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Cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of succinyl-diaminopimelate desuccinylase (Rv1202, DapE) from *Mycobacterium tuberculosis*

Succinyl-diaminopimelate desuccinylase from *Mycobacterium tuberculosis* (DapE, Rv1202) has been cloned, heterologously expressed in *Escherichia coli* and purified using standard chromatographic techniques. Diffraction-quality crystals were obtained at acidic pH from ammonium sulfate and PEG and diffraction data were collected from two crystals to resolutions of 2.40 and 2.58 Å, respectively. The crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 79.7$, $b = 76.0$, $c = 82.9$ Å, $\beta = 119^\circ$. The most probable content of the asymmetric unit was two molecules of DapE, which would correspond to a solvent content of 56%. Both examined crystals turned out to be pseudo-merohedrally twinned, with twin operator $-h, -k, h + l$ and twin fractions of approximately 0.46 and 0.16, respectively.

1. Introduction

Tuberculosis (TB) is an airborne bacterial disease that newly infects nearly ten million individuals worldwide every year. About 2–3 million people die from TB every year, thus making TB the disease with the highest death rate amongst all bacterial infectious diseases. Consequently, TB was declared a global health emergency by the World Health Organization (WHO) in 1993. The pathogen predominantly responsible for causing TB is *Mycobacterium tuberculosis* (*Mtb*). First-line drugs for TB treatment such as isoniazid, rifampin, ethambutol and streptomycin were introduced in the 1950s and 1960s. However, multi-drug-resistant (MDR), extensively drug-resistant (XDR) and, more recently, totally drug-resistant (TDR) *Mtb* strains are becoming more and more widespread, presenting a serious threat to global tuberculosis control (Raviglione & Smith, 2007) and calling for the urgent development of new antibacterial drugs. New drug targets may be found in biosynthetic pathways which are critical for survival of the pathogen but do not exist in humans. The lysine-biosynthetic pathway fulfils these criteria (Hutton *et al.*, 2007): the end product L-lysine is required for protein production and its direct precursor meso-diaminopimelate (*meso*-DAP) plays a crucial role in cell-wall biosynthesis and stability (Pavelka & Jacobs, 1996).

In mycobacteria, lysine is synthesized *via* the so-called succinyl pathway, which comprises nine successive enzymatic reactions (Umbarger, 1978). Succinyl-diaminopimelate desuccinylase (DapE, S-DAP deacylase; EC 3.5.1.18) catalyzes the seventh reaction step, in which the amide bond of *N*-succinyl-2,6-diaminopimelate is hydrolyzed, resulting in L,L-2,6-diaminopimelate (L,L-DAP) and succinate (Fig. 1). In *Mtb* strain H37Rv DapE is encoded by open reading frame Rv1202 (Cole *et al.*, 1998*a,b*).

The enzyme DapE from *Mtb* (*Mtb*-DapE) consists of 354 amino acids, resulting in a molecular weight of 37 240 Da. The enzymatic reaction is presumed to be metal-dependent. This classifies DapE as a member of peptidase family M20, which also includes a range of other zinc metallopeptidases. Currently, it is believed that DapE possesses two Zn^{2+} ions with unequal binding affinities in its active site (Kindler & Gilvarg, 1960; Cosper *et al.*, 2003). Structural information is available on DapE from *Corynebacterium glutamicum* (PDB entry 3tx8; Joint Center for Structural Genomics, unpublished work),

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Figure 1
The reaction catalyzed by DapE.

Neisseria meningitidis (PDB entry 1vgy; Badger *et al.*, 2005) and *Haemophilus influenzae* (PDB entries 3isz and 3ic1; Nocek *et al.*, 2010), which display sequence identities of 55, 27 and 24%, respectively, to *Mtb*-DapE. Structures are also known of DapE from *Staphylococcus aureus* (PDB entries 3khz and 3ki9; Girish & Gopal, 2010) and *Legionella pneumophila* (PDB entry 3pfe; Joint Center for Structural Genomics, unpublished work), but no significant sequence identity to *Mtb*-DapE could be detected. Nearly all determined DapE structures exhibit a homodimeric chain organization, with each polypeptide chain comprising a globular catalytic domain and a dimerization domain and with the active site being located at the junction between these two domains. The only exception is DapE from *S. aureus*, which exhibits a monomeric architecture.

In this report, we describe the cloning of *Mtb*-DapE (Rv1202) from genomic DNA, the heterologous overexpression of the protein in *Escherichia coli*, its purification to homogeneity and successful crystallization. As part of our long-term effort to structurally characterize all enzymes of the lysine-biosynthetic pathway in *Mtb*, diffraction-quality crystals and structures have already been obtained for the enzymes *Mtb*-AK (Schuldt *et al.*, 2011), *Mtb*-Asd (Vyas *et al.*, 2008, 2012), *Mtb*-DapA (Kefala & Weiss, 2006, 2008), *Mtb*-DapB (Kefala, Janowski *et al.*, 2005; Janowski *et al.*, 2010), *Mtb*-DapD (Schuldt *et al.*, 2008, 2009), *Mtb*-DapC (Weyand *et al.*, 2006, 2007) and *Mtb*-LysA (Kefala, Perry *et al.*, 2005; Weyand *et al.*, 2009). In addition, the structure of *Mtb*-DapF has recently been described by Usha *et al.* (2009). Hence, *Mtb*-DapE represents the last of the nine lysine-biosynthetic enzymes for which diffraction-quality crystals have been obtained.

2. Experimental methods

2.1. Cloning

A construct for *Mtb*-DapE was generated which adds a linker and a C-terminal His₆ tag to the protein sequence. The genomic DNA of *Mtb* strain H37Rv was used as a template for the polymerase chain reaction. The following oligonucleotides (synthesized by MWG Operons) were used as forward and reverse primers, respectively: 5'-TATCCA**ATGGCTGTG**CTGGATTGCGCGG-3' and 5'-CACGAAT**TTCCACCCAGG**TATCGGC-3'. In the forward primer, ATG and GCT triplets (bold) coding for Met and Ala were introduced at positions -1 and 0 to increase the efficiency of protein expression (Looman *et al.*, 1987). In the reverse primer, a silent mutation from CCG to CCC (bold) was introduced to allow the restriction site to immediately follow the coded sequence. The amplified and digested fragment containing 3'-NcoI and 5'-EcoRI restriction sites (italics) was cloned into an NcoI/EcoRI-digested pETM-13 vector. The final protein produced therefore contains the amino-acid sequences MA at the N-terminus and NSSVDKLAALAEHHHHHHH at the C-terminus immediately preceding and following its coded sequence, respectively. The total molecular weight of the expressed protein was

thus 39 382 Da. The final construct was sequenced to confirm correct cloning of the Rv1202 gene. The isoelectric point was estimated to be 5.6 and the molar extinction coefficient was 26 930 M⁻¹ cm⁻¹ as calculated by the *ProtParam* web service (Gasteiger *et al.*, 2005).

2.2. Expression and purification

The recombinant plasmid was used to transform *E. coli* BL21 (DE3) chaperone combination 3 (cc3) cells, which co-express the chaperones GroEL and GroES in order to increase the yield of soluble protein expression (de Marco *et al.*, 2007). Cells from a 5 ml overnight culture were used to inoculate 750 ml LB broth medium containing kanamycin (50 µg ml⁻¹), spectinomycin (50 µg ml⁻¹) and chloramphenicol (17 µg ml⁻¹). Cultivation was carried out at 310 K and 200 rev min⁻¹ until an OD_{600 nm} of 0.4 was reached. The temperature was lowered to 298 K and protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 500 µM. Subsequently, the temperature was lowered to 293 K. Cells were harvested by centrifugation about 20 h after induction and stored at 253 K until further processing.

1 g of wet cell pellet was dissolved in 10 ml buffer A [50 mM bis-Tris pH 6.0, 200 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol (β-ME)] supplemented with one Complete Mini EDTA-free protease-inhibitor cocktail tablet (Roche) per 20 ml and lysed by sonication three times for 3 min using 0.4 s pulses at 277 K. The cell debris was pelleted by centrifugation for 60 min at 277 K and 43 000g. The crude lysate was filtered through a 0.22 µm membrane filter and loaded onto 1.0 ml Ni²⁺-NTA beads (Qiagen) equilibrated with buffer A. In order to remove unbound protein, the column was first washed with 10 ml buffer A, then with 10 ml of the high-salt buffer B (50 mM bis-Tris pH 6.0, 700 mM NaCl, 20 mM imidazole, 5 mM β-ME) and finally again with 10 ml buffer A. The protein was eluted with a step gradient to 500 mM imidazole in buffer A immediately before proceeding to the next purification step. The protein solution was diluted to a total volume of 5 ml and filtered through a 0.22 µm membrane filter. Subsequently, the protein was purified by size-exclusion chromatography (Superdex S200 16/60 prep grade; GE Healthcare) using buffer C (50 mM bis-Tris pH 6.0, 200 mM NaCl, 0.5 mM TCEP) as a buffer system. The peak fractions were analyzed using SDS-PAGE, pooled and concentrated to 3–15 mg ml⁻¹. Protein concentrations were measured according to the absorption at 280 nm using the molecular weight and estimated extinction coefficient of the protein construct.

2.3. Crystallization

Purified protein at a concentration of 15 mg ml⁻¹ in buffer C was used for screening of initial crystallization conditions at the High Throughput Crystallization Facility at the EMBL Hamburg Outstation (Mueller-Dieckmann, 2006). In a 96-well Greiner low-profile plate, 200 nl protein sample and 200 nl precipitant were equilibrated

against 50 μ l reservoir solution. Initial crystals appeared at room temperature within 1 d in Hampton Research Crystal Screen condition No. 20 [100 mM sodium acetate pH 4.6, 200 mM ammonium sulfate, 25%(w/v) PEG 4000; Fig. 2a]. Optimization of the initial hit was carried out using the hanging-drop vapour-diffusion method. Protein solution and reservoir solution were mixed in a 1:1 ratio using either 1 or 2 μ l per drop. Equilibration occurred against 900 μ l reservoir solution in 24-well NeXtal (Qiagen) plates. Crystal growth was obtained in the presence of 5–10%(w/v) PEG 4000 or PEG 3350, 35–120 mM ammonium sulfate, 100 mM sodium acetate pH 4.1–4.6 at room temperature using protein concentrations of between 3 and 7 mg ml⁻¹ (Fig. 2b).

2.4. Diffraction data collection and processing

Diffraction data collection was performed on two single crystals. Data from crystal 1 were collected on beamline BL14.1 at the BESSY II storage ring, Berlin, Germany (Mueller *et al.*, 2012) equipped with a Rayonics MX225 3 $\times$ 3 CCD detector. The crystal was mounted in a nylon loop, cryoprotected using mother liquor [50 mM ammonium sulfate, 6%(w/v) PEG 3350, 100 mM sodium acetate pH 4.1] supplemented with 20%(v/v) glycerol, flash-cooled in liquid nitrogen and stored for transport to the synchrotron. Data collection was performed at 100 K and a total of 600 diffraction images were collected in 0.3° oscillation steps at a wavelength of 0.918 Å. For crystal 2, diffraction data were collected on the EMBL X12 beamline at the DORIS storage ring, DESY, Hamburg, Germany. The beamline was equipped with a MAR Mosaic CCD detector (225 mm). Mother liquor [80 mM ammonium sulfate, 8%(w/v) PEG 4000, 100 mM sodium acetate pH 4.6] supplemented with 20%(v/v) MPD served as a cryoprotectant. A crystal was mounted from the cryoprotectant-containing solution and flashed-cooled in a cold nitrogen stream at 100 K. A total of 200 images were then collected from this crystal in 1° oscillation steps using a wavelength of 1.278 Å.

Indexing and integration of the X-ray diffraction data from each crystal were performed using the program *XDS* (Kabsch, 1993, 2010a) and were followed by scaling with *XSCALE* (Kabsch, 2010b). Analysis of the Laue group and the space group was performed with *POINTLESS* (Evans, 2006). The *R* factors *R*_{r.i.m.} (redundancy-independent merging *R* factor) and *R*_{p.i.m.} (precision-indicating merging *R* factor) (Weiss, 2001) were calculated using the program *RMERGE* (available from MSW upon request). All relevant data-collection and processing parameters are given in Table 1. Intensities were converted to structure-factor amplitudes using the program *TRUNCATE* from *CCP4* (French & Wilson, 1978; Winn *et al.*, 2011). The optical resolution of the data set and the data twinning were

Table 1

Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	Crystal 1	Crystal 1	Crystal 2
No. of crystals	1	1	1
Wavelength (Å)	0.918	0.918	1.278
Crystal-to-detector distance (mm)	250	250	200
Rotation range per image (°)	0.3	0.3	1.0
Total rotation range (°)	180	180	200
Exposure time per image (s)	5	5	40
Space group	<i>P</i> 2 ₁	<i>C</i> 222 ₁	<i>P</i> 2 ₁
Resolution range (Å)	50.0–2.40 (2.46–2.40)	50.0–2.33 (2.39–2.33)	50.0–2.58 (2.64–2.58)
Unit-cell parameters			
<i>a</i> (Å)	79.71	79.71	80.09
<i>b</i> (Å)	76.00	145.40	75.86
<i>c</i> (Å)	82.88	75.99	82.36
$\alpha = \gamma$ (°)	90	90	90
β (°)	118.7	90	118.2
Mosaicity (°)	0.75	0.76	0.27
Total No. of reflections	118417	126420	113675
Unique reflections†	62005	34920	53004
Redundancy†	1.9 (1.7)	3.6 (2.6)	2.1 (2.1)
$\langle I/\sigma(I) \rangle$	8.42 (2.02)	9.91 (2.28)	7.62 (2.08)
Completeness (%)	92.6 (83.4)	96.1 (70.2)	97.8 (94.6)
<i>R</i> _{merge} (%)	7.6 (41.7)	10.0 (43.7)	9.9 (41.4)
<i>R</i> _{r.i.m.} (%)	10.4 (52.1)	11.7 (53.2)	13.5 (55.7)
<i>R</i> _{p.i.m.} (%)	5.4 (28.2)	4.4 (23.1)	6.5 (26.9)
Overall <i>B</i> factor from Wilson plot (Å ²)	36.5	34.4	39.1
Optical resolution (Å)	1.77	1.75	1.87

† Bijvoet pairs left unmerged.

analyzed with *SFCHECK* (Vaguine *et al.*, 1999) and *phenix.xtriage* (Adams *et al.*, 2010). Self-rotation functions were computed using the program *MOLREP* from *CCP4* (Vagin & Teplyakov, 2010; Winn *et al.*, 2011) based on structure-factor amplitudes to a maximum resolution of 3.5 Å.

3. Results and discussion

After the two-step purification procedure, approximately 0.6 mg pure protein was obtained from 1 g wet cell pellet. The purity was at least 95% as estimated by SDS–PAGE and the identity of the protein was confirmed by mass spectrometry (data not shown). Since the protein tended to precipitate even at low protein concentration, a Thermo-Fluor experiment (Pantoliano *et al.*, 2001; Nettleship *et al.*, 2008; Reinhard *et al.*, in preparation) was performed to optimize the buffer composition used in the purification and crystallization procedures (data not shown). Changing the buffer system from Tris pH 7.5 to bis-Tris pH 6.0 completely abolished the occurrence of protein

Figure 2

Crystals of DapE (Rv1202) from *M. tuberculosis*. (a) Crystals obtained from the initial screen. (b) Crystals after optimization.

precipitation at the concentrations used in this study. An apparent molecular weight of about 85 kDa was deduced from size-exclusion chromatography, which indicates that *Mtb*-DapE is a homodimer in solution. A homodimeric appearance has also been observed in the structures of DapE from other organisms, with the exception of DapE from *S. aureus*, which appears to function as a monomer (Girish & Gopal, 2010).

In the initial crystallization experiments, one condition was identified which produced an intergrown crystallite aggregate (Fig. 2*a*). For the optimization of this condition, a significant reduction of the concentration of precipitant and protein was required. The speed of crystal growth was strongly dependent on the pH, with growth being initiated after 10 min at pH 4.1 and after about 2 h at pH 4.6. The majority of the crystals were intergrown and only a few single crystals could be obtained. All attempts to date to obtain crystals from different crystallization conditions were unsuccessful.

For data collection, single crystals were mounted in nylon loops and cryoprotected using 20% (w/v) glycerol or MPD added to the reservoir solution. Many tested crystals showed anisotropic diffraction and a maximum resolution of about 3.5 Å. Only a few crystals were found to exhibit diffraction to better than 2.6 Å resolution. Complete data sets were then collected from two crystals. All relevant data-collection and processing statistics are given in Table 1. Although the data from crystal 1 could be reduced in space groups $P2_1$ and $C222_1$, the true space group is almost certainly $P2_1$. This will be explained in more detail in the next paragraphs. Despite the fact that the data-collection wavelength for crystal 2 was chosen to maximize the anomalous scattering for the presumed active-site zinc ions, no anomalous signal could be detected in the data. This suggests that either the enzyme was purified and crystallized in its apo form or

that no zinc ions are present in the active site. Based on a Matthews parameter calculation (Matthews, 1968), the most likely number of molecules in the asymmetric unit of the presumed space group $P2_1$ is two.

The self-rotation functions of both crystals are indicative of 222 symmetry (the self-rotation function based on data from crystal 2 is shown in Fig. 3). This can be explained by the presence of a non-crystallographic twofold axis parallel or perpendicular to the monoclinic x axis. The presence of a noncrystallographic twofold axis makes perfect sense in light of the fact that *Mtb*-DapE is a homodimer. The combination of the noncrystallographic twofold axis and the crystallographic twofold axis along y then results in the appearance of a third twofold axis perpendicular to these two, and 222 symmetry is observed. An unusual observation, however, is the fact that the three peaks in the self-rotation function exhibit almost the same peak height, hinting at a higher symmetry space group. Indeed, as mentioned above, the data for crystal 1 could be reduced in space group $C222_1$. However, the value of the redundancy-independent merging R factor $R_{r.i.m.}$ in the low-resolution reflection bin is 5.1%, which is slightly higher than the low-resolution $R_{r.i.m.}$ value when the data are reduced in $P2_1$ (4.2%). This already hints at $P2_1$ being the correct space group. Together with the fact that data reduction for crystal 2 data is only possible in space group $P2_1$, it may be concluded that $P2_1$ is the correct space group.

A twinning analysis of the data revealed that the situation is further complicated by twinning. The Wilson ratio and moments and the cumulative intensity distribution for the data processed in $P2_1$ clearly indicated the presence of pseudo-merohedral twinning with twinning operator $-h, -k, h + l$ or its equivalent $h, -k, -h - l$. It is sometimes observed that space group $P2_1$ is pseudo-merohedrally twinned when the unit-cell parameters of the monoclinic crystal fulfil the geometric condition $c \cos \beta = -a/2$ (Fig. 4). This is nearly exactly the case for the obtained *Mtb*-DapE crystals. In the case of perfect twinning (twin fraction $\alpha = 0.5$) the orthorhombic Laue group mmm is perfectly simulated, with the consequence that intensities of reflections which are not equal at low twinning fraction become equal. Data of crystals with high twinning fractions close to 0.5 can then also be scaled and merged in the corresponding space group

Figure 3 Self-rotation function based on data collected from crystal 2 of *Mtb*-DapE reduced in space group $P2_1$. The $\kappa = 180^\circ$ section is shown, indicating the relative locations of the twofold symmetry axes. The b axis is the unique axis in the $P2_1$ space group, causing the peaks along the y axis in the self-rotation function. The plot shows apparent 222 symmetry, which is created by a combination of the crystallographic twofold axis along y and the noncrystallographic twofold axes perpendicular to it. This figure was produced using the program *MOLREP* (Vagin & Teplyakov, 2010; Winn *et al.*, 2011).

Figure 4 Arrangement of the $P2_1$ unit cell with twinning causing simulation of space group $C222_1$. The projection of the ac plane of the monoclinic unit cell is shown in black. The b axis (76.00 Å) is perpendicular to the plane. The twinning is produced by superposition of the black cell with the green cell. The two cells share the a and b axes in opposite directions with their respective twins. The simulated orthorhombic unit cell is represented as a grey discontinuous line. The a and c axes of the orthorhombic cell are identical to the monoclinic a and b axes, respectively. The orthorhombic b axis nearly perfectly fulfils the relation $b_{\text{ortho}} = 2c_{\text{mono}} \cos(\beta_{\text{mono}} - 90^\circ)$.

Table 2Results of the twinning analysis of *Mtb*-DapE crystals 1 and 2.

Parameter	Theoretical values		Crystal 1	Crystal 2
	Untwinned	Perfect twin		
Wilson ratio and moments for acentric reflections				
$\langle I^2 \rangle / \langle I \rangle^2$	2	1.5	1.814	1.945
$\langle F^2 \rangle / \langle F \rangle^2$	0.785	0.885	0.831	0.798
$ E^2 - 1 $	0.736	0.541	0.642	0.711
Padilla and Yeates statistics (<i>L</i> -test)				
$\langle L \rangle$	0.5	0.375	0.432	0.483
$\langle L^2 \rangle$	0.333	0.2	0.258	0.312
Yeates statistics (<i>H</i> -test)				
$\langle H \rangle$	0.5	0	0.050	0.329
$\langle H^2 \rangle$	0.33	0	0.005	0.176
Estimated twinning fraction <i>via</i>				
Cumulative distribution of <i>H</i>			0.457	0.156
Britton plot			0.441	0.152
<i>L</i> -test			0.442	0.022
Twinning operator			$-h, -k, h + l$	$-h, -k, h + l$

C222₁. Based on the *H*-test (Yeates, 1988), the Britton plot (Britton, 1972; Fisher & Sweet, 1980) and the *L*-test (Padilla & Yeates, 2003), the observed twinning fractions α are about 0.45 for crystal 1 and between 0.022 and 0.156 for crystal 2 (Table 2).

It is important to note that the effects of a noncrystallographic twofold axis along or perpendicular to the monoclinic *x* axis and of pseudo-merohedral twinning as described here are identical. This may be an explanation of the fact that the peak heights for the noncrystallographic symmetry peaks observed in the self-rotation function are almost the same as that for the crystallographic peak. This may also be the reason why the various twinning indicators except the Yeates *H*-test statistic (Table 2) for the almost perfectly twinned crystal 1 data lie between the theoretical values for twinned and untwinned.

Pseudo-merohedral twinning combined with the presence of non-crystallographic symmetry in space group *P*2₁ emulating space group C222₁ has, for example, also been described in monoclinic crystals of human peroxiredoxin 5 (Declercq & Evrard, 2001) and in monoclinic crystals of $\gamma\delta$ T-cell ligand T10 (Rudolph *et al.*, 2004). In both cases the monoclinic cell settings fulfil the same geometric condition ($\cos\beta = -a/2$), resulting in similar observations to those presented in this work for *Mtb*-DapE. While the human peroxiredoxin 5 crystals only exhibited a rather low twin fraction ($\alpha = 0.24$) and reduction of the data in space group C222₁ was not possible, the $\gamma\delta$ T-cell ligand T10 crystals with a twinning fraction α of 0.46 could be reduced in space groups *P*2₁ and C222₁ with nearly equivalent data statistics.

Attempts to solve the structure of *Mtb*-DapE by molecular replacement using the structure of DapE from *C. glutamicum* (55% sequence identity) is currently in progress.

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