Crystal Structure of an Archaeal Pentameric Riboflavin Synthase in Complex with a Substrate Analog Inhibitor

STEREOCHEMICAL IMPLICATIONS

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Whereas eubacterial and eukaryotic riboflavin synthases form homotrimmers, archaeal riboflavin synthases from Methanocaldococcus jannaschii and Methanothermobacter thermoautotrophicus are homopentamers with sequence similarity to the 6,7-dimethyl-8-ribityllumazine synthase catalyzing the penultimate step in riboflavin biosynthesis. Recently it could be shown that the complex dismutation reaction catalyzed by the pentameric M. jannaschii riboflavin synthase generates riboflavin with the same regiochemistry as observed for trimeric riboflavin synthases. Here we present crystal structures of the pentameric riboflavin synthase from M. jannaschii and its complex with the substrate analog inhibitor, 6,7-dioxo-8-ribityllumazine. The complex structure shows five active sites located between adjacent monomers of the pentamer. Each active site can accommodate two substrate analog molecules in anti-parallel orientation. The topology of the two bound ligands at the active site is well in line with the known stereochemistry of a pentacyclic adduct of 6,7-dimethyl-8-ribityllumazine that has been shown to serve as a kinetically competent intermediate. The pentacyclic intermediates of trimeric and pentameric riboflavin synthases are diastereomers.

Riboflavin (vitamin B2) serves as the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), essential cofactors for several oxidoreductases that are indispensable in most living cells. The work on riboflavin biosynthesis in microorganisms has been covered extensively in recent reviews (1–4). Riboflavin is biosynthesized in plants, many bacteria, and in fungi but not in animals. Therefore, enzymes of this pathway have been proposed to be attractive targets for antimicrobial strategies (5–7).

In the final steps of the biosynthetic pathway, lumazine synthase (LS) catalyzes the condensation of the pyrimidinedione (1) with 3,4-dihydroxy-2-butanoate-4-phosphate (2) to release water, inorganic phosphate and 6,7-dimethyl-8-ribityllumazine (DMRL) (8, 9), and riboflavin synthase (RS) catalyzes a dismutation of DMRL (3) affording riboflavin (4) and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1) (Fig. 1); more specifically, that reaction involves the transfer of a four-carbon moiety between two DMRL molecules serving as donor and acceptor, respectively (10–13). Both reactions are thermodynamically irreversible (9, 14) and can proceed in the absence of a catalyst (11, 15–17).

LS from fungi are C₅-symmetric homopentamers, whereas the enzymes from plants, Archaea, and most eubacteria studied are 532 symmetric capsids of 60 identical subunits, which are best described as dodecamers of pentamers. The subunit folding patterns of pentameric and icosaehedral LS are similar. In Bacillaceae, lumazine synthase and riboflavin synthase form a complex comprising an icosaehedral capsid of 60 lumazine synthase subunits and a core of three riboflavin synthase subunits; historically, these unusual enzyme complexes were designated heavy riboflavin synthase (18, 19). Riboflavin synthases from eubacteria are homotrimers where each subunit folds into two topologically similar domains, but the protein fails to obey trigonal symmetry. An active site is formed at the interface between the C-terminal domain of one subunit (serving as the donor site with regard to transfer of a for carbon moiety) and the N-terminal domain of an adjacent subunit (serving as acceptor site) (20).

Riboflavin synthases of Archaea show sequence similarity with lumazine synthases. In line with the sequence characteristics, RS from Methanocaldococcus jannaschii (MjaRS) has been shown to be a homopentamer in solution (21). This paper reports the crystal structure of that enzyme.

MATERIALS AND METHODS

Protein Preparation, Crystallization, and Data Processing—Cloning, expression, and purification of the protein have been described elsewhere (21). Crystals of wild-type MjaRS were grown at 18 °C using the sitting drop vapor diffusion method by mixing equal amounts of protein (6 mg/ml) in 100 mM potassium phosphate, pH 7.0, containing 30 mM Tris and 2 mM dithiothreitol with a reservoir solution containing 0.1 M HEPES, pH 7.0, and 40% 2-methyl-2,4-pentanediol. Crystals appeared within several days and belonged to space group P1 with cell parameters

8 The abbreviations used are: DMRL, 6,7-dimethyl-8-ribityllumazine; SeMet, selenomethionine; Mja, M. jannaschii; RS, riboflavin synthase; LS, lumazine synthase; MjaRS, RS from M. jannaschii; SpoLS, lumazine synthase from S. pombe; DORL, 6,7-dioxo-8-ribityllumazine; r.m.s.d., root mean square deviation.

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Structure of Riboflavin Synthase of M. jannaschii

Figure 1. Terminal reactions of the pathway of riboflavin biosynthesis. 1, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione; 2, 3,4-dihydroxy-2-butanoic acid; 3, 6,7-dimethyl-8-ribityllumazine; 4, riboflavin.

$\alpha = 41.9$ Å, $b = 72.9$ Å, $c = 72.7$ Å, and $\alpha = 68.9^\circ$, $\beta = 74.9^\circ$, $\gamma = 75.1^\circ$ corresponding to five monomers per asymmetric unit. Selenomethionine (SeMet)-substituted MjaRS (6 mg/ml) was crystallized by mixing equal amounts of protein (6 mg/ml) at 18 °C with a reservoir solution containing 0.1 M HEPES, pH 7.3, 0.1 M ammonium sulfate, and 20% polyethylene glycol 4000. The composition of cryoprotectant of the SeMet-substituted crystal was: 25% glycerol, 100 mM potassium phosphate, 100 mM HEPES, pH 7.0. These crystals belonged to space group $P_{6}_3$, with cell constants $a = b = 103.8$ Å, $c = 129.4$ Å and $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$, also containing five monomers per asymmetric unit.

The protein-inhibitor complex of native MjaRS with 6,7-dioxo-8-ribityllumazine (DORL) was obtained by adding the inhibitor in excess to native crystals. After an incubation period of 2 h, the crystals were flash-frozen in mother liquor in the cryostream. All crystals were measured at 100 K.

Data sets of the native protein and the protein-inhibitor complex were collected with a Mar Research image plate on a Rigaku rotation anode. Data were processed with MOSFLM (22) and scaled and merged with the CCP4 package (23). Data collection statistics are summarized in Table 1.

An single-wavelength anomalous dispersion dataset of SeMet crystals was collected at beamline ID14-4 at the European Synchrotron Radiation Facility (Grenoble, France). Data were processed using DENZO and SCALEPACK (24). Ten selenium positions were located using SnB (25). Refinement of heavy atom parameters and phase calculation was done with SHARP (26). The resulting electron density map was modified and improved by solvent flattening and non-crystallographic averaging in RESOLVE (27). Data collection and phasing statistics are summarized in Table 1.

Model Building and Refinement—An initial model of MjaRS (SeMet) was built manually using MAIN (28) and subsequently subjected to several cycles of refinement and manual rebuilding. The structure of native MjaRS was determined by molecular replacement using the program MOLREP of the CCP4 package (23) and the pentamer of the SeMet protein as a search model.

After rebuilding of the model, energy-restrained crystallographic refinement was carried out with maximum likelihood algorithms implemented in CNS (29), using the protein parameters of Engh and Huber (30) or REFMAC (31). Bulk solvent, overall anisotropic B-factor corrections and non-crystallographic restraints were introduced depending on the behavior of the free $R$ index. The complex crystals suffered from the soaking procedure and showed a diffraction pattern with large and smeared spots which might explain the rather high $R$-values. Nevertheless, the density for the protein and the bound ligands is unambiguous.

Analysis and Graphical Representation—The pentacyclic reaction intermediates were energy minimized using SYBYL modeling software (32). Correct atom types, stereocenters, hybridization states, and bond types were defined, and Gasteiger-Hückel charges were assigned to each atom. Positioning of these intermediates in the active site formed
between adjacent monomers were carried out using the coordinates of the MjaRS-DORL complex and molecule A as the potential acceptor site. All interactions of molecule A of the complex structure, concerning its pteridine ring system and ribityl side chain persist in the model of the intermediate placed in the active site.

Stereochemical parameters were assessed with PROCHECK (33). Protein structures were three-dimensionally aligned with TOP3D (34), figures were prepared with MOLSCRIPT (35) and PYMOL (36).

**Protein Data Bank Accession Codes**—The coordinates were deposited at the RCSB Protein Data Bank under the accession numbers 2B98 (native enzyme) and 2B99 (DORL complex).

**RESULTS**

Recombinant RS of *M. jannaschii* (MjaRS) was expressed and purified as described (21). Crystals of MjaRS grew in space group P1, containing one pentamer in the asymmetric unit. The crystals diffracted to a resolution of 2.3 Å, and the structure was solved by single-wavelength anomalous dispersion using selenomethionine-substituted protein, which was crystallized in the space group P6_3. An initial model was built in the experimental electron density and was used to position the molecule in the triclinic unit cell. Both crystal forms contain identical pentamers in the asymmetric unit that presumably correspond to the solution state of the protein.

The final model of the pentameric RS of *M. jannaschii* consists of 725 residues with a well defined protein backbone. Poor electron density is present at the C-terminal region for three subunits of the pentamer (residues 143–153), but most of the side chains are clearly defined, except for some surface-exposed residues. The pentamer has a size of roughly $80 \times 80 \times 45$ Å.

The MjaRS-monomer forms a three-layered ($\alpha\beta\alpha$) structure with a
Structure of Riboflavin Synthase of M. jannaschii

| Table 1 | X-ray data processing and final refinement statistics. |
|---------|------------------------------------------------------|
| Structure | SeMet | Native | Substrate analog complex |
| Crystal data | | | |
| Space group | P6₁ | P1 | P1 |
| Cell constants | a = b = 103.83 Å, c = 129.43 Å, β = 90°, γ = 120° | a = 41.77 Å, b = 72.69 Å, c = 72.70 Å, β = 74.61°, γ = 74.90° | a = 41.94 Å, b = 72.91 Å, c = 72.78 Å, β = 74.39°, γ = 74.53° |
| Data collection | | | |
| Wavelength (Å) | 0.9793 | 15,418 | 15,418 |
| Resolution range (Å) | 20.0–3.21 | 50.0–2.30 (2.38–2.30) | 20.0–2.22 (2.31–2.22) |
| Completeness (%) | 91.7 (93.4) | 96.0 (92.2) | 90.9 (71.9) |
| Unique reflections | 23,438 | 32,165 | 34,797 |
| Redundancy | 2.2 (2.2) b | 1.9 (1.8) | 3.4 (3.2) |
| I/σ | 14.1 (3.5) b | 12.2 (3.4) | 7.9 (2.9) |
| Ramachandran statistics | | | |
| Friedel-mates treated as independent reflections. |
| Figure of merit | 0.702 | 0.220 | 0.485 |
| Resolution range (Å) | 20.0–2.30 | 25.80 | 27.56 |
| Resolution range (Å) | 50.0–2.30 | 25.80 | 1.337 |
| Resolution range (Å) | 20.0–2.22 | 29.57 | |
| Resolution range (Å) | 2.31–2.22 | | |
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molecules are located in an anti-parallel orientation in the active site formed by two adjacent subunits facing very different environments (Fig. 3).

The binding of the substrate analog inhibitors shows that one molecule lies at the bottom of the catalytic center and forms a hydrogen bond network with protein subunit C involving its extended ribityl side chain and the pteridine ring-system (Figs. 4 and 5). The ribityl side chain forms hydrogen bonds with the main chain of nitrogen Gly41, nitrogen Ile42, nitrogen and oxygen Glu97 and with the carbonyl group of Asp44 (protein subunit E), and two hydroxyl groups (OH-2, OH-3) are linked to water molecules. In addition, the pteridine ring system of molecule A is sandwiched between the aromatic ring of Phe12 on one side and Ile62 and Cys76 on the other side (Fig. 5A). The carbonyl O-2 of A is in hydrogen-bonding distance to a water molecule, which in turn makes hydrogen bonds to the side chain of Thr10 and the backbone carbonyl of Met66. The amide group of Ile62 is directed toward the carbonyl O-4 and a similar interaction is found between the carbonyl O-4 and the backbone amide group of Gly68 (Fig. 5A).

This rigid fixation of molecule A orients it to the second substrate analog inhibitor (molecule B), bound with anti-parallel orientation at subunit E. The ribityl side chain of molecule B is exposed in the direction of the solvent, and the ribityl hydroxyl groups OH-1 and OH-4 interact with the side chain of Asp57 (chain C) and a water molecule,
whereas the ribityl hydroxyl group OH-3 is directed to the side chain of Arg145 (Fig. 5B). The side chain of Arg118, which forms a salt-bridge to Glu104, lies coplanar to the pteridine ring system. The NH2-group of Arg118 and O of Glu104 are in a distance of about 3.5 Å to the N-8. Further hydrogen bonds are observed between the carbonyl O-4 and the side chain of His122. The carbonyl O-2 forms a hydrogen bond to the peptide amide group of Gln146 and to a water molecule, which is coordinated by the backbone groups of O Glu146, nitrogen Phe148, and N-1 of the pteridine ring-system (Fig. 5, B and C).

Superposition of the four well defined substrate analog inhibitor pairs show no significant differences in the coordination and geometry (data not shown). The two pteridine ring systems are oriented at an angle of −60°. The pteridine ring system of the natural substrate DMRL has two methyl groups in position 6 and 7, while the inhibitor used in this study has two carbonyl groups at these positions. In the complex structure, the 7-carbonyl-O of molecule A points to the N-5 of molecule B at a distance about 2.8 Å (3.3 Å).

The residues of the active site involved in coordination of the two substrate analog inhibitors are highly conserved in putative archaeal riboflavin synthases (Fig. 6). In all sequences, the phenylalanine stacking to molecule A is found in a position corresponding to Phe12 of the MjARS. Structural superposition of the MjARS-DORL complex with the Schizosaccharomyces pombe LS-riboflavin complex (1KYV (38)) reveals that the pteridine ring system of molecule A lies coplanar to the isoalloxazin ring of riboflavin. Likewise, the ribityl moieties of this substrate analog inhibitor and riboflavin have a similar orientation, suggesting that molecule A marks the acceptor site and molecule B the donor site (Fig. 3).

The residue Ile42 at the opposite side of the pteridine ring system is also conserved. At position 76 of MjARS, archaeal riboflavin synthases display a serine or cysteine residue. Similarly for the second binding site (molecule B) of the substrate analog inhibitor, residues Asp73, His101, Arg118, His122, and Arg145 are all invariant in putative archaeal riboflavin synthases (Fig. 6).

**DISCUSSION**

Recent studies have identified a pentacyclic intermediate in the reaction trajectories of riboflavin synthases of an eubacterium, Escherichia coli, and an Archaeon, *M. jannaschii* which have been designated as Compound Q and Compound Q′, respectively (Fig. 7). Whereas these intermediates have identical constitutions, their respective cro-
mophores are enantiotopic, and the intermediates per se are diaste-
reotopic (since the ribityl side chains invariantly obey D-configuration in
both pentacyclic intermediates). Notably, the intermediate Q produced
by the eubacterial enzyme can serve as a kinetically competent substrate
for the eubacterial but not for the archaeal enzyme; the intermediate
Q/H11541 produced by the archaeal enzyme is a kinetically competent substrate
for the archaeal enzyme and not for the eubacterial enzyme (39).

The absolute stereochemistry of the pentacyclic intermediate of the
E. coli enzyme could be determined by the comparison between the
x-ray structures of the trimeric E. coli enzyme (that had been crystal-
lized without a ligand) and an artificial monomeric form of the S. pombe
enzyme (that had been co-crystallized with 6-carboxyethyl-7-oxo-8-
ribityllumazine), and the configurations at the newly formed ring car-
cbons were assigned to be 6R and 7S (Fig. 7) (20).

The DORL used for soaking experiments in the present study is isos-
teric with the natural substrate, DMRL. Hence, it appears plausible that
the topological relation of the two inhibitor molecules at the active site
emulate the topology of the actual substrate molecules prior to the
dismutation reaction. Naturally, that topology defines the sterical con-
straints for dimer formation. Unless one assumes very far-reaching con-
formational reorganization at the active site in the course of the reaction
(which appears unlikely in light of the rather rigid active site cavity), the
only possible stereochemical outcome of the dimerization catalyzed by
the pentameric Mja riboflavin synthase is the diastereomer Compound
Q/H11541 with 6S/7R configuration (Figs. 7 and 8).

We are not aware of any other case in the literature where a given
reaction proceeds via diastereomeric intermediates under the catalytic
influence of enzymes from two different species. On the other hand, we
must assume that the uncatalyzed formation of riboflavin from DMRL is
bound to proceed via both possible stereochemical trajectories,
although the free energy difference of the two diastereomers should
result in different velocities. In any case, the stereochemical information

FIGURE 6. Sequence alignment of 16 archaeal type riboflavin synthases. Gaps (denoted as dash) were introduced to optimize alignments. The alignment was produced using the tool CLUSTALW from EMBL-EBI (www.ebi.ac.uk). Active site residues are marked by asterisks. Strains and GenBank™ accession numbers are as follow: Aeropyrum pernix K1 (NP_147650); Archaeoglobus fulgidus DSM 4304 (NP_070245); Ferroglobus acidarmanus (ZP_00306970); M. jannaschii DSM 2661 (NP_248178); Methanococcus maripaludis S2 (NP_987300); Methanopyrus kandleri AV19 (NP_613646); Methanosarcina acetivorans C2A (NP_616743); Methanococcoides burtonii DSM 6242 (ZP_00148893); Methanosarcina Barkeri str. fusaro (ZP_00296068); Methanosarcina mazei GoeI (NP_632269); Methanothermobacter thermotrophicus str. delta H (NP_75277); Micrrophilus torridus DSM 9790 (YP_023491); Pyrobaculum aerophilum str. IM2 (NP_559706); Sulfolobus solfataricus P2 (NP_341940); Sulfolobus tokodaii str. 7 (NP_376265); Sulfolobus acidocaldarius DSM 639 (YP_255481).
of the pentacyclic chromophore is lost in the final steps of the trajectory, and the stereochemical course of the reaction does not influence the stereochemistry of the products (which is exclusively determined by the chiral polyol side chains).

The fragmentation of the pentacyclic intermediates affording the products riboflavin and 5-amino-6-ribitylamino-2,4(1\(H\),3\(H\))-pyrimidine-dione (1) is believed to proceed via two consecutive and highly plausible elimination steps (Fig. 7) (40). On the other hand, the trajectory conducive to dimerization is far from clear. Early work by Plaut, Wood, Pfeiderer, and their respective groups had established that the position 7 methyl group of DMRL is acidic with a \(pK_a\) around 9 (41). This unusually high CH acidity has been attributed to the resonance stabilization of the lumazine anion (13, 42–44). The authors quoted above have proposed several variations on a common theme for the transformation of DMRL into riboflavin (11, 39, 40, 45, 46). Based on the x-ray structures reported in this work and in the paper by Gerhardt et al. (20) the hypothetical mechanism involving tricyclic adduct forms of DMRL can now be ruled out (20, 46).

Following the discovery of the pentacyclic intermediate, the consensus mechanism arising from the early work of the Wood and Plaut groups could be easily modified to incorporate the experimentally observed intermediate. Nevertheless, the revised mechanism (40) remains a matter of speculation, and the three-dimensional structures that are now available fail to resolve that dilemma. Thus, further study will be required on this highly unusual reaction that is conducive to the formation of one of the most widely used redox cofactor classes.

The riboflavin synthases of Archaea have no detectable sequence similarity with those of eubacteria, yeasts and plants. However, their sequences and structures closely resemble those of 6,7-dimethyl-8-ribityllumazine synthases. Completely sequenced archaeal genomes typically comprise sets of two similar genes, which specify a riboflavin synthase and a lumazine synthase. Sequence arguments also showed that the divergence between the paralogous lumazine synthases and riboflavin synthases occurred early in evolution (21).

The five topologically equivalent active sites of pentameric lumazine synthases are located at the interfaces between adjacent monomers of the pentamer. Two substrates, 3,4-dihydroxy-2-butanone 4-phosphate (2) and 5-amino-6-(\(\beta\)-ribitylamino)-2,4(1\(H\),3\(H\))-pyrimidinedione (1), have to be bound in the active site (38, 47–52). Recent studies with the lumazine synthase from \(B.\) \textit{subtilis} suggested that the rate enhancement by the lumazine synthase is predominantly achieved by establishing a favorable topological relation of the two substrates.

The cavity harboring the active site of the closely related pentameric riboflavin synthase of \(M.\) \textit{jannaschii} is similar to that of lumazine synthases, but the enzyme has no detectable lumazine synthase activity.
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The binding mode of lumazine molecule A in pentameric riboflavin synthase closely resembles the pyrimidinedione binding site of lumazine synthases. On the other hand, the other part of the active site cavity forming the binding site for lumazine molecule B is structurally rather different compared with lumazine synthases (Fig. 3). Hence, it is not surprising that the activity of the ancestor is lost in pentameric riboflavin synthases.

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