Biochemical and structural studies of mutants indicate concerted movement of the dimer interface and ligand-binding region of *Mycobacterium tuberculosis* pantothenate kinase

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Two point mutants and the corresponding double mutant of *Mycobacterium tuberculosis* pantothenate kinase have been prepared and biochemically and structurally characterized. The mutants were designed to weaken the affinity of the enzyme for the feedback inhibitor CoA. The mutants exhibit reduced activity, which can be explained in terms of their structures. The crystals of the mutants are not isomorphous to any of the previously analysed crystals of the wild-type enzyme or its complexes. The mycobacterial enzyme and its homologous *Escherichia coli* enzyme exhibit structural differences in their nucleotide complexes in the dimer interface and the ligand-binding region. In three of the four crystallographically independent mutant molecules the structure is similar to that in the *E. coli* enzyme. Although the mutants involve changes in the CoA-binding region, the dimer interface and the ligand-binding region move in a concerted manner, an observation which might be important in enzyme action. This work demonstrates that the structure of the mycobacterial enzyme can be transformed into a structure similar to that of the *E. coli* enzyme through minor perturbations without external influences such as those involving ligand binding.

1. Introduction

Coenzyme A (CoA) is central to various cellular metabolic processes, especially those involving fatty acids. Given the essential role of fatty acids in the survival and persistence of the tubercule bacillus, the study of this important cofactor for inhibitor design gains merit. The biosynthesis of CoA consists of five enzymatically catalysed steps, the first of which involves the conversion of vitamin B₅ (pantothenate) to 4'-phosphopantothenate by ATP-mediated phosphorylation carried out by pantothenate kinase (PanK; Begley *et al.*, 2001; Daugherty *et al.*, 2002; Fig. 1). PanK is an essential enzyme, the biosynthesis of which is feedback-regulated by CoA and its derivatives (Sasseti *et al.*, 2003). This is the primary mechanism by which the intracellular concentration of CoA is maintained (Vallari *et al.*, 1987; Leonardi *et al.*, 2005). Multiple isoforms of PanK are spread across species in a varied manner, with organisms containing from one to four enzyme isoforms (Zhou *et al.*, 2001; Choudhry *et al.*, 2003; Brand & Strauss, 2005). These isoforms exhibit differences in their regulation mechanisms and substrate affinities.

PanK from *Escherichia coli* (EcPanK) was the first to be extensively structurally characterized. The crystal structures of EcPanK in complexes with CoA, with the nonhydrolysable ATP analogue AMPPNP and with ADP and pantothenate are available (Yun *et al.*, 2000; Ivey *et al.*, 2004). EcPanK is a homodimeric protein with each subunit comprising 316

residues. It is a P-loop kinase with a Walker A motif and belongs to the mononucleotide-binding fold. It has a seven-stranded central β -sheet flanked by helices. Subsequently, *Mycobacterium tuberculosis* PanK (*MtPanK*) was thoroughly investigated in this laboratory in a long-range programme on structural studies of mycobacterial proteins (Vijayan, 2005; Krishna *et al.*, 2007; Selvaraj *et al.*, 2007; Kaushal *et al.*, 2008; Arif & Vijayan, 2012; Chandran *et al.*, 2017; Arif *et al.*, 2017) as part of an international and national effort (Terwilliger *et al.*, 2003; Murillo *et al.*, 2007; Arora *et al.*, 2011). This investigation involved, apart from biochemical studies, the structure determination of 21 independent crystals of binary and ternary complexes of *MtPanK* involving CoA, the ATP analogue AMPPCP, the GTP analogue GMPPCP, ADP, GDP, pantothenate, phosphopantothenate, citrate, pantothenol and nonyl pantothenamide (Das *et al.*, 2006; Chetnani *et al.*, 2009, 2010, 2011).

Despite the high sequence identity (52%) between *EcPanK* and *MtPanK*, the two enzymes differ in many respects. While *EcPanK* has been shown to be specific for ATP, *MtPanK* exhibits dual specificity and can make use of ATP as well as GTP for phosphorylating pantothenic acid. This has been suggested to be related to the downregulation of ATP synthesis in *M. tuberculosis* in the dormant state. Furthermore, CoA binds nearly 40% more tightly to *MtPanK* than to *EcPanK* (Chetnani *et al.*, 2009). *MtPanK* is a robust enzyme. All of the complexes of the enzyme studied in this laboratory exhibit essentially the same structure. In contrast, substantial differences exist between the complex of *EcPanK* with CoA and the corresponding complexes involving AMPPNP and ADP. On the other hand, the ligands exhibit substantial mobility within the binding region of *MtPanK*. Subsequent work on *MtPanK* showed that some of the inhibitor complexes of the mycobacterial enzyme can assume structures similar to those of *EcPanK*-nucleotide complexes (Björkelid *et al.*, 2013). There have since been brief structural communications on PanKs from other organisms (Li *et al.*, 2013; Hughes *et al.*, 2014), but they have not addressed the apparent interspecies variability of the type observed between the structures of *EcPanK* and *MtPanK*.

The differences between the nucleotide complexes of *EcPanK* and *MtPanK* outlined above appear to be related to differences in the dimer interfaces and binding regions of the two enzymes. The relation between these two regions of the structure is, however, not entirely clear. In any case, the substantial differences between the nucleotide-bound structures of *EcPanK* and *MtPanK*, which are somewhat unusual

for two closely related homologous proteins, merit further investigation. As a step in this direction, an attempt was made to disrupt the CoA-binding site of *MtPanK* by mutating two phenylalanine residues involved in interaction with the hydrophobic region of CoA. The biochemical and structural studies of two single mutants and a double mutant involving these residues, reported here, provide interesting insights into the functional relationship between the inter-subunit interface and the binding region. The studies also demonstrate how *EcPanK*-like characteristics can be introduced into *MtPanK* by simple point mutations.

2. Materials and methods

2.1. Cloning, expression and purification

The double mutant was generated using the primers given in Supplementary Table S1, along with the primers used for cloning wild-type *MtPanK* using *M. tuberculosis* genomic DNA as a template (Das *et al.*, 2005). Site-directed mutagenesis using a single primer (Shenoy & Visweswariah, 2003) was employed to generate the single mutants. The *MtPanK* clone in pET-28a(+) vector was used as the template for these reactions. The primers used for the reactions are also given in Supplementary Table S1. The mutations were confirmed by sequencing. These plasmids were then transformed into *E. coli* BL21 cells and expressed under the *lac* promoter using IPTG induction. The cells were pelleted and resuspended in buffer I (50 mM Tris-HCl pH 7.8, 600 mM NaCl, 10 mM imidazole, 10% glycerol, 0.5 mM EDTA). The cells were lysed by sonication and spun at 27 540g for 1 h to remove all cell debris. The lysate was then loaded onto an Ni-NTA affinity chromatography column pre-equilibrated with the same buffer as above. The column was further washed with buffer I. An additional wash with buffer I containing 30 mM imidazole removed all unbound protein. The protein was eluted using buffer I supplemented with 100 mM imidazole. The eluted protein was then dialysed against buffer II (100 mM Tris-HCl pH 7.8, 600 mM NaCl, 5% glycerol, 100 mM imidazole) to obtain the final pure protein. The purity of the protein was checked on an SDS gel. CoA-free wild-type *MtPanK* was purified following the method described previously (Chetnani *et al.*, 2009).

2.2. Thermal shift assay

In the present experiments, a final concentration of 5 μ M protein in 20 mM HEPES buffer pH 8.0 was used, with a final

Figure 1
The reaction catalyzed by PanK.

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Mutant	F247A/F254A	F254A	F247A
Precipitants used for crystallization	2% (w/v) PEG 400, 2.0 M (NH ₄) ₂ SO ₄ , 100 mM Na HEPES pH 7.5	10–15% (w/v) PEG 400, 1.25 M (NH ₄) ₂ SO ₄ , 100 mM Na HEPES pH 7.5	0.2 M (NH ₄) ₂ SO ₄ , 0.1 M bis-tris pH 5.5, 25% (w/v) PEG 3350
Wavelength (Å)	0.9537	0.9785	0.9537
Space group	<i>P</i> 3 ₁ 21	<i>P</i> 3 ₁ 21	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å)	<i>a</i> = 119.33, <i>c</i> = 127.44	<i>a</i> = 120.82, <i>c</i> = 125.48	<i>a</i> = 84.83, <i>b</i> = 104.72, <i>c</i> = 145.35
<i>V</i> _M (Å ³ Da ^{−1})	3.7	3.7	2.2
Solvent content (%)	66.7	67.0	43.6
No. of subunits in asymmetric unit	2	2	4
Resolution (Å)	43.55–2.26 (2.38–2.26)	24.58–1.80 (1.90–1.80)	84.96–3.20 (3.37–3.20)
No. of measured reflections	369448 (51421)	792473 (100194)	80430 (11771)
No. of unique reflections	49403 (7188)	97939 (14187)	20268 (2961)
Completeness (%)	99.7 (100)	99.9 (99.9)	89.2 (93.9)
<i>R</i> _{merge} [†] (%)	8.2 (49.8)	10.3 (99.3)	24.9 (53.7)
<i>⟨I/σ(I)⟩</i>	15.5 (3.7)	13.4 (2.1)	4.4 (2.9)
<i>CC</i> _{1/2}	0.998 (0.820)	0.998 (0.661)	0.896 (0.411)
Multiplicity	7.5 (7.2)	8.1 (7.1)	4.0 (4.0)
<i>R</i> factor (%)	19.2 (27.3)	17.8 (29.1)	29.1 (38.0)
<i>R</i> _{free} (%)	22.8 (33.8)	21.3 (33.4)	35.9 (39.1)
R.m.s. deviations from ideal			
Bond lengths (Å)	0.019	0.019	0.008
Bond angles (°)	2.0	2.0	1.2
No. of non-H atoms			
Protein atoms	4652	4761	8020
Ligands	123	216	20
Solvent atoms	178	627	33
Wilson <i>B</i> factor (Å ²)	36.72	21.57	50.34
Average <i>B</i> factors (Å ²)			
Protein	42.10	25.60	22.80
Ligand	57.30	41.50	20.80
Solvent	42.00	35.50	24.30
Ramachandran plot statistics‡			
Most favoured regions (%)	91.3	91.7	82.3
Additionally allowed regions (%)	7.6	8.2	13.8
Generously allowed regions (%)	0.8	0.2	3.0
Disallowed regions (%)	0.4	0.0	0.9

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl) \rangle$ is the weighted average intensity for all *i* observations of reflection *hkl*. [‡] Calculated for nonglycine and nonproline residues using PROCHECK.

dye concentration of 1.5× in a 25 µl reaction. Increasing concentrations of CoA (0–200 µM) were used to explore the presence or loss of binding and the effects thereof on the protein. A Bio-Rad iQ5 was used to carry out the experiments at temperatures ranging from 283 to 363 K in intervals of 0.5 K. The resultant fluorescence intensity was then plotted against temperature, the data were fitted to a sigmoidal curve using *GraphPad Prism*, and the melting temperature of the protein was thus determined. The dissociation constant was also estimated from the assay following the protocol described by Vivoli *et al.* (2014).

2.3. Radioactive assay

Activity was confirmed using a radiometric assay in which the phosphorylation of pantothenate by the radioactively labelled γ-phosphate of ATP was followed. In this assay, a reaction mixture consisting of 25 mM Tris–HCl pH 7.8, 10 mM MgCl₂, 1 mM pantothenate, 3.75 mM ATP (1:1000 hot:cold ratio) was used. The reaction was initiated by the addition of 5 µM enzyme and was stopped after 5 min by heating to 353 K for 10 min. 2 µl aliquots of the reaction mixture were then spotted onto a silica gel 60 TLC plate and dried, and the plate

was developed in a mixture containing a butanol:acetic acid:water (5:2:4) solvent system. The separated components of the reaction were then visualized using a Fujifilm phosphorimager plate employing autoradiography.

2.4. Activity assay

The enzyme activity of the mutants was measured using a coupled enzyme assay. The conversion of NADH to NAD was estimated in this assay by measuring the absorbance at 340 nm. A reaction mixture consisting of 25 mM Tris–HCl pH 7.8, 5 mM MgCl₂, 1 mM PEP, 20 units of pyruvate kinase, 30 units of lactate dehydrogenase, 1 mM pantothenate and varying concentrations of ATP was used for determination of the kinetic parameters for the conversion of ATP to ADP. The reaction was initiated by the addition of 100 nM enzyme. The absorbance at 340 nm was monitored for 10 min. Nonlinear fitting of the initial rate of reaction was used to determine the catalytic parameters.

2.5. Crystallization

The microbatch-under-oil method (Chayen *et al.*, 1990) was used in all attempts to obtain crystals of the mutants. 2 µl of

protein solution (6.5 mg ml^{-1} as determined by measuring the absorbance at 280 nm) in buffer II was mixed with an equal volume of precipitant solution. Commercially available kits from Hampton Research and Jena Biosciences were used for initial screening. Diffraction-quality crystals were obtained in 30–45 d. Crystals of the F247A/F254A and F254A mutants were hexagonal plate-shaped and approximately $0.1 \times 0.1 \times 0.05 \text{ mm}$ in size. Crystals of the F247A mutant were tetragonal prism-shaped with an approximate size of $0.1 \times 0.1 \times 0.2 \text{ mm}$. The conditions under which the different mutants crystallized are given in Table 1. All experiments were carried out at 295 K.

2.6. X-ray data collection, structure solution and refinement

Data were collected at 100 K using a CCD detector (MAR Mosaic 225) on the BM14 synchrotron X-ray beamline at the European Synchrotron Radiation Facility, Grenoble, France. The diffraction images were processed using *iMosflm* (Battye *et al.*, 2011) and merged with *SCALA* (Evans, 2006) in the *CCP4* suite (Winn *et al.*, 2011). Intensities were converted to structure-factor amplitudes using *TRUNCATE* (French & Wilson, 1978) in the *CCP4* suite.

Solvent-content analysis (Matthews, 1968) indicated the likely presence of a dimer in the asymmetric unit of two of the crystals, while the crystals of the F247A mutant were likely to contain four protomers accounting for two independent dimers. The structures were solved by molecular replacement using *Phaser* (McCoy *et al.*, 2007) employing the structure of the complex of *MtPanK* with CoA (PDB entry 2geu; Das *et al.*, 2006) as the search model, confirming the composition of the asymmetric units as indicated by the solvent-content analysis. Rigid-body refinement followed by positional and *B*-factor refinement were carried out using *REFMAC5* (Murshudov *et al.*, 2011) along with multiple rounds of manual model building

using *Coot* (Emsley & Cowtan, 2004). Tight NCS restraints were applied between the protomers in each dimer in the F247A mutant. Water O atoms were added using peaks with heights greater than 3.0σ in $F_o - F_c$ maps and 1.0σ in $2F_o - F_c$ maps at the final stages of refinement. Sulfate ions and polyethylene glycol molecules from the mother liquor were modelled into the electron density where appropriate. Data-collection, processing and refinement statistics of the three mutants are summarized in Table 1.

2.7. Analysis of the structures

The refined structures were evaluated using *PROCHECK* (Laskowski *et al.*, 1993). Structural alignments were carried out using *ALIGN* (Cohen, 1997). *NACCESS* (Hubbard & Thornton, 1993) was employed to compute buried surface areas. Helical parameters were calculated using *HELANAL* (Kumar & Bansal, 2012). *CONTACTS* from the *CCP4* suite (Winn *et al.*, 2011) and the *PIC* (*Protein Interactions Calculator*) webserver (Tina *et al.*, 2007) were used to calculate interatomic distances and interactions. *PyMOL* (DeLano, 2002) was used for viewing structures and for the generation of figures.

3. Results and discussion

3.1. Design of mutants

The activity of *PanK*, the first enzyme in the CoA-biosynthesis pathway, is regulated by product inhibition by CoA. *MtPanK* and *EcPanK* exhibit substantial differences in their interactions with ATP, ADP and pantothenate. Among the ligands that have been crystallographically characterized to date, only CoA binds to both of the enzymes in a similar manner, perhaps indicating the importance of CoA in the regulation of its own biosynthesis. A hydrophobic cluster is

Figure 2

MtPanK monomer: Structural elements involved in dimerization and ligand binding are indicated. The CoA-binding region is shown in detail on the right. CoA is shown in blue.

highly conserved in the CoA-binding regions of the two enzymes (Fig. 2), although they have somewhat different affinities for CoA. Therefore, it was felt that disruption of CoA binding was a good approach to begin an exploration of the similarities and dissimilarities between the two enzymes. Phe247 and Phe254 are prominent conserved residues in this cluster. Therefore, mutation of these residues appeared to be a reasonable strategy for partial disruption of the CoA-binding site of the enzyme. Accordingly, two point mutants involving Phe247 (F247A) and Phe254 (F254A) and the corresponding double mutant (F247A/F254A) of *MtPanK* were prepared. Biochemical and structural studies of all three mutants were carried out.

3.2. Stability and activity

Thermal shift assays were carried out on the wild-type protein and the mutants in the presence as well as the absence of CoA. The results of the experiments are given in Supplementary Fig. S1 and are summarized in Table 2. As expected, the wild-type enzyme is more stable than the mutants. As also expected, the binding of CoA to the mutants is weaker than that to the wild-type enzyme. While the T_m value of the wild-type enzyme increases by 6.4°C on binding to 200 μ M CoA, the corresponding values for the mutants range from 3.4 to 5.3°C. The binding affinity of CoA to the mutants calculated using the thermal shift assay is 2.2-fold to 3.6-fold lower than that for the wild-type enzyme. Interestingly, the mutation of Phe254 has a larger effect on binding than that of Phe247. This is presumably on account of the fact that Phe254 makes more direct interactions with CoA than Phe247.

The activities of the wild-type enzyme and the mutants were measured using a radioactivity assay following the transfer of the γ -labelled phosphate of ATP to pantothenate to form phosphopantothenate (Supplementary Fig. S2). In order to estimate the activity of the mutants relative to that of the wild-

Table 2

T_m values of *MtPanK* and its mutants and dissociation constants of CoA.

	Wild-type <i>MtPanK</i>	F247A/F254A	F254A	F247A
T_m without CoA (°C)	43.8 $\pm$ 0.42	36.7 $\pm$ 0.35	35.7 $\pm$ 0.20	37.0 $\pm$ 0.25
T_m with 200 μ M CoA (°C)	50.1 $\pm$ 1.32	40.1 $\pm$ 0.56	40.1 $\pm$ 2.52	42.4 $\pm$ 0.63
K_d (μ M)	100.0 $\pm$ 5.0	289.2 $\pm$ 14.5	358.0 $\pm$ 5.8	218.3 $\pm$ 4.4

type enzyme, k_{cat}/K_m values were obtained from a coupled assay (Supplementary Fig. S3). The mutants have nearly 50% of the activity of wild-type *MtPanK*. Considering that the mutations involved the replacement of bulky residues with alanine, the reduction in activity is surprisingly small.

3.3. Overall structural features

The crystal structures of two single mutants (F247A and F254A) and a double mutant (F247A/F254A) of *MtPanK* have been determined. Repeated attempts to produce crystals of the complexes of the mutants with CoA and the relevant nucleotides did not succeed, presumably on account of their reduced ligand affinity. The crystals of the F254A mutant and the double mutant are isomorphous to each other, but not to any other structure of PanK or its complexes. The F247A mutant has yet another crystal structure. The structure of the F247A mutant, at a resolution of 3.2 Å, is the least accurate. Also, nearly one eighth of the residues in this structure are undefined. However, the defined regions provide useful information in relation to the main theme of the paper. The crystals of the F254A mutant and the double mutant diffracted to resolutions of 1.8 and 2.26 Å, respectively; the structures are substantially well defined.

The structures of the F254A mutant and the double mutant each contain a dimeric molecule in the crystallographic asymmetric unit, while that of the F247A mutant contains two

Figure 3

Comparison of the locations of (a) Tyr182 and Phe254/Ala254 and (b) Tyr257 and Phe247/Ala247 in *MtPanK* and the double mutant. *MtPanK* is shown in green and the double mutant is in orange.

crystallographically independent dimers, referred to as molecules 1 and 2. The subunits in each dimer are related by a noncrystallographic twofold axis. In the F254A mutant and the double mutant, one of the subunits (subunit *A*) in the dimer is better ordered than the other (subunit *B*). In fact, all except a few N-terminal residues in subunit *A* of the double mutant are defined in its structure. Only six N-terminal residues and residue 27 are undefined in subunit *A* of the F254A mutant. In addition to the six N-terminal residues, about 20 residues in the region containing the mutation sites are also disordered in subunit *B* in both of the structures. The N-terminal residues and the peptide stretches 19–42, 79–87 and 243–268 are disordered to varying extents in the four subunits in the F247A mutant.

The mutants retain the structural features common to *MtPanK* and *EcPanK* in their various complexes. Structural differences are expected in the polypeptide stretch bearing the mutated residues. This stretch is wholly or partially disordered in six of the eight subunits in the three crystal structures. Interesting structural reorganizations are observed in the CoA-binding regions of subunit *A* of the double mutant and the F254A mutant, in which the stretch is fully ordered. In the double mutant, the side chain of Tyr182 flips to fill the space left behind by the mutation of Phe254 to Ala; likewise, the side chain of Tyr257 moves into the space previously occupied by the side chain of Phe247 (Fig. 3). In the well defined subunit *A*

of the F254A mutant, as is to be expected, only the side chain of Tyr182 moves. The reorganization of the CoA-binding site in the other four subunits is presumably less coherent, leading to disorder in this region.

3.4. Structural differences at the dimer interface and the ATP-binding site

Structural differences between the *MtPanK*–AMPPCP and *EcPanK*–AMPPNP complexes involve the inter-subunit interface of the dimer (Fig. 4*a*) and the nucleotide-binding region. Interactions at the dimer interface include the long 59–78 helix, the 208–217 stretch, which carries a five-residue insertion in *EcPanK*, and residues at the N- and C-termini. The long helix is in fact made up of two shorter contiguous helices (residues 45–63 and 68–78), whose axes subtend an angle close to, but less than, 180° (Fig. 4*b*). This angle is 159° in *MtPanK* and 170° in *EcPanK*. There is also a perceptible difference between the two enzymes in the disposition of the 208–217 stretch even when the insertion region in one of them is ignored (Fig. 4*c*). The stretch is farther away from the rest of the molecule in *EcPanK* than in *MtPanK*. The distance of the centroid of the two 208–217 stretches at the interface is 1.4 Å away from the centroid of the rest of the dimer in *MtPanK*. The corresponding value for *EcPanK* (the insertion is not included in the calculations) is 3.7 Å.

Figure 4

(*a*) The F254A mutant dimer. (*b*) The dimerization helix in *MtPanK* (green) and *EcPanK* (blue). The corresponding helices in the *MtPanK* F254A mutant (red) and molecule 1 of the F247A mutant (magenta) are also shown. (*c*) The insertion region in *MtPanK*, *EcPanK*, the *MtPanK* F254A mutant and molecule 1 of the F247A mutant. (*d*) The 33–43 stretch in the nucleotide-binding region of *MtPanK*, the F254A mutant and molecule 1 of the F247A mutant along with the corresponding stretch in *EcPanK*. Leu40 in *MtPanK* corresponds to Ile42 in *EcPanK*. The colour code used is the same as in (*b*).

Table 3

Summary of the parameters that delineate the mutant molecules as *MtPanK*-like or *EcPanK*-like based on structural features.

	<i>EcPanK</i>	<i>MtPanK</i>	F247A/F254A		F254A		F247A		Subunit A'	Subunit B'
			Subunit A	Subunit B	Subunit A	Subunit B	Subunit A	Subunit B		
Angle between the two components of the dimerization helix (°)	170	159	177	174	178	179	159	158	173	167
Distance between centroids of the 208–217 dimerization region and the rest of the molecule (Å)	3.7†	1.4	4.0		3.7		1.8		3.6	
Distances of 40 C α atoms from their position in <i>MtPanK</i> ‡ (Å)		0 (10.1)	10.3 (0.3)	13.1 (4.6)	10.7 (0.7)	9.8 (4.7)	12.5 (4.0)		16.0 (7.2)	

† Excluding the insertion region. ‡ The distances from 42 C α atoms in *EcPanK* are given in parentheses.

Substantial differences also exist between *MtPanK* and *EcPanK* in the regions involved in ligand binding. In the 30–45 stretch, residues 32–37 have a helical conformation in the *MtPanK*–AMPPCP complex. A longer helix (30–41 in *MtPanK* numbering) exists in the corresponding stretch in the *EcPanK*–AMPPNP complex. Furthermore, there is a lateral shift in the location of the two stretches when the structures of the *MtPanK* and *EcPanK* complexes are superposed (Fig. 4*d*). A measure of the shift is the distance between 40 C α atoms in *MtPanK* and the corresponding 42 C α atoms in *EcPanK*, which is as high as 10.1 Å. Detailed comparisons involving the crystal structures of different PanK complexes confirm that the observed structural differences between *MtPanK* and *EcPanK* are not artefacts.

The angle between the two components of the long dimerization helix, the distance between the centroids of the 208–217 stretches from that of the dimer, and the distances of 40 C α atoms from the corresponding atoms in the complexes of wild-type *MtPanK*, *EcPanK* and in the various mutants are listed in Table 3. The information presented in this table and Fig. 4 clearly show that the intersubunit interface and the ligand-binding region in the double mutant and the F254A

mutant are similar to those in *EcPanK*. The situation is not entirely clear in the F247A mutant, on account of extensive disorder, especially in the 30–45 stretch. However, the structural data clearly show that the inter-subunit interface in molecule 1 of the mutant is similar to that in *MtPanK*, while that in molecule 2 is similar to that in *EcPanK*. It would thus appear that the mutation at residue 247 tends to drive the structure from that of *MtPanK* to that of *EcPanK*, while the transition is complete in the F254A mutant. The double mutant shares the characteristics of the F254A and F247A mutants, with those of the F254A mutant predominating.

The results of *DALI* searches lend additional support to the above observations. The subunits in the F254A mutant and the double mutant show the highest structural similarity to one or another of the subunits of the *MtPanK* molecule which assume an *EcPanK*-like structure consequent to ligand binding (PDB entries 4bfu and 4bfz; Björkelid *et al.*, 2013), with Z-score values ranging from 42.9 to 45.4 and r.m.s.d.s in the range 1.0–1.3 Å. The F247A mutant presents an interesting situation. The two subunits in molecule 1 exhibit similarity to a subunit of the CoA complex of an intact *MtPanK* molecule (PDB entry 2geu; Das *et al.*, 2006; Z-score values of 37.5–38.1,

Figure 5

(a) The disposition of the 20–40 and 240–260 stretches and the dimerization helix from the other subunit in *MtPanK*. (b) The same structural elements in the F254A mutant. The dimerization helix in wild-type PanK is also shown in green for comparison.

r.m.s.d.s of 1.1–1.4 Å). The subunits of molecule 2, on the other hand, show the highest similarity to a subunit (PDB entry 4bfu) of an *EcPanK*-like molecule (Z-score values of 37.3–37.6, r.m.s.d.s of 0.9–1.0 Å). These results are entirely consistent with the observations in the crystal structures of the mutants.

Happily, a close examination of the crystal structures leads to a plausible structural rationale for the concerted changes in the nucleotide-binding region and the helix at the dimer interface. The 240–260 stretch and the 20–45 stretch are in contact with each other primarily on account of the hydrophobic interaction of Leu40 with Met242 (Fig. 5*a*). The 20–45 stretch, in turn, is in contact with the dimerization helix of the second subunit through the interactions of Leu21 with Phe77 and Leu78. The reorganization of the 240–260 stretch resulting from the mutation(s) leads to disruption of the interactions involving Leu40. Consequently, the 20–45 stretch moves away from the 240–260 stretch and also undergoes a conformational transformation (Fig. 5*b*). This in turn results in the disruption of the interaction between the 20–45 stretch and the dimerization helix, thus destabilizing the system. The interaction is restored in the mutants through straightening of the dimerization helix. The resulting arrangement in most of the molecules of the mutants, with the 240–260 and 20–45 stretches wide apart and a straight dimerization helix, is similar to that in *EcPanK*, as indicated earlier.

3.5. Structural basis for the activity of the mutants

In spite of the substantial structural transformations, the mutants are still enzymatically active, but at a lower level. The mutants bind CoA, but with reduced affinity. The structures of the mutants provide a qualitative explanation for these observations. As the mutants have structures close to that of the *EcPanK*–AMPPNP structure, it is perhaps appropriate to compare the binding sites in the mutants with that in *EcPanK*. Among the residues implicated in catalysis by *EcPanK* (Ivey *et al.*, 2004), Arg238 and His179 (Arg243 and His177 in *EcPanK*) show some changes in their locations. More conspicuously,

there is a substantial movement of the side chain of His302 (His307 in *EcPanK*; Fig. 6*a*). This side chain stacks against the base in the *EcPanK*–AMPPNP complex. It also forms hydrogen bonds to the base and the sugar in the ligand. This stacking and these hydrogen bonds are lost in the mutants. A similar situation exists in molecule 1 of the F247A mutant, which has a structure closer to that of *MtPanK*. In the initiation complex of *MtPanK*, His179 stacks against the base and forms hydrogen bonds to the ligand. This residue is ordered only in subunit *B*. Here again the interactions are lost in the mutant (Fig. 6*b*). It may be that the loss of the interactions involving the appropriate histidine residue contributes to the reduction of the activity of the mutants and their reduced affinity in CoA binding.

4. Conclusions

Two point mutants and the corresponding double mutant in *MtPanK*, which are designed to weaken the affinity for CoA, had the unintended effect of converting *MtPanK* into an *EcPanK*-like enzyme. It has been established that *MtPanK* and *EcPanK* exhibit substantial differences in terms of the plasticity of the binding region and the movement of ligands within the region, in spite of the high homology between the two enzymes (Chetnani *et al.*, 2009). The results presented here suggest that comparatively small structural perturbations are sufficient to convert *MtPanK* into an enzyme with features similar to those of *EcPanK*. In addition to the intended effect of weakening the binding to CoA, the mutants exhibit a somewhat lower activity compared with the wild-type enzyme, in a manner that is explainable in terms of their structures. All the same, it is clear that interconversion of the two structures is possible without the intervention of external agents such as that involved in ligand binding. This facile transformation between homologous proteins from two sources is in itself interesting in relation to protein folding. Furthermore, this needs to be taken into account when designing inhibitors for the enzyme from the pathogen, with a view to eventual drug

Figure 6

Stereoviews of the locations of the histidines involved in stacking and hydrogen bonds with the base of the nucleotide. (a) His307 in *EcPanK* (blue) and the corresponding His302 in the double mutant (orange). (b) His179 in *MtPanK* (green) and molecule 1 of the F247A mutant (magenta).

development. It is not sufficient that the inhibitor blocks the active site of *MtPanK*: it should be capable of dealing with the corresponding site in the transformed *EcPanK*-like enzyme as well.

An important feature of the interconversion of the structures of *MtPanK* and *EcPanK* is the correlated movement of the dimer interface and the binding region. It is satisfying that the process of this transformation can be explained on the basis of a close examination of the structures of the wild-type protein and the mutants. Concerted movement at the inter-subunit interface and at the ligand-binding site is again an observation that is of general interest in relation to the dynamics/plasticity of proteins. The correlation between the movements in the two regions could also be important for the action of the enzyme.

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