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Purification, crystallization and preliminary X-ray studies of two isoforms of Rubisco from *Alcaligenes eutrophus*

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Two different isoforms of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from *Alcaligenes eutrophus* have been purified and crystallized. Both isoforms crystallize in space group $P4_32_12$. Crystals of isoform I (unit-cell dimensions a = 112.0 and c = 402.7 Å) diffract to 2.7 Å, whereas isoform II (unit-cell dimensions a = 111.8 and c = 400.0 Å) presently diffract to 3.2 Å, using synchrotron radiation in both cases.

1. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a bifunctional enzyme which is involved both in photosynthesis and photorespiration. In photosynthesis, the enzyme catalyzes the initial step in the fixation of carbon dioxide. During photorespiration, the enzyme catalyzes a competing oxygenation reaction where carbon dioxide is released into the atmosphere, thus reducing the net efficiency of photosynthesis significantly. Due to its important but inefficient role in photosynthesis and thus in food production, Rubisco has been the subject of extensive studies for many years with the major goal of engineering Rubisco mutants with a decreased oxygenase:carboxylase ratio compared with the wild-type enzyme (Brändén *et al.*, 1991; Wildner *et al.*, 1996).

In most bacteria and higher plants the enzyme is built up from eight large and eight small subunits (type I) with M_r = 55000 and 15000, respectively, forming an L_8S_8 complex (M_r = 550000). In a few other organisms, including the photosynthetic bacterium *Rhodospirillum rubrum*, the enzyme consists of only two large subunits (type II) with less than 30% sequence identity with type I large subunits. In addition to higher plants and photosynthetic bacteria, Rubisco is found in algae and in chemosynthetic bacteria such as *Alcaligenes eutrophus*. The soil bacterium *A. eutrophus* grows in the dark with CO_2 as its carbon source, obtaining its energy from oxidation of H_2.

A comparison of the amino-acid sequences for L_8S_8 Rubisco from widely different sources shows that they fall into two main groups (types Ia and Ib). The 'green-like' group includes green plants and cyanobacteria, while the 'red-like' group includes *A. eutrophus* and many algae (Delwiche & Palmer, 1996; Watson & Tabita, 1997). Sequence identity for the large subunit within each group is about 70%, while the sequence identity between members of the two groups is only 50–60%. The small subunit exhibits the same pattern, but with lower overall sequence identities. Even though the amino acids in the active sites of Rubiscos from different species are conserved, the catalytic properties of the enzymes vary significantly. Low substrate-specificity factors ($V_{CO_2}K_{O_2}/V_{O_2}K_{CO_2}$) are characteristic of the type II Rubisco, while those of the type I enzymes show great variation from low for cyanobacteria to high for terrestrial plants and very high in some 'red-like' marine algae (Jordan & Ogren, 1981; Read & Tabita, 1994). Rubisco from *A. eutrophus* has an intermediate specificity factor (Lee *et al.*, 1991).

The three-dimensional structures of Rubisco from spinach (Andersson *et al.*, 1989), tobacco plant (Chapman *et al.*, 1988), *Synechococcus* (Newman & Gutteridge, 1993) and *Rhodospirillum rubrum* (Schneider *et al.*, 1990) have been solved. An effort was also made several years ago to solve the structure of Rubisco from *A. eutrophus* (Holzenburg *et al.*, 1987). However, subsequent photon correlation spectroscopy studies combined with sedimentation analysis concluded that the resulting model was incorrect (Choe *et al.*, 1989). Furthermore, the Rubisco preparation used probably consisted of a mixture of two isoform enzymes, as it was was purified from a strain of *A. eutrophus* that has two sets of functional Rubisco *rbc*L and *rbc*S genes (Kusian *et al.*, 1995).

A. eutrophus represents one of the simplest model systems for protein engineering of Rubisco. A large number of mutated forms of this enzyme, produced by random mutagenesis, are available. They are currently being characterized and exhibit a wide range of catalytic activities (Andersen, in preparation). Solving the structure of Rubisco from *A. eutrophus*, which evolutionarily represents a main group distinctly different from the Rubisco structures solved previously, will serve as a basis for further design and analysis of mutated forms of the enzyme. This is expected to give a more

detailed understanding of the structure–function relationship for this enzyme, especially as far as catalytic efficiency is concerned.

2. Materials and methods

2.1. Source

The *A. eutrophus* strain ATCC17707 has two sets of functional Rubisco genes, one chromosomally encoded, the other plasmid encoded (Andersen & Wilke-Douglas, 1984). Cloning and characterization of these genes has been described previously (Andersen & Wilke-Douglas, 1987; Andersen & Caton, 1987). The *A. eutrophus* strain ATCC17697, however, has only one Rubisco gene copy. Construction of the Rubisco gene-deletion derivative AE951 of strain ATCC17697 will be described in detail elsewhere (Andersen, in preparation). The Rubisco minus strain AE951 was used as a host for expressing the cloned *A. eutrophus* ATCC17707 chromosomally encoded Rubisco (plasmid pRub18-2).

A. eutrophus strain ATCC17697 (wild type) or strain AE951/pRub18-2, were grown as previously described (Andersen *et al.*, 1981), with the following modifications: cells for Rubisco purification were grown in a 2 l fermentor (Braun Biostat B) under autotrophic conditions at 303 K in mineral medium sparged with a mixture of 2–3%(*v*/*v*) CO_2, 50% H_2, with air as the balance, at a total gas flow of 1–2 l min^{-1}. Dissolved O_2 concentration was monitored with an oxygen electrode and kept above 20% of air saturation by varying the stirring rate (800–1200 rev min^{-1}). Cultures were started with 0.5 g l^{-1} NH_4Cl as the nitrogen source. Use of a pH-control system set at pH 6.80 and using 1 *M* NH_4OH as the added base ensured constant pH and adequate nitrogen supply. Trace-metal supplements were added to the growing cultures according to known requirements (Repaske & Repaske, 1976). When the cell density had reached an OD_{420} of about 15, the CO_2 concentration in the sparging gas was reduced from 2–3% to 0.6–0.8% and growth was continued for 6–8 h under CO_2 limitation, with cell densities reaching an OD_{420} of 25–30. These conditions induced a high level of Rubisco in the cells used for enzyme purification.

2.2. Purification

Rubisco was purified by PEG precipitation and column chromatography using an earlier protocol (Andersen, 1979). 40 ml batches of cell-free extract from cell suspensions with about 60 mg ml^{-1} of total *A. eutrophus* protein were produced as previously described. Protease inhibitors (Boehringer Complete tablets) were added, according to the manufacturers recommendations, to the extract immediately following cell disruption with the French press. The buffer used during the purification procedure ('enzyme buffer') contained 50 m*M* Tris–HCl pH 7.8 (at 293 K), 0.1 m*M* EDTA and 1 m*M* dithiothreitol (DTT), except where indicated. Fractions containing Rubisco were identified by activity assays (Andersen, 1979). All precipitation steps were performed for 20–40 min at 273–277 K, recovering the precipitate after centrifugation at 10000*g* for 15–30 min.

Rubisco was first recovered as a precipitate between 10 and 20%(*w*/*v*) PEG 3000 (Fluka 81227, highest purity) in 'enzyme buffer' with 1 m*M* $MgCl_2$ added to both the extract and the PEG solution. The precipitate was dissolved in 20 ml buffer, and Rubisco again recovered as a PEG 3000 precipitate, this time between 6 and 12% PEG in the presence of 10 m*M* $MgCl_2$. After an initial ammonium sulfate precipitation (at 50% saturation) to remove traces of PEG, the protein was further fractionated by $(NH_4)_2SO_4$ precipitation, recovering Rubisco as a precipitate between 26 and 39% saturation (from an initial volume of 15 ml). Anion-exchange chromatography on a Pharmacia FPLC system (at room temperature) with a Source Q 6 ml column was used for further purification. The protein was eluted using a gradient of 0–0.5 *M* NaCl in 120 ml 'enzyme buffer' with 0.1 m*M* DTT, at a flow rate of 3 ml min^{-1}, monitoring OD_{280}. The fractions containing Rubisco were pooled, and Rubisco was precipitated by adding $(NH_4)_2SO_4$ to 50% saturation. The Rubisco preparation was then rechromatographed on the same column, this time developed with Bis–Tris propane–HCl buffer pH 6.80 with 0–0.5 *M* NaCl, 0.1 m*M* EDTA and 0.1 m*M* DTT. Rubisco now eluted at a lower NaCl concentration, allowing further purification. The protein was again concentrated by $(NH_4)_2SO_4$ precipitation (at 50% saturation) and dissolved in 5 ml 'enzyme buffer'. 2 ml samples were next applied to a Pharmacia Superdex 200 (16/60) gel-filtration column, developed with 'enzyme buffer' with 0.1 m*M* DTT plus 0.1 *M* NaCl at 1 ml min^{-1} (room temperature). The purified Rubisco was concentrated from the pooled fractions by $(NH_4)_2SO_4$ precipitation (at 50% saturation). The Rubisco pellet recovered after centrifugation for 15 min at 10000*g* was dissolved in a small volume of 'enzyme buffer' with 50%(*w*/*v*) glycerol (Sigma Ultrapure). The Rubisco samples were stored at 253 or 193 K.

A. eutrophus ATCC17707 chromosomally encoded wild-type Rubisco is referred to as isoform I in the following, while *A. eutrophus* ATCC17697 wild-type Rubisco is referred to as isoform II.

2.3. Crystallization

For the initial crystallization experiments, Rubisco from *A. eutrophus* purified using a slightly modified version of the procedure described previously (Andersen, 1979) was used. The protein was isolated from the 'enzyme buffer' by dialysis overnight against a weak buffer solution, usually 20 m*M* HEPES pH 7.5 or Tris–HCl pH 7.8. A wide range of conditions for crystallization were tested using both commercial screens (Hampton Research I and II; Jancarik & Kim, 1991; Cudney *et al.*, 1994) and other non-commercial screens. Several methods were tested: hanging and sitting drops (Ducruix & Giegé, 1992), microbatch, streak and macroseeding and even crystallization in gels (Robert & Lefaucheux, 1988; Provost & Robert, 1991). Hundreds of tiny crystals were obtained both in sitting drops and in microbatch trials with ammonium sulfate, sodium acetate, sodium succinate, K_2HPO_4, PEG 4000/LiCl and PEG 4000/NaCl, but these were all too small for data collection.

The protein samples purified using the latter procedure were equilibrated against the precipitation agents listed above using the vapour-phase diffusion method. To avoid possible dissociation of the subunits at low salt concentration, the protein was isolated from the 'enzyme buffer' by precipitation with saturated ammonium sulfate and then dissolved in 20 m*M* HEPES buffer pH 7.5. The salt was removed by stepwise addition of 20 m*M* HEPES pH 7.5 during centrifugation in a Millipore ultrafree microconcentrator (molecular-weight cutoff 100 kDa). Finally, the enzyme was concentrated to about 40 mg ml^{-1}. Drops were prepared immediately by mixing 2 µl of the protein solution with 2 µl of the well solution. The only exception was for experiments set up with K_2HPO_4 as a precipitation agent where all drops were prepared in advance by mixing equal volumes of protein solution (40 mg ml^{-1}) with 0.7 *M* K_2HPO_4 pH 8.4. This mixture was spun for about 5 min to remove possible seeds before placing the drops over wells containing K_2HPO_4 with concentrations ranging from 0.5 to 1.0 *M*. All crystallization experiments were carried out at 277 K.

2.4. Data collection

For Rubisco isoform II a data set was collected at the EMBL X11 beamline at the DORIS storage ring, DESY, Hamburg (λ = 0.9117 Å). Before flash freezing, the crystals were transferred to a cryo-protectant solution containing 9% PEG 4000, 1 *M* NaCl and 25% ethylene glycol. The data set was collected at 100 K using an MAR Research image-plate detector with a crystal-to-detector distance of 560 mm and an oscillation angle of 0.5°.

For Rubisco isoform I a data set was collected at the Swiss–Norwegian beamline (BM01, λ = 0.873 Å), ESRF, Grenoble (Contribution 98–2). Using 1 *M* K_2HPO_4 and 25% ethylene glycol as a cryo-protectant solution, data were collected at 110 K on an MAR Research image-plate detector with a crystal-to-detector distance of 420 mm and an oscillation angle of 0.25°.

The data sets were processed using *DENZO* (Otwinowski, 1993), whereas the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994) was used for scaling, merging and molecular-replacement calculations.

3. Results and discussion

3.1. Purification

Cloning of the *A. eutrophus* Rubisco genes in the Rubisco minus strain AE951 allowed expression of the duplicate *A. eutrophus* Rubisco genes separately for subsequent purification and crystallization experiments. Production of large amounts of *A. eutrophus* Rubisco of high purity was facilitated by fermentor growth to high cell densities and induction of Rubisco to high cellular levels (20–40% of total cellular protein). The combination of PEG and $(NH_4)_2SO_4$ precipitations alone gave 80–90% pure Rubisco preparations, while final purity was >98–99%, as judged by SDS–PAGE. A yield of 50–100 mg of purified Rubisco per litre of culture was obtained.

Rubisco isoform I was first purified by the described procedure, but without the two final steps (ion-exchange chromatography at pH 6.8 and gel filtration). The resulting preparations were of high purity (>96–98%), as judged by SDS–PAGE. However, since numerous crystallization experiments resulted only in tiny crystals, two extra purification steps were included, as described in §2. Furthermore, Rubisco isoform II was included in the experiments.

3.2. Crystallization

Using PEG 4000 and NaCl as precipitating agents, Rubisco isoform II purified by the new procedure crystallized as square plates with more-or-less rounded edges after 4–5 d. The best crystals, with maximum dimensions 0.3 × 0.3 × 0.02 mm, were obtained in hanging drops equilibrated against 8.5% PEG 4000 and 1 *M* NaCl, HEPES buffer pH 7.5.

Later crystallization experiments with Rubisco isoform I purified by the new procedure resulted in large single crystals, using K_2HPO_4 as a precipitating agent (Fig. 1). The best crystals of isoform I (square plates with sharp edges and maximum dimensions 0.3 × 0.3 × 0.05 mm) were obtained in sitting drops equilibrated against 0.7–0.8 *M* K_2HPO_4 pH 8.4. Both isoforms crystallize in space group $P4_32_12$. The crystals of type II, unit-cell dimensions a = 111.8 and c = 400.0 Å, diffract to 3.2 Å, whereas type I (unit-cell dimensions a = 112.0 and c = 402.7 Å) diffract to 2.7 Å.

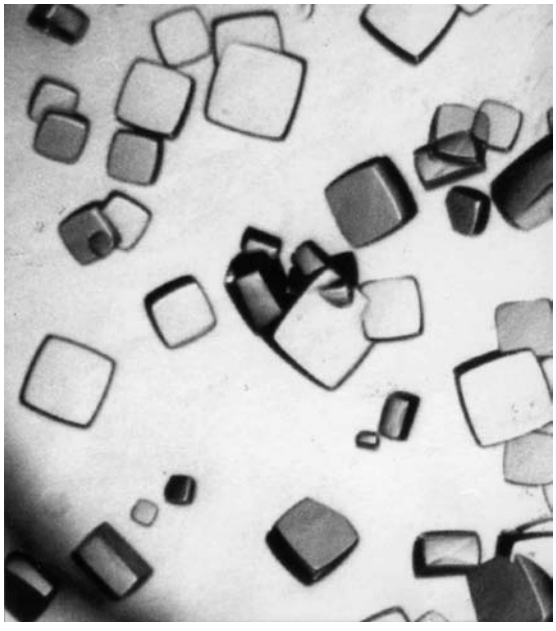

Figure 1
Crystals of *A. eutrophus* Rubisco, isoform 1. (Photograph by Gunvor Granaas, University of Tromsø.)

3.3. Data collection and analysis

The data set collected for Rubisco isoform II was 87.8% complete to 3.5 Å and contained a total of 175278 measured reflections which were reduced and merged to 28825 unique reflections, of which 81.4% had $I > 2\sigma(I)$. The multiplicity was at least 5.5 in each resolution shell and the resulting overall R factor based on intensities of both symmetry-related and reflections recorded several times ($R_{\rm merge}$) was 14.7%. The relatively high $R_{\rm merge}$ could partly be explained by the high multiplicity of the data sets, which for isoform II lies between 5.6 and 6.7. Since $R_{\rm merge}$ increases with the multiplicity, it is not necessarily a good measure of the quality of a data set (see Weiss & Hilgenfeld, 1997). The data set for Rubisco isoform I, collected later, contained 286973 reflections which were merged to give 65785 unique reflections. The $R_{\rm merge}$ was 12.5% to 2.7 Å (8.9% to 3.0 Å) and 87.5% of the possible data were collected to 2.7 Å, with an average multiplicity of 4.4. 76.3% of the reflections had $I > 2\sigma(I)$.

Rubisco from *A. eutrophus*, spinach (Andersson *et al.*, 1989) and *Synechococcus* (Newman & Gutteridge, 1993) crystallize in different space groups, but the unit-cell volume is approximately the same (5×10^6 Å^3). In the spinach crystals (space group $C222_1$, Z = 8) the asymmetric unit is L_4S_4, whereas for *Synechococcus* (space group $P2_12_12_1$, Z = 4) the asymmetric unit is a complete L_8S_8 which implies that the asymmetric unit in the *A. eutrophus* crystals is L_4S_4. This is verified by the Matthews coefficient, V_m, (Matthews, 1968) which is 2.27 Å^3 Da^{-1} corresponding to a solvent content of 45.8%, assuming L_4S_4 in the asymmetric unit.

In the molecular-replacement calculations (*AMoRe*; Navaza, 1994) a modified L_4S_4 model of Rubisco from spinach (Brookhaven PDB code 1AUS) was used as a search object; the amino-acid sequence of the spinach enzyme was replaced with the sequence of Rubisco from *A. eutrophus* (Andersen & Caton, 1987) and the geometry was regularized using *O* (Jones *et al.*, 1991). The data set for Rubisco isoform II, in the resolution range 10–4.5 Å, was used in the rotation and translation functions. The solution from the rotation search had a height of 6.3σ, whereas the next peak was at 3.7σ. The Crowther & Blow (1967) translation function gave a solution with a correlation coefficient of 0.22 and an R factor of 0.53. Rigid-body refinement using *AMoRe* resulted in a correlation coefficient and an R factor of 0.24 and 0.52, respectively. Visual inspection of the crystal packing using *O* (Jones *et al.*, 1991) revealed no bad contacts between neighbouring molecules. The results were later confirmed by using the data set collected for Rubisco isoform I and a polyalanine model.

Figure 2
Stereo diagram showing four small subunits of Rubisco from *A. eutrophus* where the C-terminal regions together form a new eight-stranded antiparallel β-barrel.

Model building and refinement is in progress and preliminary results have shown that the overall fold of the subunits and their relative location are very similar to the L_8S_8 Rubisco structures solved previously. The exception is the C-terminal regions of the small subunits, which are extended by 35 amino-acid residues compared with the spinach enzyme and which form a completely new structural motif not observed in any previous Rubisco structure (see Fig. 2).

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