Binding Modes for Substrate and a Proposed Transition-State Analogue of Protozoan Nucleoside Hydrolase†

David W. Parkin and Vern L. Schramm*

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received July 14, 1995; Revised Manuscript Received August 24, 1995⊗

ABSTRACT: The transition-state structure for inosine−uridine nucleoside hydrolase (IU-nucleoside hydrolase) from *Crithidia fasciculata* is characterized by oxycarbonium character in the ribosyl and weak bonds to the departing hypoxanthine and incipient water nucleophile [Horenstein, B. A., Parkin, D. W., Estupiñán, B., & Schramm, V. L. (1991) *Biochemistry 30*, 10788−10795]. Inhibitors designed to resemble the transition state are slow-onset, tight-binding inhibitors with observed K_m/K_i values up to 2×10^5 [Schramm, V. L., Horenstein, B. H., & Kline, P. C. (1994) *J. Biol. Chem. 269*, 18259−18262]. Although slow-onset, tight binding is consistent with transition-state stabilization, more direct evidence can be obtained by comparing the groups which interact with the substrate to provide binding and catalysis with those which interact with the putative transition-state inhibitor. The K_m value for inosine binding to IU-nucleoside hydrolase is independent of pH over the range 5.6−10.5. Dependencies of V_{max} and V_{max}/K_m on pH result in pH optima near 8.0. A single group with pK of 9.1 must be protonated for catalytic activity, and protonation of a second group with a pK of 7.1 results in loss of activity. 1-(*S*)-Phenyl-1,4-dideoxy-1,4-imino-D-ribitol (phenyliminoribitol) binds with an equilibrium K_d of 30 nM and has been proposed to be a transition-state inhibitor. The pH dependence for the competitive inhibition by phenyliminoribitol resembles the V_{max} profile with the protonation of a single group, pK 7.5, required for inhibitor binding and the protonation of a subsequent group, pK 6.6, causing loss of binding. It has been proposed that the positive charge of protonated inhibitor (pK 6.5) is a recognition feature for binding as a transition-state inhibitor. However, the pH analysis indicates that the neutral inhibitor is the preferred species for binding the active form of the enzyme. The slow-onset phase of phenyliminoribitol binding disappears at low pH, suggesting that a time-dependent protonation of the bound complex could be responsible for the slow-onset phase of inhibition.

The inosine−uridine preferring nucleoside hydrolase (IU-nucleoside hydrolase) from *Crithidia fasciculata* hydrolyzes the N-glycosidic bonds of the commonly occurring purine and pyrimidine nucleosides (Parkin et al., 1991). Kinetic isotope effect studies have established that the enzyme-stabilized transition state for inosine is distinct from the diprotonated purine which occurs during acid-catalyzed solvolysis (Horenstein et al., 1991; Garrett & Mehta, 1972; Cherian et al., 1990). Based on the geometry and electrostatic potential surfaces of inosine and inosine at the transition state (Horenstein & Schramm, 1993a), a family of inhibitors were prepared which contain various features of the transition state (Horenstein & Schramm, 1993b; Horenstein et al., 1993; Boutellier et al., 1994). A common feature of these inhibitors is the ability to be protonated to form ribooxycarbonium mimics and tight binding to the enzyme relative to the binding of substrates and substrate analogues (Figure 1). On the basis of the similarity of the inhibitors to the experimentally determined transition states, it has been proposed that these are transition-state inhibitors. However, it has been pointed out that tight binding alone cannot establish mimicry of the transition state, and in some cases strong inhibition may be due to adventitious interactions in the active site (McCarter & Withers, 1994).

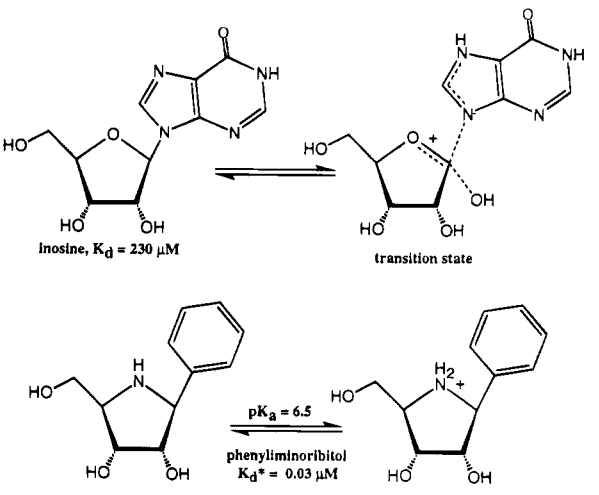

FIGURE 1: Inosine, the transition state for inosine stabilized by IU-nucleoside hydrolase, and the ionization states of phenyliminoribitol, the proposed transition-state inhibitor. The K_d for inosine is that determined here (see Figure 2). The constant K_d* for phenyliminoribitol is taken from Horenstein and Schramm (1993b) and differs slightly from that reported here as a result of different assay conditions. The value of K_d* is the overall equilibrium between enzyme and inhibitor and includes the slow-onset phase of inhibition.

Transition-state inhibitors are those which exhibit tight binding by virtue of atomic interactions with the enzymatic

† This work was supported by Research Grant GM41916 from the National Institutes of Health.

* Corresponding author. Telephone: (718) 430-2813. Fax: (718) 892-0703. E-mail: vern@aecom.yu.edu.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1995.

0006-2960/95/0434-13961$09.00/0

groups which stabilize the normal transition state. A method to establish transition-state interactions is to identify enzymatic groups which stabilize the transition state by the pH dependence of catalysis. If the catalytic groups are involved in binding the transition-state inhibitor, but are distinct from those which stabilize the enzyme−substrate complex, the hypothesis for binding as a transition-state analogue can be strengthened.

The IU-nucleoside hydrolase has favorable properties for pH analysis of inhibitor interactions. The kinetic mechanism is rapid-equilibrium, random; thus the binding of substrate is in thermodynamic equilibrium, and K_m values closely approximate dissociation constants. Observed pK values for V_{max}/K_m thus represent ionizable groups in the free enzyme or substrate required for formation of the Michaelis complex, and pK values associated with V_{max} are groups in the enzyme−inosine complex involved in catalysis, and these are required for formation of the transition state. No ionizable groups are observed in formation of the Michaelis complex. Conversion of the Michaelis complex to the transition state requires two ionizable groups, one of which must be protonated, and a second which must be unprotonated. Binding of the proposed transition-state analogue requires these groups in the same protonation states required for catalysis.

EXPERIMENTAL PROCEDURES

Initial Rate Studies. The IU-nucleoside hydrolase was purified to homogeneity from *C. fasciculata* (Parkin et al., 1991). The conversion of inosine to hypoxanthine and ribose was monitored by the change in the UV spectrum at 280 nm. Reaction mixtures were maintained at 30 °C at the desired pH and were initiated by the addition of 37−50 μl of the appropriate enzyme dilution to give 1.0 mL. The final enzyme concentration was near 5 nM. To prevent significant depletion of inhibitor during inhibition experiments, the inhibitor concentrations were always greater than 200 nM. Conversion of 1 mM inosine to hypoxanthine under these conditions gave an absorbance change of 0.72 to 0.92 cm^{-1} at 280 nm depending on pH. The inosine (recrystallized) concentration of stock solutions was calibrated by the UV absorbance. Reaction mixtures were buffered with 15 mM each of inorganic pyrophosphate, citrate, and phosphate or 15 mM each of triethanolamine, CAPSO, and inorganic pyrophosphate, adjusted to the desired pH with HCl or NaOH. The response to pH was independent of the buffer system.

Full inhibition patterns were determined with five substrate concentrations at three fixed concentrations of phenyliminoribitol. Full inhibition patterns were determined at pH 5.5, 6.5, 7.5, 8.6, and 9.6 and gave good fits to slope-linear competitive inhibition. When fit to the equation for noncompetitive inhibition, the ordinate intercept values of Lineweaver−Burke plots were insignificant, consistent with competitive inhibition. At the remainder of the pH values, the K_m, V_{max}, and V_{max}/K_m values were determined by Lineweaver−Burke plots using five substrate concentrations. The K_i values for phenyliminoribitol were established at a substrate concentration near the K_m value, using five inhibitor concentrations. The values of K_i were established in Dixon plots, using a fixed substrate concentration near the K_m value and five inhibitor concentrations.

In all cases where K_i was determined, the initial rate portion of the inhibition curve was measured prior to the tighter, slow-onset component of phenyliminoribitol inhibition (Horenstein & Schramm, 1993b). Initial rate and pH dependence of the kinetic constants were analyzed using the computer programs of Cleland (1977).

Table 1: pK_a Values for Kinetic Constants of IU-Nucleoside Hydrolase

kinetic constant	limiting value[a]	ionization constants[b] ion (pK_a)	(pK_b)
V_{max} [μmol/(min·mg)]	84 ± 7	7.1 ± 0.1	9.1 ± 0.1
k_{cat}/K_m (M^{-1} s^{-1})	(3.0 ± 0.8) × 10^5	7.3 ± 0.2	8.7 ± 0.2
K_m[c] (μM)	230 ± 80	none	none
K_i[d] (μM)	0.17 ± 0.05	6.6 ± 0.2	7.5 ± 0.1

[a] This value represents the V_{max}, k_{cat}/K_m, and K_i values which could theoretically be obtained if all of the enzyme and ligand available in the system were in the ionic state for most favorable binding. [b] pK_a values refer to groups which exhibit their catalytic or binding functions when in the deprotonated state. pK_b refers to groups which exhibit their catalytic or binding functions when in the protonated state. [c] The mean ± standard deviation of the 14 independent determinations of K_m shown in Figure 2. [d] From the pK_a values determined from the data of Figure 3.

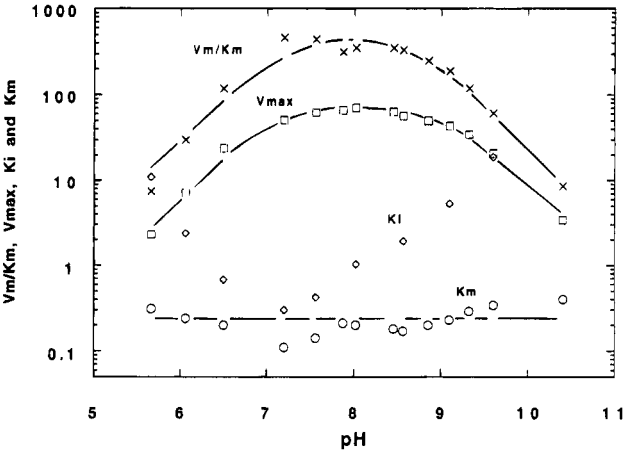

FIGURE 2: Kinetic constants for hydrolysis of inosine by IU-nucleoside hydrolase and the observed K_i values for phenyliminoribitol as a function of pH. Units on the ordinate are μmol/(min·mg of enzyme) for V_{max}, μmol/(min·mg·mM) for V_m/K_m, μM for K_i, and mM for K_m. The points are the experimental values from fits of the initial rate data to the Michaelis−Menten equation or for the equation for competitive inhibition (K_i slope values). The lines for V_m/K_m and V_{max} are the fit of the data to the equation for two ionizable groups leading to a bell shaped profile, for example; log V_{max} = log[(limiting V_{max})/(1 + H^+/K_a + K_b/H^+)] (Cleland 1977). The line drawn through the K_m data is the average of the individual determinations, since there is no systematic variation.

RESULTS

pH Dependence of Kinetic Constants for Nucleoside Hydrolase. The K_m for inosine hydrolysis by IU-nucleoside hydrolase is independent, and V_{max} is dependent on pH over the broad range from 5.6 to 10.5. Ionizable groups for V_{max} exhibited pK values of 7.1 and 9.1 with a plateau between these pH values (Table 1 and Figure 2). Since inosine has no ionizable groups over this pH range, these pK values represent ionizable groups on the enzyme which are involved in catalysis but do not influence the K_m value. The group with pK value 7.1 must be unprotonated and that at 9.1 must be protonated in order to achieve catalysis. The experimental data were fitted to the equation for two groups with distinct pK values where only the monoprotonated form is active. The data were also analyzed for their fit to the equation for

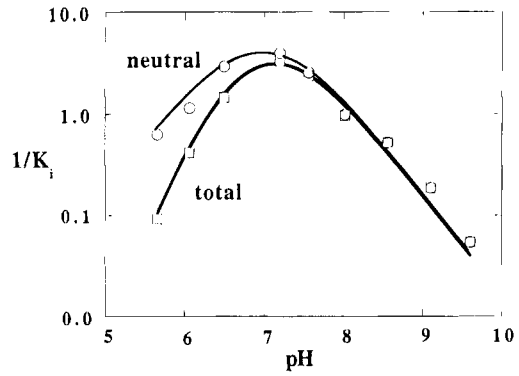

FIGURE 3: Inhibition constants for the total and neutral forms of phenyliminoribitol as a function of pH. Units on the ordinate scale are μM^{-1}. The inhibition constants for the total form of phenyliminoribitol are the observed K_i values. Inhibition constants for the neutral form were calculated using a pK_a of 6.5 for the protonation as indicated in the text and illustrated in Figure 1. The points are the observed experimental values for $1/K_i$ (*total*) or those calculated with the assumption that only the neutral form of inhibitor causes inhibition (*neutral*). The lines are the fit of the data to $(1/K_i) = [(\text{limiting } 1/K_i)/(1 + H^+/K_a + K_b/H^+)]$ (*neutral*) or $(1/K_i) = [(\text{limiting } 1/K_i)/(1 + H^+/K_a + K_b/H^+)\ (1 + H^+/K_d)]$ (*total*). For fit of the *total* data, K_a was fixed at 6.6, based on the K_a determined from the *neutral* data.

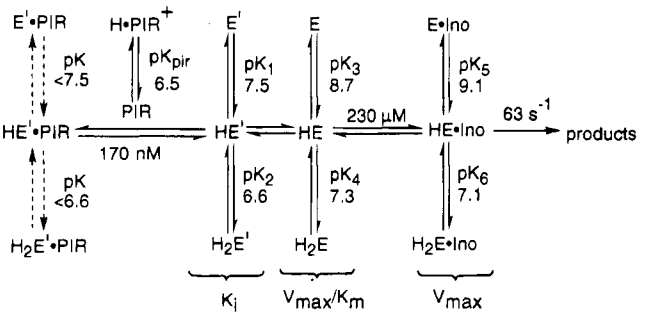

FIGURE 4: Protonation states for binding of inosine, phenyliminoribitol and for catalysis by IU-nucleoside hydrolase. E represents the ionization state of the enzyme at alkaline pH, with the catalytically significant protonations indicated as HE and H_2E, respectively. Phenyliminoribitol is indicated as PIR and inosine as Ino. HE′ is an inhibitor-binding state of the enzyme. pK_1−pK_6 are pK values for the enzyme and enzyme−substrate complexes. The dissociation constant of 170 nM for phenyliminoribitol is for the HE′•PIR complex. pK_{pir} is the pK value for free inhibitor. The dashed arrows and pK's <7.5 and <6.6 are hypothetical ionizations. The brackets labeled K_i, V_{max}/K_m, and V_{max} indicate the experimental origin for these pK values. The rate of 63 s^{-1} is the theoretical maximum rate for the enzyme if all of the enzyme could be obtained as the HE•inosine form. Since, at any pH, fractions will be in the inactive E•inosine and H_2E•inosine forms, this rate is not observed.

two ionizable groups of similar pK on the acid side and one group at alkaline pH values. The data gave better agreement with the fit to single ionizable groups at both high and low pH values. The kinetic reaction mechanism for nucleoside hydrolase is rapid-equilibrium, random (Parkin et al., 1991). Under these conditions, the pH profiles for V_{max}/K_m gave pK values for free enzyme and the pH dependence of V_{max} provides the pK values of ionizable groups in the enzyme−substrate complex which are required for formation of the transition state (Cleland, 1977). The group which appears with pK 7.1 is proposed to correspond to an enzymatic carboxylate with an elevated pK value. The other group involved in transition-state formation is an enzymatic proton donor, proposed to be a histidine or arginine (see Discussion).

The K_m values for rapid equilibrium reactions are close approximations of dissociation constants. The pH dependence of K_m identifies the pK values of ionizable groups on the enzyme or substrate required for the formation of the catalytically competent enzyme−substrate complex. No required ionizations are detected. Since the K_m value of 230 μM is unchanged across the pH range, inosine binds equally well to the active form at any pH. The results cannot eliminate the more complex possibility that substrate binds to all forms of the enzyme across this pH range.

pH Dependence of Inhibition by Phenyliminoribitol. Inhibition by phenyliminoribitol is slope-linear competitive inhibition with respect to inosine as expected for competition with the substrate when initial reaction rates are measured. After several minutes in the presence of inhibitor, the reaction rate decreases as a result of slow-onset (tight binding) inhibition and yields a net inhibition constant approximately 5-fold lower than the value calculated from the initial rate data (Horenstein & Schramm, 1993b). Experimental conditions for the results of Figure 2 were restricted to the initial rate period where competitive inhibition occurs. Competitive inhibitors, like substrates in rapid-equilibrium mechanisms, are in binding equilibrium with the enzyme, and the pK values represent the ionizable groups on both the free enzyme and inhibitor necessary for binding. A competitive inhibitor which mimics inosine interactions would be expected to show pK values only for groups on the inhibitor, since no ionizable groups appear in the K_m value for substrate binding. Direct acid/base titration of phenyliminoribitol has established a pK of 6.5 for the secondary amine (Horenstein et al., 1993; Horenstein & Schramm, 1993b; see Figure 1). If protonated and unprotonated forms bound equally, interacting with the same groups as substrate, no dependence of K_i on pH would be observed. If only the neutral form or the cationic form bound and interacted as a substrate analogue, a single pK of 6.5 would be observed. These possibilities are tested in the plot of log $1/K_i$ as a function of pH in Figure 3.

At acid pH values the limiting slope approaches 2 for the observed inhibitor binding ($1/K_i$) with a third ionizable group apparent above pH 7.0 (Figure 3, "total" curve). The binding of the inhibitor is at a maximum affinity near pH 7.2 and is strongly influenced by pH. The limiting slope at alkaline pH values is near unity, consistent with loss of inhibitor binding when this group is deprotonated. In summary, two unprotonated groups with pK values between pH 6 and 7 and one protonated group with a pK value near 7.9 are required for the binding of the proposed transition-state inhibitor. The data fit well to three ionizable groups at 6.4, 6.6, and 7.4, respectively (Figure 3).

Data for the K_i of phenyliminoribitol as a function of pH were corrected for the formation of the cationic inhibitor, and the data for inhibition by the neutral form of the inhibitor (Figure 1) were fit to the equation for two ionizable groups (Figure 3, "neutral" curve). The results establish that binding of the neutral inhibitor is dependent on pH and requires a group with a pK 7.5 to be protonated. Protonation of a second enzymatic group exhibiting a pK of 6.6 causes loss of binding of the neutral inhibitor. Binding of the inhibitor, unlike the substrate, requires the participation of a proton−donor/proton−acceptor pair similar to the ionizable groups required for catalysis. The difference between the *neutral* and *total* curves in Figure 3 is a group of pK 6.4, close to the actual pK of 6.5 for phenyliminoribitol.

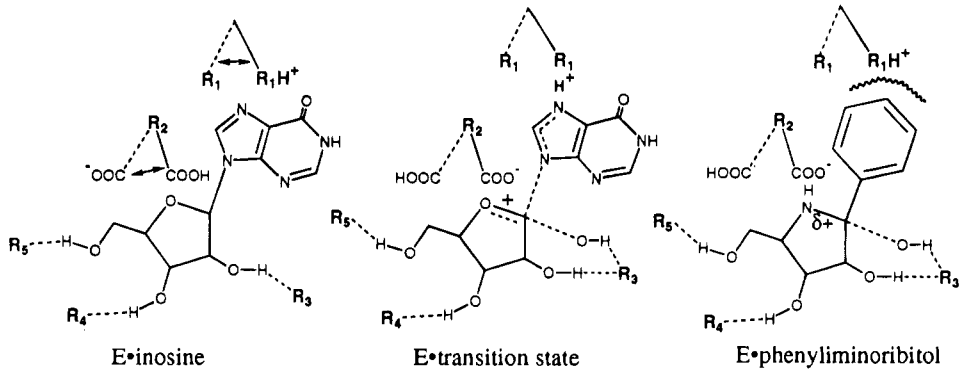

FIGURE 5: Proposal for the ionic groups which interact with substrate and inhibitor to give the pK values shown in Figure 4. Substrate-binding forms the E•inosine Michaelis complex which is insensitive to the ionization state of R_1, possibly a His residue, and R_2, a carboxylate. Inhibitor specificity studies (Horenstein & Schramm, 1993b; Boutellier et al., 1994) establish that the purine binding site also has hydrophobic character. All ribosyl hydroxyls are essential for significant enzymatic activity, and these are proposed to hydrogen-bond to three additional groups indicated as R_3, R_4, and R_5. Transition-state stabilization requires protonation of the purine by R_1 and can only occur from the protonated R_1 group. Likewise, stabilization of the oxycarbonium can only occur with the unprotonated R_2-carboxyl. The E•phenyliminoribitol complex recruits R_2 binding as the carboxylate anion. Although the phenyl group has no ionic interaction, stabilization of R_2 as the anion and filling the hydrophobic pocket (indicated by the wavy line) is apparently linked to protonation of R_1.

A log plot of the K_i value for the phenyliminoribitol cation as a function of pH gives a slope of 2 at acid pH values followed by an asymptotic approach toward a K_i of zero (infinite affinity) as pH increases (not shown). These data could not be fit to equations associated with any of the enzymatic pK values in Table 1 and are therefore inconsistent with binding of the cationic species of phenyliminoribitol. Together, these results indicate that only the neutral form of phenyliminoribitol binds. Protonation of the inhibitor, or the group with pK 6.6 causes loss of inhibitor binding.

The pH dependence of the slow-onset component of inhibition by phenyliminoribitol was followed by observing the time-dependent change in reaction rate. At pH 5.6 the slow-onset tight-binding inhibition phase was not observed. At pH values of 7.0 and above, the second component of inhibition was observed and accounted for an average increase in binding affinity of 2-fold at pH 7 and 2.6 ± 0.2-fold above pH 8.0 (not shown). In this buffer system, the second component of inhibition was less than originally reported (Horenstein & Schramm, 1993b) and was found to be pH dependent only at higher pH values.

DISCUSSION

pH Dependence of Kinetic Constants for IU-Nucleoside Hydrolase. The protonation states of IU-nucleoside hydrolase involved in catalysis are summarized in Figure 4. The independence of K_m with pH "fairly reliably establishes" that substrate binds in rapid equilibrium with the enzyme (Tipton & Dixon, 1979) in the pH range of 5.6−10.5. Since inosine has no ionizable groups over this pH range, the neutral substrate binding to the monoprotonated form of the enzyme (HE) is established. This result is consistent with steady-state kinetic studies at pH 8.0 which established a rapid-equilibrium, random kinetic mechanism (Parkin et al., 1991).

The V_{max} is dependent on the protonation of a group labeled pK_5 (Figure 4) with a pK of 9.1 and the unprotonated form of a second group with a pK of 7.1, indicated as pK_6 in Figure 4. These ionization constants are for the enzyme•-inosine complex and are the groups required to permit formation of the transition state. The pH profiles for V_{max} indicate that only the monoprotonated form of enzyme (HE•Ino in Figure 4) has significant activity, although activities of 1% or less than the observed V_{max} for the E•Ino or H_2E•-Ino species would not be easily detected over the experimentally accessible pH range.

Enzymes which show small commitments to catalysis for substrate binding give V_{max}/K_m values which approximate pK values for the free enzyme. Since K_m values are independent of pH, the V_{max} and V_{max}/K_m values are equal within experimental errors of the pK determination (Table 1 and Figure 4). The result indicates that substrate binding causes little change in the pK values. Thus pK_3 and pK_5 as well as pK_4 and pK_6 are most directly interpreted to represent the ionizations of the same groups in the absence and presence of inosine, respectively. Binding of inosine to E and H_2E and inosine release from E•Ino and H_2E•Ino cannot be determined from the results. If inosine binds to E and H_2E, the dissociation constants for these complexes would be similar to 230 μM, since these interactions would create thermodynamic equilibria of four species. The complexes cannot give rise to products at rates of >1% of V_{max}, since the limiting slopes of the V_{max} plot show no plateau regions.

Ionization State for Phenyliminoribitol Binding. Freely reversible binding of a competitive inhibitor permits the determination of pK values for groups on the free enzyme which influence inhibitor binding (Cleland, 1977). Unlike the substrate, the binding of phenyliminoribitol is dependent on the ionization state of its imino group and of two groups on the enzyme. The dependence of inhibitor binding on two enzymatic groups, which have the same protonation states as for catalysis, indicates that the inhibitor binds to the catalytically active enzyme form. This binding pattern provides evidence that the inhibitor is binding as a transition state inhibitor, rather than as a substrate analogue, since the ionizable groups, presumed to be those in catalysis, are necessary for inhibitor binding.

The ability of phenyliminoribitol to mimic the cationic nature of the transition state was originally proposed as one of the reasons for its action as a tight-binding inhibitor. In addition to tight binding, the slow-onset inhibition (Horenstein et al., 1993b) fits the characteristics of many transition-state inhibitors (Morrison & Walsh, 1988). However, the

pH profile for pK_i, corrected for pK_{pir} (Figure 4), establishes that only the neutral form binds, with a dissociation constant of 170 nM. This constant does not include the slow-onset component of inhibitor binding which occurs over a period of minutes and increases the affinity by 2.6-fold (earlier studies in a different buffer system gave a 5-fold change to 30 nM; Horenstein & Schramm, 1993b). The pH dependence of the slow-onset tight-binding phase indicated that the slow onset phase is present at higher pH ranges but was not detectable at pH 5.6. This result suggests the initial, freely reversible binding of inhibitor as the neutral species with its time-dependent rearrangement of the enzymatic groups to form a more tightly bound species. It can be speculated that this tightly bound form involves active site rearrangement to provide a more favorable bonding pattern to the enzyme. The tight-binding could include proton transfer to the imino group to form an ion pair between an enzymatic anion and the protonated imino group, as indicated by the lower broken arrows in Figure 4, $pK < 6.6$.

The rapid-equilibrium random mechanism of IU-nucleoside hydrolase and the competitive inhibition with phenyliminoribitol predicts that pK values determined from pH dependence of V_{max}/K_m and K_i would be approximately equal, i.e., $pK_1 \cong pK_3$ and $pK_2 \cong pK_4$ in Figure 4. The observed differences of 1.2 pH units between pK_1 and pK_3 and 0.7 pH unit between pK_2 and pK_4 establish that ionizations determined in the presence of phenyliminoribitol differ in a fundamental way from those determined from V_{max}/K_m. One possibility is that the enzyme exists in conformational isomers HE and HE′, in which phenyliminoribitol binding prefers HE′ (Figure 4). A second possibility is that binding of phenyliminoribitol causes shifts of the pK values to <7.5 and <6.6, respectively (Figure 4), and that the binding, at 170 nM, is not in true rapid equilibrium. The lower pK values would thus influence the experimental values of pK_1 and pK_2. Such a shift would have no effect on pK values determined from V_{max}/K_m. The k_{cat}/K_m value of 3×10^5 M^{-1} s^{-1} for IU-nucleoside hydrolase is only $\sim 10^{-3}$ of the rate for diffusion-limited catalysis; therefore, the distribution into two or more isomeric forms is well within catalytic limits. However, nonequilibrium partitioning of the enzyme into different inhibitor complexes would also be expected to cause loss of the competitive inhibition patterns. Resolution of these issues will require a detailed analysis of the stoichiometry and slow-onset kinetics of a transition-state inhibitor with more favorable properties for the second inhibitory phase [e.g., see Boutellier et al. (1994)].

Ionizable Enzymatic Groups. The pK values of 8.7 and 7.3 for pK_3 and pK_4, respectively (Figure 4), are the ionizable groups on the free enzyme. These groups are also the candidates for groups required for catalysis, since the same groups (pK_5 and pK_6) appear in the pH dependence of V_{max}.

Recent results from X-ray crystal structural analysis of the unliganded enzyme indicates that the proposed substrate binding site contains several carboxylates in the bottom of the site and a histidine, hydrophobic groups, and possibly an arginine in positions which might be expected to interact with the purine base.[1] A possible assignment of the pK_a values would be His or Arg for pK_3 and pK_5 and carboxylate(s) for pK_4 and pK_6 (Figure 4). A protonated His or Arg could act as a proton donor for the leaving group hypoxanthine, and an unprotonated carboxylate is proposed to stabilize the oxycarbonium of the transition state. This pattern can explain the interaction of the unprotonated phenyliminoribitol, since the unprotonated carboxylate would be expected to form a stable hydrogen bond to the partial positive imino hydrogen. These interactions are summarized in Figure 5. These ionization assignments correspond to the known structure of the transition state ensemble for the enzyme and explain the observed pK patterns.

Inhibitors which attempt to mimic the positive charge and hydroxyl pattern of sugar oxycarbonium ions at the enzyme-stabilized transition states have been discovered or developed for enzymes with specificities for ribosides, glucosides, arabinosides, mannosides, and galactosides (Papandreou et al., 1993; Axamawaty et al., 1990; Boutellier, 1993; Horenstein et al., 1993; Knapp et al., 1993, 1994). The ionization state for optimum binding of these inhibitors has been proposed to require an anion−cation match between a general acid catalyst and the inhibitor. Thus, optimum inhibition of an arabinofuranosidase by iminothreitol or iminoarabinitol required the pK of the inhibitor to be below the pK of the acid-catalytic group (Axamawaty et al., 1990). In this case the protonated, cationic inhibitor binds to the unprotonated anionic catalytic site. The inhibitory properties of flattened and ionizable amide, amidrazone, and amidoxime monosaccharide derivatives of glucone, mannono, and galactonolactams are less dependent on ionization state, suggesting that their inhibitory properties are primarily geometric (Papandreou et al., 1993). The binding of the neutral form of several such inhibitors is also observed, often in patterns which precludes their binding as transition-state inhibitors [e.g., Dale et al. (1985) and Axamawaty et al. (1990)]. Inhibition of IU-nucleoside hydrolase represents the case where inhibition occurs only with neutral inhibitor, but the binding pattern mimics that of catalysis and therefore qualifies as transition-state inhibition. The partial positive charge which resides on the imino hydrogen in unprotonated phenyliminoribitol provides an excellent candidate for hydrogen bonding to a carboxylate which normally ion-pairs with the oxycarbonium of the transition state (Horenstein & Schramm, 1993a,b).

ACKNOWLEDGMENT

We thank Drs. Benjamin Horenstein and John Blanchard for useful comments.

REFERENCES

Axamawaty, T. H., Fleet, G. W. J., Hannah, K. A., Namgoong, S. K., & Sinnott, M. L. (1990) *Biochem. J. 266*, 245−249.
Boullitier, M., Horenstein, B. A., Semenyaka, A., Schramm, V. L., & Ganem, B. (1994) *Biochemistry 33*, 3994−4000.
Cherian, X. M., Van Arman, S. A., & Czarnik, A. W. (1990) *J. Am. Chem. Soc. 112*, 4490−4498.
Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273−387.
Dale, M. P., Ensley, H. E., Kern, K., Sastry, K. A. R., & Byers, L. D. (1985) *Biochemistry 24*, 3530−3539.
Garrett, E. R., & Mehta, P. J. (1972) *J. Am. Chem. Soc. 94*, 8532−8541.
Horenstein, B. A., & Schramm, V. L. (1993a) *Biochemistry 32*, 7089−7097.

[1] M. Degano, D. M. Gopaul, V. L. Schramm and J. C. Sacchettini, unpublished observations.

Horenstein, B. A., & Schramm, V. L. (1993b) *Biochemistry 32*, 9917−9925.
Horenstein, B. A., Parkin, D. W., Estupiñán, B., & Schramm, V. L. (1991) *Biochemistry 30*, 10788−10795.
Horenstein, B. A., Zabinski, R. F., & Schramm, V. L. (1993) *Tetrahedron Lett. 34*, 7213−7216.
Knapp, S., Choe, Y. H., & Reilly, E. (1993) *Tetrahedron Lett. 34*, 4443−4446.
Knapp, S., Purandare, A., Rupitz, K., & Withers, S. G. (1994) *J. Am. Chem. Soc. 116*, 7461−7462.
McCarter, J. D., & Withers, S. G. (1994) *Curr. Opin. Struct. Biol. 4*, 885−892.
Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201−301.
Papandreou, G., Tong, M. K., & Ganem, B. (1993) *J. Am. Chem. Soc. 115*, 11682−11690.
Parkin, D. W., Horenstein, B. A., Abdulah, D. R., Estupiñán, B., & Schramm, V. L. (1991) *J. Biol. Chem. 266*, 20658−20665.
Schramm, V. L., Horenstein, B. A., & Kline, P. C. (1994) *J. Biol. Chem. 269*, 18259−18262.
Tipton, K. F., & Dixon, H. B. F. (1979) *Methods Enzymol. 63*, 183−234.

BI951614X

Corrections

An Equivalent Site Mechanism for Na^+ and K^+ Binding to Sodium Pump and Control of the Conformational Change Reported by Fluorescein 5′-Isothiocyanate Modification, by Irina N. Smirnova, Shwu-Hwa Lin, and Larry D. Faller*, Volume 34, Number 27, July 11, 1995, pages 8657−8667.

Page 8660. Equation 4 should read

$$[Na^+]_{1/2} = K_{Na}\left(1 + \frac{[K^+]}{K_K}\right)(\sqrt{2} - 1) \qquad (4)$$

and eq 6 should read

$$-\Delta F_0 = \left\{[K^+]^2 \Big/ \left[[K^+]^2 + \frac{2K_K}{(1 + K_C)}\left(1 + \frac{[Na^+]}{K_{Na}}\right)[K^+] + \frac{K_K^2}{(1 + K_C)}\left(1 + \frac{[Na^+]}{K_{Na}}\right)^2\right]\left[\frac{K_C}{(1 + K_C)}\right]\right\}\Delta F_{max} \qquad (6)$$

Page 8665. Equation 13 should read

$$\frac{1}{\tau} = k_f\left[\frac{[K^+]^2}{[K^+]^2 + 2K_K[K^+] + K_K^2\left(1 + \frac{[Na^+]}{K_{Na}}\right)^2}\right] + k_r \qquad (13)$$

BI9550152