complex (Suzuki *et al.*, 1967; Worrall and Tubbs, 1983), leading to the common use of the term 'CoA synthase' to describe these two activities. The mammalian enzyme complex has been purified to homogeneity from pig liver, and is reported to be a dimer of identical subunits of M_r 57 kDa (Worrall and Tubbs, 1983). In bacteria, however, PPAT and dPCoAK occur as separable enzymes. PPAT from *Brevibacterium ammoniagenes* has been characterized and used for synthesis of CoA analogues (Martin and Drueckhammer, 1993). More recently, Geerlof *et al.* have cloned, over-expressed and purified PPAT from *E.coli* (A.Geerlof, A.Lewendon and W.V.Shaw, submitted). Sequence comparison between mammalian and bacterial PPAT enzymes has not been reported to date.

The gene encoding PPAT was found originally as an open reading frame immediately downstream of *kdtA* (a gene encoding 3-deoxy-D-manno-2-octulosonic acid transferase; Clementz and Raetz, 1991) and named *kdtB*. It was suggested that the protein encoded by the *kdtB* gene would be involved in some other aspect of lipopolysaccharide assembly, namely the function of 3-deoxy-D-manno-2-octulosonic acid cytidyltransferase (Clementz and Raetz, 1991; Bork *et al.*, 1995). However, recently, Geerlof *et al.* have shown that the *kdtB* protein is a PPAT, but the enzyme did not show any activity with 3-deoxy-D-manno-2-octulosonic acid (A.Geerlof, A.Lewendon and W.V.Shaw, submitted). Thirteen PPAT amino acid sequences, all from bacteria, are currently available from DDBJ/EMBL/GenBank. Alignment of the amino acid sequences by CLUSTAL (Higgins and Sharp, 1988) reveals sequence similarity for 34 out of 159 amino acid residues in the PPAT sequence. Of the 34 homologous residues, 14 are invariant amino acids.

Here we report the crystal structure of the hexameric PPAT (M_r of the protomer 17.8 kDa) from *E.coli* in complex with its product dPCoA. This is the first structure from the CoA biosynthetic pathway to be reported. The three-dimensional structure of PPAT is compared with that of class I aminoacyl-tRNA synthetases, and likely candidate catalytic residues are identified. The molecular basis of half of sites reactivity is discussed.

Results and discussion

The structure of the protomer

The structure of PPAT consists of a five-stranded parallel β -pleated sheet folded in the manner known as the dinucleotide-binding fold (or canonical Rossmann-binding

fold; Rossmann *et al.*, 1975) of approximate dimensions $50 \times 35 \times 35 \text{ \AA}^3$ (Figure 2). This common structural motif was first observed in dehydrogenases and is found in kinases, synthetases and many other proteins that utilize ATP, GTP or other high energy nucleotide analogues.

The structures of several CoA-binding proteins of diverse functions are known in complex with their corresponding CoA derivatives (Modis and Wierenga, 1998). The folds of the 20 CoA-dependent enzymes known to date cover a wide diversity of topologies, and there are no sequence or structural motifs conserved amongst CoA-binding proteins. CoA bound in the various protein complexes shows little consensus in its mode of binding other

Fig. 1. The penultimate step in the CoA biosynthetic pathway. PPAT reversibly catalyses the adenylation by ATP of 4'-phosphopantetheine, forming 3'-dephospho-CoA and pyrophosphate.

Fig. 2. Stereo drawing (Bacon and Anderson, 1988; Kraulis, 1991; Merritt and Murphy, 1994) of the PPAT protomer: α -helices are depicted by blue helical ribbons and β -strands by red arrows. The product, dPCoA, is shown in ball-and-stick representation. As in the case of other dinucleotide-binding folds, PPAT is folded into two symmetrically related halves. The first half consists of three β -strands ($\beta 1$, $\beta 2$ and $\beta 3$) connected by intervening α -helices ($\alpha 1$ and $\alpha 2$), while the second half contains two β -strands ($\beta 4$ and $\beta 5$) related by α -helices ($\alpha 3$ and $\alpha 4$). A tandem repeated motif consisting of a short 3_{10} -helix followed by an α -helix ($\alpha 5$ – $\alpha 6$ and $\alpha 7$ – $\alpha 8$) comprises the C-terminus. A short linker sequence connects the two halves of the fold.

than its propensity for an elongated conformation (Engel and Wierenga, 1996).

Quaternary structure

The oligomeric structure of the enzyme in solution (A.Geerlof, A.Lewendon and W.V.Shaw, submitted) and in the crystal structure is consistent with that of a hexamer having point group 32. The crystallographic triad axis coincides with the oligomer's 3-fold axis (Figure 3A and B) and each protomer is tightly associated with a non-crystallographically 2-fold-related subunit (Figures 3C, and 4A and C). The intersubunit interactions are more extensive across the oligomer's dyad than triad axis, and implicate 10 intersubunit hydrogen bonds. Several hydrophobic side chains also participate in the interactions along the 2-fold axis and include residues Met28, Phe29, Val85, Leu90, Ala92, Ala94, Phe96, Met100, Ala103, Val116, Phe117, Leu118, Met119 and Pro120; of these, Phe29 and Phe96 are invariant residues. Comparison of all 13 sequences (A.Geerlof, A.Lewendon and W.V.Shaw, submitted) highlights the preservation of one salt link across the dyad interface between invariant Arg24 and Glu114 residues. The positions of the 14 invariant amino acid residues are clustered around the active site and at the dimer interface, and their C_α positions are identified by spheres in Figure 4B. Dimerization results in a total buried surface area of $2200 \text{ \AA}^2$ ($1100 \text{ \AA}^2$ per subunit).

Trimer contacts involve 10 side chains, three of which are hydrophobic. A salt bridge involving Asp72 and His138 is also used in stabilizing the triad interface. Compared with dyad interactions, triad interactions implicate fewer conserved amino acid residues. The two distinct

protomer surfaces implicated in trimer formation bury a total of 4000 Å² of the solvent-accessible surface in the trimer, which is considerably greater than in the dimer. PPAT oligomerization could thus entail a mechanism

involving association of stable trimers for hexamer formation.

The dimensions of the globular shaped hexamer are 65 Å along the triad axis and 75 Å across the 3-fold axis. A solvent channel runs through the entire hexamer along its triad axis, with a diameter of 10 Å at its most narrow cross-section at the centre of the hexamer (Figure 3A and B). The active sites in each trimer create a large cavity facing the solvent channel and away from the trimer–trimer interface, allowing unimpeded approach by the substrates to their binding pockets. The mouth of the solvent channel is lined with positively charged residues (invariant Lys42, Arg137 and His138), while negatively charged side chains (Glu97 and Asp95) pack the channel at the trimer–trimer interface. This charge distribution and the dimensions of the pore at the trimer–trimer interface suggest that the substrates cannot diffuse from one trimer to the next (Figure 3A). CoA also approaches its binding pocket in a manner similar to that proposed for the cubic and icosahedral cores (dihydrolipoamide acetyltransferase, E2p) of the pyruvate dehydrogenase multienzyme complex. However, in these structures, the radii of the solvent channels were much larger, i.e. 20 Å (Mattevi *et al.*, 1992) and 26 Å (Izard *et al.*, 1999), respectively.

Structures similar to PPAT

PPAT exhibits the same overall fold as the dinucleotide-binding domain found in class I aminoacyl-tRNA synthetases, consistent with the highest ranking C_α coordinate match using the DALI server (Holm and Sander, 1997), and is predicted in another nucleotidyltransferase (see below) that is homologous with PPAT (Bork *et al.*, 1995). Aminoacyl-tRNA synthetases covalently link, in a two-step process using ATP activation, a specific amino acid to their cognate tRNA. Amino acids are converted to aminoacyl adenylates in an activation step involving cleavage of Mg²⁺-ATP prior to coupling to their cognate tRNA. The AMP portion of dPCoA binds PPAT in the same orientation and equivalent location within the Rossmann fold as observed in ATP binding to aminoacyl-tRNA synthetases (Figure 5). In class I aminoacyl-tRNA synthetases, a conserved loop moves towards the active site during the catalytic cycle (First and Fersht, 1993; Perona *et al.*, 1993; Schmitt *et al.*, 1994), and lysine residues present in a KMSKS sequence motif in a conserved loop stabilize the transition state in the case of tyrosyl-tRNA synthetases (First and Fersht, 1993). In PPAT, an equivalent loop of different amino acid sequence contains an invariant Arg91 at its extremity that could promote transition state stabilization.

A sequence motif, TNGH (residues 15–18), located on the floor of the active site in PPAT, also makes contacts to the adenylate moiety of dPCoA in a manner reminiscent of the conserved HIGH sequence in class I aminoacyl-tRNA synthetases that contacts the adenine ring of ATP (Bork *et al.*, 1995). The δ-nitrogen of invariant His18, located in a helical region, hydrogen-bonds to the main chain nitrogen of Thr15 of the preceding helical turn. His18 is therefore neutral, with a proton localized on its ε-nitrogen. A similar helical turn stabilization through hydrogen bonding involving histidines is found within the HIGH motif of class I aminoacyl-tRNA synthetases.

Among nucleotidyltransferases, PPAT displays con-

Fig. 3. The PPAT hexamer. (A) Electrostatic surface potential (using the program GRASP; Nicholls *et al.*, 1991) of the PPAT hexamer along the triad (red, negative; blue, positive; white, uncharged). The diameter along the 3-fold axis is ~75 Å, with a channel across the entire hexamer with a diameter of at least 10 Å. The substrates and products must enter through this cavity to bind to the active site of the protein. Space-filling representations (using the program RASTER3D; Bacon and Anderson, 1988; Merritt and Murphy, 1994) looking down (B) the triad and (C) the dyad. Each of the six subunits is coloured differently.

siderable sequence identity to cytidyltransferases that cleave the α/β phosphodiester bond of CTP. Within this family, PPAT has highest sequence identity (0.24) to the glycerol-3-phosphate cytidyltransferase (TagD) from *Bacillus subtilis* (Maue *et al.*, 1991). Seven of the 18 residues implicated in dPCoA binding by PPAT are conserved in the TagD protein sequence, consistent with a predicted Rossmann fold present in TagD (Bork *et al.*, 1995). The high-resolution structure of PPAT thus points to a structural link between cytidyltransferases and class I aminoacyl-tRNA synthetases, and thus suggests a new nucleotidyltransferase superfamily of α/β phosphodiesterases containing a dinucleotide-binding fold.

Structure of PPAT-bound dPCoA

The structure of PPAT bound to its major product dPCoA is illustrated in Figure 6. Within the hexamer, only one

trimer showed electron density corresponding to dPCoA, while electron density in the other trimer indicated the absence of bound product and corresponded to several ordered water molecules. The two subunits in the asymmetric unit are, therefore, distinct, one possessing a dPCoA-bound conformation while the other is in the apo-form. PPAT binds its dPCoA ligand in a bent conformation. The elbow of the bend occurs in the middle of the pantetheine arm, bringing the sulfur atom of the β -mercaptoethylamine moiety close to the entrance of the dPCoA-binding pocket. This ligand conformation is similar to those observed for CoA interacting with enoyl-CoA hydratase (Engel *et al.*, 1996), citrate synthase (Remington *et al.*, 1982), acyl-CoA-binding protein (Kragelund *et al.*, 1983) and aminoglycoside 3-*N*-acetyltransferase (Dutnall *et al.*, 1998). The majority of bound CoA structures, however, show an extended pantetheine

conformation (Leslie *et al.*, 1988; Mattevi *et al.*, 1992; Kim *et al.*, 1993; Wolodko *et al.*, 1994; Djordjevic *et al.*, 1995; Mancia *et al.*, 1996).

dPCoA binding

The adenylate portion of dPCoA binds in a deep cleft on the enzyme proximal to the imidazole side chain of His18 and the carbonyl oxygen of Gly17; it further interacts in the PPAT sequence with Arg88, Gly89, Leu90 and Arg91 (Figure 6). The residues Gly17, His18, Gly89 and Arg91 are invariant amino acids in all known PPAT sequences, while Arg88 and Leu90 are replaced by Lys (in two species) and Ile/Val (in four of the 13 sequences), respectively (A. Geerloff *et al.*, submitted). The positively charged nature of the adenylate-binding pocket is shown in Figure 4C. The adenine ring is positioned over the conserved Gly17 residue on the floor of the active site, any larger side chain at this position causing steric interference with the adenine ring. The 2'- and 3'-OH groups of the ribose ring make hydrogen bonds to Glu99 located under the ribose as well as to the main chain carboxyl and amide groups of Gly89. The hydroxyl group of Tyr7 is hydrogen bonded (2.8 Å) to the oxygen of the ribose ring, while the backbone carbonyl oxygens of Ile127 and Trp124 make contacts with the N1 nitrogen and 6-amino group of the adenylate ring.

The adenylate α -phosphate is within hydrogen bonding distance of the hydroxyl group of Thr10 (3 Å) and the backbone amide of Phe11 (2.8 Å). The serine-rich region

at Ser129 interacts with the α -phosphate group through a water-mediated hydrogen bonding network, while Lys42 and Arg88 are almost within hydrogen bonding distance of the 4'-phosphate oxygens of phosphopantetheine. Phe11, Lys42 and Ser129 are all conserved among the PPAT sequences, while Thr10 is replaced in eight of the 13 PPAT sequences with a serine.

Several secondary structural elements contribute residues that participate in van der Waals contacts with the pantetheine arm. These include conserved Leu73 and Met74 located on α -helix α 3, Asn106 on α -helix α 4 and Glu34 from α -helix α 6 located at the 3-fold axis. Residues 38–42, located on loop β 2– α 2, and residues 92–96, located on loop β 4– α 4, also promote van der Waals interactions with the product and line the binding surface of the active site responsible for pantetheine moiety attachment. Sequence diversity of these residues would allow for conformational variability of the pantetheine moiety. A hydrogen bond is present between the main chain amide group of Met74 and the carboxylate oxygen (2.8 Å) of the pantetheine arm in dPCoA (Figure 6).

Active site binding and proposed reaction mechanism

Inspection of the active site surface curvature and electrostatic potential reveals an additional positively charged pocket adjacent to the α -phosphate group of the adenylate which provides a likely binding site for the pyrophosphate group of ATP (Figure 4C). Positioning ATP accordingly

Fig. 4. The dPCoA-bound and dPCoA-free protomer. The protomer binding dPCoA is shown in green, while the protomer in its apo-form is shown in yellow (in A and B). (A) Stereo C_{α} trace (Bacon and Anderson, 1988; Kraulis, 1991; Merritt and Murphy, 1994) of the dimer down its local dyad. This view is from the inside of the hexamer. The sulfate anions and dPCoA are shown in ball-and-stick representation. (B) Stereo C_{α} trace superposition (Bacon and Anderson, 1988; Kraulis, 1991; Merritt and Murphy, 1994) of two PPAT protomers viewed down the local dyad. Every tenth C_{α} atom is labelled. The C_{α} atoms of the 14 invariant residues are represented by a black sphere in the dPCoA-bound protomer and are Gly9, Phe11, Pro13, Gly17, His18, Phe29, Lys42, Arg51, Gly89, Arg91, Asp95, Phe96, Glu99 and Ser129. (C) Stereo electrostatic surface potential (Nicholls *et al.*, 1991) of the two PPAT protomers (red, negative; blue, positive; white, uncharged). The central negatively charged surface at the local dyad forms the narrow pore in the hexamer at the trimer-trimer interface. dPCoA bound to one protomer is shown in space filling representation, with oxygen atoms coloured in red, nitrogen in blue, sulfur in green, carbon in yellow and phosphate in grey.

Fig. 5. Putative in-line activation of 4'-phosphopantetheine. Stereo drawing (Bacon and Anderson, 1988; Kraulis, 1991; Merritt and Murphy, 1994) of the PPAT protomer (yellow; residues 3–119) superimposed onto the dinucleotide-binding domain of *E. coli* glutaminyl-tRNA synthetase (blue; residues 28–99 and 211–278) in complex with ATP (Rould *et al.*, 1991). The 4'-phosphopantetheine portion of dPCoA bound to PPAT and Mg^{2+} -ATP bound to the glutaminyl-tRNA synthetase are shown in ball-and-stick representation. The AMP portion of ATP bound to the glutaminyl-tRNA synthetase binds tRNA synthetase in the same manner as the AMP portion of dPCoA binds PPAT. Mg^{2+} is shown as a pink sphere and its position is extrapolated from Perona *et al.* (1993). The proposed in-line activation of 4'-phosphate of phosphopantetheine is shown by a dashed line. The distance between the 4'-phosphate of phosphopantetheine and the α -phosphorous of ATP is 3.5 Å.

Fig. 6. The binding of dPCoA to PPAT. Final σ_A weighted $3F_o-2F_c$ omit electron density map for the ligand bound to the enzyme. The contour level is 1σ and the resolution is 1.8 Å. Residue Glu134' resides on a 3-fold related subunit. This figure was drawn using BOBSCRIPT (Kraulis, 1991) and RASTER3D (Bacon and Anderson, 1988; Merritt and Murphy, 1994).

in its binding site would allow 4'-phosphopantetheine to bind in the active site cleft so that either substrate could bind first, thus predicting a random ordered binding mechanism. The postulated ATP conformation requires the pyrophosphate group to be bent at the α -phosphate group with respect to its mononucleotide moiety, consistent with a similar conformation observed in glutaminyl-tRNA synthetase crystal structures in complex with ATP (Perona *et al.*, 1993). Figure 5 superimposes the crystal structure

of PPAT onto the dinucleotide-binding domain of *E. coli* glutaminyl-tRNA synthetase (Perona *et al.*, 1993). Based on the pyrophosphate group conformation, observed in glutaminyl-tRNA synthetase crystal structures (Rould *et al.*, 1991), the pyrophosphate moiety in PPAT would be pointing towards the active site exterior and away from the sharp bend at the α -phosphate group in bound dPCoA (Figure 5). The β -phosphate group could form a hydrogen bond to ordered water molecules which are hydrogen

bonded to the conserved Lys133 and Lys42, while the γ -phosphate could participate in hydrogen bonds to the side chains of Ser129 and Ser130.

From this postulated mode of ATP binding in PPAT, 4'-phosphopantetheine binding positions its attacking phosphate on the side opposing the α -phosphorous relative to the pyrophosphate (ATP β and γ phosphates) leaving group. Although conserved, Lys42 is just within hydrogen bonding distance of the 4'-phosphate of phosphopantetheine; it is solvated by water molecules, suggesting a protonated state for Lys42 N ζ amine and thus not suitable for a role as a nucleophile at physiological pH. Inspection of the active site does not reveal other specific enzyme moieties proximal to the α - β phosphate bond in dPCoA capable of participating directly in acid-base or covalent catalysis. In glutamyl-tRNA synthetase, two conserved residues, His43 and Lys270, that are found analogously disposed in PPAT as conserved active site residues His18 and Lys42, stabilize the pentacovalent transition state of the α -phosphate during catalysis.

Its analogy with aminoacyl-tRNA synthetases suggests that the chemistry of PPAT is the same, with a phosphate group replacing a carboxylate as a nucleophile. The 4'-phosphate of phosphopantetheine is a potent nucleophile, and as pyrophosphate is, in addition, a good leaving group, this suggests that direct participation by active site residues through proton transfers or by formation of covalent intermediates is unnecessary for the reaction to proceed. The role of the enzyme would then be one of properly orienting the ATP and 4'-phosphopantetheine substrates and perhaps in binding the pentacovalent transition state, thus lowering the activation energy barrier for the reaction. However, our postulated reaction scheme requires verification through further structural analysis of PPAT crystal complexes capturing the transition state of the enzyme.

Asymmetric ligation

Figure 4B shows a superposition of the dPCoA-free protomer onto the complexed protomer. Binding by dPCoA disrupts a salt bridge between invariant residues Arg88 and Glu99, to allow Arg88 to interact with the α -phosphate of dPCoA. The most dramatic displacement is perhaps undergone by α -helix α 4, in particular residues 94–97 of both subunits which communicate with each other in a 2-fold-related interaction. α -Helix α 4, which is sandwiched between the lip of the active site cleft and the dyad interface, shifts upon dPCoA binding by a 1 Å r.m.s. displacement towards the mouth of the active site, away from the interface and occluding the adenine nucleoside moiety. α -Helix α 6 (residues 128–138) also shifts in response to dPCoA binding away from the active site to allow hydrogen bonding of the backbone Ile127 amide with N6 of the adenine ring. In the product-free subunit, this amide would be pointing towards N7 of the adenine ring with a distance too large to allow hydrogen bonding (3.4 Å).

Differential protomer interaction with product is also observed for residues 72–74. These residues impose steric constraints on the conformation of the pantetheine arm in dPCoA and are responsible for its bent configuration. The hydrogen bond between the amide of Met74 and the carboxylic oxygen at the elbow of the pantetheine arm stabilizes the bent configuration. In the free protomer,

residues 72–74 hinder binding of 4'-phosphopantetheine and would result in steric conflict between Met74 and carboxylic oxygen, thus entailing backbone movement prior to binding.

The liganded and unliganded subunits segregate in the hexamer such that active sites of one trimer are bound by dPCoA while only solvent molecules are bound in the other trimer (Figure 4A). The presence of dPCoA in only three of the six subunits of the hexamer of PPAT is consistent with biochemical data; during purification, 0.5 mol. of the inhibitor CoA was found to be bound per mol. of PPAT (A.Geerlof, A.Lewendon and W.V.Shaw, submitted). That dPCoA binds in an ordered manner to one set of trimers is strongly reminiscent of similar binding behaviour observed in tyrosyl-tRNA synthetases (Fersht, 1987).

Asymmetric ligation by dPCoA buries a greater subunit interface surface area of 180 Å² in dimers of heteroprotomers than in dimers of homoprotomers composed of either bound or ligand-free subunits. Hexamer segregation into bound and unbound trimers produces crystal packing contacts only between bound subunits in one hexamer and ligand-free subunits of an adjacent hexamer. Random arrangements of bound and unbound subunits in each trimer are not favoured by crystal contacts. Crystal contacts are mediated by residues 139–141 in the unbound subunit and residues 72, 73, 80 and 82 in the bound subunit. dPCoA binding displaces α -helix α 6 by 1.2 Å as well as the main chain backbone vicinal to residues 72–82 to a lesser degree. Crystal contacts between solely bound subunits would result in a large number of steric conflicts among these residues. This would disfavour hexamers composed of trimers having a random arrangement of bound and ligand-free protomers and could, in effect, promote a hexamer population wherein only sites in one trimer are occupied. The observation of a discrete half-site occupied hexamer derived from co-crystallization of dPCoA with PPAT rather than by soaking of dPCoA into apo-PPAT crystals (Izard *et al.*, 1999) suggests that the observed hexamer population is, however, not artefactual, and would argue that the hexamer observed is present in significant concentration in solution, consistent with a strong cooperative mode of product attachment.

The subunit structures of the apoenzyme and PPAT in complex with its product dPCoA suggest that the enzyme does not contribute functional groups that participate directly in the reaction mechanism during catalysis. Our postulated reaction scheme requires verification through structural analysis of the ternary complex of PPAT with 4'-phosphopantetheine and a non-hydrolysable ATP analogue. Such an analysis, possibly capturing the transition state of the enzyme, should assist in the interpretation of kinetic and other experiments that relate to the mechanism.

Materials and methods

Preparation of selenomethionyl PPAT

The seleno-L-methionine (Se-Met) isoform of PPAT was produced in the methionine auxotrophic *E.coli* strain B834 (DE3). A single colony of the latter carrying pET(*kdtB*) was added to 10 ml of NMM medium (Budisa *et al.*, 1995) containing 0.3 mM methionine and 100 µg/ml ampicillin, and incubated overnight at 37°C. A 5 ml aliquot was used to inoculate 500 ml of NMM medium, containing 0.3 mM Se-Met and 100 µg/ml ampicillin. After 15 h, protein expression was induced

Table I. Data reduction and refinement statistics

Data collection	Edge	Peak	Remote	dPCoA–PPAT
Wavelength (Å)	0.9789	0.9786	0.94	1.54
Resolution (Å)	1.8	1.8	1.8	1.8
Total data	547 185	548 477	511 129	390 875
Unique data	36 339	36 314	36 222	37 504
Redundancy	15.1	16.7	15.2	10.4
Overall completeness (100–1.8 Å)	0.947	0.944	0.94	0.975
Completeness (1.86–1.8 Å)	0.958	0.953	0.945	0.825
$F^2 > 3\sigma(F^2)$ (%)	84.4	85.6	83.7	89.7
R_{merge}^a (100–1.8 Å)	0.094	0.072	0.075	0.028
R_{merge}^a (1.86–1.8 Å)	0.239	0.217	0.214	0.141
Average $F^2/\sigma(F^2)$	27.6	29.7	27.5	37.8
Crystallographic refinement				
No. of reflections	37 423			
NCSΔB (main, side chain, Å ²)	0.98, 1.58			
NCSΔC _α positions (Å)	0.94			
Final model parameters				
No. of amino acid chains	314			
No. of protein atoms	2472			
No. of solvent molecules	255			
Resolution range (Å)	20–1.8			
R -factor ^b	0.22			
R_{free}^c	0.27			
Average main chain B -factor (Å ²)	21.2			
Average side chain/solvent B -factor (Å ²)	26.2			
Average dPCoA B -factor (Å ²)	31.5			
R.m.s. deviations from ideal geometry				
Covalent bond lengths (Å)	0.01			
Bond angles (°)	1.4			
ΔB (through bonds) (Å ²)	3.6			

^a $R_{\text{merge}} = \sum_{\text{unique reflections}} (\sum_{i=1}^N |I_i - \bar{I}|) / \sum_{\text{unique reflections}} (\sum_{i=1}^N I_i)$.

^b R -factor = $\sum_{\text{hkl}} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{\text{hkl}} |F_{\text{obs}}|$.

^cThe free R -factor is a cross-validation residual calculated using 5% of the native data which were chosen randomly and excluded from the refinement.

by adding 0.84 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested in the stationary growth phase after 41 h. Se-Met PPAT was purified to homogeneity by column chromatography as described elsewhere (A.Geerlof, A.Lewendon and W.V.Shaw, submitted). Electrospray mass spectrometry analysis (K.Lilley, unpublished results) of the Se-Met PPAT determined a relative molecular weight of 18 257 Da, indicating substitution of all nine methionine residues.

Crystallization and data collection

Crystals of Se-Met PPAT were obtained in the same way as the native form (Izard *et al.*, 1999). Vapour diffusion was used by equilibrating 8 μl of a 18 mg/ml purified protein solution in a 10 mM HEPES buffer at pH 8 with 0.5 mM dithiothreitol (DTT) against 8 μl of a reservoir solution containing 1.1 M (NH₄)₂SO₄, 0.2 M sodium chloride and 100 mM sodium acetate (pH 5). These crystals also belong to space group I23 ($a = 135.5$ Å), with a dimer in the asymmetric unit, a solvent content of 0.57 and a volume to protein mass ratio of 2.88 Å³/Da.

Crystals were cryoprotected by adding 35% glycerol to the mother liquor. A Brandeis CCD detector was used to collect data from a single, flash-frozen crystal of selenomethionyl protein at three wavelengths at beam line X12-C of the National Synchrotron Light Source, Brookhaven National Laboratory. An X-ray fluorescence spectrum was recorded and used to select the wavelength optima for subsequent MAD data collection. Data were collected at 0.9789 Å (the inflection point of the fluorescence spectrum, f' minimum), 0.9786 Å (f'' maximum) and 0.94 Å (remote high-energy wavelength). All three data sets were collected from the same crystal at a crystal–detector distance of 225 mm and using 0.5° oscillations per image. The data were collected using inverse beam geometry with each set of data measured as a single wedge of data. Data at 1.8 Å resolution at the three wavelengths were processed independently using the programs DENZO and SCALEPACK (Otwinowski and Minor, 1997). Data statistics are given in Table I.

Native data used in refinement

Co-crystals of PPAT in complex with dPCoA were grown under similar conditions as for the selenomethionyl isoform, with the inclusion of 10 mM dPCoA and 10 mM magnesium chloride in the crystallization drop (Izard *et al.*, 1999). Removal of bound CoA was achieved with an additional purification step (A.Geerlof *et al.*, submitted). X-ray data were collected at 100 K to 1.8 Å resolution on a CuKα rotating anode on an RAXIS-4 imaging plate and processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997; Table I).

Structure determination

The MAD data were scaled together with the CCP4 (1994) program SCALEIT. Sixteen Se sites of the 18 expected in the asymmetric unit were determined using SOLVE (Terwilliger and Brendzen, 1996). The N-terminal residue, which is a methionine, was disordered in both subunits within the asymmetric unit in the refined structure. Initial phases were calculated with the program MLPHARE (CCP4, 1994) and were improved by 100 cycles of 2-fold non-crystallographic symmetry (NCS) averaging with solvent flattening and gradual phase extension from 2.5 to 1.8 Å resolution using the program DM (CCP4, 1994). The final R -factor and correlation coefficient for the phase extension at 1.8 Å resolution were 0.39 and 0.729, respectively. Guidance from the Se positions allowed rapid unambiguous matching of the electron density to the sequence and construction of an atomic model using the program 'O' (Jones *et al.*, 1991). The crystal structure of PPAT in complex with dPCoA was solved by difference Fourier from the initial Se-Met PPAT model.

Crystallographic refinement

The Se-Met PPAT protein was found to include its inhibitor CoA. The Se-Met PPAT X-ray data were only used to determine the crystal structure but not for crystallographic refinement and steps to remove CoA before co-crystallizing the dPCoA–PPAT complex. The dPCoA–

PPAT model was refined with the program XPLOR (Brünger *et al.*, 1988) to 1.8 Å resolution using standard protocols. The free *R*-value (Brünger, 1992) was monitored throughout the refinement. A spherical electron-dense feature was found at the subunit dimer interface and was modelled as a sulfate anion, because of the high concentration of ammonium sulfate in the crystallization mother liquor. In the current model, the sulfate ion accepts hydrogen bonds from backbone amide nitrogen (2.9 Å) and Ne (2.8 Å) of Lys122 from one subunit, and Arg107 N ζ (3.1 Å) from the NCS 2-fold-related subunit (Figure 4A). The current model consists of 2472 protein atoms, one ligand dPCoA, two sulfate anions and 255 water molecules, and has good stereochemistry (Table I). Electron density was not observed for the first two N-terminal residues. Water molecules were placed according to strict distance and geometry criteria. The overall crystallographic *R*-factor is 0.22 and the free *R*-factor is 0.27 for all observed reflections between 20 and 1.8 Å resolution and with released NCS restraints (Table I). The poorest parts of the refined model are two regions comprising flexible loops where model building was difficult due to weak density. The flexible loop comprising residues 38–42 is well ordered in only the ligand-bound protomer, while the loop comprising residues 92–96 corresponds to weak density in the ligand-bound protomer.

Coordinates

The atomic coordinates have been deposited in the Protein Data Bank, Biology Department, Brookhaven National Laboratory, Upton, NY 11973 (PDB ID code 1b6t).

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