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Crystallization and preliminary X-ray crystallographic analysis of glutathione amide reductase from *Chromatium gracile*

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The Chromatiaceae-specific glutathione amide reductase (GAR) belongs to the well known family of the glutathione reductases, even though differences in both substrate (glutathione amide instead of glutathione) and coenzyme (NADH instead of NADPH) specificities are reported. Crystals of the GAR enzyme from *Chromatium gracile* have been grown at 294 K by the hanging-drop vapour-diffusion method using lithium sulfate as a precipitant in the presence of nickel ions. The crystals belong to space group $P4_1$, with unit-cell parameters $a = b = 71.93$, $c = 223.85$ Å, $\alpha = \beta = \gamma = 90^\circ$ and one dimer per asymmetric unit. A full set of X-ray diffraction data was collected to 2.1 Å resolution with a completeness of 95.2%. Structure determination *via* the method of molecular replacement is under way.

1. Introduction

Glutathione reductase (GR) is a member of a highly homologous family of enzymes, the flavoprotein disulfide oxidoreductases (Perham *et al.*, 1978; Carothers *et al.*, 1989), most of which function with NADPH as coenzyme. However, glutathione amide reductase (GAR) characterized from *C. gracile* (Vergauwen *et al.*, 2001) relies on NADH in order to reduce the glutathione derivative glutathione amide disulfide (GASSAG; Bartsch *et al.*, 1996). GAR (homodimer molecular weight 98 056 Da) shows 52 and 35% sequence identity with the GR from *Escherichia coli* and the trypanothione reductase (TR) from *Crithidia fasciculata*, respectively. Taking the biologically relevant substrate and coenzyme into consideration, GR, TR and GAR share similar mechanistic and biochemical characteristics (Karplus & Schulz, 1989; Hunter *et al.*, 1992; Vergauwen *et al.*, 2001). GR (Schirmer *et al.*, 1989) and TR (Hunter *et al.*, 1992) are potential drug targets and therefore of appreciable medical interest. By analyzing the structure of GAR and of its complexes with GASSAG, trypanothione disulfide (which, like GASSAG, is amidated at its C-terminus) and with glutathione disulfide, we will be able to gain a detailed knowledge of the structural features in the active site that promote the binding of the biologically relevant substrate or the close relative trypanothione disulfide and which hinder the binding of glutathione disulfide. Comparing and contrasting the structures of GR, TR and GAR may then assist in the rational design of highly specific inhibitors dealing with uncontrolled cell growth (GR) or trypanosomal infections (TR).

Furthermore, Scrutton *et al.* (1990) created an NAD-dependent mutant of *E. coli* GR by the introduction of seven point mutations (A179G, A183G, V197E, R198M, K199F, H200D and R204P) in the $\beta\alpha\beta\alpha\beta$-motif of the coenzyme-binding domain (the 'Rossmann fold'). These are the residues at the equivalent positions in the homologous NAD-dependent dihydrolipoamide dehydrogenase from *E. coli* (Stephens *et al.*, 1983). However, binding and catalysis of NADH by the mutant reductase are still 6.5- and 5.3-fold lower compared with the values for the reductase from *C. gracile* (Vergauwen *et al.*, 2001), demonstrating that structure determination of the GAR enzyme–coenzyme complex will further reveal those amino-acid changes crucial for the change in coenzyme specificity. This could become of commercial importance if biosynthetic enzymes using the expensive NADPH could be replaced by appropriate enzymes using the cheaper NADH.

Here, we report the successful crystallization and diffraction data collection of GAR from *C. gracile*, which permitted us to initiate structure determination *via* the method of molecular replacement.

2. Materials and methods

2.1. Crystallization and X-ray data collection

Recombinant *C. gracile* GAR was purified following a previously described procedure (Vergauwen *et al.*, 2001). The protein samples used for crystallization were pure according to SDS–PAGE and mass spectrometry and were stored at 193 K at a concentration of approximately 20 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.5,

1 m*M* EDTA. The same buffer was used for dilution of the protein samples to the concentration used in the crystallization trials. Crystallization experiments were performed using the hanging-drop vapour-diffusion method. Hampton Research Screens (Hampton Research, Laguna Hills, CA 92653, USA) were used for the initial screening of crystallization conditions. Drops were prepared by mixing 2 µl of protein solution (10 mg ml^{-1}) with 2 µl of reservoir solution and were allowed to equilibrate against 500 µl of reservoir solution at 294 K.

Selected crystals were transferred to a solution containing 17% glycerol in mother liquor prior to flash-cooling at 100 K in a stream of gaseous nitrogen. The X-ray diffraction data were collected at a wavelength of 0.9073 Å at the X11 synchrotron beamline (HASYLAB, DESY, Hamburg, Germany) using a MAR CCD (MAR Research) imaging-plate detector system. The crystal-to-detector distance was 170.0 mm. A total of 127 rotation images were collected, with an oscillation angle of 1.0°. Data processing was carried out using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The diffraction intensities were indexed using the program *DENZO* and were scaled and merged to 2.1 Å resolution using the program *SCALEPACK*. Intensities were converted into amplitudes using the program *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Small star-shaped crystals were obtained within 2 d from different physicochemical solutions (ammonium sulfate, MPD, PEG 4000), while two-dimensional plates (0.4 × 0.4 × ∼0.002 mm) grew within one month from a 20% 2-propanol, 0.1 *M* sodium citrate pH 5.6, 20% PEG 4000 reservoir solution. Useful small crystals, however, were obtained from 1.0 *M* lithium sulfate, 0.1 *M* Tris pH 8.5, 0.01 *M* nickel chloride (condition 41 of Crystal Screen 2, Hampton Research). Optimization of this condition, replacing the 500 µl reservoir solution by 1 ml ammonium sulfate in water, ranging in concentration from 1.5 to 3 *M*, produced large diamond-shaped (octahedral and bipyramidal) crystals (0.5 × 0.3 × 0.25 mm) within one week (Fig. 1) (reservoir solution: 2.6 *M* ammonium sulfate in water). Since the crystallization condition contained chelating nickel ions, it is interesting to recall that copper ions, at micromolar levels, are reported to significantly increase the strength of the quaternary dimer structure of GAR in solution (Vergauwen *et al.*, 2001).

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.14–2.10 Å).

Space group	$P4_1$
Unit-cell parameters (Å, °)	$a = b = 71.93$, $c = 223.85$, $\alpha = \beta = \gamma = 90$
Packing density V_M (Å^3 Da^{-1})	2.95
Solvent content (%)	56.74
Resolution (Å)	20.0–2.10 (2.14–2.10)
No. of unique reflections	62745 (3176)
Redundancy	2.70 (2.29)
R_{sym}† (%)	6.4 (15.2)
Average $I/\sigma(I)$	18.48 (4.68)
Completeness (%)	95.2 (97.1)

† $R_{sym} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the average value over multiple measurements.

A complete data set was collected to a resolution of 2.1 Å using a single crystal. The data are 95.2% complete from 20.0 to 2.1 Å resolution (completeness in the last resolution shell, 2.14–2.10 Å, is 97.1%) and the R_{sym} on intensities is 6.4% based on 430 453 measured observations. These data have been reduced to 62 745 unique reflections. The full data-reduction statistics are presented in Table 1. The space group is $P4_1$, with unit-cell parameters $a = b = 71.930$, $c = 223.850$ Å, $\alpha = \beta = \gamma = 90°$. Assuming a subunit molecular weight of 49 028 Da (as determined by electrospray ionization mass spectrometry) and two molecules in the asymmetric unit, the solvent content of the octahedral crystal is 56.74%, with a volume-to-mass ratio of 2.95 Å^3 Da^{-1}. These values are within the frequently observed ranges for protein crystals (Matthews, 1968).

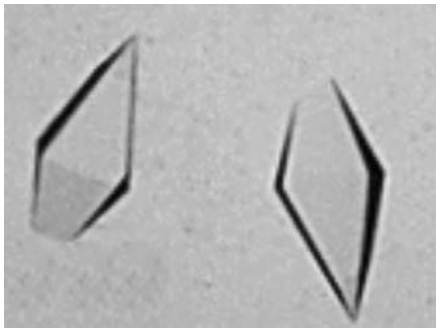

Figure 1
Two crystals of the recombinant glutathione amide reductase from the purple phototrophic bacterium *C. gracile*.

One subunit of the *E. coli* GR model (Mittl & Schulz, 1994; PDB code 1bo5) served as a starting model for solving the structure by molecular replacement. Rotation and translation searches have been performed using the software *AMoRe* (Navaza, 1994). Using a polyalanine model and using data in the resolution range 15–4.0 Å, a molecular-replacement solution was found with a correlation coefficient of 34.1% and an R factor of 47.4%. Visual inspection of the initial maps has already revealed interpretable electron density for most secondary-structural elements. The solution confirmed that there is a dimer in the asymmetric unit. The model building and refinement of this structure are under way.

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