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Crystallization and crystallographic investigations of the small subunit of mouse ribonucleotide reductase

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Abstract The R2 protein component of mouse ribonucleotide reductase has been obtained from overproducing *Escherichia coli* bacteria. It has been crystallized using NaCl as precipitant. The crystals are orthorhombic, space group $C222_1$ with cell dimensions a = 76.9 Å, b = 108.9 Å, c = 92.7 Å and diffract to at least 2.5 Å. The asymmetric unit of the crystals contains one monomer. Rotation and translation function searches using a model based on the weakly homologous *E. coli* R2 gave one significant peak. Rotation about a crystallographic 2-fold axis parallel to the a-axis produces an R2 dimer with dimer interactions very similar to those found for *E. coli* R2.

Key words: Mouse ribonucleotide reductase; DNA synthesis; Crystallization; Molecular replacement; Iron protein

1. Introduction

Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides, necessary for the synthesis of DNA [1–5]. The enzyme catalyzes the reduction of all 4 ribonucleotides. The active enzyme in eukaryotes and *E. coli* consists of 2 homodimeric subunits denoted R1 and R2. The large subunit, R1, contains substrate binding sites as well as effector binding sites for overall activity and substrate specificity. Redox active cysteines involved in the reduction of substrates are also located to R1. The small subunit, R2, contains a dinuclear iron center and a tyrosyl-free radical, which is necessary for activity. The free radical has been located to Tyr^{122} by site directed mutagenesis [6]. The mechanism of substrate reduction involves a free radical reaction, which is initiated by abstraction of a hydrogen atom at the 3′ position of the ribose [7].

The most widely studied RNR is the *Escherichia coli* enzyme for which the 3-dimensional structure of both subunits R1 and R2 have been determined [8–10]. The structure of *E. coli* R2 is 70% helical with 13 helices of which 8 long helices form a bundle. The dinuclear iron center as well as the Tyr^{122} harboring the free radical is found in the interior of the protein. The distance of Tyr^{122} to the nearest surface is 10 Å and the distance of Tyr^{122} to the nearest iron atom is 5.3 Å.

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Although *E. coli* R2 in many respects serves as a model for ribonucleotide reductases in higher organisms, differences between these enzymes exist and the sequence identity is only about 25% between the bacterial and mouse enzymes [11,12]. A study of the magnetic interaction between the radical and the iron center in *E. coli* R2 and mouse R2 indicated a stronger magnetic interaction in mouse R2 than in *E. coli* R2 [13]. The sensitivity of the *E. coli* R2 and mouse R2 towards radical scavengers and iron chelators is also different and points towards a more open structure around the iron/radical in the mouse protein [14–16]. Unlike the *E. coli* R2, the free radical-iron center in mouse R2 is labile and the protein loses 50% of its iron after 30 min at 37°C [15–17]. Therefore, it has to be continuously regenerated in vivo in a reaction which requires ferrous iron and oxygen.

In mouse R2, the C-terminal 7 residues are essential for subunit interactions while the C-terminal peptide with the same function in *E. coli* R2 is much longer [18]. The mouse C-terminal heptapeptide was shown to be highly flexible and unstructured by NMR studies, but on addition of R1 it becomes rigid and structured [19].

The differences between the *E. coli* and mammalian enzymes prompted us to solve the structure of the mammalian R2 as part of a long term project aiming at the development of specific cytostatic drugs. The mouse R2 sequence has been deduced from isolated cDNA clones encoding the mouse RNR. The sequence has 390 amino acids and corresponds to a molecular weight of 45 kDa for the monomer [11]. Mouse R2 is now available as recombinant protein in high yields [17]. In this paper we describe crystallization of mouse R2 as well as crystallographic characterization of these crystals.

2. Methods

2.1. Purification

The mouse R2 was purified as described earlier [17]. Depending on the fraction used, the protein preparation was heterogeneous because some proteolysis occurs during purification. The protein preparation may be considered as consisting of the apo mouse R2 since the purified protein contains less than 10% of iron, where 2 atoms of iron per mol of R2 polypeptide is 100%. The protein solution was concentrated to 7.5 mg/ml in 50 mM Tris/HCl buffer pH 7.5 using Centricon 30 (Amicon) filters.

2.2. Crystallization

Crystallizations were performed using the hanging drop vapor diffusion method. The best crystals were obtained by equilibration of a 8 μl drop consisting of 4 μl of a 7.5 mg/ml solution of mouse R2 and 4 μl of a reservoir solution containing 0.8–1.0 M NaCl in 50 mM acetate buffer pH 4.7, against 1 ml of the above mentioned reservoir solution

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at 20°C. NaCl concentrations of 0.8–1.2 M give crystals, while concentrations higher than 1.2 M tend to give very small crystals or simply just needles. The pH value in the crystallization solution with 1 M NaCl and 50 mM acetate buffer has been measured to 4.5 at 20°C. Crystals were grown in Petri dishes as well as in Costar culture plates depending on batch to batch variations.

2.3. Data collection

Diffraction data to 2.8 Å resolution were collected on a Nicolet multiwire area detector at 4°C using a Rigaku rotating anode. Data were evaluated using the Buddha program package [20], initially in a primitive orthorhombic space group as found by the auto-indexing program. The space group was determined by inspection of measured reflections using the program Precess (William Furey, Pittsburgh). From the systematic absences the space group was found to be $C222_1$. Data from two crystals were scaled using the programs Rotavata and Agrovata implemented in the CCP4 package (Daresbury, England) and gave R_{merge} = 6.5–11.3% (on intensity). An almost complete data set was collected on these two crystals with R_{merge} = 10.6%. A new data set was collected on the synchrotron in Daresbury, line 9.6 using a Rigaku R-axis IIC image plate detector. The data were evaluated using DENZO [21] and merged with an R_{merge} of 7.8%. The data set is 92.3% complete for the resolution range 15–2.5 Å.

2.4. Molecular replacement

A homologous model of mouse R2 based on the *E. coli* R2 structure was built using O [22]. The amino acid side chains in *E. coli* R2 were replaced by the side chains in mouse R2 according to the sequence alignment [12]. Other side chain rotamers were allowed for the substituted residues when necessary. In case of deletions, the connections between the residues were made as close as possible to the original structure by using the lego functions in the O program. The model was refined in the program package X-PLOR [23] by running 200 cycles of positional refinement in order to optimize stereochemical parameters.

Because the residues of the carboxy terminal in *E. coli* R2 cannot be located in the structure, these residues are not included in the model of mouse R2. The mouse R2 protein has an amino terminal which is 56 residues longer than the *E. coli* R2 and these amino acids have not been included in the mouse R2 model. Of the 390 amino acids in the mouse R2 sequence 299 have been included in the model.

The model of the monomer was used in the molecular replacement application with the rotation and translation functions implemented in X-PLOR. The triclinic search cell was a right angle cube of 100 Å, the maximum Patterson vector length was 30.0 Å and the resolution range was 15.0–4.0 Å. The output from the rotation function was refined using the Patterson correlation refinement of rotation function peaks also implemented in X-PLOR.

The translation function was tried out for the two highest peaks in the rotation function. Peak number 2 in the refined rotation function output gave the best translation solution. For this solution, a dimer is formed by rotation about a 2-fold axis parallel to the crystallographic a-axis, which is very similar to the R2 dimer of the *E. coli* enzyme.

The packing of the subunits in the unit cell was examined using the program O. The subunit interaction areas had only a few close contacts and the side chain conformations can be corrected before the model is subjected to rigid body refinement in X-PLOR.

In parallel, the CCP4 package program AMORE [24] was applied using a poly-alanine model but the same Patterson vector length and resolution range. The rotation and translation function produced the same solution as X-PLOR. The correlation coefficient for the highest peak in the rotation function was 13.9, and for the translation function is was 34.7. After Patterson correlation refinement, the correlation increased to 54.0. The *R*-factor for the molecular replacement solution was 50%.

Fig. 1. Rod-shaped crystals of mouse R2. The crystal is 0.14 × 0.16 × 0.8 mm.

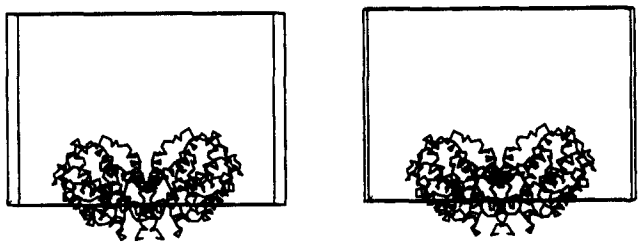

Fig. 2. Stereoscopic drawing of the packing of one mouse R2 dimer in the unit cell viewed perpendicular to the ab plane (a vertical, b horizontal).

2.5. Soaking of ferrous ions into R2 crystals

A crystal of the size 0.1 × 0.12 × 0.4 mm was soaked in a solution containing 50 mM acetate buffer pH 4.7, 1 M NaCl and 5 mM $FeCl_2$ for 18 h. Data collected on a Nicolet multiwire area detector were 91% complete in the resolution range 60–2.8 Å. The R_{sym} for these reflections were 8.9% and the isomorphous differences 16%.

3. Results

Rod-shaped crystals (Fig. 1) were obtained using the hanging drop vapor diffusion method against 0.8–1.0 M NaCl in 50 mM acetate buffer pH 4.7. Some of the crystals had holes ranging to narrow clefts going through the center of the crystals. In this

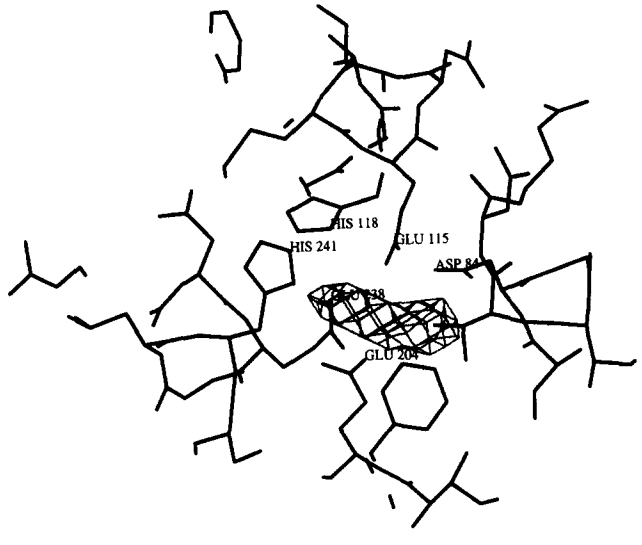

Fig. 3. A F_{Fe}–F_{Apo} difference Fourier map calculated from data collected on a crystal which had been soaked in a solution of $FeCl_2$. The map is contoured at 3 σ and shows an extended peak at the expected iron binding site. Iron liganding side chains in the model are labeled.

respect they resemble crystals of *E. coli* R2 [25]. This phenomenon seems to be coupled to a fast growth rate since crystals that appeared after 1–2 days were more prone to develop a hole. The first crystals can be observed after 1–4 days and grow larger during the following week. Crystals may also be obtained after 10 or 21 days but then they are usually small.

The inhomogeneity of the mouse R2 protein consisting of a mixture of N-terminal degradation products as well as the full length protein did not seem to be crucial for the crystallization, since crystals were obtained from degraded fractions (fractions containing increasing amounts of the 43.5, 41.5 and 39 kDa polypeptides) as well as from less degraded fractions as judged by inspection of SDS-gels of the purified protein. An SDS-gel with starting material as well as a carefully washed and dissolved crystal showed that the crystal contained predominantly the full length protein but also a mixture of peptides of different chain lengths. This inhomogeneity may hinder growth of larger crystals.

The crystals are orthorhombic, space group $C222_1$, with cell dimensions a = 76.9 Å, b = 108.9 Å, c = 92.7 Å. The unit cell parameters correspond to a V_M = 2.16 Å^3/Da, which gives a water content of about 43%, common for protein crystals [26]. There is only one monomer in the asymmetric unit in contrast to the crystals of *E. coli* R2, where the asymmetric unit contains a dimer. The crystals diffract to at least 2.5 Å at synchrotron radiation.

Molecular replacement methods gave one significant solution and the same solution using different program packages. When the monomer of R2 is placed according to this solution, a dimer is formed by crystallographic 2-fold symmetry operation. The shape and the subunit interactions of this dimer are very similar to those of the *E. coli* R2 dimer (Fig. 2).

The purified mouse R2 protein has low iron content since the iron center is unstable and it is expected to be practically iron free at the crystallization conditions at pH 4.7. Soaking experiments with $FeCl_2$ produced one positive density peak in a difference Fourier map at the expected iron binding sites (Fig. 3) demonstrating that the molecular replacement solution is essentially correct. No other significant peak was found in the $F_{Fe}-F_{Apo}$ difference Fourier, using phases calculated from the Apo-R2 model. The experiment also demonstrates that it is possible to obtain iron containing R2 in the present crystal form.

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