Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic analysis of the recombinant N-terminal domain of riboflavin synthase

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Received 11 April 2000
Accepted 21 June 2001

Riboflavin synthase catalyzes the final step in the biosynthesis of riboflavin. Animals and humans lack this enzyme, whereas many bacteria and certain yeasts are absolutely dependent on endogenous riboflavin synthesis. Riboflavin synthase is therefore an attractive target for chemotherapy. The N-terminal domain of riboflavin synthase forms a dimer in solution and is capable of strongly binding riboflavin. It can serve as a model for the binding site of the native enzyme. Structural information obtained from this domain at high resolution will be helpful in the determination of the binding mode of riboflavin and thus for the development of antimicrobial drugs. Here, the crystallization and preliminary crystallographic analysis of the N-terminal domain of riboflavin synthase are reported. The crystals belong to the space group $C222_1$, with unit-cell parameters $a = 50.3$, $b = 104.7$, $c = 85.3$ Å, $\alpha = \beta = \gamma = 90^\circ$, and diffract to 2.6 Å resolution.

1. Introduction

Riboflavin (vitamin B_2) is the precursor of coenzymes which are essential for a wide variety of enzyme-catalyzed reactions and as optical transponders. Animals are absolutely dependent on an exogenous supply of the vitamin. Plants and many microorganisms obtain it by *de novo* biosynthesis. Gram-negative bacteria and certain yeasts are absolutely dependent on endogenous synthesis as they are unable to absorb flavins from the environment owing to the absence of an uptake system. The enzymes of the riboflavin pathway have been shown to be essential in enterobacteria. They are also likely to be essential in mycobacteria and in pathogenic yeasts. The enzymes are therefore potential targets for antimicrobial chemotherapy. In the light of the rapid development of antibiotic resistance, the identification of novel antibiotic targets is urgent.

Riboflavin synthase catalyzes the final step in the biosynthesis of the vitamin. The enzyme-catalyzed reaction involves the dismutation of 6,7-dimethyl-8-ribityllumazine (3) affording riboflavin (4) and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (1) (Fig. 1). The latter product is recycled in the biosynthetic pathway *via* the enzyme 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase). Riboflavin synthases from eubacteria and fungi are homotrimers with a molecular weight around 75 kDa. In Bacillaceae, riboflavin synthase and lumazine synthase form a 1 MDa complex. More specifically, a riboflavin synthase trimer is enclosed in a capsid of 60 lumazine synthase subunits with icosahedral 532 symmetry (Bacher *et al.*, 1980, 1986; Ladenstein *et al.*, 1986). The riboflavin synthases of methanobacteria have no detectable similarity to those from eubacteria and fungi.

The riboflavin synthase subunits of eubacteria and fungi are characterized by internal sequence similarity. 26 identical amino-acid residues are shared between the N-terminal and C-terminal half of the peptide from *Bacillus subtilis* and 25 in the peptide from *Escherichia coli* (Fig. 2) (Eberhardt *et al.*, 1996; Schott *et al.*, 1990). It was therefore proposed that each subunit folds into two topologically similar domains. The homotrimeric riboflavin synthase could then be described as a quasi-hexamer with pseudo-32 symmetry. In light of the internal sequence similarity, it was proposed that each domain accommodates one lumazine substrate molecule and that the reaction proceeds at the interface between two domains whose close apposition would bring the two substrate molecules required for the dismutation reaction into close proximity with antiparallel orientation.

Riboflavin synthases of *E. coli* and *B. subtilis* have been crystallized in our laboratory. The best crystals of the *E. coli* enzyme diffracted to a resolution of 3.3 Å. A preliminary crystallographic analysis of riboflavin synthase from *E. coli* revealed non-crystallographic threefold local symmetry with additional twofold symmetry axes perpendicular to the threefold symmetry axis (Meining *et al.*, 1998). This finding supported the hypothesis of the hypothetical two-domain model. The crystallization

of riboflavin synthase of *E. coli* has also been reported by Jordan & Thompson (1997). The crystals of riboflavin synthase from *B. subtilis* did not diffract X-rays.

Recently, Eberhardt & Bacher (2000) showed that both the N-terminal and C-terminal parts of the *E. coli* riboflavin synthase subunit can be expressed as soluble proteins in a recombinant bacterial host. The N-terminal domain was found to form a homodimer which can bind riboflavin, 6,7-dimethyl-8-ribityllumazine and several analogues with high affinity. This artificial protein therefore appears to be a valid structural model for riboflavin synthase. Moreover, in the light of the ligand-binding properties, the artificial protein may be suitable for the screening of chemical libraries. In this paper, the preliminary crystallographic characterization of the artificial protein domain is reported.

2. Materials and methods

2.1. Cloning, expression and purification

A recombinant *E. coli* strain with a plasmid specifying the amino-acid residues 1–97 of *E. coli* riboflavin synthase was cultured as described previously (Eberhardt *et al.*, 1996). The bacterial cells were harvested and disrupted as described. The supernatant was passed through a column of Sepharose Q (2 × 15 cm), which was developed with a gradient of 0–1 *M* sodium chloride and 25 m*M* Tris–HCl pH 8.2. Fractions containing the desired protein were intensely yellow coloured. Fractions were combined according to colour and protein content and were concentrated by ultrafiltration. The solution was passed through a Superdex 75 column, which was developed with 70 m*M* phosphate pH 7.0 containing 100 m*M* sodium chloride. Fractions were combined and concentrated by ultrafiltration.

2.2. Crystallization and data collection

Crystallization experiments were performed *via* the vapour-diffusion method using sitting drops. A solution containing 7 mg ml^{-1} protein in 70 m*M* sodium/potassium phosphate pH 7.0 containing 100 m*M* sodium chloride was saturated with riboflavin. 2 µl aliquots of this solution were mixed with 2 µl of reservoir buffer and equilibrated against reservoir buffer containing 1.0 *M* sodium chloride and 0.1 *M* Tris–HCl pH 8.0. Monocrystalline plates with maximum dimensions of 60 × 60 × 20 µm appeared after 2 d (Fig. 3). 4 d later most of the drops contained precipitated protein.

For diffraction experiments, the crystals were flash-frozen in reservoir solution containing 15% glycerol. A complete data set was collected in angular steps of 1.5° at a wavelength of 0.8424 Å and a distance of 250 mm using a MAR image-plate system with a diameter of 180 mm (Table 1). The data were collected at the BW7B beamline of the EMBL Outstation (DESY, Hamburg). The data were evaluated with the *HKL* package (Otwinowski, 1993) and a self-rotation function was calculated using the program *AMoRe* as implemented in the *CCP*4 crystallographic package (Collaborative Computational Project, Number 4, 1994).

Table 1
Data-collection parameters and statistics.

Values in parentheses are for the outer resolution shell.

Wavelength (Å)	0.8424
Crystal-to-detector distance (mm)	250
Oscillation range (°)	1.5
Plate diameter (mm)	180
Resolution (Å)	20–2.60 (2.63–2.60)
Observations (unique) ($I_o > 0$)	68513 (7202)
Completeness (%)	99.8 (100)
$\langle I\rangle/\langle\sigma(I)\rangle$	20.1 (3.4)
Space group	$C222_1$
Unit-cell parameters (Å, °)	a = 50.3, b = 104.7, c = 85.3, $\alpha = \beta = \gamma$ = 90
$R_{\rm merge}$†	0.070 (0.455)
Subunits per asymmetric unit	2 (V_M = 2.65 Å^3 Da^{-1})

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$.

3. Results and discussion

A peptide comprising amino-acid residues 1–97 of *E. coli* riboflavin synthase was expressed in a recombinant *E. coli* host strain and was purified as described in §2. The protein was crystallized from a solution containing the recombinant domain and riboflavin using sodium chloride as precipitant. The crystals formed plates with maximum dimensions of 60 × 60 × 20 µm (Fig. 3). They were yellow coloured owing to the presence of bound riboflavin. Despite

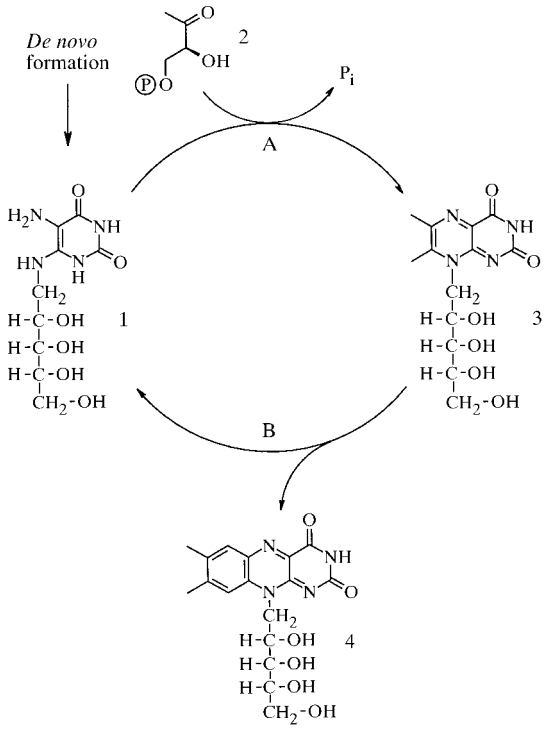

Figure 1
Biosynthesis pathway of riboflavin.

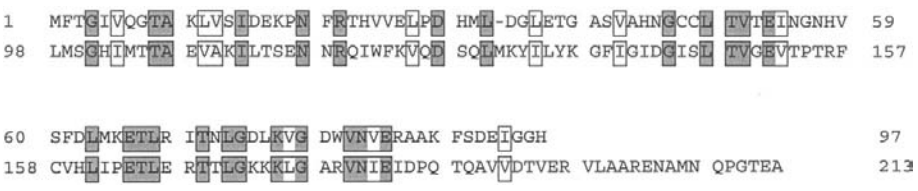

Figure 2
Intramolecular sequence alignment of *E. coli* riboflavin synthase. Identical residues are outlined and shaded; similar residues are outlined.

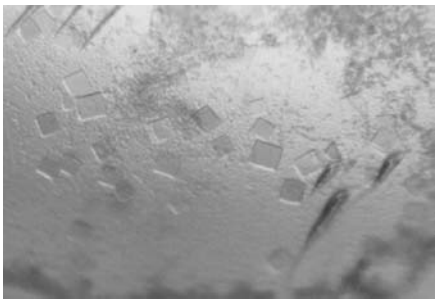

Figure 3
Crystals of the N-terminal domain of *E. coli* riboflavin synthase.

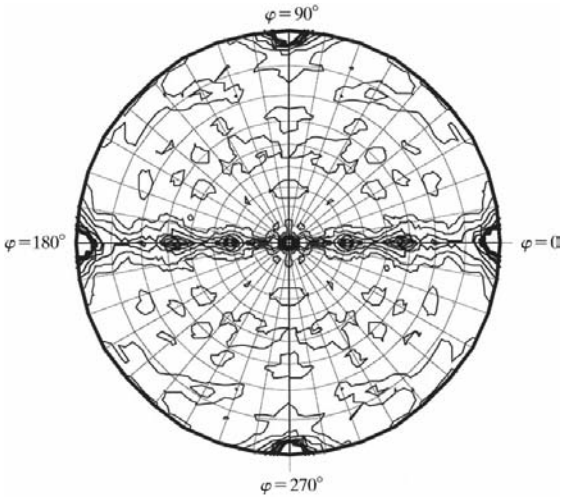

Figure 4
Stereographic projection of the self-rotation function at the section $\chi = 180^\circ$. The radius of integration was 28 Å and the resolution limits were 19.7–3.0 Å. Peaks arising from non-crystallographic symmetry are observed at $(\theta, \varphi, \chi) = (32.2, 90, 180^\circ)$ (4.4σ) and $(\theta, \varphi, \chi) = (19.5, 90, 180^\circ)$ (2.6σ). Contours are drawn in steps of $6.0/10.0\sigma$(Rf). The plot was generated with the help of *MOLREP* (Vaguine *et al.*, 1999).

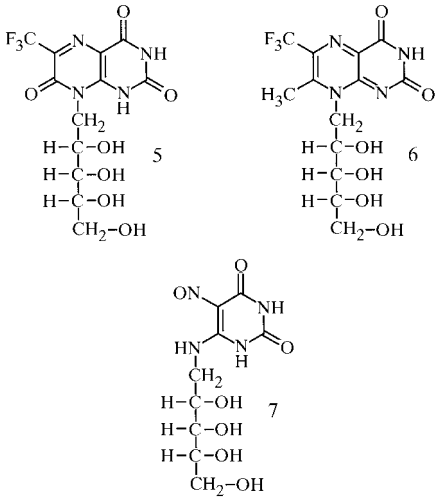

Figure 5
Molecular structures of 6-(trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine (5), 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (6) and 5-nitroso-6-(ribitylamino)-2,4-(1*H*,3*H*)-pyrimidindione (7).

their moderate size, the crystals diffracted synchrotron X-ray radiation to 2.6 Å resolution. The space group was determined to be $C222_1$, with unit-cell parameters $a = 50.3$, $b = 104.7$, $c = 85.3$ Å, $\alpha = \beta = \gamma = 90^\circ$. A search for local symmetries in the self-rotation function revealed twofold non-crystallographic symmetry at $(\theta, \varphi, \chi) = (32.2, 90, 180^\circ)$ and $(\theta, \varphi, \chi) = (19.5, 9^\circ, 180^\circ)$ (Fig. 4). Peak intensities with respect to the crystallographic twofold axes were 29.0% (Rf/σ = 4.4) and 17.6% (Rf/σ = 2.6). The observation of non-crystallographic twofold symmetry is in agreement with the assumption of one dimer per asymmetric unit. This assumption gives a Matthews coefficient $V_M = 2.65$ Å^3 Da^{-1}, which is typical for proteins (Matthews, 1968). The ability of the crystals to bind riboflavin suggests that a riboflavin-binding site is formed at the interface of two associated monomers and supports the assumption that the dimer in the crystals corresponds to the dimer in solution. Whether the riboflavin-binding site mimics the interaction of the C-terminus with the N-terminus or a homologous interaction of C- or N-terminus cannot be judged based on the present data. Otto & Bacher (1981) showed previously that riboflavin synthase from *B. subtilis* binds one molecule of riboflavin per protomer.

The formation of riboflavin by dismutation of 6,7-dimethyl-8-ribityllumazine is catalyzed by riboflavin synthase but can also proceed spontaneously. Earlier studies on the regiospecificity of the reaction suggest that it requires the simultaneous presence of two substrate molecules with antiparallel orientation at the active site of the enzyme (Plaut & Beach, 1975, 1976).

In line with this hypothesis, ligand-binding studies have shown that the homotrimeric riboflavin synthase can bind up to six molecules of the substrate analogues 6-(trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine (5) or 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (6) (Fig. 5) (Cushman *et al.*, 1992). It was demonstrated that both compounds are displaced under addition of either riboflavin (4) or 5-nitroso-6-(ribitylamino)-2,4-(1*H*,3*H*)-pyrimidindione (7), an inhibitor closely related to the second enzyme product (1), indicating the close vicinity of the binding sites for both the substrate and the two products.

Ligand-perturbation studies monitored by NMR suggested that the homotrimeric protein can accommodate bound ligands in at least four different environments (Scheuring *et al.*, 1996). This indicated that the homotrimer could be inherently asymmetric. In other words, the local symmetry elements observed by low-resolution X-ray crystallographic analysis appeared to describe an approximate rather than a perfect symmetry of the protein molecule.

On the other hand, NMR ligand-perturbation studies indicate that the two ligand-binding sites of the artificial domain dimer are equivalent. Moreover, $^1H^{15}N$ heterocorrelation NMR experiments provide evidence that the recombinant domain dimer obeys strict *C*2 symmetry in the presence as well as in the absence of bound riboflavin (unpublished data).

The binding affinity of riboflavin to the N-terminal domain exceeds that of the native enzyme. Based on its ligand-binding properties, the recombinant N-terminal domain of riboflavin synthase may be useful for the screening of chemical libraries. From future crystallographic work, we expect to gain knowledge about the binding mode of riboflavin. The elucidation of the three-dimensional structure of the recombinant N-terminal domain could also benefit the structural analysis of the full-length protein. This structural knowledge will make it possible to design compounds which can mimic the way riboflavin is bound to the enzyme. These compounds might eventually develop into potent inhibitors of the enzyme.

This work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, the Karolinska Institute and the Stockholm County Council. We gratefully acknowledge expert help by Jordi Benach with the data collection and the possibility of collecting diffraction data at the EMBL Outstation (DESY synchrotron, Hamburg).

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