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pH Studies toward the Elucidation of the Auxiliary Catalyst for Pig Heart Aspartate Aminotransferase†

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ABSTRACT: The pH variation of the kinetic parameters for aspartate aminotransferase has been measured in order to determine which enzyme residues participate in auxiliary catalytic and binding roles. When the initial rate is obtained by varying concentrations of α-ketoglutarate and several different fixed concentrations of aspartate, competitive inhibition by both substrates is observed, indicative of E–PLP–α-ketoglutarate and E–PMP–aspartate dead-end complexes (E = enzyme, PLP = pyridoxal phosphate, and PMP = pyridoxamine phosphate). Inhibition by aspartate is significantly enhanced at high pH as a result of deprotonation of the amino group of aspartate, while α-ketoglutarate inhibition is stronger when an enzyme group with a pK of 6.4 is protonated. The maximum rate is pH independent from 5.5 to 10.0. The V/K for α-ketoglutarate decreases below a pK of 5.8 and above a pK of 9.2, while the V/K for aspartate decreases below a pK of 6.4 and above pK values of 9.2 and 10.0. The K_i for inhibition by maleate, competitive with aspartate (representing maleate binding to E–PLP), exhibits a pH dependence identical with α-ketoglutarate substrate inhibition, while the K_i for inhibition by maleate, competitive with α-ketoglutarate (representing binding to E–PMP), increases above a pK of 9.3. The K_i for inhibition by α-methylaspartate competitive with aspartate and indicative of binding to E–PLP increases below a pK of 6.5 and above a pK of 9.3. The temperature dependence of V/K pK values yields ΔH_{ion} values of 5–6 kcal/mol for all groups. V/K values obtained in the presence of 30% DMF with cationic acid buffers suggest that all groups are cationic acids. In all probability the group with a pK of 9.2 reflected in both V/K pH profiles is the pyridinium moiety of the cofactor. The above data are consistent with a mechanism in which (1) the nitrogen of the Schiff base between the 6-amino of lysine-258 and PLP must accept a proton from the amino group of aspartate to start the first half-reaction, (2) lysine-258, which originally participates in the Schiff base, acts as a general base during the conversion of aspartate and E–PLP to oxalacetate and E–PMP, and (3) at the completion of the first half-reaction, the 6-amino of lysine-258 and the pyridoxamino group of PMP may be hydrogen bonded and this diamine system is positively charged.

Aspartate aminotransferase catalyzes the reaction

L-aspartate + α-ketoglutarate ⇌
oxalacetate + L-glutamate

This enzyme has been studied extensively by several groups [reviewed in Braunstein (1972)], and an abundance of information has been obtained concerning its physical, kinetic, and spectral properties. The native holoenzyme is isolated with pyridoxal phosphate bound as a Schiff base to the 6-amino moiety of lysine-258 (Braunstein, 1972). Velick & Vavra (1962) have shown that the enzyme catalyzes a ping-pong reaction; aspartate transfers its amino group to the cofactor (PLP)[1] and dissociates as oxalacetate prior to the addition of α-ketoglutarate, which accepts the amino group and dissociates as L-glutamate. During the course of conversion of aspartate to oxalacetate, pyridoxal phosphate is converted to pyridoxamine phosphate. At pH 7.3, α-ketoglutarate at high concentration is found to inhibit the reaction, and this inhibition is competitive with aspartate (Velick & Vavra, 1962). In addition, inhibition is observed by mono- and dianions (notably mono- and dicarboxylic acids), which are competitive vs. both aspartate and α-ketoglutarate (Velick & Vavra, 1962; Bonseb et al., 1975; Harruff & Jenkins, 1978).

Chemical modification studies have implicated various enzyme residues as being the auxiliary acid–base catalyst facilitating removal of the aspartate α proton. The most notable of these is lysine (Turano et al., 1963). In addition, histidine has also been implicated (Peterson & Martinez-Carrion, 1970). Recently, the three-dimensional structure has been obtained to 2.7–2.8 Å resolution for both the mitochondrial (Jansonius et al., 1981) and cytoplasmic (Arnone et al., 1981) enzymes. Structures for these enzymes are very similar, particularly with respect to the active site, and indicate that the only residues in close enough proximity to act as an acid–base catalyst (and still be consistent with the known stereochemistry of the reaction) are lysine-258 and tyrosine-70* (tyrosine-70* is located

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DMF, dimethylformamide; NADH, nicotinamide adenine dinucleotide, reduced; α-KG, α-ketoglutarate; Asp, aspartate.

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on the other subunit in the dimer). The known location of these residues in the active site greatly facilitates assignment of the acid–base catalyst(s) for the transferase.

This study presents a detailed investigation of the pH dependence of the steady-state kinetic parameters and K_i values obtained for competitive inhibition. Substrate inhibition by aspartate and the pH dependence of the inhibition are reported which requires that the kinetic mechanism be modified to take into account the formation of an abortive E–PMP–aspartate complex. In addition, carefully obtained and accurate kinetic data for the pH dependence of V/K_{Asp} and $V/K_{\alpha\text{-KG}}$ have been obtained. These data, along with the effect of temperature and organic solvent on the pK values observed from these pH dependencies, provide corroborative information concerning the residues which participate in auxiliary catalytic and binding roles. These data suggest that tyrosine-70* is most likely not the auxiliary acid–base catalyst for this reaction. A chemical mechanism consistent with the above data is proposed for aspartate aminotransferase.

Materials and Methods

Chemicals and Enzymes. Dimethylformamide was purchased from Aldrich. Pig heart malate dehydrogenase and aspartate aminotransferase were from Sigma. The α subform of pig heart aspartate aminotransferase was kindly provided by Drs. David E. Metzler and Carol M. Metzler at Iowa State University. All other substrates, inhibitors, and buffers were obtained from commercially available sources and were of the highest quality available.

Initial Velocity Studies. These studies were carried out with a Beckman DU monochromator and a Gilford OD convertor. The reaction was assayed in the direction of production of L-glutamate and oxalacetate by coupling the oxalacetate produced to the malate dehydrogenase reaction and monitoring the decrease in NADH at 340 nm. Reaction mixtures contained 100 mM buffer (neutralized with KOH), 0.2 mM NADH, 17 units/mL malate dehydrogenase, and variable concentrations of the substrates aspartate and α-ketoglutarate. In all cases aspartate aminotransferase was 3.6 pM. All reactants were obtained as the free acid and were titrated with KOH so that the only anions present in the reaction mixture were reactants. At pH values of 7 and below, NADH and malate dehydrogenase were added to the reaction mixture just prior to the addition of aspartate aminotransferase to minimize acid-catalyzed degradation of NADH and loss of malate dehydrogenase activity.

The concentration of malate dehydrogenase required to give true initial velocities of the aminotransferase reaction with no detectable lag time was determined at pH 6, 8, and 9.5 (Cleland, 1979a) and checked by varying the aminotransferase to be sure it was limiting (as shown by a linear velocity vs. enzyme curve). The highest concentration of enzyme from these three determinations was then used for the entire pH range. Whenever conditions were altered, e.g., solvent or temperature, saturation by the coupling enzyme was redetermined by checking the linearity of a plot of velocity vs. aspartate aminotransferase concentration.

Initial velocity data obtained in the presence of either maleate or α-methylaspartate were collected in a manner similar to that outlined above except the inhibitor was also included in the reaction mixture. The inhibitor was present at several different fixed concentrations (including zero) when either α-ketoglutarate (in the case of maleate) or aspartate (in the case of α-methylaspartate) was varied at a single fixed level of the remaining substrate. This will be discussed further under Results.

pH Studies. Determination of V, $V/K_{\alpha\text{-KG}}$, V/K_{Asp}, and the K_i's for maleate and α-methylaspartate will be discussed in detail under Results. Buffers at 100 mM final concentration were used over the following pH ranges: N,N'-bis(2-hydroxyethyl)piperazine, pH 4.0–5.5; Mes,[1] 5.5–6.5; Hepes, 7.5–8.1 (since rates obtained in the presence of Pipes were significantly reduced, additional points were obtained by using mixtures of Mes and Hepes which gave a final buffer concentration of 100 mM); Taps, 8.0–9.0; Ches, 9.0–10.0; Caps, 10.0–11.0. All buffers were titrated to pH with KOH. In all cases, sufficient overlaps were obtained when buffers were changed so that correction could be made for spurious buffer effects. Kinetic parameters were obtained at 12.5, 25, and 37.5 °C, and temperature was maintained with a circulating water bath with the capacity to heat and cool the thermospacers for the cell compartment. Assay mixtures were incubated for at least 10 min in the water bath and 5 min in the cell compartment prior to initiating the reaction. Assay temperatures were routinely checked using a YSI tele-thermometer with a Teflon probe while the cuvette was in the cell compartment. The pH was obtained at each temperature by using a pH electrode calibrated and maintained at the temperature.

Studies in which the pH dependence of V/K_{Asp} and $V/K_{\alpha\text{-KG}}$ were determined in the presence of 30% DMF made use of the same cationic acid buffers listed above. The pH value was recorded prior to the addition of solvent. Velocities were obtained as a function of solvent concentration at saturating (but noninhibiting) substrate concentrations and under conditions where either one or the other substrate was fixed at its Michaelis constant. Under all conditions 30% DMF produced 40% inhibition of the rate.

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations. All plots were linear except the initial velocity patterns obtained when one substrate was varied at several fixed levels of the other. Data were fitted to the appropriate rate equations by using the Fortran programs of Cleland (1979b). Individual saturation curves were fitted to eq 1. Data for initial velocity patterns which exhibited double competitive substrate inhibition were fitted to eq 2. Data for pH profiles which decreased with a slope of

$$v = \frac{VA}{(K + A)} \tag{1}$$

$$v = \frac{VAB}{K_aB(1 + B/K_{IB}) + K_bA(1 + A/K_{IA}) + AB} \tag{2}$$

$$\log y = \log \frac{C}{1 + H/K_1 + K_2/H} \tag{3}$$

$$\log y = \log \frac{C}{1 + H/K_1 + K_2/H + K_2K_3/H^2} \tag{4}$$

$$\log y = \log \frac{C}{1 + K_1/H} \tag{5}$$

$$\log y = \log \frac{Y_L + Y_H(K_1/H)}{1 + K_1/H} \tag{6}$$

$$pK = \frac{\Delta H_{ion}}{2.303RT} + B \tag{7}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{8}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \tag{9}$$

1 at low pH and a slope of −1 or −2 at high pH were fitted to eq 3 or 4, respectively. Data for pH profiles which decreased with a slope of −1 at high pH were fitted to eq 5. Data for pK_i profiles in which pK_i decreased from a constant value at one pH to another constant value at another pH were fitted to eq 6. In eq 3 and 4 K_1 and K_2 represent dissociation constants for enzyme groups, y is V/K, and C is the pH-independent value of y. In eq 6 K_1 is the dissociation constant for an enzyme or substrate group, and Y_L and Y_H are the observed values of y at low and high pH, respectively. Apparent pK values as a function of temperature were fitted to eq 7. Data for linear competitive and noncompetitive inhibition were fitted to eq 8 and 9, respectively.

Results

Initial Velocity Studies. Velick & Vavra (1962) have shown that at high concentrations, α-ketoglutarate gives inhibition which is competitive against aspartate, indicative of a dead-end E–PLP–α-ketoglutarate complex. Since this could potentially give rise to incorrectly determined values of V/K_{Asp} if α-ketoglutarate were fixed at too high a concentration, the pH dependence of substrate inhibition by α-ketoglutarate was determined. The easiest way to acquire this information is by obtaining the initial velocity pattern (varying one substrate at several different fixed levels of the other substrate) as a function of pH. Examples of these patterns obtained at pH values of 7.2 and 9.5 are shown in Figure 1. These patterns exhibit double competitive substrate inhibition in a ping-pong mechanism so that both E–PLP–α-ketoglutarate and E–PMP–aspartate complexes are formed. Inhibition by both substrates is pH dependent. From the pH dependence of patterns like those shown in Figure 1, data are obtained for the pH dependence of V, the V/K for both substrates, and the K_I values for substrate inhibition. The pH dependence of $1/K_I$ for α-ketoglutarate and aspartate is shown in Figure 2. Inhibition by the two substrates is reciprocal with pH. α-Ketoglutarate has a dissociation constant of 0.82 ± 0.06 mM from the protonated enzyme–α-ketoglutarate complex which increases to 27 ± 1 mM for dissociation from the unprotonated enzyme–α-ketoglutarate complex (α-ketoglutarate has no pK values in this pH range). The pK for free enzyme is 6.5 which is perturbed to 8.2 in the enzyme–α-ketoglutarate complex (Cleland, 1977). This is in agreement with data of Sizer & Jenkins (1963) and Fonda & Johnson (1970), who have shown that dicarboxylic acid inhibitors increase the pK of some enzyme group from 6.2 to about 8.0. This pK value has been shown to be anion dependent. Aspartate has a dissociation constant of 6.6 ± 0.7 mM when either an enzyme group or the aspartate amino group is unprotonated which increases to 420 ± 17 mM when this group is protonated. The pK for the free enzyme or substrate group is 10.2 which is perturbed to 8.4 in the enzyme–aspartate complex. All pK values are summarized in Table I.

pH Dependence of Kinetic Parameters. The V/K for one substrate is independent of the concentration of the other substrate in a ping-pong mechanism. Thus, V/K for each substrate can be determined by maintaining the concentration of the nonvaried substrate (at each pH value) at a level one-tenth its K_I (Figure 2) and varying the other substrate. Under these conditions, from pH 5.5 to 8.5 the concentrations of aspartate used were saturating when α-ketoglutarate was varied, and in the pH range 8.5–10.5, the concentrations of α-ketoglutarate used when aspartate was varied were saturating. Thus, data in addition to those determined from the initial velocity patterns were obtained for the pH dependence of V. Figure 3 shows the pH dependence of V, $V/K_{\alpha\text{-KG}}$, and

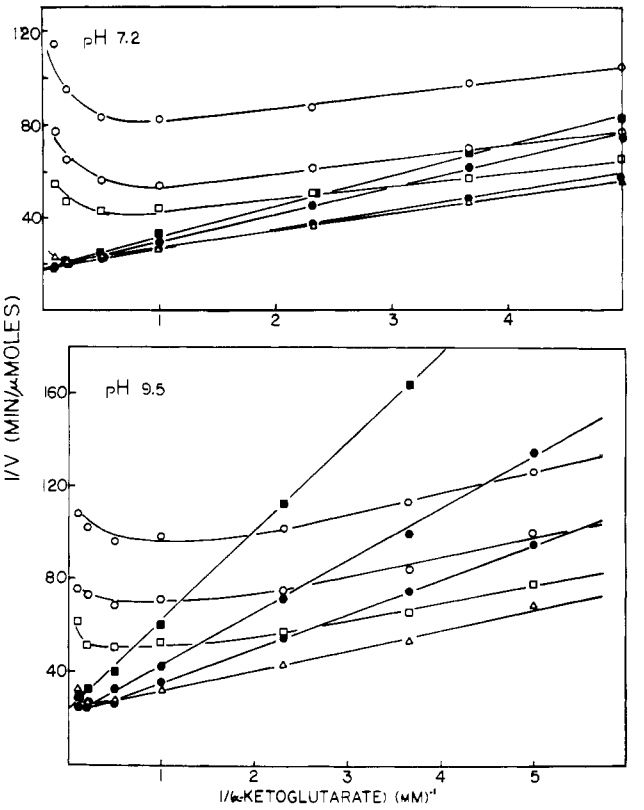

FIGURE 1: Dependence of the initial velocity of the aspartate aminotransferase reaction on substrate concentrations. (Top panel) pH 7.2, 100 mM Hepes, 0.2 mM NADH, 17 units/mL malate dehydrogenase, and 3.6 pM aspartate aminotransferase. (Bottom panel) Conditions were the same as in the top panel but 100 mM Ches, pH 9.5, was used. The α-ketoglutarate concentration was varied as indicated, and aspartate concentrations used were as follows: (○) 2.5 mM; (○) 3.57 mM; (□) 6.25 mM; (△) 40 mM; (●) 100 mM; (●) 200 mM; (■) 400 mM. Data were fit to eq 2 to produce the theoretical curves as shown.

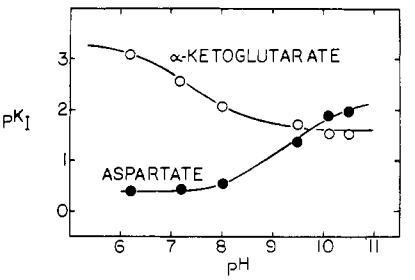

FIGURE 2: pH dependence of the $1/K_I$ for substrate inhibition. This was obtained from data similar to those shown in Figure 1; (○) α-ketoglutarate; (●) aspartate. The lines shown are theoretical curves for fits of the data of eq 6.

V/K_{Asp}. V is pH independent from 5.5 to 10.0. The V/K for α-ketoglutarate decreases below a pK of 5.8 and above a pK of 9.2, while V/K_{Asp} decreases below a pK of 6.4 and above pK values of 9.2 and 10.1. The pH-independent values of these parameters are $V/E_t = 160\ s^{-1}$, $V/(K_{Asp}E_t) = 8.3 \times 10^4\ M^{-1}\ s^{-1}$ and $V/(K_{\alpha\text{-KG}}E_t) = 4.0 \times 10^6\ M^{-1}\ s^{-1}$. Essentially identical results were obtained for the V/K profiles whether the α subform or enzyme purchased from Sigma was used.

Intrinsic pK values are usually observed in pK_i profiles since both combinations of proton and inhibitor are at thermodynamic equilibrium (Cleland, 1977). Thus, α-ketoglutarate binding to E–PLP is sensitive to the protonation state of an enzyme group with a pK of 6.4 which is essentially identical with the pK observed in the V/K_{Asp} profile. This pK has been shown to be anion dependent (see Discussion). Since both

Table I: Summary of pK Values Obtained from the pH Dependence of Kinetic Parameters

parameter	pK_a ± SE	pK_b ± SE
V/K_{Asp}	6.4 ± 0.1	9.2 ± 0.2
		10.1 ± 0.1
$V/K_{\alpha\text{-KG}}$	5.8 ± 0.1	9.2 ± 0.1
$pK_{I\ \alpha\text{-KG}}$[a]	6.5 ± 0.1	NA[b]
$pK_{ii\ maleate}$	6.5 ± 0.1	NA
$pK_{is\ maleate}$	NA	9.3 ± 0.1
$pK_{i\ \alpha\text{-methylaspartate}}$	6.5 ± 0.1	9.3 ± 0.1
$pK_{I\ Asp}$	NA	10.2 ± 0.1[c]

[a] The pK value for the E-inhibitor complex is also obtained from a fit of pK_i vs. pH for α-ketoglutarate, maleate, and aspartate. Values are 8.2 ± 0.1 for E-PLP-α-ketoglutarate, 8.4 ± 0.1 for E-PMP-aspartate, and 8.2 ± 0.2 for E-PLP-maleate. [b] Not applicable. [c] This pK is for the amino group of aspartate.

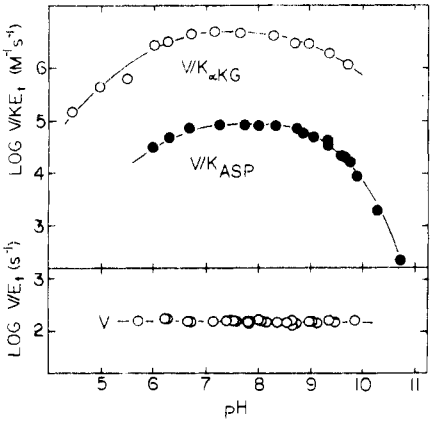

FIGURE 3: pH dependence of V, $V/K_{\alpha\text{-KG}}$ and V/K_{Asp} for aspartate aminotransferase. For determination of $V/K_{\alpha\text{-KG}}$ (○), aspartate was maintained at one-tenth its K_i for substrate inhibition, such that the concentration ranged from 40 mM at pH 5.5 to 1 mM at pH 10. Likewise, for determination of V/K_{Asp} (●), α-ketoglutarate was maintained at one-tenth its K_i for substrate inhibition, such that the concentration ranged from 0.1 mM at pH 6 to 3 mM at pH 10. The lines are theoretical curves for fits of the data to eq 3 ($V/K_{\alpha\text{-KG}}$), and eq 4 (V/K_{Asp}). For V, the line represents the average value of the parameter.

profiles were obtained by using similar reaction conditions and are identical, the pK values observed in the V/K_{Asp} profile directly reflect ionization of an enzyme group, and therefore, aspartate is not sticky.[2]

For determination of whether pK values observed in the $V/K_{\alpha\text{-KG}}$ profile directly reflect ionization of enzyme groups, the pH dependence of an inhibitor competitive with α-ketoglutarate was obtained. However, Velick & Vavra (1962) have shown that unless aspartate is maintained at a saturating concentration, inhibition will be obtained as a result of binding of inhibitor to both E–PLP and E–PMP, since the reactants resemble one another so closely (as evidenced by the competitive substrate inhibition). To take advantage of this, aspartate was fixed at or below its K_m so that, as shown in Figure 4, when α-ketoglutarate is varied at several fixed levels of maleate, noncompetitive inhibition is obtained. In this case, K_{is} is the dissociation constant for maleate from E–PMP–maleate and the K_{ii} is the apparent dissociation constant for maleate from E–PLP–maleate. The true K_{ii} is calculated from the apparent K_{ii} by dividing by 1 + [aspartate]/K_{Asp}. Thus, the pH dependence of K_i for maleate binding to E–PLP is obtained as

[2] Substrate stickiness is observed when substrate dissociates slower than it reacts to give products. This will cause the pK to be pushed to a lower (or higher since these are symmetric cases dependent on whether unprotonated or protonated enzyme is active) pH value (Cleland, 1977).

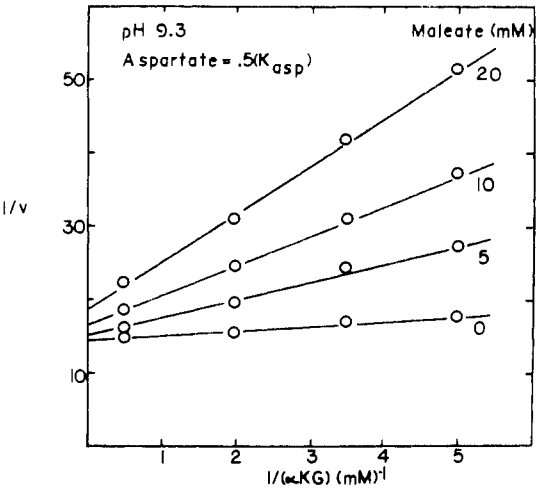

FIGURE 4: Inhibition of the aspartate aminotransferase reaction by maleate. Conditions are similar to those given in the legend of Figure 1, bottom panel, except that α-ketoglutarate and maleate concentrations are as indicated, and aspartate was fixed at half its K_m value. The data were fit to eq 9 to produce the theoretical curves as shown.

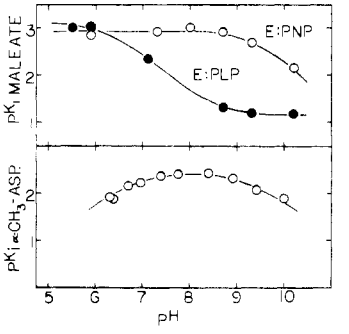

FIGURE 5: pH dependence of $1/K_{i\ maleate}$ and $1/K_i$ for α-methylaspartate. The $K_{i\ maleate}$ values for dissociation from E–PLP–maleate (●) and E–PMP–maleate (○) complexes were obtained from noncompetitive inhibition patterns similar to those shown in Figure 4 and described under Results, and $1/K_{i\ \alpha\text{-methylaspartate}}$ was obtained from competitive inhibition against aspartate. The lines shown for $1/K_{i\ maleate}$ data are theoretical curves for fits of the data to eq 5 (○) and 6 (●) while that for $1/K_{i\ \alpha\text{-methylaspartate}}$ is a theoretical curve for a fit of the data to eq 3.

well as for binding to E–PMP, and these are shown in Figure 5. Maleate binding to E–PLP mimics substrate inhibition by α-ketoglutarate not only in terms of observed pK values (6.5 for free enzyme and 8.2 for the enzyme–α-ketoglutarate complex) but also in the value of K_i. That is, a value for K_i of 1.30 ± 0.03 mM is obtained for binding to protonated enzyme and 74 ± 1 mM for binding to unprotonated enzyme. Binding of maleate to E–PMP, however, is quite different as indicated by a decrease in $1/K_i$ above a single pK of 9.3 and a K_i of 1.15 ± 0.03 mM for binding to protonated enzyme. The latter pK value is in very good agreement with that observed in the $V/K_{\alpha\text{-KG}}$ profile which indicates that maleate binding to E–PMP is dependent upon the protonation state of the same group which affects $V/K_{\alpha\text{-KG}}$. Thus, intrinsic pKs are observed in the $V/K_{\alpha\text{-KG}}$ profile, and α-ketoglutarate, like aspartate, is not sticky.

Under similar conditions (α-ketoglutarate maintained at its K_m and aspartate varied) the pH dependence of α-methylaspartate inhibition was obtained. The $1/K_{is}$ value for α-methylaspartate decreases below a pK of 6.5 and above a pK of 9.3 as shown in Figure 5. These are essentially identical with the pK values observed in the V/K_{Asp} profile, suggesting that the pK values observed in the V/K_{Asp} profile directly reflect the protonation state of enzyme and substrate. The

pH-independent value of the K_i for α-methylaspartate binding to E–PLP is 3.75 ± 0.40 mM, in good agreement with the value of 2.3 mM reported by Fasella et al. (1966) and 4.7 mM reported by Fonda & Johnson (1970). No significant intercept inhibition was observed at any pH.

Temperature Dependence of pKs and Kinetic Parameters. Since K_I values for substrate inhibition have the potential to change as the temperature is varied, initial velocity patterns were repeated at pH 6, 8, and 9.5 to be sure no large changes in substrate inhibition K_I values were observed. Thus, at the new temperature the nonvaried substrate can be fixed at one-tenth its K_I while the other substrate is varied. Very little change was observed in the K_i values as a function of temperature. The V/K's for both substrates were obtained as a function of pH at each new temperature (12.5 and 37.5 °C) in addition to those already obtained at 25 °C. At 12.5 °C the $V/K_{\alpha\text{-KG}}$ pH profile exhibited pK values of 6.1 ± 0.1 and 9.3 ± 0.1, while at 37.5 °C pK values were 5.6 ± 0.1 and 8.9 ± 0.1. At 12.5 °C the V/K_{Asp} profile gave pK values of 6.6 ± 0.1 and 9.3 ± 0.1, while at 37.5 °C the values were 6.2 ± 0.1 and 9.0 ± 0.1. The ΔH_{ion} values calculated from pK values obtained at 12.5, 25, and 37.5 °C are as follows: 5.4 ± 0.8 and 5.8 ± 0.2 kcal/mol for the low and high pKs in the $V/K_{\alpha\text{-KG}}$ pH profile; 5.1 ± 0.3 and 5.5 ± 0.8 kcal/mol for the low and high pKs in the V/K_{Asp} pH profile.

Solvent Dependence of pK Values. Initial velocity patterns were again repeated in 30% DMF. Although the K_I for α-ketoglutarate was not affected by solvent, a decrease of about 4-fold was obtained for the K_I for aspartate from E–PMP–aspartate at all pH values. This may be indicative of ion pairing between aspartate and arginine residues responsible for binding the carboxyl groups of aspartate (Gilbert & O'Leary, 1975).

Cleland (1977) suggested that solvent dependence of pK values be obtained in both neutral and cationic acid buffers in order to determine the effect of solvent on the pK of the buffers. These experiments are corroborative, however, and only one of the two is actually required. In the case of aspartate aminotransferase, the experiments in neutral acid buffers are difficult to carry out since the enzyme is inhibited by anions. Therefore, only the experiments with cationic acid buffers were carried out. In the presence of 30% DMF, $V/K_{\alpha\text{-KG}}$ decreases below a pK of 5.9 ± 0.1 and above a pK of 9.4 ± 0.1, while V/K_{Asp} decreases below a pK of 6.3 ± 0.1 and above a pK of 8.9 ± 0.1. Thus, no significant change is obtained in any of the pK values.

Discussion

Kinetic Mechanism. The kinetic mechanism for aspartate aminotransferase was determined by Velick & Vavra (1962) to be of the ping-pong type with a dead-end E–PLP–α-ketoglutarate complex. Kinetic studies to date have not shown substrate inihibition by aspartate as a result of combination with E–PMP. However, Martinez-Carrion et al. (1973) observed competition between perfluorosuccinate and aspartate binding to E–PMP using ^{1}H and ^{19}F NMR. These data are indicative of an E–PMP–aspartate complex. Initial velocity patterns shown in Figure 1 clearly indicate formation of the dead-end E–PMP–aspartate complex so that the mechanism proposed by Velick & Vavra (1962) must be modified to include this complex. The reason this inhibition has not been previously observed is the high K_I for aspartate (300–400 mM) from pH 7 to 8 where most previous studies were carried out.

Identification of Groups. Data obtained by Velick & Vavra (1962) for the pH dependence of V/K_{Asp} are similar to the data obtained in this study. However, the above authors showed that V_{max} decreased below a pK of 5.1 while $V/K_{\alpha\text{-KG}}$ increased below a pK of 7.0. In the present study no decrease in V_{max} is observed as pH is decreased to 5.5, so that the pK for decrease in V_{max} would have to be at least as low as 4.5. In addition, a decrease rather than an increase in $V/K_{\alpha\text{-KG}}$ is observed. The reason for these discrepancies is not known.

Since pK values which directly reflect titration of enzyme groups are observed in both V/K profiles, ΔH_{ion} values and solvent perturbation of pK values from these V/K profiles provide information concerning the nature of the groups responsible for the pH dependencies. Because none of the pK values are significantly perturbed when 30% DMF is present in the cationic acid buffered system, all groups most likely represent cationic acids. (Although the value of 9.4 obtained for the $V/K_{\alpha\text{-KG}}$ pK in 30% DMF is 0.2 pH unit higher than the value observed in the absence of DMF, a value of 9.33 is obtained in the pK_{is} profile for maleate, and these are not significantly different.) This rules out tyrosine-70* as a viable possibility for the auxiliary acid–base catalyst, unless it is assumed that Tyr-70* is sequestered from solvent. This is unlikely, however, given the X-ray data.

When E–PLP is titrated to low pH, an increase in absorbance at 430 nm is observed as a result of protonation of the internal aldimine nitrogen (Braunstein, 1972). The pK for this change is 6.3,[3] identical with the one observed in the V/K_{Asp} profile. It is an apparent pK since it is very sensitive to the concentration of specific anions (Jenkins & D'Ari, 1966; Bergami et al., 1968; Bonseb et al., 1975). Also, Haddad et al. (1977) have shown that this pK can be perturbed by the addition of salt ions and/or mixed solvents. Thus, V/K_{Asp} decreases as a result of protonation of the Schiff base nitrogen. In addition, the binding of maleate, α-ketoglutarate, and α-methylaspartate is apparently sensitive to the protonation state of the imine as observed in the pK_i profiles for the above inhibitors competitive with aspartate. The pK observed (6.3–6.5) in all these profiles indicates the protonation of the Schiff base since pK values obtained in the pK_i profiles directly reflect the protonation state of an enzyme or inhibitor functional group. The pK for this same group in enzyme–dicarboxylic acid inhibitor complexes is perturbed to 8.2 [unlike the effect of monovalent ions such as fluoride which only perturbs the pK about 0.6 pH unit (Jenkins, 1980)]. Ivanov & Karpeisky (1969) suggested that this is a result of neutralization of the positive charge of the group which ion pairs with the α-carboxyl group of aspartate. This group has been identified as arginine-386 which is about 4–5 Å from the aldimine nitrogen but may be hydrogen bonded through an asparagine residue to it (Ford et al., 1980; Arnone et al., 1982). In agreement with the above, model studies (Johnston et al., 1963) have shown that in the absence of a positive charge in the vicinity of the aldimine nitrogen (by using an aldimine of *N*-methylpyridoxal) the pK is 8.0. Thus, monovalent ions (Jenkins, 1980) probably do not bind to arginine-386, in close proximity to the aldimine nitrogen, and may instead bind to arginine-292 (present on the other subunit) which ion pairs with the β-carboxyl.

The amino group of aspartate is protonated at the start of the first half-reaction. A transient absorbance at 430 nm is observed on binding of aspartate, which is due to the transfer of a proton from the amino group to the Schiff base nitrogen (Fasella et al., 1966; Braunstein, 1972). However, since the

[3] The apparent pK of the phenolic hydroxyl group of cofactor on enzyme is 6.2 (Braunstein, 1972). This is actually the pK for the proton shared by the phenolic hydroxyl group of the coenzyme and the aldimine nitrogen.

keto acid is the product of this half-reaction, the positive charge remains associated with enzyme. The decrease below a pK of 5.8 observed in the $V/K_{\alpha\text{-KG}}$ profile is most likely a result of protonating the diamine system composed of the pyridoxamino group and the 6-amino of lysine-258. The 6-amino of lysine-258 which originally formed the internal aldimine with PLP is present as the free amine in E–PMP. Since the 6-amino and the pyridoxyl carbon may be in close proximity to one another (they participated in aldimine linkage prior to addition of aspartate), this proton may be shared between the 6-amino group of lysine and the pyridoxamino group. Morino et al. (1978) have shown that the pK for inactivation of aspartate aminotransferase by iodoacetate (representing the pK for protonation of the 4′-amino group of pyridoxamine phosphate) is 8.3. Slebe & Martinez-Carrion (1976) have shown that the pK obtained from the pH dependence of carbamoylation of lysine-258 in the apoenzyme is 8.0. This is quite low for either a lysine pK or the pyridoxamino group of the cofactor and suggests either an additional positively charged residue in the vicinity or a hydrophobic environment. That PLP enzymes may provide a hydrophobic environment has been suggested by O'Leary & Piazza (1981) for arginine decarboxylase based on the increase in decarboxylation rate in the presence of nonpolar solvents. The amino group of pyridoxamine phosphate in solution has a pK similar to that of the 6-amino group of lysine (pK of 10–11) (Dawson et al., 1969) so that, if it were placed in the same environment, it would most likely show a similar perturbation in the pK value. The possibility of a proton shared between the two amino groups in E–PMP will result in a marked lowering (as a result of charge repulsion) of the pK for putting an additional proton on this system. If it is assumed that all of the change in the pK of the pyridoxamino group, from its solution pK of approximately 10.9, is reflected in ΔH_{ion}, the calculated value for ΔH_{ion} is about 4.5 kcal/mol which is close to the observed value of 5.4 kcal/mol. However, accompanying large entropic effects cannot be ruled out.

The group responsible for the pKs observed at high pH in both V/K profiles may be the pyridinium nitrogen of the cofactor (as first suggested to these authors by Dr. J. F. Kirsch). The pK for this group when the phenolic oxygen of the coenzyme is ionized is about 8.4 (Johnston et al., 1963) in solution but is probably perturbed to a higher value as a result of hydrogen bonding with aspartate-222 on the enzyme (Ford et al., 1980; Arnone et al., 1982). This could account for the observed increase of approximately 1 pH unit in the pK to a value of 9.2–9.3. An average ΔH_{ion} value of about 5.6 kcal/mol is obtained for this group. There are other groups which could result in a pK of 9 which are somewhat removed from the active site, e.g., histidine, and these cannot be ruled out. The assignment of the pyridinium nitrogen is suggested as a possibility, and mechanistic implications will be discussed below.

Interpretation of Inhibition Data. Non amino acid inhibitors bind more tightly to E–PLP in which the Schiff base nitrogen is protonated. The carboxyl groups of maleate are about 6.4 Å apart, and it has been shown that most dicarboxylic acids in which the carboxyl groups can be this far apart are good inhibitors (Braunstein, 1972). Even though there are no functional groups on maleate (other than the two carboxyl groups), it binds with about the same affinity as α-ketoglutarate.[4] Ivanov & Karpeisky (1966) and Arnone et al. (1982) suggest the coenzyme moves when the Schiff base is formed. When the amino acid binds, it donates a proton to form a protonated imine which forms a chelate H bond to the ionized phenolic hydroxyl group.[3] This may be the point at which the coenzyme moves, perhaps by weakening the H bond to Tyr-225 allowing structural rearrangement. The enhanced binding of α-ketoglutarate at low pH may be a result of this same movement of the coenzyme upon protonation of the imine, thus allowing a better accommodation of the inhibitor molecule. This ionized phenolic oxygen in free enzyme (E–PLP) is hydrogen bonded to Tyr-225 (Ford et al, 1980; Arnone et al., 1982), but this hydrogen bond is probably weaker in the enzyme–inhibitor complex (Arnone et al., 1982). Thus, formation of a hydrogen bond between the Schiff base nitrogen and the phenolic oxygen of the coenzyme may facilitate movement of the coenzyme to readily accommodate the bulk of the inhibitor. Movement of the coenzyme molecule was first postulated by Ivanov & Karpeisky (1966) and has been confirmed by X-ray studies (Arnone et al., 1982), although the details are slightly different. In addition to the phosphate of the coenzyme remaining fixed as postulated by Ivanov & Karpeisky (1966), the pyridinium nitrogen also remains fixed and the coenzyme rotates around the methylene at the 5′ position, which bridges the phosphate to the pyridinium ring. While non amino acids exhibit enhanced binding at low pH, amino acids exhibit decreased affinity as a result of charge repulsion between the positively charged amino group and the positively charged Schiff base nitrogen. This is clearly shown by the increase in K_i for α-methylaspartate below a pK of 6.3.[5]

The binding of aspartate as a substrate inhibitor to E–PMP is enhanced as the amino group of aspartate is unprotonated (pK is approximately 10). This is most likely a result of elimination of charge repulsion between the amino group and the proton shared by the 6-amino of lysine-258 and pyridoxamine. The pK for the amino group decreases to 8.4 in the enzyme–inhibitor complex as a result of neutralization of the charge on the α-carboxyl group. Since only a single pK at 9.3 is observed in the $V/K_{\alpha\text{-KG}}$ profile, the pK for removal of the proton shared by pyridoxamine and the 6-amino group of lysine-258 must be above 10. The K_i for maleate, on the other hand, increases above a pK of 9.3. This is probably due to the deprotonation of the pyridinium nitrogen which ultimately destroys the binding of maleate, since the pyridinium ring is no longer fixed in the proper orientation to accommodate the inhibitor. Deprotonation of the pyridinium ring does not affect aspartate binding as a dead-end inhibitor, and thus compensating favorable interactions as a result of the presence of the amino group must be invoked.

Chemical Mechanism. The above data support previously proposed mechanisms which require only a single base catalyst (Snell, 1963; Ivanov & Karpeisky, 1966). Figure 6 shows a chemical mechanism which is consistent with the above data. L-Aspartate binds to E–PLP via its carboxyl groups with an ionized amino group (as suggested by the decrease in the V/K for aspartate above a pK of 10) to form the Michaelis complex. Neutralization of the α-carboxyl decreases the amino pK to 8.4 and increases the aldimine nitrogen pK to 8.2 (as observed

[4] At high pH, α-ketoglutarate binds about 3-fold better than maleate probably as a result of favorable interaction with the α-keto group.

[5] Both of these can be explained as a result of increased affinity of anions competitive with the amino acid as the pH decreases (Harruff & Jenkins, 1978). In the case of α-ketoglutarate and maleate, the increased affinity for these two dicarboxylic acids is directly observed, while in the case of aspartate (as seen in the V/K aspartate profile) and α-methylaspartate, the effect is observed indirectly as a result of an increase in affinity of background anions which are competitive vs. the two amino acids.

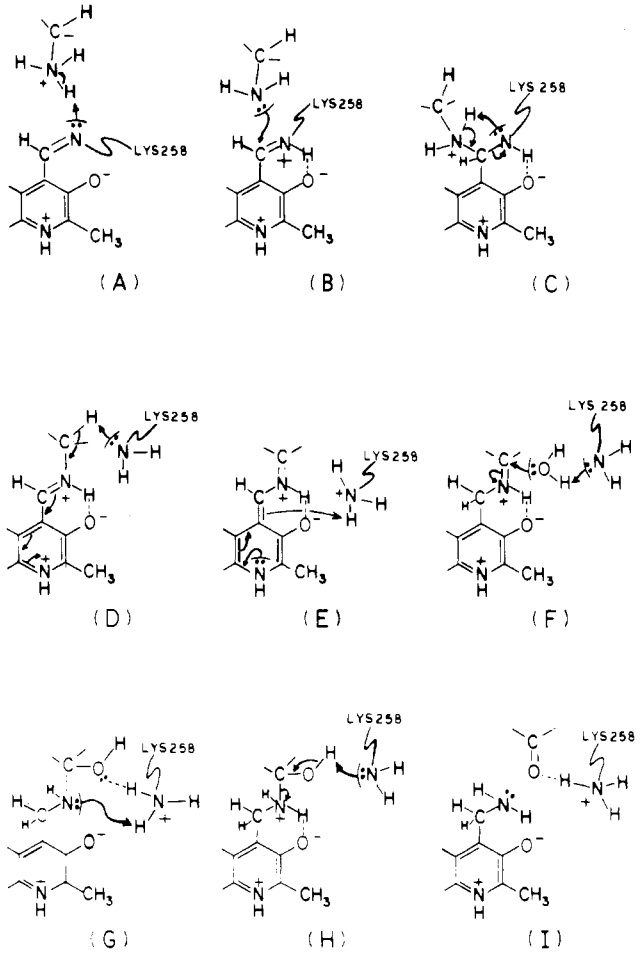

FIGURE 6: Possible mechanism for the aspartate aminotransferase reaction. The scheme is not meant to imply correct geometry or stereochemistry but simply to show the movement of protons. The large majority of proton transfers will most likely occur through the use of the ionized phenolic hydroxyl group. However, since it was difficult to properly draw the structures to show this aspect, proton transfers are shown to occur directly for simplification.

in pK_i profiles for maleate and α-ketoglutarate) which allows for proton transfer from one nitrogen to the other through the interaction of the phenolic oxygen on the coenzyme as all proton transfers will most likely occur through the use of the phenolic hydroxyl as a proton shuttle. This will require only minimal movement of the enzyme around the axis through N1 and C4. The internal aldimine nitrogen abstracts a proton from the amino group (Figure 6A), allowing the aldimine nitrogen to hydrogen bond to the phenolic oxygen of the coenzyme (Figure 6B) and perhaps facilitate movement of the coenzyme to better accommodate aspartate. In agreement with this, binding of non amino acid inhibitors, such as maleate, and α-ketoglutarate to E–PLP is much better at low pH, when the aldimine nitrogen is protonated (as judged by the 430-nm λ_{max}). The subsequent chemistry has been described previously for the formation of the *gem*-diamine (Figure 6C), which collapses to form the protonated external aldimine and free lysine (Figure 6D), followed by production of a quinonoid intermediate (Figure 6E). Consistent with this, crystals soaked with 3-hydroxyaspartate (a substrate analogue) (Jenkins, 1961) produce a species with 495-nm λ_{max} (Eichele et al., 1978; Metzler et al., 1978) which has been assigned to the semiquinoid intermediate (Jenkins, 1964). This absorbance disappears concomitant with production of the pyridoxamine form of the enzyme. The quinonoid then rearranges to a ketimine (Figure 6F) and the 6-amino of lysine-258, thus facilitating attack by water to form a carbinolamine (Figure 6G). The carbinolamine is protonated (Figure 6H) at nitrogen and subsequently collapses, aided by the lysine amino to give the α-keto acid, pyridoxamine, and protonated lysine-258 (Figure 6I). Once the keto acid is released, E–PMP is obtained in which the 6-amino group of Lys-258 and the pyridoxamino group of the coenzyme may be hydrogen bonded (Ivanov & Karpeisky, 1969). It is protonation of this system which is observed in the $V/K_{\alpha\text{-KG}}$ profile that exhibits a pK of 5.8.

Registry No. Aspartate aminotransferase, 9000-97-9; L-lysine, 56-87-1; α-ketoglutaric acid, 328-50-7; L-aspartic acid, 56-84-8; maleic acid, 110-16-7; α-methyl-L-aspartic acid, 3227-17-6.

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Adenosine Deaminase: Solvent Isotope and pH Effects on the Binding of Transition-State and Ground-State Analogue Inhibitors†

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ABSTRACT: We have studied the pH(D) effects on the deamination of adenosine catalyzed by adenosine deaminase as well as on the binding of the inhibitors purine riboside, a ground-state analogue, and 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR), a transition-state analogue. The observed pK_a value of 4.9 for the free enzyme in H_2O buffers is found to be increased by 0.6 pK unit in the enzyme–substrate complex and decreased by 0.5–1.0 pK unit in the enzyme–inhibitor complexes. In D_2O buffers, the pK_as of the free enzyme and its complexes are all found to be increased by ~0.6 pK unit relative to their position in H_2O. A small inverse solvent isotope effect is observed on V_{max} while none is observed on K_m. Substantial solvent isotope effects [$K_i(H_2O)/K_i(D_2O)$ = 1.2–1.5] are found for the dissociation of both ground-state and transition-state analogue inhibitors from the enzyme–inhibitor complex. Fluorescence titrations of the enzyme with DHMPR in H_2O and D_2O confirm the equilibrium solvent isotope effect obtained from kinetic experiments. For the transition-state analogue, a small inverse kinetic effect, similar in magnitude to that on V_{max}, is found on the association rate constant, k_{on}, while a normal effect is observed on the dissociation rate constant, k_{off}. The intrinsic protein fluorescence is quenched 70% by the transition-state analogue and only 6% by the ground-state analogue supporting the idea that a greater structural reorganization of the enzyme is required to bind the transition state effectively in comparison to the ground state. In contrast, a large UV difference spectrum is observed upon formation of the complex with purine riboside, suggesting that the binding isotope effect may be interpretable in terms of structural changes in the ligand rather than in the enzyme. Three possible structures for the complexed inhibitors are discussed which could account for the observed solvent isotope effect. The data are most consistent with protonation of the purine ring at N-1 by an active-site sulfhydryl. However, hydration of the purine ring at C-6 or formation of a covalent sulfhydryl adduct at C-2 or C-6 cannot be excluded in view of the large pK_a shifts required to accomplish purine protonation with a sulfhydryl group.

For catalysis to occur, an enzyme must have a greater affinity for its substrates in the transition state than in the ground state (Pauling, 1946). Thus, an understanding of enzyme mechanisms requires an understanding of those interactions between the enzyme and its substrate which lead to transition-state stabilization. While the factors responsible for transition-state stabilization can be investigated through a study of the response of the kinetic constants of the catalytic reaction to perturbations, the development of stable analogues of the substrate portion of the activated complex (Wolfenden, 1972) has made possible a more direct approach. In general, transition-state analogues bind more tightly to the enzyme than do ground-state analogues (or substrates), but an apparent slow rate of binding also distinguishes transition-state from ground-state analogues in many cases (Wolfenden, 1976; Frieden et al., 1980).

These points are well illustrated by the binding of adenosine deaminase (EC 3.5.4.4) to the ground-state analogue purine riboside (PR) and the transition-state analogue 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR) (Wolfenden et al., 1977). It has been proposed that DHMPR approximates the structure of the tetrahedral intermediate (or the transition state leading to it) which results from water attack on the substrate adenosine (Figure 1). In a previous study of adenosine deaminase and AMP deaminase, we concluded (Frieden et al., 1980) that the initial structure of the active site of the enzyme appears to be appropriate for binding the ground state of the substrate rather than the transition state and that considerable readjustment of the enzyme structure seems to be required to bind the transition state effectively.

The importance of proton transfer processes to catalysis and the importance of hydrogen bonding to protein structure (and to protein conformation changes) make solvent isotope effects an ideal probe since such effects will be sensitive to bonding changes involving exchangeable hydrogens. In principle, isotope effects can be interpreted in terms of changes in bond force constants which in turn can allow a detailed molecular description of the processes accompanying catalysis.

Accordingly, we report here a study of the effects of pH(D) on the kinetic and equilibrium properties of the binding of these inhibitors to adenosine deaminase. Data for the effects on the

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