Complex Structure of Bacillus subtilis RibG

THE REDUCTION MECHANISM DURING RIBOFLAVIN BIOSYNTHESIS

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Bacterial RibG is a potent target for antimicrobial agents, because it catalyzes consecutive deamination and reduction steps in the riboflavin biosynthesis. In the N-terminal deaminase domain of Bacillus subtilis RibG, structure-based mutational analyses demonstrated that Glu51 and Lys79 are essential for the deaminase activity. In the C-terminal reductase domain, the complex structure with the substrate at 2.56-Å resolution unexpectedly showed a ribitylimino intermediate bound at the active site, and hence suggested that the ribosyl reduction occurs through a Schiff base pathway. Lys151 seems to have evolved to ensure specific recognition of the deaminase product rather than the substrate. Glu290, instead of the previously proposed Asp199, would seem to assist in the proton transfer in the reduction reaction. A detailed comparison reveals that the reductase and the pharmaceutically important enzyme, dihydrofolate reductase involved in the riboflavin and folate biosyntheses, share strong conservation of the core structure, cofactor binding, catalytic mechanism, even the substrate binding architecture.

Flavin coenzymes are essential for a wide variety of physiological processes particularly the redox reactions and hence are ubiquitously found in all organisms (1). The precursor riboflavin is biosynthesized in plants and many microorganisms (2, 3). The imidazole ring of GTP is first hydrolytically opened with elimination of formate by GTP cyclohydrolase II to yield 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate (DAROPP).2 In eubacteria and plants, DAROPP is deaminated into 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (ARIPP) (Fig. 1) (4, 5). In most eubacteria, the responsible enzyme is a bifunctional protein such as Bacillus subtilis RibG (BsRibG), which is composed of an N-terminal deaminase domain (D domain) and a C-terminal reductase domain (R domain). In contrast, in fungi and some archaea, DAROPP is first reduced into 2, 5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (DARIPP) and then deaminated into ARIPP. The responsible enzymes are two separate proteins, Rib7 and Rib2 (6, 7). Furthermore, animals lack this biosynthetic pathway and hence must obtain riboflavin from dietary sources. Thus the enzymes of the riboflavin biosynthesis pathway have a strong possibility of becoming novel antimicrobial targets, particularly for the development of new chemotherapeutic agents to help defend against antibiotic-resistant pathogens (8, 9).

Recently, we have solved the tetrameric ring-like structure of BsRibG (10). The D domain belongs to the cytidine deaminase superfamily, in which the members catalyze the hydrolytic deamination of the base moiety of a variety of nucleotides including RNA, DNA, mononucleotides, and several therapeutically useful analogues (11). Despite no significant sequence similarity, the R domain is the only protein so far to share high structural homology with dihydrofolate reductase (DHFR). DHFR catalyzes the reduction of dihydrofolate into tetrahydrofolate, and many inhibitors of this enzyme have long been used clinically for the treatment of cancer, inflammation, and microbial infection (12–13).

The reduction mechanism of the ribose ring into a ribityl group is still inconclusive. Accumulation of an Amadori derivative in riboflavin-requiring B. subtilis mutants suggested that in bacteria, the reaction involves an Amadori rearrangement initiated by proton abstraction from C′2, followed by reduction of the C′2 carbonyl group (14). In contrast, feeding experiments using deuterium incorporation in the yeast Ashbya gossypii, suggested that the reduction occurs by hydride transfer to C′3, ruling out an Amadori mechanism (15). Recently, kinetic isotope studies of Escherichia coli RibD suggested a direct hydride transfer from the C′3-pro-R hydrogen of NADPH to C′2, and that this is the rate-limiting step in the overall deamination-reduction reaction (16). To gain structural insights into the substrate specificity, catalytic mechanisms, and inhibitor design, we have determined the structure of BsRibG as a complex with AROPP at 2.56-Å resolution. Together with the previous NADPH complex structure (10), a reduction mechanism...
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is proposed. In the D domain, mutational analysis of the predicted substrate binding residues was also carried out.

EXPERIMENTAL PROCEDURES

Preparation of Protein and Substrates—Site-directed mutagenesis was carried out using a QuikChange mutagenesis kit (Stratagene). Protein expression, purification, enzyme activity assay, and crystallization of the N-terminally His-tagged recombinant BsRibG were performed as previously described (10). The E51A mutant and wild-type crystals were grown in 28% polyethylene glycol 400, 200 mM CaCl₂, and 100 mM HEPES (pH 7.5) with a protein concentration of 20–25 mg/ml. The DAROPP compound was prepared from a reaction solution containing 5 mg/ml of recombinant E. coli cyclohydrolase II, 10–30 mM GTP, 8 mM MgCl₂, and 20 mM Tris-HCl (pH 8.0). The reaction was complete at around 10 min, and then 2 mg of BsRibG was added for production of AROPP. The reactions were monitored by UV absorption spectra recorded on a Beckman DU640B spectrophotometer.

Activity Assay—For measurement of the relative deaminase activity of the mutants, 20 μg BsRibG was incubated into the 1-ml reaction solution containing 0.1 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM dithiothreitol, and ~0.2 mM DAROPP for 20 min at room temperature. The deaminase activity was measured by monitoring change in absorbance at 292 nm. For the reductase assay, after the deamination was complete, 1 mM NADPH was added into the reaction mixture for 15–60 min at 37 °C. Diacetyl was added with a final concentration of 1% (v/v), and the mixture was incubated at 95 °C for 30 min. After centrifugation to remove protein precipitant, the fluorescence spectrum was recorded with an excitation wavelength of 408 nm. The relative reductase activity was estimated by the emission intensity at 485 nm.

Structure Determination—The reaction solution was added to the crystal drops for 30–60 min with a final concentration of ~10–15 mM DAROPP or AROPP. X-ray diffraction data from several crystals between 2.56–2.68 Å resolution were collected and processed at the beamlines BL13B1 at NSRRC (Hsinchu, Taiwan), and NW12 at the Photon Factory (Tsukuba, Japan). The crystals belong to the space group with one tetramer per asymmetric unit. The complex structure was determined using the molecular replacement method and refined using CNS (17). The statistics for the best data set are summarized in Table 1. Figs. 2, 3, B and C, 4, B and C, and 5 were generated by MolScript (18) and Raster3D (19), and Fig. 3A by BobScript (18).

RESULTS AND DISCUSSION

Substrate Preparation—Production of DAROPP and AROPP was monitored by UV-VIS absorption spectra because the distinct nucleobases of GTP, DAROPP and AROPP result in different spectra with different maximum wavelengths of 254, 293, and 284 nm, respectively. The DAROPP concentration was estimated using an absorption coefficient ε293 of 9600 M⁻¹ cm⁻¹ (20, 21), because GTP has hardly any absorption at this wavelength. The yields were consistent with previous studies, in which DAROPP formation accounts for ~90% of the E. coli cyclohydrolase II products (21). The AROPP concentration was estimated using NADPH consumption catalyzed by the R domain in term of the decrease in absorbance at 340 nm. The D domain of BsRibG produces AROPP as its main product at >80% of total conversion. In the absence of dithiothreitol, the reaction solution gradually turned yellow in a couple of hours with the maximum wavelength shifted from 284 to 245 nm and with a shoulder at 270 nm. Addition of 30 mM dithiothreitol into the reaction solution prevented the formation of the yellow color and the shift of the UV spectra.

Mutational Analysis in the D Domain—Asn²¹ and His⁴², His⁷⁶ and Lys⁷⁹, and Asp¹⁰¹ and Asn¹⁰³ were previously predicted to interact with the pyrimidine, phosphate and ribose of the substrate DAROPP, respectively (10). These six residues as well as the invariant glutamate in the family signature HXE, were substituted with alanine. No detectable deaminase activity was observed in the E51A and K79A mutants, whereas the other five mutants showed a decreased relative activity by 2–3-fold compared with the wild-type enzyme. As with other family members (22–26), the invariant glutamate in BsRibG, Glu⁵¹, may be also involved in the proton shuttle during the deamination process (Fig. 1), and hence substitution with alanine eliminates the deaminase activity. Previous studies showed that the dephosphorylated form cannot serve as a substrate (27). Thus as predicted, Lys⁷⁹ may interact with the phosphate group and such interactions would seem to be essential for the deaminase activity. In addition, in the soaking experiments, neither did AROPP bind to the D domain of the wild-type BsRibG, nor did DAROPP bind to the E51A mutant. In these crystal structures, the RibG unique loop containing His⁷⁶ and Lys⁷⁹ is flexible and swings away from the active site, and hence a substrate binding cavity may not be fully formed.

The Substrate Binding Site in the R Domain—BsRibG exists as a tetramer in solution (10). The four subunits (Molecules A-D) in the crystal asymmetric unit form a ring-like tetramer (Fig. 2). Significant strong electron density for the ligand was observed only in the R domain of Molecule C. The substrate DAROPP was initially modeled into the electron density, but the pyrimidine and the phosphate groups could not be modeled simultaneously into their respective electron dense areas (Fig.

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### Table 1

Statistics for data collection and structural refinement

| Data collection | Unit cell (Å) | 87.0, 108.3, 187.4 |
|-----------------|---------------|-------------------|
| Resolution range (Å) | 50–2.56 (2.65–2.56) |
| Total observations | 467764 (22291) |
| Unique reflections | 56499 (5427) |
| Completeness (%) | 97.7 (95.4) |
| R₁/σ₁<1> (%) | 26.2 (2.7) |
| Rmerge (%) | 6.2 (42.6) |

| Refinement | Resolution range (Å) | 50–2.56 (2.65–2.56) |
|-------------|----------------------|-------------------|
| Reflections (F > 0 σ) | 56277 (5283) |
| Rmerge (%) | 22.6 (34.5) |
| Nmerge (%) for 10% data | 27.9 (36.8) |
| R.m.s. deviations | Bond lengths (Å) 0.008 |
|                     | Bond angles (°) 1.38 |
|                     | Average B-factors (Å²) |
|                     | 10,970 Protein atoms 54.5 |
|                     | 4 Zinc ions 54.5 |
|                     | 191 Water molecules 51.3 |
|                     | AROPP intermediate 67.3 |

* Values in parentheses are for the highest resolution shell.
The proposed deamination and reduction mechanisms catalyzed by the D and R domains of eubacterial RibG, respectively. The inset illustrates the chemical structure of DARIPP.

FIGURE 2. The BsRibG tetramer. Molecules A-D are colored in red, blue, green, and black, respectively. Molecules A and C, as well as B and D, interact with each other via their D domains (the D-D interface), while Molecule A makes contacts with B, as well as C with D, through their R domains (the R-R interface). A ternary complex of the R domain was generated by a combination of the substrate and cofactor binary structures, with the ribitylimino intermediate and the cofactor NADPH displayed as ball-and-stick representations. The zinc ion in the D domain was shown as a magenta sphere.

3A). In addition, the carboxyl group of Glu290 would seem to form unfavorable interactions with the ribosyl O4, and the ribosyl geometry is distorted during the structure refinement.

Feeding experiments with A. gossypii suggested that the reduction occurs at C1, and the authors proposed a formation of a Schiff base intermediate (15). Interestingly, this proposed ribitylimino intermediate could be modeled nicely into the electron density (Fig. 3A) and forms more extensive interactions with the R domain than the substrate AROPP. The pyrimidine O2 interacts with Lys151 N2, the NH3 group with Thr171 O1, the O4 with Ile170 N and Thr171 N, and the NH25 with Ser167 O61 (Fig. 3B). The two ribosyl hydroxyl groups, OH2 and OH3, have close contacts with Asp199 O62, while OH4 with Glu290 O63. And the phosphate forms an extensive interaction network with Arg183 N91, Ser202 N, Leu203 N, and Arg206 N92.

Superposition of the four R domains in the tetramer showed multiple conformations that covered two regions, residues 167–171 and 202–212 (Fig. 3C). In Molecule C, upon AROPP binding, residues 167–171 and 202–212 moved a small amount to allow interaction with the pyrimidine ring and the phosphate group, respectively, particularly, the backbone of Ser167, Ile170, Thr171, Ser202, and Leu203. Similarly, when 10 mM NADPH was added into the BsRibG crystals for 4 days, strong density was also detected only in the R domain of Molecule C (10). Upon NADPH binding, residues 156–170 moved toward the cofactor and hence some conformational changes were induced, particularly affecting the backbones of Leu160 and Gly165. The backbones of residues 193–195 also shifted toward the pyrophosphate of the cofactor.

Structure Comparison within RibG, and Rib7—Several other crystal structures of R domain homologs have also been determined: E. coli RibD (EcRibD, PDB 2G6V for apo; 2OB for binary ribose 5-phosphate; and 2O7P for binary NADP+) (28), Thermotoga maritima RibG (TmRibG, PDB 2HXV for binary NADPH), Methanocaldococcus jannaschii Rib7 (MjRib7, PDB 2AZN for binary NADPH) (29) and the putative Rib7 from Corynebacterium diptheriae (PDB 2P4G). The R domains and Rib7s as well as DHFR, share diverse loop conformations but a virtually identical structure core including the central eight β-strands (βA-βH) and the four flanking helices (αB, αC, αE, and αF), with the secondary elements numbered as found in DHFR (30) (Fig. 4). The N-terminal half constitutes the majority of the active site and hence is more conserved with a sequence identity of 30–50%, whereas the C-terminal half is more diverse, with 15–30% identity (Fig. 4A). The most diverse region is the RibG/Rib7-unique insertion between the βD and αE, which has various lengths and a low sequence homology. This insertion forms a helix (αD') and a β-strand (βD') in BsRibG and MjRib7, but it is only a strand and a long loop in TmRibG and EcRibD (Fig. 4B).

The second diverse section is the R-R interfaces (the R-R interface), while Molecule A makes contacts with B, as well as C with D, through their R domains (the R-R interface). A ternary complex of the R domain was generated by a combination of the substrate and cofactor binary structures, with the ribitylimino intermediate and the cofactor NADPH displayed as ball-and-stick representations. The zinc ion in the D domain was shown as a magenta sphere.

The BsRibG tetramer. Molecules A-D are colored in red, blue, green, and black, respectively. Molecules A and C, as well as B and D, interact with each other via their D domains (the D-D interface), while Molecule A makes contacts with B, as well as C with D, through their R domains (the R-R interface).
Comparison of the Cofactor and Substrate Binding—A detailed comparison of the complex structures revealed that their cofactor adenosine-2′-phosphate moieties occupy distinct spatial positions and interact with different residues, for example Thr221 and Ser298 in BsRibG (10), Ser194, Ser234, Arg237, and His278 in EcRibD (28), Arg221, Lys285, and Glu289 in TmRibG and Ser83, Lys84, Arg86, Val134 in MjRib7 (29) (Fig. 4C). In contrast, the remaining parts of the NADPH cofactor occupy similar spatial positions and form similar interaction networks, particularly with the protein backbones. The binding architectures of the reactive nicotinamide rings in BsRibG, TmRibG, and MjRib7, and even in the DHFRs, are virtually identical.

Superposition of the AROPP intermediate in BsRibG against the ribose 5-phosphate in EcRibD reveals that the phosphate groups occupy a similar position and form virtually identical interactions with two conserved arginines and two backbone NHs (Fig. 4C). However, the sugar moieties point in opposite directions and hence distinct residues have been proposed to be involved in the proton transfer, namely Asp200 in EcRibD (28) and Glu290 in BsRibG. Ribose-5-phosphate may not be a suitable substrate analogue because it cannot mimic substrate binding due to the lack of the molecule pyrimidine ring and a ribitylimino intermediate. Moreover, the substrate AROPP could not be docked into the current EcRibD crystal structures (28), because the active site cavity is partially blocked by LβA-H9251 and hence there is no space to accommodate the pyrimidine ring (Fig. 4B).

The observed multiple “inactive” conformations of EcRibD may be due to the crystal packing effects. Unlike BsRibG and TmRibG, Molecules A and B in the dimeric EcRibD display very distinct conformations for LβA-H9251 (residues 159–173) and LβE-H9252 (residues 322–348) (Fig. 4B). These two loops form several hydrogen bonds and constitute a
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**Substrate Specificity** — During riboflavin biosynthesis, the deamination and reduction steps proceed in the opposite order when eubacteria and yeast are compared. Their substrates are distinct (Fig. 1), namely DAROPP with a cyclic ribose binding the D domain of RibGs versus DARIPP with a linear ribitol binding yeast Rib2; this has lead to the two deaminases evolving different residues for substrate recognition (10). In contrast, the reductase substrates, AROPP for the R domain and DAROPP for Rib7, display similar structures with the only difference being a carboxyl versus an amino group; in this case, these two reductases share several conserved residues for substrate binding (Fig. 4A). Particularly, Thr\(^{177}\) (or Ser), Arg\(^{183}\), Asp\(^{199}\), Arg\(^{206}\), and Glu\(^{290}\) in BsRibG are all conserved across plants, eubacteria, archaea, and fungi, indicating that RibG and Rib7 share a similar reduction mechanism through the ribitylimino intermediate. However, the R domain has to discriminate the deaminase product AROPP from the substrate DAROPP. The complex structure here clearly demonstrates that an invariant lysine (Lys\(^{151}\)) in eubacteria and plants but not in fungi and archaea, has evolved to ensure substrate specificity through favorable interactions of its amino side chain with the AROPP O2, and

major part of the R-R interfaces. In Molecule B, residues 174–177 make several direct contacts with residues 258–260 of symmetry-related Molecule A. These crystal contacts may result in the observed \(L_{\text{R}}/H^{11001}\) conformation in Molecule B (\(L_{\text{R}}/H^{11032}\)), and hence affect the \(L_{\text{R}}/H^{11001}\) conformation and hence the R-R interaction faces, even \(L_{\text{R}}/H^{11001}\) and \(L_{\text{R}}/H^{11032}\). The R-R interface in EcRibD is much less extensive than those present in BsRibG and TmRibG, with the buried areas of ~2600 Å\(^2\) versus ~4000 Å\(^2\) and ~3800 Å\(^2\), respectively, calculated by the PISA server (31). When NADP\(^+\) was soaked into the EcRibD crystal, the cofactor binding induced \(L_{\text{R}}/H^{11001}\) to move toward the cofactor allowing interaction with the nicotinamide moiety via Thr\(^{161}\), Ala\(^{164}\), and Trp\(^{170}\) (Fig. 4B); this consequently caused conformational shifts of \(L_{\text{R}}/H^{11001}\), \(L_{\text{R}}/H^{11032}\), and \(L_{\text{R}}/H^{11032}\). In BsRibG, only subtle structural shifts in \(L_{\text{R}}/H^{11001}\) were observed on binding of AROPP and NADPH. Furthermore, the current MjRib7 structure is also affected by crystal packing; MjRib7 exists as a dimer in solution, whereas a trimer is observed in the crystal (29).

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unfavorable interactions with the DAROPP NH$_2$\(^{2}\) (Fig. 3B). Furthermore, MjRib7 can only recognize DAROPP but not AROPP as substrate (6). Based on the structural comparison described above, DAROPP was modeled into the active site of MjRib7. This predicted complex structure revealed a short distance of $\sim$3 Å between Asp$^{37}$ O$^{\delta 2}$ and the NH$_2$\(^{2}\), suggesting that this aspartate residue, conserved in archaea but not fungi, may be responsible for the substrate recognition. Further mutational analyses are under way.

A Similar Substrate Binding Architecture in RibG and DHFR—The R domain and DHFR share high homology not only in core structure but also in active-site architecture. Even the R-R structure is similar to that of the dimeric DHFR from \textit{T. maritima} (TmDHFR) (32), due to the presence of similar regions for subunit association. In addition to the four conserved regions for NADPH binding, interestingly, even though their substrates are very distinct, AROPP versus dihydrofolate, the binding residues for the individual parts are located in similar spatial positions (Fig. 4A). The residues responsible for the nucleobase are located between the $\beta$A strand and the $\alpha$B helix, for example, Lys$^{151}$ and residues 167–171 for the pyrimidine ring in BsRibG are comparable to Ile$^{5}$, Met$^{20}$, Asp$^{27}$, Phe$^{31}$ and Ile$^{94}$ for the 6-methylpterin in the \textit{E. coli} DHFR. Interestingly, both reductases possess a positively charged groove for neutralization of the negatively charged group of the substrate: Two or three conserved arginines (or lysines) located at $\alpha$B and $\beta$C-$\beta$D, Arg$^{183}$ and Arg$^{206}$ in BsRibG and Arg$^{32}$, Arg$^{33}$, and Arg$^{36}$ in EcDHFR, form salt bridges with the phosphate group and the glutamyl carboxylate moiety, respectively. These salt bridge interactions are essential for the reduction activity because BsRibG cannot utilize the dephosphorylated form as substrate (27).

The Reduction Mechanism in RibG—For BsRibG, the substrate and cofactor binary complex structures were superimposed to mimic the ternary complex to obtain a geometry estimation of hydride transfer (Fig. 5). This ternary complex was then compared with those in the DHFRs where the enzymatic mechanism has been chemically and structurally studied in detail (30). In addition to the virtually identical binding of the cofactor nicotinamide ring, surprisingly, the reactive carbon atoms of the different substrates occupy a similar position and share a virtually identical orientation toward the nicotinamide C$^4$ (Fig. 5). In BsRibG, the pyrimidine is parallel to the nicotinamide ring with a distance of 3.3 Å between the C$^4$ donor and the C$^1$ acceptor with a N$^1$-C$^4$-C$^1$ angle of 115 degrees. The corresponding distance and angle are 3.3 Å and 114 degrees for the EcDHFR ternary complex (33), and 3.2 Å and 116 degrees in the DHFR complex from \textit{Pneumocystis carinii} (34). Therefore, as well as DHFR, RibG would seem to catalyze the ribosyl reduction by C$^4$-pro-R hydride transfer from NAD(P)H to the C$^1$, which is consistent with the isotope studies (16).

On the basis of the previous studies on DHFR and the complex BsRibG structures described above, a reduction mechanism for the R domain can be proposed as outlined in Fig. 1. The closest ionizable residue in the vicinity of the NH$^2$ group of AROPP is the strictly conserved Glu$^{290}$, with a distance of $\sim$4.6 Å between N$^1$ and Glu$^{290}$O$^{\epsilon 1}$. A water molecule could be placed to mediate hydrogen bonds between Glu$^{290}$ and the NH$^2$ group, and thereby together with Glu$^{290}$, may assist in proton transfer, abstracting a proton from the NH$^2$ group on the one hand and on the other hand, protonating the O$^\delta$ to yield the ribitylimino intermediate. In addition, Glu$^{290}$ would seem to repel the ribose because of a short distance between its carboxyl group and the ribosyl O$^\delta$ ($\sim$3 Å), and hence may facilitate the formation of the Schiff base intermediate. Analogous with the DHFR reaction, formation of the product ARIPP occurs by hydride transfer of the nicotinamide H$^\delta$ to the C$^1$ of the intermediate and protonation of the N$^3$ by a water molecule.

The strong conservation between the R domain and DHFR suggests that these two reductases involved in riboflavin and folate biosyntheses might have evolved by gene duplication with conservation of the core structure, catalytic mechanism, and cofactor binding, but with subsequent divergence in which the substrate binding residues were changed, although with some aspects of their recognition properties being retained.

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