Crystallographic trapping in the rebeccamycin biosynthetic enzyme RebC

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The biosynthesis of rebeccamycin, an antitumor compound, involves the remarkable eight-electron oxidation of chlorinated chromopyrrolic acid. Although one rebeccamycin biosynthetic enzyme is capable of generating low levels of the eight-electron oxidation product on its own, a second protein, RebC, is required to accelerate product formation and eliminate side reactions. However, the mode of action of RebC was largely unknown. Using crystallography, we have determined a likely function for RebC as a flavin hydroxylase, captured two snapshots of its dynamic catalytic cycle, and trapped a reactive molecule, a putative substrate, in its binding pocket. These studies strongly suggest that the role of RebC is to sequester a reactive intermediate produced by its partner protein and to react with it enzymatically, preventing its conversion to a suite of degradation products that includes, at low levels, the desired product.

flavin enzymes | x-ray crystallography | indolocarbazoles | antitumor

Rebeccamycin (1, Fig. 1*a*) is a natural product isolated from *Lechevalieria aerocolonigenes* (1) and is a prototype for a class of compounds that bind to DNA-topoisomerase I complexes (2), preventing the replication of DNA and thereby acting as antitumor compounds (3). Rebeccamycin is synthesized by the action of eight enzymes, with the overall conversion of L-tryptophan, chloride, molecular oxygen, glucose, and a methyl group to the glycosylated indolocarbazole rebeccamycin (1) (4–9). One step in this pathway is the conversion of chlorinated chromopyrrolic acid (2) to the rebeccamycin aglycone (3); this reaction involves an eight-electron oxidation, and it is carried out by the enzyme pair RebP and RebC (10). The mechanism of this reaction is not established (8).

RebP is annotated as a cytochrome P450, and it is functionally equivalent to its homologue StaP from the staurosporine biosynthetic pathway (10), which has been used in place of RebP in initial biochemical investigations. Additionally, all biochemical work on StaP and RebC has used nonchlorinated substrates because they are well tolerated by both enzymes (8). One of the most remarkable features of StaP is that when it is incubated alone with its nonchlorinated substrate, chromopyrrolic acid (4), as well as with an electron source (provided by ferredoxin, flavodoxin NADP⁺-reductase, and NAD(P)H), StaP is capable of generating a number of products, including arcyliaflavin A (5), 7-hydroxy-K252c (6), and K252c (7) at a 1:7:1 ratio (8) (Fig. 1*b*). Inclusion of RebC, an FAD-containing protein, in the reaction mixture results in near-exclusive production of arcyliaflavin A (5) and acceleration of turnover (8). One possibility to account for this phenomenon is that RebC can direct the outcome of the reaction by mediating the catalytic activity of StaP through a protein–protein interaction rather than acting catalytically itself. Although no stable interaction was found between StaP and RebC through pull-down assays (8), a transient complex cannot be ruled out. Another possibility is that RebC can affect product distribution by reacting enzymatically with an unstable compound produced by StaP. In this latter case,

when RebC is not present, the StaP product could react further with StaP or could spontaneously degrade, giving rise to a number of nonspecific products. It is known that RebC does not react with chromopyrrolic acid, arcyliaflavin A, 7-hydroxy-K252c, K252c, or arcyliaflavin A (8), but these results do not rule out an enzymatic reaction with an unidentified product produced by StaP.

To provide a framework for further biochemical investigations of RebC, we solved the structure of RebC alone as well as RebC soaked with chromopyrrolic acid and K252c. These studies have allowed for an identification of a likely function for RebC as a flavin-dependent hydroxylase and have further resulted in the crystallographic isolation of a reactive molecule, a putative substrate, in the substrate-binding pocket.

Results

Resemblance to Flavin Hydroxylases. RebC is a monomer with three distinct domains [Fig. 2*a* and *b* and supporting information (SI) Fig. 6]. Domain I (residues 1–72, 104–190, and 279–354, in red), which binds the cofactor flavin adenine dinucleotide (FAD), is intertwined through four loops and one helix with domain II (residues 73–103, 191–278, and 355–398, in blue), which forms the base of a substrate-binding pocket. Domain III (residues 399–529, in green) is connected via an extended loop (residues 399–423) to domain II. Domain III, which is distant from the active site, exhibits a typical thioredoxin fold (11), but lacks conserved cysteines and is of unknown function.

RebC exhibits overall structural homology to three proteins: phenol hydroxylase (12) (RMSD of 1.52 Å for 281 Cα carbon pairs), *meta*-hydroxybenzoate hydroxylase (13) (RMSD of 1.50 Å for 264 Cα carbon pairs), and *para*-hydroxybenzoate hydroxylase (14) (RMSD of 1.72 Å for 248 Cα carbon pairs) (Fig. 2*c*; SI Fig. 7). Structurally homologous residues are primarily located in domains I and III, with only 49 residues of domain II having structural homology to any Cα carbons of the three structural homologues. Each of these three homologues catalyze flavin-mediated hydroxylations on the ortho position of electron-rich phenol derivatives (SI Fig. 8) (13, 15, 16).

Further, the reduction potential of RebC is -179 ± 3 mV (Fig. 3), a value close to those reported for *para*-hydroxybenzoate hydroxylase (-163 mV) (17) and phenol hydroxylase (-213 mV) (18). By contrast, the reduction potentials reported for other well

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2R0C, 2R0G, and 2R0P).

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molecule, K252c (7). These molecules, which are not substrates of RebC, were chosen for soaking experiments because the substrate for RebC itself has not been identified through genetic or biochemical analysis (8). The high-resolution structures resulting from these soaks show electron density in the cleft between domains I and II of RebC, positioned above the β -sheet of domain II (Fig. 2*b*). Careful inspection of the chromopyrrolic acid-soaked structure compared with the native structure reveals that the isoalloxazine ring of the flavin of RebC undergoes a major conformational change. This so-called “mobile flavin,” previously described for *para*-hydroxybenzoate hydroxylase (14) and phenol hydroxylase (21) (Fig. 4*a*), is observed to shift from an OUT position in the RebC native structure, where the isoalloxazine ring is more accessible to solvent and can be reduced by NAD(P)H, to an IN position in the chromopyrrolic acid-soaked structure, where the flavin can form a protected C4a-hydroperoxide intermediate adjacent to a substrate molecule (22). Although the adenosyl-diphosphate moiety of the FAD remains immobile (SI Fig. 9), the isoalloxazine ring moves on the pivot of the lowest phosphate, with the C4a carbon moving 5 Å (Fig. 4*b*), leading to drastic changes in its interactions with surrounding residues (Fig. 4*c–d*). Thus, not only does RebC have an overall structure, sequence motifs, and a redox potential resembling established flavin-dependent hydroxylases, it also appears to have a similar catalytic mechanism, involving a mobile flavin, further suggesting that RebC is a true flavin-dependent hydroxylase.

Melting Helix of RebC. One major difference between RebC and its structurally homologous flavin-dependent hydroxylases is the size of its putative substrates. Whereas *para*-hydroxybenzoate hydroxylase is thought to use a third conformation of the flavin (the OPEN conformation) to allow substrate to enter (23), any putative substrates of RebC would be too large to enter the active site through this conformation. Instead, our structural studies have revealed an alternative route for substrate entry.

Density for residues 354–363, at the domain I–domain II interface, is absent in the native structure of RebC (Fig. 2*a*). In both the chromopyrrolic acid (**4**)- and K252c (**7**)-soaked structures, density for these residues appears as an extension into domain II of a helix that begins in domain I, closing off the substrate-binding pocket (Fig. 2*b*). We speculate that residues 354–363 may be a gateway for exchange of substrate and product: the exposure of the substrate-binding site to solvent in the native structure suggests that a substrate could easily enter the substrate-binding pocket when residues 354–363 are a mobile loop. Upon substrate binding, the helix could form, sealing the substrate from solvent and preventing its departure. This melting helix has not been observed in any other structural homologues of RebC and may represent a unique adaptation of this flavin hydroxylase for reaction with larger substrates.

Identification of Compound Bound. Although an authentic sample of chromopyrrolic acid (**4**) was used in our RebC crystal soaks, this compound is not a good fit to the experimental density (SI Fig. 10 *a–d*), suggesting that during the week-long soak at room temperature, the integrity of the chromopyrrolic acid sample may have been compromised. In particular, although the experimental electron density is planar, modeling studies carried out by using the program Gaussian (24) show that the energetically favorable conformation of chromopyrrolic acid would be non-planar, with the indole rings turned out from one another to avoid steric clash. To eliminate difference electron density peaks beyond $\pm 3.0 \sigma$ in RebC, the indole rings of chromopyrrolic acid must be refined as planar, with a distance of 2.2 Å or less between the two indole C-2 carbons. Such a distance would imply an energetically destabilizing van der Waals clash between these two close carbon atoms, if this molecule were indeed chromopyr-

studied nonhydroxylase flavoproteins can differ significantly from this value: for instance, lipoamide dehydrogenase (*Escherichia coli*) has a value of -317 mV, yeast glutathione reductase has a value of -327 mV (19), and the flavin of plant-type assimilatory nitrate reductase has a value of -270 mV (20). The structural homology and the close similarity of the reduction potential of RebC to flavin hydroxylase enzymes suggests that RebC is also a catalyst, functioning to carry out a flavin-mediated hydroxylation reaction on an electron-rich substrate.

Flavin Shift upon Substrate Binding. To identify the substrate-binding site of RebC, we soaked crystals with chromopyrrolic acid (**4**), the substrate for the two-enzyme system, and a related

Fig. 1. Structures of small molecules and overall reaction scheme. (a) Structures of compounds discussed in the article are shown schematically. (b) StaP (or RebP), incubated with an electron source, reacts with chromopyrrolic acid (4) to give three products, as shown. Addition of RebC leads to near-exclusive formation of arcyliaflavin A (5).

Fig. 2. RebC structural comparisons. (a) The structure of native RebC is shown with domain I (red), domain II (blue), domain III (green), and a flavin in the OUT conformation (green carbons). Density for residues 354–363 are missing in this structure; two arrows indicate the residues bordering the missing density. (b) The structure of chromopyrrolic acid-soaked RebC, also colored by domain, shows flavin in the IN conformation (green carbons), with $F_o - F_c$ omit density for the molecule captured in the substrate-binding pocket contoured at $+3 \sigma$. The “melting” helix, which traverses from domain I to II, is visible in this structure, and two arrows indicate the residues bordering this helix. (c) Chromopyrrolic acid-soaked RebC (gray) is shown with aligned portions of *para*-hydroxybenzoate hydroxylase (pale blue, 1DOC, 248 of 394 residues), phenol hydroxylase (pale pink, 1PNO, 281 of 664 residues), and *meta*-hydroxybenzoate hydroxylase (pale yellow, 2DKH, 264 of 639 residues). Aligned FAD molecules, which have been observed in the IN conformation, are shown in black (RebC), blue (*para*-hydroxybenzoate hydroxylase), and red (phenol hydroxylase).

rolic acid. Analysis of the substrate-binding site of RebC reveals no mechanism for the stabilization of chromopyrrolic acid in such a high-energy conformation. Further, refining a distance of 1.43 Å (a covalent bond) between these two carbons atoms gives an even better fit to the observed density.

Although HPLC analysis of chromopyrrolic acid, as stored in a -20°C freezer, showed the presence of only one peak, corresponding to chromopyrrolic acid, similar analysis of a chromopyrrolic acid solution left at room temperature for 1 week, as occurs during the crystal-soaking experiment, showed the presence of multiple species including a major peak corresponding to chromopyrrolic acid and minor peaks corresponding to arcyriaflavin A (**5**), 7-hydroxy-K252c (**6**), and K252c (**7**) (SI Fig. 11). These results were confirmed by mass spectrometry and UV/visible spectroscopy (data not shown). The minor peaks indicate that, at low levels, chromopyrrolic acid spontaneously degrades under aerobic conditions at room temperature. This result suggests that the bound molecule in the RebC active site might be an intermediate or product of chromopyrrolic acid degradation rather than chromopyrrolic acid itself. A molecule other than chromopyrrolic acid in the active site is consistent with observed planar density as well as with the long soak times required to obtain electron density.

Fig. 3. Reduction potential of RebC. The redox potential of RebC was determined by using the method of Massey (18) using 1-hydroxyphenazine ($E^{\circ} = -172$ mV) as a reference dye (see *SI Methods*).

Fig. 4. Mobile flavins. (a) Phenol hydroxylase (PDB ID code 1PN0) (12) has a mobile flavin, as seen from the superimposed structures of the FAD, which is found in both the OUT (light blue carbons) and IN (green carbons) conformations. The substrate phenol is also shown. (b) Two positions of FAD in RebC, manually superimposed from structures of native (light blue carbons, OUT) and chromopyrrolic acid-soaked structures (green carbons, IN), are shown. The 7-carboxy-K252c, which was refined into the chromopyrrolic acid-soaked structure, is shown with green carbons. (c) Cut-away view of FAD (light blue carbons) with a $2F_o - F_c$ omit map at 1σ shows that the flavin in the native structure is OUT, with a hydrogen bond between the isoalloxazine ring and Arg-239. The isoalloxazine ring is stacked between Arg-46 and Trp-276. Because FAD is thought to be reduced by NAD(P)H in the OUT conformation via a reaction at the *re* face of the isoalloxazine ring (23), Trp-276 would have to move aside for reduction to occur, just as proposed for the structurally homologous residue, Tyr-317 in *meta*-hydroxybenzoate hydroxylase, which also stacks on the *re* face of its isoalloxazine ring (13). (d) Cut-away view of FAD (green carbons) in the chromopyrrolic acid-soaked structure shows that the isoalloxazine ring in the IN conformation no longer stacks between Trp-276 and Arg-46, and the interaction with Arg-239 is lost. Arg-46 now interacts with FAD phosphate oxygens, and Arg-239 interacts with bound 7-carboxy-K252c (green carbons). The $2F_o - F_c$ omit map is contoured at 1σ .

Fig. 5. Substrate-binding site. (a) The 7-carboxy-K252c, which was refined in the chromopyrrolic acid-soaked structure, is bound at the interface of domain I (red) and II (blue), surrounded by a number of hydrophobic side chains, including Phe-227, which shifts conformation. Leu-358 is found on the helix that is disordered in the native structure. A loop (red, residues 303–306) from domain I interacts with one face of 7-carboxy-K252c. Hydrogen-bonding interactions with 7-carboxy-K252c include Glu-396, Arg-230, and Arg-239. An $F_o - F_c$ omit map is contoured at 3.0σ . (b) Binding site of the K252c-soaked structure shows the isoalloxazine ring of flavin (green) locked in an OUT conformation. Trp-276 stacks on the *re* face of the isoalloxazine ring, Phe-227 is not shifted, and Arg-230 is too far to interact with K252c. Charged residue Glu-396 hydrogen-bonds with the indole nitrogens, and Arg-239 hydrogen-bonds with the FAD, and it does not interact with the substrate. K252c is contoured with an $F_o - F_c$ omit map at 3.0σ . (c) The native structure of RebC shows the exposure of the substrate-binding pocket to solvent with the absence of the melting helix. Additionally, Glu-396 is disordered, Phe-227 is not shifted, and Phe-216 assumes two alternate conformations. FAD (green), as in the K252c-soaked structure, is positioned in the OUT conformation. (d) Cut-away view of 7-carboxy-K252c and FAD-binding site shows that one ring carbon of 7-carboxy-K252c is 5.1 Å from the C4a carbon of FAD. One oxygen of the carboxylate of 7-carboxy-K252c additionally has hydrogen bonds to both Arg-230 and Arg-239.

Knowing that chromopyrrolic acid underwent spontaneous degradation during the week-long soak, we tried a series of crystallographic refinements to identify whether the molecule bound was a final product of degradation (5, 6, or 7) or an intermediate such as 9–11. Compound (9), which differs from chromopyrrolic acid in having an additional carbon–carbon bond and being planar, is a better fit to the overall density. Refinement of this compound, however, results in significant negative electron difference density at the C-5 carboxylate site, suggesting a substituent smaller than a carboxylate at the C-5 ring carbon. Electron difference density, however, shows an acceptable fit for a carboxylate at the C-7 carbon, when it is positioned out-of-plane to the plane of the indolocarbazole (SI Fig. 10e). We then refined arcyriflavin A, which has ketone oxygen atoms in place of both carboxylates. The resulting $F_o - F_c$ maps show no difference density at the ketone of the C-5 carbon but show significant positive difference electron density at the ketone of the C-7 carbon, suggesting that a larger substituent, like a carboxylate, is attached to the C-7 carbon (SI Fig. 10f). Refinement of a molecule with a ketone at the C-5 carbon and

an out-of-plane carboxylate at the C-7 carbon yields no significant electron difference density at either position (SI Fig. 10g). In summary, the experimental density is consistent with a compound containing an aryl–aryl bond between the indole C-2 carbons, with a single oxygen atom as a substituent at the C-5 carbon and a substituent larger than a single oxygen atom, such as an out-of-plane carboxylate, at the C-7 carbon. This density is most consistent with two tautomers (10 and 11) of 7-carboxy-K252c. Allowing the molecule to refine against the experimental electron density gives a short C–C bond (1.3 Å) from the C-7 carbon to the carboxylate, suggesting that tautomer 10 is bound, but also gives out-of-plane carboxylate density, suggesting that tautomer 11 is bound. It is likely that RebC is able to bind both 10 and 11 forms of 7-carboxy-K252c in its active site and that our final, refined 7-carboxy-K252c molecule reflects the presence of both tautomers. Given the observed degradation of chromopyrrolic acid to the products arcyriflavin A, 7-hydroxy-K252c, and K252c, we postulate that an intermediate such as 7-carboxy-K252c must accumulate during the week-long crystal soak with chromopyrrolic acid and be “captured” by the RebC crystal.