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Crystallization and preliminary crystallographic investigations of rhodanese from *Azotobacter vinelandii*

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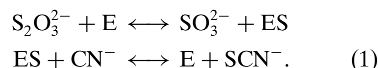
The *rhdA* gene identified in *Azotobacter vinelandii* codes for a protein, RhdA, which displays rhodanese (thiosulfate–cyanide sulfurtransferase) activity. RhdA was overexpressed and purified to homogeneity. The protein crystallized in the orthorhombic space group $P2_12_12$ with unit-cell parameters $a = 44.4$, $b = 150.8$, $c = 53.8$ Å; on a synchrotron source the diffraction patterns could be collected to a resolution limit of 1.8 Å. Evaluation of the crystal density indicates that the crystal lattice accommodates one molecule per asymmetric unit and that the solvent content is 59% of the total volume.

1. Introduction

Sulfurtransferases are a class of enzymes which are widely distributed among living organisms and catalyze the transfer of a sulfane S atom from a donor molecule to an acceptor substrate (Westley *et al.*, 1983). Bovine liver rhodanese (thiosulfate–cyanide sulfurtransferase, E.C. 2.8.1.1; M_r = 33.2 kDa), by far the best characterized sulfurtransferase to date, catalyzes the sulfur transfer reaction *in vitro* by way of a double-displacement mechanism (Westley, 1973, 1980). In the course of catalysis, the enzyme cycles between two distinct forms, the free enzyme (E) and the covalent intermediate sulfur-substituted enzyme (ES) (1). In the latter form, a divalent S atom is linked by a persulfide bond to the sulfydryl group of the active-site residue Cys247 (Westley, 1980; Ploegman *et al.*, 1978).



$$\begin{aligned} S_2O_3^{2-} + E &\longleftrightarrow SO_3^{2-} + ES \\ ES + CN^- &\longleftrightarrow E + SCN^-. \end{aligned} \quad (1)$$

In spite of its ubiquity, the biological role of rhodanese is still an object of debate and several physiological functions have been proposed (Westley, 1980; Cerletti, 1986), including the participation as a sulfur insertase in the formation of redox centres in Fe–S proteins (Bonomi *et al.*, 1977, 1985; Pagani & Galante, 1983; Pagani *et al.*, 1984, 1987), or in cysteine metabolism, where it may be coupled to the 3-mercaptopyruvate sulfurtransferase (Koj *et al.*, 1975; see also *Biochemical Pathways*, Boehringer Mannheim).

The crystallographic structure of bovine liver rhodanese (Ploegman *et al.*, 1978) has shown that the overall architecture of the enzyme consists of two domains which display very similar three-dimensional folds, in spite of negligible sequence homology. Each domain is formed by a central five-stranded parallel β-sheet surrounded by five α-helices. The sulfur-binding site in the covalent enzyme–sulfur intermediate is located in the second domain, in the proximity of the inter-domain interface. The active-site Cys247 residue is located at the bottom of a pocket whose walls are built of residues from both domains. One side of the pocket mostly contains hydrophobic residues, while the other side is mostly formed of hydrophilic residues. Both hydrophobic and hydrophilic residues are deemed important for substrate specificity (Ploegman, 1977).

Among prokaryotes, rhodanese activity has been detected in numerous bacterial strains (Westley *et al.*, 1983). In *Azotobacter vinelandii* in particular, a gene (*rhdA*) encoding for a protein homologous to eukaryotic rhodanese has been identified and cloned (Pagani *et al.*, 1993). The encoded protein, RhdA, is 271 amino acids long and has a molecular weight of 29.7 kDa. Further biochemical characterization has shown that RhdA is indeed a rhodanese, since it is capable of transferring a sulfane sulfur from thiosulfate to cyanide with a double-displacement mechanism similar to that of the bovine enzyme (Colnaghi *et al.*, 1996); additionally, RhdA is also able to transfer the sulfane sulfur from thiosulfate to the dithiol dihydrolipoate (Pagani *et al.*, unpublished work).

To better understand the functionality and catalytic mechanism of a prokaryotic rhodanese and to gain insight into the molecular bases of substrate specificity, which is maintained in spite of a widely altered amino-acid sequence, RhdA was crystallized with the aim of determining its three-dimensional structure. After more than 20 years (Ploegman *et al.*, 1978), this is the second rhodanese on which a crystallographic investigation is undertaken.

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Table 1
Data-collection statistics.

Values referring to the last resolution shell (1.83–1.8 Å) are given in parentheses.

Resolution (Å)	1.8
Mosaicity (°)	0.99
Unique reflections	30532
Average redundancy	2.8
Completeness (%)	98.5 (96.3)
$\langle I/\sigma(I)\rangle$	8.5 (6.3)
$R_{\rm merge}$ (%)	7.6 (23.6)

2. Materials and methods

A thiosulfate–cyanide sulfurtransferase enzyme was identified in the nitrogen-fixing bacterium *A. vinelandii*, as described previously (Pagani *et al.*, 1991). The gene (*rhdA*) encoding for the rhodanese enzyme was later identified and cloned (Colnaghi *et al.*, 1996). The extract (5 ml) from BL21(DE3)(pRC9T7.7) induced culture was fractionated overnight on a column of Sephacryl S-200 (Pharmacia, 3.3 × 88 cm), equilibrated in 50 m*M* phosphate buffer pH 7.0 and the fractions with thiosulfate–cyanide sulfurtransferase activity were pooled and brought to 75% saturation with ammonium sulfate. Further purification of RhdA was achieved by gel filtration of the ammonium sulfate precipitate, which had previously been solubilized in 5 ml 50 m*M* phosphate buffer pH 7.0 containing 0.1 *M* NaCl (protein concentration 5 mg ml^{-1}), by HPLC on a Superdex 75HR column (Pharmacia, 1 × 30 cm) equilibrated in the same buffer.

Rhodanese activity in the presence of cyanide as the nucleophilic acceptor was determined by a colorimetric assay based on the absorbance at 460 nm of the ferric thiocyanide complex formed by the reaction product thiocyanate and ferric nitrate (Sörbo, 1953). Enzyme unitary activity (1 U) was defined as the amount of enzyme which produces 10^{-6} mol thiocyanate per minute at 298 K. A colorimetric method based on the continuous determination, *via* nitroblue tetrazolium (NBT) reduction of the sulfite produced in the rhodanese reaction in presence of thiosulfate and cyanide (Cannella *et al.*, 1984), was also used. The protein concentration was determined by Bradford colorimetric assay (Bradford, 1976).

For crystallization purposes, the protein was brought to a final concentration of 15 mg ml^{-1} in a buffer containing 30 m*M* ammonium sulfate, 3 m*M* NaN_3, 5 m*M* Tris–HCl pH 7.5. Initial crystallization conditions were obtained with the sparse-matrix method (Jancarik & Kim, 1991). Refinement of these conditions resulted in crystals of good quality which grew to their final size after a period of nine months equilibration against a reservoir solution containing 1.6 *M* $MgSO_4$, 50 m*M* MES pH 6.0 and 5%(*v*/*v*) ethanediol. The crystallization experiments were carried out at 293 K and the hanging drops contained a mixture of 1 µl protein and 1 µl reservoir solution. Filtering all solutions prior to use improved the crystal yield and prevented the formation of crystal aggregates unsuitable for X-ray diffraction. The RhdA crystals had the shape of thin plates with typical dimensions 30 × 100 × 200 µm. Crystals were removed from the drop before data collection and stored in a stabilizing solution containing 1.8 *M* $MgSO_4$, 50 m*M* MES pH 6.0 and 5%(*v*/*v*) ethanediol. Immediately prior to X-ray collection, crystals could be frozen at 100 K following immersion in a medium identical to the stabilizing solution but containing an increased fraction of ethanediol (20%).

3. Results and discussion

HPLC gel-exclusion chromatography on the Superdex 75HR column produced a 30 kDa protein with a purity higher than 98%, as assessed by SDS–PAGE, and high (450 U mg^{-1}) thiosulfate–cyanide sulfurtransferase activity.

X-ray data collections on native protein crystals were carried out at 100 K on a MSC R-AXIS IIc image-plate system coupled to a Rigaku RU200 rotating-anode generator (Cu $K\alpha$ radiation). The crystals diffracted to a resolution limit of 3.0 Å, with a mosaic spread of 0.3°. Subsequently, a high-resolution data set was obtained at the X-31 beamline at the EMBL-DESY synchrotron radiation facility (Hamburg), where the diffraction pattern could be recorded (λ = 1.10 Å) to a resolution limit of 1.8 Å. Remarkably, despite an unusually high mosaicity of 0.99° compared with the 0.3° mosaic spread usually observed for these crystals, the data set was of very good quality (see Table 1).

From inspection of the diffraction pattern and systematic absences, it could be shown that the RhdA crystals belong to the orthorhombic space group $P2_12_12$ (*a* = 44.4, *b* = 150.8, *c* = 53.8 Å). Estimation of the crystal packing parameter (Matthews, 1968) indicated that the lattice can accommodate one molecule per asymmetric unit (V_M = 3.0 Å^3 Da^{-1}) with a solvent content of 59%, calculated assuming a specific volume of 0.74 cm^3 g^{-1} for the protein molecule.

The availability of a high-resolution (1.36 Å) bovine rhodanese structure (Gliubich *et al.*, 1998) allowed us to attempt structure solution based on a molecular-replacement approach. For this purpose, the amino-acid sequences of bovine and *A. vinelandii* rhodanese enzymes were aligned (see Fig. 1). The sequence alignment shows that 24% of the amino-acid residues are identical in the two molecules, indicating that the *A. vinelandii* rhodanese should have a three-dimensional fold very similar to that of the bovine enzyme. Molecular replacement was attempted using methods based

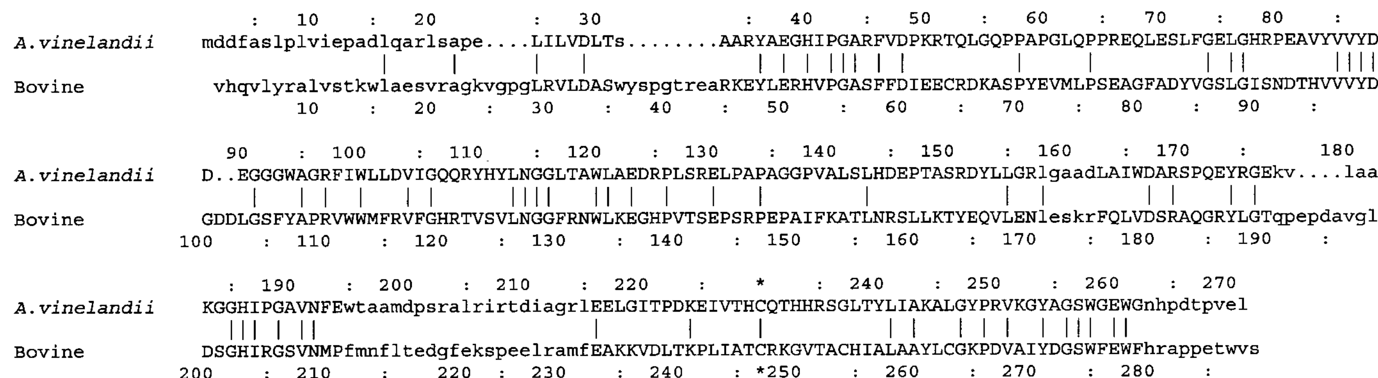

Figure 1
Amino-acid sequence alignment of *A. vinelandii* and bovine (*Bos taurus*) liver rhodanese. Conserved residues are indicated by |; amino acids for which reliable alignment could not be obtained are shown in lower case. Active-site Cys residues are indicated by *.

either on reciprocal space (Navaza, 1994) or on real space (Brünger, 1992) with both the 3.0 and 1.8 Å data sets. In spite of several search models derived either from the whole bovine rhodanese molecule or from only one of the two structural domains, and taking into account regions of higher sequence homology, no solution could be found to allow the correct orientation and translation of the template molecule in the RhdA unit cell.

The elucidation of the molecular structure of the rhodanese from *A. vinelandii* will therefore be pursued through multiple isomorphous replacement techniques. To this end, the search for heavy-atom isomorphous derivatives is currently in progress.

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