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CHARACTERIZATION OF THE ACTIVE SITE OF HOMOGENEOUS THYROID PURINE NUCLEOSIDE PHOSPHORYLASE

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Summary

Purine nucleoside phosphorylase (purine-nucleoside : orthophosphate ribosyltransferase, EC 2.4.2.1) has been purified approx. 4000-fold and to electrophoretic homogeneity from bovine thyroid glands. The isolated enzyme has a specific activity of 17 μmol · min^{-1} · mg^{-1}. The native enzyme appears to have a molecular weight of 92 000 as determined by sedimentation equilibrum ultracentrifugation and is comprised of three subunits having a molecular weight of 31 000 each as shown by sodium dodecyl sulfate gel electrophoresis. The enzyme is irreversibly denatured below pH 5 and the enzyme-substrate complex is shown to have an ionization constant (pK_a) of 9.2 which influences catalytic activity. The pH dependence of the kinetic constants identifies three amino acid ionizable protons. The binding of inosine is effected by an imidazole ring of histidine (pK_a 5.65) and a sulfhydryl group of cysteine (pK_a 8.5) and the maximal velocity is restricted by an ϵ-amino group which is essential for phosphate binding. The requirement of these residues for activity was confirmed by group-specific chemical modification. The presence of phosphate protected only the lysyl residue while inosine protected all three residues from chemical titration. A model is proposed for the catalytic mechanism of purine nucleoside phosphorylase.

Introduction

Purine nucleoside phosphorylase of the purine catabolic pathway catalyzes the reversible phosphate substitution of a purine base from the corresponding

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Abbreviations: Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

ribose nucleoside. The mammalian enzyme has been isolated to electrophoretic homogeneity from many tissues [1—8] and has been shown to have a molecular weight in the range of 80 000—90 000 [1,5—8]. A trimeric quaternary structure has been reported [5,6,8] with the chicken liver purine nucleoside phosphorylase shown to be comprised of two non-identical subunits [8]. The rabbit liver purine nucleoside phosphorylase [2] is reported to be a monomer with a molecular weight of 43 000 and the bovine brain enzyme [4] is identified as a dimer with a subunit molecular weight of 38 000.

Purine nucleoside phosphorylase kinetics follow a Bi-Bi type mechanism, in which both substrates are bound to the enzyme prior to the release of products [3,4,9—12] and exhibits a non-linear double-reciprocal plot when the nucleoside is the varied substrate [1,3,5,7,10] or when inorganic phosphate is varied [5,13]. The phenomenon of negative cooperativity drew our interest to investigate thyroidal phosphorylase because in this laboratory the binding of phosphate was observed to affect the kinetics [13]. Prior to this report only the bacterial purine nucleoside phosphorylase [14] was associated with negative cooperativity by phosphate. Recently, two additional bovine enzymes have been cited by Ikezawa et al. [15] as demonstrating negative cooperativity by inorganic phosphate.

We report the purification of thyroidal purine nucleoside phosphorylase to electrophoretic homogeneity, examine the pH dependence of the reaction, the effect of pH on the kinetic constants and identify the amino acid residues at the active site by group-specific chemical modification.

Materials and Methods

Materials. Inosine, guanosine, dithiothreitol, 2-mercaptoethanol, disodium ethylenediaminetetraacetate, ammonium sulfate, Coomassie brilliant blue G250, sodium dodecyl sulfate (SDS), Sephadex gels, standard molecular weight proteins, xanthine oxidase, Bistris, Trizma base, Tes, sodium acetate, glycine, sodium barbital, *p*-chloromercuribenzoate, *N*-ethylmaleimide, pyridoxal 5-phosphate, methylene blue and xanthine oxidase were purchased from Sigma Chemical Company. Formycin B was obtained from Calbiochem. Electrophoresis reagents were purchased from Eastman Chemical. All other reagents were reagent grade.

Acrylamide and *N,N'*-methylene bisacrylamide were recrystallized from chloroform and acetone, respectively. TEMED was redistilled. Sodium dodecyl sulfate was recrystallized from 80% ethanol.

Enzyme assay. Purine nucleoside phosphorylase activity with inosine was followed by a coupled enzyme assay with xanthine oxidase at 30°C. The reaction mixture contained 50 μmol potassium phosphate, pH 7.0, 0.33 μmol inosine and 0.1 μmol dithiothreitol or mercaptoethanol in 1 ml. Sufficient units of xanthine oxidase (1 unit converts 1 μmol hypoxanthine to uric acid/min at the pH stated) were added to insure a five-fold excess with respect to the purine nucleoside phosphorylase activity being observed. The reaction was monitored on a Perkin-Elmer Model 124 recording spectrophotometer at 292 nm in a 1 cm cuvette. When appropriate, data were subjected to linear regression analysis to determine kinetic parameters.

Protein assay. Protein content was analyzed by the biuret method [16] during the purification of the enzyme. Protein concentration to 0.5 mg/ml was determined by a modified Coomassie brilliant blue G250 procedure [17,18]. A dye reagent containing 100 mg Coomassie brilliant blue G250, 50 ml 95% ethanol, 100 ml 85% phosphoric acid and 850 ml H_2O was filtered through Whatman paper to remove particulate material. A 0.10 ml aliquot of protein was added to 5.0 ml of dye reagent and allowed to stand at room temperature for 5 min. The absorbance at 595 nm was linear to 50 μg of a bovine serum albumin protein standard.

Purification of purine nucleoside phosphorylase from bovine thyroid. Bovine thyroid glands were obtained within 15 min of slaughter and transported in cold homogenizing buffer containing 0.2 M sodium bicarbonate, 1 mM EDTA, 1 mM mercaptoethanol, pH 7.7. Glands were trimmed free of connective tissue and stored frozen in 100 g lots for as long as 3 months with no loss of enzyme activity. Glands were thawed in cold water and homogenized in the above buffer (500 g tissue/l of buffer) with a Tekmar Model SD 45 homogenizer fitted with a Model G-450 generator. The homogenate was passed through four layers of cheesecloth and centrifuged at 44 000 $\times g$ for 55 min. The non-particulate fraction which contains an appropriate amount of lipids was fractionated by the addition of a 20% (by vol.) of *n*-butanol followed by centrifugation at 10 000 $\times g$ for 20 min.

The supernatant was fractionated with ammonium sulfate with initial cuts at 6% and 30% saturation. The precipitate from 30 to 65% saturation which contained the phosphorylase activity was dialyzed against 10 mM potassium phosphate, pH 7.0, 0.1 mM mercaptoethanol before it was applied to a DEAE-Sephadex column (2.5 $\times$ 30 cm, phosphate form) equilibrated with the dialyzing buffer. Unbound proteins were washed from the column with the equilibration buffer prior to eluting the enzyme with a 2 l linear gradient of 10—100 mM potassium phosphate, pH 7.0, 0.1 mM mercaptoethanol. The active fractions were pooled and concentrated with an Amicon ultrafiltration apparatus using a PM-10 membrane prior to being chromatographed on Sephadex G-200 (2.2 $\times$ 80 cm) equilibrated with 50 mM potassium phosphate, pH 7.0, 0.1 mM mercaptoethanol. The peak fractions containing the enzyme were pooled and concentrated by ultrafiltration.

The protein was applied to a Buchler 'Polyprep 100' preparative polyacrylamide gel electrophoresis apparatus in a concentration not to exceed 5 mg of protein/cm^2 of gel surface. Optimum separation was achieved using a bilayer gel system with discontinuous buffers. A 4 cm lower gel consisting of 10% acrylamide and 0.23% bisacrylamide and a 2 cm upper gel with 5% acrylamide and 0.2% bisacrylamide were chemically polymerized in 0.75 M Tris-HCl, pH 8.9. The upper cathode buffer contained 52 mM Tris/glycine, pH 8.8, 10 mM sodium thioglycolate and the lower anode buffer contained 400 mM Tris-HCl, pH 8.1. Electrophoresis of the proteins was done at 4°C maintaining a constant current of 30 mA. Proteins eluted from the gel were collected in 100 mM Tris-HCl, pH 8.1, 10 mM mercaptoethanol.

Molecular weight estimation. Purine nucleoside phosphorylase (0.55 mg/ml) in 50 mM potassium phosphate, pH 7.0, 0.1 mM dithiothreitol was allowed to reach equilibrium at 15 220 rev./min on a Model E Spinco Ultracentrifuge at

20°C. The molecular weight was estimated by the meniscus depletion sedimentation equilibrium method of Yphantis [19].

Equilibrium constant. Purine nucleoside phosphorylase (0.026 unit) was incubated at 30°C in 1.0 mM potassium phosphate, pH 7.0, 1.0 mM inosine, 0.2 mM dithiothreitol, 25 mM each acetate, Bistris, Tris and glycine, pH 7.0 in 1.0 ml. At various times, 0.10 ml of the equilibrium mixture was removed and added to 0.30 ml of 0.1 M HCl to denature the enzyme. Hypoxanthine released from inosine was quantitated by neutralizing the acidified equilibrium mixture with 0.50 ml 0.1 M Tris and converting the purine base to uric acid by the addition of 0.05 unit of xanthine oxidase in 0.10 ml of 0.1 M potassium phosphate, pH 7.0.

Activation energy. The effect of temperature on the maximum velocity of purine nucleoside phosphorylase was studied by varying guanosine from 20 to 180 μM in 66 mM potassium phosphate, pH 7.0, 0.1 mM dithiothreitol and 0.03 unit of enzyme at a final volume of 3.0 ml. The temperature was varied from 25 to 45°C. The reaction was monitored at 257 nm.

The effect of temperature on the enzyme activity with inosine as the nucleoside substrate was evaluated between 20 and 45°C. At varying times, 0.10 ml of a 1.0 ml reaction mixture containing 100 mM potassium phosphate, pH 7.0, 1.0 mM inosine, 0.1 mM mercaptoethanol and 0.0012 unit of purine nucleoside phosphorylase was stopped by adding 0.30 ml 0.1 M HCl. After 10 min at 30°C the denatured enzyme mixture was neutralized with 0.50 ml 0.1 M Tris. Xanthine oxidase (0.02 unit) was added in 0.10 ml of 0.1 M potassium phosphate, pH 7.0, to convert the released base to uric acid.

Effect of pH on the apparent Michaelis constants. The assay mixture contained 50 mM acetate, Bistris, Tris and glycine with the pH adjusted between 6.00 and 10.00. When phosphate was the varied substrate a concentration range of 0.20—30 mM was used, the pH of the phosphate was also adjusted and inosine was saturating at 4 mM for all pH values. When inosine was varied, the substrate concentration ranged from 25 to 400 μM and the phosphate concentration was saturating at 50 mM for the pH range studied. The xanthine oxidase employed in these studies was chromatographed free of ammonium sulfate on a 5 × 70 mm column of Sephadex G-75 to exclude a possible sulfate ion effect [20] although none has been observed for the thyroid enzyme.

Active site directed chemical modification. The effect of pH on the photosensitized inhibition of purine nucleoside phosphorylase by methylene blue was studied at 4°C in 0.10 M potassium acetate (pH 5.5, 6.0), or 0.10 M potassium phosphate (pH 6.5, 7.0, 7.5, 8.0), 0.10 mg methylene blue with 2.85 units of enzyme in a final volume of 1.0 ml. The buffer was oxygenated prior to the addition of the enzyme to insure that the oxygen concentration was not limiting and reducing agents were previously removed from the enzyme. A Kodak slide projector with a 250 W lamp served as the light source 20 cm from the water-jacketed reaction vessel. Photochemical oxidation was terminated at varying times by passing a 0.10 ml aliquot of the reaction mixture through a 5 × 70 mm Sephadex G-75 column equilibrated with 0.10 M potassium phosphate, pH 7.0, 0.5 mM dithiothreitol at 4°C.

Nucleoside protection of the enzyme from photooxidation was conducted at pH 7.5 in 40 mM Tes-chloride, 50 mM potassium phosphate and 5 μM

methylene blue with 1.14 units of purine nucleoside phosphorylase at 25°C in a final volume of 0.50 ml. The nucleoside analog formycin B was present at 5.0 mM.

Catalytic inactivation by *p*-chloromercuribenzoate and *N*-ethylmaleimide was followed by incubating 0.16 unit of purine nucleoside phosphorylase in 50 mM Tes-chloride, pH 7.6, with fixed concentrations of inhibitors in a final volume of 0.25 ml of 30°C. Aliquots of 0.025 ml were quenched by dilution with 0.50 ml 0.10 M Tris-HCl, pH 7.6, at 4°C. The remaining activity was assayed as described above.

Pyridoxal 5-phosphate inhibition of purine nucleoside phosphorylase was conducted at 30°C in a foil-covered reaction vessel containing 0.15 unit of enzyme, 0.05 M sodium barbital, pH 8.5, and fixed concentrations of inhibitor in a final volume of 1.0 ml. Titration of the enzyme was stopped by diluting a 0.10 ml aliquot of the reaction mixture with 0.20 ml of 0.10 M Tris-HCl, pH 8.0, 10 mM sodium borohydride at 4°C. The reduction of the Schiff base was completed in 3 min, at which time enzymic activity was assayed.

Results

Enzyme purification

The purification procedure for bovine thyroidal purine nucleoside phosphorylase resulted in a 4000-fold increase in specific activity over the homogenate with an overall recovery of 7%. The *n*-butanol extraction of the non-particulate fraction to remove lipid material had a minimal effect on the enzyme, but prepared the supernatant for the ensuing ammonium sulfate fractionations. A summary is shown in Table I. The enzyme eluted from the preparative polyacrylamide gel electrophoresis is electrophoretically pure. Homogeneity was confirmed by a single symmetrical band obtained for 100 μg of protein electrophoresed on 10% polyacrylamide analytical gels and stained for protein and activity. Homogeneity was also confirmed by sedimentation equilibrium ultracentrifugation.

Physical characteristics of purine nucleoside phosphorylase

Using miniscus depletion sedimentation equilibrium, the thyroid purine

TABLE I

PURIFICATION DATA FOR THYROID PURINE NUCLEOSIDE PHOSPHORYLASE

Fraction	Total volume (ml)	Total activity (μmol · min^{-1})	Specific activity (μmol · $min^{-1} \cdot mg^{-1}$)	Yield (%)	Purification (-fold)
Homogenate	1100	320	0.0043	100	1
n-Butanol extraction	1160	279	0.0044	87	1
6% $(NH_4)_2SO_4$ supernatant	1170	260	0.012	83	2.8
30% $(NH_4)_2SO_4$ supernatant	1200	240	0.025	75	5.9
65% $(NH_4)_2SO_4$ precipitate	45	225	0.56	70	30
DEAE-Sephadex column	600	115	1.29	36	300
Sephadex G-200 column	80	48	4.33	15	1000
Preparative electrophoresis	6	25	17.0	7	3950

nucleoside phosphorylase was observed to be homogeneous with a molecular weight of 92 400 ± 2500 when averaged across the cell using a partial specific volume of 0.73 g · ml^{-1}. Standard molecular weight proteins gave a linear relationship when the log of the molecular weight versus the relative mobility in the SDS gel was plotted. The relative mobility was calculated as outlined by Weber et al. [21] as the ratio of the distance the tracking dye moved. Purine nucleoside phosphorylase from the thyroid stained as one symmetrical band with an apparent molecular weight of 31 000 ± 1000 averaged from four analyses. The enzyme appears to be a trimer with subunits of identical molecular weight.

The equilibrium constant was evaluated by selectively stopping the equilibrium reaction by acid denaturating the purine nucleoside phosphorylase and quantitating the free hypoxanthine by converting it to uric acid with xanthine oxidase. The K_{eq} for the thyroid enzyme is 0.021 in the direction of phosphorolysis.

A linear Arrhenius plot was obtained for the V temperature dependence of the purine nucleoside phosphorylase. When the varied substrate was inosine or guanosine an activation energy of 16.2 or 27.0 kcal · mol^{-1} was calculated, respectively.

Effect of pH on enzyme stability, activity and kinetic parameters

When purine nucleoside phosphorylase was incubated at 30°C for 10 min in a buffer at the pH specified, the enzyme irreversibly lost activity at a lower pH (Fig. 1a). Complete denaturation was obtained below pH 5 whereas between pH 6.0 and 10.0 no loss of enzymic activity was observed. A broad pH optimum ranging from 6.5 to 8.0 (Fig. 1b) was demonstrated for the enzyme which is typical for purine nucleoside phosphorylase [22].

The effect of pH on the Michaelis constants of thyroid purine nucleoside phosphorylase was evaluated by the method of Dixon [23] and the data are presented in Fig. 2. The maximum velocity for both substrates is influenced by an ionization at pH 9.2 on the enzyme-substrate complex. The $\log(V/K_m)$ plot for inosine as the varied substrate identified two additional ionizable groups which by the nature of the plot are associated with either the free enzyme or free substrate. Since inosine has no ionizable protons with a pK_a of 5.6 or 8.5 they must be assigned to the active site of the enzyme. The effect of pH on the pK_m of the enzyme for inosine also supports three protonation changes, two on the free enzyme and one on the enzyme substrate complex. Negative cooperativity was observed from pH 6.00 to 8.00 when inorganic phosphate was the varied substrate. Both regions of the double-reciprocal plot gave straight log plots with a slope of zero. Only the data from low substrate concentrations are shown. Above pH 8.00 substrate inhibition became evident, therefore the data at low concentrations of substrate could be evaluated. It is in the high pH region that a break in the $\log(V/K_m)$ versus pH plot is observed. An ionizable group on the enzyme with a pK_a of 9.2 influences the binding of phosphate as well as the overall reaction rate. The linearity of the log K_m versus pH plot suggests that the ionization of the enzyme-substrate complex and the free enzyme results from the same ionizable group. Although phosphate does have an ionizable proton within the pH range studied, no discontinuities in the

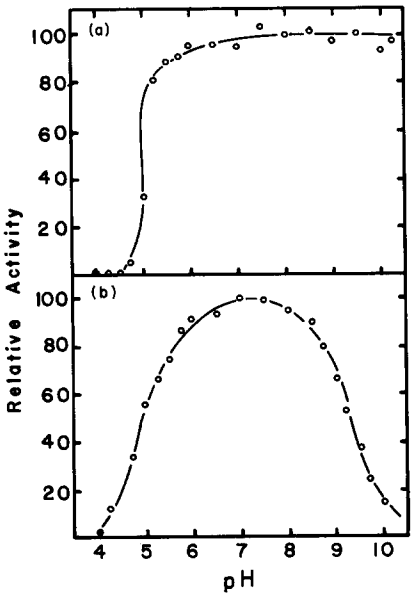

Fig. 1. (a) Effect of pH on enzyme stability. The enzyme (0.006 unit) was incubated in 0.10 ml of 50 mM acetate, 50 mM Bistris, 50 mM Tris and 50 mM glycine with the pH adjusted between 4.00 to 10.50. After 10 min at 30°C, 2.90 ml of assay mixture containing 190 μmole potassium phosphate, pH 7.0, 0.95 μmol inosine and 0.05 unit of xanthine oxidase was added and the enzyme activity was monitored at 292 nm. (b) Effect of pH on enzyme activity. The xanthine oxidase standard assay for enzyme activity was run in 3.0 ml containing 25 mM acetate, Bistris, Tris and glycine, 50 mM potassium phosphate, 1.3 mM inosine, 0.1 unit of xanthine oxidase (chromatographed free of $(NH_4)_2SO_4$ on a 5 × 40 mm column of Sephadex G-75 to eliminate any possible sulfate ion effect), and 0.01 unit of purine nucleoside phosphorylase. The pH of the buffer and the phosphate was adjusted between 4.00 and 10.25. Activity was observed at 292 nm.

plots can be ascribed to it. The derivation of a kinetic expression which defines the effect of pH-dependent ionization on the enzymic activity requires the assignment of the various proton dissociation constants to the proper enzyme form. Eqn. 1 introduces the ionization events which were identified and estimated from Fig. 2 into a velocity equation for an Ordered Bi-Bi type mechanism.

$$\frac{v}{V} = \frac{[A][B]}{\alpha K_{ia} K_{m_B} + \beta K_{m_B}[A] + \alpha K_{m_A}[B] + \alpha[A][B]} \tag{1}$$

where

$$\alpha = 1 + \frac{K'''_a}{[H]} \qquad pK'_a = 5.65$$

$$\beta = 1 + \frac{[H]}{K'_a} + \frac{K''_a}{[H]} \qquad \begin{matrix} pK''_a = 8.50 \\ pK'''_a = 9.20 \end{matrix}$$

Rearrangement of Eqn. 1 into the double-reciprocal format and simplifying by saturating the non-varied substrate, i.e. $[A] >> K_{ia}$, $[A] >> K_{m_A}$, $[B] >> K_{m_B}$ is given by

$$\frac{1}{V} = \frac{\alpha K_{m_A}}{V} \frac{1}{[A]} + \frac{\alpha}{V} \tag{2}$$

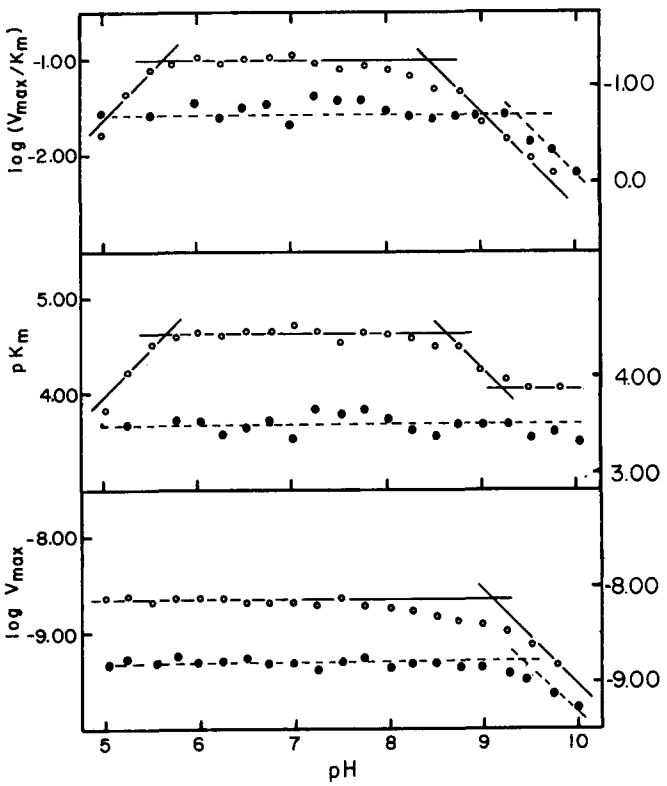

Fig. 2. Effect of pH on the apparent Michaelis constants of purine nucleoside phosphorylase. The ordinate on the left (○———○) is varying inosine and phosphate saturating. The ordinate on the right (●- - - - - -●) is varying phosphate and inosine saturating. The apparent Michaelis constants were evaluated by a linear extrapolation of the double-reciprocal plot generated at the pH stated under the conditions described in Materials and Methods.

for phosphate as the varied substrate and

$$\frac{1}{V} = \frac{\beta K_{m_B}}{V} \frac{1}{[B]} + \frac{\alpha}{V} \tag{3}$$

when inosine is the varied substrate. Theoretical plots of the Michaelis constants presented in Eqns. 2 and 3 as a function of pH gave the same pH-dependent profile as obtained in Dixon pH plots.

The range of a pK_a for an ionizable amino acid residue which may participate in the catalytic mechanism has been evaluated, thus enabling the identification of the participating moieties. The imidazole ring of histidine has been assigned the lowest pK_a of 5.6 and the pK_a of 8.5 to cysteine. These groups appear only when inosine was the varied substrate and the ionization occurs only on the free enzyme in both instances. The highest pK_a of 9.2 may be assigned to the ϵ-amino group of lysine. It is the ionization of the ϵ-amino group of the free enzyme which is affected when phosphate is the varied substrate and the enzyme-substrate complex when either inosine or phosphate is varied.

Active site directed chemical modification

The pH dependence of the photosensitized inhibition of the purine nucleoside phosphorylase by methylene blue was studied. The effect of pH on the photoinhibition of purine nucleoside phosphorylase suggests an oxidation of the non-protonated imidazole ring of histidine and is not attributed to a modification of tryptophan, tyrosine or methionine. The rate of photoinactivation of purine nucleoside phosphorylase is not affected by inorganic phosphate but may be modified by the presence of the nucleoside analog, formycin B (Fig. 3). Protection of the enzymic activity by inosine or guanosine was not studied because both nucleosides are photochemically modified in the presence of methylene blue, while formycin B is not [24,25].

Purine nucleoside phosphorylase is inactivated in the presence of *p*-chloromercuribenzoate and *N*-ethylmaleimide which react with cysteine. The rate of catalytic inactivation by *p*-chloromercuribenzoate is reduced by the presence of substrate level nucleoside (Fig. 4) but is only slightly affected by phosphate. Similar results were obtained with *N*-ethylmaleimide.

The ϵ-amino group of lysine was selectively modified by the formation of a Schiff base with the aldehyde of pyridoxal 5-phosphate. The lysine adduct was reduced with sodium borohydride to insure a permanent modification. If the modified enzyme was not reduced, dissociation of the pyridoxal 5-phosphate from the enzyme could be detected as an increase in enzymic activity with

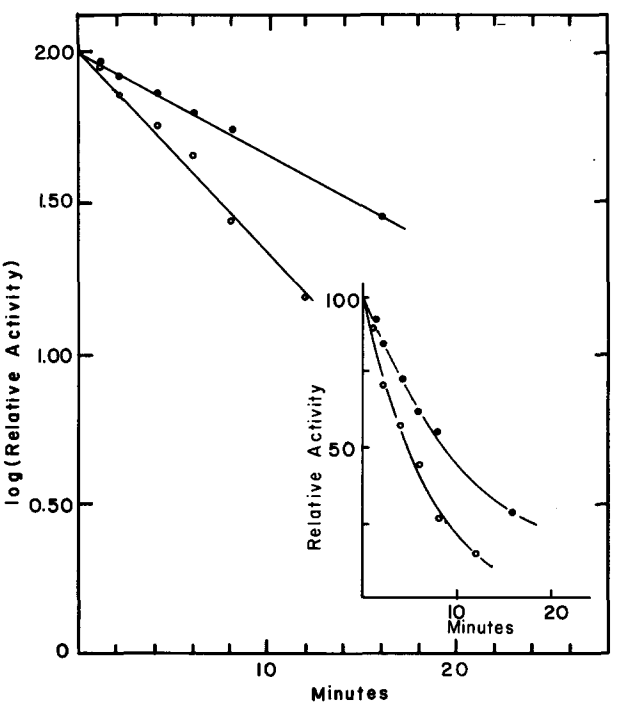

Fig. 3. Effect of formycin B on the photoinactivation of purine nucleoside phosphorylase in the presence of methylene blue. A first-order evaluation of methylene blue, light-sensitized inactivation in the absence (○———○) and presence (●———●) of formycin B (5 mM). The inset is the time course of inactivation as described in Materials and Methods.

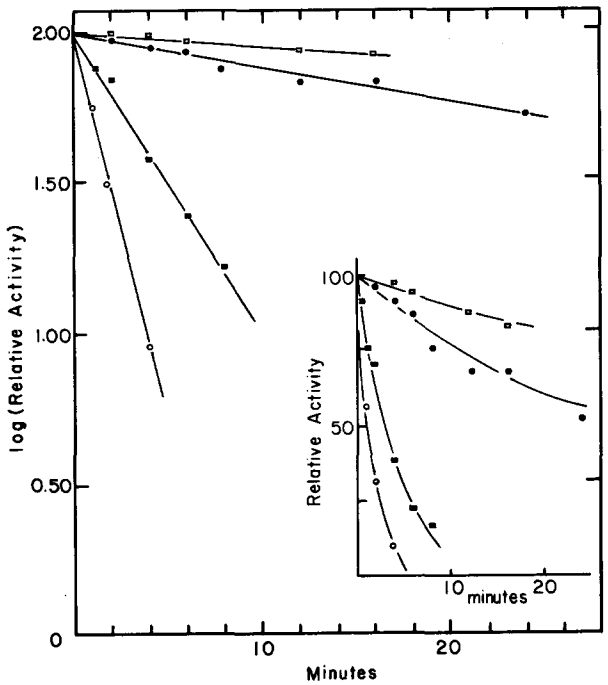

Fig. 4. Effect of *p*-chloromercuribenzoate on purine nucleoside phosphorylase activity in the presence of inosine. A first-order evaluation of inhibition by 10 μM (■———■) and 100 μM (○———○) *p*-chloromercuribenzoate and the influence of 10 μM (●———●) and 200 μM (□———□) inosine on inhibition by 10 μM *p*-chloromercuribenzoate. The inset is the time course of inactivation as described in Materials and Methods.

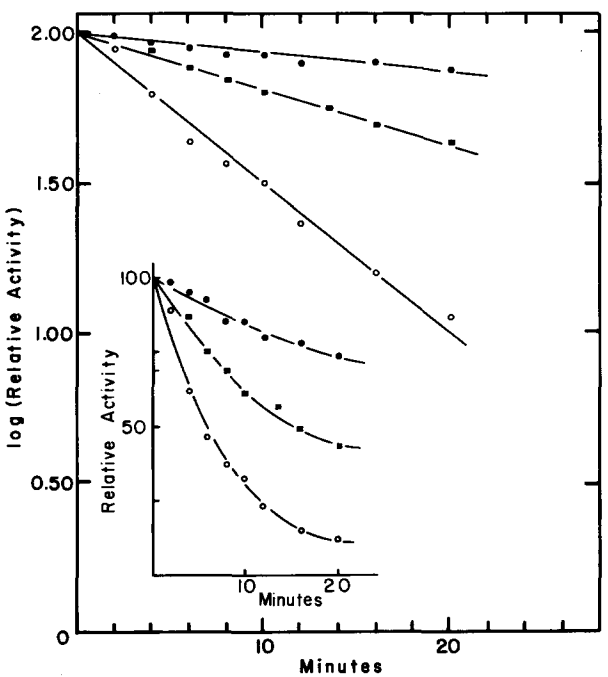

Fig. 5. Effect of pyridoxal 5-phosphate on purine nucleoside phosphorylase activity in the presence of inosine. A first-order evaluation of inhibition by 10 mM pyridoxal 5-phosphate (○———○), in the presence of 2 μM (■———■) and 20 μM (●———●) inosine. The inset is the time course of inactivation as described in Materials and Methods.

time. Purine nucleoside phosphorylase activity was not affected by sodium borohydride when incubated under the conditions specified. Therefore, permanent inactivation of the enzyme was due to alteration of the lysine residue. Phosphate will protect the lysine residue from modification when present at concentrations higher (approximately five times) than the apparent K_m for phosphate. Inosine will protect the primary amino group of lysine (Fig. 5) even though there is no evidence from the pH dependence of the reaction to suggest lysine has an effect on nucleoside binding.

Discussion

Purine nucleoside phosphorylase has been isolated to electrophoretic homogeneity from the bovine thyroid gland. The purified enzyme has a specific activity of 17 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. From other sources, purine nuceloside phosphorylase has the following specific activities (μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}): human erythrocyte 96 [26], 53.3 [37]; bovine spleen 32 [12]; rabbit liver 47.4 [2]; chicken liver 45 [3]; bovine brain 78 [4] and bovine liver 22.8 [5].

The enzyme may utilize inosine, guanosine, the corresponding deoxynucleosides, or less efficiently xanthosine for the nucleoside substrate and phosphate or arsenate as the second substrate [28]. Adenosine will not serve as a substrate [28]. The reversible enzyme-mediated reaction favors nucleoside synthesis with a K_{eq} of 49 which is characteristic of the reaction [3,22,29—32]. The Arrhenius energy of activation was calculated to be 16.2 kcal $\cdot$ mol^{-1} with inosine as the substrate between 20 and 45° C. This value is approximately two-fold higher than reported for purine nucleoside phosphorylase from other tissues [2,4,22, 30], suggesting a possible difference in the mechanism [33].

The enzyme was reported to have a molecular weight of 90 000 by gel filtration and 92 000 by sedimentation equilibrium. A trimeric subunit structure was observed by SDS gel electrophoresis with each subunit having a molecular weight of 30 000. No size heterogeneity was observed, in contrast to the chicken liver enzyme [8].

Thyroidal purine nucleoside phosphorylase is acid labile with irreversible denaturation (Fig. 1a) and loss of catalytic activity (Fig. 1b). The decrease in catalytic activity at the higher pH may be attributed to a change in the protonation of the enzyme-substrate complex such that it becomes catalytically inactive. The acid dissociation constant which restricts the optimum pH range, can be obtained by plotting the log of the apparent activity versus the pH [33]. A log plot of the data of Fig. 1b gave a pK_a of 9.2 for the enzyme-substrate complex. The pH profile of the 'aged' bovine liver and the commercial bovine spleen enzyme compare very closely [5]. Although the acid lability of the bovine enzymes is not discussed by Ikezawa et al. [15], the decline in activity at the lower pH does give an inflection point near pH 5. Purine nucleoside phosphorylase from other sources have a more restrictive pH range for activity with inflections near pH 6.0 and 8.0 [2,4,22,26,32].

The active site of purine nucleoside phosphorylase from the bovine thyroid gland has been probed to identify the acid dissociation constants which effect the enzymic catalysis by evaluating the effect of pH on the apparent Michaelis constants [23]. This method has identified the pK_a of three ionizable protons;

two affect the binding of the nucleoside substrate (Fig. 2) and the third influences the binding of phosphate (Fig. 2) in addition to restricting the maximal velocity of the overall reaction. When the acid dissociation constants are introduced into the kinetic expression for purine nucleoside phosphorylase (Eqn. 1), it becomes evident that the α-term, containing the single pK_a of 9.2, not only modifies the binding of phosphate to the free enzyme but also interacts with the ternary complex which represents the enzyme-substrate complex prior to the release of products. The α-term in the intercept (Eqn. 2) is the same as that found in the slope thus accounting for the linear pK_m versus pH plot for phosphate (Fig. 2). The formal charge on phosphate ($pK_a \approx 6.8$) does not appear to be critical for catalysis since substrate binding and purine nucleoside phosphorylase activity is pH independent of free substrate (Fig. 2).

Nucleoside binding requires two amino acid residues. The pK_a of 5.6 is assigned to the imidazole ring of histidine for which the neutral species is active and the pK_a of 8.5 is attributed to the ionization of the sulfhydryl group of cysteine. Group-specific chemical modification by a pH-dependent photosensitized oxidation with methylene blue confirms a requirement for histidine and the *p*-chloromercuribenzoate or *N*-ethylmaleimide titration of enzymic activity verifies the presence of an active cysteine. The rate of chemical modification of histidine (Fig. 3) and cysteine (Fig. 4) was reduced in the presence of a nucleoside substrate or analog, suggesting that these two amino acid residues are in the nucleoside binding site. Phosphate affords little protection for histidine and cysteine against chemical modification.

It has been previously proposed that phosphate binds in the active site by a salt bridge formation between the enzyme and substrate [28]. The ϵ-amino group of lysine, protonated at physiological pH, may be the amino acid residue which affects the kinetics observed when phosphate is the varied substrate. Arginine modification has recently been shown to influence the catalytic activity of calf spleen and human erythrocyte purine nucleoside phosphorylase [34]. Since arginine is also cationic at physiological pH, it was proposed to be the residue which forms a salt bridge, binding the inorganic phosphate. However, kinetically no ionization could be attributed to arginine ($pK_a \approx 11.6$) on the bovine thyroid enzyme. Titration of lysine with pyridoxal 5-phosphate demonstrates a requirement of a primary amine for activity which is protected by inosine but only slightly by phosphate.

The influence of lysine on the maximum velocity can be explained by using the kinetic mechanism. Since phosphate is the first substrate to bind, a decrease of the enzyme-phosphate species resulting from the increase of non-protonated lysine upon an increase in pH will lead to a parallel decrease in catalytically active enzyme and thus a change in the V regardless of whether inosine or phosphate is the varied substrate (Fig. 2). This is also observed when inosine is the first substrate to bind in an ordered reaction. The human erythrocyte purine nucleoside phosphorylase activity is influenced by the ionization of two amino acid residues having a pK_a of 6.5 and 8.1 when the nucleoside was the varied substrate [26,35]. Purine nucleoside phosphorylase isolated from rabbit liver [36] and brain [5] and bovine brain [4] are catalytically restricted by the identification of two amino acid residues with a pK_a of 5.5 and 8.4 for the liver enzyme and 5.3 and 7.8 for the brain enzyme. The difference in the pK_a

reported [36] at low pH for inosine versus guanosine as the substrate may be explained by reviewing the method of assay. The xanthine oxidase assay employed for inosine as the varied substrate is limited below pH 7 where the catalytic activity of the coupling enzyme is significantly hindered. The lower pK_a may be a reflection of the decreasing activity of xanthine oxidase and not of purine nucleoside phosphorylase. Therefore, the ionizations restricting nucleoside binding to purine nucleoside phosphorylase are similar among those reported with the differences explained kinetically by the order of substrate addition.

Specific protein-substrate interactions and the orientation of the bound nucleoside and phosphate have been proposed from nucleoside analog studies by Carlson and Fischer [28]. The amino acid residues implicated in this paper can now be assigned to the specific interactions alluded to in the earlier investigation. The active site of thyroid purine nucleoside phosphorylase may best be described as a pocket in which the lysine residue is located at the base. In this manner both phosphate and nucleoside will protect the amino acid moiety from modification. The neutral histidine may hydrogen bond with the substituent at the 6-position. The imidazole ring of histidine is not, however, limited to only hydrogen bonding with the 6-hydroxyl group of the nucleoside substrate but may also interact with the N-1 of the heterocycle. This provides an additional means of dispersing the electron density of the purine as suggested by our earlier observations. The protonated cysteinyl residue may be assigned to protonate the N-7, which induces an electron-poor imidazole ring of the purine base which is believed to be the rate-limiting step for the bovine thyroid enzyme.

This model appears to hold for purine nucleoside phosphorylases which

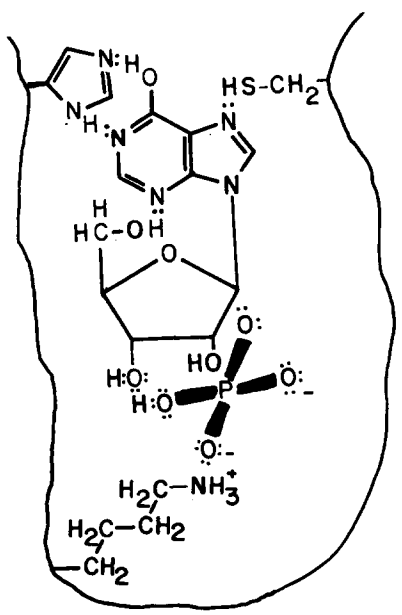

Fig. 6. Model of the active site of thyroid nucleoside phosphorylase.

kinetically follow a reversed ordered addition of substrate. Nucleoside analog studies with the human erythrocyte enzyme [36] have the same inhibition and substrate pattern for the analogs as was reported for the bovine thyroid purine nucleoside phosphorylase [28] and the same amino acid residues have been demonstrated earlier to be required for binding the nucleoside substrate. An active site may, therefore, have the essential histidine and cysteine residues located in the pocket so as to expose the ribose of the bound nucleoside to attack by phosphate.

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