Purine Nucleoside Phosphorylase

KINETIC MECHANISM OF THE ENZYME FROM CALF SPLEEN*

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Ribose 1-phosphate, phosphate, and acyclovir diphosphate quenched the fluorescence of purine nucleoside phosphorylase at pH 7.1 and 25 °C. The fluorescence of enzyme-bound guanine was similar to that of anionic guanine in ethanol. Guanine and ribose 1-phosphate bound to free enzyme, whereas inosine and guanosine were not bound to free enzyme in the absence of phosphate. Thus, synthesis proceeded by a random mechanism, and phosphorylosis proceeded by an ordered mechanism. Steady-state kinetic data for the phosphorylisis of 100 μM guanosine were fitted to a bifunctional kinetic model with catalytic rate constants of 22 and 1.3 s⁻¹. The dissociation rate constants for guanine from the enzyme-guanine complex at high and low phosphate concentrations were similar to the catalytic rate constants. Fluorescence changes of the enzyme during phosphorylisis suggested that ribose 1-phosphate dissociated from the enzyme ribose 1-phosphate-guanine complex rapidly and that guanine dissociated from the enzyme-guanine complex slowly. The association and dissociation rate constants for acyclovir diphosphate, a potent inhibitor of the enzyme (Tuttle, J. V., and Krencisky, T. A. (1984) J. Biol. Chem. 259, 4065–4069), were also dependent on phosphate concentration. The effects of phosphate are discussed in terms of a dual functional binding site for phosphate.

Purine nucleoside phosphorylase has emerged as a potential target for chemotherapeutic intervention for immunomodulation (1, 2), for malaria (3), and for the potentiation of antineoplastic nucleosides (4). Consequently, considerable effort has been directed toward discovering inhibitors of the enzyme (5–11). Acyclovir (9-[2-hydroxyethoxy)methyl]guanine) diphosphate, a bisubstrate analogue, is one of the most potent reversible inhibitors (12, 13). Alternatively, site-specific inactivators of the enzyme have been designed. For example, 9-(3,4-dioxopentyl)hypoxanthine inactivates the enzyme by covalent modification of an active-site arginyl residue (14), and a quinazoline-based analogue inactivates the enzyme by cross-linking catalytically essential residues (15).

Even though the importance of purine nucleoside phosphorylase in nucleoside catabolism is recognized (16) and the x-ray crystal structure for the erythrocyte enzyme is known to 3.2 Å (17), the kinetic mechanism of the enzyme has not been completely characterized. Early kinetic data for erythrocyte and calf spleen purine nucleoside phosphorylases were consistent with an Ordered Bi Bi mechanism (18, 19). Subsequently, initial velocity rate data from brain and erythrocyte purine nucleoside kinases were interpreted in terms of a Theorell-Chance mechanism (20, 21) with the nucleoside or nucleobase adding to the enzyme prior to phosphate or ribose 1-phosphate. In contrast to these results, highly purified purine nucleoside phosphorylase from bovine thyroid catalyzes an Ordered Bi Bi mechanism with phosphate binding prior to the nucleoside (22, 23). Enzymes from these sources do not obey simple Michaelis-Menten kinetics over an extended range of substrate concentrations (16, 20, 21, 24–27). This result has been attributed to nonequivalent interacting active sites on the trimeric enzyme (16) or to substrate-induced dissociation of the enzyme into monomers (20). Since the order of substrate addition to the enzyme was based on product inhibition data, which were collected over a limited range of substrate concentrations, errors in assigning the order for substrate addition to the enzyme from these data were possible.

Initial velocity data for calf spleen and erythrocyte purine nucleoside phosphorylases exhibit nonlinear double reciprocal plots when the phosphate concentration is varied at a fixed inosine concentration (27). In contrast to the erythrocyte enzyme (16), however, the double reciprocal plots of initial velocity data for the calf spleen enzyme are linear when the phosphate concentration is fixed and the inosine concentration is varied (28). Since the calf spleen enzyme is commercially available as a homogeneous preparation (26) and appears more amenable to kinetic analysis, it was chosen for the studies described herein. Equilibrium constants and kinetics for substrate binding to purine nucleoside phosphorylase were monitored by the fluorescence changes of the enzyme that occurred upon ligand binding. Data from these experiments were consistent with a random mechanism for the synthetic reaction and an ordered mechanism for the phosphorylolytic reaction with obligate binding of phosphate prior to the nucleoside.

EXPERIMENTAL PROCEDURES

Materials

Calf spleen purine nucleoside phosphorylase was purchased as an ammonium sulfate suspension from Sigma. The enzyme was collected by centrifugation and desalinated on a column of Bio-Rad P-6 resin equilibrated in 50 mM PIPES¹ at pH 7.1. The enzyme was a single band on 10% sodium dodecyl sulfate gel electrophoresis and had an Mr of 31,000. Guanine, guanosine, inosine, hypoxanthine, and ribose 1-phosphate were purchased from Sigma or Aldrich. Guanosine, which was crystallized from hot water, was shown by reverse-phase high pressure liquid chromatography to be free of guanine (<1%). Acyclovir diphosphate was synthesized as described previously (13).

¹ The abbreviations used are: PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); Hyp, hypoxanthine.
Kinetics of Purine Nucleoside Phosphorylase

General Methods

The concentrations of substrates and inhibitors were calculated from the absorbance of the sample in 0.1 N HCl and the following extinction coefficients: inosine, ε_{280} = 12.2 mm⁻¹ cm⁻¹; guanine, ε_{280} = 12.2 mm⁻¹ cm⁻¹; hypoxanthine, ε_{280} = 10.6 mm⁻¹ cm⁻¹; and acyclovir diphosphate, ε_{280} = 12.2 mm⁻¹ cm⁻¹. Ribose 1-phosphate and phosphate solutions were prepared gravimetrically. The concentration of purine nucleoside phosphorylase was determined spectrophotometrically with ε_{280} = 61 mm⁻¹ cm⁻¹ (see "Results"). The enzyme concentration was 310 nM in all experiments, except for those that were designed to determine the K_d for acyclovir diphosphate ([E] < 100 nM). This minimized the effects that enzyme dissociation could have on the kinetic properties of the enzyme (26). All reactions were in 50 mM PIPES at pH 7.1 and 25 °C.

Acquisition of Spectral Data

Spectral data were collected on a UVIKON 880 spectrophotometer (Kontron Analytical, Everett, MA) with 10-mm cuvettes. Fluorescence spectra were collected on a Kontron SFM 25 spectrofluorometer. Rapid kinetic data were collected with an SF 17MV stopped-flow spectrofluorometer (Applied Photophysics Ltd., Leatherhead, United Kingdom). The time constant for the instrument was set to one-tenth of the fastest relaxation time monitored. The entrance and exit slits were 2 mm. Other parameters were adjusted as described in the manual from the manufacturer. Fluorescence (scattering) data were collected with an excitation wavelength of 280 nm. The path length for the excitation beam was 2 mm, and the path length for emission was 10 mm. Absorbance data were collected with a 10-mm path length. All reactions were initiated by mixing equal volumes of reactants. Five or more experiments were averaged for data fitting.

Steady-state Kinetic Data

Steady-state rate data were collected on the stopped-flow spectrophotometer. The synthesis or the phosphorolysis of guanosine was followed at 252 nm. These data were corrected for the nonlinearity of the spectrophotometer at this wavelength by calibration of the instrument with solutions of known absorbance. The corrected absorbance values were converted to product concentration with a measured difference extinction coefficient between guanosine and guanine of 4.0 mm⁻¹ cm⁻¹. The synthesis or the phosphorolysis of inosine was measured at 278 nm. The absorbance values were converted to product concentration with a difference extinction coefficient between inosine and hypoxanthine of 1.2 mm⁻¹ cm⁻¹. Steady-state data for the inhibition of purine nucleoside phosphorylase (<10 nM) by acyclovir diphosphate were collected with inosine and phosphate as substrates. The product was oxidized by xanthine oxidase (0.2 unit/ml) to uric acid, which was monitored spectrophotometrically at 293 nm (Δε_{293} = 12.5 mm⁻¹ cm⁻¹) (29).

Data Analysis

Steady-state Kinetic Analysis—Steady-state kinetic data for the enzymatic phosphorylation of inosine or guanosine with the nucleoside concentration fixed and the phosphate concentration ([A]) varied over a wide range of concentrations were fitted to a bifunctional kinetic model (Equation 1):

\[
\frac{v}{[E]} = \frac{k_1[A]}{[A] + K_{a1}} + \frac{k_2[A]}{[A] + K_{a2}}
\]

(1)

Steady-state kinetic data for the phosphorylation of inosine or guanosine over a limited range of substrate concentrations were fitted to Equation 2:

\[
\frac{v}{[E]} = \frac{k_3[A][B]}{K_c + K[B] + K_0[A] + [A][B]}
\]

(2)

where A is phosphate, and B is the nucleoside. Steady-state kinetic data for the synthesis of inosine or guanosine over a limited range of substrate concentrations were fitted to Equation 3:

\[
\frac{v}{[E]} = \frac{k_3[P][Q]}{K_c + K[Q] + K_0[P] + [P][Q]}
\]

(3)

where P is ribose 1-phosphate, and Q is the nucleobase.

Titration of Purine Nucleoside Phosphorylase—Fluorescence—Changes in the fluorescence of purine nucleoside phosphorylase (excitation, 280 nm; emission, 335 nm) were used to monitor ligand (S) binding to the enzyme. When the dissociation constant of the titrant was much greater than the enzyme concentration, the titration data were fitted to Equation 4:

\[
\text{Fluorescence} = A + (B - A) \cdot \left[\frac{[S]}{K_d + [S]}\right]
\]

(4)

where A is the fluorescence of free enzyme, B is the fluorescence of complexed enzyme, and K_d is the concentration of ligand that results in one-half of the total fluorescence change. When the concentration of the enzyme was comparable to the dissociation constant (K_d) for the titrant, the titration data were fitted to Equation 5:

\[
\text{Fluorescence} = A + (B - A) \cdot f
\]

(5)

where A and B are defined as above and f is the fraction of the enzyme complexed to the titrant. The fraction of complexed enzyme (f) was calculated from the total enzyme concentration ([E]) and the total ligand concentration ([S]) with the quadratic equation and the assumption that the stoichiometry for ligand binding is 1 (Equation 6).

\[
f = \frac{1}{2} \left(\frac{(E + S + K_d)}{E} \right)^2 - \frac{1}{2} \left(\frac{(E + S + K_d)}{E} \right) - \frac{4S^2}{E}
\]

(6)

In a typical titration, fluorescence measurements were made at 20–30 concentrations of the titrant.

Time course for Quenching of Purine Nucleoside Phosphorylase—Fluorescence—Single and double exponential time courses (400 data points) were fitted with the software provided with the SF 17MV stopped-flow spectrophotometer. The dependencies of these rate constants on the concentration of ligand ([S]) were fitted to the appropriate equation (Equations 7–9):

\[
k_{obs} = k_o + k[S]
\]

(7)

\[
k_{obs} = \frac{k[S]}{K_c + [S]}
\]

(8)

\[
k_{obs} = \frac{(k - k_0)[S]}{[S] + K_{r}} + k_0
\]

(9)

where k_o and k are the values of k_{obs} extrapolated to zero and infinite concentrations of S, respectively; k is the slope of a linear plot of k_{obs} versus [S]; and K_c is the concentration of S that yields a half-maximal effect on k_{obs}.

The pseudo-first-order rate constant (k_{obs}) for acyclovir diphosphate binding to purine nucleoside phosphorylase was decreased by phosphate and ribose 1-phosphate. The K_i for these substrates was determined from the effect that they had on the pseudo-first-order rate constant for acyclovir diphosphate binding. These data (eight or more concentrations of inhibitor) were fitted to Equation 10:

\[
k_{obs} = \frac{A}{K_i + [S]}
\]

(10)

where K_i is the concentration of competing ligand that produces one-half the maximal effect, and the ratio of A to K_i is equal to the value of k_{obs} in the absence of a competing ligand.

Time Course for Formation of Active Enzyme from Enzyme-Acyclovir Diphosphate Complex—The phosphorylation of inosine by the enzyme-acyclovir diphosphate complex was initially zero. As acyclovir diphosphate dissociated from the enzyme-acyclovir diphosphate complex, the rate of product formation increased. This process was fitted to an exponential increase in the rate of product formation to a steady-state rate as acyclovir diphosphate dissociated from the enzyme-acyclovir diphosphate complex (Equation 11):

\[
[\text{Product}] = A + B \cdot t + \frac{B}{C} \cdot \exp(-C \cdot t)
\]

(11)

where A is a function of the initial product concentration and B/C is the final steady-state velocity, and C is the first-order rate constant for dissociation of acyclovir diphosphate from the enzyme-acyclovir diphosphate complex.

The constants defined by these equations were estimated by the iterative nonlinear least-squares fitting routine outlined by Bevington (30).
RESULTS

Steady-state Kinetics—Double reciprocal plots of initial velocity data for phosphorolysis of inosine by calf spleen nucleoside phosphorylase have been reported to be nonlinear over extended ranges of phosphate concentrations (26). Similar results were observed in this study when the phosphate concentration was varied at a fixed concentration of inosine or guanosine (Fig. 1). These data were fitted to Equation 1 with $K_a$ values for phosphate of 600 or 1000 and 15 or 30 μM, respectively. When the phosphate concentration was either >200 μM or <30 μM, the initial velocity data appeared to be linear in a double reciprocal analysis. Consequently, initial velocity data for the phosphorolytic reaction were collected with phosphate concentrations between 200 and 8000 μM and with nucleoside concentrations between 10 and 100 μM. The results from fitting these data to Equation 2 are tabulated in Table I. Initial velocity data for the synthetic reaction yielded linear double reciprocal plots (data not shown) and were fitted to Equation 3 (Table I).

The turnover number of calf spleen purine nucleoside phosphorylase is dependent on enzyme concentration under certain experimental conditions (26). Whenever feasible, the data reported herein were collected with 310 nM enzyme. However, lower concentrations of the enzyme for studies with acyclovir diphosphate were necessary because its $K_i$ is <100 nM (13). The validity of comparing data obtained at different enzyme concentrations was determined by comparing the steady-state kinetic parameters at different enzyme concentrations. The steady-state data for phosphorolysis of inosine with 8.2 nM enzyme were fitted to Equation 2 with $k_p = 13.5$ ± 0.8 s⁻¹, $K_p = 1100 ± 200$ μM, $K_n = 13 ± 3$ μM, and $K_d = 39,000 ± 8000$ μM. These kinetic parameters were similar to those determined with 310 nM enzyme (Table I). Thus, the kinetic parameters for the phosphorolysis of inosine were similar at 310 and 8.2 nM enzyme, and a comparison of kinetic data determined at these enzyme concentrations was probably valid.

The equilibrium constant for the synthesis of inosine was $56 ± 6 (n = 10)$, which agreed with the previously reported equilibrium constant of 54 (31).

Effect of Ligands on Fluorescence of Purine Nucleoside Phosphorylase—Phosphate, ribose 1-phosphate, and acyclovir diphosphate quenched (5-15%) the protein fluorescence of purine nucleoside phosphorylase (Fig. 2A). The fluorescence of the enzyme was slightly quenched (3%) by hypoxanthine. When guanine bound to the enzyme, the protein fluorescence was enhanced (Fig. 2A). These fluorescence changes were used to monitor binding of these ligands to the enzyme. The fluorescence of the enzyme was not affected significantly by inosine or guanosine.

![Fig. 1. Dependence of turnover number of purine nucleoside phosphorylase on phosphate concentration.](image)

![Fig. 2. Effects of substrates and acyclovir diphosphate on fluorescence emission spectrum of purine nucleoside phosphorylase.](image)

| Table I | Steady-state kinetics for phosphorolysis and synthesis of guanosine and inosine |
|-----------------|------------------|------------------|------------------|
| **Phosphorolysis** | **Inosine** | **Guanosine** |
| $k_p$ | 13 ± 3 s⁻¹ | 12 ± 3 s⁻¹ |
| $K_p$ (phosphate) | 470 ± 60 μM | 700 ± 50 μM |
| $K_p$ (nucleoside) | 34 ± 3 μM | 13 ± 2 μM |
| $K_d$ | 12,000 ± 3,000 μM² | 2,500 ± 800 μM² |
| **Synthesis** | | |
| $k_s$ | 8 ± 2 s⁻¹ | 22 ± 6 s⁻¹ |
| $K_s$ (ribose-1-P) | 66 ± 7 μM | 18 ± 4 μM |
| $K_s$ (nucleoside) | 7.1 ± 0.8 μM | 6 ± 1 μM |
| $K_s$ | 180 ± 30 μM² | 30 ± 14 μM² |
Guanine bound sufficiently tightly to purine nucleoside phosphorolase that the concentration of the enzyme and the dissociation constant for guanine could be calculated from titration data such as that shown in Fig. 2B. These data were fitted to Equation 5 to give a $K_d$ of 0.08 ± 0.02 μM for guanine at pH 7.1 and a binding site concentration of 310 ± 70 nM for the enzyme with an $A_{280}$ of 0.019 cm$^{-1}$ (Table II). The active-site concentration of purine nucleoside phosphorylase was calculated from these data with the assumption that guanine bound to the active site of the enzyme. The extinction coefficient of the enzyme at 280 nm was 61 μM$^{-1}$ cm$^{-1}$.

The dissociation constants for phosphate, ribose 1-phosphate, and guanosine were also determined by spectrofluorometric titration (Table II). Inosine did not affect the binding of ribose 1-phosphate, and guanosine did not affect the binding of guanine to the enzyme. Thus, these nucleosides did not bind to the ribose-binding site or to the nucleoside-binding site and probably did not bind to free enzyme. The nucleobases, hypoxanthine and guanine, inhibited the binding of phosphate to the enzyme (Table II). Moreover, the dissociation constant of the enzyme-guanine complex was increased from 0.98 to 1.2 μM by 2 μM hypoxanthine. If hypoxanthine and guanine competed for the same binding site, the apparent catalytic formation of guanine that could bind to the enzyme to form the enzyme-guanine complex. Furthermore, the rate constant for this reaction was too slow (<2 s$^{-1}$ with 200 μM guanosine) for a binary complex between the enzyme and guanosine to be an obligate intermediate in catalysis (turnover number was 32 s$^{-1}$).

Dissociation constants were determined by titration of enzyme fluorescence with the indicated ligands as described under “Experimental Procedures.” There was no significant fluorescence change upon addition of inosine or guanosine to purine nucleoside phosphorylase. The absorbance of the enzyme at 280 nm was 0.019 cm$^{-1}$.

Furthermore, the rate constant for this catalytic formation of guanine that could bind to the enzyme to form the enzyme-guanine complex. Furthermore, the rate constant for this reaction was too slow (<2 s$^{-1}$ with 200 μM guanosine) for a binary complex between the enzyme and guanosine to be an obligate intermediate in catalysis (turnover number was 32 s$^{-1}$).

TABLE II  Dissociation constants of purine nucleoside phosphorylase for substrates and acyclovir diphosphate

<table>
<thead>
<tr>
<th>Titrant</th>
<th>Competing ligand</th>
<th>Apparent $K_a$ μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>None</td>
<td>170 ± 10</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>290 ± 20</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>230 ± 10</td>
</tr>
<tr>
<td></td>
<td>2.4 μM Hyp</td>
<td>480 ± 40</td>
</tr>
<tr>
<td></td>
<td>194 μM Hyp</td>
<td>760 ± 70</td>
</tr>
<tr>
<td></td>
<td>1 μM Gua</td>
<td>540 ± 30</td>
</tr>
<tr>
<td></td>
<td>20 μM Gua</td>
<td>1000 ± 50</td>
</tr>
<tr>
<td>Ribose-1-P</td>
<td>None</td>
<td>250 ± 20</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>240 ± 10</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>340 ± 20</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>260 ± 20</td>
</tr>
<tr>
<td></td>
<td>324 μM Ino</td>
<td>270 ± 30</td>
</tr>
<tr>
<td>Guanine</td>
<td>None*</td>
<td>0.043 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>None*</td>
<td>0.081 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>None*</td>
<td>0.098 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>None*</td>
<td>0.059 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>None*</td>
<td>0.010 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>100 μM Gua</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>2 μM Hyp</td>
<td>1.19 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>50 mM phosphate</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>Acyclovir diphosphate</td>
<td>None</td>
<td>&lt;0.010$^b$</td>
</tr>
</tbody>
</table>

$^a$ The concentrations of guanine-binding sites for these samples of the enzyme with an $A_{280}$ of 0.019 cm$^{-1}$ were calculated from titration data such as that of Fig. 2B to be 410 ± 10, 280 ± 10, 250 ± 10, 270 ± 10, and 340 ± 20 nM, respectively.

$^b$ The dissociation constant for acyclovir diphosphate was too small to determine accurately by this titration method (i.e. the concentration of acyclovir diphosphate-binding sites was much greater than the dissociation constant). The concentration of acyclovir diphosphate-binding sites for the enzyme with an $A_{280}$ of 0.019 cm$^{-1}$ was 420 nM.

Dissociation Rate Constants for Ribose 1-Phosphate and Phosphate—Since ribose 1-phosphate and phosphate quenched the fluorescence of purine nucleoside phosphorylase (Fig. 2A), we attempted to measure spectrophotometrically the kinetics for binding of these ligands to the enzyme. There was, however, no observable fluorescence change upon mixing 310 nM purine nucleoside phosphorylase with 300 μM ribose 1-phosphate or 300 μM phosphate on the stopped-flow spectrophotometer. Thus, the dissociation rate constants for these substrates were probably >500 s$^{-1}$. These values were estimated with the following considerations. First, the fluorescence changes upon binding of these substrates to purine nucleoside phosphorylase were sufficiently small that a reaction with a first-order rate constant $>1000$ s$^{-1}$ would not have been observed on the stopped-flow instrument (i.e. the fluorescence change would have occurred within the dead time of the instrument). Second, the measurements were made at a substrate concentration equal to its $K_d$ so that the observed rate constant for equilibration of substrate with the enzyme was twice the dissociation rate constant.

Association Rate Constant for Acyclovir Diphosphate—In contrast to the very rapid equilibration of ribose 1-phosphate or phosphate with purine nucleoside phosphorylase, the equilibration of the enzyme with acyclovir diphosphate was readily observable on the stopped-flow spectrophotometer. The first-order rate constants for fluorescence quenching in the absence of phosphate were linearly dependent on acyclovir diphosphate concentrations (0–30 μM) (data not shown). A linear plot of these data had a slope of (6.6 ± 0.4) × $10^6$ M$^{-1}$ s$^{-1}$, which was the association rate constant. The ordinate intercept of this plot was too small to obtain an accurate estimate for the dissociation rate constant.

Effect of Phosphate and Ribose 1-Phosphate on Association Rate Constant for Acyclovir Diphosphate—The pseudo first-order rate constant for binding of 5 μM acyclovir diphosphate to purine nucleoside phosphorylase decreased from 38 to 1 s$^{-1}$ as the phosphate concentration was increased from 0 to 50 mM. The $K_d$ for phosphate was 140 ± 20 μM (Fig. 3). Since the association rate constant did not approach zero at high concentrations of phosphate (Fig. 3), acyclovir diphosphate bound not only to free enzyme, but also to the enzyme-phosphate complex. Ribose 1-phosphate also decreased the dissociation constant ($K$) for guanine was given by Equation 12:

$$K = K_{Gua} \left(1 + \frac{[Hyp]}{K_{hyp}}\right)$$

where $K_{Gua}$ is the dissociation constant for guanine, and $K_{hyp}$ is the dissociation constant for hypoxanthine. $K_{hyp}$ was estimated from these data to be 0.14 μM.

FIG. 3. Effect of phosphate concentration on pseudo first-order rate constant for binding of 5 μM acyclovir diphosphate to 310 nM purine nucleoside phosphorylase. The data were fitted to Equation 9 with $K_m = 140 ± 20$ μM, $b_0 = 38 ± 1$ s$^{-1}$, and $k = 1 ± 1$ s$^{-1}$.

$I_2$ $\alpha$ $I_1$ $I_0$
association rate constant for acyclovir diphosphate. The apparent $K_i$ for ribose-1-phosphate was 80 ± 10 μM (Table III).

Effect of Inosine and Guanosine on Association Rate Constant for Acyclovir Diphosphate—Inosine (240 μM) and guanosine (100 μM) did not inhibit the rate for binding of 5 μM acyclovir diphosphate to the enzyme (Table III). However, inosine in the presence of 5 mM phosphate inhibited the rate of acyclovir diphosphate binding with an apparent dissociation constant of 12 ± 4 μM (Table III), which was similar to the $K_i$ (34 ± 4 μM) for inosine (Table I). These results and those of Table II demonstrated that inosine and guanosine did not bind to the enzyme in the absence of phosphate.

Dissociation Rate Constant for Acyclovir Diphosphate—As acyclovir diphosphate dissociated from the purine nucleoside phosphorylase-acyclovir diphosphate complex in a reaction mixture containing phosphate and inosine, the rate of hypoxanthine formation increased exponentially to a final steady-state rate (Fig. 4A). The first-order rate constant for the delayed onset of complete catalytic activity was the first-order rate constant for dissociation of acyclovir diphosphate from the enzyme-acyclovir diphosphate complex. This rate constant was linearly dependent on the concentration of phosphate (Fig. 4B) and extrapolated to a dissociation rate constant that was small (0.001 ± 0.002 s⁻¹) at zero phosphate concentration.

The steady-state $K_i$ for acyclovir diphosphate for purine nucleoside phosphorylase measured in the presence of 50 mM phosphate was 99 ± 5 nM. The association rate constant ($k_a$) under these conditions was 2 × 10⁶ M⁻¹ s⁻¹ (Fig. 3), and the dissociation rate constant ($k_d$) was 0.025 s⁻¹ (Fig. 4B). The value of $K_i$ calculated from these rate constants was 125 nM (0.025 s⁻¹ divided by 2 × 10⁶ M⁻¹ s⁻¹). Since the measured and calculated $K_i$ values were similar, the quenching of fluorescence by acyclovir diphosphate that was used to measure the individual rate constants reflected the inhibition of the enzyme by acyclovir diphosphate.

Association Rate Constant for Guanine—The fluorescence of guanine bound to purine nucleoside phosphorylase was enhanced relative to the fluorescence of guanine or the enzyme (Fig. 2A). Guanine reacted with purine nucleoside phosphorylase in a biphasic reaction with ~70% of the total fluorescence change occurring with the rapid phase of the reaction (Fig. 5A). The pseudo first-order rate constants for the fast phase of the reaction were linearly dependent on the concentration of guanine (Fig. 5B) and were fitted to Equation 7 with an ordinate intercept of 140 ± 20 μM and a slope ($k_i$) of (7.8 ± 0.2) × 10⁵ M⁻¹ s⁻¹. The first-order rate constants for the slow phase of the reaction increased to a limiting value at high concentrations of guanine (data not shown). These data were fitted to Equation 8 with a maximum first-order rate constant ($k_i$) of 11 ± 0.8 s⁻¹ and an apparent dissociation constant ($K_i$) of 3.2 ± 0.8 μM.

Effect of Phosphate on Association Rate Constant for Guanine—The rate of guanine binding was enhanced by phosphate. Furthermore, the time courses for guanine binding were essentially monophasic at high concentrations of phosphate. The pseudo first-order rate constants for the reaction at high phosphate concentrations were linearly dependent on guanine concentration and were fitted to Equation 7 with $k_0$ (dissociation rate constant) equal to 24 ± 7 s⁻¹ and $k_i$ (association rate constant) equal to (20 ± 1) × 10⁵ M⁻¹ s⁻¹ (Fig. 5B). The rate constant for the binding of 5 μM guanine increased from 31 to 103 s⁻¹ as the phosphate concentration was increased from 0 to 5 mM. These data were fitted to Equation 9 with $K_i$ for phosphate of 140 ± 30 μM. This value was in reasonable agreement with the $K_i$ of phosphate for free enzyme (Table II) and for the effect of phosphate on acyclovir diphosphate binding (Table III).

Dissociation Rate Constant for Guanine—The data of Fig. 4B suggested that the rate constant for dissociation of guanine

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**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Effect on $k_{on}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>Inhibition ($K_i = 140 ± 20$ μM)²</td>
</tr>
<tr>
<td>Ribose-1-P</td>
<td>Inhibition ($K_i = 80 ± 10$ μM)²</td>
</tr>
<tr>
<td>Ino</td>
<td>None⁴</td>
</tr>
<tr>
<td>Ino + 5 mM phosphate</td>
<td>Inhibition ($K_i = 12 ± 4$ μM)⁴</td>
</tr>
<tr>
<td>Guo</td>
<td>None⁴</td>
</tr>
<tr>
<td>Hyp</td>
<td>Biphase reaction⁴</td>
</tr>
</tbody>
</table>

² Data were taken from Fig. 3.

³ Values for $k_{on}$ were determined at eight concentrations of the competing substrate and were fitted to Equation 10.

⁴ $k_{on}$ was 36.4 ± 0.4 s⁻¹ with 5 μM acyclovir diphosphate alone versus 37.5 ± 0.8 s⁻¹ with 5 μM acyclovir diphosphate and 240 μM inosine. There was no effect of inosine on the amplitude of the fluorescence change.

⁵ $k_{on}$ was 39.3 ± 0.5 s⁻¹ with 5 μM acyclovir diphosphate alone versus 54.4 ± 0.7 s⁻¹ with 5 μM acyclovir diphosphate and 100 μM guanosine. There was no effect of guanosine on the amplitude of the fluorescence change.

⁶ $k_{on}$ was 14.4 ± 0.1 s⁻¹ with 2.5 μM acyclovir diphosphate. The time course for fluorescence quenching became biphasic in the presence of 5 μM hypoxanthine. The first-order rate constants for the fast and slow phases were 23.1 ± 0.7 and 0.63 ± 0.02 s⁻¹, respectively. The fast phase accounted for 45% of the total fluorescence change.
Phase changes during binding of 1 pM guanine to the enzyme were fitted to a biphasic process with rate constants of 8.8 ± 0.1 and 1.0 ± 0.2 s⁻¹. The amplitude of the fast phase of the reaction was 74% of the total fluorescence change. The time courses for fluorescence changes during binding of 30 μM guanine to the enzyme were also biphasic with rate constants of 206 ± 5 and 9.2 ± 3 s⁻¹. The amplitudes of the fast phase of the reaction was 58% of the total fluorescence change.

The rate constants for guanine binding to the enzyme-guanine complex and ribose 1-phosphate had concentrations that agreed with the dissociation constant for guanine in the absence of phosphate. The rate constants for the fast phase of guanine binding to the enzyme-guanine complex and ribose 1-phosphate had concentrations that agreed with the dissociation constant for guanine in the absence of phosphate.

The rate constants for the dissociation of guanine from the enzyme-guanine complex to a limiting value of $k_d$ were also biphasic with rate constants of 20 ± 5 and 5.0 ± 0.1 s⁻¹. Guanine binding was monophasic in the presence of 5 mM phosphate. These rate constants (Table II) were fitted to Equation 7 with $k_d = 54 ± 7$ s⁻¹ and $k_s = (39.6 ± 1) × 10^{9}$ M⁻¹ s⁻¹.

(k₀) from the enzyme-guanine complex in the absence of phosphate ($k_0 = 1.4$ s⁻¹) was small. A more accurate estimate of this rate constant was made by directly measuring the rate of formation of free enzyme as guanine dissociated from the enzyme-guanine complex. Since purine nucleoside phosphorylase bound acyclovir diphosphate rapidly (Table II), free enzyme that formed by dissociation of the enzyme-guanine complex was effectively trapped as the enzyme-acyclovir diphosphate complex. The larger difference between the fluorescence of the enzyme-acyclovir diphosphate and enzyme-guanine complexes (Fig. 2A) was used to monitor the dissociation of guanine. The effectiveness of acyclovir diphosphate as a reagent to sequester free enzyme was validated by demonstrating that the rate constant for the reaction was not changed by halving or doubling the concentration of acyclovir diphosphate. The first-order rate constant for dissociation of guanine ($k_d$) in the absence of phosphate was calculated from the association and dissociation rate constants to be 0.14 μM (1.1 s⁻¹ divided by $7.8 × 10^{9}$ M⁻¹ s⁻¹), which agreed with the dissociation constant (0.08 ± 0.02 μM) measured by spectrophotometric titration of the enzyme (Table II).

Effect of Phosphate and Ribose 1-Phosphate on Dissociation Rate Constant for Guanine—Phosphate increased the rate constant for dissociation of guanine from the enzyme-guanine complex to a limiting value of 23 ± 2 s⁻¹ at high phosphate concentration (Fig. 6). This value was similar to the estimate for the dissociation rate constant ($k_d = 24 ± 7$ s⁻¹) extrapolated from data collected with different phosphate concentrations and were fitted to Equation 9 with $k = 23 ± 2$ s⁻¹, $k_0 = 1.1 ± 0.1$ s⁻¹, and $K_m = 2.0 ± 0.3$ mM.

The amplitude of the fluorescence change associated with dissociation of guanine from the enzyme decreased as the phosphate concentration was increased. When the dissociation of guanine from the enzyme was determined in the presence of 5 mM phosphate, 75% of the fluorescence change occurred within the mixing time of the stopped-flow spectrophotometer. This suggested that a ternary enzyme-phosphate-guanine complex with reduced fluorescence formed at a rate that was too rapid to be followed by the stopped-flow spectrophotometer. The dissociation of guanine from this ternary complex was the event monitored on the stopped-flow spectrophotometer.

Ribose 1- phosphate also enhanced the apparent dissociation of guanine from the enzyme-guanine complex. The limiting first-order rate constant was 115 ± 6 s⁻¹, and the concentration of ribose 1-phosphate that caused one-half the maximal effect was 130 ± 30 μM. The initial complex between the enzyme-guanine complex and ribose 1-phosphate had 25% of the fluorescence of the enzyme-guanine complex alone, and it formed too rapidly to be measured on the stopped-flow spectrophotometer. Since ribose 1-phosphate could react with guanine in the enzyme-guanine complex, the release of guanine (the event monitored on the stopped-flow spectrophotometer) was due to dissociation of guanine and/or to the formation of guanosine.

Association Rate Constant for Hypoxanthine—When 10 μM hypoxanthine was mixed with 310 nM purine nucleoside phospho-
Phosphorylase in the stopped-flow spectrophotometer, there was no detectable fluorescence change of the enzyme. However, hypoxanthine affected the rate of binding of acyclovir diphosphate to the enzyme (Table III). This suggested that the pseudo first-order rate constants for binding of acyclovir diphosphate and hypoxanthine to the enzyme were comparable and that hypoxanthine bound to the acyclovir diphosphate-binding site without altering the fluorescence of the enzyme. The rate constant for binding of hypoxanthine to this site was estimated from competition experiments in which the rate constant for binding of acyclovir diphosphate was measured as a function of hypoxanthine concentration. Since the dissociation rate constants for hypoxanthine (see below) and acyclovir diphosphate were small, the observed rate constant ($k_{obs}$) for equilibration of acyclovir diphosphate (ACVDP) and hypoxanthine with the enzyme was the sum of the pseudo first-order rate constants for binding of hypoxanthine and acyclovir diphosphate (Equation 13).

$$k_{obs} = k_{ACVDP[ACVDP]} + k_{Hyp[ACVDP]}$$

The slope of a linear plot of $k_{obs}$ for acyclovir diphosphate binding versus hypoxanthine (0–5 μM) at a fixed concentration of acyclovir diphosphate was equal to the bimolecular rate constant for hypoxanthine binding ($k_{hyp}$). $k_{hyp}$ had a value of (1.5 ± 0.4) × 10^6 M⁻¹ s⁻¹ (data not shown). A similar set of experiments (data not shown) with guanine (2 μM) substituting for acyclovir diphosphate and with the hypoxanthine concentration varied between 0 and 10 μM yielded an association rate constant for hypoxanthine of (1.8 ± 0.4) × 10^6 M⁻¹ s⁻¹. These two independent determinations for the association rate constant for hypoxanthine were in good agreement.

**Dissociation Rate Constant for Hypoxanthine**—The rate constant for release of hypoxanthine from the enzyme-hypoxanthine complex was estimated by a method similar to that used for estimating the rate constant for release of guanine from the enzyme-guanine complex (Fig. 5B). When the enzyme-hypoxanthine complex (2 μM hypoxanthine and 0.62 μM enzyme) was mixed with an equal volume of 100 μM acyclovir diphosphate, the dissociation rate constant was 0.66 ± 0.02 s⁻¹. The dissociation constant calculated from the association and dissociation rate constants was 0.39 μM (0.66 s⁻¹ divided by 1.7 × 10^6 M⁻¹ s⁻¹), which was in reasonable agreement with the dissociation constant (0.14 μM) (see above) calculated from the titration of the enzyme by guanine in the presence of 2 μM hypoxanthine.

**Effect of Phosphate and Ribose 1-Phosphate on Dissociation Rate Constant for Hypoxanthine**—The first-order rate constant for formation of free enzyme from the enzyme-hypoxanthine complex was also enhanced by phosphate and ribose 1-phosphate. In the presence of phosphate, the dissociation rate constant increased to a limiting value of 10.6 ± 0.8 s⁻¹ with 200 ± 40 μM phosphate giving a one-half maximal effect. Ribose 1-phosphate increased the dissociation rate constant to a limiting value of 12.3 ± 0.6 s⁻¹ with 50 ± 10 μM ribose 1-phosphate producing one-half the maximal effect. Since ribose 1-phosphate could react with the enzyme-hypoxanthine complex to generate inosine, the enhanced rate of formation of free enzyme by ribose 1-phosphate could be due to an enhanced rate of dissociation of hypoxanthine and/or to formation of inosine.

**Effects of Inosine and Guanosine**—The first-order rate constant for binding of 5 μM acyclovir diphosphate was not affected by 240 μM inosine (37.5 ± 0.8 s⁻¹ in the presence of 240 μM inosine versus 37.5 ± 0.8 s⁻¹ in its absence). Likewise, the rate constant for binding of 1.5 μM guanine to purine nucleoside phosphorylase was not affected by 100 μM guanosine (9.35 ± 0.04 s⁻¹ in the presence of 100 μM guanosine versus 9.29 ± 0.05 s⁻¹ in its absence). These results and the titration data (Table II) suggested that guanosine and inosine did not bind to purine nucleoside phosphorylase in the absence of phosphate.

**Correlation of Product Formation with Changes in Fluorescence of Enzyme during Turnover**—The enzyme-guanine and enzyme-ribos 1-phosphate-guanine complexes had enhanced fluorescence (excitation wavelength, 280 nm) relative to free enzyme. If these species were on the catalytic pathway, the changes in enzyme fluorescence during turnover should be related to changes in the rate of product formation. Thus, when 310 nM enzyme was reacted with 10 μM guanine and 500 μM ribose 1-phosphate, there was a rapid increase in enzyme fluorescence followed by a slow decrease in fluorescence (Fig. 7). Concomitantly, the rate of guanosine formation (Aₚ₄) slowly decreased from an initially rapid rate. Thus, the rate of formation of guanosine was large when the fluorescence of the enzyme was high and small when the fluorescence of the enzyme was low (Fig. 7).

**Approach to Steady State**—The fluorescence changes associated with turnover of the enzyme (see above) were used to monitor the approach to the steady state. The kinetic parameters for the approach to the steady state for the phosphorolysis and synthesis of guanosine are summarized in Table IV.

The magnitude of the fluorescence change for the approach to the steady state during turnover with 100 μM guanosine and 50 μM phosphate was comparable to that for formation of the enzyme-guanine complex (Fig. 8). Since the fluorescence of the enzyme-ribose 1-phosphate-guanine complex was ~25% of that for the enzyme-guanine complex, these results suggested that ribose 1-phosphate dissociated rapidly from the ternary complex during turnover and that the enzyme-guanine complex accumulated.

**Comparison of Fluorescence Spectrum of Guanine Bound to Purine Nucleoside Phosphorylase with That of Guanine Anion**—Purine nucleoside phosphorylase bound guanine with a large increase in fluorescence (excitation wavelength, 280 nm). The other ligands used in this study caused a decrease in fluorescence intensity of the enzyme (Fig. 2A). Guanine is not fluorescent at neutral pH (32), but becomes highly fluorescent as the pH is raised above 9 (32–34). The fluorescence increase of guanine titrated with a pKa of 9.4 (data not shown). Consequently, the increase in fluorescence upon binding of guanine to purine nucleoside phosphorylase could be due to preferential binding of the anionic form of guanine. The difference fluorescence spectrum between the binary complex of purine nucleoside phosphorylase and guanine (270 nM) and
The kinetics of ligand binding to purine nucleoside phosphorylase were measured spectrofluorometrically as described under "Experimental Procedures."

Since the equilibration of ribose 1-phosphate with $E$ or $E_{\text{Gua}}$ was too rapid to be observed on the stopped-flow spectrophotometer, these species were in rapid equilibrium with ribose 1-phosphate. These rapid equilibrium conditions were due to the large dissociation rate constant ($k_1$), which was calculated to be $\approx 3000$ s$^{-1}$ from a dissociation constant ($K_d = k_{-1}/k_1$) of $300 \mu M$ (Table I) and a lower limit for the association rate constant ($k_1$) of $10^6$ M$^{-1}$ s$^{-1}$. Thus, if the substrates were at concentrations equal to their dissociation constants, the flux through the upper ($k_3$) and lower ($k_5$) pathways in Scheme I would be approximately in the ratio 7:8:13 (i.e., $k_3$ to $k_5$).

The enhanced fluorescence of the enzyme in the synthetic reaction with guanine as a substrate (Fig. 8) suggested that breakdown of an enzyme intermediate containing guanine, and not guanosine, was rate-limiting. Possible intermediates (Scheme I) were the enzyme-guanine complex ($E_{\text{Gua}}$) and the enzyme-ribose 1-phosphate-guanine complex ($E_{\text{Gua}}$). Since the concentration of ribose 1-phosphate in these experiments was greater than the $K_m$ for ribose 1-phosphate, $E_{\text{Gua}}$ must be the predominant intermediate. Consequently, the rate-limiting step for the synthesis of guanosine at high concentrations of ribose 1-phosphate and guanine was the bond-
making step (Scheme I, $k_\text{diss}$. Thus, this rate constant was equal to $22 \pm 6$ s$^{-1}$, which was the maximum turnover number of the enzyme (Table I, $k_\text{cat}$ for guanine).

Inosine and guanosine did not affect the fluorescence of purine nucleoside phosphorylase, and they did not affect the dissociation constants of the enzyme for ribose 1-phosphate and guanine (Table II). Furthermore, the association rate constants for acyclovir diphosphate and guanine were not diminished by guanosine (Table III). Thus, guanosine and inosine did not bind to free enzyme. Since phosphate equilibrated rapidly with free enzyme, the phosphorolytic reaction was ordered with phosphate binding before the nucleoside (Scheme II). The initial velocity data for phosphorylation of 100 $\mu$M guanosine with phosphate as the variable substrate, however, did not fit a simple ordered mechanism (Fig. 1). The data were fitted to a bifunctional kinetic model (Equation 1) with catalytic rate constants of $22 \pm 1$ and $1.3 \pm 0.4$ s$^{-1}$ and $K_m$ values of 1000 $\pm$ 200 and 16 $\pm$ 8 $\mu$M, respectively.

The fluorescence intensity of purine nucleoside phosphorylase during phosphorylation of guanosine was similar to that of $E^{\text{Gua}}$. Since the fluorescence intensity of $E^{\text{Gua}}$ was unique among the ligands studied, this species was probably accumulating during turnover, which indicated that the rate-limiting step was the dissociation of guanine from $E^{\text{Gua}}$. The measured rate constant for release of guanine was $1.1 \pm 0.1$ s$^{-1}$ ($k_5$) in the absence of phosphate and $23 \pm 2$ s$^{-1}$ ($k_6$) in the presence of high phosphate concentrations (Fig. 6). These rate constants were in good agreement with the catalytic rate constants determined from a fit of the initial velocity data to Equation 1. Consequently, they were probably the rate-determining steps in the phosphorolytic reaction. The $K_m$ of phosphate for the enzyme-guanine complex was $2 \pm 0.3$ mM (Fig. 6), which agreed with the larger $K_m$ for the steady-state data of Fig. 1. The nonlinear double reciprocal plots of initial velocity data were likely due to enhancement of the release of phosphate by phosphate (Scheme II, $k_5 > k_6$). The value of $K_m$ for phosphate in the steady-state analysis (Table I) with phosphate concentrations varied between 200 and 8000 $\mu$M was determined by the binding of phosphate to $E^{\text{Gua}}$ and was not influenced significantly by the binding of phosphate to free enzyme as a substrate. Furthermore, the nearly parallel lines for the double reciprocal plot of these initial velocity data were due to the apparently irreversible product release step (dissociation of ribose 1-phosphate ($K_p$) in the absence of free ribose 1-phosphate) between the addition of guanosine to the enzyme and the second addition of phosphate. The obligatory addition of phosphate followed by the nucleoside observed herein for calf spleen purine nucleoside phosphorylase in the phosphorolytic reaction was the same as that for the thyroid enzyme (22, 23), but was the reverse of that reported for the human erythrocyte (20) and bovine brain (21) enzymes.

The validity of the steady-state kinetic parameters was confirmed by comparing the measured equilibrium constant with that calculated from the Haldane relationship (Equation 14).

$$K_{eq} = \frac{k_K}{k_k K_c}$$

(14)

The equilibrium constants for the inosine and guanosine reactions were calculated from the data in Table I and Equation 14 to be 48 and 57, respectively. Since these values agreed with the measured equilibrium constant of 56 $\pm$ 6 for the inosine reaction at pH 7.1 in 50 mM PIPES at 25°C, the steady-state kinetic parameters are consistent with the overall equilibrium constant for the reaction.

The equilibrium constant for the synthesis of guanosine on the enzyme (Scheme II, $k_{-3}/k_5$) was compared to $K_{eq}$ for the overall reaction. The value of $k_{-3}$ (Scheme II) was $22 \pm 6$ s$^{-1}$. The value of $k_5$ was estimated from the data for the approach to the steady state with guanosine and 5 mM phosphate (Fig. 8) as follows. The value for the first-order rate constant for the approach to the steady state at a saturating concentration of guanosine was $250 \pm 20$ s$^{-1}$ (Fig. 8). Since the concentration of ribose 1-phosphate was zero and the concentration of phosphate was 5 mM and these species were in rapid equilibrium with the enzyme, this rate constant was approximately the sum of $k_5$ and $k_6$ (Scheme II). Consequently, $k_5$ was 227 s$^{-1}$ (250 s$^{-1}$ minus 23 s$^{-1}$), and the equilibrium constant for the synthesis reaction on the enzyme was 0.1. Thus, the equilibrium for synthesis was less favorable on the enzyme ($K_{eq} = 0.1$) than it was free of the enzyme ($K_{eq} = 48$).

The association and dissociation rate constants for acyclovir diphosphate were dependent on phosphate concentration (Scheme III). The association rate constant was $6.6 \times 10^4$ M$^{-1}$ s$^{-1}$, and the dissociation rate constant was small ($0.001$ s$^{-1}$) in the absence of phosphate (Fig. 4). Phosphate decreased the association rate constant to $2 \times 10^4$ M$^{-1}$ s$^{-1}$ (calculated from the data of Fig. 3) with a binding constant of 140 $\mu$M. This binding constant was similar to the $K_d$ for phosphate (230 $\mu$M) determined by spectrofluorometric titration of the enzyme. Since the apparent dissociation rate constant was linearly dependent on phosphate concentration (Fig. 4), only the ratio (k in Equation 15) of the first-order dissociation constant ($k_{\text{eff}}$) to the binding constant of phosphate for the enzyme-acyclovir diphosphate complex ($K_e$) can be estimated from the data of Fig. 4B.

The value of k was $0.58$ M$^{-1}$ s$^{-1}$. If $k_{\text{eff}}$ for acyclovir diphosphate was similar to the dissociation rate constants for hypoxanthine or guanine determined at high phosphate concentrations (~15 s$^{-1}$), then the dissociation constant of phosphate for the enzyme-acyclovir diphosphate complex ($K_p$) was calculated with Equation 15 to be 28 M. The dissociation rate constant for acyclovir diphosphate in the absence of phosphate was...
calculated from these constants to be 0.004 s\(^{-1}\). This value was in reasonable agreement with the dissociation rate constant extrapolated from the data of Fig. 4A (0.001 ± 0.002 s\(^{-1}\)). Since the binding constant of phosphate for the enzyme-guanine complex (1030 \(\mu M\) (Table I) was comparable to that for free enzyme (230 \(\mu M\) (Table I), the large binding constant of phosphate (28 M) for the enzyme-acyclovir diphosphate complex was consistent with phosphate and the phosphate moiety of acyclovir diphosphate competing for the same site on the enzyme.

The \(K_i\) for acyclovir diphosphate in the absence of phosphate was calculated to be 0.6 nM from a dissociation rate constant of 0.004 s\(^{-1}\) and an association rate constant of 6.6 \(\times 10^{9}\) M\(^{-1}\) s\(^{-1}\). This value agreed with the \(K_i\) of 0.35 nM estimated for the erythrocyte enzyme by steady-state methods (13). It has been suggested that acyclovir diphosphate and 9-(phosphonoalkyl)hypoxanthines are bisubstrate analogues for purine nucleoside phosphorylase (9, 13). If acyclovir diphosphate were a bisubstrate analogue that utilized the full binding potentials of phosphate and guanine moieties, its dissociation constant should be approximately equal to the product of the dissociation constants for guanine (80 nM) and phosphate (230 \(\mu M\)), which was 0.02 nM. Since the dissociation constant for acyclovir diphosphate was 30-fold larger than the predicted value, the phosphate and guanine moieties of acyclovir diphosphate were not bound by the enzyme as effectively as free guanine and free phosphate.

The increase in the fluorescence of guanine upon binding to purine nucleoside phosphorylase was a unique property of guanine that was not observed with the other ligands studied. The fluorescence spectrum of the anion of guanine in ethanol was similar to that of the fluorescence spectrum of guanine bound to the enzyme at pH 7.1 (Fig. 9). These results suggested that the enzyme preferentially bound anionic guanine. This would be consistent with a chemical mechanism for synthesis in which the N-9 anion of guanine displaced the phosphate of ribose 1-phosphate.

In summary, the effects of phosphate on the enzyme ketinics and ligand binding were readily explained in terms of a dual functional phosphate-binding site. Thus, the biphasic double reciprocal plots of initial velocity data for phosphorolyis with phosphate as the variable substrate, which were suggested previously to be due to a cooperative interaction between enzyme subunits (16), could be due to phosphate binding as a participant (substrate) in the reaction and binding to the same site after release of ribose 1-phosphate as an effector to enhance the rate-limiting release of nucleobase from the enzyme. The effects of phosphate on the association and dissociation rate constants for acyclovir diphosphate were consistent with phosphate and the terminal phosphate of acyclovir diphosphate competing for the same binding site.

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