The structure of *Saccharomyces cerevisiae* Met8p, a bifunctional dehydrogenase and ferrochelatase

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Sirohaem is a tetrapyrrole-derived prosthetic group that is required for the essential assimilation of sulfur and nitrogen into all living systems as part of the sulfite and nitrite reductase systems. The final two steps in the biosynthesis of sirohaem involve a β -NAD⁺-dependent dehydrogenation of precorrin-2 to generate sirohydrochlorin followed by ferrochelation to yield sirohaem. In *Saccharomyces cerevisiae*, Met8p is a bifunctional enzyme that carries out both of these reactions. Here, we report the 2.2 Å resolution crystal structure of Met8p, which adopts a novel fold that bears no resemblance to the previously determined structures of cobalt- or ferro-chelatases. Analysis of mutant proteins suggests that both catalytic activities share a single active site, and that Asp141 plays an essential role in both dehydrogenase and chelatase processes.

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Introduction

The tetrapyrrole-derived cofactor sirohaem is a vital component of sulfite and nitrite reductases that perform the six-electron reduction of sulfite to sulfide or nitrite to ammonia. Although this process occurs only in plants and microorganisms, it is essential for the incorporation of sulfur and nitrogen into all forms of life (Cole and Ferguson, 1988; Wray and Kinghorn, 1989). In structural terms, sirohaem is the simplest of the modified tetrapyrroles, a family of metalloprosthetic groups and coenzymes that includes haem, chlorophyll, coenzyme F₄₃₀ and cobalamin (vitamin B₁₂).

All modified tetrapyrroles are synthesized via a branched biosynthetic pathway, in which the last common intermediate is a macrocyclic structure called uroporphyrinogen III (Scott, 1993; Jordan, 1994). Sirohaem is synthesized from this mutual precursor in three steps. Step 1 involves the donation of two *S*-adenosyl-L-methionine (SAM)-derived methyl groups to carbons 2 and 7 of the tetrapyrrole to produce the intermediate precorrin-2. Step 2

is an NAD-dependent dehydrogenation that yields sirohydrochlorin, and step 3 is the chelation of ferrous iron to give sirohaem. In *Saccharomyces cerevisiae* the last two steps are carried out by a single bifunctional enzyme called Met8p (Figure 1) (Hansen *et al.*, 1997; Raux *et al.*, 1999). In some bacteria, steps 1–3 are catalysed by a single multifunctional protein, called CysG, which appears to have arisen from a gene fusion between a uroporphyrinogen III methyltransferase and a Met8p-type protein (Warren *et al.*, 1990, 1994; Spencer *et al.*, 1993). Indeed, studies on CysG from *Escherichia coli* have determined that the dehydrogenase and ferrochelatase activities lie in the N-terminal half of the protein and the SAM-dependent methyltransferase activity resides in the C-terminal half. Although the family of Met8p and CysG homologues share only 3% identity (~20% similarity), both proteins contain a typical nucleotide-binding motif, GxGxxG/A, consistent with the NAD-dependent dehydrogenase activity (Figure 2). In contrast, *Bacillus megaterium* produces separate enzymes for all three sirohaem biosynthetic reactions, termed SirA (methyltransferase), SirB (chelatase) and SirC (dehydrogenase). SirC is the only one of these three enzymes to share sequence similarity with Met8p, although it functions solely as a dehydrogenase, lacking any chelatase activity (M.J. Warren, unpublished data).

The chelatases associated with the biosynthesis of tetrapyrrole-derived cofactors fall into two classes. Class 1 chelatases exist as a large multimeric complex that requires ATP hydrolysis for metal ion insertion; examples include the enzymes for chlorophyll/bacteriochlorophyll (ChlH, I, D) (Walker and Willows, 1997) and aerobic cobalamin biosynthesis (CobN, S, T). Class 2 chelatases exist as homomeric species that do not require ATP and are exemplified by the enzymes for haem (HemH) and anaerobic cobalamin (CbiK) biosynthesis (Raux *et al.*, 1997). By this definition Met8p is a class 2 chelatase, although there is no sequence similarity between Met8p and any other known chelatase.

In an effort to understand how the dehydrogenase and chelatase activities of Met8p are accommodated in one protein, we have determined the X-ray crystal structure of Met8p to 2.2 Å resolution. The protein forms an unusual, tightly intertwined homodimer that is comprised of three structural domains per monomer. The N-terminal domain, which binds the cofactor NAD, is followed by a central dimer-interface domain and a helical C-terminal domain. Most of the residues conserved between Met8p and CysG cluster in the cleft formed between the NAD-binding domain and the dimer interface domain. We propose that this cleft contains both the dehydrogenase and ferrochelatase active sites and we have performed mutagenesis experiments which suggest that the invariant residue Asp141 is required for both functionalities.

Results and discussion

Overall structure of Met8p

The structure of Met8p was determined using multi-wavelength anomalous diffraction (MAD) data collected from a single crystal of selenomethionine (SeMet)-substituted protein. The structure was refined to 2.2 Å resolution to a final *R*-factor of 22.1% (*R*_{free} = 28.7%) with

Fig. 1. Met8p catalyses the last two steps in sirohaem synthesis. The NAD-dependent dehydrogenation of precorrin-2 to produce sirohydrochlorin is followed by the ferrochelation of sirohydrochlorin to produce sirohaem. The proposed role of Asp141 as a general base in the dehydrogenase mechanism is shown in the first half of the figure. Side chain designation: A, acetate; P, propionate.

reasonable geometry (Table I). The asymmetric unit of the crystal contains one and a half dimers, where molecule A dimerizes with molecule B, and molecule C dimerizes with a symmetry related molecule across a crystallographic 2-fold axis. The coordinates are available from the Protein Data Bank (PDB) under the accession code 1KYQ.

Met8p is a homodimer comprised of two NAD-binding domains, an intertwined central domain and two helical C-terminal domains. The entire shape resembles a large 'X', with one polypeptide crossing the other (Figure 3). The N-terminal NAD-binding domain contains many of the familiar aspects of all nucleotide-binding domains, including the GxGxxG/A sequence, which forms the C-terminal end of the domain's initial β -strand, β 2. An initial $\beta\alpha\beta$ unit, β 2- α 1- β 3, is mirrored in pseudo 2-fold symmetry by a second, β 6- α 5- β 7, to comprise the core of the NAD binding site (Figure 3). A comparison of the N-terminal domain of Met8p with structures in the protein database indicates similarity to members of the tyrosine-dependent oxidoreductase family of NAD-binding domains, although Met8p contains an additional anti-parallel strand (β 4). There is no structural similarity between any region of Met8p and the known structures of the anaerobic cobalt chelatase, CbiK, or protoporphyrin IX

Fig. 2. Sequence alignment of Met8p with the bifunctional dehydrogenase and chelatase domains of CysG. This figure shows the Met8p sequences from *S. cerevisiae* (S20155, with four deviations in sequence identified during cloning as described in the Materials and methods section), *Saccharomyces pombe* (T38797) and CysG from *E. coli* (P11098). Alignment and definition of sequence conservation was performed using Clustal_W (Higgins and Sharp, 1989), and also considered sequences of CysG from *Vibrio anguillarum* (JC4347), *Pseudomonas aeruginosa* (AAG05999), *Salmonella typhimurium* (B39200), *Buchnera* sp. APS (BAB13123) and *Rhizobium leguminosarum* bv. *viciae* (AAF87214). Invariant residues are coloured orange, conserved residues yellow. The secondary structure elements of the *S. cerevisiae* Met8p structure are shown above the sequence (α -helices pink; β -strands purple). The predicted secondary structure of the beginning of the methylase domain of CysG is shown in white, based on sequence homology to the known structure of precorrin-4 methylase, CbiF (Schubert *et al.*, 1998). A solidus (/) before or after a sequence indicates additional sequence N- or C-terminal to what is shown.

Table I. Crystallographic statistics

Data collection statistics	High resolution	Se-peak	Se-remote	Se-inflection point	Mn ²⁺
Wavelength (Å)	0.97	0.97969	0.95372	0.97990	0.97
Resolution (Å) ^a	20–2.2 (2.28–2.2)	20–2.7 (2.8–2.7)	20–2.7 (2.8–2.7)	20–2.7 (2.8–2.7)	20–3.3 (3.4–3.3)
Observed reflections	117 894	58 227	57 961	58 549	57 585
Unique reflections	56 148	29 993	29 706	30 123	16 103
% Completeness ^a	98.0 (85.6)	98.0 (87.8)	98.3 (85.4)	98.3 (85.7)	98.3 (85.4)
<i>R</i> _{merge} ^{a,b}	7.0 (30.4)	8.4 (24.9)	6.8 (33.9)	6.9 (39.4)	7.5 (21.2)
<i>I</i> / <i>σI</i> ^a	15.5 (3)	23 (5)	18 (2.7)	19.6 (3)	15 (5.6)
Mosaicity (°)	0.730	0.793	0.756	0.742	1.3
<i>R</i> _{factor} ^c	0.221				
<i>R</i> _{free} ^d	0.287				
R.m.s.d. (bond lengths) (Å)	0.007				
R.m.s.d. (bond angles) (°)	2.1				

^aNumbers in parentheses are for the high-resolution bin.
^b $R_{\text{merge}} = \sum I - \langle I \rangle / \sum I$, where *I* is the intensity of an individual measurement and $\langle I \rangle$ is the average intensity from multiple observations.
^c $R_{\text{factor}} = \sum ||F_{\text{obs}}| - k|F_{\text{calc}}|| / \sum |F_{\text{obs}}|$
^d*R*_{free} equals the *R*-factor against 5% of the data removed prior to refinement.

Fig. 3. Structure of Met8p. **(A)** The intertwined homodimer is shown with molecule A coloured according to secondary structure: helices pink; strands and loops purple, molecule B coloured white and the secondary structure elements labelled (Kraulis, 1991). The NAD is shown in green in ball-and-stick representation. The catalytic residue, Asp141, and the Mn²⁺-binding residue, His237, are shown in yellow ball-and-stick representation. **(B)** Secondary structure topology diagram coloured as in (A), dashed lines indicate a disordered loop in molecule A, residues 59–71.

ferrochelatase (HemH) (Al-Karadaghi *et al.*, 1997; Schubert *et al.*, 1999; Wu *et al.*, 2001).
The central domain of the dimer is formed by close association of both polypeptides (Figure 3). This domain

consists of a three layer structure, in which the top two layers are each comprised of a four-stranded antiparallel β -sheet, and the bottom layer is comprised of two α -helices. The two inner strands (β 1) of the top β -sheet

Fig. 4. NAD-binding site and active site cleft. (A) The adenosine portion of NAD is shown in green bonds covered by 1σ solvent-flattened MAD-phased experimental electron density. Hydrogen bonds are shown in black. The nicotinamide ring, which is disordered in the crystal structure, has been positioned from comparison with other structures (e.g. 2HDH), and is shown in white. (B) Stereo diagram of the active site cleft. NAD and nearby residues are labelled. Asp141 may play a crucial role in the mechanism of both the dehydrogenase and chelatase activities.

are formed by residues near the N-terminus of each of the monomers. Each polypeptide then forms an entire NAD-binding domain before returning to the central domain to complete the top two outer strands ($\beta 8$) of the top β -sheet, and the rest of the central domain. Although numerous intermolecular contacts in the homodimer arise from the intertwined polypeptides of the central domain, the two N-terminal NAD-binding domains also contribute significantly to the large dimer interface ($3481 \text{ \AA}^2$) (Hubbard *et al.*, 1991). The C-terminal domain consists of five α -helices and one large extended loop between $\alpha 11$ and $\alpha 12$. Individual comparison of the interface and C-terminal domains with the protein database did not identify any structures with significant structural similarity (Holm and Sander, 1993).

The three domain structure of Met8p appears to allow some conformational flexibility between domains through hinging motions around residues 148 and 192, which are located immediately before and after the central domain. Although overlap of any individual domain against other molecules in the asymmetric unit (using main-chain atoms) typically gives an average root mean square deviation (r.m.s.d.) of $0.5 \text{ \AA}$, alignment of the NAD-binding domains of any two molecules results in a relative $\sim 9^\circ$ rotation and a maximum displacement of $\sim 5.4 \text{ \AA}$ between their C-terminal domains. Domain motions may

be necessary for substrate binding, positioning catalytic residues or sequestering the active site from solvent.

The NAD-dependent dehydrogenase active site

Crystals of Met8p were grown in the presence of $5 \text{ mM } \beta\text{-NAD}^+$, which is seen bound to the enzyme in the crystal structure. The adenosine half of the NAD binds above the $\beta 2$ strand, where it forms many van der Waals and hydrogen bonding interactions with the protein (Figure 4A). The electron density for the adenine nucleotide, sugar and both phosphates of the NAD is strong, but the catalytic β -nicotinamide moiety and corresponding sugar lack defined electron density. The conformation of the adenosine portion of the ligand is almost identical to several other NAD-bound structures (including L-3-hydroxyacyl CoA dehydrogenase, PDB code 2HDH; and UDP-galactose 4-epimerase, PDB code 1XEL), in which the β -nicotinamide moiety is positioned such that a hydrogen bond forms between the nicotinamide amide group and an NAD phosphate oxygen (Figure 4, white model). If this conformation is also adopted by Met8p-bound NAD in the presence of substrate, then the reactive carbon of the β -nicotinamide would point towards a cleft between the NAD-binding and interface domains (Figure 4B) that we propose contains the active site. This proposal is supported by the proximity to NAD, the distribution of

Fig. 5. Surface distribution of conserved residues and electrostatic potential. (A) Conserved residues are coloured according to the scheme in Figure 2, with invariant residues in orange. The proposed active site is indicated by a dashed green line. Many conserved residues are involved in the dimer interface, both in the NAD-binding and central domains. (B) Positive electrostatic potential is displayed in blue (+13.288 kT) and negative potential in red (−6.308 kT) (Nicholls *et al.*, 1991). The NAD is shown as a stick representation, with the modelled nicotinamide ring shown coloured white. The eight carboxylate groups of the substrate and product would complement the positive charge of this pocket, although the arginine and lysine side chains that stem from the C-terminal domain are unlikely to make specific interactions, since they are non-conserved.

conserved residues that cluster around this cleft (Figures 2 and 5A), and the positive electrostatic potential of the cleft, which would complement the negatively charged substrate (Figure 5B). Remarkably, the absence of clusters of conserved residues or positive potential in other regions of Met8p suggests that the bifunctional Met8p enzyme may contain a single active site, a proposal that is supported by site-directed mutagenesis (below). There are two independent active sites per dimer, which are separated from each other by ~50 Å across the protein surface (Figure 3A). Each active site cleft is bordered by the NAD-binding and central domains of one monomer, and the C-terminal domain of the other monomer.

The active site cleft houses conserved residues that appear suited to perform catalytic or structural roles. The invariant residue Asp141, which is located on the edge of the cleft and adjacent to the proposed location of the

Table II. Dehydrogenase and chelataze activities of Met8p

Met8p variant	Dehydrogenase specific activity (nmol/min/mg)	Chelataze specific activity (nmol/min/mg)	Complementation ^a
Wild type	11.2 ± 0.8	31.1 ± 2.5	++
Gly22Asp	0	32.5 ± 2.5	+
Asp141Ala	0	0	—
His237Ala	13.3 ± 1.1	35.4 ± 3.2	+++

^aComplementation of *E.coli* strain 302Δa in M9 media after 24 h. All strains grew well in the presence of exogenous cysteine.

β-nicotinamide ring, appears poised to participate in catalysis. Additional invariant residues, Asn138, Gly168, Pro171 and Arg178, lie in the interface domain, are solvent accessible to the cleft, and could potentially interact with the bound substrate or provide structural support. A number of additional semi-conserved hydrophobic (Ala140, Tyr149 and Phe150) and charged residues (Lys31 and Arg28) surround the cleft (Figure 4B). This distribution of residues, hydrophobics on one side and charged residues on the other, is reminiscent of other known chelataze structures (Al-Karadaghi *et al.*, 1997; Schubert *et al.*, 1999; Lecerof *et al.*, 2000; Wu *et al.*, 2001), including that of a simple catalytic antibody that catalyses chelation using a single aspartic acid positioned across the binding cleft from a tyrosine residue (Romesberg *et al.*, 1998).

Dehydrogenase and chelataze active sites overlap

The importance of specific residues in catalysis was determined using site-directed mutant enzymes, which were analysed *in vivo* by complementation of a defined *E.coli* (*cysG*) mutant and *in vitro* by assaying independently the dehydrogenase and chelataze activities of the enzyme (Table II; Figure 6). The *in vivo* complementation is based on the cysteine auxotrophy of the *E.coli cysG* strain 302Δa, which has no methyltransferase, dehydrogenase or chelataze activity associated with sirohaem synthesis (Warren *et al.*, 1994), and which requires either functional gene homologues for these activities or exogenous cysteine for growth. Wild-type *MET8* cloned in tandem with a uroporphyrinogen III methyltransferase (*cobA* from *Pseudomonas denitrificans*) complements this strain, while constructs encoding inactive variants do not sustain growth. *In vitro*, the dehydrogenase activity of the enzyme was monitored by measuring the conversion of precorrin-2 into sirohydrochlorin at 37°C in the presence of NAD⁺ by UV/VIS spectroscopy (see Figure 6). The chelataze activity was assayed by incubating sirohydrochlorin with enzyme and cobalt, since cobalt is more stable than ferrous iron and also gives a larger spectral change on formation of a cobalt-isobacteriochlorin (Figure 6). For both the dehydrogenase and chelataze assays, the clear isosbestic points are indicative of the reaction proceeding without the release of any intermediates during the respective transformations.

The role of NAD was tested by disrupting its binding site through mutation of Gly22, which contacts the cofactor phosphates, to Asp. Gly22Asp Met8p, like the analogous substitution in CysG (Woodcock *et al.*, 1998), has no measurable dehydrogenase activity *in vitro* (Figure

Fig. 6. Spectra of substrates and products of the reactions catalysed by Met8p. (A) Spectra of precorrin-2 (light grey line), sirohydrochlorin (λ_{max} 376) and cobalt-sirohydrochlorin (λ_{max} 414). (B) Reaction profile of the dehydrogenase reaction catalysed by Met8p under the conditions described in Materials and methods. The spectra were recorded every 30 s and clearly show an increase in the λ_{max} at 376 nm with time. (C) Reaction profile of the chelatase reaction catalysed by Met8p under the conditions described in Materials and methods. Spectra were recorded every 30 s and show a shift in the λ_{max} from 376 to 414 nm.

6; Table II). Gly22Asp Met8p did, however, function efficiently as a chelatase, with a similar rate to that of wild-type enzyme. This indicates that sirohydrochlorin can associate and dissociate with Met8p during the course of sirohaem synthesis, that the chelatase activity can function independently of the dehydrogenase activity, and that

chelation does not depend on the presence of NAD. *In vivo*, Gly22Asp Met8p was able to complement the *E. coli* CysG mutant, albeit poorly, presumably due to spontaneous oxidation of the highly unstable precorrin-2, which has a half life of ~2 min under aerobic conditions.

Asp141 was selected as a potential catalytic residue because it is invariant and lies adjacent to the modelled nicotinamide ring (Figure 4). Like Gly22Asp Met8p, the Asp141Ala protein had no measurable dehydrogenase activity (Table II). Unlike Gly22Asp Met8p, however, the Asp141Ala protein failed to function as a chelatase. This result suggests that Asp141 plays a role in both the dehydrogenase and chelatase activities, and that both reactions catalysed by this bifunctional enzyme are performed in a single active site. As discussed above, this proposal is consistent with the distribution of conserved residues and electrostatic potential (Figure 5). It seems unlikely that Asp141Ala Met8p adopts an altered protein conformation, since this side chain is solvent exposed and makes no hydrogen bonding interactions with other groups on the protein. Moreover, this protein is overproduced like native protein, and is dimeric as judged by sizing chromatography. Interestingly, Asp141Ala Met8p copurifies with a fluorescent ligand, whose emission spectrum is consistent with that of the precorrin-2 substrate or sirohydrochlorin intermediate (data not shown). This suggests that Asp141Ala Met8p is correctly folded and is competent to bind substrate, but is unable to complete catalysis. Similar observations were made with murine ferrochelatase, where substitution of Glu287 for Ala or Gln resulted in an enzyme that maintained binding to its substrate, protoporphyrinogen IX, throughout purification (Franco *et al.*, 2001). Unfortunately, our attempts to grow crystals of the Asp141Ala Met8p–ligand complex have not yet been successful. Our preferred model is that Asp141 functions as a general base in both dehydrogenase and chelatase reactions, by abstracting protons from the pyrrole nitrogens (Figure 1). A similar role in proton abstraction from the pyrrole nitrogens has been assigned to Glu314 in *S. cerevisiae* ferrochelatase (Gora *et al.*, 1996).

We attempted to identify a metal substrate-binding site, by soaking crystals of Met8p in 10 mM MnCl_2 or CoCl_2 . No metal binding site was observed in data obtained from the CoCl_2 -soaked crystals, but a clear 8σ positive difference peak was observed in 3.3 Å resolution data collected from a crystal soaked in MnCl_2 (Table I). This feature indicates that Mn^{2+} binds to just one Met8p residue, His237, which lies in the C-terminal domain of Met8p external to the active site. His237 is not important for Met8p activity, however, since substitution of this residue by Ala gave a protein with properties indistinguishable from the wild-type protein in our *in vitro* and *in vivo* assays (Table II). We thus do not believe that His237 represents the metal binding site for the chelation reaction.

In summary, these results suggest that both the dehydrogenation and ferrochelation activities of Met8p are catalysed in a single active site cleft formed between the N-terminal NAD-binding domain and the central domain. We propose a mechanism of enzyme catalysed NAD-dependent dehydrogenation, whereby the invariant Asp141 functions as a catalytic general base to abstract a proton from the pyrrole nitrogen of ring C with concomitant hydride transfer from the prochiral bridge carbon to

the nicotinamide ring of the NAD (Figure 1). Asp141 may also function as a general base during ferrochelation, since additional protons must be removed from the pyrrole nitrogens during the course of metal ion insertion.

Materials and methods

Purification and crystallization of Met8p

Recombinant Met8p, with a 20 residue N-terminal extension containing six histidine residues, was overproduced, purified and crystallized according to Schubert *et al.* (2001). Four deviations from the published sequence, gi:6319690, were identified upon sequencing several independent PCR-produced clones from *S.cerevisiae*, and include Lys15Arg, Ile33Met, Glu61Lys and Asp102Asn (residues in gi:6319690 shown first). The clone containing these deviations was used for the expression construct and is the sequence listed in Figure 2. Briefly, Met8p was purified from bacterial lysates using metal chelate affinity chromatography (using standard protocols) followed by dialysis into 300 mM sodium formate (pH 6.5) and size exclusion chromatography. Crystals were grown at room temperature using the hanging drop method, where 2 µl of Met8p concentrated to 7 mg/ml containing 2.5 mM NAD was mixed with 2 µl of well solution containing 16–18% polyethylene glycol (mol. wt 4000), 0.1 M CaCl₂, 0.1 M Tris-HCl (pH 8.5) and 2 mM dithiothreitol. Crystals grew overnight in the form of thin flat plates, space group C2 ($a = 156.1$ Å, $b = 81.2$ Å, $c = 104.2$ Å and $\beta = 121.8^\circ$), with three molecules per asymmetric unit, corresponding to 60% solvent (Matthews, 1968). Fifteen percent methylpentane diol was added to the crystallization solution as a cryoprotectant, and the crystals were vitrified by plunging into liquid nitrogen.

SeMet-substituted protein was prepared using the methionine inhibition method (Van Duyne *et al.*, 1993) and purified identically to the native protein. The presence of SeMet was confirmed by amino acid analysis (data not shown). MAD data were collected at the Advanced Light Source, LLNL, station 5.0.2 (Table I). High-resolution data to 2.2 Å were collected on an additional SeMet-substituted protein crystal on beamline 9-1 at the Stanford Synchrotron Radiation Laboratory, SSRL. All data were processed and scaled using the HKL set of programs (Otwinowski and Minor, 1997). Attempts to obtain a metal-bound Met8p complex were undertaken by soaking native crystals in well and cryoprotectant solutions supplemented with 10 mM MnCl₂ and CoCl₂. A full set of data was collected on one crystal from each soak and the statistics for the Mn²⁺-bound structure are shown in Table I.

Phasing and refinement of Met8p

The unmerged reflections were processed with SOLVE to identify the selenium substructure (Terwilliger and Berendzen, 1999). Twelve sites with heights over 10.7 σ (Z-score of 76.24) were easily identified and the resultant phases were solvent flattened using RESOLVE. The resulting electron density map was easily interpretable and essentially the entire polypeptide chain was traced in this map. The model was refined through a series of building and refinement cycles using the programs O (Jones *et al.*, 1991) and REFMAC (Murshudov and Dodson, 1997). The final model was produced by refining the coordinates against the high-resolution data (20–2.2 Å) and the MAD phases (20–2.7 Å), in the form of Henderson–Lattman coefficients. Medium main-chain and loose side-chain non-crystallographic restraints were used during refinement, and the domains were treated as separate groups to accommodate the differences between molecules. The final model contains residues 1–58 and 72–273 of molecule A, residues 1–273 of molecule B, residues 1–58 and 72–273 of molecule C, three partial NAD molecules and 648 water molecules. The 20-residue N-terminal histidine tag is not visible in the structure and the protein model begins with the native initiator methionine as Met1.

Site-directed mutagenesis

The following mutations were introduced into pER259 (pET14b-wild-type MET8 plasmid) (Raux *et al.*, 1999) using the GeneEditor™ *in vitro* site-directed mutagenesis system from Promega, such that the following substitution were made: Gly22Asp, Asp141Ala and His237Ala. All mutations were verified through DNA sequencing.

In vitro dehydrogenase and chelataze assay

All MET8 variant proteins were overproduced and purified by metal chelate chromatography using the same protocol as for wild type, and were diluted to 0.2 mg/ml for the assay in 20 mM Tris-HCl (pH 8.0) and

100 mM NaCl. Porphobilinogen (PBG) deaminase (*hemC*) was purified as described in Jordan *et al.* (1981). *Bacillus megaterium* uroporphyrinogen III synthase (*hemD*), *P.denitrificans* uroporphyrinogen III methyltransferase (*cobA*) and the *B.megaterium* precorrin-2 dehydrogenase (*sirC*) were overproduced with N-terminal His tags and purified. HemD and SirC were subsequently dialysed against 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl, and CobA against 50 mM Tris-HCl (pH 8.0). PBG was synthesized from 5-aminolaevulinic acid (ALA) using purified ALA-dehydratase (Jordan *et al.*, 1981).

Precorrin-2 was generated *in situ* under an atmosphere of nitrogen in a glove box, with <2 p.p.m. oxygen. This was accomplished by incubating 2.5 mg PBG in a total volume of 40 ml containing 5 mg of purified PBG deaminase, 1 mg purified uroporphyrinogen III synthase, 5 mg of purified uroporphyrinogen III methyltransferase and 15 mg SAM. The reaction was left overnight at room temperature to allow it to reach completion, and was filtered prior to use. Sirohydrochlorin was generated using the same enzyme cocktail as described above, except that 5 mg of SirC and 10 mg of NAD were added to the incubation. The rate for the dehydrogenase reaction was calculated by monitoring the appearance of sirohydrochlorin (Figure 6B) at a $\lambda_{\max}$ of 376 nm, using an extinction coefficient of 2.4×10^5 M/cm. The rate of the chelataze reaction was calculated by measuring the rate of disappearance of sirohydrochlorin (Figure 6C), using the extinction coefficient given above.

The dehydrogenase assay was monitored by incubating precorrin-2 (2.5 µM) with 100 µg of Met8p in a reaction volume of 3 ml with 1 mM NAD in 0.05 M Tris-HCl buffer, pH 8. The chelataze activity was measured with sirohydrochlorin (2.5 µM), Co²⁺ (20 µM) and 25 µg of Met8p in a 1 ml reaction volume in 0.05 M Tris-HCl buffer, pH 8. For both reactions, initial rates were recorded on a Hewlett Packard 8452A photodiode array spectrophotometer and assays were performed in duplicate.

Complementation assay

All MET8 clones were subcloned into pETac. This plasmid is a modified pET14b in which the T7 promoter has been substituted with a *Ptac* promoter, allowing the expression of MET8 variants in *E.coli* strain 302Aa. This strain is an *E.coli* *cysG* mutant that is unable to produce sirohaem and, therefore, unable to synthesize cysteine. The cysteine auxotrophies were studied on minimal media as described previously (Raux *et al.*, 1997).

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