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Single- and Double-Headed Analogs of Pyridoxamine 5′-Phosphate as Probes for Pyridoxamine 5′-Phosphate Utilizing Enzymes[1]

DELORES M. BOWERS-KOMRO AND DONALD B. MCCORMICK[2]

Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322

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Single- and double-headed analogs of pyridoxamine 5′-phosphate were synthesized as cleft-depth probes for the catalytic sites of two types of enzymes, viz. those which use pyridoxamine 5′-phosphate as a substrate, i.e., pyridoxamine (pyridoxine) 5′-phosphate oxidase and those which use pyridoxamine 5′-phosphate as coenzyme, i.e., aspartate aminotransferase. These probes were prepared by borohydride reduction of the Schiff's bases formed by the interaction of pyridoxal 5′-phosphate with one or both ends of 1,*n*-diaminoalkanes (where n = 2, 4, 6, 8, or 12 carbons). Kinetic studies using these analogs as substrates for the oxidase revealed that the single-headed analogs exhibit values ranging from 3 K_m to K_m for pyridoxamine 5′-phosphate in a curvilinear relationship with chain lengths for n = 2 to 12. The short-chain substrates (n = 2, 4) exhibit V_m values greater than for pyridoxamine 5′-phosphate, whereas the long-chain ones exhibit lesser V_m values. These observations may indicate the increasing hydrophobicity of the substrate with increasing chain length and thus poorer turnover. Lineweaver–Burk plots of the double-headed substrates were bilinear for the shorter chains (n = 2, 4, 6) and linear for the longer chains (n = 8, 12) over the concentration range of study (1 to 500 μM). This indicates that the catalytic cleft may impede reorientation for further substrate action of the single-headed amine released from the double-headed substrate. In both cases (>50 μM), the high K_m values may reflect the ionic repulsion forces established by the different secondary amine functionalities, $-(CH_2)_n-NH_3^+$ for single-headed and 5′-phosphopyridoxyl for the double-headed, with active site residues thus effecting binding and ultimate turnover. These same analogs do not act as coenzymes for apo-aspartate aminotransferase or compete with pyridoxamine 5′-phosphate at the micromolar level. They do appear to bind weakly to the enzyme as observed by fluorescence quenching.

Characterization of pyridoxamine (pyridoxine) 5′-phosphate oxidase (PM(PN)P-oxidase,[3] EC 1.4.3.5), from rabbit liver has revealed that the 54,000 M_r enzyme

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[2] To whom correspondence should be addressed.

[3] Abbreviations: PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; PNP, pyridoxine 5′-phosphate; PM(PN)P-oxidase, pyridoxamine (pyridoxine) 5′-phosphate oxidase; AAT, aspartate aminotransferase; FMN, flavin mononucleotide; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; NADH, reduced β-nicotinamide adenine dinucleotide; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; Lys, lysine; RT, retention time.

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is composed of two seemingly identical, N-terminal blocked 27,000 M_r chains (*1*, *2*) which associate ionically and hydrophobically to generate a catalytic cleft wherein the coenzyme, FMN, is bound. The holoenzyme catalyzes the oxidation of both amine (PMP) and alcohol (PNP) types of natural substrates with relatively narrow specificity for the 5'-phospho-4'-pyridoxyl structure. This specificity extends to the product, PLP, which binds as the free nonhydrated aldehyde (*3*) at least as tightly as substrate (*4*). Moreover, sizeable secondary amine substituents on the 4'-methylene of substrate can be oxidatively removed to liberate amine and PLP (*5*, *6*).

Cytosolic aspartate aminotransferase (c-AAT, EC 2.6.1.1), from pig heart is also composed of two seemingly identical chains of 412 residues, which associate to form a 93,000 M_r dimer (*7*). Crystallographic studies (*8*) indicate that each 412-residue monomer folds into two domains, a small domain (that is loosely structured and composed of the NH_2-terminal and COOH-terminal peptides) and a large domain (that is highly structured and composed of largely alternating segments of 7 β-helices and 7 α-strands from residues 75 to 300). The catalytic site containing the coenzyme, either PLP or PMP, is located in a pocket formed between these two domains. Thus each dimer contains two coenzyme molecules. The small domain also provides the mobility whereby an "oscillatory rotor" mechanism of action has been proposed for the reversible transamination reaction between amino acids and keto acids.

The systematic study of c-AAT using spectroscopic and crystallographic techniques has led to a relatively clear picture of the 3-dimensional structure of the catalytic-cleft during turnover. However, little is known about the 3-dimensional structure (no crystallographic data) of the catalytic-cleft of PM(PN)P-oxidase. Thus, we designed cleft-depth probes to use as substrates for PM(PN)P-oxidase and as coenzyme for apo-AAT in order to learn more about the flexibility of these different catalytically involved binding sites.

MATERIALS AND METHODS

Syntheses and structures of PMP analogs. Pyridoxamine 5'-phosphate analogs having the two general structures 1-(5'-phospho-4'-pyridoxyl)-1,*n*-diaminoalkane[4] (single-headed) and 1,*n*-bis(5'-phospho-4'-pyridoxyl)-1,*n*-diaminoalkane (double-headed) were synthesized by a slight modification of the method reported by Collier and Kohlhaw (*9*) using sodium borohydride reduction of Schiff's bases formed by the interaction of PLP and one or both ends of 1,*n*-diaminoalkanes where n = 2, 4, 6, 8, or 12 carbons (Fig. 1). To form the single-headed analogs, 1 mmol of PLP was dissolved in 20 ml of methanol : water (1 : 1) containing 1.1 mmol of the respective 1,*n*-diaminoalkane. To form the double-headed analogs, 2.2

[4] Chemical abstract nomenclature for the single-headed series is 4-[[(*n*-aminoalkyl)amino]methyl]-5-hydroxy-6-methyl-3-pyridine methanol, α-dihydrogen phosphate where the corresponding (*n*-aminoalkyl) groups are 2-aminoethyl, 4-aminobutyl, 6-aminohexyl, 8-aminooctyl, and 12-aminododecyl.

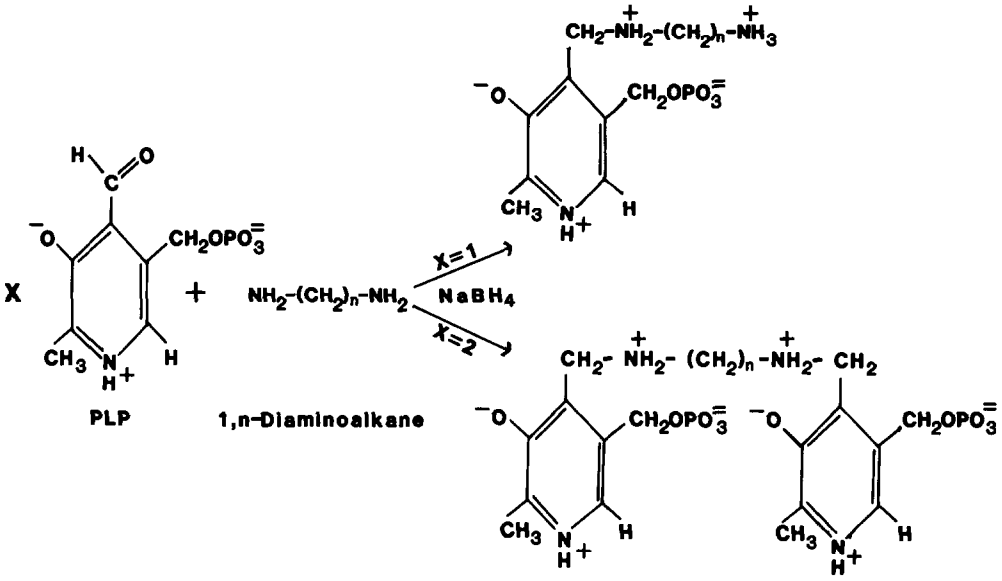

FIG. 1. Reaction of PLP with various 1,*n*-diaminoalkanes to form single- and double-headed analogs of PMP. Compounds were synthesized by borohydride reduction of Schiff's bases formed in alkaline methanol : water (1 : 1) solutions (pH 8) by the interaction of 1 mmol or >2 mmol of PLP with 1 mmol of the various 1, *n*-diaminoalkanes where n = 2, 4, 6, 8, or 12 carbons.

mmol of PLP was dissolved in 20 ml of methanol : water (1 : 1) containing 1 mmol of the respective 1,*n*-diaminoalkane. The solutions were made alkaline (pH ≥ 8) by dropwise addition of 1 M LiOH. After 1 h at room temperature or 12 h at 4°C in the dark, a molar equivalent plus 10% excess of sodium borohydride (1.1 mmol for single-headed and 2.4 mmol for double-headed) was added. Upon completion of the reduction (disappearance of yellow color) the solutions were acidified with formic acid to destroy excess borohydride, then neutralized to pH 7 with LiOH and flash evaporated at low temperature. Residues were taken to dryness with methanol to volatilize borate. The material was applied to a cation-exchange column, Amberlite CG-50 (2.5 × 15 cm, H^+ form), and eluted in the dark with water to remove unreacted PLP and any side product PNP. The amine compounds were then eluted by displacement with dilute ammonium hydroxide and concentrated by flash evaporation at low temperature. Separation of the single- and double-headed analogs was accomplished using an anion-exchange column, DEAE–Sephadex (2.5 × 20 cm), equilibrated with 0.02 M ammonium bicarbonate and eluted with a linear gradient to 1.0 M ammonium bicarbonate. The purity of the compounds was monitored by TLC using Kodak cellulose chromagram sheets and two different solvent systems: (I) 1-butanol : acetic acid : water (3 : 2 : 2) and (II) methylethylketone : ammonia : ethanol : water (3 : 1 : 1 : 1) with visualization by Gibb's reagent (2,6-dichloroquinone-4-chloroimide) or ninhydrin spray and by HPLC using a Brownlee Aquapore AX-300 anion-exchange column (4.6 mm × 25

cm with guard) and isocratic elution with 40 mM ammonium bicarbonate at a flow rate of 0.8 ml/min with detection at 315 nm on a Waters HPLC system.[5]

To further characterize the new analogs, absorption spectra from 230 to 400 nm were obtained in 0.1 M HCl, in 0.1 M potassium phosphate buffer (pH 7.0), and in 0.1 M NaOH using a Cary 14 spectrophotometer interfaced to a North Star Horizon computer with hardware and software provided by On-Line Instrument Systems (Jefferson, GA). Fluorescence measurements both in 0.1 M Hepes buffer (pH 7.5) and in 0.1 M Tris buffer (pH 8.2) were obtained using a Perkin–Elmer MPF-44B fluorescence spectrophotometer. Proton NMR spectra of compounds in acidified deuterium oxide were recorded using a Varian EM360L NMR spectrophotometer and further substantiated which compounds were the single- and double-headed analogs.

Kinetic studies using PMP analogs as substrate. Concentrations of the purified PMP analogs were determined in 0.1 M potassium phosphate buffer (pH 7.0) by using the 325-nm molar extinction coefficient of PMP, 8300 M^{-1} cm^{-1} (*10*), for the single-headed analogs and a value of 16,600 M^{-1} cm^{-1} for the double-headed analogs. Incubations were performed with varying amounts of each of the analogs (2 μM to 2 mM) in 0.2 M Tris buffer (pH 8.0) containing 0.5 μM FMN and 10 μg/ml of BSA at 37°C under air-saturation using 40 units[6] of PM(PN)P-oxidase purified from rabbit liver by the modified method of Kazarinoff and McCormick (*1, 11*). The initial velocities were monitored by the determination of PLP formation after 30 min as the phenylhydrazone derivative (*12*). Measurements were also made at 27 and 47°C with air-saturation and at 37°C with oxygen-saturation for PMP and four selected single- and double-headed analogs where $n = 2$ and 8.

Kinetic and binding studies using PMP analogs as coenzyme. Commercially available L-aspartate aminotransferase (AAT, Sigma Chemical Co., St. Louis, MO) was converted to phosphate-free apoenzyme using the published cysteine sulfinate procedure (*13*). The enzyme was shown to be 30 to 40% AAT by specific activity measurements compared to literature values of 300 μmol NADH oxidized min^{-1} mg^{-1} (*14*). Of that AAT, 97% was determined to be apo by reconstituting with PLP and assaying spectrophotometrically using the coupled malate dehydrogenase–NADH assay (*15*). Reconstitution of holoenzyme from the apoenzyme with PMP and with each of the 10 PMP analogs was also monitored by this method. All incubations contained 5 μg of active apo-AAT [0.5 μM based on a 280-nm extinction coefficient of 130,000 M^{-1} cm^{-1} and M_r of 93,000 (*7*), and/or Lowry protein assay using BSA as standard and percentage active AAT], 0.2 mM NADH, 100 mM L-aspartate, 4 units of malate dehydrogenase, and a given amount of coenzyme or analog in 50 mM sodium phosphate buffer, pH 7.5. The solutions were preincubated at room temperature for 27 min (in the dark) then for 3 min at assay temperature, 30°C, before the reaction was initiated by addition of α-ketoglutarate (6.0 mM final concentration). Activity of reconstituted apo-AAT with

[5] The Waters HPLC systems consisted of a 6000A and M45 solvent delivery system with 720 system controller, a 450 variable-wavelength detector, a U6K injector, and a M730 data module.

[6] One unit of activity is defined as one nanomole of PLP released per hour under assay conditions (*1*).

varying concentrations of PMP (2 to 200 μM) was also monitored in the presence of selected analogs at concentrations of 1, 2, and 5 times that of PMP.

Binding of PMP and the four selected analogs (single and double-headed analogs where $n = 2$ and 8) was also monitored by spectrofluorometric procedures. To conserve enzyme, different concentrations of the analogs ranging from 10 nM to 1 mM were added to a constant amount of active apo-AAT and allowed to equilibrate for 1 h at room temperature in the dark. The fluorescence of these solutions was compared to the fluorescence observed for the same concentration of unbound analog. The excitation slit width was very narrow and exposure of solutions to radiation was only a few seconds to minimize photodecomposition. These experiments were performed using buffers at two different pH values (0.1 M Hepes, pH 7.5, and 0.1 M Tris, pH 8.2).

RESULTS

PMP analogs. Single-headed analogs of PMP were synthesized by sodium borohydride reduction of Schiff's bases formed by the 1 : 1 interaction of PLP and the respective 1,*n*-diaminoalkanes, whereas the double-headed analogs were isolated from reactions containing >2 : 1 ratios of the same reactants (see Fig. 1). Isolation and purification was monitored by TLC and HPLC techniques with mobility values listed in Table 1. The detection of trace impurities of the single- and/or double-headed analogs in each reaction mixture was best monitored using either TLC and solvent II or by HLPC. These basic systems led to greater R_f values and shorter retention times for the single-headed analogs, thus making them easy to distinguish from the slower moving and more retained double-headed analogs. Changing the TLC solvent to acidic conditions allowed for a double check on the purity of the isolated analog.

Spectroscopic studies in the uv/vis region revealed that the analogs had similar absorption maxima at 295 nm in acid solution, 251 nm and 325 nm at neutral pH, and 243 and 308 nm in basic solution within ± 1 nm deviation and a tight isobestic point at 266 nm upon changing pH. In addition the relative intensities of A_{325}/A_{251} at neutral and A_{308}/A_{243} at basic conditions agreed within ± 0.06 and ± 0.03, respectively, of each other. Thus the 325-nm molar extinction coefficients of 8300 M^{-1} cm^{-1} and 16,600 M^{-1} cm^{-1} at pH 7.0 were employed for the single- and double-headed analogs throughout the study to determine concentration levels. PMP excited at 330 nm fluoresces at 397 nm. The four selected analogs also exhibit these same characteristics. Table 1 lists the relative intensities of the four selected analogs in two different buffer systems, 0.1 M Hepes (pH 7.5) and 0.1 M Tris (pH 8.2).

Proton NMR of the compounds in acidified deuterium oxide revealed that the 2-CH_3 and 4′-CH_2 (4′-COH for PLP) proton chemical shifts, δ (ppm), observed for the pyridoxyl groups were similar to those reported by Korytnyk and Ahrens (*16*) for PMP (2.75 and 4.49) and unlike those for PNP (2.65 and 5.05) or PLP (2.43 and 10.5 or 6.53 if hydrated). In fact, binding to the amine function had no significant effect on chemical shift positions of any of the pyridoxyl protons from that ex-

TABLE 1

PROPERTIES OF SINGLE- AND DOUBLE-HEADED ANALOGS OF PMP

Substance	R_f[a]	R_f[b]	RT[c] (min)	Relative fluorescence[d] pH 7.5	Relative fluorescence[d] pH 8.2
PMP	0.32	0.18	6.45	1.00	1.00
PNP	0.43	0.10	9.30		
PLP	0.60	0.26			
Single-headed					
$n = 2$	0.26	0.13	4.9	1.0	0.47
$n = 4$	0.50	0.13	6.2		
$n = 6$	0.43	0.18	6.7		
$n = 8$	0.65	0.33	6.8	1.5	1.55
$n = 12$	0.80	0.76	7.0		
Double-headed					
$n = 2$	0.20	0.03	6.6	1.26	0.34
$n = 4$	0.25	0.03	14.5		
$n = 6$	0.24	0.05	14.0		
$n = 8$	0.56	0.03	14.6	2.69	1.02
$n = 12$	0.72	0.10	13.0		

[a] TLC on cellulose sheets using 1-butanol : acetic acid : water (3 : 2 : 2).

[b] TLC on cellulose sheets using methylethylketone : ethanol : ammonia : water (3 : 1 : 1 : 1).

[c] HPLC retention times observed after separation on a Brownlee Aquapore A-300 anion-exchange column (4.6 mm × 25 cm with corresponding guard) eluted isocratically with 40 mM ammonium bicarbonate at a flow rate of 0.8 ml/min and peak detection at 315 nm.

[d] Relative fluorescence intensities in 0.1 M Hepes buffer (pH 7.5) and in 0.1 M Tris buffer (pH 8.2) with excitation at 330 nm and emission at 397 nm.

pected for PMP since δ (ppm) positions were found to be 2.75 for singlet 2-CH_3, 4.53 for singlet 4'-CH_2, 5.02 and 5.15 for doublet 5'-CH_2, and 8.12 for singlet C_6-H. Comparison of the integrated areas for protons associated with amine methylene-chain carbons to those for pyridoxyl protons further substantiated which compounds were the single- and double-headed analogs.

Analogs as substrates. To learn more about the character of the catalytic cleft of PM(PN)P-oxidase, kinetic studies at 37°C under conditions of air saturation in 0.2 M Tris buffer (pH 8.0) were initiated using all the newly synthesized analogs. All act as substrates for PM(PN)P-oxidase and release PLP as product.

The single-headed analogs exhibit linear Lineweaver–Burk plots and have K_m values ranging from 3 K_m to K_m for PMP in a curvilinear relationship with chain length from $n = 2$ to 12 (Table 2). The short-chain analogs ($n = 2, 4$) exhibit V_m values greater than PMP, whereas the long-chain analogs ($n = 6, 8, 12$) exhibit V_m values equal to or less than PMP (see Fig. 2). Increasing the temperature increases the V_m values for the compounds in the same order and influences the K_m values by less than an order of magnitude. Increasing the oxygen supply also increases the rate of product released, since reaction with amine substrates follows a ter-

TABLE 2

KINETIC CONSTANTS FOR OXIDATION OF PMP AND ITS ANALOGS BY PM(PN)P-OXIDASE[a]

Substrate	K_m (μM)	V_m (nmol PLP/h)	Ratio V_m (analog/PMP)
PMP[b]	7.7	54	1.0
Single-headed[b]			
$n = 2$	26	142	2.6
$n = 4$	17	140	2.6
$n = 6$	15	59	1.1
$n = 8$	8.2	49	0.9
$n = 12$	7.9	38	0.7
Double-headed[c]			
$n = 2$	124 (16)	64 (31)	1.2 (0.6)
$n = 4$	21 (4.4)	77 (56)	1.4 (1.0)
$n = 6$	52 (8.1)	56 (24)	1.0 (0.4)
$n = 8$	9.1	32	0.6
$n = 12$	2.2	24	0.4

[a] Incubations which contained 40 units of active enzyme, 2 μM to 2 mM substrate, 0.5 μM FMN, and BSA (10 μg/ml) in 0.2 M Tris buffer (pH 8.0) were assayed at 37°C in a shaking water bath under dim light for 30 min before the PLP released was assayed as the phenylhydrazone derivative.

[b] Calculated from Lineweaver–Burk plots of kinetic data using substrate concentrations from 2 μM to 2.0 mM. Data represent the means of three or more determinations.

[c] Calculated from linear portions of Lineweaver–Burk plots at substrate concentrations >50 μM with values in parentheses for those analogs that exhibit a biphasic nature at <50 μM.

nary complex mechanism (*17*) involving molecular oxygen as the second substrate.

Lineweaver–Burk plots for the double-headed analogs were biphasic for the short-chain analogs ($n = 2, 4, 6$) and linear for the long-chain ones ($n = 8, 12$) over the concentration range from 1 to 500 μM (see Fig. 2). Values for K_m and V_m are listed in Table 2. Since one of the initial products is the single-headed analog which is still a substrate for the enzyme, the reaction continues to consume O_2. To test the possibility that the biphasic nature might reflect depletion of molecular oxygen at this usual temperature (37°C), the kinetic study was repeated for the double-headed analog $n = 2$ at lower temperature, 27°C, with air saturation and again at 37°C under an oxygen atmosphere. The Lineweaver–Burk plots were still biphasic under both these conditions. At substrate concentrations >50 μM, the apparent K_m values for all double-headed analogs indicate that they are poorer substrates than PMP for the PM(PN)P-oxidase.

Analogs as coenzymes. Aspartate aminotransferase uses the 4′-aldehyde group of PLP and the 4′-aminomethyl group of PMP during productive interactions with amino and keto acids. However, the 5′-phospho-4′-pyridoxyl moiety of other PMP analogs such as phosphopyridoxyl-trifluoroethylamine, -trifluoromethyl methionine, and -α-trifluoromethyl-DL-alanine (*18, 19*) have been shown to bind to the apo-AAT blocking coenzyme · substrate complex turnover. Even larger phos-

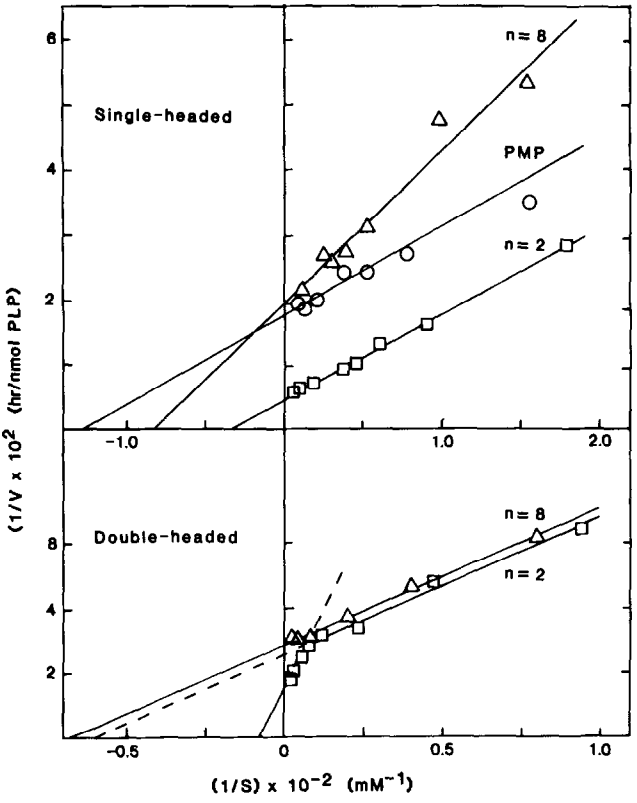

FIG. 2. Representative Lineweaver–Burk plots of the kinetic studies using PMP-analogs as substrates for PM(PN)P-oxidase in 0.2 M Tris buffer, pH 8.0, as described in text. PMP (○); analogs with n = 8 (△); analogs with n = 2 (□).

phopyridoxylated oligosaccharides (*20*) can bind to apo-AAT and become partially masked from α-mannosidase cleavage by the coenzyme binding cleft of AAT. To ascertain which of our analogs would fit into the cleft and affect activity, kinetic studies were initiated. All 10 analogs do not appear to activate apo-AAT in the coupled malate dehydrogenase–NADH assay at analog concentrations as great as 1 mM. At coenzyme concentrations greater than 1 mM, this method becomes prohibitive to monitor since the absorbance of PMP and its analogs contributes significantly to the assay wavelength, 340 nm. One can increase the sensitivity of the assay by using 360 nm, but this does not allow for measurement of any coenzyme activity. Four analogs that reflect the general characteristics of the single- and double-headed series (n = 2 and 8) were used to check for binding competitive with PMP to apo-AAT. The apo-AAT was reconstituted with varying concentrations of PMP (2 to 500 μM) in the presence of each analog at concentrations 1, 2, and 5 times that of PMP (method limited to total B_6 concentrations of 1 mM). The activity of the reconstituted enzyme at these levels was no different from that observed for PMP alone, thus none of the analogs seem to provide competitive inhibition with PMP.

Fluorescence measurements of PMP (excitation around 330 nm and emission around 400 nm) have been shown to decrease upon binding to apo-AAT at pH values between 7.4 and 8.5 (*19–24*). Churchich and Farrelly (*22*) and Arrio-Dupont (*23*) have used fluorescence quenching of PMP upon binding to apo-AAT to evaluate association constants of the binding strength. Relimpio *et al.* (*19*) have shown that PMP analogs act as competitive inhibitors, bind to the active site of apo-AAT, reduce its activity, and exhibit fluorescence quenching of the pyridoxyl derivatives. Seeing no evidence for significant binding by kinetics measurements with the present analogs, the more sensitive fluorescence property of these analogs was examined upon addition of apo-AAT. To conserve enzyme, solutions contained a constant amount of active apo-enzyme and varying amounts of PMP or one of the four selected analogs. At very low analog concentrations (small analog to enzyme ratios) the fluorescence should be quenched if binding occurs, and as the concentration increases the amount of free analog should increase as was found to be the case in 0.1 M Hepes buffer, pH 7.5 (Fig. 3A) and in 0.1 M Tris buffer, pH 8.2 (Fig. 3B). Scatchard plots of the binding data gave curves which indicate more than one binding site similar to those reported by Arrio-Dupont (*23*). This is expected since apo-AAT is known to be a dimer with two PMP binding sites. For comparison, the PMP data at pH 8.2 was extrapolated to $r = 0$ (where r = average moles PMP bound per mole of active dimeric apo-enzyme) to evaluate the intrinsic dissociation constant. Our data indicate a value of 175 nM which is in fair agreement with the value of 63 nM reported by Churchich and Farrelly (*22*) and the values of 20 and 200 nM reported by Arrio-Dupont (*23*). In order to access the relative binding constants and the degree of cooperativity exhibited by these analogs as coenzymes for apo-AAT the fluorescence data was evaluated using the well-established Hill equation. The dissociation constants from this method (Table 3) reflect the differences in binding at pH 7.5 and 8.2, which these analogs express toward apo-AAT.

DISCUSSION

In designing the cleft-depth probes, a major consideration was that they must contain the 5′-phospho-4′-pyridoxyl moiety known to bind to both the PM(PN)P-oxidase and apo-AAT. The second consideration was that two such rings attached through a methylene chain of varying lengths (thus double-headed analogs) might serve to bridge the gap between the two active sites of apo-AAT. Some measured binding property of these compounds to both enzymes might then correlate with known crystallographic data for AAT. In doing so, this structural information regarding AAT might infer some further information regarding the nature of the catalytic cleft of the oxidase. For comparisons it was necessary to synthesize the corresponding single-headed analogs as well.

The principle features of PM(PN)P-oxidase relating to the catalytic cleft reveal that FMN is bound by ring association with a tryptophanyl residue of the apoenzyme (*25*), by hydrogen binding of flavin side-chain hydroxyls, and by cationic interaction of the dianionic 5′-phosphate terminus (*1, 26*). This binding orients the

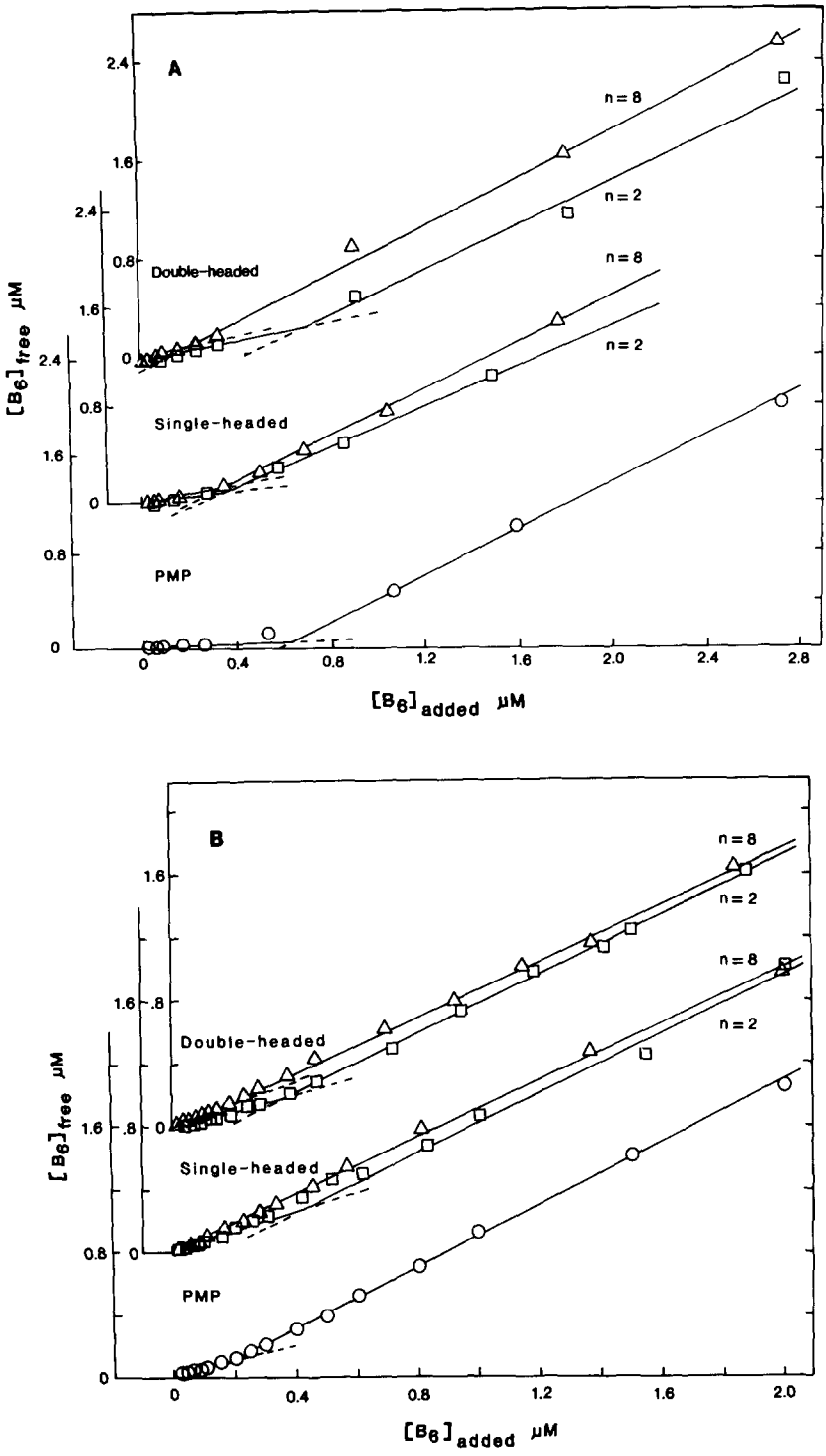

FIG. 3. Binding curves of PMP-analogs with apo-AAT in 0.1 M Hepes buffer, pH 7.5 (A) and in 0.1 M Tris buffer, pH 8.2 (B). The protein concentration was kept constant at 0.33 μM (pH 7.5) and 1.9 μM (pH 8.2). The amount of free analog was calculated from fluorescence data as described in the text. PMP (○); analogs with $n = 8$ (△); analogs with $n = 2$ (□).

TABLE 3

BINDING CONSTANTS AND MEASURED COOPERATIVITY FOR PMP AND ITS ANALOGS WITH apo-AAT USING THE HILL EQUATION[a]

Ligand	K_d (M)		n_H	
	pH 7.5	pH 8.2	pH 7.5	pH 8.2
PMP	8.3×10^{-8}	3.5×10^{-8}	0.84	1.14
Single-headed				
$n = 2$	2.5×10^{-5}	2.9×10^{-3}	0.55	0.42
$n = 8$	1.4×10^{-5}	1.8×10^{-1}	0.62	0.20
Double-headed				
$n = 2$	5.7×10^{-6}	4.5×10^{-3}	0.54	0.32
$n = 8$	1.2×10^{-7}	9.7×10^{-3}	0.98	0.41

[a] Values were calculated using the Hill equation with a value of $n = 1$ in the $r/(n - r)$ term.

FMN with its dimethylbenzoid edge toward solvent (*27*). The holoenzyme binds the dianionic 5′-phosphate of substrates and product through ionic interaction with a guanidinium function of an arginyl residue (*28*). Catalysis involves the base function of a histidyl residue (*29*) in hydrogen abstraction and an arginyl residue near the N(1)–O(2) region of FMN, which stabilizes the dihydroflavin anion (*27, 30*). The oxidase action proceeds through a ternary complex (sequential) mechanism with PMP from which hydrogen is abstracted more easily than with PNP as substrate; however, a release of product PLP from the imine–enzyme complex is slower than reaction of O_2 with the reduced flavoprotein (*17*).

The oxidase had been shown to catalyze the oxidation of a variety of primary and secondary amines as long as they possessed the 5′-phospho-4′-pyridoxyl moiety (*1, 5, 6*). This is extended by the present findings with the new analogs which release PLP and act as substrates for the enzyme. The observations that the long-chain substrates ($n = 12$) have lower K_m and V_m values than PMP and that the single-headed analogs show a curvilinear relationship between K_m values and chain length may reflect in part an increase in hydrophobicity with increase in chain length and thus poorer turnover. Since putrescine (1,4-diaminobutane) has been shown to be a "nonessential activator" of PMP-oxidase purified from baker's yeast (*31*), it is not surprising that increased activity for the short-chain analogs was noted. The biphasic nature of the short-chain, double-headed analogs may reflect the interference of product release or further substrate binding due to the close proximity of the charge on the product amine. Comparing such secondary amines as *N*-(5′-phospho-4′-pyridoxyl)-*N*′-(1-napthyl)ethylenediamine and the more highly charged *N*-(5′-phospho-4′-pyridoxyl)-*N*′-(1-napthyl-5-sulfonate)ethylenediamine, indicates that the added charge due to the sulfonate group on the leaving group (amine product) from the latter lowers the relative substrate activity by 160/76 or 2.1-fold (*6*), whereas changing the chain length of otherwise identical secondary amines only lowered the relative activity by 102/89 or 1.1-fold. The short-chain single- and double-headed substrates (>50 μM) have high K_m

and high V_m values relative to substrate PMP. Repulsive forces of an ionic nature [$-(CH_2)_n-NH_3^+$ for single-headed and 5′-phosphopyridoxyl for double-headed] might account for the increases observed in both K_m and V_m values. That is, those factors that make binding unfavorable might also make the rate-limiting step (release of PLP from imine–enzyme complex) faster. Hence, the binding step (through distortion, strain, position of catalytic groups, etc.) may contribute to catalysis by lowering the overall energy of activation of the rate-limiting step.

The four-selected analogs of PMP (where $n = 2$ and 8) do not behave as coenzymes for apo-AAT or act strongly as competitive inhibitors of PMP binding as measured in the malate dehydrogenase–NADH assay. They do tend to bind to the enzyme as indicated by fluorescence quenching. From the apparent dissociation constants, all the analogs bind more tightly at pH 7.5 (near physiological pH) than at pH 8.2, which may reflect the influence of localized charges. In general, the lower binding constants for these analogs further supports the kinetic measurements in that they all exhibit weaker binding than natural coenzyme, PMP.

The generally weak binding of the present analogs of PMP to apo-AAT does not permit correlation to the crystallographic detail now available for this enzyme. However, interaction of the analogs with PM(PN)P-oxidase extends visualization of the active-site cleft for the oxidase. Though narrowly specific for the 5′-phospho-4′-pyridoxyl moiety, the cleft must be rather shallow and significantly solvent exposed to accommodate the diverse geometries and charges of structures adherent to the secondary amine positions of these analogs. Moreover, this active site is necessarily at the locus of the catalytically involved FMN which itself is exposed to solvent along the benzenoid edge (*27*).

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