Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression and preliminary crystallographic studies of R1E, the large subunit of ribonucleotide reductase from *Salmonella typhimurium*

Malin Uppsten,[a] Mathias Färnegårdh,[a]† Albert Jordan,[b] S. Ramaswamy[a]‡ and Ulla Uhlin[a]*

[a]Swedish University of Agricultural Sciences, Department of Molecular Biology, Box 590, Uppsala Biomedical Center, S-751 24 Uppsala, Sweden, and [b]Department of Genetics and Microbiology, Autonomous University of Barcelona, E-08193 Barcelona, Spain

† Formerly Mathias Eriksson (until 24 July 1999). Present address: Karo Bio AB, Novum, S-141 57 Huddinge, Sweden.
‡ Present address: Department of Biochemistry, The University of Iowa, Iowa City, IA 52242, USA.

Correspondence e-mail: ulla@xray.bmc.uu.se

Received 6 January 2003
Accepted 26 March 2003

The *nrdE* gene product R1E, the large subunit of the class 1b *Salmonella typhimurium* ribonucleotide reductase, has been overexpressed, purified and crystallized. Initially, the protein crystallized in two orthorhombic space groups, $C222_1$ and $P2_12_12$, using tartrate and PEG 6000 as precipitants, respectively. Better diffracting crystals belonging to the tetrahedral space group $P4_32_12$ were obtained using sodium malonate as precipitant. The $P4_32_12$ crystals could only be obtained after seeding from a drop containing $C222_1$ crystals grown in sodium tartrate. Thus, streak-seeding resulted in crystals of a supergroup to $C222_1$. Data to 2.8 Å resolution have been collected on the $P4_32_12$ crystals which contained one R1E subunit in the asymmetric unit.

1. Introduction

Ribonucleotide reductase (RNR) catalyses the *de novo* synthesis of all four deoxyribonucleotide building blocks for DNA synthesis by reducing the corresponding ribonucleotides. This synthesis is carefully transcriptionally and allosterically regulated in most organisms. The substrate-reduction mechanism involves the use of a radical (Ehrenberg & Reichard, 1972; Reichard & Ehrenberg, 1983). Depending on the availability of cofactors and oxygen, three different types of probably unrelated radical-generation mechanisms have evolved and define three classes of RNRs (Reichard, 1993, 1997).

The most well studied enzyme is that from *Escherichia coli*, which is the prototype for class I reductases, which are present in eukaryotes, several viruses and bacteria. It consists of two homodimeric proteins, R1 and R2. Protein R2 contains the radical-generating machinery. It contains a diferric iron centre and a buried tyrosyl radical in its active form (Larsson & Sjöberg, 1986). R1 contains the active site, redox-active cysteinyl residues and two allosteric binding sites. The binding of ATP or dATP to one of the allosteric effector sites, called the overall activity site, determines whether the enzyme is active or inhibited, respectively (Thelander & Reichard, 1979). The second effector-binding site changes the substrate specificity by binding different dNTPs or ATP. This site is tuned to produce balanced levels of dNTPs appropriate for the particular organism. The structures of R1 (Uhlin & Eklund, 1994) and R2 (Nordlund *et al.*, 1990) from *E. coli* have been determined.

In this paper, we describe the expression, purification and crystallization of the large subunit of a differently regulated class I ribonucleotide reductase. This enzyme has now been discovered in many organisms, particularly pathogenic bacteria; the enzymes from different sources share limited sequence similarities and have a similar molecular composition. The most interesting difference compared with the normal class I enzymes is the lack of negative regulation by high concentrations of dATP (Eliasson *et al.*, 1996). This suggests that the enzymes lack the allosteric overall activity site. From the structure of *E. coli* R1 in complex with the ATP analogue AMPPNP, the overall activity site was shown to be located in the first 100 residues of the N-terminal domain (Eriksson *et al.*, 1997). The R1 chains of the new class I are considerably shorter in the N-terminus.

To distinguish the new type from the *E. coli* class I prototype, the old enzymes with two allosteric binding sites were named class Ia and the new enzymes with only one effector-binding site were named class Ib. Both types of reductases are present in *E. coli* and *S. typhimurium*. The class Ib enzyme is fully functional in these organisms but seems not to be essential, while the Ib form is the active RNR in *Mycobacterium tuberculosis* (Yang *et al.*, 1994), *Lactococcus lactis* (Jordan *et al.*, 1996) and *Bacillus subtilus* (Scotti *et al.*, 1996); class Ib genes have also been found in *Mycoplasma genitalium* (Fraser *et al.*, 1995) and *M. pneumonia* (Himmelreich *et al.*, 1997).

The *S. typhimurium* genes *nrdE* and *nrdF* were the first to be sequenced and cloned and the *S. typhimurium* enzyme is the prototype of

the class Ib enzymes (Jordan *et al.*, 1994). The gene products of the class Ia ribonucleotide reductases are named R1A and R2B and those of class Ib are named R1E and R2F. The structure of R2F has been determined to 2.0 Å resolution with the iron site in both the ferrous and ferric state (Eriksson *et al.*, 1998). We have developed high-level expression systems and protein-purification procedures for the class Ib proteins from *S. typhimurium*. Below, we describe the expression and purification of the catalytic subunit R1E and the crystallization and data collection in three different space groups.

2. Material and methods

2.1. Expression of the *S. typhimurium* R1E protein

In order to express the *nrdE* gene of *S. typhimurium* at high levels, an *Nde*I restriction site was engineered in the translation start codon. The entire gene was introduced into the *Nde*I-restricted pET24a (Novagen). Cloning of genes with a translation start codon into the *Nde*I site allows high-level expression when T7 RNA polymerase is induced by addition of IPTG in the host strain BL21(DE3) (Novagen).

In *S. typhimurium,* translation of the *nrdE* gene starts with a TTG codon which is read as methionine; this residue is subsequently removed by N-terminal processing (Jordan *et al.*, 1994). To achieve good overproduction of the R1E protein, substitution of the start codon TTG by an ATG codon was required. By introducing an *Nde*I restriction site into the start codon (5′-CATATG-3′), this was also accomplished. For this purpose, the primers PrTTGup (5′-**CATATG**GCAACA-ACTACC-3′), containing an *Nde*I site (in bold), and PrTTGlow (5′-TCTTCCAG-CATTTTCAT-3′) (antisense), annealing to the start region of the *nrdE* gene and 0.7 kbp downstream from this position, respectively, were used to amplify the 5′ extreme of the gene by PCR. Plasmid pUA725 containing the *S. typhimurium nrdHIE* genes (3 kbp), obtained by 3′ exonuclease III deletion from plasmid pUA335 (Jordan *et al.*, 1994), was used as a template. 30 cycles of amplification were used with annealing at 323 K. The 0.7 kbp product was gel-purified and cloned into the pGEM-T (Promega Corp.) vector according to the manufacturer's protocol. The original 5′ extreme of the *nrdE* gene present in plasmid pUA725 containing the TTG start codon was substituted by the PCR-generated fragment using a *Hin*cII restriction site internal to the gene. The reconstituted gene (2.2 kbp) was obtained after *Nde*I restriction of the resulting plasmid and cloned into the pET24a plasmid previously digested with the same restriction enzyme and dephosphorylated with alkaline phosphatase. The ligation mix was transformed into *E. coli* DH5aF′ (ClontechLab. Inc.). The plasmid (pUA726) from one clone unambiguously confirmed to code for *nrdE* was transformed to *E. coli* BL21(DE3) cells. General cloning procedures were carried out by standard methods (Sambrook *et al.*, 1989). Restriction enzymes and other enzymes were from Boehringer Mannheim. Oligonucleotide primers were from MWG-Biotech (Germany).

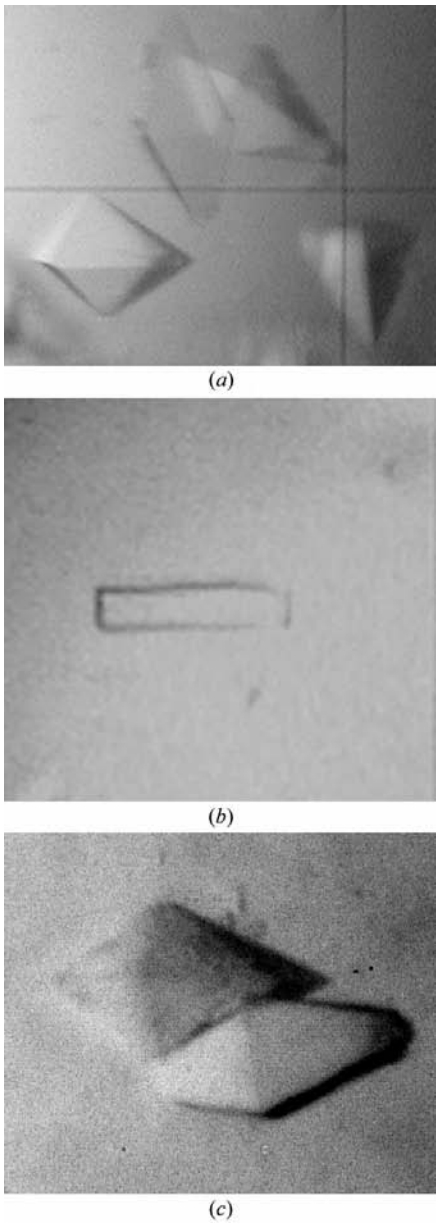

Figure 1
Three crystal forms of R1E. (*a*) Space group $C222_1$, size 0.5 × 0.2 × 0.2 mm. (*b*) Space group $P2_12_12$, size 0.5 × 0.1 × 0.05 mm. (*c*) Space group $P4_32_12$, size 0.4 × 0.15 × 0.15 mm.

The pUA726 clone containing the nrdE protein was grown aerobically to the logarithmic phase (OD_{600} = 0.7) in 8 × 1 l Luria broth with 50 mg l^{-1} kanamycin at 303 K. The cultures were cooled to 288 K by transferring the shakers to 277 K. 30 min after the change in temperature, the cells were induced with 0.8% IPTG and left for 10–12 h. The bacteria were harvested by centrifugation (3000*g*) at 277 K, resulting in about 30 g (wet weight) of bacteria.

2.2. Purification

Bacteria were suspended in Tris–HCl pH 7.5, 50 m*M* KCl, 1 m*M* phenylmethanesulfonyl fluoride (PMSF) and 10 m*M* dithiothreitol (DTT) containing lysozyme at 0.6 mg ml^{-1} (Eliasson *et al.*, 1992). The purification was carried out at 277 K. After disruption of the cells by sonication, the cell debris was removed by centrifugation at 10 000*g*. Streptomycin sulfate was added to a final concentration of 1%(*w*/*v*) and the turbid solution was centrifuged. Finally, proteins were precipitated by slow addition of solid ammonium sulfate to 70% saturated concentration. The precipitate was dissolved in 50 m*M* Tris–HCl pH 8.0, 10 m*M* PMSF and 10 m*M* DTT and desalted on a pd10 Sepharose G25 (Pharmacia Biotech) column. The desalted fractions were applied to a POROS-20 HQ column (Pharmacia). R1E protein interacts weakly with the column material and elutes in a delayed peak without a salt gradient. The eluted fractions were pooled, concentrated and the buffer carefully changed to MES pH 6.5 with Centriprep 30 (Amicon). The concentrated samples were absorbed onto a POROS-20 HS column and eluted with a 0–0.5 *M* KCl gradient. The fractions containing R1E protein were pooled and the pH was increased to 7.5 before concentration with Centricon-30 (Amicon). Protein concentrated to 20–30 mg ml^{-1} in 50 m*M* HEPES pH 7.5 and 10 m*M* DTT was rapidly frozen in appropriate aliquots before use.

2.3. Crystallization setup

R1E was crystallized using the hanging-drop vapour-diffusion method in 24-well boxes (Falcon). The hanging drops contained a 1:1 mix of protein solution (20 mg ml^{-1} R1E protein) and reservoir solution. The drops varied in size from 3 to 9 µl and the wells contained 500 µl. Crystal Screen I from Hampton Research was used. Chemicals were purchased from Sigma.

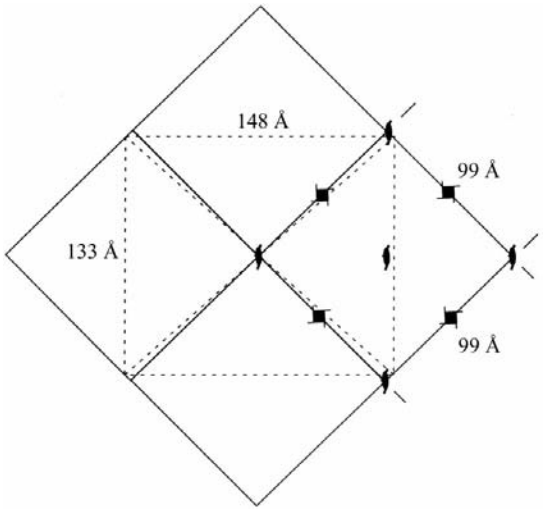

Figure 2
Relationship between the orthorhombic $C222_1$ and tetragonal $P4_32_12$ or $P4_12_12$ lattices in the R1E crystals, showing a minor rotation difference in the *c* plane. The $C222_1$ cell (dotted lines) is inscribed in four $P4_32_12$ or $P4_12_12$ cells. The lengths of the *a* and *b* axes are marked.

Table 1
Statistics of data collection.

Values in parentheses are for the last resolution shell. Prior to data collection, the crystals were cryocooled in liquid nitrogen using 25–30% ethylene glycol as cryoprotectant together with the same concentration of precipitating agent as in the crystallization setup. All data were collected at 100 K.

Space group	$C222_1$	$P2_12_12_1$	$P4_32_12$
Unit-cell parameters			
a (Å)	133	59	99
b (Å)	148	158	99
c (Å)	291	170	289
$\alpha = \beta = \gamma$ (°)	90	90	90
Solvent content (%)	68	44	73
Monomers per AU	2	2	1
Synchrotron beamline	Lund	ESRF BM14	ESRF ID14-1
Resolution (Å)	30–3.2	2.5/3.5 (anisotropic)	40–2.8
R_{merge} (%)	8.2 (28.2)		7.7 (41.4)
No. observations	115496		178638
No. unique reflections	38952		37293
Redundancy	2.97		4.79
Average $I/\sigma(I)$	12.5 (3.2)		17.4 (2.7)
Completeness (%)	97.4 (95.5)		98.0 (99.9)

3. Results and discussion

3.1. Crystallization of R1E and data collection

Three crystal forms were obtained. Two of the crystal forms appeared after 3–6 months and continued growing for another three months. The third form appeared after a few days but could only be obtained after streak-seeding from drops containing one of the other crystal forms.

The initial crystal form $C222_1$ grew from a reservoir containing 0.8 *M* tartrate (sodium/potassium or potassium salt) pH 7.5 (No. 29 in Crystal Screen I, Hampton Research). Seeding decreased the crystal-growth time and crystals started to appear after one week and finished growing after one month. A data set to 3.2 Å could be collected at the Max II lab synchrotron in Lund from a crystal of dimensions of ∼0.2 mm. The unit-cell parameters were $a = 133$, $b = 148$, $c = 291$ Å (Fig. 1*a*). There were two R1E monomers in each asymmetric unit and the solvent content was calculated as 68%. The data were indexed and scaled using the *HKL* program suite (Otwinowski, 1993).

The space group of the second crystal form was $P2_12_12_1$, with unit-cell parameters $a = 59$, $b = 158$, $c = 170$ Å (Fig. 1*b*). The crystals grew with an elongated shape ($0.5 \times 0.1 \times 0.05$ mm) in 8% PEG 6000 pH 7.5. This form had a solvent content of 44%, with two R1E monomers per asymmetric unit. A data set was collected on a double crystal at ESRF station BM14, giving two superimposed diffraction patterns. The diffraction patterns could be separated by manual picking using *MOSFLM* (Leslie, 1994). Unfortunately, the data were significantly anisotropic, with diffraction to around 2.5 Å resolution along one axis but only to 3.5 Å resolution along the other.

The third and best-diffracting crystal form, space group $P4_32_12$ or its enantiomorph, had unit-cell parameters $a = 99$, $b = 99$, $c = 289$ Å (Fig. 1*c*). Subsequent studies using molecular replacement have revealed $P4_32_12$ to be the correct space group. The crystals contain only one R1E monomer per asymmetric unit and have a solvent content of 73%. They grow in 1 *M* sodium malonate with 50 m*M* TES pH 7.2 after seeding with material from drops containing $C222_1$ crystals of R1E. Material was transferred with a cat whisker by streak-seeding from a drop containing 0.6–0.8 *M* potassium/sodium tartrate pH 7.5. Seeding was performed after 2–7 d in hanging drops with an approximate protein concentration of 17 mg ml^{-1}. Crystals appear within a week of seeding.

When comparing the unit-cell axes of the two space groups, the following can be stated. The lengths of the $a = b$ cell axes (99 Å) of the $P4_32_12$ space group correspond to a $C222_1$ subgroup with unit-cell axes $a = b = 140$ Å, showing an increase in the *a* axis of 7 Å and a decrease in the *b* axis of 8 Å. The *c* axes in the two space groups are of approximately the same length. Fig. 2 illustrates the relationship between the $C222_1$ and the $P4_32_12/P4_12_12$ space groups. Data from the $P4_32_12$ crystals were collected to 2.8 Å at ESRF station ID14-1. Data collection and statistics for the different space groups are summarized in Table 1.

This work was supported by the Swedish Research Foundation and the Swedish Cancer Foundation. We thank Hans Eklund for useful comments on the manuscript.

References

Ehrenberg, A. & Reichard, P. (1972). *J. Biol. Chem.* **247**, 3485–3488.
Eliasson, R., Pontis, E., Fontecave, M., Gerez, C., Harder, J., Jörnvall, H., Krook, M. & Reichard, P. (1992). *J. Biol. Chem.* **267**, 25541–25547.
Eliasson, R., Pontis, E., Jordan, A. & Reichard, P. (1996). *J. Biol. Chem.* **271**, 26582–26587.
Eriksson, M., Jordan, A. & Eklund, H. (1998). *Biochemistry*, **37**, 13359–13369.
Eriksson, M., Uhlin, U., Ramaswamy, S., Ekberg, M., Regnström, K., Sjöberg, B. M. & Eklund, H. (1997). *Structure*, **5**, 1077–1092.
Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavagne, A. R., Sutton, G., Kelley, J. M., Fritchman, J. L., Weidman, J. F., Small, K. V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T. R., Saudek, D. M. & Venter, J. C. (1995). *Science*, **270**, 397–403.
Himmelreich, R., Plagens, H., Hilbert, H., Reiner, B. & Herrmann, R. (1997). *Nucleic Acids Res.* **25**, 701–712.
Jordan, A., Pontis, E., Åslund, F., Hellman, U., Gibert, I. & Reichard, P. (1996). *J. Biol. Chem.* **271**, 8779–8785.
Jordan, A., Pontis, E., Atta, M., Krook, M., Gibert, I., Barbe, J. & Reichard, P. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 12892–12896.
Larsson, A. & Sjöberg, B.-M. (1986). *EMBO J.* **5**, 2037–2040.
Leslie, A. G. W. (1994). *MOSFLM* User Guide: *MOSFLM* version 5.20. Cambridge: MRC Laboratory of Molecular Biology.
Nordlund, P., Sjöberg, B. M. & Eklund, H. (1990). *Nature (London)*, **345**, 593–598.
Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Reichard, P. (1993). *Science*, **260**, 1773–1777.
Reichard, P. (1997). *Trends Biochem. Sci.* **22**, 81–85.
Reichard, P. & Ehrenberg, A. (1983). *Science*, **221**, 514–519.
Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. New York: Cold Spring Harbor Laboratory Press.
Scotti, C., Valbuzzi, A., Perego, M., Galizzi, A. & Albertini, A. M. (1996). *Microbiology*, **142**, 2995–3004.
Thelander, L. & Reichard, P. (1979). *Annu. Rev. Biochem.* **48**, 133–158.
Uhlin, U. & Eklund, H. (1994). *Nature (London)*, **370**, 533–539.
Yang, F. D., Lu, G. Z. & Rubin, H. (1994). *J. Bacteriol.* **176**, 6738–6743.