Conserved residue His-257 of \textit{Vibrio cholerae} flavin transferase \textit{ApbE} plays a critical role in substrate binding and catalysis

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The flavin transferase \textit{ApbE} plays essential roles in bacterial physiology, covalently incorporating FMN cofactors into numerous respiratory enzymes that use the integrated cofactors as electron carriers. In this work we performed a detailed kinetic and structural characterization of \textit{Vibrio cholerae} WT \textit{ApbE} and mutants of the conserved residue His-257, to understand its role in substrate binding and in the catalytic mechanism of this family. Bi-substrate kinetic experiments revealed that \textit{ApbE} follows a random Bi Bi sequential kinetic mechanism, in which a ternary complex is formed, indicating that both substrates must be bound to the enzyme for the reaction to proceed. Steady-state kinetic analyses show that the turnover rates of His-257 mutants are significantly smaller than those of WT \textit{ApbE}, and have increased \(K_m\) values for both substrates, indicating that the His-257 residue plays important roles in catalysis and in enzyme-substrate complex formation. Analyses of the pH dependence of \textit{ApbE} activity indicate that the pK\textsubscript{a} of the catalytic residue (pK\textsubscript{ESI}) increases by 2 pH units in the His-257 mutants, suggesting that this residue plays a role in substrate deprotonation. The crystal structures of WT \textit{ApbE} and an H257G mutant were determined at 1.61 and 1.92 Å resolutions, revealing that His-257 is located in the catalytic site and that the substitution does not produce major conformational changes. We propose a reaction mechanism in which His-257 acts as a general base that deprotonates the acceptor residue, which subsequently performs a nucleophilic attack on FAD for flavin transfer.

Flavins are vitamin B\textsubscript{2} derivatives broadly used in redox-active enzymes, participating as cofactors that mediate electron-transfer reactions (1, 2). In most cases, flavin cofactors are attached via noncovalent interactions that include hydrogen bonding, electrostatic interactions, and aromatic stacking (3–6). In addition, a significant number of enzymes contain covalently-bound flavins (7). The covalent interactions confer several advantageous properties, such as increased redox potentials and higher stabilities of the holoenzyme (8, 9). In most of these cases, the covalently-bound flavins are attached autocatalytically through the isoalloxazine flavin ring to Cys, His, and Tyr residues (7, 10–16).

It has been recently reported that several families of respiratory enzymes contain covalently-bound FMN attached through a phosphoester bond between FMN’s phosphate moiety and a threonine residue located within the semiconserved motif SGAT (17–20, 23), which is the first respiratory enzyme and the main sodium pump for \textit{Vibrio cholerae} (24–29). This type of covalent attachment of the FMN cofactor has also been observed in the membrane-bound ferredoxin:NAD\textsuperscript{+} oxidoreductase (RFN) (21), urocanate reductase (30), and nitrate reductase regulatory protein NosR (22, 31). These respiratory enzymes, in particular NQR, are essential for pathogenic bacteria including \textit{V. cholerae}, \textit{Pseudomonas aeruginosa}, \textit{Chlamydia trachomatis}, and \textit{Klebsiella pneumoniae}, sustaining the metabolism, and in some cases supporting antibiotic-resistance development (24–26, 32–35). Although this covalent bond was considered exclusive to bacterial enzymes, it was recently reported that eukaryotic fumarate reductases also carry this type of covalently-bound FMN (36). Remarkably, the incorporation of the FMN cofactor through this phosphoester bond is not autocatalytic, as in all other reported cases, but is carried out by \textit{ApbE} (alternative pyrimidine biosynthesis protein, subunit E) (20, 22, 23), the only flavin
transferase found in nature, so far. ApbE transfers FMN to diverse respiratory enzyme subunits, using FAD as substrate (20, 22, 23). In previous work, the conserved residue His-257 was identified as part of the *V. cholerae* ApbE active site (23). Mutagenesis studies indicated that this residue plays a critical role in enzyme catalysis and we proposed a Ping–Pong type reaction mechanism that involved the formation of an imidazole-FMN covalent intermediate. To understand the role of this residue, detailed structural and kinetic characterizations of WT ApbE and His-257 mutants Gly, Thr, Asp, and Lys were performed. Our results indicate that the mutants retain significant activity, suggesting that the reaction does not undergo covalent catalysis through His-257, as previously proposed. Bi-substrate and substrate inhibition kinetics show that the flavin transfer catalyzed by ApbE follows a Random Bi Bi sequential reaction, and not a Ping–Pong reaction, indicating the formation of a ternary complex in the process. Steady-state kinetics characterizations show that the turnover rates of the mutants are significantly lower compared with the WT enzyme and that the *Km* values for the substrates increase substantially, indicating that His-257 has a direct role in catalysis and in the formation of the productive complex. pH titrations of the WT and the mutants suggest that His-257 is involved in the deprotonation of the catalytic residue. Based on the data obtained, a reaction mechanism is proposed in which His-257 acts as a general base that deprotonates the acceptor residue (Thr-225) in the protein substrate. Upon deprotonation, Thr-225 becomes a nucleophile that subsequently attacks the pyrophosphate group of FAD through which the FMN cofactor is covalently attached to the protein. This work clarifies the kinetic and reaction mechanisms of the novel flavin transferase family ApbE, and the role of the conserved His-257 residue in catalysis.

**Results**

*Flavin-transfer activity measurement*

The flavin transferase activity of ApbE was studied using NqrC as the protein substrate. NqrC is one of the two subunits of NQR that carry the covalently-attached FMN (17–20). For these experiments the transmembrane segment of NqrC was eliminated for optimal protein expression, as reported previously (23). In addition, ApbE activity was investigated in the presence of potassium, which was recently shown to specifically activate the enzyme (23), to mimic the physiological conditions that *V. cholerae* encounters during infection.

ApbE flavin-transfer activity can be measured by following the fluorescence of covalently-bound FMN to NqrC in SDS-PAGE gels exposed to UV light (23). However, this method has many limitations, such as moderate accuracy and a high amount of sample required. Here, we report a new spectrophotometric method to follow the flavin-transfer activity. This method exploits the absorbance difference between the free FAD and the covalently-bound FMN. As shown in Fig. 1A, the fully flavinylated NqrC (solid line) displays a distinctive absorption spectrum, which is different from that of free FAD (gray line). The difference spectrum shows a peak at 395 nm with an isosbestic point at 366 nm (Fig. 1A, inset). By following the absorbance change at 395 nm, using 366 nm as reference (Fig. 1B), the activity of ApbE can be measured accurately and inexpensively. It should be pointed out that the change in absorption spectra of the flavin is probably due to the interactions of FMN with the folded NqrC protein. Indeed, upon denaturation with 0.1% SDS, the absorption spectrum of flavinylated NqrC resembles that of free FMN (Fig. 1A, dashed line). As can be seen in Fig. 1B, the in-gel activity data points (squares) match very well with the activity trace obtained with the spectrophotometric method developed in this work. Furthermore, the spectrophotometric method allows the collection of substantially more data points, compared with the SDS-PAGE gel method, and thus the activity rates can be calculated more accurately.

*Bi-substrate kinetics*

To investigate the kinetic mechanism of ApbE, bi-substrate kinetic experiments were carried out measuring ApbE activity under different fixed concentrations of NqrC, while varying the concentration of FAD. Data were globally fitted to the three bi-substrate kinetic models: Random, Ordered, and Ping–Pong (Equations 1–3). Statistical analyses show that the data were best fitted to either Random or Ordered models, whereas the fitting to the Ping–Pong model yielded a higher *χ²* (Fig. 2, A and B). Moreover, the double-reciprocal plots show intercepting patterns, also consistent with sequential mechanisms, in which a ternary complex (ApbE–FAD–NqrC) is formed (Fig. 2E).
Steady-state kinetic characterization of His-257 mutants

Previously, the conserved residue His-257, which directly faces the pyrophosphate moiety of FAD, was proposed as part of the catalytic site of ApbE (23). The mutation to glycine completely abolished ApbE activity, suggesting that this residue plays an essential role in enzymatic catalysis. We proposed a covalent catalysis mechanism in which His-257 could react with FAD, producing a covalent intermediate that mediates the incorporation of FMN to the acceptor residue NqrC-Thr-225 (23). To understand the role of His-257, different mutants were obtained and characterized in this work. In particular, His-257 was mutated to Gly, Thr, Asp, and Lys, to characterize the effects of the charge and size of the residue on the flavin-transfer activity. The results show that all mutants are inactive at pH 7.0 (Fig. 3C, inset), but when the activity is measured at pH 9.0, the mutants H257G and H257T have 4–5 times lower turnover rates compared with WT ApbE (Table 1). It should be pointed out that we previously reported that the mutant H257G was completely inactive (23). However, in the previous experiments the results were obtained in the absence of the physiologic regulator potassium, which greatly activates the enzyme. On the other hand, mutants H257K and H257E showed negligible activities at all pH values assayed, probably due to the net charges interfering with substrate binding (see below). This result indicates that His-257 is not essential for enzyme function, and that it does not form a covalent intermediate with FMN during the reaction, and thus His-257 may participate in a different mechanism. This is consistent with the Random Bi Bi sequential mechanism of the WT enzyme, because the formation of a covalent flavin-imidazole intermediate would be required for a Ping–Pong type mechanism. Mutants H257G and H257T were further characterized. Both mutants show a significant increase in the $K_{in}$ values for FAD and NqrC compared with the WT enzyme, respectively (Fig. 3, A and B, and Table 1), indicating that His-257 plays a major role in the formation of the enzyme–substrate complex and participates directly in catalysis.

Characterization of catalytic residues $pK_a$ and $pK_e$ predictions

To understand the role of His-257 in the reaction mechanism of ApbE, a characterization of the effects of pH on the activity was carried out, which allows the study of the $pK_a$ of residues involved in the reaction process. In a previous work we showed that the catalytic residue of WT ApbE has a $pK_a$ of 8.4 (23), corresponding to $pK_{E3}$ in Equation 5, which was assigned His-257. To study the pH dependence of ApbE activity, NqrC titrations were performed under different pH values (Fig. 3C). In these experiments 50 $\mu$M FAD was used, which is near-saturating at all pH values tested (Fig. S1). Global analyses of the data using Equation 5 show that ApbE displays nearly identical $pK_{E3}$ of 7.2 and 6.8, close to the $pK_a$ of a histidine residue. These $pK_a$ values indicate the dissociation constants of residues whose protonation determine substrate binding ($pK_{E1}$) and catalysis ($pK_{E3}$), i.e. $pK_{E1}$ and $pK_{E3}$ are the $pK_a$ values at which the deprotonated enzyme is able to bind the substrate and become catalytically active, respectively (37). Thus, the data...
Table 1

Kinetic parameters of ApbE wildtype and mutants

|        | \(k_{\text{cat}}\) | \(K_m\) FAD | \(K_m\) NqrC |
|--------|-------------------|-------------|-------------|
| Wildtype | 0.16 ± 0.007      | ≤0.1 ± 0.011 | 11 ± 2     |
| H257G   | 0.045 ± 0.001     | 0.20 ± 0.08 | 87 ± 5     |
| H257T   | 0.036 ± 0.001     | 0.19 ± 0.03 | 119 ± 3    |
| H257E   | 2.6 \times 10^{-4} ± 4.6 \times 10^{-5} | NA          | NA         |
| H257K   | 9.5 \times 10^{-5} ± 8.4 \times 10^{-6} | NA          | NA         |

* This value is not reported due to the detection limit of the method. Experimental measurements could not be performed accurately at FAD concentrations lower than 0.1 μM.

* NA, not applicable.

Table 2

pK\(_{\text{ESI}}\) values for the pH kinetic parameters of ApbE wildtype and mutants

|        | pK\(_{\text{ESI}}\) |
|--------|-----------------|
| Wildtype | 6.8 ± 0.1 |
| H257G   | 9.3 ± 0.2 |
| H257T   | 9.4 ± 0.1 |

The shift in pK\(_{\text{ESI}}\) strongly suggests that His-257 acts as a general base that deprotonates the FMN acceptor residue Thr-225, which could allow a nucleophilic attack on FAD and the subsequent incorporation of the FMN moiety (Fig. 4D). It should be pointed out that experiments could not be carried out at pH values higher than 10.5, due to the formation of precipitates in the reaction buffer (probably MgOH\(_2\)).

Deka et al. (38) demonstrated that conserved Lys-207 in NqrC is important for flavin transfer, and that this residue points directly to the acceptor residue Thr-225. It is likely that Lys-207 may lower the pK\(_a\) of Thr-225, as threonine generally does not undergo deprotonation under physiologic conditions, which could allow flavin transfer assisted by His-257. To gain insight into this hypothesis, theoretical pK\(_a\) predictions of NqrC residues 225 and 207 were carried out using the program PROPKA3.1 (39, 40). Because PROPKA3.1 only predicts pK\(_a\) values of conventional ionizable residues, Thr-225 was mutated in silico to cysteine, a structurally similar but ionizable residue, using UCSF Chimera (41). Furthermore, to examine the effects of Lys-207 on residue 225, Lys-207 was also mutated to Ala, Gln, Glu, and Arg using the same method. PROPKA 3.1 also calculates how much nearby residues contribute to a pK\(_a\) change. As listed in Table 3, an Ala or Gln residue at position 207 is predicted to have no effect on the pK\(_a\) of Cys-225. On the other hand, Arg and Glu shift the pK\(_a\) of Cys-225 by about 0.3–0.4 pH units, consistent with the previously suggested hypothesis (38) that a positive charge can help activate Thr-225 but it is not sufficient to carry out catalysis.

Crystal structures of ApbE

To fully understand the role of His-257, the crystal structures of WT ApbE and H257G were investigated. Crystals grown under conditions supplemented with FAD and MgCl\(_2\) (as described under "Experimental procedures") were used for data collection. Resolutions for WT and H257G were determined to be 1.61 and 1.92 Å, respectively. Overall, ApbE WT structure shows a significant similarity to previously published ApbE structures from Salmonella enterica (42), Pseudomonas stutzeri (22), Treponema pallidum (43), Escherichia coli (38), and Tho-
motoga maritima (44) (Fig. S2). Superposition of the two structures showed that no significant changes occurred due to the mutation (r.m.s. deviation = 0.418 Å, calculated by UCSF Chimera, Fig. 4A). Electron densities corresponding to FAD in both WT and H257G structures were clearly defined around all FAD atoms up to 2σ of 2Fo − Fc (difference density) maps. In the FAD-binding pocket, there are 14 hydrogen bonds with FAD formed by Met-31, Tyr-69, Ala-110, Asp-112, Asp-168, Ser-170, Thr-171, Lys-174, Glu-200, Ser-241, Ile-259, Asp-285 and Thr-289 residues (Fig. S3). In addition, His-257 in the WT also forms a hydrogen bond through the N1 of its imidazole side chain with the 3′-OH of the FAD ribose moiety (Fig. 4B) and locks FAD inside the pocket (Fig. 4C). Thus, the crystallographic data reveal that mutation of His-257 did not result in significant changes in the enzyme structure, but it could have an effect on FAD binding, further demonstrating that His-257 does not play a structural role. Instead, it is an important residue in catalysis.

**Discussion**

The newly discovered family ApbE is the only known flavin transferase that covalently integrates FMN cofactors into different respiratory enzymes of various pathogenic bacteria. In this work we characterized the kinetic and reaction mechanisms of flavin transfer carried out by ApbE, providing insight to understand its catalytic mechanism.

**Structural insight into ApbE mechanism**

High-resolution X-ray crystallographic structures were obtained for ApbE WT and H257G. Superposition of the WT and mutant structures show that the mutation does not cause significant conformational changes. Examination of the active site in both crystal structures demonstrate that the FAD-binding pocket remains undisturbed upon mutation of His-257, in terms of both the protein-FAD interactions and the active site conformation (Fig. 4A and Fig. S2). However, kinetic studies show that the mutation substantially changed the \( K_m \) values for both substrates, which in the case of FAD can be explained by the fact that His-257 forms a hydrogen bond with this molecule. The data indicate that His-257 does not contribute to the overall structural integrity of the enzyme. Rather, it is directly...
involved in the catalytic process and in the formation of a ternary complex with the substrates.

**Random Bi Bi kinetic mechanism of ApbE**

ApbE uses FAD as a substrate and covalently attaches the FMN moiety to the protein substrate (NqrC), following a bi-substrate kinetic mechanism. There are three basic bi-substrate models: Random, Ordered, and Ping–Pong. To determine the ApbE mechanism, steady-state kinetics were investigated. Both the Random and Ordered mechanisms are sequential processes in which the substrates are bound simultaneously before they can be converted into the products (37). On the other hand, in the Ping–Pong mechanism, one of the substrates reacts with the enzyme, leading to the formation of an intermediate and the release of the first product. Subsequently, the second substrate is bound and reacts with the intermediate and the enzyme catalyzes the formation of the final product. The Ping–Pong mechanism is common in many group transfer reactions (37), in which the first substrate carries the group that is later on transferred to the second substrate. Therefore, it would be reasonable to postulate that ApbE flavin-transfer reaction also follows a Ping–Pong mechanism in which FAD provides the FMN moiety and NqrC is the acceptor substrate. However, bi-substrate kinetic analyzes show that the kinetic behavior of ApbE is best explained by a sequential mechanism in which a ternary enzyme–substrates complex is formed during the reaction, suggesting that both ApbE and NqrC participate in enzyme catalysis.

To distinguish between Random and Ordered mechanisms, product inhibition kinetic experiments were performed, using AMP. The results reveal that AMP behaves as a mixed inhibitor against NqrC. As illustrated in Fig. 2E, AMP competes with NqrC for the free form of the enzyme (E), and it is also bound “uncompetitively” to the modified form (E-NqrC-FMN), explaining the mixed behavior. On the other hand, if ApbE followed an ordered mechanism with either FAD or NqrC binding first, AMP would act as an uncompetitive inhibitor with respect to NqrC.

Interestingly, AMP inhibition kinetics have also demonstrated that the ternary complex with the bound inhibitor remains catalytically active, yet to a lesser extent. Our previous study has shown that ApbE is activated by ADP, probably through an allosteric site (23). Thus, it is possible that at high concentrations AMP can be bound to the regulatory site, de-inhibiting the enzyme and producing the partial inhibitory behavior. Further work is being conducted to clarify the location of this site and its role.

**Role of His-257 in catalysis**

In our previous report His-257 was found to be essential for catalysis, the pH profiles of the activity of H257G and H257T under near-saturating concentrations of both substrates were examined, which can provide insight into the apparent pK\textsubscript{ES1} of the mutants. The mutants displayed pH dependence explained by Equation 5 that results in a sigmoidal curve (37), from which the critical kinetic parameter pK\textsubscript{ES1} was obtained. Both mutants display increased pK\textsubscript{ES1} values by more than 2 units, demonstrating that His-257 helps in the deprotonation of the catalytic residue, and that it likely acts as a general base. Consequently, it is possible that His-257 deprotonates the FMN acceptor residue Thr-225 in NqrC, which activates this residue. However, the canonical pK\textsubscript{a} of threonine found in aqueous solution is above 13 (45), significantly higher than the pK\textsubscript{ES1} values found in the mutants. Interestingly, a conserved Lys-207 in NqrC was discovered to be essential for flavin transfer (38). It was proposed that this residue serves as a general base that deprotonates Thr-225 due to its proximity to the threonine residue. Our data suggest that Lys-207 may have a different role. pK\textsubscript{a} predictions by PROPKA3.1 show that the pK\textsubscript{a} of residue 225 is clearly influenced by the charge of residue 207, i.e. a positive charge will shift the pK\textsubscript{a} down. In addition, the predictions showed that the pK\textsubscript{a} of residue 207 is within 1 pH unit. In general, such change does not support the role of Lys-207 as the catalytic residue. We propose that Lys-207 lowers the pK\textsubscript{a} of Thr-225, which allows its deprotonation by His-257 and/or stabilizes the alkoxy form through its positive charge (46).
Kinetic and reaction mechanisms of V. cholerae ApbE

Table 4

Mutagenesis primers

| Primer sequence                      |
|-------------------------------------|
| ApbE H257G sense                    |
| ApbE H257G antisense                |
| ApbE H257T sense                    |
| ApbE H257T antisense                |
| ApbE H257E sense                    |
| ApbE H257E antisense                |
| ApbE H257K sense                    |
| ApbE H257K antisense                |

Proposed mechanism of flavin transfer

Based on the data obtained in this study, a reaction mechanism of ApbE-catalyzed flavin transfer is proposed and illustrated in Fig. 4D. In the first step of the reaction, the two substrates, FAD and NqrC, are bound to the enzyme in a random order, forming the ternary complex. The binding of FAD seems to be mediated through the coordination with the Mg\(^{2+}\) ions, as shown in the crystal. When both substrates are bound, Lys-207 lowers the \(pK_a\) of Thr-225 to a physiologically feasible value as its positive charge shifts the ionization equilibrium of Thr-225 toward the alkoxide form. His-257 subsequently acts as a general base, removing a proton from Thr-225. The deprotonated Thr-225 then performs a nucleophilic attack on the pyrophosphate group of FAD and eventually forms the phosphoester bond between FMN and NqrC, finalizing the flavin-transfer process.

Similar cases have been reported in other protein families such as the Fic domain (47, 48) and serine protease (49). The Fic domain is an adenylyltransferase that integrates AMP to either Thr or Tyr residues of the Rho-family GTPases, which then inhibits downstream signaling pathways (50, 51). Two independent studies by Luong et al. (47) and Xiao et al. (48) have demonstrated that a conserved His residue in the Fic domain is essential for the adenylyltransferase activity. The authors proposed that the His residue functions as a base that deprotonates the AMP acceptor Thr/Tyr residue, leading to a subsequent nucleophilic attack on ATP by the deprotonated residues and hence AMPylation of the protein substrate. In the case of the well-studied serine proteases, the classic catalytic triad is composed of Asp, His, and Ser residues. Peptide hydrolysis is initiated by His, which as a base abstracts a proton from Ser, allowing it to attack the peptide bond, whereas Asp stabilizes the protonated His (49). These mechanisms are analogous to the one proposed in this work, describing a general base catalysis mechanism in which the His residue deprotonates the hydroxyl groups of Thr/Ser/Tyr, activating these residues for subsequent nucleophilic attacks.

Conclusions

The flavin transferase family ApbE catalyzes the incorporation of flavin cofactors into a variety of bacterial respiratory enzymes and hence plays a critical physiological role in the survival and pathogenicity development of these bacteria. In this work, we have demonstrated that ApbE follows a random sequential mechanism and that the conserved His-257 residue participates in the catalytic mechanism of this enzyme as a general base. These results allow us to gain a deeper understanding of the flavin-transfer mechanism catalyzed by ApbE and the interplay between this enzyme and a variety of essential bacterial respiratory enzymes.

Experimental procedures

Recombinant plasmid construction

Recombinant V. cholerae ApbE and NqrC, as a substrate of the flavin-transfer reaction, were heterologously expressed in E. coli (23), as previously described. Briefly, the apbe and nqrc genes were engineered to carry a His\(_6\) tag at the C terminus for protein purification. The leader sequence of ApbE, and the N-terminal transmembrane section of NqrC, were eliminated during the cloning procedure, allowing high yields of protein expression (20, 23). The engineered fragments were inserted into the pBAD/HisB vector, transformed into E. coli DH5\(\alpha\) for plasmid production, and subsequently used for transformation of E. coli BL21 for protein expression.

Site-directed mutagenesis

To study the role of His-257, mutants Gly, Thr, Asp, and Lys were obtained as previously described using the QuikChange site-directed mutagenesis kit (Agilent Technologies). The primers used are listed in Table 4 (23).

Protein expression and purification

Recombinant proteins were expressed and purified as described previously (23). Briefly, E. coli cells carrying the proteins of interest were grown in terrific broth media (supplemented with 0.4% glycerol). 0.05% arabinose was added to induce the expression of the proteins. The cells were then harvested by centrifugation and lysed by sonication in washing buffer, containing 300 mM NaCl, 1 mM MgCl\(_2\), 5 mM imidazole, 50 mM Na\(_2\)HPO\(_4\), pH 8.0. Subsequently, the soluble cytosolic proteins were obtained by ultracentrifugation, after which the supernatant was collected and subject to two steps of chromatographic purification: nickel-nitrotriacetic acid affinity chromatography and DEAE-Sepharose cation exchange chromatography. Purities >95% were obtained for both proteins, as determined by SDS-PAGE.

Protein crystallization

Prior to crystallization, protein samples were subject to size exclusion chromatography with a Superdex 75 10/300 column (GE Healthcare) in buffer containing 10 mM HEPES, 150 mM NaCl, pH 8.0, to remove possible protein aggregates. Protein samples were supplemented with 2 mM FAD and 5 mM MgCl\(_2\), and subsequently subject to size exclusion chromatography with a Superdex 75 10/300 column (GE Healthcare) in buffer...
containing 10 mM HEPES, 150 mM NaCl, 500 µM FAD, 5 mM MgCl$_2$, pH 8.0.

The initial crystallization conditions were determined with a sparse crystallization matrix at 16 °C temperatures using the sitting-drop vapor-diffusion technique using MCSG crystallization suite (MicroArt) and PEG/Ion HT screen (Hampton Research). The crystals grew in multiple conditions after a couple weeks of incubation. The best crystals of WT and H257G mutant ApbE protein were obtained from MCGS-4 screen, reagent formulation number 86 (0.1 M sodium acetate, 25% PEG 4000, 8% isopropyl alcohol) and MCSG-4 screen, reagent formulation number 66 (0.1 M sodium acetate, 22% PEG 4000, 0.1 M HEPES buffer, pH 7.5), respectively. Crystals selected for data collection were soaked in the crystallization buffer supplemented with 15% ethylene glycol and flash-frozen in liquid nitrogen.

**Data collection, structure determination, and refinement**

Single-wavelength X-ray diffraction data were collected at 100 K temperature at the 19-ID beamline (52) of the Structural Biology Center at the Advanced Photon Source at Argonne National Laboratory using the program SBCcollect. The intensities were integrated and scaled with the HKL3000 suite (53). The structures were determined by molecular replacement using the HKL3000 suite (53) incorporating the following programs, MOLREP (54), SOLVE/RESOLVE (55), and ARP/wARP (56). The coordinates of *S. enterica* ApbE protein (PDB 3PND) (42) were used as the starting model for the structure solution. Several rounds of manual adjustments of structure models using COOT (57) and refinements with the Refmac program (58) from the CCP4 suite (59) were done. The stereochemistry of the structure was validated with PHENIX suite (60) incorporating MOLPROBITY (61) tools. A summary of data collection and refinement statistics is given in Tables 5 and 6.

### Coordinates

The atomic coordinates and structure factors of WT and H257G mutant ApbE protein were deposited into the Protein Data Bank as 6NXI and 6NXJ, respectively.

### Flavin-transfer activity measurement

We are reporting a completely novel method of flavin-transfer activity measurement catalyzed by ApbE, based on the change in UV-visible absorption spectrum of free FAD versus covalently-bound FMN. The flavin transferase activity of ApbE was followed at 395-minus-366 nm as the difference spectrum of holo-NqrC-minus-FAD shows that the most significant difference is observed at 395 nm, whereas 366 nm is the isosbestic point (Fig. 1A, inset). The molar extinction coefficients of free FAD and free FMN were 12.50 mM$^{-1}$ cm$^{-1}$ at 450 nm (62) and 12.02 mM$^{-1}$ cm$^{-1}$ at 445 nm, respectively (63). In addition, the molar extinction coefficient of the flavinylated NqrC at 450 nm was determined to be 13.91 mM$^{-1}$ cm$^{-1}$.

### Steady-state kinetics

To investigate the kinetic properties of WT ApbE and the mutants, steady-state kinetic experiments were performed in reaction buffer containing 100 mM KCl, 1 mM EDTA, 5 mM MgCl$_2$, 50 mM Tris, pH 9.0. NqrC titrations were carried out at a fixed concentration of FAD (50 µM), using an ApbE concentration of 0.5 µM. On the other hand, FAD titrations were run at a fixed concentration of NqrC (100 µM), at an enzyme concentration of 0.01 µM to allow a more accurate measurements of the reaction rates.

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**Table 5**

| Crystal data collection statistics | Wildtype | H257G |
|----------------------------------|----------|-------|
| X-ray wavelength (Å)             | 0.9792   | 0.9792|
| Space group                       | $P_2_1$  | $P_2_1$|
| Unit cell dimensions              | $a = 39$ Å, $b = 71$ Å, $c = 107$ Å, $\alpha = \beta = \gamma = 90^\circ$ | $a = 49$ Å, $b = 72$ Å, $c = 105$ Å, $\alpha = \beta = \gamma = 90^\circ$, $\beta = 93^\circ$ |
| Resolution* (Å)                  | 42.7–1.62 (1.65–1.62) | 36.9–1.92 (1.95–1.92) |
| No. of unique reflections         | 38,465 (1667) | 53,702 (2614) |
| Completeness                      | 98.9% (88.1%) | 97.0% (95.0%) |
| Rmerge                           | 0.097 (0.695) | 0.107 (0.640) |
| CC1/2 (Å$^2$)                    | 0.985 (0.804) | 0.992 (0.707) |
| I/α                              | 22.9 (1.74) | 13.4 (1.53) |
| Redundancy                       | 7.7 (6.1) | 3.3 (2.9) |
| Molecules per asymmetric unit    | 1        | 2     |
| No. of protein residues           | 336      | 672   |

* Numbers in parentheses are shown for the highest resolution shell.

**Table 6**

| Structure refinement statistics | Wildtype | H257G |
|--------------------------------|----------|-------|
| PDB accession code             | 6NXI     | 6NXJ  |
| Resolution range (Å)           | 42.7–1.61 (1.65–1.614) | 36.9–1.92 (1.97–1.92) |
| Rmerge                          | None     | None  |
| R-value (all) (%)              | 17.46    | 19.63 |
| R-value (R-work) (%)           | 17.25 (25.5) | 19.42 (29.7) |
| Free R-value (%)               | 21.39 (25.9) | 23.68 (32.0) |
| Mean R-factor (Å$^2$)          | 0.011    | 0.010 |
| Angle (degrees)                | 1.70     | 1.57  |
| Chiral (Å)                     | 0.088    | 0.075 |
| No. of atoms                   | 2,444    | 4,649 |
| Protein                        | 53       | 106   |
| Magnesium ions                 | 2        | 4     |
| 1,2-Ethanediol                 | 4        | –     |
| Water                           | 249      | 334   |
| Mean B-factor (Å$^2$)          | 31.2     | 36.9  |
| All atoms                      | 30.6     | 36.9  |
| Protein atoms                  | 28.6     | 34.9  |
| Protein main chain             | 32.7     | 39.0  |
| Protein side chain             | 25.2     | 28.8  |
| FAD                            | 20.7     | 27.4  |
| Magnesium ions                 | 37.1     | –     |
| 1,2-Ethanediol                 | 38.4     | 39.3  |
| Water                           | 1.03     | 1.18  |

**Kinetic and reaction mechanisms of V. cholerae ApbE**

We are reporting a completely novel method of flavin-transfer activity measurement catalyzed by ApbE, based on the change in UV-visible absorption spectrum of free FAD versus covalently-bound FMN. The flavin transferase activity of ApbE was followed at 395-minus-366 nm as the difference spectrum of holo-NqrC-minus-FAD shows that the most significant difference is observed at 395 nm, whereas 366 nm is the isosbestic point (Fig. 1A, inset). The molar extinction coefficients of free FAD and free FMN were 12.50 mM$^{-1}$ cm$^{-1}$ at 450 nm (62) and 12.02 mM$^{-1}$ cm$^{-1}$ at 445 nm, respectively (63). In addition, the molar extinction coefficient of the flavinylated NqrC at 450 nm was determined to be 13.91 mM$^{-1}$ cm$^{-1}$.
Kinetic and reaction mechanisms of V. cholerae ApbE

To understand the kinetic mechanism of ApbE, bi-substrate kinetic experiments were carried out. Initial rates were measured through FAD titrations (0, 0.1, 0.2, 0.5, 1, and 2 μM) at different fixed concentrations of NqrC (5, 10, 20, and 100 μM). Rates calculated were then plotted and fitted to different bi-substrate kinetic models to determine the kinetic mechanism. Three bi-substrate kinetic models used were: 1) Random, 2) Ordered, and 3) Ping–Pong, and are described below.

Random

\[ \frac{v}{[E_i]} = \frac{k_{cat}[A][B]}{\alpha K_a K_b + \alpha K_a [B] + \alpha K_b [A] + [A][B]} \]  

(Ordered)

\[ \frac{v}{[E_i]} = \frac{k_{cat}[A][B]}{K_a K_b + K_a [A] + [A][B]} \]  

(Ping–Pong)

\[ \frac{v}{[E_i]} = \frac{k_{cat}[A][B]}{K_b [B] + K_a [A] + [A][B]} \]  

where \(k_{cat}\) is the turnover rate, \([A]\) and \([B]\) are the concentrations of FAD and NqrC, respectively, \(K_a\) and \(K_b\) are the Michaelis constants of the enzyme–substrate complexes, and \(\alpha\) is the factor by which the binding of one substrate changes the Michaelis constant of the other (37).

To investigate if ApbE fits in either the random or ordered kinetic model, ApbE inhibition kinetics were carried out. In particular, NqrC titrations at a fixed concentration of FAD (50 μM) and different fixed concentrations of AMP (0, 0.01, 0.2, and 5 mM) were performed. Rates were calculated and globally fitted to the partial mixed inhibition equation shown below.

\[ \frac{v}{[E_i]} = \frac{k_{cat}[S] + k_{cat,R}[S][I]}{K_m + [S][I]} \]  

where \(k_{cat}\) is the turnover rate, \(k_{cat,R}\) is the turnover rate obtained at saturating concentrations of AMP, \([S]\) is the concentration of NqrC, \([I]\) is the concentration of AMP, \(K_m\) is the Michaelis constant of the enzyme–substrate complex, \(K_a\) and \(K_b\) are the Michaelis constants of the enzyme–inhibitor complex and enzyme–substrate–inhibitor complex, respectively (37).

ApbE pH-dependence assay

To inspect the pH dependence of ApbE WT and the mutants, pH-dependence assays were carried out in buffer containing 100 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 50 mM MES, 50 mM Tris, 50 mM CAPS, at a series of pH values (6.0–10.5). Initial rates at near saturating concentrations of the substrates were obtained and plotted against pH. For WT ApbE the concentrations of substrates used were: 50 μM FAD (>1000 × \(K_m\)) and 100 μM NqrC (10 × \(K_m\)). For the mutants these concentrations were: 50 μM FAD (250 × \(K_m\)) and 500 μM NqrC (5 × \(K_m\)). The data were fitted to the following equation describing the kinetic behavior of the enzyme at varying pH values.

\[ \frac{v}{[E_i]} = \frac{k_{cat}[S]}{K_m \left(1 + \frac{pH}{pK_{E1}}\right) + [S] \left(1 + \frac{pH}{pK_{ES1}}\right)} \]  

where \(k_{cat}\) is the turnover rate, \([S]\) is the concentration of NqrC, \(K_m\) is the Michaelis constant of the enzyme–substrate complex, \(pK_{E1}\) is the negative logarithm of \(K_{E1}\), the equilibration constant of the enzyme deprotonation, and \(pK_{ES1}\) is the negative logarithm of \(K_{ES1}\), the dissociation constant of the protonated enzyme–substrate complex (37).

\(pK_a\) predictions

To predict the effect of the Lys-207 residue on Thr-225, the \(pK_a\) values for all ionizable residues in NqrC were predicted using PROPKA3.1 (39, 40). Protein models were protonated using UCSF Chimera (41). However, due to limitations of the software, \(pK_a\) predictions are not possible for residues other than the standard ionizable residues. To combat the inability of PROPKA3.1 to predict the \(pK_a\) of Thr-225, Thr-225 was mutated in silico to Cys-225 using UCSF Chimera (41). Although the predicted \(pK_a\) of Cys-225 will not reflect the \(pK_a\) of Thr-225 in an absolute manner, relative changes in calculated Cys-225 \(pK_a\) can be expected to be mirrored by Thr-225. Four mutants at position 207 (K207A, K207R, K207E, K207Q) were created in silico again using UCSF Chimera (41) to elucidate the role of residue 207 on the \(pK_a\) of residue 225. These mutants were chosen due to their diverse nature in charge, size, and polarity. With these four mutants and the WT, the effects of positive charge, negative charge, polarity, and hydrophobicity of residue 207 on residue 225 were determined.

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