Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Optimization of Met8p crystals through protein-storage buffer manipulation

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Received 11 January 2001
Accepted 14 March 2001

Sirohaem, the prosthetic group of assimilatory sulfite and nitrite reductases, is a modified tetrapyrrole that belongs to the same fraternity of metallo-prosthetic groups as haem, chlorophyll, cobalamin and coenzyme F430 [Warren & Scott (1990), *Trends Biochem Sci.* **15**, 486–491]. In *Saccharomyces cerevisiae*, the last step in the biosynthesis of sirohaem involves Met8p, a bifunctional enzyme responsible for both the NAD^+-dependent dehydrogenation of the corrin ring and ferrochelation. Optimization of the protein storage buffer according to the results of crystallization trials resulted in a more monodisperse protein solution. Crystals were grown that diffracted to 2.1 Å.

1. Introduction

The six-electron reduction process associated with assimilatory sulfite and nitrite reductases requires sirohaem as a prosthetic group (Crane & Getzoff, 1996). Some dissimilatory sulfite reductases also require sirohaem, where sulfite represents the terminal redox couple of a respiratory electron-transfer chain. Sirohaem is structurally related to other metallo-chelated macrocyclic compounds including haem, chlorophyll and cobalamin (vitamin B_{12}) and indeed is biosynthesized from a common primogenitor molecule called uroporphyrinogen III (uro'gen III). The transformation of uro'gen III into sirohaem requires four steps: two methyl additions at positions 2 and 7 which generate the intermediate precorrin-2, an NAD^+-dependent dehydrogenation which yields sirohydrochlorin and ferrochelation which produces sirohaem.

In *S. cerevisiae*, the synthesis of sirohaem from uro'gen III requires the actions of only two enzymes, Met1p and Met8p. It has been shown that Met1p acts as an *S*-adenosyl-L-methionine dependent uro'gen III methyltransferase capable of transferring two methyl groups from SAM to generate precorrin-2 (Raux *et al.*, 1999). Met8p has been shown to be a bifunctional enzyme capable of converting precorrin-2 into sirohaem *via* an NAD^+-dependent dehydrogenation and also to be capable of ferrochelation (Hansen *et al.*, 1997; Raux *et al.*, 1999). Moreover, in heterologous systems Met8p can also act as a cobalt chelatase in the synthesis of cobalamin, although yeast is no longer is able to make this nutrient *de novo* (Raux *et al.*, 1999).

Met8p contains 274 amino acids. The N-terminus of the protein contains a G*x*G*xx*G sequence, which is a motif commonly associated with nucleotide-binding domains and is consistent with the role of Met8p as a dehydrogenase. There is some similarity between the N terminus of Met8p and the N terminus of CysG, a multifunctional protein found in bacteria such as *Escherichia coli* that contains all the activities associated with the transmogrification of uro'gen III into sirohaem (Spencer *et al.*, 1993; Warren *et al.*, 1994). However, there is no similarity between Met8p and any of the known tetrapyrrole chelatases, suggesting that Met8p may adopt a different unique fold for the purpose of chelation. Thus, Met8p has the potential to yield a novel chelation mechanism coupled to an NAD^+-dependent dehydrogenation. The operons of many tetrapyrrole genes contain numerous examples of gene fusions which result in multiple catalytic activities associated with the expressed polypeptide. What is not clear is whether Met8p represents such a fusion containing two active sites or whether it is a protein with a single catalytic site. The crystal structure of Met8p will provide a clearer insight into this fascinating problem.

Crystallization of any protein requires both sufficient purity and solubility such that incubation of this the protein with a variety of salts and precipitants can push the protein gently out of solution into a crystalline state (McPherson, 1999). Many proteins may form high molecular-weight aggregates but still remain soluble. The buffer in which the protein is stored has a large effect on the protein's aggregation and can be varied to produce a more monodisperse solution. Initial studies to determine general pH and salt conditions where the protein remains in solution can be augmented with information from both

successful and unsuccessful crystallization trials. We describe the manipulation of the protein-storage buffer of purified recombinant Met8p to obtain high-quality crystals diffracting to 2.1 Å.

2. Materials and methods

2.1. Protein purification and crystallization

Recombinant N-terminus His-tagged Met8p was overexpressed in BL21(DE3)-LysS cells behind the T7 promoter of the pET14b vector. Approximately 10 mg was purified per litre of bacterial culture using a combination of metal-chelate affinity chromatography with size-exclusion chromatography (Pharmacia S75). After the Ni^{2+}-affinity chromatography step the protein was dialysed against a stabilizing buffer which was then used for subsequent size exclusion. The nature of this buffer evolved over the course of the project. The initial protein that eluted from the Ni^{2+} column was separated and dialysed into four different buffers all containing 20 m*M* buffer, 100 m*M* NaCl and 1 m*M* dithiothreitol (DTT): sodium acetate pH 5.6, sodium citrate pH 6.5, HEPES pH 7.5 and Tris–HCl pH 8.5. A slight degree of precipitation was observed at the two extreme pH values 5.6 and 8.5. Consequently, the second protein preparation was dialysed directly against buffer containing 0.02 *M* sodium citrate pH 6.5 and 0.1 *M* NaCl, 1 m*M* DTT and exposed to gel filtration. Though the protein did not precipitate in the buffer, it did aggregate as shown by native polyacrylamide gel analysis (PAGE) and dynamic light scattering (data not shown). This preparation was nonetheless screened for crystallization using hanging-drop vapor diffusion with 4 µl drops formed by equal amounts of protein solution (7 mg ml^{-1}) and well solution (screens were carried out using Crystal Screen I from Hampton Research). Spherical globules resulted from screen solutions containing high concentrations (1 *M*) of sodium citrate (Fig. 1*a*).

The globules disappeared with increasing concentrations of citrate, indicating that the protein might be becoming more soluble. Based on this observation, the next protein preparation was dialysed against 0.3 *M* citrate pH 6.5 and subjected to gel-filtration chromatography. Two peaks emerge from the column. The smaller peak corresponds to a molecular mass of 60 kDa, indicating that Met8p might form a homodimer. The larger of the two peaks was interpreted as a high molecular-mass aggregate and was discarded. Large curved crystals grew over wells containing 0.9 *M* sodium citrate and 0.1 *M* Tris–HCl pH 8.5, 1 m*M* DTT (Fig. 1*b*). The crystals diffracted to 3.3 Å and proved to be tetragonal, with unit-cell parameters $a = b = 104.0$, $c \simeq 286.6$ Å. The crystals were not fully characterized, but depending on the final space group the crystals could contain from ten to 20 molecules in the asymmetric unit. Structure determination by any method would be difficult for this crystal form.

To explore additional crystallization conditions, the Met8p stored in citrate buffer was fully rescreened. In addition to the known citrate conditions, crystalline material was observed in a well solution containing 3.6 *M* sodium formate and 0.1 *M* HEPES pH 7.5. For all subsequent preparations, the protein was dialysed against 0.3 *M* sodium formate, 1 m*M* DTT and purified by gel filtration. In contrast to the initial sodium citrate and NaCl conditions, the protein stabilized in formate appeared as a single species on a native PAGE gel. The protein was once again screened for crystallization and resulted in another new crystal form from a well solution containing 18–22% polyethylene glycol (MW = 4000), 0.2 *M* Li_2SO_4, 0.1 *M* Tris pH 8.5, 3% methylpentanediol (MPD), 1 m*M* DTT. These crystals had much sharper edges but still lacked sufficient quality for diffraction experiments. The addition of 5 m*M* β-NAD^+ resulted in crystals of substantially improved quality in the form of thick flat plates (Fig. 1*c*).

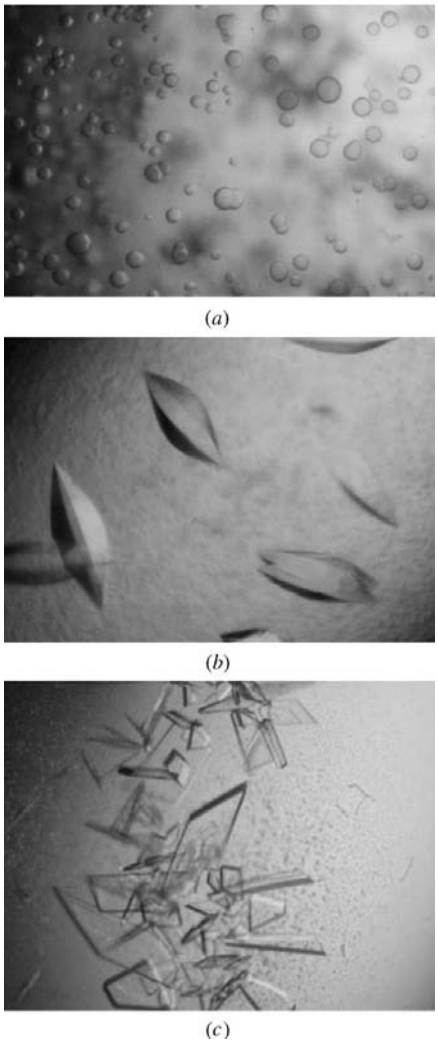

Figure 1
(*a*) Initial crystalline globules from Met8p stored in 0.02 *M* sodium citrate pH 6.5, 0.1 *M* NaCl. (*b*) Poorly formed tetragonal crystals from Met8p stored in 0.3 *M* sodium citrate pH 6.5. (*c*) Optimum monoclinic crystals from protein stored in 0.3 *M* sodium formate pH 7.0.

Table 1
Met8p native data collected at the ESRF, station BW7B.

Values in parentheses refer to the outer resolution shell.

Space group	*C*2
Wavelength (Å)	0.8445
Resolution (Å)	20–2.1 (2.17–2.1)
Observed reflections	1017319
Unique reflections	64822
$\langle I/\sigma(I)\rangle$	31.6 (3.2)
Completeness (%)	99.0 (90.4)
R_{merge}†	0.045 (0.408)
Mosaicity	0.523

† $R_{merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the intensity of an individual measurement and $\langle I\rangle$ is the average intensity from multiple observations.

2.2. Data collection and analysis

Using 15% MPD as cryoprotectant, these crystals were vitrified at 110 K. They diffract to 2.1 Å (Table 1) and complete native data were collected at the Hamburg EMBL Outstation on station BW7B and processed with the *HKL* suite of programs (Otwinowski & Minor, 1997). The crystals belong to space group *C*2, with unit-cell parameters $a = 158.0$, $b = 80.6$, $c = 104.1$ Å, $\beta = 121.8^\circ$. The unit cell can accommodate from two to four molecules per asymmetric unit, corresponding to 70–47% solvent (Matthews, 1968). A self rotation reveals a strong peak (7.4σ) at $\chi = 180^\circ$ indicating the presence of at least two molecules in the asymmetric unit (Vagin & Teplyakov, 1997). There is an additional 7.4σ peak at $\chi = 110^\circ$, with two 4.1σ peaks at $\chi = 180^\circ$ and $\chi = 110^\circ$ suggesting the presence of additional independent molecules.

3. Results and discussion

Most proteins that crystallize share a series of positive characteristics. The most impor-

tant feature of a protein is its stability over time, both in terms of its catalytic activity and also its oxidation and aggregation state. In addition, the protein must be pure from both additional protein contaminants and microhomogeneitgy caused by a mixture of post-translational modifications, isoforms or proteolytic breakdown products. Finally, the inherent flexibility of the protein is crucial, though less easily discerned or prevented, for repetitive crystal-lattice formation. The addition of ligands can sometimes cause the protein to adopt a more compact conformational state.

While size-exclusion chromatography can separate distinct species that are separated by large variations in molecular weight, the protein will slowly retain equilibrium between monomeric and aggregated states. To reduce the amount of aggregated species, changes must be made to the protein's local and long-term environment. The storage conditions of a protein, namely its buffer solution, protein concentration and storage temperature, directly impact on its stability and aggregation. Ionic strength, pH, the presence of ligands and the protein concentration can all be optimized (Bergfors, 1999).

Although Met8p remains soluble in solutions with a wide variety of pH values and salt concentrations, the protein often forms high molecular-mass aggregates. Optimally, a protein should remain soluble and monodisperse in a particular solution under non-saturating conditions but should begin to crystallize out of solution as supersaturation occurs. Using this rationale, storage buffers were designed containing dilute amounts of the crystallization conditions which yielded positive results. After each purification with a new storage buffer the protein was rescreened for additional crystallization conditions and the storage buffer further optimized. Eventually, and in the presence of the NAD^+ cofactor, conditions were identified which produced diffraction-quality crystals.

These results reinforce the concept that the stability and monodispersity of a protein are critical for crystallization and that the conditions of both positive and negative crystallization experiments can lead an investigator on a path to optimal diffraction-quality crystals.

We acknowledge the help of G. Davies, S. Charnock, V. Ducros and the support staff of the EMBL Hamburg Outstation, and the provision of funds for data collection from the European Community Access to Research Infrastructure Action of the Improving Human Potential Programme to the EMBL Hamburg Outstation, contract number HPRI-CT-1999-00017.

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