九重子Counting Protein ApbE Is a Flavin Transferase Catalyzing Covalent Attachment of FMN to a Threonine Residue in Bacterial Flavoproteins*

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Background: The ApbE protein with unknown function is widespread in bacteria.
Results: ApbE catalyzes Mg$^{2+}$-dependent FMN transfer from FAD to Thr residues of flavoproteins in vitro and in vivo.
Conclusion: ApbE is a novel modifying enzyme involved in the maturation of flavoproteins.
Significance: Broad distribution of ApbE suggests a wide utilization of flavoproteins containing FMN attached via a phosphoester bond.

Na$^+$-translocating NADH:quinone oxidoreductase (Na$^+$-NQR) contains two flavin residues as redox-active prosthetic groups attached by a phosphoester bond to threonine residues in subunits NqrB and NqrC. We demonstrate here that flavinylation of truncated Vibrio harveyi NqrC at Thr-229 in Escherichia coli cells requires the presence of a co-expressed Vibrio apbE gene. The apbE genes cluster with genes for Na$^+$-NQR and other FMN-binding flavoproteins in bacterial genomes and encode proteins with previously unknown function. Experiments with isolated NqrC and ApbE proteins confirmed that ApbE is the only protein factor required for NqrC flavinylation and also indicated that the reaction is Mg$^{2+}$-dependent and proceeds with FAD but not FMN. Inactivation of the apbE gene in Klebsiella pneumoniae, wherein the nqr operon and apbE are well separated in the chromosome, resulted in a complete loss of the quinone reductase activity of Na$^+$-NQR, consistent with its dependence on covalently bound flavin. Our data thus identify ApbE as a novel modifying enzyme, flavin transferase.

Na$^+$-translocating NADH:quinone oxidoreductase (Na$^+$-NQR)$^2$ is a redox-driven sodium pump that generates a transmembrane difference in electrochemical Na$^+$ potential (1). This enzyme has been shown to operate in the respiratory chain of various bacteria, including several pathogenic microorganisms (2, 3). Na$^+$-NQR consists of six subunits (NqrA–F) (4) encoded by six genes of the nqr operon (5, 6) and has a unique amino acid sequence and set of prosthetic groups, some of which are covalently bound. The enzyme contains one [2Fe-2S] cluster, one noncovalently bound FAD and riboflavin, and two covalently bound FMN residues (1, 7). The [2Fe-2S] cluster and FAD are found in subunit NqrF (8, 9), riboflavin is present in subunit NqrB (10), and covalently bound FMN residues are located in subunits NqrB and NqrC (2, 11).

Bacteria may additionally contain a Na$^+$-NQR homolog, the so-called RNF complex. It has been proposed that RNF catalyzes electron transfer from ferredoxin to NAD$^+$ (12, 13) or methanophenazine (14) and, depending on the direction of the catalyzed redox reaction, can act as a Δμ$_{\text{Na}^+}$ generator (forward reaction) (13) or consumer (reverse reaction) (12). The RNF complex is also formed by six subunits, five of which are homologous to the corresponding NqrA–E subunits. The RNF subunits RnfD and RnfG (paralogs of NqrB and NqrC, respectively) have been shown to contain covalently bound FMN residues as redox-active prosthetic groups (15).

Several types of bonds between flavins and proteins are known. In Na$^+$-NQR (11), RNF (15), and closely related proteins, such as regulator of NO reductase transcription (NosR) (16) and urocanate reductase (UrdA) (17), FMN is bound by a phosphoester bond through a Thr residue. Other acceptors of flavins in flavoproteins include His, Tyr, and Cys residues that form C-N, C-O, and C-S bonds, respectively, mostly through coupled reductase, sarcosine oxidase, trimethylamine dehydrogenase, and p- cresol methylhydroxylase (18).

It has generally been thought that these types of covalent bonds between flavin and proteins form in autocatalytic reactions (18, 19). However, flavinylation of succinate dehydrogenase (at His) was recently reported to require a specific protein (SdhE) as an FAD chaperone (20, 21). Heterologous expression of the Vibrio cholerae nqrC gene in Escherichia coli results in production of a flavin-deficient apo-form of NqrC (22), also suggesting that some unknown protein factor, absent in E. coli, is required for NqrC flavinylation. Similar results have been recently obtained for Shewanella oneidensis urdA expressed in E. coli (17). Although Na$^+$-NQR subunits NqrB and NqrC and related FMN-binding proteins have completely different overall primary and tertiary structures (23), they contain a common pattern around the FMN-carrying Thr residue (Fig. 1) reminis-
FIGURE 1. Sequence alignment of FMN-binding motifs in several prokaryotic proteins. Vh_NqrB and Vh_NqrC, V. harveyi Na+-NQR subunits NqrB and NqrC (accession numbers Q9RFW0 and Q9RFV9, respectively); Vc_RnFD and Vc_RnFG, V. cholerae RNF subunits RnFD and RnFG (ACP0041 and ACP0040, respectively); Pd_NosR, P. denitrificans regulator of NO reductase transcription NosR (Pden_4220); So_UrdA, S. oneidensis MR-1 urocanate reductase (YP_002238733). The position of the experimentally identified FMN acceptor Thr residue (11, 15) is marked by an asterisk.

FIGURE 2. Typical arrangements of genes for ApbE, Na+-NQR, RNF, and other FMN-binding proteins in bacterial chromosomes. Genes for Na+-NQR and RNF subunits are indicated by the last letters in their standard designations. apbE genes together with their accepted alternative designations are shown as rectangles, genes for FMN-binding Na+-NQR and RNF subunits, UrdA, NosR, and other FMN-binding proteins (fmb) are indicated by gray shading. The connecting lines refer to intercalating sequences. The numbers above the genes indicate their numbers in genomes according to the Kyoto Encyclopedia of Genes and Genomes. The genes were detected as hits in a BLAST search of complete prokaryotic genomes using the V. harveyi proteins as the queries. Genes for FMN-binding proteins were identified using search for Pfam motifs PF04205 and PF03116.
Undamaged cells and cell debris were removed by centrifugation at 22,500 \( \times \) g (10 min), and the supernatant was further centrifuged at 180,000 \( \times \) g (60 min). The membrane pellet was suspended in medium 2 (20–30 mg of protein ml\(^{-1}\)) and immediately used for activity measurements.

**Activity Assays**—NADH and dNADH oxidation by membrane vesicles isolated from *K. pneumoniae* strains were measured at 30 °C using a Hitachi 557 spectrophotometer at 340 nm. The reaction medium contained 20 mM HEPES-Tris, 5 mM MgSO\(_4\), and 100 mM KCl or NaCl (pH 8.0). For measurement of dNADH:menadione oxidoreductase activity, the reaction medium was supplemented with 50 mM menadione. The extinction coefficient, \( \varepsilon_{340} \), of 6.22 mM\(^{-1}\) cm\(^{-1}\) was used for NADH and dNADH quantitation.

**Isolation of Recombinant His\(_6\)-tagged NqrC**, ApbE', and UrdA Proteins—For nqrC, apbE', and urdA induction, *E. coli* cells bearing the appropriate plasmid(s) were grown at 32 °C to mid-exponential phase (\( A_{600} = 0.3–0.4 \)), after which the growth medium was supplemented with 0.2% (w/v) i-arabinose, and cells were grown for additional 3 h. The cells were harvested by centrifugation (10,000 \( \times \) g, 10 min) and washed twice with medium containing 300 mM NaCl, 10 mM Tris-HCl, and 5 mM MgSO\(_4\) (pH 8.0). The cell pellet was suspended in medium containing 300 mM NaCl, 20 mM Tris-HCl, 5 mM MgSO\(_4\), 1 mM phenylmethylsulfonyl fluoride, and 5 mM imidazole HCl (pH 8.0), and the suspension was passed twice through a French press (16,000 p.s.i.). Cell debris and membrane vesicles were removed by centrifugation at 180,000 \( \times \) g (60 min). His\(_6\)-tagged proteins were purified from the supernatant using affinity chromatography. This was accomplished by loading the supernatant onto a nickel-nitrilotriacetic acid column equilibrated with solution A containing 300 mM NaCl, 10 mM Tris-HCl, and 5 mM MgSO\(_4\) (pH 8.0). The cell pellet was suspended in medium containing 300 mM NaCl, 20 mM Tris-HCl, 5 mM MgSO\(_4\), 1 mM phenylmethylsulfonyl fluoride, and 5 mM imidazole HCl (pH 8.0), and the suspension was passed twice through a French press (16,000 p.s.i.). Cell debris and membrane vesicles were removed by centrifugation at 180,000 \( \times \) g (60 min). His\(_6\)-tagged proteins were purified from the supernatant using affinity chromatography. This was accomplished by loading the supernatant onto a nickel-nitrilotriacetic acid column equilibrated with solution A containing 300 mM NaCl, 10 mM Tris-HCl, and 5 mM MgSO\(_4\) (pH 8.0). 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with the flow rate of 0.1 ml/min was used. Flavins were detected at 360 nm.

**Mass Spectrometry**—Mass spectra were recorded on an Ultraflex Extreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a neodymium laser. Aliquots of the sample were mixed on a steel target with a solution of 2,5-dihydroxybenzoic acid (20 mg/ml) in 30% (v/v) TFA. The [MH]+ molecular ions were analyzed in linear (proteins) or reflector (peptides) mode; the values of m/z were accurate to 30 ppm. Fragment ion spectra were obtained in Lift mode with a mass accuracy of better than 1 Da.

**In Vitro Flavinylation of apoNqrC** Protein—The apoNqrC protein (4 mg/ml) was incubated for 45 min at 30 °C in medium containing 100 mM NaCl, 0.1 mM EDTA, 1 mM FAD or FMN, 10 mM Tris–HCl (pH 8.0) in the presence or absence of 0.1 mg/ml ApbE and/or 5 mM MgSO4. After the incubation, the reaction mixtures were separated by SDS-PAGE.

**Electrophoresis**—SDS-PAGE was performed using 12.5% (w/v) polyacrylamide gels (29). Covalently bound flavins were detected by photographing gels under UV illumination with a GelDoc-It M-26XV gel documentation system using a SYBR Green emission filter.

**RESULTS**

**Genomic Context Analysis of the nqr and rnf Operons**—Bacterial genes are known to often cluster according to their functions (30), a feature that facilitates identification of functionally coupled proteins (31). Genome context analysis of the nqr operons in marine bacteria of the genus *Vibrio*, a popular source of Na+-NQR, revealed the gene *apbE*, encoding the putative “alternative pyrimidine biosynthesis” protein ApbE, immediately downstream of the nqr operon in all *Vibrio* species with known genome sequences (Fig. 2). In contrast to this conservation at downstream positions, upstream positions are occupied by a variety of genes. This same regularity holds throughout the entire set of ~1600 sequenced bacterial genomes, most of which, primarily proteobacteria, contain the *apbE* gene adjacent to the nqr operon. In the genomes of Na+-NQR-containing bacteria of the Chlamydiae/Verrucomicrobia group, Deferribacteres, Fusobacteria, Spirochaetes, Thermotogae, and some enterobacteria (*e.g.*, *K. pneumoniae*), the *apbE* gene is also invariably present, but at more distant positions from the nqr operon (Fig. 2). Thus, all nqr-containing bacterial genomes contain the *apbE* gene, which is most commonly adjacent to the nqr operon.

Similarly, the genomes of bacteria containing a homologous RNF complex with covalently bound FMN residues as prothetic groups (15) always carry an *apbE* gene (Fig. 2), which is frequently located adjacent to the rnf operon; sometimes proteins containing an ApbE-like domain are even designated RnF (12). We found only one exception to this rule: some species of the genus *Buchnera* (obligate endosymbionts of aphids) possess the full rnf operon, but their *apbE* genes contain an authentic frameshift. The nqr and rnf operons are thus genetically coupled to *apbE*, suggesting a functional link between the Na+-NQR (RNF) and ApbE proteins. Even when an *apbE*-like gene is clustered in bacterial genomes with genes for proteins other than NqrB, NqrC, RnfD, and RnfG, their encoded proteins are also often shown or predicted to contain a covalently bound FMN residue. Such examples include the FMN-containing NosR protein (Pden_4220) and ApbE-like protein NosX (Pden_4214) of *Paracoccus denitrificans*; the presumptive FMN-containing UrdA-like urocanate reductases (Shal_2847, Ssed_0695, and ZP_02156482) and ApbE-like proteins (Shal_2846, Ssed_0695, and ZP_02156483) of *Shewanella halifaxensis*, *Shewanella sediminis*, and *Shewanella benthica* KT99, respectively; and the FAD/FMN-binding/flavocytochrome c (KPK_2907) and ApbE-like protein (KPK_2905) of *K. pneumoniae* (Fig. 2). In addition, there are several examples in genomic databases where the genes of an ApbE-like protein and a putative FMN-binding protein are fused, resulting in one elongated gene (CAJ73234, ZP_03630453, and ZP_10101304).

**ApbE Catalyzes Flavinylation of NqrC When Both Are Co-produced in *E. coli* Cells**—The above analysis suggested that the ApbE protein is the sought-after “flavin-attaching” enzyme. To test this hypothesis, we measured the effect of co-expressing *V. harveyi* nqrC and *V. cholerae* apbE in *E. coli* cells on flavin incorporation into NqrC.

As originally reported by Barquera et al. (22), heterologous expression of the *V. cholerae* nqrC gene in *E. coli* cells results in the production of flavin-deficient NqrC. A similar result was obtained in the present work using a truncated *V. harveyi* NqrC (NqrC') protein in which the N-terminal transmembrane α-helix was eliminated by genetic manipulations to make NrqC water-soluble. The NqrC' protein isolated from *E. coli/pMshC* cells showed no bands characteristic of bound flavin in absorption spectra (Fig. 3A, red line), and no fluorescent band was detected in SDS-PAGE analyses (Fig. 4A, lane 1).

To construct an *E. coli* cell line co-expressing *V. harveyi* nqrC' and *V. cholerae* apbE, we introduced a second plasmid (*pΔhis3*) containing a gene encoding a cytoplasmic variant of *V. cholerae* ApbE lacking its leader sequence, ApbE', into *E. coli/pMshC*. Upon induction of simultaneous synthesis of NqrC' and ApbE', the cells became yellow-colored. Purification of NqrC' from these cells yielded a bright yellow protein, which was detected as a ~27-kDa fluorescent band (calculated mass of the His6-tagged NqrC' protein, 27.5 kDa) in SDS-polyacrylamide gels (Fig. 4A, lane 2), confirming formation of holoNqrC'. Similar results were obtained for another FMN-containing protein, urocanate reductase UrdA, from *S. oneidensis* MR-1, which was produced as a flavinylated protein when co-expressed with the ApbE-encoding plasmid *pΔhis3* (Fig. 4C) but was detected as an apoprotein when expressed alone in *E. coli* (17).

The absorption spectrum of the presumed holoNqrC' (Fig. 3A, blue line) exhibited three peaks with maxima at 273, 392, and 452 nm, and a shoulder ~475 nm. All three peaks were significantly red-shifted compared with the spectra of free flavin in water solutions (32), indicating strong interaction of the flavin isoalloxazine moiety with NqrC'. Interestingly, the short wavelength band of the spectrum (392 nm) was more intense than the longer wavelength band (452 nm), which is not typical for flavoproteins. A nearly identical spectrum was reported for RnfG, an NqrC paralog from *V. cholerae* (15), suggesting that such a perturbation in the spectrum may be an intrinsic prop-
An anionic form of flavosemiquinone (33) is stabilized in reduced holoNqrC obtained from the data in Fig. 3.

The flavin content of holoNqrC’ was determined in two different ways. First, the masses of the major protein peaks seen in mass spectra (Fig. 5A) differed by 437 Da between the apo- and holoNqrC’, which compares well with the mass of an FMN residue (438.3 Da). Thus, the spectra indicated that a single FMN residue was attached per NqrC’ molecule with a nearly quantitative yield. Second, the amounts of covalently and noncovalently bound flavins were semiquantitatively estimated using a classical acid/alkali treatment procedure (28). The NqrC’ protein obtained in co-expression experiment was precipitated with 7.5% (v/v) TFA and sedimented by centrifugation. Essentially no flavins were detected in the supernatant, indicating that this protein does not contain flavin bound covalently or by acid-labile bonds. Subsequent treatment of the protein pellet with LiOH caused a complete release of bound flavin into solution, consistent with its being bound to NqrC’ by a phosphoester bond (28). HPLC analysis of the alkaline extract revealed FMN (97%), traces of riboflavin (3%), and no FAD (Fig. 5B).

The site of the modification in NqrC’ was identified by mass spectral analysis of the tryptic digests of both NqrC’ proteins. There was only one signal, with an m/z of 4254 in apoNqrC’, that was shifted to an m/z of 4693 in holoNqrC’ (Fig. 6, A and B). This signal corresponded to the peptide occupying positions 212–254 (GGAPEGEHGVDLSGATLTGNGVQGTDFWLGDMFGPFLAK). The position of the FMN residue within this peptide was determined by analysis of the fragmentation spectra of modified and unmodified peptide variants (Fig. 6, C and D). With the unmodified peptide, a complete series of expected N-terminal (b-ions) and C-terminal (y-ions) fragments was observed, whereas the series ended at Thr-229 in the spectrum of the flavinylated peptide, identifying Thr-229 as the site of the modification.

These data show that ApbE is indeed required for flavin incorporation in NqrC and that the binding mode of the incor-
porated flavin is identical to that in authentic NqrC isolated from Vibrio alginolyticus or V. cholerae cells (11, 28). Interestingly, the E. coli genome contains its own apbE-like gene (Fig. 2) and, presumably, the corresponding ApbE protein. The inability of E. coli proteins to flavinylate V. cholerae (22), S. oneidensis MR-1 (17), and V. harveyi NqrC proteins suggested that ApbE proteins are species-specific.

**Isolated ApbE Can Flavinylate apoNqrC in vitro**—To test whether other cellular factors are required for NqrC flavinylation, we carried out the reaction with isolated V. cholerae ApbE’ and flavin-free V. harveyi NqrC’. The genes for both proteins were separately expressed in E. coli, and the produced proteins were isolated by affinity chromatography.

Remarkably, incubation of apoNqrC’ with ApbE’ (at a molar ratio of 55:1) for 45 min in the presence of FAD and Mg²⁺ resulted in the appearance of a ~27-kDa fluorescent band on gels after SDS-PAGE of the reaction mixture (Fig. 7B, lane 3), indicative of covalent bond formation between NqrC’ and flavin (2, 21). Using the holoNqrC’ protein purified from the E. coli/pMshC3 + pΔhis3 strain as a fluorescent band standard, we were able to estimate that the yield of flavinylated NqrC’ upon incubation with ApbE’ was ~20%. Each molecule of ApbE’ thus produced ~10 molecules of holoNqrC’. In contrast, incubation of apoNqrC’ in the same medium but without ApbE did not produce holoNqrC’ (Fig. 7B, lane 1). Hence, the flavinylation reaction requires the presence of ApbE and is therefore not an autocatalytic process. The data in Fig. 7B (lanes 4 and 5) additionally indicate that flavinylation did not occur without Mg²⁺ or when FAD was replaced by FMN. An obvious corollary is that ApbE acts as a Mg²⁺-dependent FAD:protein FMN transferase.

**Activities of the Na⁺-NQR Complex in an ApbE1-deficient Strain of K. pneumoniae**—The above data clearly demonstrated that ApbE can flavinylate apoNqrC’ in vitro and in recombinant E. coli cells. To verify the physiological significance of this activity with a full-length apoNqrC that allows formation of a complete Na⁺-NQR complex in authentic cells, we constructed a mutant strain of K. pneumoniae with a disrupted apbE1 gene (YP_002237370) and measured the activities of Na⁺-NQR produced by this strain. Na⁺-NQR exhibits two main enzymatic activities in vitro: quinone reductase and NADH dehydrogenase. Quinone reductase activity is coupled with the transfer of Na⁺ across the membrane, is specifically activated by sodium ions, is inhibited by low HQNO concentrations, and is observed only with a complete form of the Na⁺-NQR complex (37). NADH dehydrogenase activity results in a single-electron reduction of soluble quinones (menadione, for example) and other artificial electron acceptors (38), does not depend on the concentration of sodium ions, is resistant to low HQNO concentrations, and is not energy-coupled. Only the FAD-binding domain of the Nqrf subunit is apparently required for this activity (8, 9).

There are several reasons that K. pneumoniae KNU210 (24) is the strain of choice for mutagenesis of apbE. First, the nqr operon and apbE1 are separated by 2,500,000 bp in the K. pneumoniae chromosome, making a polar effect of an apbE1 lesion on the nqr operon unlikely (Fig. 2). Second, Na⁺-NQR is the sole dNADH (reduced nicotinamide hypoxanthine dinucleotide)-oxidizing complex of the respiratory chain in the K. pneumoniae KNU210 strain because of disruption of the nuoB gene encoding a subunit of H−-translocating NADH:quinone oxidoreductase (NDH-1) (24). The other NADH:quinone oxidoreductase (NDH-2) of K. pneumoniae oxidizes only NADH, and not dNADH (39), whereas Na⁺-NQR acts on both substrates (40). This allowed us to estimate quinone reductase and NADH dehydrogenase activities of Na⁺-NQR in K. pneumoniae KNU210 membrane vesicles from measurements of dNADH oxidase and dNADH:menadione oxidoreductase activities, respectively (24, 40).

As Table 2 makes clear, the inactivation of apbE1 in K. pneumoniae strain KNUAE11 only slightly decreased the dNADH: menadione oxidoreductase activity of membrane vesicles, whereas the Na⁺-stimulated and HQNO-sensitive dNADH oxidase activity was completely lost. NDH-2-linked respiration (the difference between the NADH and dNADH oxidase activities) was not affected by apbE1 deletion (Table 2), indicating that complexes of the respiratory chain other than Na⁺-NQR were not affected in the ΔapbE1 K. pneumoniae strain. Importantly, introduction of a plasmid bearing the K. pneumoniae apbE1 gene (pATAE15) into the KNUAE11 strain resulted in a complete recovery of dNADH oxidase activity (Table 2).
FIGURE 6. MALDI mass spectral identification of the modification site in NqrC'. A and B, tryptic digests of apoNqrC' and holoNqrC', respectively. C and D, MS/MS spectra of the selected tryptic peptide of apoNqrC' (m/z = 4254) and holoNqrC' (m/z = 4693), respectively. The deduced protein sequences are shown.
finding ruled out the possibility of any polar effects of the \textit{apb}E1 lesion. These experiments thus demonstrated that ApbE1 is necessary for Na\textsuperscript{+}-NQR to exhibit quinone reductase activity, which requires covalently bound flavins, but not NADH dehydrogenase activity, which has no such requirement.

**DISCUSSION**

Covalently bound flavin is a common redox-active prosthetic group that can be attached to proteins by different bond types, yet the mechanisms of the post-translational modification remain largely unknown. In many flavoproteins, flavin is attached via a bond involving the 8\textalpha{}C of the flavin residue and the N1 or N3 of a protein His residue (18). The widely accepted view has been that this bond forms by an autocatalytic reaction (i.e., the modification does not require additional protein factors). However, it has been recently demonstrated that this type of flavin attachment to succinate dehydrogenase requires the presence of an SdhE chaperone protein (20, 21).

Here we report that formation of a different type of flavin-protein bond, a phosphoester bond involving a protein Thr residue, requires the presence of a specific enzyme, ApbE. The \textit{apb}E gene was originally identified in a study of the genes involved in thiamine synthesis in \textit{S. enterica}. Lesions in \textit{apb}E resulted in a conditional thiamine auxotrophy in this bacterium (41). The product of the \textit{apb}E gene, ApbE, was found to be a lipoprotein anchored to the periplasmic side of the inner membrane in \textit{S. enterica} (25). The principal difference between ApbE and SdhE is that the latter delivers a complete FAD molecule to the target protein, whereas ApbE catalyzes transfer of only the FMN moiety and thus acts as an Mg\textsuperscript{2+}-dependent flavin transferase (Fig. 8). ApbE is not homologous to SdhE, emphasizing the different flavinylation mechanisms utilized by these proteins.

Quite recently, the three-dimensional structures of four ApbE-like proteins have been solved (Protein Data Bank codes 1VRM, 2O18, 2O34, and 3PND) (42, 43). Remarkably, one of the structures (\textit{S. enterica} ApbE) contains a FAD molecule bound to a unique flavin-binding motif that was not previously observed in flavoproteins (43). The ability of ApbE to bind FAD is fully consistent with the proposed function of this protein as a flavin transferase. The adenosine and flavin moieties of FAD are embedded in the protein globule in the ApbE-FAD com-

![FIGURE 7. In vitro flavinylation of apoNqrC’.](image)

**TABLE 2**

Rates of NADH and dNADH oxidation by membrane vesicles isolated from different \textit{K. pneumoniae} strains

| Strain | NADH oxidase | dNADH oxidase | dNADH:menadione oxidoreductase |
|--------|--------------|---------------|---------------------------------|
| \textit{K. pneumoniae} KNU210 (nuoB::Cm) | 125\textsuperscript{a} | 255 | 235 |
| \textit{K. pneumoniae} KNUAE11 (nuoB::Cm apbE1::Km) | 1.6\textsuperscript{b} | 170 | 145 |
| \textit{K. pneumoniae} KNUAE11 (nuoB::Cm apbE1::Km)/pATAE15 (apbE1) | 100\textsuperscript{a} | 270 | 250 |

\textsuperscript{a} Activity was stimulated by sodium ions and inhibited by HQNO (4 \textmu{}M).

\textsuperscript{b} Activity was not stimulated by sodium ions or inhibited by HQNO (4 \textmu{}M).

![FIGURE 8. The reaction catalyzed by ApbE. HO-Pr, the protein to be modified.](image)
plex, whereas the pyrophosphate group of FAD is accessible to attack by an acceptor protein Thr (Fig. 9). Notably, FAD does not bind to *S. enterica* ApbE very tightly, and the major fraction of the complex dissociates during its purification (43). This is expected behavior for FAD as a substrate, rather than a prosthetic group, of ApbE.

The catalytic activity of ApbE described in the present work is in good agreement with previously determined phenotypes of mutant strains of different bacteria with lesions in apbE-like genes. For example, impaired nitrogen fixation in a Δ*nrfE* strain of *Rhodobacter capsulatus* (12) can be explained by an inability to flavinylate subunits of the RNF complex, given that this complex was proposed to be involved in electron transport to nitrogenase (12). Defective iron-sulfur cluster metabolism in a Δ*apbE* strain of *S. enterica* (44), leading to thiamine auxotrophy (41), can also be accounted for by impaired activity of the RNF complex that participates in maturation of iron-sulfur clusters (45, 46). Simultaneous disruption of the genes encoding two ApbE orthologs (NosX and NirX; Fig. 2) in *P. denitrificans* cells eliminates their NO reductase activity (47), which can now be explained by defective maturation of the regulator of NO reductase transcription NosR, which contains a covalently bound FMN residue (16).

*apbE*-like genes are found in the majority of known bacterial genomes, including many pathogens, such as *Yersinia pestis*, *V. cholerae*, *Shigella dysenteriae*, *S. enterica*, *Neisseria meningitidis*, and *Treponema pallidum*. These genes are completely absent only in Cyanobacteria. In Archaea, *apbE*-like genes have been found in only a few species of Methanosarcinaceae and Halobacteriaceae. Among Eukaryota, *apbE*-like genes are present in some Kinetoplastida (*Trypanosoma* and *Leishmania* species), wherein they form the N-terminal part of NADH-dependent fumarate reductase (48). The number of the *apbE*-like genes may vary from one (e.g., in *Chlamydia* spp.) to five (in some *Shewanella* spp.). This fact may mean that the corresponding bacteria require a spectrum of flavin transferases specificities to flavinylate different proteins. This inference is further supported by the observation that different ApbE-like proteins of a bacterium are sometimes only distantly related to each other. Thus, ApbE1 from *K. pneumoniae* (YP_002237370) clusters with ApbE-like proteins from other *Gammaproteobacteria* in a phylogenetic tree, whereas ApbE2 (YP_002238733) is close to the ApbE proteins from Firmicutes.

Although *apbE*-like genes are present in all *nqr* (*rnf*)-containing bacterial genomes, many *apbE*-containing microorganisms, including *Bacilli*, *Actinobacteria* (except for *Coriobacteriidae*), *Aquificae*, *Chloroflexi*, *Deinococcus-Thermus*, *Fibrobacteres-Acidobacteria*, *Kinetoplastida*, and *Halobacteriaceae*, contain no *nqr* or *rnf* genes in their genomes. This may mean that the corresponding organisms have as yet unrecognized ApbE-dependent proteins that also use a covalently bound FMN residue as a prosthetic group.

In summary, we have shown that covalent binding of an FMN residue to proteins via a phosphoester bond is not an autocatalytic process and instead requires a specific flavin transferase, ApbE. ApbE-like proteins are widespread in the bacterial world, including among many pathogens, but are relatively rare in Archaea and Eukaryota. Because these proteins appear to be species-specific, they may represent good targets for the design of antibacterials.

When our manuscript was under revision, a paper by Deka et al. (52) appeared online on an ApbE-like protein from *T. pallidum*, reporting that this protein catalyzes a “single-turnover” hydrolysis of FAD to yield FMN and AMP. Although such an unusual activity is feasible as a side reaction of flavin transferase, ApbE. The reported structure of *T. pallidum* ApbE and its ability to interact with other proteins are consistent with the flavin transfer function demonstrated herein for the *V. cholerae* protein.

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