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High-resolution structures of *Thermus thermophilus* enoyl-acyl carrier protein reductase in the apo form, in complex with NAD⁺ and in complex with NAD⁺ and triclosan

Enoyl-acyl carrier protein reductase (ENR; the product of the *fabI* gene) is an important enzyme that is involved in the type II fatty-acid-synthesis pathway of bacteria, plants, apicomplexan protozoa and mitochondria. Harmful pathogens such as *Mycobacterium tuberculosis* and *Plasmodium falciparum* use the type II fatty-acid-synthesis system, but not mammals or fungi, which contain a type I fatty-acid-synthesis pathway consisting of one or two multifunctional enzymes. For this reason, specific inhibitors of ENR are attractive antibiotic candidates. Triclosan, a broad-range antibacterial agent, binds to ENR, inhibiting fatty-acid synthesis. As humans do not have an ENR enzyme, they are not affected. Here, high-resolution structures of *Thermus thermophilus* (*Tth*) ENR in the apo form, bound to NAD⁺ and bound to NAD⁺ plus triclosan are reported. Differences from and similarities to other known ENR structures are reported; in general, the structures are very similar. The cofactor-binding site is also very similar to those of other ENRs and, as reported for other species, triclosan leads to greater ordering of the loop that covers the cofactor-binding site, which, together with the presence of triclosan itself, presumably provides tight binding of the dinucleotide, preventing cycling of the cofactor. Differences between the structures of *Tth* ENR and other ENRs are the presence of an additional β -sheet at the N-terminus and a larger number of salt bridges and side-chain hydrogen bonds. These features may be related to the high thermal stability of *Tth* ENR.

1. Introduction

Enoyl-acyl carrier protein reductase (ENR) is an important enzyme that is involved in the type II fatty-acid-synthesis (FAS) pathway in bacteria, plants, apicomplexan protozoa and mitochondria (Massengo-Tiassé & Cronan, 2009). Harmful pathogens such as *Mycobacterium tuberculosis* and *Plasmodium falciparum* use the FAS II system, but not mammals or fungi, which have an FAS I system that consists of one or two multifunctional enzymes, respectively (Leibundgut *et al.*, 2008). For this reason, specific inhibitors of ENR are attractive antibiotic candidates. ENR is encoded by the *fabI* gene (Bergler *et al.*, 1994) and catalyses the last step of the elongation cycle in the synthesis of fatty acids. Structures of ENR from the bacteria *M. tuberculosis* (Dessen *et al.*, 1995), *Escherichia coli* (Baldock *et al.*, 1996), *M. leprae* (Wang *et al.*, 2007), *Helicobacter pylori* (Lee *et al.*, 2007), *Bacillus anthracis* (Tippiraju *et al.*, 2008), *Francisella tularensis* (Lu *et al.*, 2009), *Staphylococcus aureus* (Priyadarshi *et al.*, 2010), *B. cereus* (Kim *et al.*, 2010) and *B. subtilis* (Kim *et al.*, 2011), oil seed rape (*Brassica napus*; Roujeinikova, Sedelnikova *et al.*, 1999), the malaria parasite *P. falciparum* (Perozzo *et al.*, 2002), the mammalian parasite *Toxoplasma gondii* (Muench *et al.*, 2007) and the chicken parasite *Eimeria tenella* (Lu *et al.*, 2007) have been described either alone or in complex with cofactors and/or inhibitors. Structures of

ENR from other species such as the parasite *P. berghei* (PDB entry 3f4b; M. Yu *et al.*, unpublished work) and the bacteria *Aquifex aeolicus* (PDB entry 2p91; Southeast Collaboratory for Structural Genomics and RIKEN Structural Genomics/Proteomics Initiative, unpublished work), *Burkholderia pseudomallei* (PDB entry 3ek2; Seattle Structural Genomics Center for Infectious Disease, unpublished work) and *Anaplasma phagocytophilum* (PDB entries 3k2e and 3k31; Seattle Structural Genomics Center for Infectious Disease, unpublished work) have also been submitted to the Protein Data Bank.

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a broad-range antibacterial agent that is used in soaps, mouthwashes, toothpaste and other products; bathing in 2% (w/v) triclosan is an accepted regime for decolonizing methicillin-resistant *Staphylococcus aureus* patients. Triclosan binds to ENR, increasing its affinity for NAD⁺ and resulting in the formation of a stable ternary ENR–NAD⁺–triclosan complex which is unable to participate in fatty-acid synthesis (Levy *et al.*, 1999; Roujeinikova, Levy *et al.*, 1999). As humans do not have an ENR enzyme, they are not affected.

Here, we present the structure of ENR from *Thermus thermophilus*, which is an extremely thermophilic, Gram-negative, aerobic, rod-shaped bacterium. *T. thermophilus* was isolated from a natural geothermal environment in Japan and can grow at temperatures of up to 385 K, with an optimal growth temperature of 343 K (Oshima, 1974). Although structures of *Tth* ENR in the apo form (PDB entry 1ulu; 2.00 Å resolution; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) and bound to NADP⁺ (PDB entry 2yw9; 2.50 Å resolution; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) are available from the PDB, they have not been described. Here, we report the high-resolution structures of *Tth* ENR in the apo form, bound to NAD⁺ and bound to NAD⁺ plus triclosan. These structures were solved by molecular replacement and refined at 1.50, 1.86 and 1.90 Å resolution, respectively.

2. Materials and methods

2.1. Expression, purification and crystallization

The *Tth* ENR gene sequence was amplified from *T. thermophilus* HB8 genomic DNA using the polymerase chain reaction. The resulting DNA fragment encoding *Tth* ENR (UniProt accession code Q5SLI9) was cleaved with *Nde*I and *Sal*I and ligated into the expression vector pET30a (Novagen/Merck, Nottingham, England) between the *Nde*I and *Xho*I restriction sites (*Sal*I and *Xho*I produce compatible 5'-TCGA overhangs). The resulting plasmid pET30-*Tth* ENR encoded all 261 residues of *Tth* ENR. The sequence of the insert was confirmed by DNA-sequence analysis (Eurofins MWG Operon, Ebersberg, Germany). *E. coli* strain Rosetta (DE3), obtained from Novagen, was freshly transformed with pET30-*Tth* ENR and a 0.5 l culture in LB (10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g sodium chloride per litre) supplemented with 20 mg l⁻¹ chloroamphenicol and 25 mg l⁻¹ kanamycin was grown aerobically at 310 K to an optical density of 0.9–1.1 at 600 nm. The culture was cooled on ice and expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, after which growth was allowed to continue overnight (12–16 h at 293 K). The cells were harvested by centrifugation at 3500g for 20 min at 277 K and the resulting pellet was resuspended in 10 ml buffer A [20 mM Tris–HCl pH 7.5, 1 mM ethylenediamine tetraacetate (EDTA)] and frozen at 253 K. The frozen pellet was thawed on ice, phenylmethanesulfonyl fluoride was added to a final concentration of 1 mM and the bacteria were lysed by a triple pass through an emulsifier (Avestin Emulsifier

C5, Avestin Europe GmbH, Mannheim, Germany). From this point onwards, all procedures were carried out at 277 K unless stated otherwise. The lysate was centrifuged at 18 000g for 30 min and the insoluble fraction was removed. The clear supernatant was incubated for 15 min at 343 K in a water bath with constant shaking and was then immediately cooled on ice for 15 min. The resulting suspension was centrifuged at 18 000g for 15 min and the insoluble fraction was removed. Ammonium sulfate was added slowly to the supernatant fraction to 55% saturation and the resulting suspension was centrifuged at 18 000g for 30 min. Ammonium sulfate was then added to the supernatant to 75% saturation and after centrifugation as before the pellet was resuspended in a minimal amount of buffer A and dialysed three times against 1 l of the same buffer. The sample was then loaded onto a strong anion-exchange column (Uno-Q6, 6 ml bed volume; Bio-Rad, Madrid, Spain), which had previously been equilibrated with buffer A, at a flow rate of 1.0 ml min⁻¹. The protein was eluted with a linear gradient of 0.0–1.0 M sodium chloride in buffer A. *Tth* ENR eluted right at the start of the gradient and the purified fractions containing *Tth* ENR were pooled and dialysed twice against 1 l buffer B [20 mM 2-(*N*-morpholino)ethanesulfonic acid–NaOH pH 6.0, 1 mM EDTA, 10% (v/v) glycerol]. The sample was loaded onto a strong cation-exchange column (BioRad Uno-S6, 6 ml bed volume), which had previously been equilibrated with buffer B, at a flow rate of 1.0 ml min⁻¹. The protein was eluted with a linear gradient of 0.0–1.0 M sodium chloride in buffer B. The protein eluted at around 100 mM sodium chloride and the pooled fractions were concentrated in Amicon Ultra-15 concentrators (Millipore, Madrid, Spain), incorporating three washes with buffer A to remove low-molecular-weight impurities. The purity of the protein was established by Coomassie Brilliant Blue stained 15% SDS–PAGE according to the method of Laemmli (1970). The protein concentration was determined by spectrophotometric measurements at 280 nm using an estimated extinction coefficient of $\epsilon = 0.75 \text{ l g}^{-1} \text{ cm}^{-1}$ (NanoDrop ND-1000, Wilmington, Delaware, USA).

2.2. Crystallization, structure solution and refinement

For crystallization, drops consisting of 2 µl *Tth* ENR at approximately 4 mg ml⁻¹ mixed with 2 µl reservoir solution were equilibrated against 0.15 ml reservoir solution at 291 K. Plate-shaped crystals of apo *Tth* ENR were obtained by sitting-drop vapour diffusion using 20% (w/v) polyethylene glycol 4000 in 100 mM sodium citrate pH 5.4 as the precipitant solution. NAD⁺ was dissolved to 2 mM in *Tth* ENR solution at 4 mg ml⁻¹ and *Tth* ENR–NAD⁺ crystals were obtained using 32% (w/v) polyethylene glycol 4000 in the same buffer as the precipitant solution. To obtain *Tth* ENR–NAD⁺–triclosan crystals, *Tth* ENR–NAD⁺ crystals were soaked for 48 h in crystallization solution (*Tth* ENR–NAD⁺ solution plus precipitant solution) containing 3.2 mM triclosan (Sigma–Aldrich Quimica SA, Madrid, Spain). Crystals were flash-cooled in liquid nitrogen using crystallization solution with 10% (w/v) glycerol as a cryoprotectant. Crystallographic data were collected at 100 K on beamline ID14-4 at ESRF, Grenoble, France for the apo *Tth* ENR and *Tth* ENR–NAD⁺ complex crystals and on beamline X11 at DESY, Hamburg, Germany for the *Tth* ENR–NAD⁺–triclosan complex crystals.

Crystallographic data were processed using *MOSFLM* (Powell, 1999; Leslie, 2006), scaled using *SCALA* (Evans, 2006) and further treated using other programs from the *CCP4* program suite (Winn *et al.*, 2011). The structure of the apo form was solved by molecular replacement using the program *Phaser* (McCoy *et al.*, 2007) with a monomer of PDB entry 1ulu as a search model. For the NAD⁺ and

NAD⁺/triclosan complex structures *MOLREP* (Vagin & Teplyakov, 2010) and monomer *A* of the apo form (PDB entry 2wyu) were used. Atomic coordinates, molecular topologies and geometric restraints for NAD⁺ and triclosan were generated with the *PRODRG2* server (Schüttelkopf & van Aalten, 2004). The resulting models were rebuilt with *Coot* (Emsley & Cowtan, 2004) and refined with *REFMAC* (Murshudov *et al.*, 2011), and ligand fitting was performed manually in the electron-density maps. A few water molecules were replaced by Na atoms as suggested by the *WASP* application (Nayal & Di Cera, 1996). Validation was performed with *MolProbity* (Chen *et al.*, 2010) and figures were created with *PyMOL* (Schrödinger LLC, New York,

USA). Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 2wyu for apo *Tth* ENR, 2wyv for *Tth* ENR bound to NAD⁺ and 2wyw for *Tth* ENR bound to NAD⁺ and triclosan.

3. Results and discussion

Full-length nontagged *T. thermophilus* enoyl-acyl carrier protein reductase (*Tth* ENR) was expressed in *E. coli* and purified. For purification, advantage was taken of the thermal resistance of the protein by incorporating a heat-treatment step to denature and precipitate endogenous *E. coli* proteins. After centrifugation, supernatants were further purified by ammonium sulfate precipitation and anion-exchange and cation-exchange chromatography as described in §2. The purified protein was crystallized at pH 5.4 using polyethylene glycol 4000 as precipitant. The crystals belonged to space group *P*2₁ with one tetramer in the asymmetric unit. To obtain the complex structures, the protein was cocrystallized with NAD⁺ as described in §2 and triclosan was soaked into pre-existing crystals that already contained NAD⁺. Data were collected to better than 2.0 Å resolution for all three crystal forms using synchrotron radiation. All of the structures were solved by molecular replacement and refined to good geometry and good agreement with the experimental data (Table 1).

Some residues belonging to the C-termini of all of the structures could not be modelled owing to a lack of electron density; presumably these are flexible parts of the protein. Also disordered were residues Thr195–Gly203 of chain *B* and Arg194–Met207 of chain *D* on the surface of the apo *Tth* ENR structure and Ser200–Thr205 of chain *D* and Val196–Met207 of chain *B* on the surface of the *Tth* ENR–NAD⁺ structure. It is likely that these are flexible and inspection of the crystal packing shows that in monomers *A* and *C* of those two structures the equivalent residues are held in place by crystal contacts. One Ramachandran outlier, Arg194 in chain *D*, is present in the *Tth* ENR–NAD⁺ structure; this residue is located next to one of the disordered surface stretches. In the *Tth* ENR–NAD⁺–triclosan structure the same regions are ordered in all four monomers, presumably owing to triclosan binding to the active site, which provides a link between the NAD⁺ and the loop that covers the active site. The *Tth* ENR–NAD⁺–triclosan structure contained 40% solvent, while the other two structures contained 45% solvent. Remarkably, the triclosan-soaking process appears to have caused significant changes in the unit-cell parameters (see Table 1). The tighter packing

Figure 1

Structure of the *T. thermophilus* enoyl-acyl carrier reductase apo form. (a) Structure of a tetramer. Green, cyan, magenta and yellow colours are used for chains *A*, *B*, *C* and *D*, respectively. (b) Monomer with secondary-structure elements labelled. (c) Topology diagram. α -Helices are coloured red and β -strands yellow.

Table 1

Crystallographic data and refinement statistics.

Values in parentheses are for the highest resolution bin (where applicable).

	Apo form	NAD ⁺ complex	NAD ⁺ /triclosan complex
Data statistics			
Wavelength (Å)	0.939	0.939	0.815
Detector	ADSC Q315r CCD	ADSC Q315r CCD	MAR 555 flat panel
Crystal-to-detector distance (mm)	230.6	295.7	300.0
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 54.3, <i>b</i> = 107.8, <i>c</i> = 86.0, α = γ = 90.0, β = 95.9	<i>a</i> = 53.5, <i>b</i> = 107.4, <i>c</i> = 84.8, α = γ = 90.0, β = 94.9	<i>a</i> = 57.7, <i>b</i> = 127.5, <i>c</i> = 66.5, α = γ = 90.0, β = 108.2
Unique observations	151147 (19108)	77770 (10441)	65244 (8528)
Resolution range (Å)	15.0–1.50 (1.58–1.50)	40.0–1.86 (1.96–1.86)	30.0–1.90 (2.00–1.90)
Multiplicity	2.9 (2.3)	2.9 (2.5)	3.5 (3.5)
Completeness (%)	96.4 (83.8)	95.9 (89.2)	91.2 (82.1)
Mean <i>I</i> /σ(<i>I</i>)	8.7 (2.0)	10.1 (2.2)	10.5 (2.9)
<i>R</i> _{merge} [†]	0.062 (0.299)	0.064 (0.407)	0.071 (0.393)
Wilson <i>B</i> (Å ²)	18.9	21.5	21.5
Refinement			
Resolution range (Å)	15.0–1.50	40.0–1.86	30.0–1.90
No. of reflections used	149093	76657	62991
No. of reflections for <i>R</i> _{free}	2001	1098	2198
<i>R</i> factor	0.170	0.182	0.155
<i>R</i> _{free}	0.200	0.239	0.196
No. of protein atoms	7754	7716	7876
No. of ligand atoms	0	88	244
No. of solvent atoms	1079	548	675
Average <i>B</i> factors (Å ²)			
Protein	21.8	27.7	22.5
Ligand	—	49.3	18.3
Solvent	35.4	36.2	32.6
R.m.s.d. values			
Bonds (Å)	0.015	0.016	0.014
Angles (°)	1.5	1.5	1.5
Ramachandran statistics			
Favoured	984	978	997
Allowed	16	23	28
Outliers	0	1	0

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th measurement of the same reflection and $\langle I(hkl) \rangle$ is the mean observed intensity for that reflection.

of the protein in the crystal lattice is related to the ordering of the protein active-site lid presumably caused by the binding of triclosan. A degree of drying out of the crystallization drop owing to the extra manipulations involved in soaking may also have had some influence.

3.1. Overall protein structure

In the three structures that we have determined, *Tth* ENR forms homotetramers with internal 222 symmetry (Figs. 1*a*, 3*a* and 4*a*). This is the same as has been observed for ENRs from other species such as *M. tuberculosis* (Dessen *et al.*, 1995). The four monomers lie in a ring, adopting alternate ‘up’ and ‘down’ orientations. Each monomer interacts with its neighbours with two energetically favourable interfaces. One interface (between chains *A* and *B* and between chains *C* and *D*) comprises 1.8×10^3 Å² and has a calculated energy gain of -84 kJ mol⁻¹, while the other (between chains *A* and *D* and between chains *B* and *C*) comprises 1.7×10^3 Å² and has a calculated energy gain of -117 kJ mol⁻¹ (Krissinel & Henrick, 2007). Each *Tth* ENR monomer contains a central seven-stranded parallel β-sheet covered on each face by three α-helices (Figs. 1*b* and 1*c*). The N-terminal residues 2–4 form a short additional β-strand (B0) that interacts with a counterpart from a neighbouring monomer (*A* with *D* and *B* with *C*), potentially stabilizing the tetramer. On top of the NAD⁺-binding site, two α-helices are present (A4*b* and A6). The C-termini of each *Tth* ENR monomer interact with those from opposite monomers (*A* with *C* and *B* with *D*); this may also be a tetramer-stabilization interaction.

The topology of the monomer is similar to those of other dehydrogenases (Rossmann *et al.*, 1975) and is virtually identical to those of *M. tuberculosis* (Dessen *et al.*, 1995), *H. pylori* (Lee *et al.*, 2007) and *E. coli* (Rafi *et al.*, 2006) ENRs (Figs. 2*a* and 2*b*). Principal differences arise from the lid that covers the nicotinamide-binding site (Gly191–Gly203 in *Tth* ENR), apart from the now resolved N-terminal β-strand B0 (Fig. 2*c*). The four monomers of the apo structure are also very similar to each other (r.m.s.d. of 0.13–0.14 Å for 201–208 C^α-atom pairs), with the only significant differences occurring in the residues flanking the disordered region spanning residues 194–207. When we structurally compare *Tth* ENR (subunit *A* of the apo structure) with the apo structure of ENR from *H. pylori* (PDB entry 2pd3; Lee *et al.*, 2007), the r.m.s.d. is 0.72 Å for 214 superposed C^α-atom pairs. The r.m.s.d. increases to 0.79 Å for 206 superposed C^α-atom pairs when we compare the *Tth* ENR monomer with the apo structure of ENR from *E. coli* (PDB entry 2fhs; Rafi *et al.*, 2006) and to 1.05 Å for 195 superposed C^α-atom pairs with the apo structure of ENR from *M. tuberculosis* (PDB entry 1eny; Dessen *et al.*, 1995).

The crystallographic and likely biological units of *Tth* ENR are homotetramers, which are packed in the crystal lattice forming parallel layers. Similar homotetramers have been observed in the crystallographic packing of ENRs from *H. pylori*, *M. tuberculosis*, *P. falciparum*, *F. tularensis*, *E. tenella*, *A. phagocytophilum*, *B. anthracis* and *P. berghei*. The main interactions between monomers involved in the formation of the tetramer are 20 salt bridges between chains *A* and *B* and between chains *C* and *D* (Asp70 with Arg111, Arg105 with Glu133 and Arg111 with Glu119), between chains *B* and *D* and between chains *A* and *C* (Glu151 with Lys152)

and between chains *A* and *D* and between chains *B* and *C* (Glu244 with His253), and 12 side-chain hydrogen bonds between chains *A* and *B* and between chains *C* and *D* (Tyr106 with Ser170), between chains *B* and *D* and between chains *A* and *C* (Tyr148 with Lys152) and between chains *A* and *D* and between chains *B* and *C* (Glu225 with Ser239) (Table 2). There are no important differences in the quaternary structure between the apo, NAD⁺-bound and NAD⁺/triclosan-bound *Tth* ENR forms. The inclusion of NAD⁺ in the active site of *Tth* ENR does not cause considerable differences with respect to the apo form in the crystallographic unit-cell parameters and the atomic unit-cell content. However, the inclusion by soaking of triclosan in the active site of *Tth* ENR–NAD⁺ causes a tighter packing of the tetramers in the crystallographic lattice. When we compare subunit *A* of the *Tth* ENR apo structure with subunit *B* of

the *Tth* ENR–NAD⁺ structure and subunit *A* of the *Tth* ENR–NAD⁺–triclosan structure, the r.m.s.d.s are 0.45 and 0.37 Å for 215 and 209 C α -atom pairs, respectively. When subunit *B* of the *Tth* ENR–NAD⁺ structure and subunit *A* of the *Tth* ENR–NAD⁺–triclosan structure are compared, the r.m.s.d. is 0.31 Å for 242 C α -atom pairs.

3.2. Cofactor and triclosan binding

In the structure of *Tth* ENR cocrystallized with NAD⁺ (Fig. 3*a*), density for the cofactor was only observed in monomers *B* and *D*. In monomer *B*, the density is well defined and the dinucleotide temperature factors refined to an average value of 27 Å² (Fig. 3*b*). However, the dinucleotide in monomer *D* had an average temperature factor of 71 Å² and may therefore be more disordered and/or have partial occupancy. Apart from their NAD⁺ content, the four crystallographically independent monomers were very similar, with only residues 39–67 showing differences between monomers without NAD⁺ and with NAD⁺: they move 'inwards' to make more contacts with the adenosine part of NAD⁺ (Fig. 3*c*). Fig. 3(*d*) details the interactions that NAD⁺ makes with the protein: Gln42, Asp66 and the N atom of Val67 form weak hydrogen bonds to the adenosine moiety (distances of between 2.9 and 3.1 Å). Residues of the loop between strand B1 and helix A1 (Thr17) and of helix A1 itself (Ser21 and Leu22), of the top of helix A5 and of the flexible region (Val193, Thr195 and Ala197) also provide polar contacts and van der Waals interactions with the nicotinamide diphosphate moiety. There are no significant differences in the structure bound to NADP⁺ (PDB entry 2yw9) and the cofactor is bound in exactly the same way (Fig. 3*e*). The nearby Arg45 would be well placed to interact with the extra phosphate of NADP⁺, but is disordered in the 2yw9 structure. It is not known whether *Tth* ENR prefers NADH or NADPH as cofactor.

The active site of the ENRs is a large pocket placed in the middle of the protein and covered by a flexible lid (amino acids 191–215 in *Tth* ENR). There are two cavities, which are capable of being filled by a nicotinamide cofactor and the aliphatic chain of a fatty acid. Triclosan and other diaromatic derivatives can effectively bind in the fatty-acid binding site only when NAD⁺ is also present, promoting inhibition of the enzyme. The formation of the ENR–NAD⁺–triclosan complex is highly effective and forces NAD⁺ to be retained in the active site. This became clear in previously described crystallographic

Figure 2
(*a*) Alignment of ENR sequences from *T. thermophilus*, *E. coli*, *H. pylori* and *M. tuberculosis*. Secondary structure as determined for the *T. thermophilus* structure is indicated (*a*, α -helix; *b*, β -strand). The sequence of the lid of the active site is indicated in bold. (*b*) Superposition of ENR from *T. thermophilus* (red; PDB entry 2wyu), *E. coli* (green; PDB entry 2fhs), *H. pylori* (yellow; PDB entry 2pd3) and *M. tuberculosis* (cyan; PDB entry 1eny). The region of the most pronounced structural variation is indicated by an ellipse. (*c*) Detailed view of the N-terminal interactions between dimers of ENR from *T. thermophilus* (cyan/magenta) and *E. coli* (red/orange).

Table 2
Salt bridges and side-chain hydrogen bonds in *T. thermophilus*, *E. coli* and *M. tuberculosis* ENR.

Homologous interactions are shown on the same row. An asterisk indicates an intermolecular interaction between monomers.

Salt bridges			Side-chain hydrogen bonds		
<i>T. thermophilus</i> ENR	<i>E. coli</i> ENR	<i>M. tuberculosis</i> ENR	<i>T. thermophilus</i> ENR	<i>E. coli</i> ENR	<i>M. tuberculosis</i> ENR
	Lys7/Asp235	Lys8/Asp248	Asp5/Ser7		
Lys10/Glu36				Arg9/Asn86	Arg9/Gln35 Thr17/Ser19
Lys29/Glu32 Lys29/Glu224		His24/Arg27 Arg27/Glu31			
	Arg30/Glu31			Arg30/Asn227 Glu34/Trp82	
Tyr41/Arg47			Tyr41/Glu44		
		Asp42/Arg43	Gln42/Asp66	Gln40/Asp64	
Arg47/Glu51					Gln48/Asp52
Arg64/Glu72		Arg53/Glu220*		Gln62/Ser70	
			Asp66/Thr68		Gln66/Lys118
Asp70/Arg111* Asp70/Arg131	Asp68/Arg110*		Thr68/Gln69		
Asp74/Arg131 Asp88/Arg137					Ser73/Arg77
			His92/Ser124 His92/Thr144	Asp86/Asn136 His90/Ser123 His90/Thr143	Asp89/Asn139 His93/Ser126
Arg99/Glu103					Thr101/Asp115
Arg105/Glu133* Arg105/Asp108	Asp103/Arg132*				
			Tyr106/Trp114 Tyr106/Ser170*	Tyr104/Asn169* Asn106/Arg132	
Arg111/Glu119* Arg111/Asp70*	Arg110/Asp118* Arg110/Asp68*			His117/Ser165	
Glu119/Arg111*	Asp118/Arg110*			Ser121/Ser165	
					Tyr127/Asn172 Tyr127/Ser186
Glu133/Arg105*	Arg132/Glu180 Arg132/Asp103*		Thr146/Glu168 Tyr148/Lys152*	Ser145/Glu167	Asp148/Glu169 Asp150/Ser152
			Ser150/Glu168 Glu151/Tyr247		
Glu151/Lys152* Lys152/Glu151*	Glu150/His246		Lys152/Tyr148* Ser170/Tyr106*	Asn175/Asn257* Asn169/Tyr104*	
				Arg183/Ser237	Asn172/Ser186* Arg177/Gln267* Glu178/Lys181*
Arg195/Glu219					Lys181/Glu178* Ser186/Asn172* Asn187/Thr253
Arg219/Glu225	Arg218/Asp224	Glu220/Arg53*	Arg210/Gln213		
			Thr222/Glu224 Thr222/Glu225 Glu225/Ser239* Ser239/Glu225*	Thr221/Glu223 Thr221/Asp224	
Glu244/His253* His253/Glu244*	Glu243/His246* His246/Glu243*			Asn257/Asn175*	Thr266/Gln267 Gln267/Arg177*

structures of binary (ENR–NAD⁺) or ternary (ENR–NAD⁺–triclosan) complexes of ENRs. For instance, the *A. phagocytophilum* ENR structure contained only one cofactor per dimer in the ENR–NAD⁺ crystallographic complex (PDB entry 3k31). The *E. coli* ENR–NAD⁺ complex (PDB entry 1dfi; Baldock *et al.*, 1996) contained four cofactors per tetramer, but two of these cofactors showed high average temperature factors compared with the surrounding protein, suggesting that they were bound much more loosely. However, in the structures of all of the ENR–NAD⁺–

triclosan complexes both the cofactor and the inhibitor occupy the active sites of all of the monomers and have similar average temperature factors to the surrounding amino acids. Therefore, as previously suggested by Kuo *et al.* (2003), the inhibition of ENRs by triclosan or its derivatives is uncompetitive and triclosan can only effectively bind to the active site in the presence of NAD⁺. These observations also suggest that ENR tetramers may show an allosteric effect in the reaction mechanism in which the conformation of one monomer may influence the conformations of its neighbours.

Figure 3

(a) Structure of the *T. thermophilus* ENR tetramer with NAD⁺ bound in chains B (cyan) and D (yellow). (b) Unbiased electron density for NAD⁺. A maximum-likelihood weighted $2F_o - F_c$ map calculated before including ligands in the model contoured at 2σ (black) is shown up to $1.6\text{ \AA}$ around the ligand (green). Chain B of the final model (grey), including the ligand, is superposed onto the map. (c) Comparison of *Thh* ENR with (light blue) and without (light green) NAD⁺. Residues 39–67 are highlighted in dark colours. (d) NAD⁺ contacts.

When we structurally compare the binary complex *Tth* ENR–NAD⁺ (subunit *B* of the NAD⁺-bound structure) with the NAD⁺-bound structure from *M. tuberculosis* (PDB entry 1eny; Dessen *et al.*, 1995), the r.m.s.d. is 0.96 Å for 187 paired C^α atoms. NAD⁺ is bound in a very similar way in both structures despite the active-site lid of *M. tuberculosis* ENR adopting a more distant position than in *Tth* ENR, preventing the stabilizing interactions between the lid and the cofactor that we observe (Fig. 3f). Another difference is that in *M. tuberculosis* ENR the loop around residue 200 is extended by nine residues, presumably to allow it to accept precursors of mycolic acids, the long-chained α -branched β -hydroxy fatty acids characteristic of mycobacterial cell walls.

The *Tth* ENR–NAD⁺ crystals soaked with triclosan show clear density for NAD⁺ and triclosan in all four monomers of the structure (Figs. 4a and 4b). The triclosan molecules make hydrophobic contacts

with Met160, Ala197, Ala198 and Ile201 of the protein; the monochloro-substituted aromatic ring of triclosan stacks onto the nicotinamide group of NAD⁺, while the hydroxyl O atom is at a hydrogen-bonding distance from the hydroxyl O atom of Tyr157 and the C2 hydroxyl of the nicotinamide ribose (Fig. 4c). There are no significant differences between the protein structures with and without triclosan. There are also no changes in the NAD⁺ conformation. The inhibitor occupies a site in the structure next to the cofactor previously occupied by solvent. The loop around residue 200 becomes more ordered, as shown by significantly lower temperature factors, which makes it much harder for the dinucleotide cofactor to leave, thus leading to tighter binding.

When we structurally compare the ternary complex *Tth* ENR–NAD⁺–triclosan (subunit *A* of the NAD⁺/triclosan complex) with the NAD⁺/triclosan complex structures of ENRs from *P. falciparum* (PDB entry 1uh5; Pidugu *et al.*, 2004), *E. coli* (PDB entry 1c14; Qiu *et al.*, 1999), *M. tuberculosis* (PDB entry 1p45; Kuo *et al.*, 2003) and *H. pylori* (PDB entry 2pd3; Lee *et al.*, 2007), the r.m.s.d.s are 0.52 Å (for 136 C^α-atom pairs of structurally aligned residues), 0.76 Å (for 231 C^α-atom pairs), 0.97 Å (for 185 C^α-atom pairs) and 0.60 Å (for 221 C^α-atom pairs), respectively. Triclosan binds to each of them in the same way (Fig. 4d).

3.3. Reasons for thermostability

Kumar *et al.* (2000) performed a statistical comparison of homologous high-resolution structures from thermophilic and mesophilic organisms. They found that thermostability is related to an increased number of salt bridges, an increased number of side-chain hydrogen bonds, a higher proportion of arginine and tyrosine residues and a lower proportion of cysteine and serine residues in the sequence, a higher proportion of residues in α -helical conformation and fewer prolines in α -helices. When compared with the *E. coli* and *M. tuberculosis* ENRs, there are indeed more arginines (18 *versus* 12 and 13) and tyrosines (nine *versus* seven and six) and fewer cysteines (zero *versus* three and one) and serines (ten *versus* 15 and 16) in the sequence. However, *Tth* ENR does not contain more α -helical residues than the *E. coli* and *M. tuberculosis* ENRs; also, there are not fewer prolines in these α -helices. As mentioned above, the presence of an extra N-terminal small β -strand (B0) in the structure, which forms a small two-stranded β -sheet with an equivalent partner from a neighbouring protein chain in the tetramer, is likely to stabilize the tetramer and may also contribute to thermostability. The *Tth* ENR structure contains many more salt bridges than the *E. coli* and *M. tuberculosis* structures (22 *versus* 13 and six salt bridges, respectively) and an almost equal number of side-chain hydrogen bonds interactions (20 *versus* 18 and 21 side-chain hydrogen bonds, respectively) (see Table 2). The thermostability of the *Tth* ENR may thus arise owing to the higher number of salt bridges that are present in its structure. In *Tth* ENR ten salt bridges are involved in tetramer formation and 12 salt bridges are involved in folding of the monomer, while in *E. coli* ENR eight salt bridges are involved in tetramer formation and five salt bridges stabilize the structure of the monomer.

4. Conclusion

The high-resolution structure of *T. thermophilus* enoyl-acyl carrier protein reductase has been described. The lid that covers the active site has been completely described in the apo protein for the first time, but no significant differences were found between the apo and the cofactor-coordinated dispositions of this loop. The structure of *Tth* ENR is quite similar to those of mesophilic strain ENRs. The

Figure 3 (continued)

(e) Comparison of NAD⁺ (blue) and NADP⁺ (yellow) in the active site. (f) Comparison of ENR from *T. thermophilus* (green) and *M. tuberculosis* (cyan) bound to NAD⁺.

Figure 4

(a) Structure of *T. thermophilus* ENR with NAD⁺ and triclosan. The monomers are coloured differently. (b) Unbiased electron density for NAD⁺ and triclosan. A maximum-likelihood weighted $2F_o - F_c$ map calculated before including ligands in the model contoured at 2σ (black) is shown up to 1.6 Å around the ligands (yellow). Chain A of the final model (grey), including the ligands, is superposed onto the map. (c) NAD⁺ and triclosan contacts. (d) Overlap of NAD⁺ and triclosan pairs from known ENR structures: *T. thermophilus* (C atoms in cyan), *E. coli* (C atoms in brown), *M. tuberculosis* (C atoms in yellow), *P. falciparum* (C atoms in salmon) and *H. pylori* (C atoms in grey).

main differences are related to the thermostability of the former: a higher number of arginine and tyrosine residues, a lower number of cysteine and serine residues, the presence of an extra intramolecular two-stranded β -sheet interaction and a higher number of salt bridges. Additionally, the high-resolution structures of NAD⁺ and NAD⁺/triclosan complexes with *Tth* ENR have been described. The binding mode of NAD⁺ and triclosan to the *Tth* ENR active site is similar to that in previously described ENRs. No binary complex between ENR and triclosan could be determined, which is consistent with an uncompetitive inhibition mode of triclosan with this protein. The

binding of triclosan to the *Tth* ENR–NAD⁺ complex appears to prevent the release of NAD⁺.

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