Structure of an Atypical Epoxide Hydrolase from Mycobacterium tuberculosis Gives Insights into its Function

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Epoxide hydrolases are vital to many organisms by virtue of their roles in detoxification, metabolism and processing of signaling molecules. The Mycobacterium tuberculosis genome encodes an unusually large number of epoxide hydrolases, suggesting that they might be of particular importance to these bacteria. We report here the first structure of an epoxide hydrolase from M. tuberculosis, solved to a resolution of 2.5 Å using single-wavelength anomalous dispersion (SAD) from a selenomethionine-substituted protein. The enzyme features a deep active-site pocket created by the packing of three helices onto a curved six-stranded β-sheet. This structure is similar to a previously described limonene-1,2-epoxide hydrolase from Rhodococcus erythropolis and unlike the α/β-hydrolase fold typical of mammalian epoxide hydrolases (EH). A number of changes in the mycobacterial enzyme create a wider and deeper substrate-binding pocket than is found in its Rhodococcus homologue. Interestingly, each structure contains a different type of endogenous ligand of unknown origin bound in its active site. As a consequence of its wider substrate-binding pocket, the mycobacterial EH is capable of hydrolyzing long or bulky lipophilic epoxides such as 10,11-epoxystearic acid and cholesterol 5,6-oxide at appreciable rates, suggesting that similar compound(s) will serve as its physiological substrate(s).

Introduction

Mycobacterium tuberculosis continues to claim more lives worldwide than any other pathogenic bacterium. Tuberculosis is the major cause of death in HIV-infected individuals. The recent resurgence of the disease has been accompanied by an increased frequency of strains resistant to two of the most important anti-tubercular agents, isoniazid and rifampicin. Thus, the need for efficient new drugs is urgent. The sequence of the M. tuberculosis genome has recently been reported,1 paving the way for a functional genomics approach in fighting the pathogen. The genome displays a number of peculiarities, one of them being that it codes for an unusually large number of potential epoxide hydrolases (EHs),2 with a total of nine separate genes (M. A. & A. C., unpublished results).

EHs are important to many organisms because of their ability to convert chemically reactive and thus harmful epoxides into less reactive diols. The enzymes often process the products of cytochrome P-450-dependent monoxygenases, which can produce epoxides from a broad range of lipophilic substrates. The M. tuberculosis genome includes sequences for at least 20 such monoxygenases, the largest number so far reported in a single bacterium.1,3 The unusual number of EHs in the bacterium, combined with the potential toxicity of EH substrates in general, suggests a vital function for this enzyme family in the physiology of the pathogen. Thus, blocking of EH function may

Abbreviations used: EH, epoxide hydrolase; LEH, limonene epoxide hydrolase from Rhodococcus erythropolis; Se-Met, selenomethionine; SAD, single-wavelength anomalous dispersion; RMS, root-mean-square; NCS, non-crystallographic symmetry.

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represent a promising new approach for antitubercular therapy. Elucidation of the structure of the M. tuberculosis enzymes would greatly assist the efforts to identify EH inhibitors that could be developed into drugs. To date, no such structures are available.

The majority of the mycobacterial EHs are related to eukaryotic α/β-hydrolase fold epoxide hydrolases. However, one gene, rv2740, annotated as coding for a conserved hypothetical protein in TubercuList†,4 is related to the radically different bacterial limonene 1,2-epoxide hydrolase, LEH.5 In Rhodococcus erythropolis, LEH is part of a metabolic system that allows growth of the bacterium on limonene as a sole source of carbon and energy.6 Such enzymes are completely absent from mammals, suggesting that Rv2740 may be a particularly good target for drug design.

We report here the first crystal structure of an M. tuberculosis epoxide hydrolase, namely Rv2740 (EC 3.3.2.8), and explore its relationship to LEH, as well as to sequences from other bacteria. This structure, together with kinetic studies, is a first step towards understanding the biological role of this enzyme in M. tuberculosis and the development of specific inhibitors.

†http://www.pasteur.fr/Bio/TubercuList/

Figure 1. Ligands and enzymatic mechanism of Rv2740 and LEH. Substrates of potential interest for Rv2740 activity are shown in (a) and the generic EH inhibitor, valpromide, in (b). The proposed catalytic mechanism of Rv2740 is shown in (c).

Results

Specificity

Purified Rv2740 enzyme expressed recombinantly in Escherichia coli showed moderate activity with three structurally different substrates: styrene 7,8-oxide, cis-9,10-epoxysearic acid and cholesterol 5α,6α-epoxide (Figure 1(a)); kinetic experiments are summarized in Table 1. The enzyme showed rather low affinity for the generic epoxide hydrolase substrate, styrene 7,8-oxide, and so only the catalytic efficiency, expressed as $k_{\text{cat}}/K_m$, could be determined in this case. The two other substrates displayed affinities of the order of 50 mM, with cis-9,10-epoxysearic acid being processed appreciably faster. Interestingly, when using excess enzyme, the reaction with a racemic mixture of this latter substrate always stopped at 50% turnover, indicating a strong preference for one of the two epoxysearic acid enantiomers.

In a first screening for inhibitors, which included a range of urea and amide derivatives, valpromide (Figure 1(b)) was found to have a reasonably efficient $K_i$ value of $\sim 100$ μM. This anti-epileptic drug has been shown to competitively inhibit a number of structurally different EHs.5,7
Overall structure

Statistics for the X-ray data and the final refined model of the selenomethionine (Se-Met) substituted form of Rv2740 are summarized in Table 2. The structure is characterized by a curved six-stranded β-sheet that packs against three antiparallel α-helices, creating a wide pocket lined primarily with hydrophobic side-chains. A fourth long helix completes the fold (Figure 2). The asymmetric unit contains three chemically equivalent molecules with very similar conformations; residues 16–140 of each chain can be superimposed with a root-mean-square (RMS) distance of less than 0.1 Å. Two of the molecules form a dimer with subunits related by a nearly perfect non-crystallographic (NCS) 2-fold axis. The third forms a dimer with a neighboring molecule, its 2-fold rotational axis coinciding with a crystallographic dyad.

The subunit interface of each dimer has a total buried surface area of 2000 Å², within the range expected for homodimers (1700 (± 250) Å²). Three major segments are involved in quaternary interactions between the two subunits. W105, Y123, W105’ and Y123’ form a hydrophobic surface; the prime indicates that a residue belongs to the adjacent polypeptide chain. Two sets of double bidentate salt-bridges, R79–E111′–E90–R121′, also stabilize the interaction. Although C107 and C107’ are close enough to form a disulfide bridge across the dimer interface, they are clearly reduced in the crystal structure, contributing instead to the hydrophobic interactions between the subunits.

Table 2. Data collection and refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>Native data</th>
<th>Se-Met data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Data collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beamline (wavelength, Å)</td>
<td>MaxLab 711 (1.134)</td>
<td>ESRF ID14-EH2 (0.934)</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>30.0–3.0 (3.05–3.0)</td>
<td>70.9–2.50 (2.64–2.50)</td>
</tr>
<tr>
<td>Unit cell dimensions (Å)</td>
<td>80.6, 80.6, 118.7</td>
<td>81.9, 81.9, 117.0</td>
</tr>
<tr>
<td>Number of observed/unique reflections</td>
<td>70,441/9338</td>
<td>348,194/16,247</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.8 (99.8)</td>
<td>100.0 (100.0)</td>
</tr>
<tr>
<td>Multiplicity/anomalous multiplicity</td>
<td>7.5 (7.5)/–</td>
<td>21.2 (21.5)/10.8 (11.4)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.042 (0.136)</td>
<td>0.095 (0.33)</td>
</tr>
<tr>
<td>D(h)/σ(h)</td>
<td>26.4 (7.7)</td>
<td>29.0 (8.8)</td>
</tr>
<tr>
<td><strong>B. Refinement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R cryst (%)</td>
<td>–</td>
<td>22.2</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>–</td>
<td>25.4</td>
</tr>
<tr>
<td>Number of atoms (Average B (Å²))</td>
<td>–</td>
<td>3285 (42.1)</td>
</tr>
<tr>
<td>Number of waters (Average B (Å²))</td>
<td>–</td>
<td>90 (35.9)</td>
</tr>
<tr>
<td>Bond RMSD from ideal values (Å)</td>
<td>–</td>
<td>0.023</td>
</tr>
<tr>
<td>Angle RMSD from ideal values (deg.)</td>
<td>–</td>
<td>1.96</td>
</tr>
<tr>
<td>Ramachandran plot outliers (%)</td>
<td>–</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest resolution shell.

* Rfree, calculated from a randomly chosen 5% of unique reflections.
* Ideal values from Engh & Huber.36
* Defined according to Kleywegt & Jones.37

Table 1. Kinetic characteristics of Rv2740 with different substrates

<table>
<thead>
<tr>
<th>Substrate/enzyme</th>
<th>V max (nmol mg⁻¹ min⁻¹)</th>
<th>K m (μM)</th>
<th>k cat/K m (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene 7,8-oxide (LEH)</td>
<td>n.d. (1700±250)</td>
<td>&gt;5000 (1400±280)</td>
<td>3 ± 0.6 (340±30)</td>
</tr>
<tr>
<td>Cholesterol 5,6-oxide</td>
<td>1.1 ± 0.4</td>
<td>60 ± 30</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>9,10-Epoxystearic acid</td>
<td>30 ± 10</td>
<td>45 ± 15</td>
<td>210 ± 65</td>
</tr>
</tbody>
</table>

V max for styrene 7,8-oxide is recorded as not determined (n.d.); saturation was not obtained at substrate concentrations as high as 5 mM, and so only k cat/K m could be estimated with confidence. Values measured for LEH with the same substrate are shown in parentheses for comparison. No turnover could be detected for LEH with cholesterol-5,6-oxide and 9,10-epoxystearic acid as substrates.
Comparison with known structures

A DALI search of the Protein Data Bank (PDB), using the Rv2740 A subunit as a probe, identified several structural homologues (Table 3). Rather surprisingly, the most similar structure is that of Bal32a, a thermostable protein of unknown function from an unidentified bacterium. Strong similarity to Rv2740 is observed in both the subunit and dimer. However, Bal32a has a long loop that covers the entrance of the active-site pocket, turning it into a closed cavity. Robinson et al. speculate that the interior of the protein may be accessible to substrates via movements of a flexible cap. As for Rv2740, a pair of cysteine residues is observed in the reduced state at the subunit–subunit interface in the crystal structure. Oxidation results in an increase in the melting temperature of Bal32a from 65°C to 80°C, strongly suggesting a role for the disulfide in stabilization of its dimer.

As expected, Rv2740 was found to be similar in structure to LEH. Similarities were also noted to Δ5-3-ketosteroid isomerase, scytalone dehydratase, and a carotenoid-binding protein of unknown function. The Δ5-3-ketosteroid isomerase forms a dimer with a similar construction to that of Rv2740, LEH and Bal32a. The scytalone dehydratase forms a trimer along similar principles, while the corresponding region of the carotenoid-binding protein represents a single domain of a larger protein.

Active site

The three residues mainly responsible for catalysis in LEH have been identified through site-directed mutagenesis. In the mycobacterial epoxide hydrolase, these side-chains correspond to R91, D93 and D122, which are situated at the bottom of the active-site pocket (Figure 3(a)). Two other residues, Y46 and N48 (in Rv2740 numbering), have been suggested to be associated with the active water molecule of the hydrolase reaction. The active sites of LEH and the mycobacterial enzyme have almost identical hydrogen-bonding networks, the only exception being N55 in LEH, which is stabilized by a single hydrogen bond. The corresponding side-chain of Rv2740, N48, is coordinated both by Y46 and S52. In Bal32a, the equivalents of R91, D93 and D122 are changed to H105, N107 and E136, respectively, suggesting that its function is different from that of Rv2740. Furthermore, the counterparts of Y46 and N48 are completely absent.

Although the catalytic residues of LEH and Rv2740 are very similar, the shape of the pocket that contains them is quite different (Figure 3(b)). The deeper substrate-binding site of the mycobacterial structure is in part explained by the differing orientations of L35 and L28, as well as I80 and F73, in the LEH and Rv2740 structures, respectively. Substitutions such as M78 to V71 and L74 to M67, combined with the different packing of the third and fourth helices, make room in the mycobacterial enzyme for substrates bulkier than limonene-1,2-epoxide. The side-chains of L95, M129, I97, L140, V138, L137, L134 and F130 form a large solvent-exposed hydrophobic patch at the upper part of the pocket; this feature seems to be well conserved throughout Mycobacteriaceae.

As in LEH, an endogenous ligand of unknown origin was found in the active-site cavity of each of the three molecules in the asymmetric unit, bound close to the catalytic residues (Figure 3(c) and (d)). The electron density is essentially identical in averaged and unaveraged maps at 2.5 Å resolution, suggesting a single compound with a single well-defined binding mode. The character of this ligand is markedly different from the heptanamide modeled in the LEH case, being more consistent with a cyclohexane ring with a single attached aliphatic chain. The shape of the density does not resemble that of any chemical used in either purification or crystallization.

Related sequences

In addition to the group of sequences previously identified as potential LEHs, at least four more sequences from actinobacteria and proteobacteria have a highly conserved catalytic center (Figure 4). A few differences from the originally defined LEH family are evident. For example, Y46 in the mycobacterial enzyme is replaced by a tryptophan in both Ralstonia solanacearum (GenBank accession number 17547309; partial sequence) and Erwinia caratovar (50120522). The latter protein also lacks a hydrogen bond between N48 and S52 (Rv2740 numbering), leaving the residue corresponding to N48 solely responsible for positioning the putative catalytic water molecule.

Four additional sequences are more distantly related, but likely to have similar structure. The

Table 3. Comparisons with DALI

<table>
<thead>
<tr>
<th>PDB entry</th>
<th>Z</th>
<th>RMS difference</th>
<th>Residues aligned</th>
<th>Length of sequence</th>
<th>Identical (%)</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1TUH</td>
<td>15.8</td>
<td>2.7</td>
<td>122</td>
<td>131</td>
<td>19</td>
<td>Bal32a</td>
</tr>
<tr>
<td>1OPY</td>
<td>14.9</td>
<td>2.1</td>
<td>115</td>
<td>123</td>
<td>16</td>
<td>Δ5-3-Ketosteroid isomerase</td>
</tr>
<tr>
<td>1NLE3</td>
<td>13.9</td>
<td>2.2</td>
<td>117</td>
<td>141</td>
<td>28</td>
<td>LEH</td>
</tr>
<tr>
<td>3STD</td>
<td>13.3</td>
<td>2.4</td>
<td>121</td>
<td>162</td>
<td>5</td>
<td>Scytalone dehydratase</td>
</tr>
<tr>
<td>1M98</td>
<td>12.0</td>
<td>2.2</td>
<td>106</td>
<td>316</td>
<td>11</td>
<td>Unknown function, orange carotenoid protein</td>
</tr>
</tbody>
</table>

Only hits with Z-scores greater than 12 are listed.
protein from *Burkholderia cepacia* (46319940), as well as two from the *Mycobacterium avium* subspecies *para-
tuberculosis* (41407700 and 41409656), is missing one of
the catalytic aspartate residues (D93). In the *M. tuber-
culosus* Rv0141c (15607283), as well as both of the
*M. avium* sequences, R91 is substituted by histidine or
asparagine. All four have replaced N48 with various
other hydrophilic residues, suggesting that their
function may be different from that of Rv2740.

**Discussion**

Studies of LEH suggest that it uses a single-step,
push–pull mechanism that is distinct from the
two-step mechanism used by the α/β-hydrolase
class of EHs. The single-step mechanism features
an Asp-Arg-Asp triad, i.e. residues R91, D93 and
D122 in Rv2740 (Figure 1(c)). We suggest that D122
activates a water molecule for its attack on the
epoxide ring, while the ring is simultaneously
encouraged to open by a proton donated by D93.
The arginine side-chain interacts with and orients
the carboxylate groups of the catalytic aspartate
residues, while Y46 and N48 help position the
catalytic water.

We show here that Rv2740 can hydrolyze three
structurally diverse epoxides, albeit with moderate
catalytic efficiency (Table 1). If Rv2740, like LEH,
metabolizes carbon compounds obtained from the
surroundings, it would be expected to have a higher
activity on one or more preferred substrates. This is
in contrast to the broad specificity and slower
reaction rates associated with a function in detox-
ification (a major function of mammalian α/β EHs).
Obviously, the physiological substrates for Rv2740
have yet to be identified, and the observed
efficiency on these non-optimal substrates is under-
standably lower. The hydrophobic character of the
substrate binding cavity and the low $K_m$ value with
epoxystearic acid and cholesterol 5,6-epoxide
suggest that the most relevant substrates may be
fatty acid or steroid derivatives. However, the
metabolism of such compounds in *M. tuberculosis*
remains largely unexplored. In this context, the
observed electron density for an endogenous ligand
Figure 4. Structure-based sequence alignment of Rv2740 and LEH, together with alignment of other related sequences. The last five residues of the first *M. avium* sequence are omitted for simplicity. Asterisks denote residues lining the active-site cavity.
in the active site of Rv2740 is extremely interesting. Although the compound probably originated from the E. coli expression host, it provides leads for the task of identifying the true substrates for Rv2740. This ligand could only be partially displaced by valpromide (K_1 ~ 100 μM), even at the highest soluble concentration, 10 mM; its apparent persistence throughout purification also indicates tight binding. The shape of the electron density is suggestive of a cyclohexane ring with an attached aliphatic chain (Figure 3(c) and (d)). Limonene itself, one of the physiological substrates of LEH, has some similarities in shape (Figure 1), but the wider site of Rv2740 (Figure 3(b)) would allow a larger substrate. The relevant portion of the carotenoid in the related structure described above (Table 3) is placed in a very similar location and orientation within that binding-site pocket. However, residues lining the corresponding site in Rv2740 are not conserved; indeed, sequence identity between the two proteins is very low, and the similarities could only be uncovered based on comparisons of the three-dimensional structures.

The ability of Rv2740 to hydrolyze cholesterol 5,6-epoxide is intriguing for reasons of broader interest. On the basis of an analysis of LEH, we have previously speculated that it could be a homologue of the human cholesterol epoxide hydrolase, a key enzyme in the metabolism of cholesterol oxidation products which has, so far, evaded attempts at purification and cloning. Although LEH itself did not show any turnover with cholesterol epoxide, the present study shows that the wider active site of Rv2740 does allow such a reaction to occur.

Another interesting finding is the high enantioselectivity of Rv2740 with a fatty acid epoxide substrate. Although the enantioselectivity of this class of enzymes has been shown for the rigid substrate limonene 1,2-epoxide, the phenomenon is rather unexpected with 9,10-epoxystearic acid, which is rather unexpected with 9,10-epoxystearic acid, (Figure 1). In recent years, there has been a strong interest in epoxide hydrolases for the production of enantio-pure epoxides and diols, mainly as building blocks in the fine chemical and drug industries. Indeed, mining for such novel enzymes was a goal in the studies with Bal32a referred to here as well as similar ones with α/β-type epoxide hydrolases. Given its unusual properties, Rv2740 will provide a useful addition to the toolbox of enzymes available for biocatalytic production of fine chemicals.

**Experimental Procedures**

**Radiolabeled compounds**

[26-14C]Cholesterol 5,6-oxide (53 mCi/mmol), racemic [1-14C]9,10-epoxystearic acid (56 mCi/mmol) and racemic [14C]styrene 7,8-oxide (15 mCi/mmol) were synthesized as described. The sequence corresponding to the open reading frame of the Rv2740 gene was amplified by PCR from total DNA of M. tuberculosis strain H37Rv, using the high-fidelity polymerase Pfu Turbo (Stratagene) with the primers ATGGCTCATGATCATGATCTGTGGGGAGGCTGACC GAAAACATC (forward) and CTACAGCCGTGCTTCA CAGCGTAG (reverse). The forward primer included the sequence for a histidine-tag immediately upstream of the gene. After addition of a 3’ A overhang by incubation with Taq polymerase (Roche), the DNA fragment was ligated into the pCR T7 TOPO vector (Invitrogen), and used to transform E. coli TOP10 cells (Invitrogen); the construct was verified by DNA sequence analysis. Expression was performed in E. coli Rosetta (DE3) cells with a pLacI plasmid (Novagen) or in E. coli BL21-AI cells (Invitrogen). The cells were cultured in LB medium at 37 °C. At A_{600} = 0.5–1.0, the temperature was lowered to 24 °C and expression of the target gene induced with IPTG (100 mg/l) for three hours. After harvesting by centrifugation, the cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100 (pH 8.0)) with 1 mg/ml of lysozyme, 1 mM PMSE, 0.01 mg/ml of RNase A and 0.02 mg/ml of DNase I, and lysed using a One Shot cell disruptor (Constant Systems Ltd.). The soluble fraction was incubated with 1 ml of pre-equilibrated Ni-NTA agarose (Qiagen) slurry for 4 °C for one hour, then poured into a column. After washing with 20 column volumes of buffer (20 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, 10% (v/v) glycerol (pH 8.0)), the protein was eluted with four column volumes of 250 mM imidazole in the same buffer. Fractions containing the protein were pooled and further purified on a HiLoad 16/60 Superdex 75 prep grade column (Amersham Biosciences) equilibrated in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol. The protein eluted in two peaks, apparently a monomer and a dimer. The two samples were concentrated separately to 4–5 mg/ml using Vivaspin 6 concentrators (10,000 MWCO).

For expression of the Se-Met labeled protein, it was necessary to efficiently suppress the background level of expression. Therefore, the gene was transferred to the pET101D vector (Invitrogen) and the expression performed in BL21-AI cells. Se-Met was introduced into the protein by metabolic inhibition. Fermentation was performed in minimal medium supplemented with Se-Met, lysine, threonine, phenylalanine, leucine, isoleucine and valine according to published procedures. Expression was performed at room temperature overnight. The purification was carried out as described for the native protein, except that 10 mM β-mercaptoethanol was included in all buffers to prevent Se-Met oxidation.

**Enzyme assays**

The enzymatic activity of native Rv2740 with racemic [14C]styrene 7,8-oxide as the substrate was determined essentially as described. For the assay with [1-14C]-cis-9,10-epoxystearic acid, 10–15 μg of pure enzyme was incubated with the substrate in 100 mM sodium phosphate, (pH 7.4), 50 mM NaCl in a total volume of 50 μl and incubated for 15 minutes at 37 °C. The substrate was added from a stock solution in acetonitrile, resulting in a final solvent concentration in the assay mixture of 2%; the organic solvent has no effect on the enzyme activity. The reaction was terminated and 200 μl of ethylacetate was
added. After vigorous shaking and centrifugation, 50 μl of the organic (upper) phase was applied to thin-layer chromatography on silica gel 60 F254 sheets and developed using n-hexane/diethyl ether/formic acid (70:30:2, by volume) as the eluent. Product formation was quantified with an InstantImager (Packard Instruments). Activity towards [26-14C]-cholesterol 5,6-epoxide (70:30:2, by volume) as the eluent. Product formation was identical with those used with epoxyxystearic acid. The enzymatic reaction was terminated by addition of 30 μl of tetrahydrofuran, and 40 μl of the resulting mixture was applied directly to thin-layer chromatography on silica gel 60 F254 sheets and developed with tetrahydrofuran/dichloromethane (1:1, by volume) as the eluent. Product formation was quantified as described above.

**Crystallization**

A search for crystallization conditions for the native protein was conducted at both room temperature and 4 °C using the 67 conditions of the Core Screen.21 The best crystals were grown at room temperature as follows: 10 mg/ml protein solution in 10 mM Hepes buffer (pH 7.0), was mixed 1:1 with 26%(w/v) polyethylene glycol 6000, 0.15 M CaCl2 then equilibrated by vapor diffusion. Crystals achieved maximum dimensions of 0.1 mm × 0.05 mm in two to three weeks. Se-Met-substituted crystals with dimensions in the range of 0.04–0.07 mm could be grown under similar conditions.

**X-ray data collection and processing**

For data collection purposes, native and Se-Met crystals of Rv2740 were flash-cooled in liquid nitrogen after addition of 5% polyethylene glycol 400 to the mother liquor. X-ray data were collected at beamline I711, MAX Laboratory, Lund, Sweden and at beamlines ID14-EH1 and EH2 ESRF, Grenoble, France. Native and Se-Met crystals diffracted to resolutions of approximately 3.0 Å and 2.5 Å, respectively (Table 2). Indexing, integration and scaling of the data were carried out using the HKL suite.22 Both crystal types belonged to space group P321. Assuming three molecules in the asymmetric unit gives a Vm value of 2.3 Å3 Da−1, which corresponds to a solvent content of 45%.23

Numerous attempts to solve the structure using heavy-atom replacements with the native structure failed. Subsequent work therefore focused on the Se-Met crystals. The six selenium positions were determined by Patterson methods, using the program RSPS.24 Initial SAD phases to a resolution of 2.5 Å were determined with SHARP.25 The six selenium positions were determined by Patterson methods, using the program RSPS.24 Initial SAD phases to a resolution of 2.5 Å were determined with SHARP.25 The best crystal types were grown at room temperature as follows: 10 mg/ml protein solution in 10 mM Hepes buffer (pH 7.0), was mixed 1:1 with 26%(w/v) polyethylene glycol 6000, 0.15 M CaCl2 then equilibrated by vapor diffusion. Crystals achieved maximum dimensions of 0.1 mm × 0.05 mm in two to three weeks. Se-Met-substituted crystals with dimensions in the range of 0.04–0.07 mm could be grown under similar conditions.

In an attempt to obtain an enzyme–inhibitor complex, native crystals were soaked with 10 mM valpromide dissolved in 0.1 M 2-(N-morpholinio)ethanesulfonic acid buffer for one minute prior to flash-cooling. Diffraction data to a resolution of 2.5 Å were collected at beamline ID14-EH2 ESRF, Grenoble, France. However, the electron density indicated that valpromide was only able to partially displace the endogenous ligand.

**Other methods**

Similar sequences were identified by searching UNIPROT iteratively using WU-BLAST24 together with the least similar positive hit of the prior search. Structural alignments were performed using the Lsq tools of O, and structure-based sequence alignments using the INDONE-SIA package (D. Madsen, P. J. & G. J. Kleywegt, unpublished computer program)†. Figures were made in O and rendered in Molray,‡ or produced using ChemDraw (CambridgeSoft Corp.). Protein–protein interactions were assessed with the PPI server.†

**Protein Data Bank accession code**

Coordinates and structure factors for the Se-Met protein have been deposited at the RCSB PDB with entry code 2BNG.

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