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RESEARCH LETTER

Characterization of *Mycobacterium tuberculosis* diaminopimelic acid epimerase: paired cysteine residues are crucial for racemization

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Abstract

Recently, the overproduction of *Mycobacterium tuberculosis* diaminopimelic acid (DAP) epimerase MtDapF in *Escherichia coli* using a novel codon alteration cloning strategy and the characterization of the purified enzyme was reported. In the present study, the effect of sulphydryl alkylating agents on the *in vitro* activity of *M. tuberculosis* DapF was tested. The complete inhibition of the enzyme by 2-nitro-5-thiocyanatobenzoate, 5,5′-dithio-bis(2-nitrobenzoic acid) and 1,2-benzisothiazolidine-3-one at nanomolar concentrations suggested that these sulphydryl alkylating agents modify functionally significant cysteine residues at or near the active site of the epimerase. Consequently, the authors extended the characterization of MtDapF by studying the role of the two strictly conserved cysteine residues. The putative catalytic residues Cys87 and Cys226 of MtDapF were replaced individually with both serine and alanine. Residual epimerase activity was detected for both the serine replacement mutants C87S and C226S *in vitro*. Kinetic analyses revealed that, despite a decrease in the K_M value of the C87S mutant for DAP that presumably indicates an increase in nonproductive substrate binding, the catalytic efficiency of both serine substitution mutants was severely compromised. When either C87 or C226 were substituted with alanine, epimerase activity was not detected emphasizing the importance of both of these cysteine residues in catalysis.

Introduction

Despite the availability of appropriate chemotherapy for approaching 60 years, tuberculosis remains an enormous and universal healthcare concern (Dye, 2006; Kaufmann & Parida, 2007). The lack of a universally effective vaccine (Barreto *et al.*, 2006) and the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of the *Mycobacterium tuberculosis* complex, the aetiological agents for the disease, compound the problem. There is an urgent need for the development of new antituberculosis vaccines and drugs. Additionally, the definition of unexplored essential enzymes that may represent effective nodes of mycobacterial physiology for chemical intervention must be pursued.

The integrity of the complex mycobacterial envelope is essential with even minor changes compromising intracellular survival and virulence (Gao *et al.*, 2003; Bhatt *et al.*, 2007) and inhibitors of cell wall biosynthesis, such as isoniazid and ethambutol, have been clinically exploited as specific antimycobacterial drugs (Winder *et al.*, 1970; Takayama & Kilburn, 1989). More generally, the inhibition of cross-linking of bacterial peptidoglycan by β-lactam antibiotics has proven extremely successful. The physical strength of peptidoglycan is related to its architecture; linear glycan chains are cross-linked by short stem peptides that vary in structure according to taxonomy (Schleifer & Kandler, 1972). Although mycobacterial peptidoglycan is unremarkable, penicillins are not clinically useful in treating tuberculosis. During exponential growth, mycobacteria cross-link stem peptides between the third [*meso*-diaminopimelic acid (DAP)] residue and the fourth (D-Ala) of another (Schleifer & Kandler, 1972; Wietzerbin *et al.*, 1974). However, on entering stationary phase, *M. tuberculosis* incorporates *meso*-DAP → *meso*-DAP linkages between stem peptides, a penicillin resistant mode of ligation (Wietzerbin *et al.*, 1974). As mycobacterial infection is complicated by the bacterium's ability to persist by entering a

dormant phase, this altered mode of peptidoglycan cross-linking is likely relevant here (Goffin & Ghuysen, 2002). Importantly, however, *meso*-DAP is essential for both types of mycobacterial peptidoglycan cross-linking and thus plays a key role in mycobacterial cell wall biosynthesis.

DAP epimerase (DapF) catalyses the interconversion of LL-DAP and *meso*(DL)-DAP (Wiseman & Nichols, 1984) and has been identified and characterized in *Escherichia coli* (Wiseman & Nichols, 1984; Richaud *et al.*, 1987; Higgins *et al.*, 1989), *Haemophilus influenzae* (Cirilli *et al.*, 1998; Koo & Blanchard, 1999; Lloyd *et al.*, 2004; Pillai *et al.*, 2006) and *Corynebacterium glutamicum* (Hartmann *et al.*, 2003). Two active site cysteine residues of *H. influenzae* DAP epimerase, Cys73 and Cys217, are critical components in a two base mechanism; an active site thiolate deprotonates the α-carbon while the neighbouring thiol acts as an acid, protonating the resulting carbanion intermediate (Cirilli *et al.*, 1998; Koo & Blanchard, 1999).

The authors recently cloned and overexpressed *M. tuberculosis dapF* using a novel codon alteration strategy, to enable its purification and characterization (Usha *et al.*, 2006). It was reported here that the effect of alkylating agents on the wild-type MtDapF activity, the biochemical characterization of mutants of MtDapF generated by site-directed mutagenesis to examine the significance of cysteine residues to catalysis.

Materials and methods

The QuikChange II XL site-directed mutagenesis kit was procured from Stratagene. Oligonucleotide primers were synthesized by MWG Biotech, Germany. A newly-synthesized batch of [2,6-^{3}H] DAP, 60 Ci mmol^{-1} was obtained from American Radiolabeled Chemicals. Cation exchange resin AG 50W-X8, 200–400 mesh, H^+ form was obtained from BioRad Laboratories. All other chemicals were of reagent grade and purchased from Sigma. *Escherichia coli* C41(DE3) (Imaxio, France) was used as a host for protein production. HisTrap Ni^{2+} Sepharose high performance 1-mL column was obtained from GE Healthcare.

DAP epimerase assay

The assay measures the release of ^{3}H to H_2O from [2,6-^{3}H] DAP (Wiseman & Nichols, 1984; Richaud *et al.*, 1987). DAP epimerase activity was assayed as described previously (Usha *et al.*, 2006) with minor modifications. The reaction, in a final volume of 100 μL, contained 0.1 M Tris-HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 μCi [2,6-^{3}H] DAP, 64 μM DL-DAP and 125 ng of recombinant wild type *M. tuberculosis* DapF. The reactions were incubated at 30 °C for 30 min and terminated by addition of 10% tricarboxylic acid. The quenched reaction mixture was applied to a 2-mL AG 50W-X8 ion exchange column (H^+ form) packed in a syringe, which was washed with 3 × 2 mL of water, the eluates were combined and radioactivity was quantified by liquid scintillation counting. The quantity of LL-DAP formed in each sample was determined after subtraction of ^{3}H release from identical reactions without added enzyme from those containing the DapF. Where alkylating agents were used these were incubated with the enzyme at 37 °C for 10 min before initiating the assay through the addition of *meso*-DAP.

The variable concentrations of the wild-type enzyme used for the assay ranged from 32 to 250 ng and that for the C87S or C226S mutants ranged from 0.5 to 20 μg and C87A or C226A mutants ranged from 4 to 24 μg. All measurements were carried out in duplicate.

Table 1. Primers used for the site-directed mutagenesis of *mtdapF*

Mutation	Primer sequence (5′ → 3′)
C87A	Forward: ggcagcccagatggccggcaacggtgtgc
	Reverse: gcacaccgttgccggccatctgggctgcc
C87S	Forward: ggcagcccagatgagcggcaacggtgtgc
	Reverse: gcacaccgttgccgctcatctgggctgcc
C226A	Forward: ggaaacccgctcggctggcaccgggacgg
	Reverse: ccgtcccggtgccagccgagcgggtttcc
C226S	Forward: ggaaacccgctcgtctggcaccgggacgg
	Reverse: ccgtcccggtgccagacgagcgggtttcc

Site-directed mutagenesis

Mycobacterium tuberculosis DapF mutants were constructed by the individual replacement of two cysteine residues (cysteine 87 and 226) with either alanine or serine residues using QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturers' instructions. The primers used for mutagenesis are described in Table 1. The pET 28b-*dapFca* vector (Usha *et al.*, 2006) was used as the template for the mutagenic PCR. After PCR, the template plasmid DNA was digested with Dpn I and the nonmethylated mutated plasmid DNA was used to transform *E. coli* XL10-Gold. Transformants were cultured on Luria–Bertani (LB) agar containing 25 μg mL^{-1} kanamycin sulphate. Single transformant clones were cultured and plasmid DNA extracted. The validity of the constructs was confirmed *via* nucleotide sequencing.

Expression, purification and characterization of MtDapF mutants

The mutants of *M. tuberculosis dapF* were identically expressed like the wild-type gene in *E. coli* C41 (DE3) cells. A single colony was used to inoculate 10 mL overnight culture that subsequently provided the inoculum for 1 L cultures in Terrific broth containing 25 μg mL^{-1} kanamycin sulphate. The cultures were incubated at 37 °C until it reached an $OD_{600\,nm}$ of 0.6, induced with 1 mM isopropyl-β-D-

thiogalactopyranoside and incubated at 16 °C overnight. The cells were harvested and stored in −80 °C. The wild-type *M. tuberculosis* DapF and the mutant enzymes were purified as described previously (Usha *et al.*, 2006) with minor modifications. Cell pellets were resuspended in binding buffer (20 mM HEPES pH 8.0, 500 mM NaCl, 50 mM imidazole, 10% glycerol, 5 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride, 1 mM benzamidine and 1 mg mL^{-1} lysozyme) and incubated for 30 min at 4 °C. After lysis by French press, 5 μg mL^{-1} of DNAse and RNAse were added to the crude extract kept on ice for 10 min and centrifuged at 27 000 ***g*** for 45 min at 4 °C to remove cell debris and the supernatant applied to a 1 mL HisTrap Ni^{2+} Sepharose high performance column (GE Healthcare) equilibrated with binding buffer without the protease inhibitors and lysozyme. The column was extensively washed with binding buffer to remove contaminating proteins. MtDapF was recovered by elution with 2 mL of elution buffer containing 20 mM HEPES pH 8.0, 500 mM NaCl, 10% glycerol and 150 mM imidazole. The purified protein was dialysed against 20 mM HEPES pH 8.0, 10% glycerol, 10 mM dithiothreitol and 1 mM EDTA, concentrated using Centricon YM-10 filter units and stored as aliquots in −80 °C.

Determination of K_M app for MtDapF mutants and wild-type enzyme

The kinetic parameter, apparent K_M (K_M app) of the mutant and wild-type enzyme was determined by measuring initial velocity in the presence of varying concentrations of *meso*-DAP. All values were obtained from duplicate readings. Kinetic constants, K_M app and V_{max} were obtained from Lineweaver–Burke plots.

Results

In silico analyses of MtDapF primary structure

Alignment of the amino acyl residue sequence of MtDapF with the characterized DAP epimerases of *H. influenzae*, *E. coli* and *C. glutamicum* using the CLUSTALW algorithm revealed a significant degree of sequence similarity; the mycobacterial enzyme shares 28% amino acid identity with its *H. influenzae* orthologue (Fig. 1a). The primary structure of MtDapF contains five cysteine residues, two of which are strictly conserved in all of its characterized orthologues (Fig. 1a) and are crucial for DAP epimerase activity (Koo & Blanchard, 1999; Koo *et al.*, 2000). These two cysteine residues have been shown to be close neighbours in

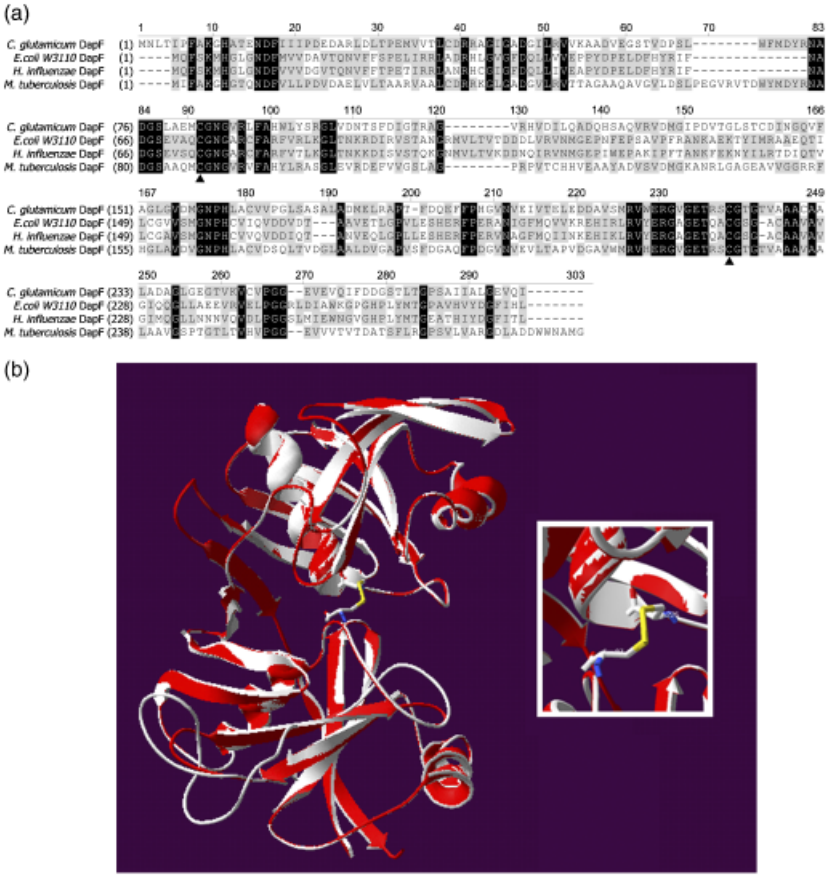

Fig. 1. *In silico* analyses of MtDapF structure. (a) Multiple sequence alignment of MtDapF with characterized diaminopimelate epimerases. The cysteine residues 87 and 226 are indicated by the triangles. (b) Superposition of modeled MtDapF structure (white) on 1BWZ (red), the crystal structure of DAP epimerase from *Haemophilus influenzae*. The inset provides a more detailed view of the close superposition of Cys87 and Cys226 (MtDapF numbering) on the corresponding residues in 1BWZ (Cys73 and Cys217): atoms from both structures here are coloured according to the CPK scheme.

structural studies and readily form a disulphide linkage (Born & Blanchard, 1999). The authors' homology modelling studies (Fig. 1b) suggest that MtDapF likely adopts a very similar fold to that of the *H. influenzae* enzyme. In this model Cys residues 87 and 226 superimpose with their *H. influenzae* counterparts, which were determined as cystine (Lloyd *et al.*, 2004). The potential for the formation of a similar disulphide linkage in MtDapF appears consistent with its often rapid oxidative inactivation (data not shown).

Inhibition of *M. tuberculosis* DAP epimerase with sulphydryl alkylating agents

The apparent conservation in MtDapF of catalytically active cysteine residues (Fig. 1) associated with pyridoxyl-5-phosphate (PLP)-independent amino acid racemization (Rudnick & Abeles, 1975; Gallo & Knowles, 1993; Tanner *et al.*, 1993; Koo *et al.*, 2000) led to the investigation of the effects upon catalysis of three commercially available sulphydryl alkylating agents, 2-nitro-5-thiocyanatobenzoate (NTCB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 1,2-benzisothiazolidine 3-one (BIT). The *Bacillus subtilis* glutamate racemase was inactivated on incubation with 0.2 mM NTCB at 37 °C for 10 min (Ashiuchi *et al.*, 1998) and DTNB rapidly and completely inactivates the *Lactobacillus fermenti* glutamate racemase (Gallo & Knowles, 1993). Here the panel of agents were tested as inhibitors of DAP epimerase activity by preincubating for 10 min at 37 °C with MtDapF over a range of concentrations before initiation of each assay by the addition of *meso*-DAP. Pretreatment with all three alkylating agents at 30 nM for ten minutes resulted in complete inhibition of the DAP epimerase activity of MtDapF (Fig. 2). The sulphydryl specificity of these alkylating agents strongly suggests an essential role for at least one cysteine residue in the catalytic mechanism.

DAP epimerase activity of *M. tuberculosis* DapF mutants

As part of this ongoing investigation of MtDapF as a potential novel antimycobacterial drug target and in order to assess whether these residues are important elements of the catalytic mechanism here, a series of four mutants of MtDapF by the individual replacement of these two cysteine residues (Cys87 and Cys226) with either alanine or serine residues were generated.

Like the wild-type allele, optimal expression of *M. tuberculosis dapF* mutants was achieved with 1 mM isopropyl β-D-1-thiogalactopyranoside at 16 °C for 24 h. The mutant proteins were purified in a single step by metal chelate affinity chromatography on Ni^{2+} Sepharose in an identical fashion to the wild-type enzyme. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of mutant enzymes showed a major band with mobility identical to that

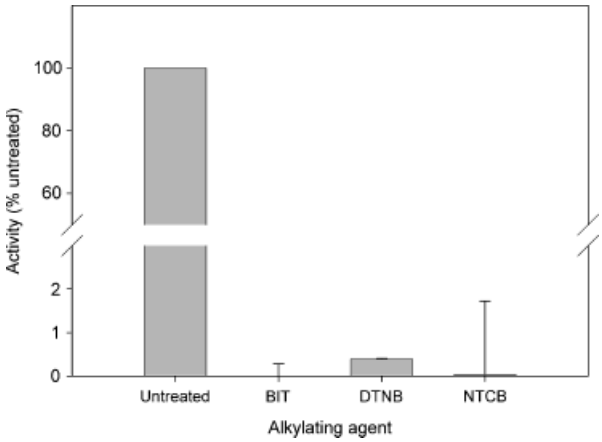

Fig. 2. Inhibition of MtDapF activity by sulphydryl alkylating agents. The purified wild-type MtDapF was incubated with 30 nM concentration of the sulphydryl alkylating agent as described in Materials and methods.

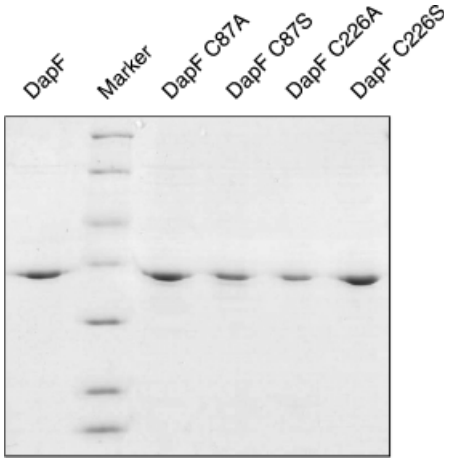

Fig. 3. Purification of wild-type MtDapF and mutants. The proteins were purified by Ni^{2+} Sepharose high performace chromatography as described in Materials and methods and stained with Coomassie blue. Lanes 1, purified wild-type MtDapF; lane 2, molecular weight marker of 116, 66, 45, 35, 25, 18 and 14 kDa; lane 3, C87A mutant; lane 4, C87S mutant; lane 5, C226A mutant and lane 6, C226S mutant.

of the wild-type enzyme although relative yields of mutant protein recovered following purification were decreased (Fig. 3).

The DAP epimerase activity of purified wild-type *M. tuberculosis* DapF and mutants was assayed *in vitro* by following the release of ^3H from ^3H-DAP to ^3H_2O (Usha *et al.*, 2006). Since a freshly synthesized batch of ^3H-DAP was procured for this present study, the activity profile of the wild type MtDapF was different from that reported in the authors' earlier paper (Usha *et al.*, 2006). The apparent K_M value for the wild-type *M. tuberculosis* DapF with *meso*-[^3H]DAP was calculated at 166 μM, somewhat lower than the 1217 μM reported previously (Usha *et al.*, 2006). Here, K_{cat} was determined as 0.1465 s^{-1} (Table 2), a turnover rate

Table 2. Kinetic constants for wild-type MtDapF and serine substitution mutants

	K_{cat} (s^{-1})	K_M (μM)	K_{cat}/K_M (s^{-1} M^{-1})
Wild type	0.1465	165.93	882.71
C87S	0.0010	70.51	14.87
C226S	0.0025	214.91	11.70

that is much lower than those described previously for DAP epimerases (Higgins *et al.*, 1989; Koo & Blanchard, 1999). However, this is consistent with an expected kinetic isotope effect $^3(V/K) \approx 5.9$ (Wiseman & Nichols, 1984) and the use of a mixture of ^3H and ^1H isotopes in these assays. The values for K_{cat} and K_{cat}/K_M derived herein can only be used for comparative purposes within this study.

The analyses of the DAP epimerase activity of each of the various mutants revealed that substitution of either cysteine residue significantly compromises the activity of the enzyme (Table 2). Unlike the Cys → Ser substitutions, the DAP epimerase activity of their alaninyl counterparts C87A and C226A was undetectable *in vitro* at all concentrations of *meso*-DAP and enzymes tested indicating that the two cysteine residues occupy critically important space that requires resident residues to accept and donate protons for catalysis. Consistently and like the glutamate racemase of *L. fermenti* (Glavas & Tanner, 1999), the Cys → Ser substitutions retained residual activity (∼1% of K_{cat}) despite the substitution of the more stable alcohol (pKa∼16) for a thiol (pKa∼10) (Fig. 4).

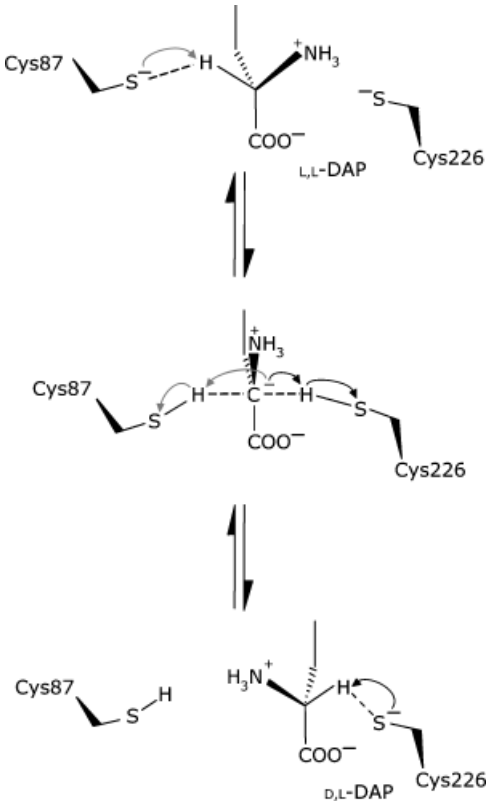

Fig. 4. Proposed mechanism of *Mycobacterium tuberculosis* diamianopimelic acid epimerase. The two catalytic cysteine residues, Cys87 and Cys226 interconvert LL-DAP and DL-DAP through a planar carbanion intermediate (central panel). This intermediate can be derived from either stereoisomer. Essentially the base (thiolate), Cys87 when considering LL-DAP (upper panel) and Cys226 for DL-DAP (lower panel), deprotonates the substrate C^{α} which can then be protonated *via* the acid (thiol) represented by the other Cys residue. Minimal mobility of the substrate atoms is possible and, excluding H^{α} the amino group and C^{α} are among the most mobile entities, thus facilitating the epimerization. The substrate carboxyl and amino functions interact with other polar active site residues via hydrogen bonds, holding them rigidly away from the H^{α} in order to limit their ability to donate a proton to the thiolate base and hence interfere with epimerization. The grey and black arrows illustrate electronic rearrangements relating to the epimerization of LL-DAP and DL-DAP respectively.

Discussion

The continuing global prevalence of tuberculosis and the alarming emergence of strains of the *M. tuberculosis* complex exhibiting multiple and extensive drug resistance profiles demand that new chemotherapeutic options are addressed. Historically, the biosynthesis of the mycobacterial cell wall has provided a useful chemotherapeutic target with component enzymes inhibited by isoniazid, ethionamide and ethambutol (Takayama & Kilburn, 1989; Banerjee *et al.*, 1994; Kremer *et al.*, 2003; Vilcheze *et al.*, 2006). Despite the relatively unremarkable peptidoglycan structure, penicillins are not clinically effective against mycobacterial infections. Mycobacteria combine β-lactamase production (Chambers *et al.*, 1995) with their ability to adopt an alternative, penicillin-insensitive stem peptide cross-linking mode in stationary phase, a strategy that is likely relevant *in vivo* (Goffin & Ghuysen, 2002). Other than the use of D-cycloserine, which inhibits stem peptide synthesis (Cáceres *et al.*, 1997), as a second-line agent in drug-resistant cases (Ruiz, 1964), the inhibition of mycobacterial peptidoglycan has not been exploited clinically. The biosynthesis of *meso*-DAP represents a potentially useful node of mycobacterial cell wall physiology for chemical intervention as the di-amino acid is strictly required for all peptidoglycan stem peptide cross-linking (Wietzerbin *et al.*, 1974). Thus, DAP epimerase was considered a good candidate for further investigation towards the development of new antimycobacterial agents. Recently, the authors overcame the particularly poor yields of recombinant DapF produced in *E. coli* by introducing several silent S → W mutations in the 5′ end of the mycobacterial sequence and began to characterize the enzyme (Usha *et al.*, 2006).

Here, the study was extended by confirming the likely mechanism of the epimerization reaction. The enzyme

shows significant sequence similarity to its *H. influenzae* orthologue with 23.1 % identity. Of particular significance is the conservation of two cysteine residues that are catalytically active in other DAP epimerases as well as other PLP-independent amino acid racemases (Koo & Blanchard, 1999; Koo *et al.*, 2000; Tanner, 2002). Consistent with their involvement here, treatment of MtDapF with nanomolar concentrations of three different thiol-specific alkylating agents resulted in the total ablation of DAP epimerase activity.

The authors were thus encouraged to construct substitution mutants at both of these residues; the wild-type Cys residues were replaced individually by both serine and alanine residues. The activities that were measured were completely consistent with the observations of Koo *et al.* (2000) using equivalent mutants of DapF from *H. influenzae*. In all cases, the epimerase activity of the mutant enzymes was severely impaired, the extent of the impairment being dependent on the nature of the amino acid replacing the Cys. Marked decreases in K_{cat}/K_M revealed that replacement of either Cys87 or Cys226 with Ser produced mutant enzymes that were at least 50-fold less efficient than wild-type despite retaining similar or even improved substrate affinities indicated by the K_M values exhibited for DAP.

As the catalytic efficiency of this mutant was severely diminished, the moderately improved K_M for *meso*-DAP apparent with the C87S mutant indicates nonproductive substrate binding. Presumably here, the deprotonation of C^{α} and the formation of the planar carbanion intermediate is likely to be supported by the presence of Cys226, but its subsequent protonation by S87 is compromised. This engagement of the catalytic machinery may culminate in an increased residency of substrate in the active site without efficient epimerisation, thus explaining the decrease in the value of the apparent K_M, i.e. the off-rate is slowed. Such a phenomenon is not apparent using the D,L-DAP substrate with C226S as the initial deprotonation is less favoured, although a similar effect might occur if L,L-DAP were used as the roles of the Cys residues are interchangeable.

In conclusion, all the observations are consistent with the hypothesis that MtDapF and DapF from *H. influenzae* achieve the epimerisation of DAP through an identical mechanism. Thus it can be confidently stated that the structural models produced are accurate enough to drive structure-based drug design.

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References

Ashiuchi M, Tani K, Soda K & Misono H (1998) Properties of glutamate racemase from *Bacillus subtilis* IFO 3336 producing poly-gamma-glutamate. *J Biochem (Tokyo)* **123**: 1156–1163.

Banerjee A, Dubnau E, Quemard A *et al.* (1994) InhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263**: 227–230.

Barreto ML, Pereira SM & Ferreira AA (2006) BCG vaccine: efficacy and indications for vaccination and revaccination. *J Pediatr (Rio J)* **82**: S45–S54.

Bhatt A, Fujiwara N, Bhatt K *et al.* (2007) Deletion of *kasB* in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. *Proc Natl Acad Sci USA* **104**: 5157–5162.

Born TL & Blanchard JS (1999) Structure/function studies on enzymes in the diaminopimelate pathway of bacterial cell wall biosynthesis. *Curr Opin Chem Biol* **3**: 607–613.

Cáceres NE, Harris NB, Wellehan JF, Feng Z, Kapur V & Barletta RG (1997) Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. *J Bacteriol* **179**: 5046–5055.

Chambers HF, Moreau D, Yajko D *et al.* (1995) Can penicillins and other beta-lactam antibiotics be used to treat tuberculosis? *Antimicrob Agents Chemother* **39**: 2620–2624.

Cirilli M, Zheng R, Scapin G & Blanchard JS (1998) Structural symmetry: the three-dimensional structure of *Haemophilus influenzae* diaminopimelate epimerase. *Biochemistry* **37**: 16452–16458.

Dye C (2006) Global epidemiology of tuberculosis. *Lancet* **367**: 938–940.

Gallo KA & Knowles JR (1993) Purification, cloning, and cofactor independence of glutamate racemase from *Lactobacillus*. *Biochemistry* **32**: 3981–3990.

Gao LY, Laval F, Lawson EH *et al.* (2003) Requirement for *kasB* in *Mycobacterium* mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. *Mol Microbiol* **49**: 1547–1563.

Glavas S & Tanner ME (1999) Catalytic acid/base residues of glutamate racemase. *Biochemistry* **38**: 4106–4113.

Goffin C & Ghuysen JM (2002) Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins versus lack of efficiency of penicillin as therapeutic agent. *Microbiol Mol Biol Rev* **66**: 702–738.

Hartmann M, Tauch A, Eggeling L, Bathe B, Mockel B, Puhler A & Kalinowski J (2003) Identification and characterization of the last two unknown genes, *dapC* and *dapF*, in the succinylase

branch of the L-lysine biosynthesis of *Corynebacterium glutamicum*. *J Biotechnol* **104**: 199–211.

Higgins W, Tardif C, Richaud C, Krivanek MA & Cardin A (1989) Expression of recombinant diaminopimelate epimerase in *Escherichia coli*. Isolation and inhibition with an irreversible inhibitor. *Eur J Biochem* **186**: 137–143.

Kaufmann SH & Parida SK (2007) Changing funding patterns in tuberculosis. *Nat Med* **13**: 299–303.

Koo CW & Blanchard JS (1999) Chemical mechanism of *Haemophilus influenzae* diaminopimelate epimerase. *Biochemistry* **38**: 4416–4422.

Koo CW, Sutherland A, Vederas JC & Blanchard JS (2000) Identification of active site cysteine residues that function as general bases: diaminopimelate epimerase. *J Am Chem Soc* **122**: 6122–6123.

Kremer L, Dover LG, Morbidoni HR *et al.* (2003) Inhibition of inhA activity, but not kasA activity, induces formation of a kasA-containing complex in mycobacteria. *J Biol Chem* **278**: 20547–20554.

Lloyd AJ, Huyton T, Turkenburg J & Roper DI (2004) Refinement of *Haemophilus influenzae* diaminopimelic acid epimerase (dapF) at 1.75 A resolution suggests a mechanism for stereocontrol during catalysis. *Acta Crystallogr D Biol Crystallogr* **60**: 397–400.

Pillai B, Cherney MM, Diaper CM, Sutherland A, Blanchard JS, Vederas JC & James MN (2006) Structural insights into stereochemical inversion by diaminopimelate epimerase: an antibacterial drug target. *Proc Natl Acad Sci USA* **103**: 8668–8673.

Richaud C, Higgins W, Mengin-Lecreulx D & Stragier P (1987) Molecular cloning, characterization, and chromosomal localization of *dapF*, the *Escherichia coli* gene for diaminopimelate epimerase. *J Bacteriol* **169**: 1454–1459.

Rudnick G & Abeles RH (1975) Reaction mechanism and structure of the active site of proline racemase. *Biochemistry* **14**: 4515–4522.

Ruiz RC (1964) D-cycloserine in the treatment of pulmonary tuberculosis resistant to the standard drugs: a study of 116 cases. *Dis Chest* **45**: 181–186.

Schleifer KH & Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**: 407–477.

Takayama K & Kilburn JO (1989) Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* **33**: 1493–1499.

Tanner ME (2002) Understanding nature's strategies for enzyme-catalyzed racemization and epimerization. *Acc Chem Res* **35**: 237–246.

Tanner ME, Gallo KA & Knowles JR (1993) Isotope effects and the identification of catalytic residues in the reaction catalyzed by glutamate racemase. *Biochemistry* **32**: 3998–4006.

Usha V, Dover LG, Roper DL, Lloyd AJ & Besra GS (2006) Use of a codon alteration strategy in a novel approach to cloning the *Mycobacterium tuberculosis* diaminopimelic acid epimerase. *FEMS Microbiol Lett* **262**: 39–47.

Vilcheze C, Wang F, Arai M *et al.* (2006) Transfer of a point mutation in *Mycobacterium tuberculosis* inhA resolves the target of isoniazid. *Nat Med* **12**: 1027–1029.

Wietzerbin J, Das BC, Petit JF, Lederer E, Leyh-Bouille M & Ghuysen JM (1974) Occurrence of D-alanyl-(D)-*meso*-diaminopimelic acid and *meso*-diaminopimelyl-*meso*-diaminopimelic acid interpeptide linkages in the peptidoglycan of Mycobacteria. *Biochemistry* **13**: 3471–3476.

Winder FGA, Collins P & Rooney SA (1970) Effects of isoniazid on mycolic acid synthesis in *Mycobacterium tuberculosis* and on its cell envelope. *Biochem J* **117**: P27.

Wiseman JS & Nichols JS (1984) Purification and properties of diaminopimelic acid epimerase from *Escherichia coli*. *J Biol Chem* **259**: 8907–8914.