The TP0796 Lipoprotein of *Treponema pallidum* Is a Bimetal-dependent FAD Pyrophosphatase with a Potential Role in Flavin Homeostasis

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**Background:** The TP0796 lipoprotein of *Treponema pallidum* belongs to the poorly characterized ApbE superfamily.

**Results:** TP0796 hydrolyzed FAD into FMN and AMP, consistent with the general enzymatic mechanism of an FAD pyrophosphatase.

**Conclusion:** This novel metal-dependent enzyme probably plays an essential role in flavin homeostasis in *T. pallidum*.

**Significance:** This is the first description of a metal-dependent FAD pyrophosphatase in bacteria.

*Treponema pallidum*, an obligate parasite of humans and the causative agent of syphilis, has evolved the capacity to exploit host-derived metabolites for its survival. Flavin-containing compounds are essential cofactors that are required for metabolic processes in all living organisms, and riboflavin is a direct precursor of the cofactors FMN and FAD. Unlike many pathogenic bacteria, *Treponema pallidum* cannot synthesize riboflavin; we recently described a flavin-uptake mechanism composed of an ABC-type transporter. However, there is a paucity of information about flavin utilization in bacterial periplasms. Using a discovery-driven approach, we have identified the TP0796 lipoprotein as a previously uncharacterized Mg$^{2+}$-dependent FAD pyrophosphatase within the ApbE superfamily. TP0796 probably plays a central role in flavin turnover by hydrolyzing exogenously acquired FAD, yielding AMP and FMN. Biochemical and structural investigations revealed that the enzyme has a unique bimetal Mg$^{2+}$-catalytic center. Furthermore, the pyrophosphatase activity is product-inhibited by AMP, indicating a possible role for this molecule in modulating FMN and FAD levels in the treponemal periplasm. The ApbE superfamily was previously thought to be involved in thiamine biosynthesis, but our characterization of TP0796 prompts a renaming of this superfamily as a periplasmic flavin-trafficking protein (Ftp). TP0796 is the first structurally and biochemically characterized FAD pyrophosphate enzyme in bacteria. This new paradigm for a bacterial flavin utilization pathway may prove to be useful for future inhibitor design.

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The atomic coordinates and structure factors (codes 4IFU, 4IG1, 4IFZ, 4IFX, and 4IFW) have been deposited in the Protein Data Bank (http://wwpdb.org/).

This article contains supplemental Fig. 1.

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of the ApbE superfamily (Pfam02424). This prokaryotic family of proteins is related to the periplasmic ApbE lipoprotein of *Salmonella typhimurium* (16, 17). In *S. typhimurium*, ApbE has been shown to be involved in thiamine biosynthesis and may serve in the conversion of aminomimidazole ribotide to 4-amino-5-hydroxymethyl-2-methylpyrimidine (16). *T. pallidum* is predicted to lack the thiamine pathway as well as the enzymes involved in aminomimidazole ribotide metabolism (3, 4, 18). Second, the periplasmic location of ApbE prompts questions concerning how it could participate in a cytoplasmic pathway. Third, ApbE proteins are relatively understudied biochemically, and representative crystal structures (PDB<sup>3</sup> entries 1VRM and 2018) have failed to definitively elucidate their functions. A recent crystal structure of the *Salmonella enterica* homolog of TP0796 (ApbE<sub>Se</sub>), classified it merely as an FAD-binding protein (19).

These intriguing information gaps regarding TP0796 and ApbE-like proteins piqued our interest in TP0796 as a putative ApbE homolog. As an initial approach, we cloned, expressed, and purified TP0796 that had been heterologously hyperexpressed. We then determined the crystal structures of recombinant TP0796 with constitutively bound Mg<sup>2+</sup>, in a substrate complex with Mg<sup>2+</sup>-FAD, in a product complex with Mg<sup>2+</sup>-AMP, or in an inhibitor complex with Mg<sup>2+</sup>-ADP. Crystallographic and solution biochemical analyses revealed TP0796 to be a previously uncharacterized Mg<sup>2+</sup>-dependent FAD pyrophosphatase that hydrolyzed FAD into AMP and FMN. Although ApbE family members have been predicted to bind FAD, none have been reported to hydrolyze it. Moreover, whereas the FAD biosynthetic pathway in bacteria has been well characterized, there is a paucity of information regarding bacterial FAD degradation pathways. Thus, the FAD pyrophosphatase activity of TP0796 is potentially to generate a flavin pool via its product inhibition mechanism (by AMP), probably playing an important role in flavoprotein biogenesis. Our combined studies have allowed us to present a new conceptual flavin turnover model that serves to satisfy flavin cofactor requirements within the bacterial periplasm.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich or Hampton Research.

**Protein Preparation**—A DNA fragment corresponding to amino acids 2–340 (without the lipid-modified N-terminal Cys residue; the numbering reflects the assignment of this Cys as residue 1 of the processed protein) of the mature form of TP0796 from *T. pallidum* was generated by PCR amplification of *T. pallidum* genomic DNA using primers with flanking restriction sites for BsaI and XbaI. The digested PCR fragment was ligated into the pE-SUMOpro fusion vector with kanamycin resistance (LifeSensors), which had been digested with the same two enzymes, to generate the expression vector. The plasmid was then transformed into *Escherichia coli* BL21 (DE3). Bacteria were grown at 37 °C in LB medium supplemented with 40 μg/ml kanamycin until the *A*<sub>600</sub> reached ~0.5. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.6 mM for 3 h to induce expression of the recombinant protein. Cells were harvested by centrifugation, resuspended in the lysis buffer (50 mM NaPi, pH 8.0, 0.2 mM NaCl, 10 mM imidazole), and lysed by sonication (6). The supernatant was applied to a 4-mL Ni<sup>2+</sup>-agarose column (Qiagen), immobilizing the tagged protein. The elution of the TP0796 followed a previously developed methodology (8). The eluted protein was further purified by size exclusion chromatography on a Superdex 200 (16/60) column (GE Healthcare) equilibrated with buffer A (20 mM Hepes, pH 7.5, 0.1 mM NaCl, 2 mM β-octyl glucoside). Pooled fractions were digested with SUMO protease 1 (LifeSensors) to remove the His-SUMO tag, and the digestion mixture was separated by size exclusion chromatography (described above).

The purity of the eluted fractions was determined by SDS-PAGE. Fractions containing pure TP0796 were pooled and concentrated using an Amicon Ultra filter device (Millipore) to ~10 mg/ml for crystallization and biochemical analyses. Protein concentration was determined spectrophotometrically using an extinction coefficient of 21,680 M<sup>−1</sup> cm<sup>−1</sup> at 280 nm, which was calculated using the ProtParam utility of ExPASy.

The *E. coli* ApbE (ApbE<sub>Ec</sub>) fusion construct (in pET-21 NESP vector) was obtained from the DNASU plasmid repository. The plasmid was transformed into *E. coli* BL21 (DE3), and the recombinant protein was hyperproduced as described above. Both liganded and ligand-free ApbE<sub>Ec</sub> were prepared as described for PnrA (8). The concentration was determined at 280 nm using an extinction coefficient of 46,410 M<sup>−1</sup> cm<sup>−1</sup> (calculated as above). All purified proteins were stored at 4 °C and were stable for 2 weeks.

**Crystallization and Data Collection**—All crystals were obtained using the hanging drop vapor diffusion method. One small plate crystal of apo-TP0796 (no bound nucleotide) appeared in 7 weeks in 0.7 M sodium acetate, 0.1 M sodium cacodylate (pH 6.5) at 20 °C. The crystal was cryoprotected by successive transfer over the course of 10 min at 20 °C in increasing steps of 5% ethylene glycol to a final solution of 20% (v/v) ethylene glycol, 0.1 M MES (pH 6.5), 0.8 M sodium acetate, 0.1 M NaCl. The crystal was mounted into a nylon loop, flash-cooled in liquid nitrogen, and then used for data collection. TP0796 crystals exhibited the symmetry of space group C2 with unit cell parameters of *a* = 117.6 Å, *b* = 47.3 Å, *c* = 57.6 Å, and β = 102.4° and contained one molecule of TP0796 per asymmetric unit and ~40% solvent. The crystals of ternary complexes were routinely obtained in 2–3 days by co-crystallizing TP0796 in the presence of 5 mM concentration of the chloride salt of the divalent metal ion and 5 mM AMP or ADP using 0.1 M MES (pH 6.5), 0.7 M sodium acetate as precipitant; these co-crystals were isomorphous to the apo-crystals. The complex crystals were generally larger than the apo-crystal and were cryoprotected as above for data collection with a final concentration of 35% ethylene glycol. The crystals for the FAD-Mg<sup>2+</sup>- complex were obtained by the addition of 5 mM FAD to the protein prior to crystallization, with no added Mg<sup>2+</sup> ion. Data for the Mn<sup>2+</sup>-AMP complex were collected using incident radiation at the
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TABLE 1  
Data collection, phasing, and refinement statistics for TP0796 structures  

| Data collection | Apo | FAD-Mg2+ | AMP-Mg2+ | AMP-Mn2+ | ADP-Mg2+ |
|-----------------|-----|----------|----------|----------|----------|
| Crystal         | Apo | FAD-Mg2+ | AMP-Mg2+ | AMP-Mn2+ | ADP-Mg2+ |
| Energy (eV)     | 12.684.5 | 12.684.1 | 12.804.1 | 8.280.4 | 12.684.1 |
| Resolution range (Å) | 36.6–1.83 (1.86–1.83) | 28.6–1.45 (1.48–1.45) | 28.6–1.45 (1.48–1.45) | 35.8–1.90 (1.93–1.90) | 36.5–2.30 (2.34–2.30) |
| Unique reflections | 27,231 (1,354) | 53,215 (2,571) | 53,906 (2,616) | 24,023 (1,156) | 13,766 (667) |
| Multiplicity | 4.0 (3.9) | 4.0 (3.3) | 4.9 (3.9) | 6.0 (5.9) | 3.9 (3.4) |
| Data completeness (%) | 99.7 (99.9) | 98.4 (95.3) | 99.4 (96.7) | 99.4 (98.6) | 99.0 (94.9) |
| Rmerge (%) a | 7.6 (57.4) | 4.0 (62.8) | 6.0 (55.9) | 4.6 (23.2) | 3.2 (13.8) |
| I/σ(I) | 18.4 (2.0) | 33.0 (1.79) | 19.5 (1.88) | 42.1 (7.16) | 8.9 (1.94) |
| Wilson B-value (Å²) | 19.1 | 17.4 | 16.0 | 20.4 | 18.3 |

Refinement statistics

| Data collection | Apo | FAD-Mg2+ | AMP-Mg2+ | AMP-Mn2+ | ADP-Mg2+ |
|-----------------|-----|----------|----------|----------|----------|
| Crystal         | Apo | FAD-Mg2+ | AMP-Mg2+ | AMP-Mn2+ | ADP-Mg2+ |
| Resolution range (Å) | 28.6–1.45 (1.48–1.45) | 28.6–1.45 (1.48–1.45) | 28.6–1.45 (1.48–1.45) | 28.6–1.45 (1.48–1.45) | 28.6–1.45 (1.48–1.45) |
| No. of reflections Rmerge | 27,219/1,364 (2,446/129) | 50,440/2,687 (3,488/195) | 53,888/2,731 (808/43) | 24,011/1,187 (2,762/141) | 13,721/673 (2,518/135) |
| Data completeness (%) | 99.4 (96.0) | 98.0 (92.4) | 96.0 (31.0) | 99.3 (98.0) | 98.7 (96.0) |
| Atoms (non-hydrogen protein/solvent/metal/nucleotide) | 2,538/169/1/0 | 2,610/256/2/53 | 2,594/264/2/23 | 2,601/160/2/23 | 2,501/161/2/27 |
| Rwork (%) | 16.9 (21.6) | 17.2 (30.2) | 17.7 (29.8) | 15.9 (15.3) | 17.7 (21.3) |
| Rfree (%) | 21.7 (26.4) | 20.3 (32.4) | 21.1 (35.4) | 20.3 (21.6) | 23.8 (28.9) |
| r.m.s. deviation, bond length (Å) | 0.018 | 0.022 | 0.014 | 0.015 | 0.013 |
| r.m.s. deviation, bond angle (degrees) | 1.28 | 2.23 | 1.28 | 1.27 | 0.80 |
| Mean B-value (Å²) | 28.4/31.8/11.6/NA | 25.1/32.0/19.7/35.0 | 25.4/36.7/19.8/15.7 | 30.1/32.0/16.4/16.7 | 19.2/22.9/14.8/14.7 |
| (protein/solvent/metal/nucleotide) | 99.4/0.6/0.0 | 98.2/0.9/0.9 | 98.5/1.2/0.3 | 97.6/2.1/0.3 | 97.5/2.5/0.0 |
| Ramachandran plot (%) | 0.19 | 0.05 | 0.14 | 0.17 | 0.25 |
| (favorable/additional/disallowed) a | 2–4, 201–206 | 2–4, 201–206 | 2–7, 201–206 | 2–4, 201–206 | 2–5, 114–115, 201–206 |

| Missing residues | 2–4, 201–206 | 2–4, 201–206 | 2–7, 201–206 | 2–4, 201–206 | 2–5, 114–115, 201–206 |

Accession Codes—The coordinates and structure factors for apo-TP0796 (4IFU), and its complexes (4IG1, the Mg2+-AMP complex; 4IFZ, the Mn2+-AMP complex; 4IFX, the FAD complex; and 4IFW, the ADP complex) have been deposited in the Protein Data Bank.

FAD Pyrophosphatase Activity of TP0796—Flavin stock solutions were prepared in MilliQ (Millipore) water and stored at −20 °C. The concentrations of flavin solutions were determined spectrophotometrically using extinction coefficients (ε450 = 11.3 mm2 cm−1 for FAD and 12.2 mm2 cm−1 for FMN) (30, 31).

The assay for FAD pyrophosphatase (EC 3.6.1.18) activity was adapted from the method of Forti and Sturani (32). It exploits the fact that fluorescence of FMN is higher than that of an FAD solution of the same concentration and that pyrophosphatase catalyzes the conversion of FAD to FMN and AMP. Thus, FAD hydrolysis (or FMN formation) was monitored using a differential fluorescence (Fproduct formation, expressed in arbitrary rel-M enzyme in buffer A and 5 mM MgCl2, and allowed the reactions to incubate at 37 °C. After 11 min of incubation, 10 μM FAD was added, and the reactions were allowed to incubate for an additional 5 min at 37 °C. Increasing fluorescence intensity was monitored at excitation...
and emission wavelengths of 430 and 510 nm, respectively, with a gain setting of 60, and at room temperature. Data for all experiments were collected in duplicate or triplicate, and the background signal, which constitutes the reaction mixture without enzyme, was subtracted.

Activity was also measured with various divalent metal cations in the reaction buffer. To determine the pH dependence of the reaction, individual assays were performed in 50 mM BIS-TRIS propane containing 0.1 M NaCl at various pH values.

**Inhibition Studies**—Inhibition was assayed by incubating the enzyme reaction mixture either with 1 mM AMP, ADP, or ATP prior to the addition of FAD. EDTA inhibition was examined by adding 5 mM EDTA prior to the addition of metal cofactor to the reaction mixture.

**Quantitation of FMN**—FMN fluorescence values were quantitated on the basis of an FMN standard curve. The standard curve was prepared by measuring the fluorescence emission of a solution containing increasing concentrations of FMN in buffer A. Based on measurements of FMN standards, 1 μM FMN was assumed to be equivalent to 700 RFU.

**Thin Layer Chromatography (TLC) Analysis**—TLC of the products of FAD hydrolysis by TP0796 was performed on plates of silica gel (20 × 20 cm; thickness, 0.25 mm; pore size, 60 Å; Sigma) (33, 34). For TLC analysis, reactions were performed in buffer A as described for the enzyme assay, except that 20 μM protein was incubated with 50 μM FAD. Following incubation at 37 °C, the reactions were boiled for 10 min and centrifuged to remove protein precipitates. Approximately 20 μl of the yellow supernatant was applied to the TLC plate. Equal volumes of individual flavin-containing standards were loaded at concentrations of 50 μM, and the plate was allowed to air-dry. The mobile phase was a solution of butanol-acetic acid/water (12:3.5) and developed for 6 h at room temperature. The fluorescence of flavin-containing compounds on air-dried TLC plates was photographed with UV illumination and compared with the standards. Some FMN bands were scraped from the TLC plate and extracted with 50% (v/v) ethanol for MALDI-TOF mass analyses.

**FAD Binding Activity**—Ligand-free ApbE_Ec was prepared by an on-column denaturation/renaturation method (8). To determine the FAD binding affinity to the ligand-free protein, changes in fluorescence (ΔF) upon binding of different concentrations of FAD were measured (as described above), and the binding affinity was calculated using the GraphPad software. The relative ΔF at emission wavelength of 510 nm upon FAD binding was defined as ΔF = F[ FAD] - F[ApbE] where ΔF is the changes in fluorescence due to FAD binding, F[ FAD] is fluorescence of the added FAD, and F[ApbE] is the fluorescence upon FAD binding to the ApbE protein. The experiment was performed as described above for the enzyme assay by incubating a 1 μM concentration of ligand-free ApbE with 0.00, 0.05, 0.10, 0.30, 0.50, 1.00, 2.00, 5.00, or 10 μM FAD. Titration in the absence of protein were performed as a reference.

**Analytical Ultracentrifugation**—All analytical ultracentrifugation studies were performed at 4 °C in a Beckman-Coultier Optima XL-I centrifuge. TP0796 or ApbE_Ec was diluted to the experimental concentration with Buffer A. The protein solution was injected into an assembled ultracentrifugation cell that featured a charcoal-filled Epon centerpiece sandwiched between two sapphire windows. The cells were placed in an An50-Ti rotor (Beckman-Coultler, Indianapolis, IN) and allowed to incubate at the experimental temperature under vacuum overnight. Centrifugation at 50,000 rpm was commenced and continued until the sedimentation was complete (~9 h). Absorbance optics were used to acquire radial concentration profiles at a wavelength of 280 nm from the cells at an ~10-min interval. These data were analyzed using the continuous c(s) model (35, 36) in SEDFIT. The figure derived from this analysis was made using GUSSI.

**In Vivo Cross-linking of Associated Proteins**—Treponema pallidum protein cross-linking was performed according to the method described previously (12, 37). Briefly, ~1 × 10⁸ freshly harvested bacteria from rabbit tissue in phosphate-buffered saline (PBS) were incubated with 1% (v/v) methanol-free formaldehyde (Thermo Scientific) for 90 min at room temperature with gentle rocking. To stop the cross-linking reaction, glycerol was added to a final concentration of 125 mM and incubated for 5 min at room temperature. A control reaction was performed similarly but without adding formaldehyde. Both cross-linked and non-cross-linked treponemal proteins were harvested by centrifugation at 16,000 × g and rinsed twice with ice-cold PBS to remove excess unreacted cross-linker. They were frozen at −70 °C until needed for analysis. Cross-linked complexes were detected by immunoblotting with TP0796-specific antibodies (12). Antiserum recognizing TP0796 was obtained from rats by injecting purified recombinant antigens. Antibodies from the antiserum were then affinity-purified using an antibody purification kit (Thermo Scientific). The specificity of the purified antibodies was documented against recombinant test and control antigens (not shown).

**RESULTS**

**Purification and Biophysical Properties of the Proteins**—Recombinant TP0796 and ApbE_Ec were hyperexpressed in *E. coli* and purified to homogeneity. Unlike the TP0796 solution, the solution containing ApbE_Ec was bright yellow, suggesting that the protein bound a flavin-containing compound. Further, this yellow protein solution displays a UV-visible flavin spectrum that was characteristic for flavin-containing molecules (not shown).

To investigate the oligomeric statuses of both TP0796 and ApbE_Ec, analytical ultracentrifugation was performed. Sedimentation velocity demonstrated that ApbE_Ec was a dimer under our solution conditions but that TP0796 was mostly monomeric (Fig. 1). The estimated molar mass of ApbE_Ec was 77,900 g/mol; this value is consistent with a dimer of the protein, which was expected to be 75,206 g/mol. The sedimentation coefficient of ApbE_Ec (s20, w = 4.8 S) was insensitive to varying its concentration. TP0796, however, had a significantly smaller s-value (s20, w ~3.1 S) and estimated molar mass (32,700 g/mol). The expected molar mass for a monomer of TP0796 calculated from its sequence is 36,593 g/mol. Thus, the majority of the protein is monomeric. However, there is a weak tendency for the sedimentation coefficient to increase with increasing concentrations of TP0796 (Fig. 1). This observation indicates that TP0796 apparently dimerizes under our
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FIGURE 1. Hydrodynamic behavior of TP0796 and ligand-bound AbpE_Ec. Shown are c(s) distributions for two concentrations (5.4 and 21.6 μM, red and blue lines, respectively) of TP0796 and one (gray line) of AbpE_Ec. The distributions have been normalized by the total amount of absorbance found in each respective experiment.

solution conditions, but the dimerization constant must be very high (>>20 μM).

TP0796 Apo-structure—The crystal structure of apo-TP0796 (Fig. 2A) demonstrates that it adopts the canonical ApbE superfamily fold observed in the previously determined structural homologs from S. enterica, T. maritima (18, 19), and E. coli (PDB entry 2018). Whereas AbpE_Se and AbpE_Ec are dimers in the solid state, TP0796 is a monomer in the crystalline lattice as determined via analysis with the Protein Interfaces, Surfaces, and Assemblies Service (PIASA) at the European Bioinformatics Institute (38, 39), a distinction it shares with T. maritima AbpE (AbpE_Tm), with which it shares the highest primary sequence homology (32%).

Structure-based sequence alignments of TP0796 to the ApbE homologs (Table 2) reveal that they cluster into two closely related subgroups (Fig. 2B and supplemental Fig. 1). TP0796 and AbpE_Tm both contain an additional β-hairpin loop not found in the AbpE_Se and AbpE_Ec structures. This β-hairpin loop, which comprises residues 244–256 in TP0796, positions several residues close to the respective nucleotide-binding sites in these proteins. The analogous regions of polypeptide in the proteins of the other subgroup are either a long, flexible, and partially disordered loop in the AbpE_Ec structure or a helix and long flexible loop in the AbpE_Se structure, in which these residues are not in close proximity to the respective ligand-binding sites. The Desulfovibrio vulgaris Hildenborough hypothetical protein adopts an ApbE-like fold but appears to retain only 60% of the secondary structural elements found in the other homologs. For this reason, at present we do not consider this protein to be a true member of the ApbE family.

Inspection of the electron density map for apo-TP0796 revealed an atom octahedrally coordinated to the OD1 carboxylate and main-chain oxygens of Asp284, the main-chain oxygen of Ala162, the OG1 oxygen of Thr288, and two water molecules (Fig. 3A). This atom has been assigned as a Mg2+ ion due to the coordination geometry, the nature of the coordinated ligands, the average bond distance of 2.3 Å (Table 3), and the refined electron density. These geometric parameters correspond well with those found for crystallographically refined Mg2+ ions in other protein structures (40, 41). The metal-binding residues Asp284 and Thr288 are conserved across the ApbE structural homologs. The presence of the bound metal ion in the structure was unexpected because Mg2+ was not specifically added to the apoprotein during purification or crystallization. The region in the apo-TP0796 structure that has been identified as an FAD binding site in ApbE_Se is occupied by water molecules, with no clear pattern that might indicate any other type of bound ligand. This corresponds with the observation that the TP0796 protein used for crystallization screening was colorless upon purification.

Superposition of the backbone coordinates for all TP0796 structures reported herein range from 0.3 to 0.5 Å root mean square deviations for 327 C-α atoms, indicating the high level of isomorphism among the various complexes. The primary structural differences are reflected in the active site coordination of the bound ligands by protein residues, metals, and waters (see below).

Characterization of FAD Pyrophosphatase Activity of TP0796—The crystal structure of TP0796 in the presence of FAD (no added Mg2+) (see below) showed the presence of a bimetal center near the phosphate moieties of bound FAD. These observations led us to investigate whether TP0796 could act as a hydrolase. TP0796 was incubated with FAD in the presence of divalent metal ions, and the formation of FMN was monitored via its differential fluorescence compared with FAD (32). TP0796 hydrolyzed FAD under these conditions (Fig. 4). When the effects of different cations at 5 mM concentrations were tested (Fig. 4A), both Mg2+ and Mn2+ most efficiently supported FAD hydrolysis. Other cations (Ca2+ or Ni2+) could replace Mg2+/Mn2+ but with lower catalytic efficiencies. To verify whether divalent metal ions are essential for FAD hydrolysis by TP0796, we assayed the enzyme in the presence of EDTA. As shown in Fig. 4B, EDTA inhibited the enzyme activity; thus, the activity of TP0796 is dependent on the presence of divalent metal ions. AMP, ADP, and ATP all strongly inhibited the hydrolysis reactions (Fig. 4B).

The optimum pH for the FAD pyrophosphatase activity of TP0796 was determined using a series of BIS-TRIS propane buffers ranging from pH 6.5 to 9.5. The enzyme showed a broad pH activity profile, with an optimum at pH 7.5 (Fig. 4C), which is physiologically relevant.

The hydrolysis of FAD by TP0796 produced a fluorescent compound when the reaction mixture was separated by TLC. Although the detection method used above assumed the formation of FMN (and AMP), we sought confirmation of this assumption. The flavin-containing reaction product was identified as FMN based on its identical migration to an FMN standard on a TLC plate (Fig. 5A). Additionally, MALDI-TOF mass analyses of the TLC-purified product gave the same mass as an FMN standard (457.72 m/z) (Fig. 5B). Based on the crystallographically observed Mg2+-AMP-bound structure (Fig. 3F) and in-solution identification of the reaction product (Fig. 5), it can be concluded that TP0796 hydrolyzes FAD into FMN and AMP, consistent with the general enzymatic mechanism of an FAD-pyrophosphatase (EC 3.6.1.18).
We attempted to examine the kinetics of the reaction catalyzed by TP0796 to determine the rate constant of FAD hydrolysis. However, the reactions were characterized by an initial burst of product followed by a linear phase, indicating a single-turnover reaction (Fig. 6A). This observation is consistent with the crystallographically observed product-bound structure and inhibition of the pyrophosphatase activity by AMP. The rate of burst is dependent on the enzyme concentration. These reactions were too fast for manual assays on a Tecan plate reader, so that even the earliest points in the time traces were at the end of the exponential phase. For this reason, we measured the extent and kinetics of FMN formation in single turnover FAD pyrophosphatase reactions that were catalyzed by TP0796. As shown in Fig. 6B, the plot of FMN formation against the enzyme concentration is strongly correlated, suggesting a single turnover mechanism. A calculated ratio of ~0.7 μmol of FMN generated per μmol of enzyme was obtained. Given the possibility that a fraction (~30%) of the “as-purified” protein is either liganded or inactive, we conclude that one molecule of FMN is generated by one molecule of protein under our assay conditions. Together, the results indicate that TP0796 can quantitatively hydrolyze FAD to FMN and AMP, and we propose that it be designated an FAD pyrophosphatase (EC 3.6.1.18).

TABLE 2
Native TP0796 alignments to ApbE homologs
All alignments were performed using the DaliLite server.

| Organism    | PDB entry | Resolution | Sequence ID | r.m.s. deviation | C-α atoms |
|-------------|-----------|------------|-------------|------------------|-----------|
| T. maritima | 1VRM      | 1.58       | 32          | 2.0              | 298       |
| E. coli     | 2O18      | 2.20       | 24          | 2.3              | 286       |
| S. enterica | 3PND      | 2.75       | 25          | 2.5              | 295       |
| D. vulgaris | 2O34      | 1.95       | 18          | 2.8              | 186       |

a Chain A for each ApbE homolog was used for alignment to TP0796.

b Number of C-α atoms aligned.
To investigate the hydrolytic activity of homologs of TP0796, we compared its activity with those of ApbE-Se and ApbE-Ec. Although ApbE-Se was reported to be an FAD-binding protein, no FAD hydrolytic activity was associated with ApbE-Se (19). Our own studies on ligand-free ApbE-Ec demonstrated a decrease in the emission fluorescence of FAD in our assays (data not shown). This result indicated that ApbE-Ec might be an FAD-binding protein but not a hydrolase. Fluorescence titrations were thus used to determine the FAD binding affinity ($K_d$ value) to ApbE-Ec. As shown in Fig. 7, the $K_d$ of FAD binding to ApbE-Ec was $\sim 0.7 \mu M$. This high value of $K_d$ could explain why there is no bound FAD in the reported ApbE-Ec crystal structure. We attempted to crystallize fully reconstituted FAD-bound ApbE-Ec under previously reported conditions and obtained colorless ApbE-Ec crystals. Analysis of x-ray diffraction data collected from one of these crystals revealed no discernible FAD electron density in the nucleotide-binding site (data not shown). Unlike the TP0796-catalyzed reaction, the binding event to ApbE-Ec was metal-independent (data not shown). Further, no metal was included in the FAD-bound ApbE-Se crystallographic model (19), although the amino acid residues involved in the metal binding of TP0796 are conserved among the known ApbE structures. This observation suggests that the catalytic function of TP0796 is related to the presence of the bimetal center, and FAD pyrophosphatase activity may have evolved as an additional trait in a subset of ApbE proteins.

**TP0796 Substrate Complex (FAD-Mg$^{2+}$)—**To assess whether TP0796 is an FAD-binding protein, TP0796 was incubated with 5 mM FAD prior to crystallization, and complex crystals isomorphous to the apo were grown under similar conditions; the structure was refined to a resolution of 1.45 Å. Despite the bright yellow color of the initial protein preparation, the crystals were only slightly yellow in color, and upon initial inspection of the electron density map, it appeared as if the protein contained bound ADP and two Mg$^{2+}$ atoms and not FAD-Mg$^{2+}$. Further refinement revealed weak electron density for the FMN portion of the bound substrate. This led us to consider the possibility that TP0796 had enzymatically cleaved the FAD, with the most likely activity being that of an FAD pyrophosphatase that would produce one molecule each of AMP and FMN as products. Crystallographic refinement of the bound ligands as a mixture of FAD (substrate) and AMP (product) resulted in a large amount of positive difference electron density in the $\beta$-phosphate region of the substrate, and there was no observation of multiple conformations for residues that surround the diphosphate-binding site. Because it was not expected that there was a significant amount of ADP as an impurity in the freshly made FAD solutions used for crystal growth, in the final cycles of refinement, the ligand was modeled as an FAD at 100% occupancy, and the $B$-factors of the ligand atoms were allowed to vary. As a result, there is no longer a large amount of positive (or negative) difference density in the diphosphate region of the FAD (Fig. 3B).

The conditions for crystal growth of the FAD-Mg$^{2+}$ complex included 5 mM FAD, but no Mg$^{2+}$ was added during the crystallization or purification of TP0796. Apo-TP0796 crystallization also did not include the explicit addition of Mg$^{2+}$ to the purification or crystallization buffer. Thus, the metal ions
observed in these structures were probably obtained as trace elements from the purification and crystallization chemicals or were adsorbed to the laboratory glassware and were thus at submicromolar (or lower) concentrations. Because the concentration of the nucleotide in the crystallization buffer was much higher than that of the catalytically required metal ion, significant amounts of FAD hydrolysis were not expected to occur in the time frame of crystal nucleation and growth.

TP0796 binds FAD in a bent conformation, centered around the diphosphate (Fig. 3C), which is in an eclipsed conformation, such that the ribose O3B atom is 2.8 Å from the ribitol O3’ atom. The overall bent conformation is similar to that found in the ApbE_Sc structure but differs greatly in the central portion of the molecule (Fig. 3D). Whereas the adenine and isoalloxazine rings in the two structures align quite well, the central diphosphate and ribitol portions of the FAD adopt quite different conformations in the two structures. It is presumed that the coordination of two Mg2+ ions to the FAD diphosphate and to the protein side chains in the TP0796 structure is the cause of these differences because there are no metal ions identified in the ApbE_Sc structure. The AMP portion of the FAD in TP0796 is probably due to the dearth of specific protein contacts for the ribitol and isoalloxazine rings in the spacious FMN region of the substrate-binding cavity. In comparison with the AMP portion of the FAD molecule, the FMN portion is relatively solvent-exposed, with a solvent-accessible surface area of 31.1%. In the ApbE_Sc FAD-bound structure, specific hydrogen-bonding interactions occur between protein side chains and the ribitol hydroxyls, and the isoalloxazine ring is sandwiched between the side chains of Tyr-78 and Met-41. The equivalent residues in TP0796 are Asn-55 and Ile-17, respectively. Whereas these side chains contact the isoalloxazine ring in the TP0796 complex, the amount of buried surface area is smaller than in the ApbE_Sc complex, with an average solvent-accessible surface area of 24.2% for the FMN portion of the FAD. The polar carboxamide of Asn-55 contact with this ring is undoubtedly less stabilizing than the

### Table 3

| Active site geometric parameters for TP0796 structures | Apo | FAD-Mg2+ | AMP-Mg2+ | AMP-Mn2+ | ADP-Mg2+ |
|------------------------------------------------------|-----|----------|----------|----------|----------|
| Resolution (Å)                                       | 1.83| 1.48     | 1.45     | 1.90     | 2.30     |
| Me1 identity (Å)                                     | Mg2+| Mg2+    | Mg2+    | Mg2+    | Mg2+    |
| Me1 β-factor (Å²)                                    | 15.1| 15.2    | 15.1    | 15.2    | 3.2     |
| Me2 identity                                        | Mg2+| Mg2+    | Mn2+    | Mg2+    | Mg2+    |
| Me2 β-factor (Å²)                                    | 24.3| 16.8    | 18.7    | 26.4    |          |
| Me1 C.N.                                            | 6   | 6       | 5       | 5       | 6        |
| Me2 C.N.                                            | 5   | 6       | 6       | 6        |          |

**Bond distances (Å)**

| Bond distances | Apo | FAD-Mg2+ | AMP-Mg2+ | AMP-Mn2+ | ADP-Mg2+ |
|----------------|-----|----------|----------|----------|----------|
| Me1–Me2        | 3.30| 3.14     | 3.24     | 3.27     |          |
| Me1–T284OD1    | 2.42| 2.31     | 2.32     | 2.42     |          |
| Me1–T284O      | 2.38| 2.26     | 2.24     | 2.26     |          |
| Me1–A162O      | 2.45| 2.42     | 2.41     | 2.44     |          |
| Me1–T288OG     | 2.38| 2.29     | 2.31     | 2.39     |          |
| Me1–bWAT       | 2.21| 3.99d    | 3.64     | 2.23     |          |
| Me1–H2O        | 2.20|          |          |          |          |
| Average Me1 distances | 2.34| 2.33     | 2.33     | 2.21     |          |
| Me2–T284OD1    | 2.34| 2.10     | 2.15     | 2.01     |          |
| Me2–αpO        | 2.24| 2.07     | 2.20     | 2.60     |          |
| Me2–βpO        | 2.23|          |          |          |          |
| Me2–bWAT       | 3.49| 2.13     | 2.23     | 3.31     |          |
| Me2–H2O        | 2.17| 2.12     | 2.24     | 2.29     |          |
| Me2–H3O        | 2.61| 2.03     | 2.26     | 2.15     |          |
| Me2–H4O        | 2.18|          |          |          |          |
| Average Me2 distances | 2.32| 2.09     | 2.14     | 2.26     |          |
| Lys165 NZ–βpO | 2.33|          |          |          |          |
| Ser146G–αpO    | 3.42| 2.72     | 2.75     | 3.06     |          |
| Ser146G–bWAT  | 2.95| 2.91     | 3.03     | 2.83     |          |
| PA–bWAT        | 3.74| 3.64     | 3.68     | 3.55     |          |
| nWAT–αpO      | 2.41|          |          |          |          |
| Glu244 OE2–nWAT| 2.64|          |          |          |          |
| PA–nWAT       | 3.04|          |          |          |          |

**Bond angles (degrees)**

| Bond angles | Apo | FAD-Mg2+ | AMP-Mg2+ | AMP-Mn2+ | ADP-Mg2+ |
|-------------|-----|----------|----------|----------|----------|
| bWAT–PA–O5B | 154| 148      | 152      | 150      |          |
| nWAT–PA–O5B | 156|          |          |          |          |

**a** Me, metal.

**b** C.N., coordination number.

**c** bWAT, bridging water.

**d** Distances in italic type are not assumed to represent first sphere metal ion coordination but are included for reference.

**nWAT**, nucleophilic water.
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...interaction of the aromatic ring of Tyr-78 in the ApbE_Se structure. The only contact to the ribitol portion of the FAD in the TP0796 structure is with the guanadinium moiety of Arg245, although the electron density for this side...
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formation, and release. One or both of the metals may help to preconfigure protein active site residues for catalysis, bind or assist in activation of the attacking nucleophile, and position the proton donor for efficient proton transfer to the transition state. In addition to the two Mg\(^{2+}\) ions, three protein residues in the FAD-binding site are noteworthy in their interactions near the site of phosphate hydrolysis. The NE2 of His\(^{256}\) is 3.2 Å from the bridging O3P of the FAD diphosphate and could assist in neutralization of the negatively charged phosphoanion transition state or could serve as the proton donor. The OG of Ser\(^{240}\) is within hydrogen-bonding distance of the Me1-coordinated water molecule, and the carboxylate of Glu\(^{244}\) coordinates a water molecule that bridges between the Ser\(^{240}\) OG and the FAD α-phosphate (Table 3). This water molecule is situated 3.0 Å from the site of hydrolysis at the α-phosphate atom, and it forms an angle of 156° to the PA-O5B atoms of the FAD. Thus, because it is in position for an in-line nucleophilic attack on the α-phosphate and is potentially activated as a nucleophile by its interaction with Glu\(^{244}\), we have designated this water as "nWAT." Another water coordinated to Me1 is also positioned to form an angle of 154° to the PA-O5B atoms of the FAD, but it is significantly farther (3.7 Å) from the phosphorus atom site of attack and is thus a less likely candidate as a nucleophile. In the other TP0796 ligand complex structures, this water appears to switch coordination from Me1 to Me2 (see below); therefore, we have designated this water as "bWAT" to designate it as the water that “bridges” the metal sites. Stabilization of the transition state and leaving group phosphates could also occur through phosphoryl oxygen interactions with the NZ atom of Lys\(^{165}\) and the guanadinium group of Arg\(^{245}\). His\(^{256}\) and Ser\(^{240}\) are conserved over all ApbE homologs, and Glu\(^{244}\) is an arginine and Lys\(^{165}\) is a glutamate in the ApbE_Se and ApbE_Ec structures.

**TP0796 Product Complex (AMP-Mg\(^{2+}\))**—After obtaining the high resolution FAD-Mg\(^{2+}\) complex structure, an attempt was made to obtain a higher resolution apo-structure from crystals grown from “as purified” TP0796. Inspection of the electron density map calculated from a 1.5 Å data set collected on one of these crystals revealed that there was a substoichiometric amount of AMP-Mg\(^{2+}\) in the ligand-binding site (data not shown). Subsequently, the protein was incubated with 5 mM AMP and 5 mM Mg\(^{2+}\), and complex crystals isomorphous to the apo were grown under similar conditions; this structure was refined to a resolution of 1.45 Å. As in the substrate complex, there are two metal ions bound in sites Me1 and Me2, which have been assigned as Mg\(^{2+}\) ions (Fig. 3F and Table 3). In contrast to the apo and substrate FAD complex structures, Me1 is 5-coordinate and has no waters in its first coordination sphere. The OD1 of Asp\(^{284}\), an AMP phosphate oxygen, and four water molecules coordinate Me2.

Compared with the substrate FAD-Mg\(^{2+}\) complex, NE2 of His\(^{256}\) is at approximately the same distance (3.1 Å) from the O3P of AMP, the OG of Ser\(^{240}\) is closer to the Me1-coordinated water by 0.7 Å, and Glu\(^{244}\) has adopted a rotamer that places it farther away from the AMP α-phosphate, probably due to charge repulsion.

**TP0796 Metal Identification**—Potential identities of the ions in sites Me1 and Me2 that are consistent with the bond lengths

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**FIGURE 7. FAD binding to ApbE_Ec.** Changes in fluorescence emission (ΔF) of ligand-free ApbE_Ec protein upon exposure to FAD were plotted as a function of FAD concentration. The solid line reflecting the best fit curve was used to derive the K_d value using GraphPad software.

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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and coordination numbers observed include Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\). In an attempt to unambiguously identify the bound metal ions, AMP-Mn\(^{2+}\) complex crystals were generated by incubation of TP0796 with 5 mM AMP and 5 mM Mn\(^{2+}\). The coordination of the nucleotide and metals in this structure was isomorphous to the AMP-Mg\(^{2+}\) complex structure. X-ray diffraction data to 1.90 Å resolution were collected at the Mn\(^{2+}\) K-edge energy, and an anomalous difference map was calculated (Fig. 3G). A strong peak at the Me2 site was observed as well as a smaller peak at the AMP phosphate that is due to the small amount of anomalous signal expected for phosphorus at this energy (48). No anomalous signal was observed at the Me1 site, which is probably a reflection of the high affinity of this site for a non-exchangeable metal ion that does not absorb x-rays at the incident wavelength. This is in agreement with our observation that TP0796 protein has a tendency to precipitate when stored for long periods when isolated without added metal ion but is stable for weeks at 4 °C when stored in a buffer containing 5 mM Mg\(^{2+}\) (not shown). This instability may be a function of less than 100% incorporation of Mg\(^{2+}\) in the Me1 site. The absence of anomalous signal at site Me1 indicates that K\(^+\) is not a likely candidate ion because the expected anomalous signal at the manganese K-absorption edge for potassium is almost 3 times that expected for phosphorus. Attempts to refine the metal in site Me1 as a Ca\(^{2+}\) in all structures presented in this work resulted in a large amount of negative difference electron density at Me1. This negative electron density disappeared in the difference density maps once Me1 was refined as a Mg\(^{2+}\) ion. B-Factors of the refined metal ions are reported in Table 3. The low value of 3.2 Å\(^2\) for the Mg\(^{2+}\) in Me1 for the ADP-Mg\(^{2+}\) complex is not unexpected, given that the B-factors for the first coordination sphere atoms at this metal site range from 3.4 to 11.5 Å\(^2\) for non-solvent atoms, with an average value of 7.0 Å\(^2\). Coordination numbers of 5 and 6 are most common for Mg\(^{2+}\) ions and less likely for Ca\(^{2+}\) and Na\(^+\) ions (40). We conclude that the data reported throughout this paper are most consistent with Mg\(^{2+}\) ions bound at sites Me1 and Me2 (when present). The lack of Mn\(^{2+}\) exchange described above at site Me1 therefore probably indicates that the dissociation of Mg\(^{2+}\) from Me1 is very slow on the time scale of our incubation and crystallization protocols.

**TP0796 Inhibitor Complex (ADP-Mg\(^{2+}\))**—To investigate the nature of inhibition of the FAD pyrophosphatase activity of TP0796, protein was incubated with 5 mM ADP and 5 mM Mg\(^{2+}\), complex crystals isomorphous to the apo were grown under similar conditions, and the structure was refined to a resolution of 2.30 Å. The two metal ions bound in sites Me1 and Me2 were assigned as Mg\(^{2+}\) ions (Table 3) and were coordinated in a similar fashion as observed in the substrate FAD-Mg\(^{2+}\) complex (Fig. 3H). The main differences between the two structures are that the OG of Ser\(^{246}\) is closer to the Me1-coordinated water by 0.4 Å, the lack of a Glu\(^{244}\)-coordinated water that is within 3 Å of the α-phosphate of the ADP, and the ~1-Å lengthening of the distance between the Lys\(^{165}\) NZ and the β-phosphate of the ADP. Glu\(^{244}\) has adopted a rotamer that removes it from the ADP phosphate-binding site, probably due to charge repulsion. The inability of TP0796 to hydrolyze ADP or ATP probably stems from the lack of both an appropriate nucleophile and the catalytic residue Glu\(^{244}\) in the active site. These results demonstrate that the mechanism of ADP inhibition of TP0796 is competitive; by occupying part of the FAD binding site, it sterically excludes the binding of the substrate.

**Enzymatic Mechanism**—Recent studies of the ApbE protein from Salmonella spp. (17, 19) have proposed an FAD-binding role for this class of bacterial periplasmic lipoproteins and implicated it in the thiamine biosynthetic pathway for this organism. We initiated a detailed molecular study of TP0796, inasmuch as the T. pallidum genome does not include genes for thiamine biosynthesis, and thus the role of this protein was unclear. We have obtained solution evidence that TP0796 rather functions as a bimetal Mg\(^{2+}\)-dependent FAD pyrophosphatase and not simply as a periplasmic binding protein. The broad pH profile for enzyme activity is consistent with a solvent-derived hydroxide ion nucleophile for which we have structural evidence in a substrate complex with FAD. There are reports of this enzyme activity associated with plant chloroplasts and mitochondria (49), yeast mitochondria (50), rat liver mitochondria (51), and human mitochondria (52–54). It has been reported that purified 5'-nucleotidase from human placental mitochondria possesses metal-dependent FAD pyrophosphatase activity (53), which is inhibited by EDTA and stimulated by Co\(^{2+}\) and to a lesser degree by Mn\(^{2+}\). This glycoenzyme, which is present as an ectoenzyme bound to the outer plasma membrane, was specific for FAD and did not hydrolyze ADP, ATP, NAD(H), or FMN. Several structures are available for human soluble and mitochondrial 5'-nucleotidases, which are oligomeric multidomain proteins that contain a Rossmann fold core and a cap domain and belong to the haloacid dehalogenase superfamily of hydrolases (44, 55, 56). It is tempting to speculate that, due to the correlations in metal dependence and specific activity between TP0796 and the human placental mitochondrial enzyme, they may share some similarities in active site configurations despite any detectable sequence or overall structural similarities.

**Native TP0796 Is Associated with Other Proteins in Vivo**—As mentioned earlier, AMP, a product of the TP0796-catalyzed reaction, inhibited the enzyme activity of TP0796. However, it remains unclear how TP0796 releases the AMP product. It is possible that an interaction with an unidentified protein(s) results in a conformational change that may facilitate AMP release. Therefore, to examine the potential in vivo interactions of native TP0796 with other partner proteins, treponemes were either untreated or treated with formaldehyde and then processed for immunoblot analysis using antibody against recombinant TP0796. As shown in Fig. 8, when treponemes were incubated under conditions without formaldehyde, only monomeric TP0796 was observed (lane TP). Cross-linking treatment of treponemes with formaldehyde resulted in a disappearance of the TP0796 monomer, with concomitant formation of multiple, diffuse, high molecular mass protein bands (lane CL, above ~100 kDa). This result probably reflected the interaction of TP0796 with unknown protein partners. The larger complexes revealed by formaldehyde cross-linking were essentially undetected after boiling (TP0796 monomer was the dominant band, lane CL\(_2\)). These results strongly support the contention that the high molecular mass protein bands observed on immu-
FAD pyrophosphatase enzymatic activity, recombinant proteins from *Treponema denticola*, *Enterococcus faecalis*, and *Listeria monocytogenes* exhibit the same *in vitro* activity.4

We have obtained the highest resolution x-ray crystal structures to date for any member of the ApbE protein family and herein report the structures of TP0796 in multiple forms, including the apo-form and substrate-, product-, and inhibitor-bound. Metal ions are found in all forms of the protein and have been identified as Mg2+ based upon solution studies, geometric considerations, and crystallographic refinement results. Catalytically important residues involved in substrate binding, solvent nucleophile activation, and proton donation include Ser240, Glu244, and His256.

Subtle structural differences between TP0796 and ApbE_Se are probably responsible for their functional differences. The positioning of the Glu244-Arg245-His256 residues of the β-hairpin loop in the active site, the presence of bound metal ions, a solvent molecule hydrogen-bonded to Ser240 and Glu244 in a conformation suitable for in-line nucleophilic attack, and the absence of stabilizing hydrophobic residues near the isoalloxazine ring in TP0796 all contribute to placing the FAD in a conformation suitable for phosphoester hydrolysis and FMN product release. We predict that the close structural and sequence homolog ApbE_Tm (see above) also is likely to be an FAD pyrophosphatase, but this remains to be verified experimentally.

Although no metal ions have been identified in the ApbE_Se structure, the other ApbE homolog structures contain various mono- or divalent cations in sites analogous to Me1. In the ApbE_Ec structure (PDB entry 2018), this ion has been identified as a Ca2+, probably based upon the average bond lengths of around 2.7–2.8 Å. Because this structure was solved and refined by a National Institutes of Health Protein Structure Initiative (PSI) group (Northeast Structural Genomics Consortium), there is no publication that accompanies this structure, and it can thus be presumed that no solution or crystallographic experiments were conducted to prove or disprove the metal identity. This structure was refined using the CNS crystallographic software package, which is known to tightly restrain metal-ligand bond distances, so it is possible that the actual metal ion in this structure is Mg2+, and the long bond distances are simply a refinement artifact. In the ApbE_Tm structure (18) (PDB entry 1VRM), there is a water molecule modeled in the Me1 site, but it is octahedrally coordinated with an average bond length of 2.4 Å to the main chain and OD1 oxygens of Asp303, the O1G of Thr307, the main chain oxygen of Gly191, and two water molecules. The B-factor of this atom is 4.8 Å2, which is significantly lower than the average B-factor of 31.0 Å2 for all of the water molecules in this structure and lower than the average B-factor of 12.0 Å2 for the protein. Once again, this is a structure that was solved and refined by a PSI group (Joint Center for Structural Genomics), and there is no primary citation for the work. Thus, we feel this atom probably was mistakenly assigned as water and should be Mg2+.

The *D. vulgaris* Hildenborough structure (PDB entry 2034) was solved and refined by yet another PSI group (New York SGX Research Center for

4 R. K. Deka, C. A. Brautigam, W. Z. Liu, D. R. Tomchick, and M. V. Norgard, unpublished data.
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Structural Genomics) with no primary citation, and this structural model contains Na\(^+\) ions in the analogous Mc1 site of both monomers in the asymmetric unit, with average bond lengths of 2.3–2.4 Å. The B-factors and bond lengths of this ion are reasonable, but due to the one-electron difference between Na\(^+\) and Mg\(^{2+}\), it is difficult to identify the bound ion with certainty from the data presented. Despite the structural similarity of this protein to the other ApbE homologs, the relatively small size of the protein and the absence of a clear binding pocket for nucleotides (either AMP, ADP or FAD) call its function into question as either an FAD-binding protein or FAD pyrophosphatase.

FMN product release from TP0796 could be facilitated by interaction with Arg\(^{245}\) and Lys\(^{165}\) and also by the accessibility of the bulk solvent to this product. It is unclear how TP0796 releases the product AMP, although it is possible that an interaction of native TP0796 with a yet unidentified protein (TP0796 apparently interacts with several unidentified proteins in vivo; see Fig. 8) results in a conformational change that facilitates release. This could occur, for example, by an interaction that causes the \(\beta\)-hairpin loop to become poorly ordered and thus removes important ligand binding residues from the enzyme active site.

It is not unusual for enzymes to exhibit single turnover in vitro due to the absence of interacting partners that are required for the in vivo activity. Prominent examples include the multienzyme E. coli MutM-MutY-MutT DNA repair system that removes the adenine in 7,8-dihydro-8-oxo-2'-deoxyguanosine (OG); A base pairs. In the absence of the other two enzymes, MutY exhibits single turnover, product inhibition, and slow product release with the OG:A mispair enzyme, which would result in a DNA double-strand break. Dissociation of the product from MutY in vitro is enhanced by the inclusion of either of the apurinic-apyrimidinic endonucleases III or IV in the reaction mixture, and the C-terminal domain of MutY is required for this enhancement, presumably through protein-protein interactions (61).

Another example of in vitro single turnover and product inhibition is that of E. coli chorismate lyase, which converts chorismate to 4-hydroxybenzoate in the ubiquinone biosynthesis pathway. The crystal structure of the enzyme reveals the tightly bound product in a hydrophobic pocket completely shielded from the bulk solvent by two helix-turn-helix loops that would be required to make large conformational changes for substrate binding and product release (62). This sequestration of product by the cytosolic chorismate lyase may be biologically relevant because association with the next enzyme in the pathway, the membrane-bound enzyme 4-hydroxybenzoate octaprenyltransferase, may be required for delivery of the 4-hydroxybenzoate substrate (63). The chorismate lyase fold has also been found to serve an effector binding function in the C-terminal domain of the E. coli PhnF transcriptional regulator, where the crystal structure of the domain contains a \(\beta\)-vfructoppyranose molecule in the hydrophobic binding pocket (64). Thus, the chorismate lyase fold is an example of a common fold adapted for regulatory as well as enzymatic function.

We propose to rename the ApbE superfamily as a periplasmic “flavin-trafficking protein” (Ftp) to reflect its proper biochemical function. Genome analyses, along with the available limited structural data, have failed to identify this previously uncharacterized FAD pyrophosphatase activity within the ApbE superfamily. This is primarily due to the lack of precise biochemical characterization of the deposited structures (65), which has led to the misannotation of the ApbE superfamily as members of a thiamine biosynthetic pathway (16). Some of the family members bind FAD, as in the case of S. enterica and E. coli, whereas others have evolved to catalyze the hydrolysis of FAD, but they both traffic flavin. Although Ftp is predominantly a prokaryotic protein superfamily, it is also found in lower eukaryotes, such as Trypanosoma spp. (the causative agents of sleeping sickness and Chagas disease) and Leishmania spp. (the causative agent of leishmaniasis), where it is fused or coupled with a mitochondrial NADH-dependent fumarate reductase, a flavin-requiring multimeric enzyme associated with the respiratory chain for electron transfer from either NADH or FMNH\(_2\) to fumarate (66–68).

Our discovery of a treponemal FAD pyrophosphatase implies that flavin homeostasis could play an important role in maintaining periplasmic flavin-containing compounds for utilization by putative periplasmic flavoproteins. The Universal Protein Resource Knowledgebase comprehensive resource for protein sequence and annotation data predicts that the *T. pallidum* genome encodes several flavoproteins in both the periplasm and cytoplasm. These are TP0081 (a putative FAD-binding protein), TP0171 (a putative periplasmic FMN-binding protein, also known as TP15), TP0298 (RfuA, a periplasmic riboflavin-binding protein), TP0572 (a putative FMN-binding protein), TP0735 (GltA, an FAD requiring glutamate synthase), TP0736 (HydG, a putative FAD-binding protein), TP0814 (trxB, an FAD thioredoxin reductase), TP0888 (RibF, an FAD synthetase), TP0921 (Nox, a flavin-requiring NADH oxidase), and TP0925 (fldA, a flavodoxin). *T. pallidum* may utilize some of these flavoproteins either in redox processes or in flavin-dependent non-redox reactions as described herein. Nonetheless, flavin-containing compounds (FMN and FAD) are the cofactors of flavoproteins, and their reported functions include transferring electrons from and to reactive redox centers (69). In Gram-negative bacteria, genes encoding the RnfABCDGE system are believed to be involved in a redox-driven ion pump. Topological analyses indicate that the RnfF cofactor in RnFABC system of the RnfABCDGE system are exposed on the outer leaflet of the cytoplasmic membrane (70, 71). *T. pallidum* is thought to lack an Rnf-type electron transport complex (3, 4), other treponemes, such as *T. denticola* (71), *Treponema phagedenis* (Weinstock et al., NCBI database), *Treponema brennaborense* (Lucas et al., NCBI database), *Treponema azotonutricium* (Tetu et al., NCBI database), and *Treponema succinifaciens* (72), are reported to have a complete RnFABC system. However, their biochemical characterization is lacking, and therefore, most of the available information is derived only from bioinformatics. Our UniProtKB search revealed that the *T. pallidum* genome encodes at least two likely Rnf homologs: tp0151 (RnfD), which probably requires an FMN cofactor, and tp0152 (RnfC), a protein with sequence signatures suggesting that it contains an Fe-S cluster.
The presence of these components in the colinear gene cluster tp0147–tp0153 (3) suggests that T. pallidum could have a membrane-bound electron transfer complex that employs these proteins, at least one of which apparently requires FMN. Thus, in T. pallidum and other bacteria, there very likely are periplasmic proteins that require FMN for their function. The apparent absence of quinone-synthesizing enzymes in T. pallidum (4) suggests that its Rnf components (and thus Ftp) play a critical role in energy transduction (73) in this bacterium.

These data raise the question of how bacteria supply FMN to periplasmic proteins. There are reports that bacteria of the genus Shewanella secrete their endogenously synthesized FAD via an unknown cytoplasmic membrane exporter to be processed in the periplasm by an UshA nucleotidase for extracellular flavin-requiring electron shuttles (59, 74, 75). However, this mechanism is not likely to be applicable to all bacteria utilizing an Rnf redox system as the physiological electron donor; further studies of the Ftp proteins and their in vivo interactions will broaden our overall understanding of flavin homeostasis and turnover in bacteria (Fig. 9). Finally, Ftp is found both in pathogenic bacteria and in the human parasites Trypanosoma and Leishmania. Given that the mammalian FAD pyrophosphatase is probably a member of a different superfamily, it is tempting to speculate that the Ftp family may offer potential promising targets for drug therapy, without serious side effects to the mammalian host.

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