CRYSTALS OF HUMAN ERYTHROCYTE GLUTATHIONE REDUCTASE

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1. Introduction

The enzyme glutathione reductase (EC 1.6.4.2.) catalyses the reaction between oxidised glutathione and NADPH [1,2]:

$$GSSG + NADPH + H^+ \rightleftharpoons 2\ GSH + NADP^+$$

The enzyme is a flavoprotein and the catalytic process is thought to proceed through single electron transfer mediated by the prosthetic group FAD [3]. Glutathione reductase is widely distributed in animals, plants and bacteria [4–7]. The ubiquity of the enzyme seems to be related to the requirement of all cells for a high level of reduced glutathione, which stabilises cellular enzymes and components of cell membranes [8,9]. Despite the interest in glutathione reductase as a flavin enzyme and its apparent importance to various tissues, there is little information on its physical properties. This has been mostly due to difficulties in purifying sufficient amounts of the enzyme. Recently, these difficulties have been overcome for the human erythrocyte enzyme [10]. This species yields well-shaped crystals, which have been examined by X-rays. They belong to space group C2 with one subunit of the molecule in the asymmetric unit. This indicates that the two subunits of the molecule are identical and related to each other by a two fold axis.

2. Materials and methods

The isolation procedure of glutathione reductase has been reported earlier [10]. The method requires ten steps with an overall purification of 40 000-fold and a yield of 36%, providing 40 mg of enzyme from 40 litres of blood. The specific activity of this material was 240 units per mg. The material was homogeneous by zone electrophoresis in starch and polyacrylamide and by ultracentrifugation. At the final stage of purification the enzyme was stored in potassium phosphate buffer pH 7.0, $I = 0.1$, with 0.2 M KCl, 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA [10].

The protein could be readily crystallised either at low ionic strength by dialysis against deionised water, or at high ionic strength by addition of ammonium sulphate. For X-ray analytical purposes, we preferred high ionic strength because it allows for much better control of the crystallization process.

For crystallization the enzyme solution at 8 mg/ml in the above buffer was initially dialyzed against 1000 volumes of 20% satureated ammonium sulphate. A drop (approx. 50 μl) of the enzyme solution was placed on the underside of a siliconised glass cover slip, which stood on a plastic well containing 0.5 ml of 30% saturated ammonium sulphate. The rim of the well had previously been brushed with thin silicone oil to provide a seal. Glutathione reductase crystals formed over 24 hr at room temperature. For X-ray analysis the crystals were transferred to a storage solution which contained potassium phosphate buffer pH 7.0, $I = 0.3$, with 1 mM EDTA and 50% saturated ammonium sulphate.

The 'hanging drop method', which was applied

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here, has the advantage that the salt concentration is raised gradually, so that only a few, rather large crystals will grow. Furthermore, because of gravity, the crystals grow on the droplet surface and not on a glass surface where they tend to stick even when the glass has been siliconised. Thus, there is no breakage problem when transferring the crystals to the storage solution.

3. Results and discussion

A view of the crystals has been shown earlier [10]. They form elongated polyhedra without any apparent external symmetry. Crystal dimensions are approximately 400 × 150 × 150 μm. Due to FAD the crystals have a light yellow colour.

The crystals were examined by X-ray diffraction on a precession camera. The diffraction patterns show that the molecules have crystallized in space group C2 with unit cell dimensions a = 12.0 nm, b = 6.3 nm, c = 8.5 nm, $\beta = 58.2°$. The crystals are mechanically stable and they are rather stable in the X-ray beam. They diffract to a resolution higher than 0.25 nm. A precession photograph of the h0l-plane is shown in fig.1.

Physico-chemical studies [11] of human erythrocyte glutathione reductase have shown that it is a dimer of mol. wt 100 000, consisting of two, probably identical subunits. With this information one can determine the number of molecules in the asymmetric unit of the crystals, and thus assess the magnitude of the problems posed in an X-ray structure analysis. For this purpose we measured the crystal density by sedimentation of crystals in a calibrated toluene-carbontetrachloride density gradient. It can be derived [12] from crystal density (1.25 ± 0.02 $g \cdot cm^{-3}$), storage solution density (1.12 $g \cdot cm^{-3}$), protein density (assumed as 1.35 $g \cdot cm^{-3}$), and mol. wt (100 000) that there are 0.47 ± 0.06 molecules in the asymmetric unit. This indicates that there is half a molecule in the asymmetric unit, the small deviation from 0.50 pointing to a mol. wt slightly less than 100 000. The result is corroborated by the fact that the corresponding crystal volume per molecule mass of $V_M = 0.0029\ nm^3$/dalton fits well into the range found with other protein crystals [13].

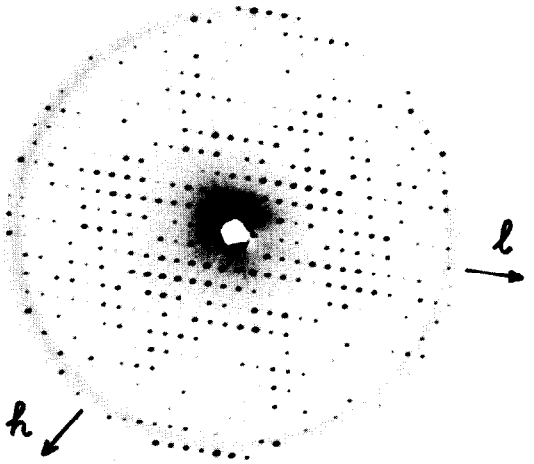

Fig.1. X-ray precession photograph of the h0l-plane of a glutathione reductase crystal irradiated with nickel-filtered Cu K_α radiation. The precession angle was 9°.

Since there is one monomer in the asymmetric unit, the monomers in the molecule must be related to each other by crystallographic symmetry, implying that they are exactly identical. Furthermore, these findings indicate that the subunits in solution are related to each other by a two-fold axis.

For X-ray analysis it is crucial to prepare heavy atom derivatives of the crystalline protein. One can expect that the thiol groups present in glutathione reductase will be suitable for this purpose. Therefore, we soaked the crystals in storage solution containing 500 μM methyl mercury nitrate for half a day. The resulting crystals showed marked changes in the reflection intensity pattern, indicating that the methyl mercury group has been attached to the protein, probably to thiols. Furthermore, the crystals are still well ordered and rather stable in the X-ray beam. Thus, we expect the methyl mercury nitrate derivative to be useful for structural analysis.

It has been shown that glutathione reductase contains two classes of free thiols [11]. One class is involved in the association of the enzyme to higher aggregates by formation of intermolecular disulphide bridges. Since these aggregates are still active, this class of thiols is distinct from the other one which comprises the thiols in the active centre [11]. It is hoped that heavy atom compounds, for instance bulky mercury compounds, can be attached selectively to one or the other class, thus providing further

crystallographically distinct heavy atom derivatives of the crystalline protein.

In conclusion we can state that the crystals seem to be amenable to an X-ray analysis of the molecular structure. Of particular interest would be the location of the FAD moiety, its interaction with NADPH and the location of the neighbouring thiols which take part in the reactions with oxidised glutathione [3]. Furthermore, it would be interesting to see whether or not the topology of the glutathione reductase polypeptide chain corresponds to those of other nucleotide binding proteins [14–17,18,19], thus providing essential additional information on the structural correspondence within this group of proteins.

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