Purine Nucleoside Phosphorylase. Inosine Hydrolysis, Tight Binding of the Hypoxanthine Intermediate, and Third-the-Sites Reactivity†

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ABSTRACT: Purine nucleoside phosphorylase from calf spleen is a trimer which catalyzes the hydrolysis of inosine to hypoxanthine and ribose in the absence of inorganic phosphate. The reaction occurs with a turnover number of 1.3×10^{-4} s^{-1} per catalytic site. Hydrolysis of enzyme-bound inosine occurs at a rate of 2.0×10^{-3} s^{-1} to form a stable enzyme–hypoxanthine complex and free ribose. The enzyme hydrolyzes guanosine; however, a tightly-bound guanine complex could not be isolated. The complex with hypoxanthine is stable to gel filtration but can be dissociated by acid, base, or mild denaturing agents. Following gel filtration, the E·hypoxanthine complex dissociates at a rate of 1.9×10^{-6} s^{-1} at 4 °C and 1.3×10^{-4} s^{-1} at 30 °C. The dissociation constant for the tightly-bound complex of enzyme–hypoxanthine is estimated to be 1.3×10^{-12} M at 30 °C on the basis of the dissociation rate. The stoichiometry of the reaction is 1 mol of hypoxanthine bound per trimer. The reaction is reversible since the same complex can be formed from enzyme and hypoxanthine. Addition of ribose 1-phosphate to the complex results in the formation of inosine without release of hypoxanthine. Thus, the complex is catalytically competent. Inorganic phosphate or arsenate prevents formation of the tightly-bound E·hypoxanthine complex from inosine or hypoxanthine. Direct binding studies with hypoxanthine in the presence of phosphate result in 3 mol of hypoxanthine bound per trimer with a dissociation constant of 1.6 μM. In the absence of phosphate, three hypoxanthines are bound, but higher hypoxanthine concentrations cause the release of two of the hypoxanthines with an apparent inhibition constant of 130 μM. The results establish that enzymatic contacts with the nucleoside alone are sufficient to destabilize the N-glycosidic bond. In the absence of phosphate, water attacks slowly, causing net hydrolysis. The hydrolytic reaction leaves hypoxanthine stranded at the catalytic site, tightly bound to the enzyme with a conformation related to the transition state. In the phosphorolysis reaction, ribose 1-phosphate causes relaxation of this conformation and rapid release of hypoxanthine.

Purine nucleoside phosphorylase (EC 2.4.2.1) catalyzes the reversible phosphorolysis of purine nucleosides according to the reaction:

purine nucleoside + $P_i \rightleftarrows$ purine base + α-ribose 1-phosphate

A genetic deficiency in the enzyme leads to an immunodeficiency disorder characterized by a lack of T-cells (Giblett et al., 1975). Thus, T-cell-mediated immune responses are deficient. Inhibitor design programs targeted to purine nucleoside phosphorylase have the goals of reducing T-cell-mediated events including tissue rejection, the inflammation in rheumatoid arthritis, and as chemotherapeutics in T-cell proliferative disorders (Stoeckler et al., 1986; Kazmers et al., 1981). As a prelude to transition-state analysis of the enzyme, it is necessary to characterize the reactivity of substrates and the role of phosphate in committing the nucleoside substrate to catalysis. During these studies, a hydrolytic reaction was observed in the absence of phosphate. This work characterizes the hydrolytic reaction. The identification of a complex of enzyme with tightly-bound hypoxanthine has significance in the rational design of inhibitors for the enzyme.

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Purine nucleoside phosphorylase is specific for purine ribo- and 2′-deoxyribonucleosides and does not react with pyrimidines. Purine nucleosides having the β configuration at C1′ are substrates, with the reaction causing inversion of stereochemistry at C1 of ribose 1-phosphate (Parks & Agarwal, 1976). A recent report has indicated that the N3 isomer of inosine is also a substrate for the purine nucleoside phosphorylase isolated from *Escherichia coli* (Lehikoinen et al., 1989). The enzyme has been proposed to undergo a sequential mechanism with both nucleoside and phosphate required to form the reactive complex (Krenitsky, 1967). The enzymes from both human erythrocytes and bovine spleen have trimeric structures with apparent molecular weights of 84 000 and subunit molecular weights near 28 000 (Stoeckler et al., 1978; Edwards et al., 1973). The DNA sequence encoding for purine nucleoside phosphorylase from human erythrocytes has been sequenced and contains 291 amino acids with a computed molecular weight of 32 003 (Williams et al., 1984).

The crystal structure for the human erythrocyte enzyme indicates that each monomer of the trimer contains an active site and each site binds substrate analogues (Ealick et al., 1990). The catalytic sites are located at the interfaces of two subunits; however, only Phe159 from the neighboring monomer is in contact with the substrate analogue at the active site. A phosphate binding site is located near the ribose binding site. A second anion binding site is located near the entrance to the active sites; however, this binding site may be a weak site occupied by the sulfate ions used in the crystallization medium.

This study characterizes the kinetics, intermediates, and products of the hydrolytic activity of purine nucleoside

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phosphorylase in the absence of phosphate. A unique feature of this activity is the formation of a stable enzyme–hypoxanthine complex which is not observed during the phosphorolysis reaction. The chemical nature and stoichiometry of this complex have been established, the kinetics of complex formation have been determined, and direct binding studies have defined the dissociation constants for hypoxanthine. The results indicate that an enzymatic intermediate with characteristics of the transition state accumulates when phosphate is not present to permit the normal catalytic steps to operate. The formation of a similar complex from both inosine and guanosine with human erythrocyte purine nucleoside phosphorylase was reported by Kim et al. (1968). However, in these experiments, the complex was assumed to be an artifact formed from contaminants in the labeled nucleosides. Quantitative analysis of this complex defines its kinetic properties, stoichiometry, reactivity, and relationship to the transition-state structure.

Experimental Procedures

Materials

Inosine, guanosine, hypoxanthine, [8-^{14}C]inosine (50.9 μCi/μmol), [8-^{14}C]hypoxanthine (51.4 μCi/μmol), [8-^{14}C]-adenine (50.8 μCi/μmol), and [6-^{3}H]glucose were obtained from Sigma Chemical Co. α-Ribose 1-phosphate (monocyclohexylammonium salt) and [8-^{14}C]guanosine (56 μCi/μmol) were purchased from ICN Biochemicals. Phosphate-free xanthine oxidase was purchased from Boehringer-Mannheim. Sephadex G-25 resin was obtained from Pharmacia Co. Super Select-D, G-50 spin columns were purchased from 5 Prime → 3 Prime Inc. Purine nucleoside phosphorylase (bovine spleen) in 3.2 M ammonium sulfate was also obtained from Sigma Chemical Co. The enzyme was desalted by gel filtration chromatography on a column of Sephadex G-25 (2.5 × 16.5 cm) eluted with 50 mM triethanolamine, pH 7.5, containing 1 mM dithiotreitol. The purity of the desalted enzyme was determined by denaturing polyacrylamide gel electrophoresis. The bovine spleen enzyme was found to be >99% pure. Enzymes in the desalting buffer were stored at 4 °C and used within 48 h after being desalted.

[8-^{14}C,5′-^{3}H]AMP was synthesized from [6-^{3}H]glucose and [8-^{14}C]adenine as starting materials (Parkin et al., 1984). Labeled AMP was converted to [8-^{14}C,5′-^{3}H]inosine by simultaneous treatment with 2 units of calf intestine alkaline phosphatase and 3 units of adenosine deaminase in 50 mM triethanolamine, pH 8.0, with 1.5 mM $MgCl_2$ at 30 °C. The reaction was monitored by reverse-phase HPLC and quenched by heating to 100 °C for 4 min. The nucleoside was purified by HPLC on a Waters μBondapak C_{18} column eluted with 5% MeOH at 1 mL/min. Fractions containing [8-^{14}C,5′-^{3}H]-inosine were pooled, concentrated, and stored at −70 °C in 50% aqueous ethanol. Synthesis of [1′-^{14}C]inosine used a similar procedure except that [1-^{14}C]ribose was used as starting material.

Methods

Phosphate assays were carried out using a malachite green–molybdate assay (Hess & Derr, 1975; Lanzetta et al., 1979). Radioactive samples were dissolved in 10 mL of LiquiScint (National Diagnostics) and counted for 5 min on a TM Analytic BetaTrac liquid scintillation counter.

Protein Concentrations. Protein concentrations were determined by quantitative dry weight analysis (Schramm, 1976). Purine nucleoside phosphorylase (approximately 20 μg/μL) was dialyzed against 1 mM triethanolamine, pH 7.5. Samples of the dialyzed protein and dialysate were spotted on aluminum disks (previously dried to a constant weight under vacuum) and dried at 120 °C under vacuum to a constant weight. The disks were weighed on a Cahn Model 4600 electrobalance. Protein mass was calibrated against the Bradford dye binding assay for protein (Bradford, 1976) which was then used for routine analysis.

Formation of Complex. The purine nucleoside phosphorylase–hypoxanthine complex was formed by incubating the enzyme from bovine spleen in the presence of nucleoside at 30 °C for 20–40 min. The reaction mixture was applied to a Sephadex G-25 column (2.5 × 16.0 cm) and eluted with 50 mM triethanolamine, pH 7.5. Fractions were collected, and aliquots were removed for analysis of protein and bound hypoxanthine. The pooled complex was then used for additional experiments. The enzyme–hypoxanthine complex could be stored for several hours on ice before use.

UV Spectroscopy. Absorbance spectra were recorded at room temperature on a Perkin Elmer Lambda 6 UV/VIS spectrophotometer interfaced with a Dell System 220 computer. Spectra of purine nucleoside phosphorylase and hypoxanthine or inosine were first recorded in a tandem cell with the beam passing sequentially through the enzyme and hypoxanthine or inosine compartments. The enzyme and inosine solutions were mixed and the spectra recorded immediately after mixing and at 20 min after mixing.

Identification of Reaction Products. The purine nucleoside phosphorylase–hypoxanthine complex was formed as described above using [8-^{14}C,5′-^{3}H]inosine as the substrate. After 40 min, the reaction mixture was loaded on a Sephadex G-25 column (1.5 × 85 cm) and eluted with distilled water. Fractions were collected, and aliquots were removed and assayed for protein and radioactivity. Double-channel liquid scintillation counting was used to determine counts in the ^{3}H and ^{14}C channels. The distribution of ^{14}C radioactivity appearing in each channel was determined with [6-^{14}C]glucose as the ^{14}C standard. The enzyme-hypoxanthine peak was denatured with 10% trichloroacetic acid and the protein removed by centrifugation. Samples were analyzed by HPLC as described below. The peak containing ^{3}H but no ^{14}C (the second peak in Figure 4A) was concentrated at 4 °C. This procedure prevents hydrolysis of ribose 1-phosphate formed from adventitious phosphate. This peak was analyzed on a DEAE-Sephadex A-50 column (acetate form, 2.5 × 15 cm). The column was washed with 50 mL of 50 mM sodium acetate, pH 5.5, and eluted with a linear gradient of 50–500 mM sodium acetate, pH 5.5 (500 mL). Fractions were collected and aliquots were removed for scintillation counting. The third peak (fractions 31–51) from the Sephadex G-25 column (Figure 4A) contained both ^{14}C and ^{3}H. It was concentrated under reduced pressure at 30 °C and chromatographed on a reverse-phase Waters C_{18} μBondapak column. The column was eluted with 5% MeOH at 1 mL/min, and the components were identified by their retention times compared to authentic ribose, ribose 1-phosphate, inosine, and hypoxanthine.

Mass Spectral Analysis of Ligands Released from the Complex. The purine nucleoside phosphorylase complex was formed using inosine as described above. The complex was loaded on a Sephadex G-25 column (1.5 × 45.8 cm) and eluted with 50 mM triethanolamine, pH 7.5. Fractions containing protein were pooled and allowed to dissociate for 48 h at 30 °C or were dissociated by 1 N HCl. The reaction mixture was loaded on a second Sephadex G-25 column (1.5 × 45.8 cm) and the small molecule peak pooled, lyophilized, and analyzed by fast atom bombardment in a glycerol–thioglycerol matrix on a Finnigan MAT 90 mass spectrometer.

The complex was formed from inosine and purified on a Sephadex G-25 column (1.5 × 45.8 cm). α-Ribose 1-phosphate (100 μM) was added and the reaction mixture incubated at 30 °C for 10 min. The reaction mixture was loaded on a Sephadex G-25 column (1.5 × 45.8 cm) and eluted with 50 mM triethanolamine, pH 7.5. The peak containing small molecules was concentrated under reduced pressure and was chromatographed on a Waters μBondapak C_{18} HPLC column eluted with 5% MeOH. Peaks were pooled, lyophilized, and analyzed by mass spectrometry.

Determination of Complex Stability. The purine nucleoside phosphorylase–hypoxanthine complex was prepared and purified by gel filtration as described above. The complex (8 nmol) was dialyzed against 1 L of 50 mM triethanolamine, pH 7.5, at 4 °C and at 30 °C. Aliquots of the reaction mixture (20 μL) were removed and counted until 95% of the radioactivity had dissociated.

Stoichiometry of the Purine Nucleoside Phosphorylase–Hypoxanthine Complex. Purine nucleoside phosphorylase (1 nmol of trimer) was incubated for 30 min at 30 °C in the presence of 3, 5, 10, 25, and 50 nmol of [8-^{14}C]inosine in a total volume of 250 μL. Reaction mixtures were loaded on Sephadex G-50 spin columns and centrifuged for 6 min. The effluent solutions were analyzed for protein and radioactivity. Control reaction mixtures without enzyme were treated in the same manner, and the radioactivity was subtracted from the reaction mixtures with enzyme. Similar experiments used [8-^{14}C]hypoxanthine as the substrate for complex formation.

Effect of Xanthine Oxidase on Complex Formation. Purine nucleoside phosphorylase (30 μM), [8-^{14}C]inosine (150 μM) (5 × 10^4 cpm), and xanthine oxidase (3 units) in 1 mL of 50 mM triethanolamine, pH 7.5, were incubated for 30 min at 30 °C. The reaction mixture was loaded on a Sephadex G-25 column (1.5 × 45.8 cm) and eluted with the triethanolamine buffer. Fractions were assayed for radioactivity and protein. The protein peak was concentrated using an Amicon Centricon 10 microconcentrator and was denatured by the addition of trichloroacetic acid. Denatured protein was removed by centrifugation, and the supernate was resolved on a Waters μBondapak C_{18} HPLC column eluted with 1% methanol in H_2O. The radioactive peak was concentrated to 1 mL. The UV absorbance spectrum was compared to that of inosine, hypoxanthine, xanthine, and uric acid in the same solvent.

In a control experiment, 30 μM hypoxanthine in a reaction mixture without purine nucleoside phosphorylase resulted in >95% oxidation of hypoxanthine in the same incubation period. Conversion was monitored by thin-layer chromatography of the reaction mixture. The labeled products were quantitated by Ambis-scanner radioanalytic counting.

Catalytic Competence of the Purine Nucleoside Phosphorylase–Hypoxanthine Complex. The purine nucleoside phosphorylase–hypoxanthine complex was prepared and purified by chromatography on a Sephadex G-25 column as described above. The purified complex (2 nmol) was mixed with 3 units of xanthine oxidase, followed by 21 nmol of α-ribose 1-phosphate. The reaction mixture was incubated for 5 min at 30 °C and the reaction quenched by addition of trichloroacetic acid to 10%. Denatured protein was removed by centrifugation and the supernatant analyzed by HPLC on a Waters C_{18} μBondapak column eluted with 1% methanol in H_2O. The products were identified by their retention times. In control experiments, 2 nmol of [8-^{14}C]hypoxanthine and 21 nmol of ribose 1-phosphate were treated with a mixture of 2 nmol of purine nucleoside phosphorylase and 3 units of xanthine oxidase. No inosine was formed, and >95% of the labeled hypoxanthine was oxidized by xanthine oxidase.

Effect of Hypoxanthine on Complex Stability. The complex was formed with [8-^{14}C]inosine as described above and isolated on a Sephadex G-50 spin column. The purified complex was chromatographed on a Sephadex G-25 column (2.5 × 15.6 cm) equilibrated with 50 mM triethanolamine, pH 7.5, 1 mM dithiothreitol, and 1 mM hypoxanthine. Fractions were collected and assayed for radioactivity and protein. The results were compared to similar experiments in which hypoxanthine was excluded from the Sephadex G-25 column.

Rate Constant for Formation of the Purine Nucleoside Phosphorylase–Hypoxanthine Complex. A reaction mixture containing 15.4 μM [8-^{14}C]inosine and 1.2 μM purine nucleoside phosphorylase in 50 mM triethanolamine, pH 7.5, was incubated at 30 °C. At specified times, samples were placed on Sephadex G-50 spin columns and centrifuged for 6 min. The effluent containing the purine nucleoside phosphorylase–hypoxanthine complex was collected and counted. Reaction mixture without enzyme was treated in the same way as a control experiment to correct for the fraction of counts that eluted in the absence of enzyme. The rates of complex formation and decomposition were determined by incubating 1.2 μM purine nucleoside phosphorylase with 15.4 μM [8-^{14}C]inosine as described above. The reaction was quenched by the addition of 50 μL of 1 N HCl, followed by chromatography on a reversed-phase Waters μBondapak C_{18} HPLC column eluted with 5% MeOH. The first peak contained [8-^{14}C]hypoxanthine, and the second contained [8-^{14}C]inosine. The content of free hypoxanthine in the reaction mixture was calculated by subtracting enzyme-bound hypoxanthine from the total hypoxanthine formed under the same conditions.

Ultrafiltration Binding Study. Binding stoichiometries used a modification of the ultrafiltration method (Paulus, 1969; Schramm, 1976). The ultrafiltration apparatus was assembled with dialysis membranes (molecular weight retention = 10000–14000) and dried under vacuum. Reagents, unlabeled hypoxanthine, [8-^{14}C]hypoxanthine, and enzyme solutions were added by syringe, and the system was pressurized to 20 psi with N_2. After 5 h, the ultrafiltration was complete, and the lower portion of the membranes was thoroughly washed with cold ethylene glycol. The radioactivity retained with enzyme on each membrane was determined by scintillation counting. Controls corrected for amounts of label imbibed in the volume of the membrane and for nonspecific binding to the enzyme and membrane (Schramm, 1976).

RESULTS

Formation of the Purine Nucleoside Phosphorylase–Hypoxanthine Complex and Identification of Components. In the presence of phosphate, purine nucleoside phosphorylase catalyzes the phosphorolysis of purine nucleosides to yield the corresponding base and α-ribose 1-phosphate. It has been reported that purine nucleoside phosphorylase does not hydrolyze nucleosides in the absence of phosphate (Parks et al., 1976). However, with stoichiometric amounts of inosine or guanosine and enzyme, a slow hydrolysis of the nucleoside occurs in the absence of phosphate. The rates are sufficiently rapid to preclude chemical decomposition of the nucleosides or cycling with trace amounts of phosphate. When [8-^{14}C]-inosine was incubated with purine nucleoside phosphorylase in the absence of phosphate and chromatographed on a column of Sephadex G-25, a radioactive component coeluted with the protein peak (Figure 1A). An equivalent experiment with [8-^{14}C]guanosine did not result in labeling of the enzyme peak (Figure 1B).

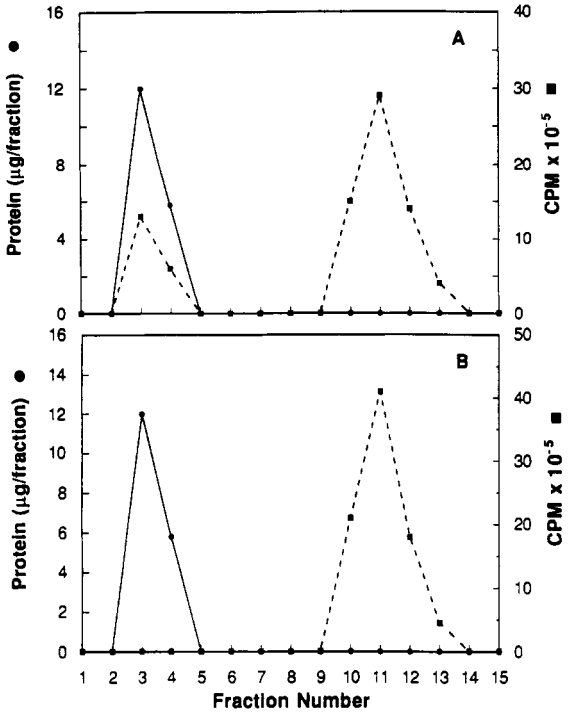


FIGURE 1: Formation of the purine nucleoside phosphorylase–hypoxanthine complex. (A) The enzyme (5 μM) was incubated with 25 μM [8-^{14}C]inosine at 30 °C for 30 min. The reaction mixture was chromatographed on a Sephadex G-25 column with 50 mM triethanolamine hydrochloride, pH 7.5. The equivalent experiment using [8-^{14}C]guanosine is shown in (B).

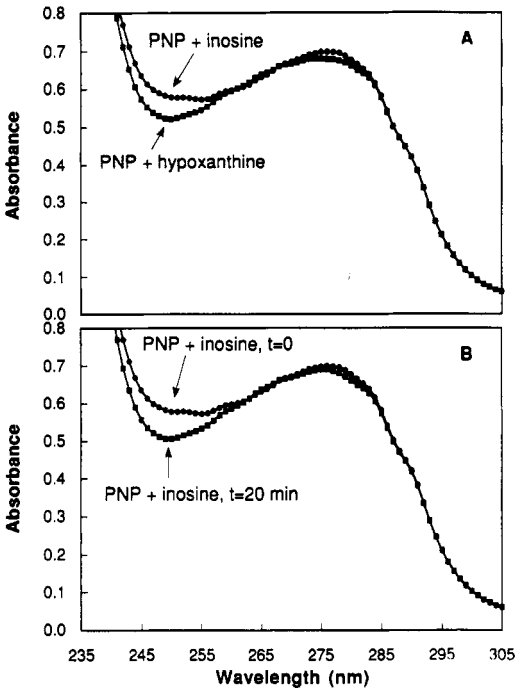


FIGURE 2: Ultraviolet absorbance spectra of purine nucleoside phosphorylase (PNP) with inosine or hypoxanthine. In (A), the enzyme, inosine, and hypoxanthine were each present at 53 μM in the tandem compartments of a divided cuvette. The tandem cell prevents interaction of the enzyme with substrates. In (B), the ultraviolet spectra were recorded immediately after 53 μM purine nucleoside phosphorylase was mixed with 53 μM inosine (t = 0) and after 20-min incubation at room temperature.

The ultraviolet absorbance spectra of purine nucleoside phosphorylase plus hypoxanthine and of purine nucleoside phosphorylase plus inosine were recorded and compared in an effort to determine if a complex is formed with altered spectral characteristics (Figure 2A). Spectra were recorded using a tandem cuvette with purine nucleoside phosphorylase in one chamber and hypoxanthine or inosine in the other. The spectra were first recorded before mixing. Purine nucleoside phosphorylase and inosine were then mixed and the spectra recorded as the complex formed. Immediately after mixing, the spectra resemble purine nucleoside phosphorylase plus inosine. As the reaction progressed, the ultraviolet absorbance spectrum changed to resemble purine nucleoside phosphorylase plus hypoxanthine (Figure 2B). This result indicated that hydrolysis of the N-glycosidic bond occurs without the formation of a covalent complex with ultraviolet spectral characteristics different from the combined properties of enzyme and hypoxanthine.

The ultraviolet absorbance spectrum which indicated the hydrolysis of the N-glycosidic bond of inosine does not resolve whether hypoxanthine and/or ribose remains bound to the enzyme. When [1′-^{14}C]inosine was incubated with purine nucleoside phosphorylase, no radioactivity eluted with the protein peak after gel filtration chromatography, and all ^{14}C eluted with the small molecular weight peak, similar to the results of Figure 1B. The ribose moiety was therefore released rapidly from purine nucleoside phosphorylase relative to the rate of hypoxanthine release.

The products of the reaction between purine nucleoside phosphorylase and inosine were further characterized by mass spectrometry. The complex was formed from purine nucleoside phosphorylase and inosine, purified by chromatography on a Sephadex G-25 column, and dissociated for 48 h at 30 °C as described under Methods. The dissociation products were resolved on a second Sephadex G-25 chromatography step. The fractions containing small molecules were pooled, lyophilized, and subjected to analysis by mass spectrometry. The mass spectrum of the ligand released from the complex with purine nucleoside phosphorylase was identified as hypoxanthine (Figure 3A). The same results were obtained when the enzyme·hypoxanthine complex was dissociated by 1 N HCl and analyzed as described above.

Stoichiometry of the Enzyme·Hypoxanthine Complex. Purine nucleoside phosphorylase is a trimer of 28 000 molecular weight subunits with each monomer containing one active site (Stoeckler et al., 1978; Edwards et al., 1973). Formation of the tightly-bound complex of enzyme·hypoxanthine from enzyme and inosine gave a stoichiometry of 0.96 ± 0.07 mol of hypoxanthine bound/mol of purine nucleoside phosphorylase trimer (Table I). The stoichiometry is independent of enzyme concentration over the range from 1 to 49 μM and inosine concentrations where inosine is present at molar ratio of >1 relative to the trimer of purine nucleoside phosphorylase. Thus, only one of the three active sites in the enzyme is occupied by hypoxanthine in the complex. Since excess inosine is present and does not react, the neighboring sites do not function following complex formation at the first site.

A stoichiometry of 0.98 mol of hypoxanthine bound/trimer was obtained when xanthine oxidase was included at 3 units/mL in the presence of purine nucleoside phosphorylase and inosine. The tightly-bound material was characterized as hypoxanthine by UV spectroscopy as described under Methods. Formation of free hypoxanthine from inosine followed by binding to the enzyme could not occur in the presence of excess xanthine oxidase.

Catalytic Competence of Bound Hypoxanthine. The catalytic competence of tightly-bound hypoxanthine in the complex was established by the addition of α-ribose 1-phosphate. The products were resolved by C_{18} HPLC chromatography. The peaks which absorbed in the ultraviolet were identified

Table I: Formation of the Purine Nucleoside Phosphorylase·[8-^{14}C]Hypoxanthine Complex[a]

reactants			product		
	[8-^{14}C]inosine		enzyme·[8-^{14}C]hypoxanthine		mol of hypoxanthine
enzyme trimer (μM)	μM	cpm	μM	cpm	bound/mol of enzyme trimer
4.0	12	3.39×10^5	3.68	1.04×10^5	0.92
4.0	20	5.65×10^5	3.80	1.07×10^5	0.95
4.0	40	1.13×10^6	3.64	1.03×10^5	0.91
4.0	100	2.82×10^6	3.88	1.10×10^5	0.97
4.0	200	5.64×10^6	3.72	1.05×10^5	0.93
1.7	20	2.26×10^6	1.6	1.77×10^5	0.94
8.1	39	4.44×10^6	7.4	8.32×10^5	0.91
11.1	39	4.44×10^6	10.9	1.23×10^6	0.98
49	100	1.13×10^7	55.5	6.27×10^6	1.13
					av ± SD 0.96 ± 0.07

[a] The enzyme·[8-^{14}C]hypoxanthine complex was formed at 30 °C in 50 mM triethanolamine, pH 7.5, for 30 min using the reactant concentrations in the table. The enzyme-hypoxanthine complex was isolated on columns of Sephadex G-25 eluted with the same buffer. The columns gave base-line resolution of the complex from free hypoxanthine and inosine (see Figures 1A and 4A). The stoichiometry of bound hypoxanthine per mole of enzyme was analyzed by the protein content of the peak and the ^{14}C content associated with the protein peak.

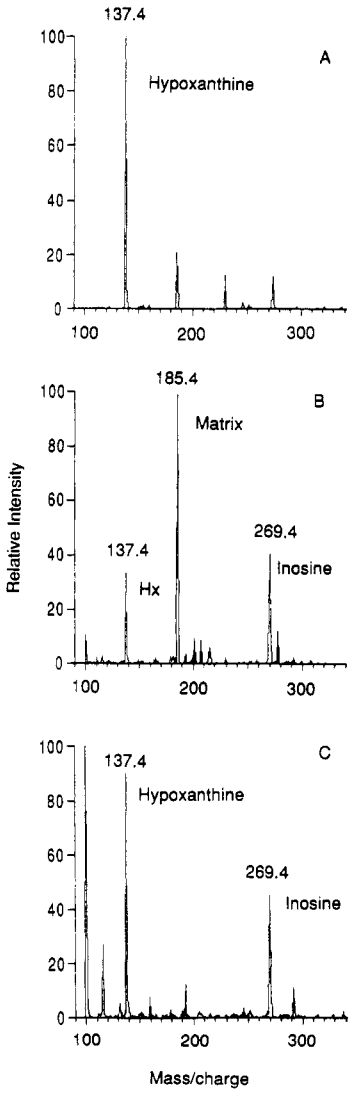


FIGURE 3: Fast atom bombardment mass spectrometry (FAB-MS) of ligand released from purine nucleoside phosphorylase complex. In (A), the major peak at m/z 137.4 is the hypoxanthine M + 1 peak. In (B), FAB-MS of the small molecular weight molecules released from the complex of purine nucleoside phosphorylase and hypoxanthine (Hx) after incubation with ribose 1-phosphate and chromatography on a Sephadex G-25 column is shown. The peak at m/z 269.4 is the M + 1 peak for inosine. Hypoxanthine at mass 137.4 results from incomplete conversion of enzyme-bound hypoxanthine to inosine and some from glycoside bond hydrolysis of inosine during mass analysis. The peak labeled "Matrix" comes from medium used to introduce the sample. An authentic sample of inosine was subjected to FAB-MS and is shown in (C).

as hypoxanthine and inosine by mass spectrometry (Figure 3B). In a similar experiment, the complex was mixed with xanthine oxidase and ribose 1-phosphate incubated for 5 min, and denatured with trichloroacetic acid, and the products were analyzed by HPLC. In three such experiments, the conversion of bound hypoxanthine to inosine was 73 ± 24%. In experiments with xanthine oxidase, no xanthine or uric acid could be detected. However, incubation of the same amount of free hypoxanthine with xanthine oxidase for 5 min resulted in >95% oxidation of hypoxanthine. The results indicate that hypoxanthine is not released from the enzyme prior to conversion to inosine.

Effect of Phosphate on Complex Formation. All reagents were analyzed for residual phosphate, and none was detected at a sensitivity of 5 pmol/mL. The possibility that phosphate was bound to enzyme or was present in covalent form in enzyme preparations was investigated by digesting the enzyme in concentrated nitric acid and assaying for phosphate. The level of phosphate was below the detection level of the assay which would have detected less than 0.3 mol of phosphate/mol of enzyme subunit. From these results, low levels of phosphate cannot account for the stoichiometric formation of the enzyme–hypoxanthine complex. In addition, the phosphorolysis reaction does not lead to the formation of a stable enzyme–hypoxanthine complex.

If phosphate were responsible for the observed cleavage of the N-glycosidic bond of inosine, a product of the reaction would be α-ribose 1-phosphate. The complex with purine nucleoside phosphorylase was formed from enzyme and [8-^{14}C,5′-^{3}H]inosine, and the reaction mixture was chromatographed on a Sephadex G-25 column (Figure 4A). The peak containing the enzyme contained only ^{14}C, indicating that hypoxanthine was bound in the complex. The peak of small molecular weight material which eluted after the enzyme–hypoxanthine complex (Figure 4A) contained only tritium. This peak was chromatographed on a column of DEAE-Sephadex, and the ^{3}H-labeled peak consisted only of ribose with no detectable ribose 1-phosphate (Figure 4B). From the specific radioactivity of the nucleoside, 0.01 nmol of α-ribose 1-phosphate would have been detected in experiments which formed 11 nmol of enzyme–hypoxanthine complex. Thus, less than 0.1% of the ribose released from inosine is present as α-ribose 1-phosphate following hydrolysis of inosine by purine nucleoside phosphorylase. The third peak resolved from the reaction of purine nucleoside phosphorylase with inosine contained a mixture of ^{3}H and ^{14}C (Figure 4A). Reverse-phase HPLC identified two peaks with retention times consistent with hypoxanthine and inosine (Figure 4C). The hypoxanthine peak

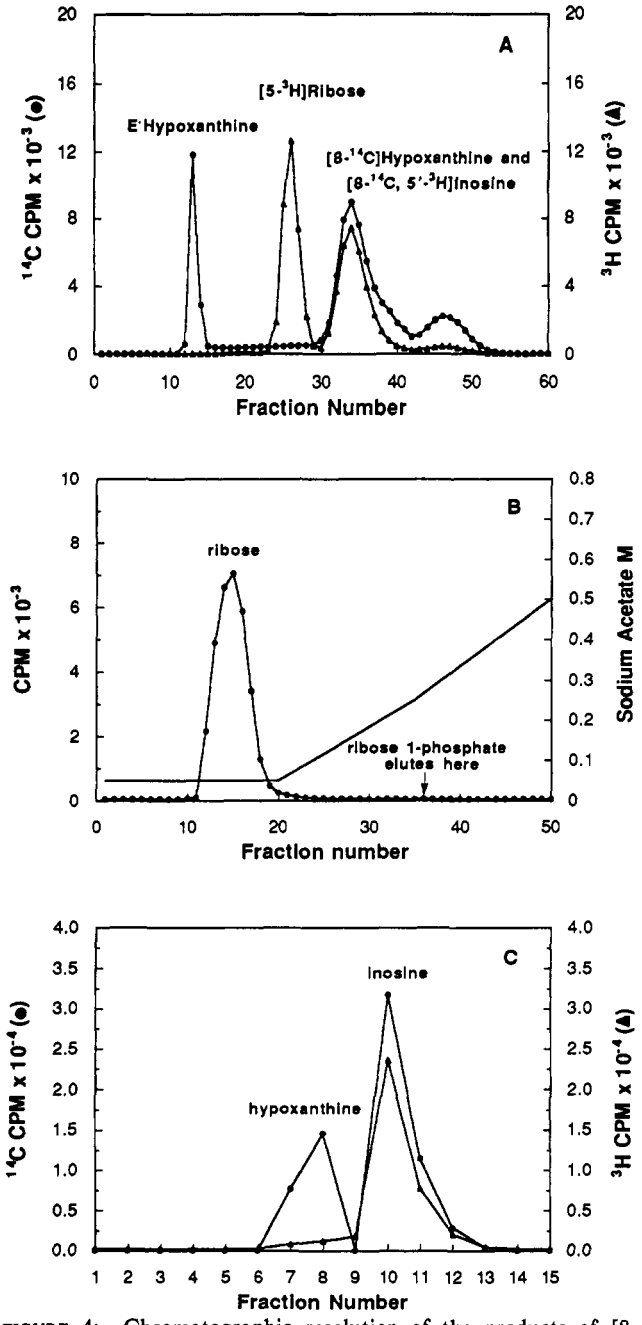


FIGURE 4: Chromatographic resolution of the products of [8-^{14}C,5'-^{3}H]inosine hydrolysis by purine nucleoside phosphorylase. (A) Elution profile of the Sephadex G-25 column after incubating 13 μM purine nucleoside phosphorylase with 75 μM [8-^{14}C,5'-^{3}H]inosine in the absence of phosphate. Radioactivity was determined by scintillation counting. Protein was determined by the Bradford dye binding method (see Methods). The peak of protein eluted in fraction 13 and is labeled "E·Hypoxanthine" in panel A. (B) Elution profile of peak 2 from the Sephadex G-25 column (A) on a DEAE-Sephadex A-50 column. The arrow indicates the elution position of ribose 1-phosphate at fractions 30–40. (C) Elution profile of peak 3 from the Sephadex G-25 column on a reverse-phase C_{18} HPLC column.

contained only ^{14}C, and both ^{3}H and ^{14}C were associated with the unreacted inosine peak.

The effect of phosphate on formation of the purine nucleoside phosphorylase–hypoxanthine complex was established by measuring the amount of complex formed at various phosphate concentrations. The results of Table II demonstrate that addition of phosphate at stoichiometries of 0.08 and 0.8 mol/mol of purine nucleoside phosphorylase trimer had no effect on formation of the enzyme–hypoxanthine complex. At a stoichiometry of 8 mol of phosphate/mol of trimer, phosphorolysis predominates, and less than 3% of the enzyme forms the complex with hypoxanthine.

Table II: Effect of Phosphate on the Formation of the Complex of Purine Nucleoside Phosphorylase with Hypoxanthine[a]

phosphate concn (μM)	purine nucleoside phosphorylase concn (μM)	bound hypoxanthine concn (μM)
0	1–49	0.96 ± 0.07[b]
0.1	1.3	1.16
1	1.3	1.14
10	1.3	0.26

[a] Reaction mixtures in 50 mM triethanolamine hydrochloride, pH 7.5 (250 μL), containing 15 μM [8-^{14}C]inosine, 1.3 μM purine nucleoside phosphorylase and the indicated concentration of potassium phosphate were incubated for 20 min at 30 °C. The samples were loaded on a Sephadex G-50 spin column and centrifuged for 6 min. Effluent was analyzed for protein and radioactivity. [b] The reaction mixtures used to determine the stoichiometry in the absence of phosphate used a range of enzyme concentrations. The average values from experiments using different enzyme preparations and [8-^{14}C]inosine were calculated from Table I.

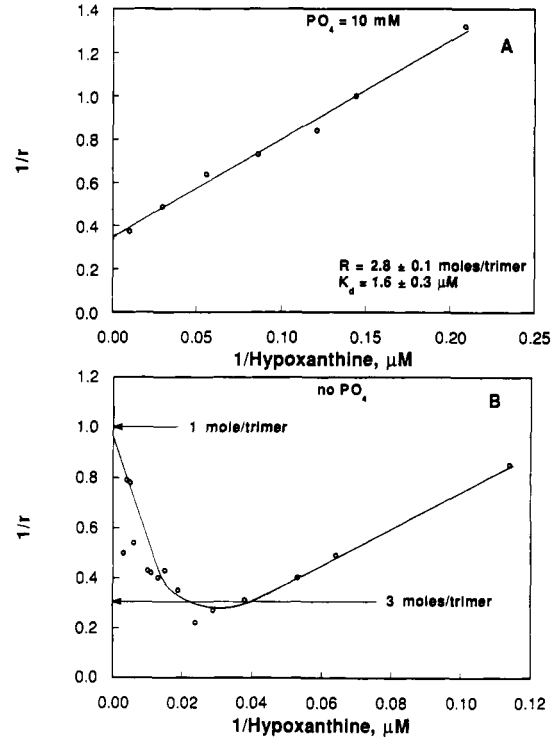


FIGURE 5: Equilibrium binding of [8-^{14}C]hypoxanthine to purine nucleoside phosphorylase. (A) Binding of hypoxanthine to purine nucleoside phosphorylase in the presence of 10 mM potassium phosphate. Each chamber contained approximately 4 μM purine nucleoside phosphorylase and 0.07 μCi [8-^{14}C]hypoxanthine. The line is the linear least-squares fit to the equation describing a normal binding isotherm. (B) Binding of hypoxanthine to purine nucleoside phosphorylase in the absence of phosphate. The line is drawn by eye to fit the experimental points. The concentrations of purine nucleoside phosphorylase and the quantity of [8-^{14}C]hypoxanthine were the same as in (A). In all experiments, the total hypoxanthine concentration was established by the addition of variable amounts of unlabeled hypoxanthine. All binding experiments were at room temperature. Additional details are provided under Methods.

Equilibrium Binding of Hypoxanthine. Binding of hypoxanthine in the presence of phosphate was a hyperbolic function of the free hypoxanthine concentration and was fit to the equation:

$$1/r = (K_d/R)(1/A) + 1/R$$

where r = the moles of ligand bound per mole of enzyme, R = the moles of ligand bound per mole of enzyme at saturating

Table III: Formation of Purine Nucleoside Phosphorylase·[8-^{14}C]Hypoxanthine from Hypoxanthine[a]

reactants			product		
	[8-^{14}C]hypoxanthine		enzyme[8-^{14}C]hypoxanthine		
enzyme trimer (μM)	μM	cpm	μM	cpm	mol of hypoxanthine bound/mol of enzyme trimer
4.0	4.0	1.14×10^5	3.92	1.12×10^5	0.99
4.0	20.0	5.70×10^5	3.80	1.08×10^5	0.95
4.0	60.0	1.71×10^6	4.40	1.26×10^5	1.10
					av ± SD 1.01 ± 0.08

[a] The tightly-bound complex of enzyme·hypoxanthine was formed by incubation for 30 min at 30 °C. The enzyme complex was separated from unbound hypoxanthine by spun gel filtration columns as described under Methods.

ligand concentration, K_d = the dissociation constant for the enzyme–ligand complex, and A = the free ligand concentration. In the presence of 10 mM phosphate, three binding sites were found per trimer (Figure 5A). This stoichiometry agrees with the crystal structure data in which each monomer contains a single active site saturated with substrate analogue (Ealick et al., 1990). The dissociation constant was found to be 1.6 μM, which is equal to the value of 1.6 μM reported for the human enzyme (Agarwal & Parks, 1969). In the absence of phosphate, all three binding sites are occupied at intermediate concentrations of hypoxanthine near 30 μM (Figure 5B). As the hypoxanthine concentration increased from 30 to 300 μM, only a single hypoxanthine remained bound. The hypoxanthine binding curve in the absence of phosphate could not be fit to the simple equation for total substrate inhibition [$r = RA/K_d + A(1 + A/K_i)$] or to third-order binding functions. The variability in the results at high substrate concentration, where binding data become less accurate and/or there is complexity of the binding isotherm, can account for this observation. Note that in Scheme II six enzyme intermediates can exist (see Discussion). The observed inhibition of binding occurs at an apparent inhibition constant of 130 μM, a concentration 80-fold greater than the measured K_d of 1.6 μM for hypoxanthine in the presence of phosphate.

Stability of the Enzyme–Hypoxanthine Complex. The purified purine nucleoside phosphorylase–hypoxanthine complex was dialyzed and the loss of hypoxanthine measured as a function of time. The dissociation of hypoxanthine occurred with a first-order rate constant of 1.9×10^{-6} s^{-1} at 4 °C, while at 30 °C the rate constant was 1.3×10^{-4} s^{-1}. The radioactive product released in these experiments was identified as hypoxanthine by HPLC chromatography (see Methods). Addition of arsenate to the purified complex of enzyme–hypoxanthine doubled the first-order rate constant for hypoxanthine release. At 5 mM arsenate and 7 μM enzyme–hypoxanthine complex, the rate was approximately 3×10^{-4} s^{-1} at 30 °C.

The chemical stability of the complex was determined in the presence of denaturing reagents, including ethanol, trichloroacetic acid, urea, guanidine hydrochloride, and NaOH. Purified complex was incubated for 5 min with the reagents, and the reaction mixture was resolved by gel filtration. In every case, the fractions containing the small molecular weight components contained hypoxanthine equivalent to the amount bound in the enzyme complex. These results demonstrate that the complex was rapidly dissociated from the enzyme by the addition of denaturing reagents. Enzymatic digestion of the purified complex with trypsin also resulted in the destruction of the complex. Reduction of enzyme–hypoxanthine with sodium borohydride also resulted in release of hypoxanthine.

Effects of Hypoxanthine on Formation and Dissociation of the Complex. The complex of purine nucleoside phosphorylase and hypoxanthine was formed from enzyme and [8-^{14}C]inosine and isolated by chromatography on a column of Sephadex G-25. The complex contained one [8-^{14}C]-hypoxanthine per trimer. In a parallel experiment, the complex was passed through a column previously equilibrated with 1 mM hypoxanthine. The enzyme was isolated without bound [8-^{14}C]hypoxanthine, demonstrating that tightly-bound hypoxanthine exchanges with unlabeled hypoxanthine during chromatography.

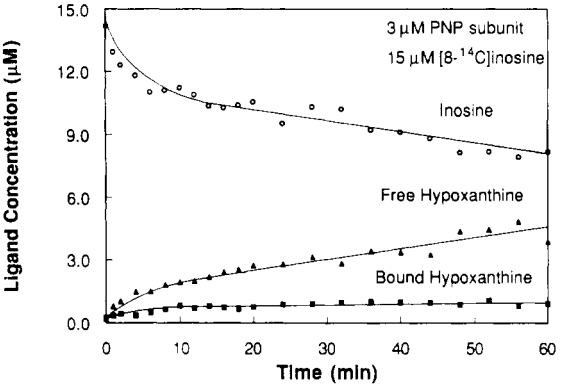
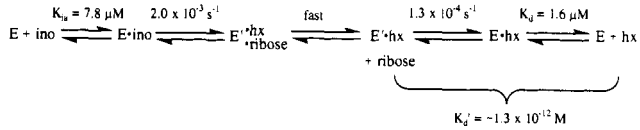


FIGURE 6: Kinetics of formation of the purine nucleoside phosphorylase–hypoxanthine complex. Hypoxanthine bound to the enzyme was determined by incubating [8-^{14}C]inosine with purine nucleoside phosphorylase followed by rapid chromatography on a Super Select-D G-50 spin column. Total hypoxanthine concentration was determined by quenching the reaction with 1 N HCl followed by chromatography of the mixture on a reverse-phase C_{18} HPLC column as described under Methods. Free hypoxanthine concentration was determined by subtracting bound hypoxanthine concentration from total hypoxanthine concentration. The lines were drawn by eye to fit the experimental data points.

Incubation of 4 μM enzyme with 4, 20, and 60 μM [8-^{14}C]hypoxanthine for 30 min at 30 °C was followed by G-25 column chromatography as described under Methods. Radioactive hypoxanthine was tightly bound to the enzyme with an average of 1.01 ± 0.08 mol of hypoxanthine per trimer of purine nucleoside phosphorylase. All concentrations of [8-^{14}C]hypoxanthine gave a stoichiometry of 1 mol of hypoxanthine bound per trimer (Table III).

Kinetics of Enzyme–Hypoxanthine Formation. The time course for formation of the enzyme–hypoxanthine complex demonstrates a relatively rapid formation of the complex to a steady-state level of 1 mol of hypoxanthine bound to each trimer of purine nucleoside phosphorylase (Figure 6). In separate experiments which characterized the rate of enzyme–hypoxanthine formation, a rate of 2.0×10^{-3} s^{-1} was determined for complex formation. In these experiments, excess [8-^{14}C]inosine was used to provide pseudo-first-order conditions. Once the complex formed, the concentration of free hypoxanthine increased as hypoxanthine was slowly released from the complex. The rate-limiting step of the reaction is the dissociation of hypoxanthine which occurs at 1.3×10^{-4} s^{-1} at 30 °C following isolation of the complex. If hydrolysis of inosine were slower than dissociation of hypoxanthine, the concentration of complex would maintain a steady-state level of less than 1 mol/mol of enzyme, and a tightly-bound complex

Scheme I: Kinetic, Rate, and Equilibrium Constants for the Hydrolytic Reaction of Purine Nucleoside Phosphorylase with Inosine[a]



[a] The purine nucleoside phosphorylase trimer is abbreviated E, inosine = ino, hypoxanthine = hx, and the tight-binding conformation of enzyme is E′. K_{ia} is the kinetic dissociation constant for the E·ino complex. K_d is the dissociation constnt for the E·hx complex. K_d' is an apparent dissociation constant for dissociation of the E′·hx complex.

would not be observed.

Discussion

Kinetics of Inosine Hydrolysis. The kinetic steps of inosine hydrolysis by purine nucleoside phosphorylase from calf spleen are summarized in Scheme I. The K_{ia} value of 7.8 μM is the apparent dissociation constant for the enzyme–inosine complex from steady-state kinetic studies extrapolated to zero concentration of arsenate. This value compares to apparent K_m values of 13 and 19 μM from other studies of the calf spleen enzyme (Agarwal et al., 1975; Bzowska et al., 1988). The rate of the hydrolytic step on the enzyme is 2.0×10^{-3} s^{-1} compared to the turnover number of 12 s^{-1} for phosphorolysis at a specific activity of 22 μmol min^{-1} mg^{-1}. Recent reports indicate that the calf spleen enzyme exists as a trimer at concentrations above 0.5 μM and exhibits a 50-fold decrease in specific activity in this form (Ropp & Traut, 1991). Concentrations above 0.5 μM were used in the hydrolytic experiments described here, so the pertinent turnover numbers are 2.0×10^{-3} and 0.24 s^{-1} for comparison of the hydrolytic step of catalysis and the phosphorolysis turnover number for trimer enzyme. Phosphorolysis thus occurs 114-fold more efficiently than hydrolysis in the trimeric form of the enzyme.

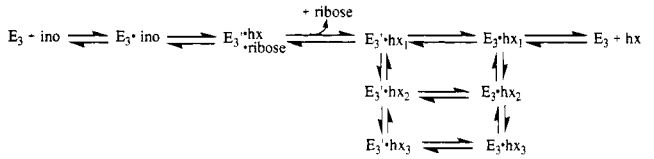
Inosine hydrolysis and the release of ribose are rapid relative to the release of hypoxanthine, since the isolated enzyme·hypoxanthine complex contains only hypoxanthine. The slowest step in the hydrolytic reaction is hypoxanthine release, as evidenced by the pre-steady-state burst of hypoxanthine during inosine hydrolysis. Hypoxanthine release also occurs in the phosphorolysis reaction and is proposed to follow release of ribose 1-phosphate in an ordered sequential mechanism (Lewis & Glantz, 1976; Krenitsky, 1967; Agarwal & Parks, 1969). The release of hypoxanthine in the hydrolytic reaction is too slow to accommodate the rate of the phosphorolysis reaction and indicates that a conformational change is required in the enzyme to permit hypoxanthine release (E′·hx → E·hx in Scheme I). The release of ribose 1-phosphate in the normal reaction sequence is proposed to cause a rapid structural relaxation which facilitates hypoxanthine release. Since the trimer is capable of phosphorolysis at rates 114-fold and the monomer at rates 5700-fold greater than the observed rate of hypoxanthine release for the hydrolytic reaction, this structural change lowers the energy barrier for hypoxanthine release by a minimum of 2.9–5.2 kcal/mol. The 3.2-Å crystal structure of the trimeric enzyme from human erythrocytes with bound 5′-iodoformycin B indicates only a small number of specific contacts to the ribose. In contrast, the proposed phosphate binding site is reported to be in hydrogen-bonding distance to Ser33, Arg84, His86, Ser220, Ser33, Ala116, and the 3′-hydroxyl of the ribosyl group (Ealick et al., 1990). Phosphate alone is inefficient in causing the conformational relaxation required for rapid hypoxanthine release. Data from the crystal structure suggest that inosine binding causes a disordered loop to be converted to a helix which then blocks release of products from the catalytic site. Ribose 1-phosphate exit is proposed to return the helix to the loop. We propose that ribose escapes without changing the blocking helix. This causes tight binding of hypoxanthine. This hypothesis is supported by the independent experimental observation that ribose 1-phosphate but not phosphate causes the rapid release of tightly-bound hypoxanthine.

Stoichiometry of Hypoxanthine Binding and Catalytic Turnover. Equilibrium binding studies of [^{14}C]hypoxanthine to purine nucleoside phosphorylase indicated the independent binding of 3 mol per trimer of enzyme in the presence of inorganic phosphate. The observed dissociation constant of 1.6 μM for hypoxanthine agrees well with kinetic studies which have provided a value of 3 μM as a product inhibition constant (Bzowska et al., 1988). The identification of three sites per trimer is expected on the basis of the X-ray crystal structure which clearly locates one substrate analogue inhibitor at each of three structurally equivalent binding sites. A dissociation constant of 1.6 μM cannot account for the slow release of hypoxanthine observed for inosine hydrolysis. Thus, an enzymatic isomerization (E′·hx ⇄ E·hx in Scheme I) exists between the tightly-bound complex and the complex which is capable of binding 3 mol of hypoxanthine at 1.6 μM.

The catalytic competence of tightly-bound hypoxanthine requires that titration of enzyme with hypoxanthine leads to the tight binding of one hypoxanthine per trimer subsequent to the binding of three hypoxanthines per trimer. This is an unusual and diagnostic prediction for the mechanism of Scheme I. In direct binding studies at concentrations of hypoxanthine 30–200-fold above the K_d, partial reversal of hypoxanthine binding from 3 → 1 mol/trimer occurs in the absence of phosphate. Reversal of binding as hypoxanthine increases can only occur if already-saturated sites are disrupted, causing ligand release. This step is represented in Scheme I by the thermodynamic reversal of the step E′·hx → E·hx. The slow kinetic rate of conversion from E′·hx to E·hx demonstrates that the transition-state barrier between these complexes is high. However, only a stoichiometric concentration of hypoxanthine is required to convert the enzyme to the E′·hx complex. This conversion reflects the tight binding of hypoxanthine by the enzyme, even in the absence of ribose. In the presence of phosphate, conversion from E·hx → E′·hx was not observed over the experimentally accessible range of hypoxanthine concentrations. Phosphate thus acts to stabilize the E·hx complex, or to increase the rate of conversion of E′·hx → E·hx.

Hydrolysis of inosine leaves one catalytic site per trimer filled with tightly-bound hypoxanthine. Third-the-sites stoichiometry in the hydrolytic reaction is confirmed with the quantitation of three hypoxanthine binding sites by equilibrium methods using the same enzyme preparations (Figure 5). The complex of purine nucleoside phosphorylase and hypoxanthine is tightly bond and survives gel filtration and dialysis but is not a covalent complex. A variety of protein denaturing agents cause release of hypoxanthine. Retention of a single hypoxanthine per trimer indicates third-the-sites reactivity, rather than the three subunits acting independently. Third-the-sites reactivity occurs over a wide range of inosine concentrations and appears to be an intrinsic property of the reaction. A similar example is the F_oF_i ATP synthase in which the substrates ADP and PO_4 are in equilibrium with ATP on the surface of the enzyme and a single ATP molecule is tightly bound ($\sim 10^{-12}$ M) at one of three catalytic centers (Grubmeyer et al., 1982). Tightly-bound ATP is negatively coop-

Scheme II: Pathways for Stoichiometric Interactions of Hypoxanthine and Ribose with Purine Nucleoside Phosphorylase[a]



[a] The trimer of purine phosphorylase is indicated as E_3. Binding of one, two, or three molecules of hypoxanthine is represented E_3·hx, E_3·hx_2, and E_3·hx_3, respectively. Purine nucleoside phosphorylase which binds tightly to hypoxanthine is E'_3. One hypoxanthine binds tightly to this complex.

erative with subsequent interactions occurring with much lower affinity (Penefsky & Cross, 1991). Tightly-bound ATP exchanges with added excess ATP. A similar exchange occurs with purine nucleoside phosphorylase. Addition of excess hypoxanthine causes rapid exchange of the tightly-bound molecule.

Microscopic Reversibility of Inosine Hydrolysis. Microscopic reversibility requires that reversible pathways must exist for both the hydrolysis of inosine and its synthesis from hypoxanthine and ribose. The stoichiometry of one tightly-bound hypoxanthine per trimer on the hydrolytic route and three bound hypoxanthines per trimer in the presence of phosphate requires steps to connect these enzyme species. Scheme II outlines a mechanism which accounts for these stoichiometries and is consistent with all experimental data. Formation of the Michaelis complex of E_3·hx_3 proceeds rapidly with equivalent binding of hypoxanthine and a dissociation constant of 1.6 μM. Increasing the mean occupancy time of hypoxanthine on the protein by excess hypoxanthine concentration causes formation of the E'_3·hx_3 complex, where the tight-binding E′ conformation has occurred at one of the three subunits. One tightly-bound hypoxanthine causes release of hypoxanthine from the neighboring subunits, and on gel filtration, the E'_3·hx_1 complex is observed. This pathway is experimentally verified by the formation and isolation of the E'_3·hx_1 complex following incubation of enzyme with radiolabeled hypoxanthine. Independent verification comes from the unusual and characteristic hypoxanthine binding pattern of three hypoxanthines bound at intermediate hypoxanthine concentrations but one hypoxanthine bound at saturating hypoxanthine concentration. The interconversion of the E_3·hx_2 ⇄ E'_3·hx_2 forms is a minor pathway since binding studies (Figure 5B) clearly show a species with three hypoxanthines bound as the major form before conversion to E'_3·hx.

Catalytic Competence of Tightly-Bound Hypoxanthine. Addition of ribose 1-phosphate to the enzyme–hypoxanthine complex causes conversion of bound hypoxanthine to inosine. The bound hypoxanthine therefore has catalytic competence and is on the normal pathway of catalysis. Conversion of bound hypoxanthine to inosine occurs more rapidly than the rate constant for dissociation of the E′·hx complex. Hydrolysis of the N-glycosidic bond of inosine therefore leaves hypoxanthine in the purine binding region of the catalytic site, but does not cause relaxation of the bonds that promote purine group binding.

The crystal structure of human erythrocyte purine nucleoside phosphorylase with 5′-iodoformycin B (Ealick et al., 1990) shows relatively limited bonding between the enzyme and the purine ring. The tight binding of hypoxanthine to this protein configuration of the crystal structure would be unlikely based on the reported contacts between the purine ring and enzyme. Thus, the tightly-bound hypoxanthine is proposed to interact with a different protein conformation which arises as a transient structure on the catalytic pathway. The release of ribose 1-phosphate normally causes the relaxation of this structure to permit the facile departure of hypoxanthine. Without ribose 1-phosphate formation and release, a portion of the transition-state conformation remains to prevent the rapid release of hypoxanthine. This release is structure-specific, since guanosine is also hydrolyzed by the enzyme, but a bound guanosine complex does not survive chromatography.

Hypoxanthine Binding. The slow release of hypoxanthine implies tight binding to an intermediate form of purine nucleoside phosphorylase. The affinity of the E′·hx complex can be estimated by assuming diffusion-control for formation of the initial complex of enzyme and hypoxanthine. The expression

$$\text{E′·hypoxanthine} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E} + \text{hypoxanthine}$$

describes the release of hypoxanthine from the tight intermediate, including steps which relax the protein conformation as shown in Scheme I. The apparent dissociation constant, $K_d = k_2/k_1$, is defined by the release rate of hypoxanthine, 1.3×10^{-4} s^{-1}, and the diffusion-controlled encounter rate between enzyme and hypoxanthine, which is approximately 10^8 M^{-1} s^{-1} (Eigen & Hammes, 1963). From this ratio, an estimate of the dissociation constant is 1.3×10^{-12} M. This tight binding is a reflection of a fraction of the transition-state binding energy which is normally released by dissociation of ribose 1-phosphate. It may be possible to design inhibitors which take advantage of this conformation by the use of purine analogues which provide additional stabilization of the E′ complex.

Registry No. Purine nucleoside phosphorylase, 9030-21-1; inosine, 58-63-9; phosphate, 14265-44-2; hypoxanthine, 68-94-0.

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