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Kinetic Limitation and Cellular Amount of Pyridoxine (Pyridoxamine) 5′-Phosphate Oxidase of *Escherichia coli* K-12

GENSHI ZHAO AND MALCOLM E. WINKLER*

Department of Microbiology and Molecular Genetics, University of Texas Houston Medical School, Houston, Texas 77030

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We report the purification and enzymological characterization of *Escherichia coli* K-12 pyridoxine (pyridoxamine) 5′-phosphate (PNP/PMP) oxidase, which is a key committed enzyme in the biosynthesis of the essential coenzyme pyridoxal 5′-phosphate (PLP). The enzyme encoded by *pdxH* was overexpressed and purified to electrophoretic homogeneity by four steps of column chromatography. The purified PdxH enzyme is a thermally stable 51-kDa homodimer containing one molecule of flavin mononucleotide (FMN). In the presence of molecular oxygen, the PdxH enzyme uses PNP or PMP as a substrate (K_m = 2 and 105 μM and k_{cat} = 0.76 and 1.72 s^{-1} for PNP and PMP, respectively) and produces hydrogen peroxide. Thus, under aerobic conditions, the PdxH enzyme acts as a classical monofunctional flavoprotein oxidase with an extremely low k_{cat} turnover number. Comparison of k_{cat}/K_m values suggests that PNP rather than PMP is the in vivo substrate of *E. coli* PdxH oxidase. In contrast, the eukaryotic enzyme has similar k_{cat}/K_m values for PNP and PMP and seems to act as a scavenger. *E. coli* PNP/PMP oxidase activities were competitively inhibited by the pathway end product, PLP, and by the analog, 4-deoxy-PNP, with K_i values of 8 and 105 μM, respectively. Immunoinhibition studies suggested that the catalytic domain of the enzyme may be composed of discontinuous residues on the polypeptide sequence. Two independent quantitation methods showed that PNP/PMP oxidase was present in about 700 to 1,200 dimer enzyme molecules per cell in *E. coli* growing exponentially in minimal medium plus glucose at 37°C. Thus, *E. coli* PNP/PMP oxidase is an example of a relatively abundant, but catalytically sluggish, enzyme committed to PLP coenzyme biosynthesis.

Pyridoxal 5′-phosphate (PLP) is a ubiquitous, essential coenzyme that participates in many aspects of amino acid metabolism (2). In addition, PLP is the indispensable cofactor of glycogen phosphorylases, including bacterial maltodextrin phosphorylase (19). The final steps of PLP biosynthesis start with pyridoxine (PN; vitamin B_6) and PN 5′-phosphate (PNP [Fig. 1]), which are synthesized by many bacteria, plants, and fungi (13, 23, 50). In *Escherichia coli* K-12, PN and PNP are thought to be synthesized by a branched pathway from the committed precursors 4-phosphohydroxy-L-threonine and D-1-deoxyxylulose (13, 15, 22, 32, 33). It is not yet known whether 4-phosphohydroxy-L-threonine or its dephosphorylated derivative, 4-hydroxy-L-threonine, is condensed with D-1-deoxyxylulose to form the pyridine ring of the vitamin; the first case would give PNP and the second PN as the initial B_6 vitamer synthesized. If PN is formed directly, then the next step in the pathway is phosphorylation catalyzed by the PN/pyridoxal (PL)/pyridoxamine (PM) kinase (Fig. 1). Alternatively, if PNP is the first vitamer synthesized, then the PN/PL/PM kinase would be present in *E. coli* cells as part of a scavenger pathway, as it is in mammalian cells that cannot synthesize PN (28, 54).

The final step in the biosynthetic pathway is oxidation of PNP to PLP (Fig. 1). In mammalian cells, this step is catalyzed by a classical flavin mononucleotide (FMN)-containing flavoprotein oxidase that uses molecular oxygen as an electron acceptor and produces hydrogen peroxide (27, 38, 43). The mammalian oxidase uses both PNP and PM 5′-phosphate (PMP) as substrates and has approximately the same substrate specificity constant (k_{cat}/K_m) for PNP and PMP, consistent with a role in a scavenger pathway (5, 27, 38, 52). Mammalian PNP/PMP oxidases have been purified from several different types of tissues, and their enzymologies have been characterized extensively (5, 6, 27, 38, 52). The presence of PMP and PNP oxidase activities in *E. coli* crude extracts was first reported about 30 years ago (20, 51). These preliminary studies suggested that the *E. coli* PNP/PMP oxidase, like the mammalian enzyme, might be a flavoprotein that utilizes molecular oxygen as its electron acceptor. However, the *E. coli* PNP/PMP oxidase was never purified, and those studies never addressed the issue of how facultative anaerobes, such as *E. coli*, carry out the last step of PLP biosynthesis under anoxic growth conditions.

Point mutations in the *E. coli* B and K-12 genes encoding the aerobic PNP/PMP oxidase, which were designated *pdxH*, were identified by exploiting the fact that *pdxH* mutants can grow on minimal medium plus glucose supplemented with PL or PM but not with PN (Fig. 1) (10, 24). However, a gene-enzyme relationship between *pdxH* mutations and loss of PNP/PMP oxidase was not conclusively established in these early studies, partly because of an inability to assay enzyme activity in crude extracts (11). Recently, we cloned and sequenced the *pdxH* gene from *E. coli* K-12, and we also isolated mutants containing insertions in *pdxH* (31, 33). Structural analyses showed that *pdxH* is in a complex operon with the essential *tyrS* gene, which encodes the tyrosyl-tRNA synthetase (33). Likewise, all of the other PLP (*pdx*) biosynthetic genes have been found in complex superoperons in *E. coli* (1, 31, 46). Loss of *pdxH* function seriously disrupted cellular metabolism in several ways, including the excretion of significant amounts of L-glutamate and possibly α-ketoisovalerate in minimal growth medium plus glucose plus PL (33).

pdxH function was required for PLP biosynthesis in both

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Texas Houston Medical School, 6431 Fannin, JFB 1.765, Houston, TX 77030-1501. Phone: (713) 794-1744, ext. 1526. Fax: (713) 794-1782. Electronic mail address: mwinkler@utmmg.med.uth.tmc.edu.

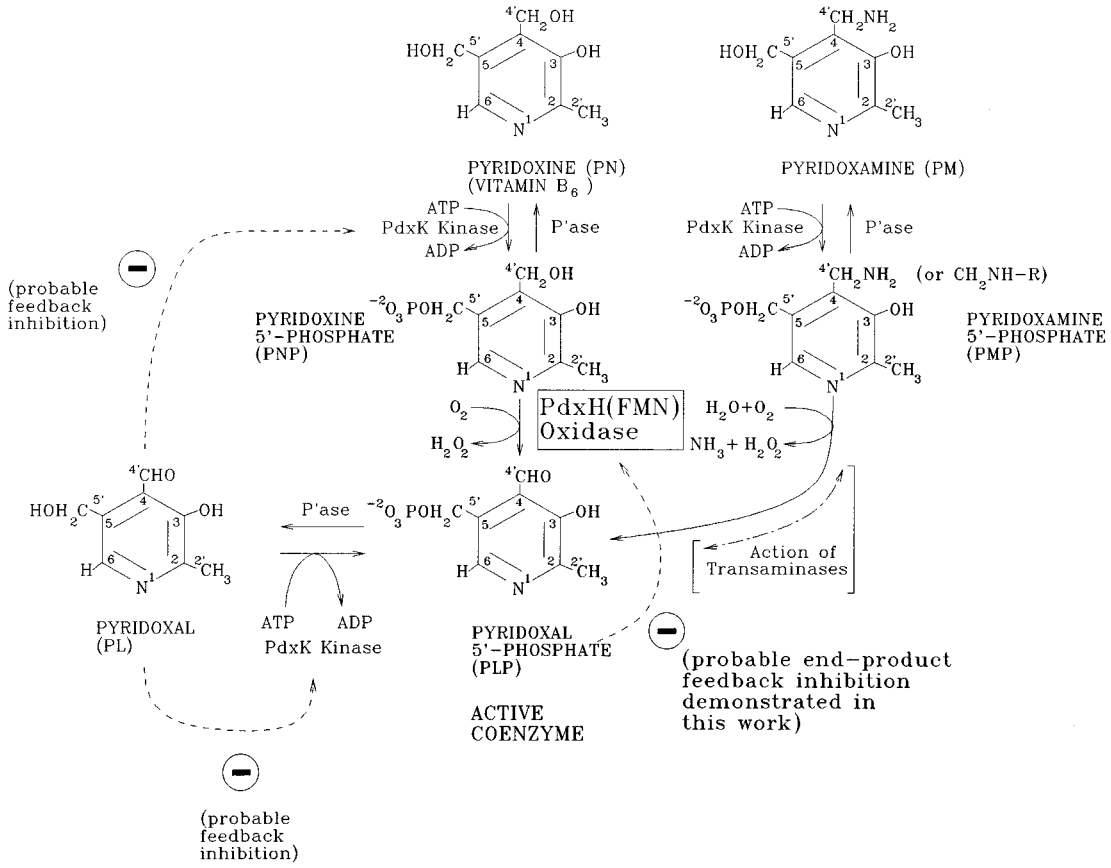

FIG. 1. Biosynthetic pathway leading from PN (vitamin B_6) and PNP to the active coenzyme PLP and allowing interconversions of the six B_6 vitamers (PL, PM, PN, PLP, PMP, and PNP). The pathway is described in the text. The reactions established in this paper that are catalyzed by *E. coli* PNP/PMP (PdxH) oxidase under aerobic conditions are boxed.

aerobically and anaerobically growing *E. coli* K-12 cells (33). It was also possible to identify second-site suppressor mutations that allowed *pdxH* mutants to grow aerobically and anaerobically in media lacking PL (33). These suppressors suggested that there might be a second pathway for PLP biosynthesis in anaerobically growing *E. coli* cells. To understand its role in PLP biosynthesis, we overexpressed, purified, and enzymologically characterized the *pdxH* gene product. The results in this paper show that *pdxH* does indeed encode a classical PNP/PMP flavoprotein oxidase, which uses molecular oxygen as an electron acceptor and produces hydrogen peroxide under aerobic assay conditions. However, unlike its eukaryotic counterpart, the *E. coli* PNP/PMP oxidase greatly prefers PNP over PMP as a substrate. We also measured the cellular concentration of PNP/PMP oxidase in *E. coli* cells growing aerobically in minimal medium plus glucose at 37°C. Together, our results suggest that *E. coli* PNP/PMP oxidase is a relatively abundant, but extremely sluggish, enzyme whose activity is possibly regulated in vivo through feedback inhibition by its product, PLP.

MATERIALS AND METHODS

Materials. PMP, 4-deoxy-PNP, PLP, PL, PM, PL, 4-pyridoxic acid, 4-aminoantipyrine, 3,5-dichloro-2-hydroxybenzenesulfonic acid, flavin adenine dinucleotide (FAD) (95% pure), FMN (98% pure), riboflavin (99% pure), nitroblue tetrazolium, NAD, NADP, horseradish peroxidase, and Sephadex G-200 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Molecular mass standards for gel filtration (bovine carbonic anhydrase, 31 kDa; bovine serum albumin [BSA], 66.2 kDa; alcohol dehydrogenase, 150 kDa; and β-amylase, 200 kDa) and for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (rabbit muscle phosphorylase *b*, 97.4 kDa; BSA, 66.2 kDa; hen egg white ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and hen egg white lysozyme, 14.4 kDa) were purchased from Sigma and Bio-Rad (Richmond, Calif.), respectively. Hydroxylapatite and Bio-Gel A-1.5m were obtained from Bio-Rad. DEAE-cellulose and carboxymethyl (CM)-cellulose were obtained from Whatman. PNP was synthesized by reducing PLP with sodium borohydride (49). The purity of the resulting PNP preparation was greater than 99.99% as determined by scanning spectrophotometry (250 to 800 nm), which showed no detectable Schiff base formation between residual PLP and Tris-HCl buffer (pH 8.5) at 400 to 420 nm.

Bacterial strains, plasmids, and culture conditions. The following strains of *E. coli* were used: JM109 [F′ *traD36 lacI*^{q} Δ(*lacZ*)M15 *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)] (Bethesda Research Laboratories), JM109 (pTX335) (this study), JM109(pUC19), NU1707 (NU816 *pdxH*::Mud1-8-1) (pTX293) (33), TX1708 (NU816 *pdxH*::Mud1-8-2) (33), TX2768 (Δ*pdxH*::ΩCm^r), which contains a deletion of the middle two-thirds of *pdxH* joined by a Ω(Cm^r) cassette (37), and NU816 (W3110 *lacU169 tna2 sup*^{o}) (C. Yanofsky). The plasmids used are pTX293, which carries a 5.4-kb *Bam*HI fragment containing *pdxH*$^+$ (*pdxH*$^+$ Ap^r Cm^r) (33), and pTX335 (P_{lac}-*pdxH*$^+$ Ap^r), which contained *pdxH*$^+$ under the control of the P_{lac} promoter of pUC19 (P_{lac}-*lacZ*α Ap^r) (this study). Expression plasmid pTX335 was constructed by ligating a 1.5-kb *Hin*cII *pdxH*$^+$ fragment from pTX293 (33) to pUC19 digested with *Sma*I restriction enzyme. Clones containing plasmids with *pdxH*$^+$ in the same orientation as the P_{lac} promoter were identified by restriction analysis and isopropyl-β-D-thiogalactopyranoside (IPTG) inducibility of PNP and PMP oxidase activities in *E. coli* JM109.

Luria-Bertani (LB) medium (10 g of NaCl per liter) was used as a rich medium (Bio 101). Vogel-Bonner (1 X E) minimal salts medium was prepared as described elsewhere (8). MMG was Vogel-Bonner minimal (1 X E) salts medium (8) containing 0.4% (wt/vol) α-D-glucose and 10 mM $FeSO_4$. Ampicillin (50 μg/ml), IPTG (1 mM), or potassium phosphate (40 mM) was added to the medium as indicated.

Enzyme assays and kinetics. Crude extracts were freshly prepared as described below for Western blotting (immunoblotting). PNP and PMP oxidase activities in crude extracts of wild-type and *pdxH* mutant strains were assayed by adding 1 to

4 mg of total protein from crude extracts to 1.5-ml reaction mixtures containing 0.2 M Tris-HCl, 0.2 M KPO_4 (pH 8.5), and 2.0 mM PMP or 0.2 mM PNP. Following incubation at 37°C for 45 to 60 min, reactions were stopped by the addition of 0.15 ml of chilled 50% (wt/vol) trichloroacetic acid, and precipitated protein was removed by centrifugation at 1,400 × *g* for 10 min at 4°C. PLP formation was measured by determining the A_{410} following the addition of 0.1 ml of 2% (wt/vol) phenylhydrazine in 10 N H_2SO_4 (52). In each experiment, linearity of enzyme assays was confirmed by adding different amounts of crude extracts.

PNP and PMP oxidase activities were measured in samples purified beyond crude extracts by monitoring PLP formation in Tris-phosphate buffer, in which PLP and Tris form a Schiff base that absorbs at 414 nm (29). Reaction mixtures (1.0 ml) contained 0.2 M Tris-HCl, 0.2 M KPO_4 (pH 8.5), 2.0 mM PMP or 0.15 mM PNP, 100 μg of BSA per ml, 2.0 μM FMN, and a suitable amount of enzyme (usually 1 to 2 μg). Initial velocities were measured by monitoring the increase in A_{414} for 5 min at 37°C. One unit of enzyme activity was defined as the formation of 1 nmol of PLP per min at 37°C. Protein concentrations were determined with the Bradford protein assay kit (Bio-Rad) with BSA as the standard (3).

Hydrogen peroxide (H_2O_2) formation by *E. coli* PNP/PMP oxidase was measured in reaction mixtures (1.0 ml) containing 0.2 M Tris-HCl, 0.2 M KPO_4 (pH 8.5), 2.0 mM PMP or 0.15 mM PNP, 100 μg of BSA per ml, 2.0 μM FMN, 1.0 μg of pure PNP/PMP oxidase, 1.0 mM 4-aminoantipyrine, 6.0 mM dichloro-2-hydroxybenzenesulfonic acid, and 20 μg of horseradish peroxidase. Initial velocities of H_2O_2 formation were measured by monitoring the increase in A_{510} at 37°C for 10 min (18). PLP formation in the same reaction mixtures was then measured by monitoring A_{414}.

Purification of PNP/PMP oxidase. A 250-ml overnight culture of JM109 (pTX335) grown in LB plus 50 μg of ampicillin per ml was used to inoculate 7.0 liters of LB containing 40 mM potassium phosphate, 50 μg of ampicillin per ml, and 1 mM IPTG and divided into seven 2-liter flasks. The cultures were grown with rapid shaking at 37°C and harvested in early stationary growth phase (90 to 110 Klett [660 nm] units). All subsequent steps were carried out at 4°C. Cells were added to 500-ml bottles, collected by low-speed centrifugation at 4,420 × *g* for 10 min, and washed with 15 ml of 20 mM KPO_4 (pH 8.2)–1 mM dithiothreitol (DTT) (buffer A) per bottle. Half of the cell pellets were frozen at −80°C and worked up the next day for DEAE chromatography. Half of the cell pellets were immediately resuspended in 2 to 3 ml of buffer A per bottle, pooled (14 to 21 ml total), and disrupted by two passages through a 20K French pressure cell at 20,000 lb/in^2. The resulting suspension was centrifuged at 150,000 × *g* (Beckman TLA 100.3 rotor) for 60 min. The crude extract supernate was loaded onto a DEAE-cellulose column (2.6 by 22 cm) equilibrated with buffer A. The column was washed with 600 ml of buffer A and then eluted with a linear 0 to 800 mM KCl gradient in buffer A. Ten 6-ml fractions showing high levels of PNP/PMP oxidase activity were pooled and reduced to 2 to 3 ml using 2-3 Centriprep 30 (30-kDa cutoff) concentrators (Amicon Corp., Beverly, Mass.) by sequential centrifugation at 1,240 × *g* (Beckman JA-17 rotor) for about 60 min. This concentrated sample was stored overnight at 4°C. The next day, the remaining frozen cells were resuspended and disrupted, and the resulting extract was chromatographed on the same DEAE column, which had been washed with 500 ml of buffer A plus 1.0 M KCl and equilibrated overnight with buffer A. Pooled column fractions were concentrated as described above. Meanwhile, the buffer was changed in the concentrated sample from the first day by adding 12 ml of 40 mM KPO_4 (pH 7.2)–1 mM DTT (buffer B) and repeating the centrifugal concentration. This dilution-concentration was repeated two more times, and the final sample was stored overnight at 4°C on ice in the cold. On the third day, the buffer of the concentrated sample from the second column was changed, and the final concentrated samples from both DEAE column runs were pooled. This 4- to 6-ml sample was applied to a hydroxylapatite column (2.5 by 6 cm) equilibrated with buffer B. The column was washed with 90 ml of buffer B and eluted with a linear 40 to 600 mM KPO_4 gradient in buffer B. Ten 5-ml fractions showing high levels of PNP/PMP oxidase activity were pooled and concentrated, and the buffer was changed to 20 mM KPO_4 (pH 5.8)–1 mM DTT (buffer C) by three rounds of dilution-concentration as described above, and applied to a CM-cellulose column (1.5 by 7 cm) equilibrated with buffer C. The column was washed with 60 ml of buffer C and eluted with a linear 0 to 800 mM KCl gradient in buffer C. Five 5-ml fractions exhibiting high levels of PNP/PMP oxidase activity were pooled, concentrated to 2 to 3 ml, and loaded (without buffer change) onto a Bio-Gel A-1.5m column (1.5 by 50 cm) equilibrated with buffer A. The column was eluted with buffer A, and five 3-ml fractions showing high levels of PNP/PMP oxidase activity were pooled, adjusted to 15% (vol/vol) glycerol, divided into 1-ml samples, and stored at −75°C. Pure enzyme samples were thawed on ice and used within 2 to 3 days without refreezing.

Biochemical characterization of purified *E. coli* PNP/PMP oxidase. The native molecular mass of purified PNP/PMP oxidase was determined on a calibrated Sephadex G-200 column (2.6 by 70 cm) equilibrated with 20 mM KPO_4 (pH 8.2) and eluted with 20 mM KPO_4 (pH 8.2)–1 mM DTT. Subunit molecular mass was determined by SDS–15% PAGE (5.6% stacking) (30). Native gel electrophoresis of purified PNP/PMP oxidase was performed on a 5.6% stacking and 7.5% polyacrylamide gel lacking SDS as described elsewhere (30). Half the gel was stained with Coomassie brilliant blue R-250, and half was incubated in an activity-staining solution containing 0.2 M Tris-HCl, 0.2 M KPO_4 (pH 8.5), 2 mM PMP, 0.1 mg of nitroblue tetrazolium per ml, and 2 μM FMN at 37°C as described previously (27). A control gel was incubated in the staining solution lacking PMP.

The amino terminus of purified PNP/PMP oxidase was determined following electrophoresis and electrotransfer. Purified PNP/PMP oxidase was subjected to SDS-PAGE (30) and then transferred electrophoretically to a polyvinylidene difluoride membrane in 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 8.0) at 90 V for 3 h. The membrane was stained in 0.1% (wt/vol) Coomassie brilliant blue R-250 in 50% (vol/vol) methanol for 5 min, destained in 50% (vol/vol) methanol–10% (vol/vol) acetic acid for 3 min, rinsed with water several times for 5 min (each), and air dried. The PNP/PMP oxidase spot was eluted directly into an Applied Biosystems 470A Protein Sequencer with On-Line 120 A PTH-Analyzer.

PNP/PMP apoenzyme lacking bound flavocoenzyme was prepared by extensive dialysis in 8-kDa-cutoff tubing against 1 M KBr–50 mM KH_2PO_4 (pH 4.0) for 5 days with eight changes (1 liter each) at 4°C and then against 50 mM KPO_4 (pH 8.2) for 3 days with six changes (1 liter each) at 4°C (7).

Antibody production and quantitative Western blotting. Polyclonal antibodies against SDS-denatured and native PNP/PMP oxidase were prepared by Bethyl Laboratories, Inc. (Montgomery, Tex.). Purified PNP/PMP oxidase was denatured by SDS-PAGE as described above, and bands containing 70 μg of PNP/PMP oxidase were visualized by incubating the gel in 0.2 M KCl–50 mM KPO_4 (pH 7.2) (21). Cut-out SDS-PAGE bands or 0.4 mg of native PNP/PMP was injected biweekly or weekly (21) into New Zealand White rabbits from which preimmune serum had been taken. After the fourth injection, serum was collected and tested weekly.

For determination of PNP/PMP oxidase amounts in crude extracts by quantitative Western blotting, strains NU816 (*pdxH*$^+$) and TX2768 (Δ*pdxH*::ΩCm^r) were grown in 400 ml of MMG with shaking at 37°C to a density of 63 Klett (660 nm) units (≈5 × 10^8 cells per ml). Cultures were chilled, and cells were collected by centrifugation at 5,000 × *g* for 10 min. Pellets were washed with 15 ml of 20 mM KPO_4 (pH 7.2) and resuspended in 2.5 ml of the same buffer. Cells were disrupted by two passages through a French pressure minicell at 20,000 lb/in^2. Suspensions were centrifuged at 150,000 × *g* (Beckman TLA 100.3 rotor) for 60 min, and 2.8 ml of 23.5-mg/ml protein was recovered, where protein concentrations were measured with the Bradford assay as described above. This procedure yielded about 330 μg of cytosolic protein per 10^9 cells. Concentrations of total protein in extracts of NU816 (*pdxH*$^+$) in the range of 30 to 120 μg per gel lane were found to give linear responses of stained PNP/PMP oxidase in the Western blots. The same amounts of extract from TX2768 (Δ*pdxH*::ΩCm^r) were mixed with 4.5, 9.0, and 18.0 ng of pure PNP/PMP and used to calibrate the blots. Pure protein alone was not used for calibration, because six to eight times more pure PNP/PMP oxidase was detected in the absence of the TX2768 (Δ*pdxH*::ΩCm^r) extract than in its presence.

Protein samples were electrotransferred from SDS-polyacrylamide gels to polyvinylidene difluoride membranes in 25 mM Tris–192 mM glycine (pH 8.5)–20% (vol/vol) methanol at 4°C for 2.5 to 3 h at 100 V (21). All subsequent steps were performed at room temperature with gentle shaking. Membranes were incubated in blocking solution (30 to 50 ml of 20 mM Tris-HCl [pH 8.2], 0.9% [wt/vol] NaCl, 4% [wt/vol] BSA) for 1 to 2 h. Membranes were then incubated for 1 h in the same volume of blocking solution containing a 1:2,500 dilution of antiserum against SDS-denatured PNP/PMP oxidase. Membranes were rinsed three times for 7 min each in washing solution (200 ml; 20 mM Tris-HCl [pH 8.2], 0.9% [wt/vol] NaCl) and incubated in the same volume of fresh blocking solution containing a 1:3,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antiserum. Membranes were rinsed three times in washing solution as described above and incubated for 5 min in 100 ml of 100 mM Tris-HCl (pH 9.5)–100 mM NaCl. Finally, the membranes were incubated in 30 to 50 ml of a solution of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 4 mM $MgCl_2$, 0.11 mg of nitroblue tetrazolium per ml, and 0.07 mg of the *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate per ml until sufficient purple color developed. Membranes were rinsed with water and air dried. The intensities of the PNP/PMP oxidase bands were quantitated using the reflective mode of a Shimadzu flying-spot densitometer at 550 nm.

RESULTS

Overexpression of *E. coli* PdxH oxidase. We constructed recombinant plasmid pTX335 by cloning the *pdxH*$^+$ gene, along with its endogenous P_{pdxH} promoter, downstream from the P_{lac} promoter of pUC19 (Materials and Methods). We then measured PNP and PMP oxidase activities in crude extracts from strains JM109(pUC19) and JM109(pTX335), which were grown to a density of about 30 Klett (660 nm) units in LB containing 50 μg of ampicillin per ml and 40 mM KPO_4 at 37°C, induced by 1 mM IPTG, and grown further to early stationary phase (90 to 100 Klett [660 nm] units). JM109 (pUC19) and JM109(pTX335) gave PNP and PMP oxidase specific activities of 0.11 and 0.25 or 8.9 and 21 U/mg, respec-

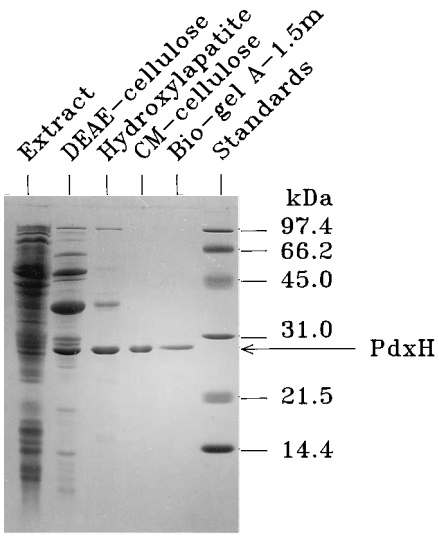

FIG. 2. SDS-PAGE analysis of samples from different stages of the purification of the *E. coli* K-12 *pdxH* gene product, PNP/PMP oxidase. The purification scheme is summarized in Table 1 and described in Materials and Methods. SDS-PAGE on 15% (wt/vol) polyacrylamide gels and Coomassie blue staining were performed as described in Materials and Methods. Lanes (from left) contained crude extract (270 μg), pooled DEAE-cellulose column fractions (100 μg), pooled hydroxylapatite column fractions (25 μg), pooled CM-cellulose column fractions (10 μg), pooled Bio-Gel A-1.5m column fractions (5 μg), and polypeptide molecular mass standards (4 μg each).

tively. Thus, we were able to overexpress both PNP and PMP oxidase activities about 80-fold from pTX335, which contained only the single reading frame identified previously as the $pdxH^+$ gene (33). Consistent with this confirmation that the $pdxH^+$ reading frame encodes PNP/PMP oxidase, crude extracts of strains NU1708 and TX2768, which contain chromosomal *pdxH*::Mud1 and Δ*pdxH*::Ω mutations, respectively (Materials and Methods), showed no detectable PNP or PMP oxidase activity. Finally, in parallel cultures, we omitted IPTG induction and still found about a 40-fold increase in PNP and PMP oxidase activities in JM109(pTX335) compared with those in JM109(pUC19) (data not shown). Therefore, most of the PNP/PMP oxidase overexpression was probably driven by the P_{pdxH} promoter on the high-copy-number pUC19 vector rather than by transcription from P_{lac}.

Crude extracts of the strain overexpressing the PdxH PNP/PMP oxidase from plasmid pTX335 were intensely yellow. A similar yellow color was observed when the *Myxococcus xanthus fprA* gene product was overexpressed in *E. coli* and was ascribed to increased flavin coenzyme production (47). Previously, we showed that the *fprA* and *pdxH* gene products are homologs (17, 33), and we show elsewhere that *fprA* does indeed encode *M. xanthus* PNP/PMP oxidase (56). Thus, the yellow color of JM109(pTX335) crude extracts was the first indication that *E. coli* PNP/PMP oxidase, like its *Myxococcus* and mammalian counterparts (7, 27, 47), is probably an FMN-containing flavoprotein.

Purification of *E. coli* PNP/PMP oxidase. We purified the *E. coli* PNP/PMP oxidase to apparent electrophoretic homogeneity by a series of four chromatography steps (Fig. 2; Table 1). We made several noteworthy observations in devising this purification scheme, which included some steps used previously to purify mammalian PNP/PMP oxidases from different tissues (27). The *E. coli* enzyme bound weakly to DEAE-cellulose in 20 mM KPO_4 (pH 8.2) but strongly to CM-cellulose in 20 mM KPO_4 (pH 5.8). This behavior on anion- and cation-exchange columns was consistent with the enzyme having a 9.6 isoelectric point (pI), calculated previously (33). The ratio of PMP to PNP oxidase activity remained at about 2.3:1 throughout all purification steps, and the two activities eluted with identical profiles from each chromatography column (data not shown). These observations and the cooverexpression of both activities described above supported the view that the *pdxH* gene product is a PNP/PMP oxidase. Throughout the purification, the peak fractions containing PNP and PMP oxidase activities were yellow; below, we confirm that the coenzyme FMN is bound to the PNP/PMP oxidase. Finally, we noted that the enzyme was tightly bound to hydroxylapatite and eluted as two partially resolved peaks, both with PNP and PMP oxidase activities (data not shown). This peak splitting of the bacterial enzyme on hydroxylapatite parallels behavior observed earlier during purification of mammalian PNP/PMP oxidase (27). When fractions from the two peaks were pooled and carried through the rest of the purification scheme (Fig. 2; Table 1), only one protein peak coincident with the PNP/PMP oxidase activity was detected.

The purity of the enzyme preparations was gauged by SDS-PAGE (Fig. 2). Following CM-cellulose or Bio-Gel A-1.5m chromatography, only a single, Coomassie blue-stained, 28-kDa polypeptide band was detected (Fig. 2). The final purified enzyme was also resolved by SDS-PAGE and silver stained; again only a single 28-kDa species was observed (data not shown). This molecular mass is consistent with the value of 25,545 Da predicted from the DNA sequence of the *pdxH* reading frame (33). The identity of the purified protein as PNP/PMP oxidase was further confirmed by native gel activity staining. The purified enzyme gave a single coincident band following electrophoresis on a native polyacrylamide gel and staining with Coomassie blue or with an activity stain containing nitroblue tetrazolium, PMP, and FMN (Materials and Methods; data not shown). The activity-stained band was cut out, denatured with SDS, and reanalyzed by SDS-PAGE; a single 28-kDa polypeptide was observed. Control gels stained with nitroblue tetrazolium and FMN without PMP failed to show a detectable band. We determined the sequence of the amino terminus of the purified PNP/PMP oxidase (Materials

TABLE 1. Purification of the *pdxH*-encoded PNP/PMP oxidase of *E. coli* K-12[a]

Fraction	Total protein (mg)	Sp act (U/mg)		Total activity (U)		Activity ratio (PMP/PNP)	Fold purification	Yield (%)
		PNP	PMP	PNP	PMP			
Crude extract	749	7.4	17.3	5,540	12,960	2.3	1	100
DEAE-cellulose	52	56	132	2,910	6,860	2.5	8	53
Hydroxyapatite	6.3	303	730	1,910	4,600	2.4	42	35
CM-cellulose	2.7	485	1,020	1,300	2,730	2.1	69	21
Bio-Gel A-1.5m	2.2	622	1,370	1,380	2,790	2.2	79	22

[a] Details about enzyme overexpression, enzyme assays, and this purification scheme are in Materials and Methods.

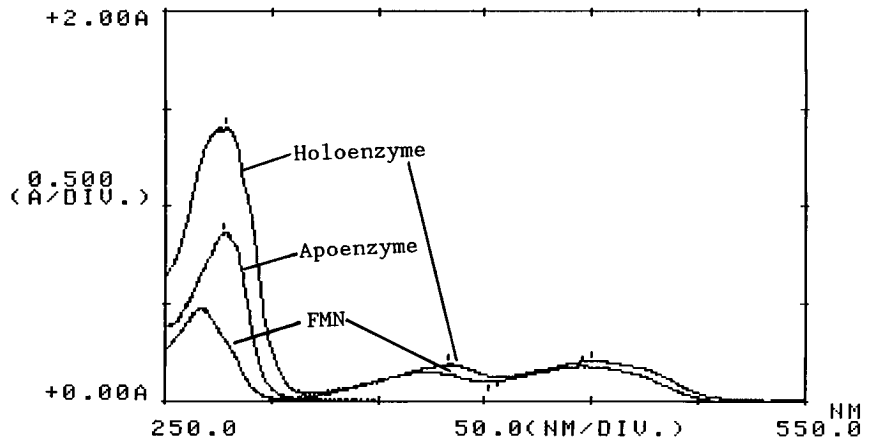

FIG. 3. Spectral analysis demonstrating that the *E. coli pdxH* gene product is a flavoprotein. Absorption spectra of the holoenzyme (13.4 μM), apoenzyme (13.4 μM), and coenzyme FMN (13.4 μM) were recorded at room temperature with a Shimadzu UV-160 recording spectrophotometer. The purified holoenzyme and apoenzymes were prepared as described in Materials and Methods.

and Methods). The 10 amino-terminal residues were Ser-Asp-Asn-Asp-Glu-Leu-Gln-Gln-Ile-Ala, which corresponded to the favored translation start deduced from the nucleotide sequence (33), except that the terminal Met residue was removed.

Native molecular mass and coenzyme bound by *E. coli* PNP/PMP oxidase. We determined the molecular mass of the native protein by gel filtration (Materials and Methods). Native PNP/PMP oxidase had a molecular mass of 51 kDa (data not shown), which corresponds to a dimer of identical subunits.

As noted above, the intense yellow color of the enzyme preparation during purification suggested that it is a flavoprotein oxidase. We used several different approaches to demonstrate that the bound coenzyme was FMN and not FAD. The purified enzyme exhibited three absorption maxima at 275, 386, and 450 nm (Fig. 3), indicative of bound FMN or FAD (16). These absorbance maxima are shifted to slightly longer wavelengths than those of pure FMN or FAD at 267, 374, and 445 nm. These spectral properties are characteristic of mammalian PNP/PMP oxidases and other flavoprotein oxidases (27, 36). To establish the identity of the bound coenzyme, we measured the fluorescence intensity at pH 7.7 and 2.6 of the coenzyme released by boiling from a known amount of *E. coli* PNP/PMP oxidase (Table 2) (16). The fluorescence intensity of the released coenzyme, like that of the FMN standard, decreased about 50% when the pH was reduced from 7.7 to 2.6 (Table 2). In contrast, the fluorescence intensity of FAD changed in the opposite direction over this pH range.

We further established that FMN is the coenzyme of *E. coli* PNP/PMP oxidase by preparing apoenzyme by extensive dialysis (Materials and Methods). The apoenzyme exhibited only one absorption maximum at 275 nm, indicating that the dialysis had removed the bound flavin coenzyme (Fig. 3). The apoenzyme preparation was totally inactive (data not shown). Addition of FMN, but not FAD or flavin, to reaction mixtures containing the apoenzyme restored both the PNP and the PMP oxidase activities (data not shown). Thus, FMN is the coenzyme of *E. coli* PNP/PMP oxidase.

We determined the molar ratio of FMN bound in two ways. Table 2 shows that the florescence intensities from 100 and 150 nM solutions of denatured purified native enzyme corresponded to the fluorescence from 85 and 140 nM solutions of pure FMN, respectively. This result implies that 1 mol of FMN is bound per mol of PNP/PMP dimer. Consistent with this interpretation, 13.4 μM of pure FMN and the native dimer enzyme had an A_{445} of 0.18 and an A_{450} of 0.2 (Fig. 3). Again, it appears that one FMN molecule is bound to each PNP/PMP oxidase dimer.

Stability, pH, and temperature optima. Purified *E. coli* PNP/PMP oxidase was stable for weeks at 4°C without significant loss of activity and for months at −75°C in 15% glycerol (Materials and Methods). The thermal stability of the purified enzyme was determined by incubating 50-μl mixtures containing purified enzyme in 20 mM KPO_4 (pH 8.2)–1 mM DTT–15% (vol/vol) glycerol at various temperatures up to 65°C for 15 min and then assaying remaining PNP and PMP oxidase activities at 37°C (Materials and Methods). No significant activity loss (<10 to 20%) was observed when the enzyme was incubated at temperatures below 50°C, and incubation at 65°C for 15 min caused only a 30 to 40% activity loss (data not

TABLE 2. Fluorescence determination of the coenzyme bound to the *E. coli pdxH* gene product[a]

Coenzyme (concn [nM])	Fluorescence intensity (arbitrary units)	
	pH 7.7	pH 2.6
FMN		
100	120.6	64.7
200	230.6	136.3
400	449.0	268.0
FAD		
100	14.2	49.3
200	23.8	92
400	42.6	176
Coenzyme released from purified protein[b]		
100	104	56
150	140	79

[a] Fluorescence intensities (excitation, 450 nm; emission, 535 nm) were measured at room temperature in 0.1 M KPO_4 (pH 7.7)–0.1 mM EDTA in a Hitachi F-2000 recording fluorescence spectrophotometer.

[b] The indicated amount of purified enzyme in the buffer was boiled for 3 min, rapidly chilled on ice for 5 min, and centrifuged in a microcentrifuge for 10 min. The supernate was equilibrated to room temperature before fluorescence was measured.

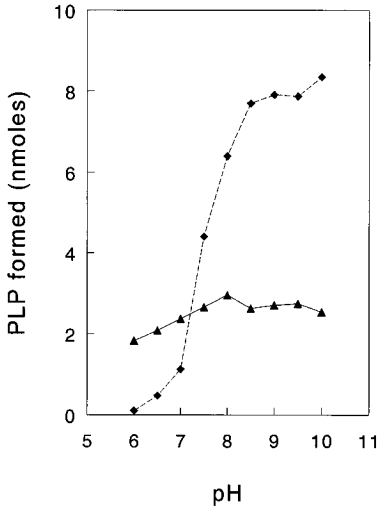

FIG. 4. pH profiles of the PNP and PMP oxidase activities of *E. coli* PNP/PMP oxidase. PNP (triangle) and PMP (diamond) oxidase activities were measured in aerobic (air; ≈0.26 mM O_2) reaction mixtures containing 0.2 M Tris-HCl, 25 mM potassium sulfate, 100 μg of BSA per ml, 2 mM PMP or 0.15 mM PNP, 2 μM FMN, and 0.9 μg of purified PNP/PMP oxidase adjusted to the pH values shown. Initial rates of PLP formation were monitored (Materials and Methods), and results are expressed as the amount of PLP formed in 8 min.

shown). Thus, the PNP/PMP oxidase is relatively thermally stable. Consistent with this stability, the optimal temperature for the PNP and PMP oxidase reactions was 50°C in the reaction mixtures used (data not shown).

The PMP oxidase activity showed a sharp pH profile (Fig. 4). The PMP oxidase enzyme activity was absent at pH 6, rose sharply as the pH increased from 6 to 8.5, and then remained nearly constant at pH values between 8.5 and 10 (Fig. 4). In contrast, the PNP oxidase activity showed a very broad pH profile (Fig. 4). The enzyme was active throughout the pH range and exhibited a slight optimum at pH 8.5. These completely different pH profiles suggest that protonation is involved in the reaction with PMP but not PNP (see Discussion).

Kinetic properties. We wanted to determine whether *E. coli* PNP/PMP oxidase uses molecular oxygen as an electron acceptor and produces H_2O_2 under aerobic assay conditions. Therefore, we assayed enzyme activities aerobically and anaerobically. All assay solutions were degassed under vacuum and immediately added to the side arms of small, stoppered Warburg reaction flasks. Anaerobic assay conditions were generated by evacuating the reaction flasks and then flushing them with ultrapure argon gas (15 lb/in^2) for 15 min on ice before the purified enzyme and substrates were mixed by tipping. Throughout the assay, the reaction flasks were gently shaken at 37°C and continuously flushed with argon gas. Reactions were stopped by chilling mixtures to 4°C. The A_{414} was recorded (see Materials and Methods) immediately after the flasks were opened. The purified enzyme failed to show any PNP or PMP oxidase activity under anaerobic assay conditions. Thus, *E. coli* PNP/PMP oxidase seems to use molecular oxygen as an electron acceptor under aerobic assay conditions, similar to other classical flavoprotein oxidases (4, 53).

The latter conclusion was supported by the generation of H_2O_2 by *E. coli* PNP/PMP oxidase under aerobic assay conditions (Table 3). The addition of horseradish peroxidase to the reaction mixtures led to an increase in PLP formation, suggesting that the oxygen radicals generated by the peroxidase were able to oxidize PNP and PMP to PLP (44) (Table 3).

TABLE 3. H_2O_2 production by *E. coli* PdxH enzyme[a]

Assay components[b]	H_2O_2 formed[c] (nmol)	PLP formed[c] (nmol)
PNP + AAP + DCHBSA (no peroxidase)	0	6.5
PNP + AAP + DCHBSA + peroxidase	6.4	11.5
PMP + AAP + DCHBSA (no peroxidase)	0	5.5
PMP + AAP + DCHBSA + peroxidase	5.1	8.6

[a] The experiment is detailed in Materials and Methods.
[b] AAP, 4-aminoantipyrine; DCHBSA, 3,5-dichloro-2-hydroxybenzenesulfonic acid; peroxidase, horseradish peroxidase.
[c] Results are expressed as amounts of products formed in 10 min at 37°C (Materials and Methods).

Subtraction of the amount of PLP formed in the absence of peroxidase from that formed in its presence (Table 3) shows that approximately 1 mol of H_2O_2 was formed for every mol of PNP or PMP oxidized.

Steady-state kinetic analyses were carried out on the PNP/PMP oxidase (data not shown). K_m values of 105 and 2 μM were obtained for PMP and PNP, respectively, from Lineweaver-Burk (double-reciprocal) plots. V_{max} values of 0.889 μmol/min/mg (k_{cat} = 0.76 s^{-1}) and 2.022 μmol/min/mg (k_{cat} = 1.72 s^{-1}) were obtained for the PNP and PMP oxidase reactions, respectively. k_{cat} values were calculated assuming full activity of the purified enzyme and one active site per enzyme dimer. The assumption of nearly full activity is supported by the results of quantitation of cellular enzyme concentration presented below. The assumption of one active site is supported by the single FMN bound per dimer and by the results of the monofunctionality experiments presented below. On the basis of these considerations, we estimate that k_{cat} values are off at most by a factor of two, which would not change any of the conclusions presented here. Double-reciprocal plots for the PNP oxidase reaction showed an upward curvature at high concentrations of PNP, indicative of substrate inhibition (data not shown). On the other hand, no substrate inhibition was observed for PMP oxidase activity. The PNP inhibition of the PNP oxidase reaction decreased when the oxygen concentration was increased from air to 30% (vol/vol) (data not shown). This type of substrate inhibition is characteristic of mammalian PNP/PMP oxidases and other kinds of protein flavooxidases (see Discussion) (4, 5, 25).

The PNP and PMP oxidase activities were not inhibited by nonphosphorylated B_6 vitamers and derivatives, including PL, PN, PM, and 4-pyridoxic acid (data not shown). Because the oxidase assay measures Schiff base formation between Tris and PLP, this lack of inhibition suggests that the enzyme does not appreciably bind to these nonphosphorylated compounds. On the other hand, the PNP and PMP oxidase activities were competitively inhibited by PLP and 4-deoxy-PNP (data not shown). K_i values of 8 and 105 μM were obtained for PLP and 4-deoxy-PNP, respectively, for both the PNP and PMP oxidase reactions. These combined K_m and K_i show that *E. coli* PNP/PMP oxidase has higher apparent affinities for phosphorylated vitamers with a 4′-alcohol or 4′-aldehyde group compared with those with a 4′-deoxy or amine group. The physiological implications of these kinetic parameters are considered in the Discussion.

Finally, we tested whether *E. coli* PNP/PMP oxidase is monofunctional or bifunctional with regard to its binding to the two substrates. If the enzyme is bifunctional, then an ad-

TABLE 4. Monofunctionality of *E. coli* PNP/PMP oxidase[a]

PMP concn (mM)	PNP concn (μM)	Oxidase activity (nmol of PLP formed)
2.5	0	3.9
2.5	12.5	2.8
2.5	25	2.6
2.5	50	1.9
5.0	50	2.0
0.5	50	1.5
0	50	1.7

[a] Enzyme assays were carried out as described in Materials and Methods. Reaction mixtures contained the amounts of the two substrates indicated. Initial rates of PLP formation were measured, and the results are expressed as the amount of PLP formed in 5 min.

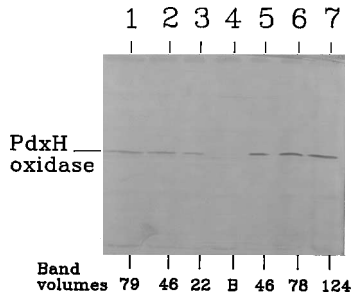

FIG. 5. Quantitative Western blotting to estimate the intracellular PNP/PMP oxidase concentration in bacteria growing exponentially in MMG medium at 37°C. Growth of bacteria, preparation of cytosolic extracts, and Western blotting were done as detailed in Materials and Methods. Lanes 1 to 3, extracts of TX2768 (Δ*pdxH*::ΩCm^r) to which purified enzyme was added to serve as a standard; lane 1, 120 μg of extract plus 18 ng of purified enzyme; lane 2, 60 μg of extract plus 9 ng of purified enzyme; lane 3, 30 μg of extract plus 4.5 ng of purified enzyme; lane 4, 120 μg of TX2768 (Δ*pdxH*::ΩCm^r) extract alone; lanes 5 to 7, extracts of NU816 (*pdxH*$^+$); lane 5, 30 μg; lane 6, 60 μg; lane 7, 120 μg. Increased volumes loaded into lanes 1 to 3 caused the spreading of the lanes. The original blots were scanned as described in Materials and Methods, and volumes of the PNP/PMP bands (in arbitrary units) are shown below each lane. B, background from 120 μg of TX2768 (Δ*pdxH*::ΩCm^r) extract.

ditive effect in activity should be observed when the two substrates with different apparent binding affinities are present in the same reaction mixture. However, for a monofunctional enzyme, inhibition or activation should then be observed, depending on the relative concentrations of the two substrates. The results show that *E. coli* PNP/PMP oxidase, like its mammalian counterparts (38, 52), is a monofunctional enzyme (Table 4). Increasing PNP (K_m = 2 μM) concentrations in reaction mixtures containing a high concentration of PMP (K_m = 105 μM) inhibited PLP formation. Increasing PMP concentrations in the reaction mixtures containing PNP increased PLP formation. Clearly, no additive effect in activity for the two substrates was observed.

Inhibition of *E. coli* PNP/PMP oxidase with polyclonal antibodies. The PNP and PMP oxidase activities of the purified enzyme were completely inhibited by the antiserum prepared against the purified native enzyme (data not shown). In contrast, antibody prepared against SDS-denatured enzyme did not affect PNP and PMP oxidase activities. Control experiments established that lack of inhibition was not due to a significantly lower titer of the antiserum against SDS-denatured PNP/PMP oxidase compared with that against the native enzyme. In fact, equivalent (1:2,500 to 3,000) dilutions of antiserum against the denatured protein gave stronger signals on Western blots than did antiserum against native protein (data not shown) (Materials and Methods). The implications of these experiments to PNP/PMP oxidase structure are taken up in the Discussion.

Cellular concentration of *E. coli* PNP/PMP oxidase. We estimated the number of PNP/PMP oxidase molecules per cell in two independent ways. The first method utilized the specific activity of the pure PNP/PMP oxidase to estimate the number of enzyme molecules present in crude extracts. The specific activities of the purified *E. coli* oxidase were 622 and 1,370 U/mg for PNP and PMP, respectively (Table 1). The specific activities of PNP/PMP oxidase in crude extracts of cells grown aerobically in MMG were 0.11 and 0.27 U/mg for PNP and PMP, respectively (Materials and Methods). The extraction procedure we used yielded about 330 μg of cytosolic protein per 10^9 cells grown under these conditions (Materials and Methods), and the molecular mass of the active dimer was 51 kDa (above). Assuming that all of the pure enzyme was active, there are about 680 PNP/PMP oxidase dimers (5.8×10^{-11} μg) per cell grown in MMG medium at 37°C. We also estimated cellular PNP/PMP oxidase concentration by quantitative Western blotting (Fig. 5) (Materials and Methods). In these cases, pure PNP/PMP oxidase that was mixed with crude extracts of a Δ*pdxH*::Ω deletion/insertion mutant was used as a standard for enzyme amounts (Materials and Methods). Quantitation of Western blots showed that 30 μg of extract from a *pdxH*$^+$ strain (Fig. 5, lane 5) gave approximately the same signal as 9 ng of purified PNP/PMP oxidase added to an extract of a Δ*pdxH* mutant (Fig. 5, lane 2). This amount corresponds to about 1,200 PNP/PMP oxidase dimers (9.9×10^{-11} μg) per cell grown in MMG at 37°C. Thus, both methods gave similar results within a factor of 2 and show that PNP/PMP oxidase is a moderately abundant *E. coli* protein (Discussion). The lower estimate based on specific activities may reflect some inactive protein in the purified enzyme preparations.

DISCUSSION

The enzymology of mammalian PNP/PMP oxidases has been determined in part because of the important metabolic roles of PLP in tissues like brain (6, 7, 29) and liver (5, 27, 38). Moreover, PNP/PMP oxidases are of intrinsic biochemical interest, because they are the only class of flavoprotein oxidases that catalyze two-electron transfer to either the primary alcohol of PNP or the primary amine of PMP to form the same product, PLP (5, 38). In this paper, we show that there are both shared and different properties between the *E. coli* PdxH PNP/PMP oxidase and its mammalian counterparts. Under aerobic assay conditions, the bacterial and mammalian enzymes act as monofunctional oxidases that use molecular oxygen as an electron acceptor and produce H_2O_2 (Tables 3 and 4). The bacterial and mammalian enzymes are homodimers containing one molecule of bound FMN (Table 2; Fig. 3) (Results). Moreover, substrate inhibition by PNP, but not PMP, that is reversed by increased oxygen concentration shows that *E. coli* PNP/PMP oxidase uses different catalytic mechanisms depending on the substrates, just like mammalian PNP/PMP oxidase and other flavoprotein oxidases (4, 5, 25). For PNP/PMP oxidases, PNP or PMP oxidation is thought to follow a binary, ping-pong or a ternary, sequential mechanism, respectively (5). Thus, the bacterial enzyme acts as a classical flavoprotein oxidase under aerobic growth conditions.

There are also some notable differences between the *E. coli* and mammalian PNP/PMP oxidases. The bacterial enzyme appears to be much more stable at high temperatures than the eukaryotic enzyme (see Results; 52), which is largely inactive at 50°C. By comparison, the *E. coli* PNP/PMP shows maximum

activity at 50°C under the reaction conditions used (see Results). The *E. coli* enzyme showed completely different pH profiles for PNP and PMP (Fig. 4) compared with those of the mammalian enzyme, which showed slightly increasing activity for both substrates with increasing pH (52). The difference in the pH profiles of the *E. coli* PNP/PMP oxidase for PNP and PMP does not simply reflect removal of a proton from the 4′ primary amine of PMP at higher pH values, because the pK_a' of this deprotonation is 10.9 in solution (48). However, it is possible that the pK_a' of this deprotonation is lower for PMP bound to the oxidase. Alternatively, the reaction mechanism for the oxidation of PMP may be different from that of PNP, as suggested for the mammalian oxidases (5). Another significant difference between the *E. coli* and mammalian enzymes is their substrate (k_{cat}/K_m) affinity constants. The k_{cat}/K_m for the *E. coli* enzyme is 38×10^4 M^{-1} s^{-1} for PNP and 1.6×10^4 M^{-1} s^{-1} for PMP. In contrast, the k_{cat}/K_m for the mammalian enzyme is 8.5×10^4 M^{-1} s^{-1} for PNP and 2.8×10^4 M^{-1} s^{-1} for PMP (5). Compared with the eukaryotic enzyme, *E. coli* PNP/PMP oxidase greatly prefers PNP over PMP as a substrate. This finding is consistent with the notion that PNP is normally on the de novo PLP biosynthetic pathway, whereas the mammalian enzyme is used in a scavenger pathway. The preference of the *E. coli* enzyme for PNP over PMP may be even larger in vivo. Apparent k_{cat}/K_m values were determined at the optimal pH of 8.5 (Materials and Methods). However, *E. coli* cells maintain their internal pH value close to 7.6, regardless of the pH of the growth medium (26). At pH 7.6, the activity of *E. coli* PNP/PMP oxidase is close to the maximum value for PNP as a substrate but is less than half of the maximum value for PMP (Fig. 4).

Although extensive enzymological and kinetic analyses have been performed on mammalian PNP/PMP oxidases (5–7, 27, 29, 38, 52), relatively little is known about their molecular structures. In fact, no DNA sequence of a mammalian PNP/PMP oxidase has been reported to date. By contrast, the enzymological characterization presented here and the DNA and deduced amino acid sequences reported previously make the *E. coli* enzyme an ideal model system for future studies of PNP/PMP oxidase biochemistry. As a start in this direction, we established that the amino terminus of *E. coli* PNP/PMP oxidase matches the predicted sequence, except that the terminal Met residue is removed (Results). We also showed that the PNP and PMP oxidase activities are strongly inhibited by antiserum against native, but not SDS-denatured, *E. coli* PNP/PMP oxidase (Results). This finding is consistent with the idea that the catalytic domain of the enzyme is composed of discontinuous amino acids from different segments of the polypeptide chain. This interpretation is supported by the three-dimensional structures of the FMN-binding and catalytic domains of some oxidases (34, 35, 45).

We found that about 700 to 1,200 dimer molecules of PNP/PMP oxidase are present per *E. coli* K-12 cell growing exponentially in MMG medium at 37°C (Results) (Fig. 5). In comparison, the tryptophan biosynthetic enzymes are present in 26 to 157 and 624 to 996 molecules per cell in wild-type *trpR*$^+$ bacteria growing in the same medium containing and lacking 50 μg of L-tryptophan per ml, respectively (40, 41). Thus, the PdxH oxidase is a relatively abundant biosynthetic enzyme. On the other hand, both the *E. coli* and mammalian PNP/PMP oxidases are catalytically sluggish, with k_{cat} values ranging from 0.1 to 1.72 s^{-1} for the two substrates (Results) (5, 38). Many enzymes have k_{cat} values 1 to 5 orders of magnitude greater than those of the PNP/PMP oxidases (53). Our results support the view that oxidases are kinetically sluggish despite the thermodynamic favorability of the reaction (53).

Dempsey showed that *E. coli* B contains about 3.6×10^{-10} mol of B_6 vitamers per mg (dry weight) of bacteria grown exponentially in minimal medium plus glucose at 30°C ($\mu \approx 1$ h) (9). This amount is far less (150 to 250 times) than those of the least abundant amino acids, tryptophan and histidine, which are mostly incorporated into protein (12, 42, 55). The large difference in amounts is thought to reflect a relatively low net rate of B_6 vitamer biosynthesis compared with that of amino acids and certain other cellular compounds (12, 42). In view of this relatively low net biosynthetic rate, it was suggested early on that vitamin biosynthetic enzymes might be present in relatively low cellular concentrations, if they are catalytically efficient (12, 39). To the contrary, White and Dempsey reported that PN/PM/PL (PdxK) kinase (Fig. 1) is a moderately abundant (≈360 molecules per cell), but catalytically sluggish [k_{cat}(PL) = 5.8 s^{-1}], enzyme (54). However, Dempsey pointed out that the PdxK kinase may not be a proper test of models about cellular concentrations of coenzyme biosynthetic enzymes (12), because this kinase is not established as a committed enzyme in B_6 vitamer biosynthesis (see the introduction) (12). It was also noted that this one example would not necessarily rule out low enzyme concentrations for other B_6 vitamer biosynthetic enzymes (54). Results reported here for *E. coli* PNP/PMP oxidase demonstrate for the first time the relationship between kinetic limitation and abundant cellular amount for an enzyme strictly committed to de novo PLP biosynthesis.

The total B_6 vitamer amount (above), dry mass per cell (270 μg/9×10^8 cells) (9), and Avogadro's number (6.023×10^{23} molecules/mol) give a net rate of B_6 vitamer biosynthesis of about 6.5×10^4 molecules per cell per h in bacteria growing in minimal medium plus glucose at 30°C (12). On the basis of the k_{cat}(PNP) and the number of enzyme molecules per cell (above), PNP/PMP oxidase has the catalytic potential to synthesize 1.9×10^6 to 3.3×10^6 PLP molecules per cell per h. Therefore, there is about a 40× excess of PNP/PMP oxidase catalytic potential in vivo in the absence of end product inhibition (below), assuming that in vitro kinetic values apply to in vivo conditions.

About 15% of the intracellular B_6 vitamer amount was extractable by toluene-saturated minimal medium and represents an estimate of the free B_6 vitamer concentration in the cytoplasm (9). On the basis of the total B_6 vitamer amount (above), of which about 25% was reported as extracellular (9), and an average volume of 0.8×10^{-12} ml for cells growing with a doubling time of 1 h (14), the intracellular concentration of total and free B_6 vitamers can be estimated as 101 and 15 μM, respectively. Although it is unknown how much of the free B_6 vitamer is PLP, it is noteworthy that the K_i of PNP/PMP oxidase for PLP is 8 μM (see Results). Thus, end product, feedback inhibition of the PNP/PMP oxidase by PLP could play a significant role in pathway regulation of PLP biosynthesis in vivo.

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