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

Heidi L. Schubert1,2, Evelyne Raux3, Amanda A. Brindley3, Helen K. Leech3, Keith S. Wilson4, Christopher P. Hill1 and Martin J. Warren2,3

1Department of Biochemistry, University of Utah, Salt Lake City, UT 84132, USA, 2School of Biological Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS and 3Structural Biology Laboratory, Chemistry Department, University of York, York YO10 5DD, UK

2Corresponding authors
e-mail: heidi@biochem.utah.edu or m.j.warren@qmul.ac.uk

Sirohaem is a tetrapyrrrole-derived prosthetic group that is required for the essential assimilation of sulfur and nitrogen into all living systems as part of the sulfate 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.

**Keywords:** chelatase/CysG/dehydrogenase/sirohaem synthase/X-ray crystallography

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**Introduction**

The tetrapyrrrole-derived cofactor sirohaem is a vital component of sulfate and nitrite reductases that perform the six-electron reduction of sulfate 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 tetrapyrrroles, a family of metalloprosthetic groups and coenzymes that includes haem, chlorophyll, coenzyme F430 and cobalamin (vitamin B12).

All modified tetrapyrrroles 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 tetrapyrrrole 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 tetrapyrrrole-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 (ChbK) 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<sub>free</sub> = 28.7%) with 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, β2. An initial β2-α1-β3 unit, β2-α1-β3, is mirrored in pseudo 2-fold symmetry by a second, β6-α5-β7, 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 (β4). There is no structural similarity between any region of Met8p and the known structures of the anaerobic cobalt chelatase, CbiK, or protoporphyrin IX.

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.

Fig. 2. Sequence alignment of Met8p with the bifunctional dehydrogenase and chelatase domains of CysG. This figure shows the Met8p sequences from <i>S. cerevisiae</i> (S20155, with four deviations in sequence identified during cloning as described in the Materials and methods section), <i>Saccharomyces pombe</i> (T38797) and CysG from <i>E. coli</i> (P11098). Alignment and definition of sequence conservation was performed using Clustal W (Higgins and Sharp, 1989), and also considered sequences of CysG from <i>Vibrio anguillarum</i> (JSC347), <i>Pseudomonas aeruginosa</i> (AAG05999), <i>Salmonella typhimurium</i> (B39200), <i>Buchnera sp. APS</i> (BAB13123) and <i>Rhizobium leguminosarum</i> bv. <i>viciae</i> (AAF87214). Invariant residues are coloured orange, conserved residues yellow. 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.
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 \( \beta \)-sheet, and the bottom layer is comprised of two \( \alpha \)-helices. The two inner strands (\( \beta 1 \)) of the top \( \beta \)-sheet

![Fig. 3. Structure of Met8p.](image)

**Table I. Crystallographic statistics**

<table>
<thead>
<tr>
<th>Data collection statistics</th>
<th>High resolution</th>
<th>Se-peak</th>
<th>Se-remote</th>
<th>Se-infection point</th>
<th>Mn({}^{2+})</th>
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<td>Wavelength (( \text{\AA} ))</td>
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<td>0.9769</td>
<td>0.9572</td>
<td>0.97990</td>
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<td>Resolution (( \text{\AA} ))(^p)</td>
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<td>20–2.7 (2.8–2.7)</td>
<td>20–2.7 (2.8–2.7)</td>
<td>20–2.7 (2.8–2.7)</td>
<td>20–3.3 (3.4–3.3)</td>
</tr>
<tr>
<td>Observed reflections</td>
<td>117 894</td>
<td>58 227</td>
<td>57 961</td>
<td>58 549</td>
<td>57 585</td>
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<tr>
<td>Unique reflections</td>
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<td>29 993</td>
<td>29 706</td>
<td>30 123</td>
<td>16 103</td>
</tr>
<tr>
<td>% Completeness(^a)</td>
<td>98.0 (85.6)</td>
<td>98.0 (87.8)</td>
<td>98.3 (85.4)</td>
<td>98.3 (85.7)</td>
<td>98.3 (85.4)</td>
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<tr>
<td>( R_{\text{merge}} )(^{b,b})</td>
<td>7.0 (30.4)</td>
<td>8.4 (24.9)</td>
<td>6.8 (33.9)</td>
<td>6.9 (39.4)</td>
<td>7.5 (21.2)</td>
</tr>
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<td>( \text{I/\text{sigma}}^{c} )</td>
<td>15.5 (3)</td>
<td>23 (5)</td>
<td>18 (2.7)</td>
<td>19.6 (3)</td>
<td>15 (5.6)</td>
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<tr>
<td>Mosaicity (( ^\circ ))</td>
<td>0.730</td>
<td>0.793</td>
<td>0.756</td>
<td>0.742</td>
<td>1.3</td>
</tr>
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<td>( R_{\text{factors}} )(^d)</td>
<td>0.221</td>
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<tr>
<td>( R_{\text{free}} )(^d)</td>
<td>0.007</td>
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<tr>
<td>R.m.s.d. (bond lengths) (( \text{\AA} ))</td>
<td>2.1</td>
<td></td>
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<tr>
<td>R.m.s.d. (bond angles) (( ^\circ ))</td>
<td>2.1</td>
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</tbody>
</table>

\(^a\)Numbers in parentheses are for the high-resolution bin.

\(^b\)\( R_{\text{merge}} = \Sigma |I - \langle I\rangle|/\Sigma I\), where \( I \) is the intensity of an individual measurement and \( \langle I\rangle \) is the average intensity from multiple observations.

\(^c\)\( R_{\text{factors}} = \Sigma |F_{\text{obs}}| - |k|F_{\text{calc}}|/\Sigma |F_{\text{obs}}|\)

\(^d\)\( R_{\text{free}} \) equals the \( R \)-factor against 5% of the data removed prior to refinement.
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 (β8) 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 Å²) (Hubbard et al., 1991). The C-terminal domain consists of five α-helices and one large extended loop between α11 and α12. 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 Å, alignment of the NAD-binding domains of any two molecules results in a relative ~9° rotation and a maximum displacement of ~5.4 Å 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 mM β-NAD⁺, which is seen bound to the enzyme in the crystal structure. The adenosine half of the NAD binds above the β2 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 adenine 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

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**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.
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). 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.

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 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 chelatase structures (Al-Karadaghi et al., 1997; Schubert et al., 1999; Leecero 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 chelatase active sites overlap**

The importance of specific residues in catalysis was determined using site-directed mutant enzymes, which were analysed in vitro by complementation of a defined *E.coli* (cysG) mutant and in vitro by assaying independently the dehydrogenase and chelatase 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 chelatase 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 chelatase 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 chelatase 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

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**Table II. Dehydrogenase and chelatase activities of Met8p**

<table>
<thead>
<tr>
<th>Met8p variant</th>
<th>Dehydrogenase specific activity (nmol/min/mg)</th>
<th>Chelatase specific activity (nmol/min/mg)</th>
<th>Complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>11.2 ± 0.8</td>
<td>31.1 ± 2.5</td>
<td>++</td>
</tr>
<tr>
<td>Gly22Asp</td>
<td>0</td>
<td>32.5 ± 2.5</td>
<td>+</td>
</tr>
<tr>
<td>Asp141Ala</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>His237Ala</td>
<td>13.3 ± 1.1</td>
<td>35.4 ± 3.2</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Complementation of *E.coli* strain 302Δa in M9 media after 24 h. All strains grew well in the presence of exogenous cysteine.
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 MnCl2 or CoCl2. No metal binding site was observed in data obtained from the CoCl2-soaked crystals, but a clear 8σ positive difference peak was observed in 3.3 Å resolution data collected from a crystal soaked in MnCl2 (Table I). This feature indicates that Mn2+ 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 the 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 (α = 156.1 Å, β = 81.2 Å, c = 104.2 Å and β = 121.8°), 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 processed and scaled using the HKL set of programs (Otwinowski and Minor, 1997). Attempts to obtain a metal-bound Met8p complex were processed and scaled using the HKL set of programs (Otwinowski and Minor, 1997). All data were collected on an additional SeMet-substituted protein crystal on beamline 5.0.2 at the Stanford Synchrotron Radiation Laboratory, SSRL. The presence of SeMet was confirmed by amino acid analysis (using standard protocols) followed by dialysis into 300 mM sodium formate as a cryoprotectant, and the crystals were vitrified by plunging into liquid nitrogen.

**Phasing and refinement of Met8p**

The unmerged reflections were processed with SOLVE to identify the selenium substructure (Terwilliger and Berendzen, 1999). Twelve sites with positive residuals in the σ2 (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 whole polyepitope chain was traced in this map. The model was refined though 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 (pET1b-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 chelatase assay**

All Met8p 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 λmax of 376 nm, using an extinction coefficient of 2.4 × 105 M/cm. The rate of the chelatase 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 190 µg of Met8p in a reaction volume of 3 ml with 1 mM NAD in 0.05 M Tris–HCl buffer, pH 8. The chelatase 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 Pac promoter, allowing the expression of MET8 variants in *E. coli* strain S302A. This strain is an *E. coli* cysG mutant that is unable to produce sirohaem and, therefore, unable to synthesize cytochrome. The cytochrome axotrophies were studied on minimal media as described previously (Raux et al., 1997).

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**References**


