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The structure of flavin-dependent tryptophan 7-halogenase RebH

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INTRODUCTION

Enzyme catalyzed regio- and stereo-specific halogenations influence the biological activity of a diverse array of therapeutically important natural products, including the antibiotics vancomycin and chloramphenicol as well as the anticancer agents calicheamicin and rebeccamycin.[1-5] The major class of enzymes responsible for this challenging synthetic reaction, the flavin-dependent halogenases, catalyzes the formation of carbon-halogen bonds using flavin, a halide ion (Cl^-, Br^- or I^-), and O_2.[6] Recent mechanistic and structural advances achieved with the model flavin-dependent tryptophan 7-halogenases PrnA and RebH[7-10] have greatly enhanced the level of understanding of this unique reaction. According to these studies, the mechanism for tryptophan halogenation proceeds via FAD(C4a)—OOH activation of a chloride ion into the transient chlorinating species HOCl.[11-14] The key evidence for the requirement of a transient chlorinating species is the discovery that a ~10-Å-long tunnel separates FAD and tryptophan in the ligand-bound form of PrnA.[12] In a recent compelling study to elucidate the strategy by which RebH controls this highly reactive and indiscriminant oxidant, a Lys79-ϵNH-Cl chloramine intermediate was implicated as the actual chlorinating species within RebH and a structural investigation of RebH was reported.[10] Here we report our independent structural analysis of *Lechevalieria aerocolonigenes* RebH (UniProt accession

Cloning and expression

The *rebH* gene was amplified from Cosmid DNA pJST23019 using the primer pair 5′-GGTACGTCATATGTCCGGCAAGA-3′ and 5′-GACGTAAGCTTCCGTCTGTCAGC-3′. After restriction digestion with *NdeI*/*HindIII* (Promega, Madison, WI), the PCR products were ligated into *NdeI*/*HindIII*-linearized pET28a (Novagen, San Diego, CA) containing an amino-terminal His_{10}-tag. The cloned expression plasmid was confirmed by sequencing and subsequently transformed into *E. coli* strain BL21(DE3) (Stratagene, La Jolla, CA) for expression. Cells were grown at 37°C until reaching an optical density at 600 nm of 0.9 and then induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside and grown for additional 40 h at 16°C.

Protein purification

Cells were resuspended in binding buffer (50 m*M* sodium phosphate, 300 m*M* NaCl, 20 m*M* imidazole pH 8) and disrupted by sonication. Insoluble debris was removed by centrifugation at 10,000*g* for 1 h, and the supernatant with the His_{10}-fusion construct was captured on a HiTrap Chelating HP column charged with Ni^{2+} (Amersham Biosciences, Piscataway, NJ). The recombinant protein was eluted with a linear 20–500 m*M* imidazole gradient, desalted using a PD-10 column (Amersham Biosciences, Piscataway, NJ). The final storage buffer contained 50 m*M* potassium phosphate, pH 8, 0.2 m*M* dithiothreitol (DTT) and 20% glycerol. Protein used for crystallization trials was dialyzed into the solution containing 50 m*M* NaCl, 10 m*M* tris(hydroxymethyl) aminomethane (TRIS) pH 8.0. Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

Activity assay

Typical assays were conducted at 303 K in 100 µL total volume 20 m*M* potassium phosphate buffer (pH 8.0) containing 0.6 m*M* L-Trp, 0.2 mg/mL RebH, 50 µ*M* FAD and 100 m*M* halide (NaCl or NaBr) and an in situ flavin reduction system. In situ flavin reduction was accomplished using either 20 m*M* NADH and 0.2 unit/mL NADH oxidase (Sigma-Aldrich) or 20 m*M* DTT. At given time points, a reaction aliquot was removed, diluted with an equal volume of MeOH, centrifuged to remove precipitated protein, and analyzed by reverse-phase HPLC (Phenomenex LUNA C18, 100 Å, 4.6 × 250 mm; 1 mL/min; isocratic for the first 0–10 min, 15% B followed by a gradient of 15–80% B from 10–17 min; A, H_2O/0.1% TFA; B, acetonitrile; A_{280}). RebH specific activity was consistent with previous reports14 and all halogenated products were confirmed by LC-MS.

Protein crystallization

precipitate in several months. Crystals were cryoprotected at 277 K by soaking in well solutions containing 10, 20, and 30% (v/v) glycerol and were flash frozen in a stream of cryogenic nitrogen gas at 100 K. To prepare the complex, the RebH crystals were placed for 22 h in the soaking solution containing 0.3 *M* NaH_2PO_4, 0.6 *M* K_2HPO_4, ≈5 m*M* FAD, ≈3 m*M* tryptophan, and 30 m*M* NaCl. Crystals were cryoprotected by stepwise addition of 100% (v/v) glycerol to the soaking solution until ≈30% (v/v) final concentration of glycerol was achieved.

Diffraction data collection

X-ray diffraction data for both the apo-structure and the substrate complex were collected at the General Medicine and Cancer Institute Collaborative Access Team (GM/CA-CAT) 23-ID-D beamline at the Advanced Photon Source at Argonne National Laboratory. Each of the 200 diffraction images for the apo-structure was collected at a crystal-to-detector distance of 325 mm and exposed for 4 s with 100-fold attenuation of the incident beam. The data were collected in a single pass with 1° oscillations per frame. Each of the 360 diffraction images for the complex was collected at a crystal-to-detector distance of 250 mm and exposed for 6 s with 200-fold attenuation of the incident beam. The data were collected by inverse-beam strategy with 30° wedges and 1° oscillation per frame. The diffraction images were integrated and scaled using HKL2000.[15] Crystals belong to the space group $P6_2$ with unit cell parameters $a = b = 114.8$ Å, $c = 230.6$ Å.

Structure determination

The apo-structure of RebH was solved by molecular replacement in MOLREP[16] using the structure of *Pseudomonas fluorescens* PrnA as a search model (PDB ID 2aqj, 55% identity). The outstanding solution with R-factor of 0.46 and a correlation coefficient of 0.49 was obtained after two molecules were placed in the asymmetric unit. Prerefinement of the molecular replacement solution without noncrystallographic symmetry constraints in REFMAC5[17] resulted in a model with $R = 32.5\%$ ($R_{free} = 37.5\%$). To reduce the model bias and improve the initial map quality, σ_A-weighted model phases were density modified in DM[18] with 2-fold noncrystallographic symmetry averaging constraints. The atomic model was then built based on the resulting phases using an automatic building procedure implemented in ARP/WARP.[19] The initial model obtained from this procedure had $R = 21.2\%$ ($R_{free} = 26.3\%$) and contained 978 residues of which 956 had side-chains assigned. The structure was completed with multiple cycles of manual building in COOT[20] and refinement in REFMAC5.[17] Final refinement protocol included TLS refinement[21] with 10 TLS-groups per molecule[22] and used medium noncrystallographic symmetry restraints to relate the main-chain atoms of the two molecules in the asymmetric unit. The final refined model has $R = 16.1\%$ ($R_{free} = 20.8\%$). The structure of

Structure quality

The structure of *Lechevalieria aerocolonigenes* RebH was determined by molecular replacement in MOLREP using the structure of the PrnA monomer[12] as a search model (PDB ID 2aqj) and was refined to a resolution of 2.5 Å. The structure of the complex of RebH with bound tryptophan, FAD, and chloride was refined to a resolution of 2.15 Å. Data collection, phasing, and refinement statistics for both the apo- and complex structures are summarized in Table I. The final model of apo-RebH contains residues 3–526 of chains A and residues 3–528 of chain B; terminal residues were not observed in the electron density and therefore not included in the model. Similarly, the final model of the RebH complex contains residues 2–528 of chains A and residues 2–528 of chain B. The chloride anion was modeled in the active site of molecule A of the RebH complex based on the structural similarity to the PrnA-FAD-chloride complex.[12] This interpretation is further supported by the existence of a 4.5σ-peak in the σ_A-weighted 2*Fo-Fc* electron density map at the expected position, compared to 2.0σ, 2.2σ, 2.6σ, 3.4σ peaks of the nearby water molecules. Clear electron density was observed for bound tryptophans in both modeled molecules. In addition, well defined electron density for FAD was observed in molecule A. However, the electron density corresponding to the cofactor in molecule B showed a significant disorder and only allowed for unambiguous modeling of the adenosine portion of FAD.

Table I. Crystal Parameters, Data Collection, Phasing, and Refinement Statistics

	Apo-RebH	RebH complex
Space group	$P6_2$	$P6_2$
Unit-cell parameters (Å; °)	*a* = *b* = 114.8, *c* = 230.6; α = β = 90, γ = 120	*a* = *b* = 114.5, *c* = 231.9; α = β = 90, γ = 120
Data collection statistics		
Wavelength (Å)	0.97919	0.97931
Energy (eV)	12,662	12,660
Resolution range (Å)	29.13–2.50 (2.59–2.50)	49.58–2.15 (2.20–2.15)
No. of reflections (measured/unique) [a]	762,860/59,677	1,881,583/92,078

R_{merge}	0.131 (0.432)	0.102 (0.334)
Redundancy	12.8 (10.3)	20.4 (9.4)
Mean *I*/sigma(*I*)	24.5 (8.1)	24.9 (3.6)
Phasing statistics [c]		
Correlation coefficient	0.49	
R factor	0.46	
Refinement and model statistics		
Resolution range	29.13–2.49 (2.56–2.49)	19.95–2.15 (2.21–2.15)
No. of reflections (work/test)	56,611/3008	87,284/4630
R_{cryst} [d]	0.161 (0.221)	0.152 (0.259)
R_{free} [e]	0.208 (0.267)	0.194 (0.362)
r.m.s.d. bonds (Å)	0.014	0.015
r.m.s.d. angles (°)	1.407	1.412
ESU from R_{free} (Å)	0.209	0.141
B factor (Å^2): Wilson/average [f]	43.2/31.9	35.2/33.4
No. of protein molecules/all atoms	2/8902	2/9627
No. of waters/ions	455/2 phosphates	1056/0
No. of substrates and/or cofactors		1 chloride, 2 tryptophans, 1 FAD, 1 adenosine portion of FAD
Ramachandran plot by PROCHECK (%)		
Most favorable region	91.5	91.3
Additional allowed region	8.5	8.7
Generously allowed region	0.4	0.0

a Values in parentheses are for the highest resolution shell.

b $R_{merge} = \sum_h \sum_i | I_i(h) - \langle I(h) | / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the intensity of an individual measurement of the reflection and $\langle I(h) \rangle$ is the mean intensity of the reflection.

c Phasing by molecular replacement in MOLREP using the PrnA monomer as a search model (PDB ID 2aqj).

d $R_{cryst} = \sum_h ||F_{obs}| - |F_{calc}|| / \sum_h |F_{obs}|$ where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively.

e R_{free} was calculated as R_{cryst} using ~5.0% of the randomly selected unique reflections that were omitted from structure refinement.

f Reported values for models refined in REFMAC5 by restrained refinement with no TLS-refinement.

Comparison of RebH and PrnA structures

RebH and PrnA share 55% sequence identity and their structures align closely with a root mean square deviation (rmsd) of 0.68 Å for 3238 structurally equivalent atoms; this value is consistent with these proteins having a very similar fold. Similarly to PrnA, the crystal structure of RebH revealed that this protein forms a dimer with a buried surface area of 1630 $Å^2$. In addition to van der Waals contacts, 14 hydrogen bonds and a single salt bridge (Arg387–Glu432) stabilize the dimer interface. Each monomer of RebH folds into a single domain with a complex topology described previously for PrnA.[12] As in the case of the PrnA structure, flavin and tryptophan binding sites are separated by ≈10 Å [Fig. 1(a)].

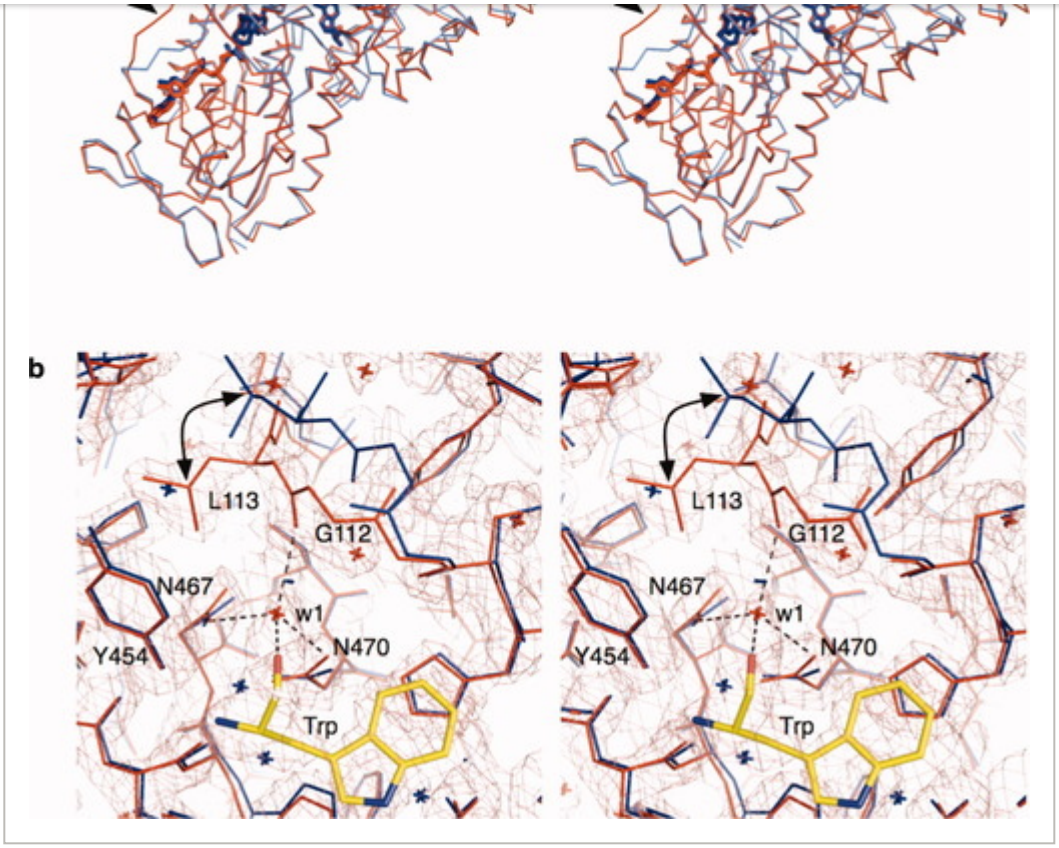

Figure 1

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Structure of RebH. (**a**) Stereo image of the C_α-trace of structurally superposed RebH (red) and PrnA (cyan, PDB ID 2ar8) complexes with FAD and tryptophan (sticks). (**b**) Stereo image of the tryptophan-binding cavity of the RebH complex with bound tryptophan (yellow sticks). The loop harboring Gly112 and Leu113 undergoes a conformational change upon tryptophan binding as seen from the difference between the structures of apo-RebH (blue) and the RebH-complex (red). Total omit map23 of the RebH complex (salmon) is shown at contour level of 1.0σ. Hydrogen bonds to a stabilizing water molecule (w1) are shown as black dashed lines.

Caption

Two notable structural differences exist between structures of these proteins: (i) a surface-exposed loop that spans residues 86–105 in RebH harbors an eight-residue insertion [Fig. 1(a), red arrow] compared to the analogous loop in PrnA (residues 87–97), (ii) the conserved loop that spans residues 40–48 in both RebH and PrnA adopts entirely different conformation [Fig. 1(a), black arrow]. In PrnA, the loop forms multiple direct hydrogen bonds to FAD as well as

quality in both apo-RebH and the RebH complex structures than for the rest of protein, indicating that this loop is quite flexible. The loop was partially stabilized in the observed conformation by the crystallographic contact with the RebH molecule related by a noncrystallographic twofold axis located in the proximity of Pro43. Despite the structural differences in this loop, the conformation of both the cofactor and the bound tryptophan are very similar in both RebH and PrnA [Fig. 1(a), sticks].

Comparison of apo-RebH and the RebH complex

The apo-RebH structure and the RebH complex align with a rmsd of 0.216 Å for 3866 structurally corresponding atoms from chains A and a rmsd of 0.187 Å for 3836 structurally corresponding atoms from chains B. The dimers of apo-RebH and the RebH complex align with rmsd of 0.276 for 7752 structurally corresponding atoms. These low values confirm that no large-scale changes in tertiary and/or quaternary structure took place upon binding of substrate and cofactor. The only notable change between the structures of apo-RebH and the RebH complex is related to the ordering of the loop comprising residues 111–114, which are involved in formation of the tryptophan binding site. In apo-RebH crystals, the electron density corresponding to this loop is of poor quality, indicating that the loop is flexible. Upon tryptophan binding, the loop undergoes a conformational change that flips the peptide carbonyl of Gly112 and displaces sidechain of Leu113 by as much as 5 Å. Substrate binding results in an apparent stabilization of the loop as judged by the well-defined electron density [Fig. 1(b)]. Upon the conformational change, the bound tryptophan becomes completely buried within the interior cavity of RebH. In addition to tryptophan, a well ordered water molecule becomes trapped in the substrate-binding cavity; this water molecule participates in a hydrogen bond network that further stabilizes the bound tryptophan [Fig. 1(b)].

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a Values in parentheses are for the highest resolution shell.

b $R_{merge} = \sum_h \sum_i | I_i(h) - \langle I(h) | / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the intensity of an individual measurement of the reflection and $\langle I(h) \rangle$ is the mean intensity of the reflection.

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