Kinetic Analysis and Chemical Modification of Vitamin B₆ Phosphatase from Human Erythrocytes*

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The specificity and active site properties of vitamin B₆ phosphatase purified from human erythrocytes were studied by kinetic analyses with vitamin B₆ compounds and derivatives and chemical modification with group-specific reagents. The kinetic constants for pyridoxal phosphate (PLP), 4-pyridoxic acid phosphate, pyridoxine phosphate, and pyridoxamine phosphate were determined from pH 5 to 9. The values of V_{max}/K_{m} and pK_{m} were highest for PLP and 4-pyridoxic acid phosphate and lowest for pyridoxamine phosphate. V_{max}/K_{m} and pK_{m} for the four substrates were between pH 6 and 8. Ionizable groups with pK_{m} values about 6 and 8 affected substrate binding to the enzyme. V_{max} values for all the substrates gradually decreased with increasing pH. The enzyme also catalyzed the dephosphorylation of 4'-secondary amine derivatives of vitamin B₆ phosphate. The phosphatase had greatest catalytic efficiency with substrates that contained a negatively charged group on the 4'-position of the pyridine ring. It is concluded that there are one or two positively charged groups at the active site of the enzyme that interact with the substrate's phosphate ester and 4'-substituent. The phosphatase was inactivated by phenylglyoxal, and PLP protected the enzyme against this inactivation. Phenylglyoxal did not modify Lys or Cys residues or an α-amino group since the enzyme's NH₂ terminus is blocked, and it did not affect the quaternary structure of the phosphatase. The enzyme was inactivated by the incorporation of 1 mol of phenylglyoxal/subunit. Diethyldipyrocarbonate inactivated the enzyme by reacting with a group with a pK_{d} of 6.7, and pyridoxine phosphate protected the enzyme against this inactivation. These data suggest that Arg and His residues are at or near the active site and may play roles in substrate binding and/or catalysis.

Dietary pyridoxine (PN) and pyridoxamine (PM) are phosphorylated by pyridoxal (PL) kinase (EC 2.7.1.35) and then converted to pyridoxal 5'-phosphate (PLP) by pyridoxamine 5'-phosphate (PMP) oxidase (EC 1.4.3.5). Most PLP is bound to proteins or hydrolyzed to pyridoxal by phosphatases. Although the kinase activity occurs in all mammalian tissues, the oxidase activity is very low or absent in many tissues. Since human erythrocytes have the kinase, oxidase, and phosphatase activities, they readily synthesize PLP and PL from PN (1). Therefore, it has been proposed that erythrocytes may play a role in providing PL to tissues containing low oxidase activity.

An inverse relationship between phosphatase activities and in vivo PLP levels has been found in some disease states. Subjects with alcoholism or liver disease often have low concentrations of plasma PLP which are inversely related to phosphatase activities (2-6). Plasma alkaline phosphatase (EC 3.1.3.1) activities are high and PLP concentrations are low in Paget's disease (2) and hypophosphatemic rickets (7). Conversely, the alkaline phosphatase activities are low and PLP concentrations are high in plasma of patients with hypophosphatasia (5). The PL kinase and PMP oxidase activities are normal in these diseases. These observations suggest that the activity of alkaline phosphatase may regulate the concentration of PLP in the circulation; however, since alkaline phosphatases are on the external surface of plasma membranes of tissues, they probably contribute little to the intracellular catabolism of vitamin B₆. Therefore, soluble intracellular phosphatases may be of importance in regulating intracellular PLP concentrations.

Despite the apparent importance of phosphatase activities in maintaining PLP homeostasis, little is known about phosphatases which hydrolyze phosphorylated B₆. PLP and PMP are hydrolyzed by alkaline phosphatases which have broad substrate specificity for phosphomonoesters (4, 8). An alkaline phosphatase was found in human brain that cleaves PLP better than any of the other nine substrates tested (9). Non-specific acid phosphatases (EC 3.1.3.2) that hydrolyze PLP have been found in liver and kidney (10, 11). An acid phosphatase that may be specific for phosphorylated B₆ compounds was partially purified from the nuclear fraction of mouse liver (12); however, this enzyme was not characterized.

We have identified and purified a vitamin B₆-specific phosphatase from human erythrocytes (13). It is a dimer with a molecular mass of 64 kDa. The phosphatase requires Mg²⁺ and hydrolyzes PLP, pyridoxine 5'-phosphate (PNP), PMP, and 4-pyridoxic acid phosphate (4-PAP). It is most active near physiologic pH and has a K_{m} value of approximately 1 μM for PLP, which is at least 10-fold lower than that reported for PLP with other phosphatases. We are interested in characterizing the role of this enzyme in the metabolism of vitamin B₆ and in comparing its catalytic properties with those of other vitamin B₆-metabolizing enzymes and with other phosphatases. In the present study, we determined the kinetics of the purified enzyme with vitamin B₆ compounds and derivatives with a variety of substituents in the 4'-position of the pyridine ring to evaluate further the substrate specificity and used chemical modification with group-specific reagents to obtain information.
about amino acid residues that may be at the active site of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—**PLP, PMP, diethylylcarbamoyl (DEPC), phenylglyoxal (PG), tetranitromethane (TNM), and reactive yellow 86-agarose were obtained from Sigma. DEAE-Sepharose CL-6B and Sepharose 4-B 200 High Resolution were purchased from Pharmacia LKB Biotechnology Inc., and hydroxylapatite high resolution was from Behring Diagnostics. 4-PAP was synthesized according to the procedure of Wada and Snel (14). Sodium [3H]borohydride (specific activity 548 Ci/mol) was purchased from DuPont-New England Nuclear. [3H]PNP and [3H]PLP were purified by chromatography on AG 1-X2 acetate (16). The purity of the [3H]PNP and [3H]PLP was determined by high performance liquid chromatography (HPLC) as described previously (17). [7-14C]Phenylglyoxal (specific activity 23.1 Ci/mol) was purchased from Amersham Corp.

N-([5'-Phospho-4'-pyridoxyl]glycine (P-Pxy-Gly), N-([5'-phospho-4'-pyridoxyl]ethanolamine (P-Pxy-Et), and N-([5'-phospho-4'-pyridoxyl]phenylalanine (P-Pxy-Phe) were synthesized by incubating 180 µmol of glycine, ethanolamine, or phenylalanine, respectively, with 80 µmol of PLP in 0.5 ml of 0.5% KOH for 10 min at room temperature. The mixture was then incubated with 160 µmol of KBH, on ice for 15 min. The unreacted KBH, was destroyed by adding five drops of concentrated formic acid. Each derivative was found to be about 99% pure by L3H1PLP were purified by chromatography on AG 1-X2 acetate (16). The purity of the [3H]PNP and [3H]PLP was determined by high performance liquid chromatography (HPLC) as described previously (17). [7-14C]Phenylglyoxal (specific activity 23.1 Ci/mol) was purchased from Amersham Corp.

**Enzyme**—The vitamin B₆ phosphatase was purified using a modification of the procedure described previously (13). The changes included starting with two units of outdated packed red cells supplied by the American Red Cross (Louisville, KY) and scaling up the volumes of buffers and columns. A second DEAE-Sepharose column was added, the order of chromatography on the reactive yellow-86 and hydroxylapatite columns was reversed, and the reactive yellow-86 column was eluted with buffer containing 10 and 20 mM P, rather than 100 and 200 mM NaCl, respectively. Prior to all modification studies, the phosphatase was dialyzed in a Pierce Chemical Co. microdialyzer against four changes of buffer A (2 mM MgCl₂, 0.002% Triton X-100, and 10 mM triethanolamine-HCl, pH 7.0) for 18-24 h to remove P, and dithiothreitol.

Protein concentrations were determined by the Coomassie Brilliant Blue assay using the reagent and micro procedure from Pierce Chemical Co. Bovine serum albumin was used as a standard.

**Enzyme Assays and Kinetic Analysis**—Vitamin B₆ phosphatase activity was routinely assayed by measuring the production of [3H]HPN from [3H]PNP as previously described (13). The 200-µl assay mixture contained 40-60 µM [3H]PNP, 4 mM MgCl₂, 20 µg of bovine serum albumin, and 0.001% Triton X-100 in 37 mM triethanolamine-HCl, pH 7.4. The release of free phosphate from putative substrates was determined colorimetrically as the molybdate complex with malachite green (13, 14) and the reaction mixture was read at 620 nm.

**RESULTS**

The vitamin B₆-specific phosphatase was purified 170,000-fold from human erythrocytes (Table I). The purified phosphatase migrated as a single band on SDS-polyacrylamide (12%) gel electrophoresis and was stable for at least 6 months when stored at concentrations of 0.3 mg/ml or greater at 4°C in the presence of 30 mM phosphate.
Kinetic Studies—The kinetics of hydrolysis of several important substrates by the highly purified phosphatase were studied in detail. The effect of pH on $K_m$ and $V_{max}$ values of the enzyme for PLP, PNP, and 4-PAP was determined from pH 5.0 to 9.0 at 0.5-unit increments, and for PMP from pH 6.0 to 8.5 (Fig. 1). The phosphatase lost no activity when incubated for 60 min at these pH values. The rate of hydrolysis of PMP was low; therefore, the data for PMP were not as reliable as for the other $B_6$ substrates and are not shown. PMP had the lowest $V_{max}$ and highest $K_m$ and PLP and 4-PAP were the best substrates at all pH values studied. The effect of pH on the kinetic parameters of all four substrates was qualitatively the same. pH did not affect the $V_{max}$ as much as $K_m$. In general, $V_{max}$ decreased with increasing pH. The lowest $K_m$ and highest $V_{max}/K_m$ values for all the substrates were obtained at pH 7. The $K_m$ and $V_{max}/K_m$ profiles were bell-shaped indicating that ionization of groups of the enzyme and/or substrate with $pK_a$ values around 5.5-6 and 8 play a role in substrate binding.

Since the group in the 4'-position of the natural substrates affected the $K_m$ and $V_{max}$ values, the enzyme active site environment was probed further with PMP derivatives containing 4'-secondary amines of different sizes and charges. The kinetic parameters for these derivatives were measured at pH 7.5 and compared with those for PLP, PNP, PAP, and PMP (Table II). Despite the different chemical groups in the 4'-position, these derivatives were all relatively good substrates. Among these derivatives, the enzyme exhibited the highest $V_{max}$ and catalytic efficiency for P-Pxy-Phe, followed by P-Pxy-Gly, P-Pxy-Et, and P-Pxy-Benz. These were hydrolyzed at a similar catalytic efficiency by the enzyme. The compounds were also studied as potential inhibitors. Fifty $\mu$M PNP, P-Pxy-Gly, P-Pxy-Et, and P-Pxy-Phe inhibited the phosphatase-catalyzed hydrolysis of 50 $\mu$M $[^{3}H]P$NP by 45, 32, 12, and 51%, respectively. The relative inhibition of the enzyme by these compounds was consistent with their $K_m$ values, which implies that these PLP derivatives bind to the same active site as the physiological substrates do. It is interesting to note that P-Pxy-Phe had a much higher catalytic efficiency than PNP, although a bulky Phe group was in the 4'-position. It appears that a negative charge on the group in the 4'-position contributes to binding since the enzyme hydrolyzed 4-PAP, P-Pxy-Phe, and P-Pxy-Gly, all of which contain a net negative charge on the 4'-substituent. The group in the 4'-position did not interfere with binding since similar efficiencies were obtained with the pair P-Pxy-Et and P-Pxy-Benz and P-Pxy-Phe bound better than P-Pxy-Gly. Phenylglyoxal Modification of Vitamin $B_6$ Phosphatase—The substrate's negatively charged phosphate group, which has a $pK_a$ value of 5.7-6.1 (21), probably contributes to the binding of substrate to the phosphatase since $V_{max}/K_m$ decreases with decreasing pH below 6. One or more positively charged groups on the enzyme may interact with the anionic phosphate group and a negatively charged group in the 4'-position of the substrates. Arginine residues frequently function as anion-binding sites. PG reacts specifically with Arg residues and was used to determine whether any Arg residues play an important role in either substrate binding or catalysis. When excess PG was incubated with the purified phosphatase, the inactivation of the enzyme was dependent on time and PG concentration and followed pseudo-first-order kinetics (Fig. 2). The rate of inactivation was greater at pH 9 than at pH 7. The presence of either 0.3 mM PLP or 30 mM P$_i$ protected the enzyme against inacti-
Diethylpyrocarbonate Modification of Vitamin B<sub>6</sub> Phosphatase—A histidine residue has been shown to play an important role in vitamin B<sub>6</sub> phosphatase structure and catalysis. We attempted to identify the B<sub>6</sub> phosphatase-modified amino acid residues by isolating and sequencing radioactively labeled tryptic peptides; however, the [14C]PG label was not stable enough to survive the trypsin digestion and HPLC separation.

Diethylpyrocarbonate Modification of Vitamin B<sub>6</sub> Phosphatase—A histidine residue has been shown to play an important role in several acid phosphatases (22–25). The effect of DEPC, a reagent which reacts preferentially with His residues at neutral pH, on vitamin B<sub>6</sub> phosphatase was studied. The inactivation of the enzyme by DEPC was time dependent and followed pseudo-first-order kinetics (Fig. 3). The dependence of inactivation on pH from 5.5 to 8.0 shown in Fig. 4 indicates that DEPC modified an amino acid residue with a pK<sub>a</sub> value of 6.7, which is consistent with the pK<sub>a</sub> of His residues in proteins. When 0.5 mM PNP was included in the incubation mixture, the extent of enzyme inactivation by 0.5 mM DEPC was reduced from 30 to 0% after 6 min of incubation and from 50 to 20% after 12 min of incubation. When DEPC-inactivated enzyme was treated with 100 mM neutral hydroxyamine for 20 min at 22 °C the activity of the phosphatase was increased from 50 to 70%.

Tetranitromethane Modification of Vitamin B<sub>6</sub> Phosphatase—TNM reacts with tyrosine residues with reasonable specificity. The phosphatase was inactivated by TNM in a time-dependent manner (Fig. 5). The plot of log (% activity) versus time is not linear indicating that 2 or more tyrosinyl residues may react with TNM at different rates during the enzyme inactivation. In a separate experiment, 10 mM TNM inactivated the enzyme 70% in the absence of PLP and only 30% in the presence of PLP. In the absence of PLP, incubation of 2 mM [14C]PG with the enzyme for 60 min resulted in the incorporation of 2.3 mol of [14C]PG/mol of enzyme subunit and 65% activity loss. While in the presence of 0.5 mM PLP, 1.8 mol of [14C]PG was incorporated per mole of enzyme subunit, which led to 20% activity loss. The presence of PLP prevented the incorporation of 0.5 mol of [14C]PG and 45% activity loss. These data suggest that PG modified only a few of the 31 Arg residues/subunit of PLP phosphatase in the absence of substrate and that PLP protected against the modification of 1 Arg residue/subunit and inactivation. We attempted to identify the [14C]PG-modified amino acid residues by isolating and sequencing radioactively labeled tryptic peptides; however, the [14C]PG label was not stable enough to survive the trypsin digestion and HPLC separation.

**DISCUSSION**

The specificity of this human erythrocyte phosphatase for phosphorylated vitamin B<sub>6</sub> is confirmed in this study. The purified enzyme exhibited high activity for all phosphorylated B<sub>6</sub> compounds (except for PMP due to the positive charge at its
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4'-position) and very low activity with p-nitrophenyl phosphate, a commonly used substrate for non-specific phosphatases and phosphotyrosyl protein phosphatases. The purified PLP phosphatase has the lowest Kₘ for PLP of phosphatases reported to hydrolyze phosphorylated B₆ compounds. Substrate binding was optimal at pH 6–8. At pH values above 8 and below 6, Kₘ values increased and Vₘₐₓ/Kₘ values decreased. This indicates that a chemical group of the substrate and/or amino acid residue of the enzyme with a pKₐ value of about 6 must be ionized and another group with a pKₐ of 8 must be protonated for substrate binding. The substrates’ phosphate moiety and pyridine ring nitrogen have pKₐ values of 5.7–6.1 and 8.3–8.5, respectively (21). These pKₐ values are similar to the pK values estimated from the Kₘ and log Vₘₐₓ/Kₘ versus pH plots. On the other hand, substrate binding and/or catalysis may involve active site His and Cys residues of the enzyme. Since the pKₐ values for His and Cys side chains are approximately 6.5 and 8.5, respectively, these residues could also contribute to substrate binding. The Vₘₐₓ values are less pH dependent than the Kₘ values, especially from pH 5.5–7.5. pKₐ values could not be estimated from the Vₘₐₓ versus pH profiles.

The introduction of relatively bulky chemical groups at the 4'-position of PMP derivatives did not affect the Kₘ and Vₘₐₓ values of the phosphatase for those derivatives. PL kinase (26, 27) and PMP oxidase (28, 29) act on diverse vitamin B₆ analogs differing at position 4. 4'-Secondary amine derivatives of PM are transported into renal cells and converted to PLP by PL kinase and PMP oxidase as effectively as PN is (30). Therefore, the ability to tolerate bulky groups at the 4'-position of vitamin B₆ is a common property for vitamin B₆-metabolizing enzymes and the B₆ transporter. There is no indication of Schiff base formation between PLP and an active site residue of PLP phosphatase. Likewise, PL kinase does not form a Schiff base with PL during catalysis.

The phosphate moiety of the substrates is extremely important for substrate binding since PL and PN bind poorly and PLP is an effective competitive inhibitor (13). Furthermore, PMP derivatives with a net negative charge on the 4'-substituent were very efficient substrates. There may be one or more positively charged groups at the enzyme active site that interact with negative charges on the 4'- and 5'-substituents of the substrates. Our chemical modification results with PG suggest that an Arg residue may be located at the active site of the enzyme. It is not known whether the same positively charged residue(s) at the enzyme active site interacts with the phosphate moiety and the negative charge at the 4'-position of the substrate.

Inactivation of PLP phosphatase by PG followed pseudo-first-order kinetics, was not due to a change in the quaternary structure of the enzyme, and was prevented by PLP. Although the reaction of PG with the guanidinium group of Arg is quite specific (31–33), PG can react slowly with highly reactive Cys and Lys residues and the a-amino group of some proteins (31). PG could not have reacted with the NH₃ terminus of PLP phosphatase because it is blocked. Amino acid analysis and thiol group titration of both native and PG-modified enzymes revealed that PG modification did not result in a measurable loss of other amino acids including Lys and Cys. These results strongly suggest the presence of an Arg residue at the active site of PLP phosphatase.

The stoichiometry of the reaction of PG with Arg residues in proteins varies and is thought to depend on the microenvironment of the modified Arg. Reported ratios of PG/Arg for many proteins range from 1:1 (34–36) to 2:1 (31, 37). Our data suggest that incorporation of one molecule of PG/subunit results in inactivation of PLP phosphatase, which is consistent with a 1:1 stoichiometry. The 1:1 adduct of PG and Arg forms reversibly and is very unstable (33). Therefore, it is not surprising that [³⁵S]PG-labeled tryptic peptides from the PG-modified phosphatase could not be purified.

Arg residues often serve as anion-binding sites, particularly for phosphate and carboxylate groups (33). The positively charged guanidinium group of Arg has been implicated in binding the phosphate moiety of PLP in aspartate aminotransferase (38, 39) and PMP in PMP oxidase (40). Alkaline phosphatases

from *Escherichia coli* (41) and pig kidney (42) acid phosphatases from human prostate (36) and *E. coli* (25) and bovine heart low molecular weight phosphophorysyl protein phosphatase (43) are reported to contain essential active site Arg residues. Two of the oxygens of P, interact with the guanidinium group of an Arg in *E. coli* alkaline phosphatase (41). The guanidinium group of the essential Arg residue in PLP phosphatase and other phosphatases may interact with the substrate’s phosphate group and enhance its susceptibility to nucleophilic attack.

The pH profile of the inactivation of PLP phosphatase by DEPC strongly suggests the modification of a His residue(s) at the active site because DEPC reacts with unprotonated imidazole. Carboxylation of His and Tyr by DEPC can be reversed by hydroxylamine (44). Treatment of the DEPC-modified phosphatase with neutralized hydroxylamine led to partial reactivation with hydroxylamine. His is phosphorylated during catalysis. The His is not involved in the essential Arg residue in PLP phosphatase and other phosphatases may interact with the substrate’s phosphate group stabilizing the transition state of the reaction.

Histidine residues can play a variety of roles in phosphatases. Histidine residues can be involved in metal cation binding in some phosphatases not functioning as a nucleophile because a residue other than Tyr residues. The pH profile of the inactivation of PLP phosphatase by DEPC is not consistent with the modification of essential Lys residues. Two His residues at the amino terminus of the enzyme is blocked and could not be modified by DEPC. We conclude that modification of 1 or more His residues at or near the active site inactivates the phosphatase.

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