Bacterial RibG is a potent target for antimicrobial agents because it catalyzes the two consecutive deamination and reduction steps in the riboflavin biosynthesis, and because it is the only protein to date to share a similar active-site architecture to the pharmaceutically important dihydrofolate reductase. The complex structures of Bacillus subtilis RibG at 2.56-Å resolution revealed four distinct interaction networks of the product/substrate with the deminase and reductase domains, which may be useful in guiding drug design. Upon the product binding to the deaminase domain, significant conformational changes were induced in two loops moving toward for interaction with the ribosyl and phosphate moieties, respectively. The phosphate forms hydrogen bonds with Asn\textsuperscript{23}, His\textsuperscript{49}, His\textsuperscript{76}, Lys\textsuperscript{79}, and Thr\textsuperscript{80}, while the ribosyl group contacts with Asp\textsuperscript{101} and Asn\textsuperscript{103}. Mutational analyses reveal that Glu\textsuperscript{51} and Lys\textsuperscript{79} are essential for deaminase activity. Unexpectedly, the electron density map demonstrates a ribitylimino intermediate bound at the reductase domain. Both the pyrimidine ring and phosphate form extensive interactions with Lys\textsuperscript{151}, Ser\textsuperscript{167}, Ile\textsuperscript{170}, and Thr\textsuperscript{171}, and Arg\textsuperscript{183}, Ser\textsuperscript{202}, Leu\textsuperscript{203}, and Arg\textsuperscript{206}, respectively, while the ribityl group with Asp\textsuperscript{199} and Glu\textsuperscript{290}. Lys\textsuperscript{151} had evolved to ensure specific recognition of the deaminase product through its amino group interacting with the carbonyl moiety, but repelling the amino of the deaminase substrate.

Flavin coenzymes are essential cofactors for a wide variety of physiological processes including redox reactions, DNA repair, circadian time keeping, light sensing, and bioluminescence, and hence are ubiquitously found in all organisms. The precursor riboflavin is biosynthesized in plants and many microorganisms. The imidazole ring of GTP is first hydrolytically opened with elimination of formate by cyclohydrolase II to yield 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate (DAROPP). In eubacteria and plants, DAROPP is deaminated into 5-diamino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate (AROPP) and subsequently reduced into 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate (ARIPP) (Fig. 1). In most eubacteria, the responsible enzyme is a bifunctional protein RibG, which is composed of an N-terminal deaminase domain (D domain) and a C-terminal reductase domain (R domain). On the other hand, animals lack this biosynthetic pathway and hence must obtain riboflavin from dietary sources. Thus the enzymes of the riboflavin biosynthesis have an important possibility to become novel antimicrobial targets, particularly for development of new defenses against antibiotic-resistant pathogens.

Recently, we solved a tetrameric ring-like structure of Bacillus subtilis RibG (BsRibG). 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. The R domain...
shares high structural homology to dihydrofolate reductase (DHFR). DHFR catalyzes the dihydrofolate reduction into tetrahydrofolate, and many of its inhibitors have long been used clinically in the treatment of cancer, inflammation, and microbial infection. Therefore, RibG is a potential target for new drug design. To gain structural insights into the substrate specificity, catalytic mechanisms, and inhibitor design, we have solved the BsRibG structure in complex with AROPP at 2.56-Å resolution.

The DAROPP-binding Site in the D Domain

BsRibG exists as a tetramer in solution as well as in the crystal asymmetric unit (Fig. 2). In Molecule C, AROPP was firmly embedded in the active site (Fig. 3A). Upon AROPP binding, residues 75-82 and 101-103 move toward AROPP for direct interactions, and hence significant conformational changes are induced. The pyrimidine base interacts with His$^{42}$, Ala$^{50}$, and Glu$^{51}$, the ribose has close contacts with Asp$^{101}$ and Asn$^{103}$, and the phosphate moiety hydrogen bonds with Asn$^{23}$, His$^{49}$, His$^{76}$, Lys$^{79}$, and Thr$^{80}$. These substrate-binding residues are all highly conserved in the eubacterial RibGs. Mutational analyses displayed that no detectable deaminase activity was observed in the E51A and K79A mutants, whereas the H42A, H76A, D101A, and N103A mutants showed a decreased relative activity by 1.5-2 folds, compared with the wild-type enzyme. Thus interactions between Lys$^{79}$ and the phosphate group are essential for the substrate binding and hence for the deaminase activity. This is consistent with previous studies showing that the dephosphorylated form cannot serve as a substrate.

The Deaminase Mechanism

Superposition of the D domain with the available CDA members reveals a virtually identical active-site architecture with a conserved interaction network surrounding the target amino group, suggesting a similar zinc-assisted hydrolytic deamination mechanism. On the basis of the tightly bound AROPP, the substrate DAROPP and the transition state intermediate were modeled into the active site and then a deamination mechanism was proposed (Fig. 1). Glu$^{51}$ assists in proton transfer, abstracting a proton from the zinc-activated water, and protonating the pyrimidine N$^1$ to form the intermediate. Subsequently, the proton shuttling from OH$^-$ to NH$^+$ facilitates the cleavage of the carbon-nitrogen bond. Steric hindrance between the carbonyl groups of Leu$^{71}$ and Glu$^{72}$ and the newly formed carbonyl group of the product AROPP allows its release from the active site, and binds to the reductase domain.
**The AROPP-binding site in the R Domain**

Feeding experiments with deuterium incorporation in *Ashbya gossypii* followed by NMR analysis suggests that the ribosyl group is reduced into a ribityl group through formation of a Schiff base intermediate at C¹, by proton abstraction from the amine nitrogen and direct hydride transfer to C¹. Interestingly, this proposed ribitylimino intermediate could be modeled nicely into the electron density including both the pyrimidine and the phosphate moieties, and forms more extensive interactions with the R domain than AROPP (Fig. 3B). The pyrimidine ring interacts with Lys¹⁵¹, Ser¹⁶⁷, Ile¹⁷⁰, and Thr¹⁷¹, the ribityl group with Asp¹⁹⁹ and Glu²⁹⁰, while the phosphate with Arg¹⁸³, Ser²⁰², Leu²⁰³, and Arg²⁰⁶. Upon the intermediate binding, residues 167-171 and 202-212 moved a little bit toward for interaction with the pyrimidine ring and the phosphate group, respectively. Similarly, the cofactor NADPH binding induces residues 156-170 and 193-195 moving toward the cofactor.

**The Reductase Mechanism**

The substrate and cofactor binary complex structures were superimposed to mimic the ternary complex for the geometry estimation of hydride transfer from the nicotinamide C⁴ of NADPH to the C¹ of AROPP. The R domain and DHFR share a similar active-site architecture, and hence a similar reduction mechanism and a close evolutionary relationship. They share a similar nicotinamide binding, indicating the same stereospecificity of the pro-R hydride transfer. Even though their substrates are very distinct, the reactive carbon atoms occupy a similar position and share a similar orientation toward the C⁴ atom of the nicotinamide ring. On the basis of previous studies on DHFR and the complex BsRibG structures described above, a reduction mechanism for the R domain was proposed (Fig. 1). The closest ionizable residue in the vicinity of the NH¹ group of AROPP is the strictly conserved Glu²⁹⁰, with a distance of 4.6 Å between N¹ and Glu²⁹⁰O⁻¹. A water molecule could be placed to mediate hydrogen bonds between Glu²⁹⁰ and the NH¹ group. This water molecule and Glu²⁹⁰ may assist in proton transfer, abstracting a proton from the NH¹ on the one hand, and on the other hand, protonating the O⁴ to yield the ribitylimino intermediate. In analogy to the DHFR reaction, formation of the product ARIPP occurs by hydride transfer of the nicotinamide H⁴ to the C¹ of the intermediate and protonation of the N¹ by a water molecule. Replacement of Glu²⁹⁰ with glutamine results in a decrease in the reaction rate by 2-3 folds, consistent with its assistance in proton transfer.