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pH Kinetic Studies of Bovine Brain Hexokinase†

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ABSTRACT: The variation of kinetic parameters with pH was examined for bovine brain hexokinase with glucose and MgATP as substrates. The $-\log V_1$ and $-\log (V_1/K_m)$ profiles for both substrates were examined and seen to decrease below pH 6.5. All profiles asymptotically approached slopes of −1, indicating that the loss of activity in each instance was due to the protonation of a single group on the enzyme. Analysis of the data indicated two ionizable groups were involved in the reaction. One functions in the binding of ATP and in catalysis while the other participates in the binding of glucose. The $-\log V_1$ profiles both showed a "hump" attributed to a loss of activity in the pH region 7.5–5.5. Addition of aluminum ions to the reaction mixture increased the magnitude of the hump, but the inhibition was abolished by the addition of citrate. Kinetic studies carried out at pH 7 indicated that aluminum was a competitive inhibitor with respect to ATP and noncompetitive with respect to glucose. However, secondary plots of the kinetic data were nonlinear, concave downward, indicating that the inhibition is not of a simple type. Possible explanations for this phenomenon are presented.

Brain hexokinase (hexokinase I) is now recognized as a primary control point of glycolysis in the brain. Because of its metabolic importance, much time and study have been devoted in attempts to acquire a thorough understanding of the kinetic mechanism of hexokinase (Purich et al., 1973). However, it has only been within the last few years that attention has been directed to the chemistry of this enzyme's active site. Recent studies have shown that sulfhydryl groups are required for activity in both yeast (Jones et al., 1975; Otieno et al., 1975) and mammalian (Redkar & Kenkare, 1972; Chou & Wilson, 1974; Subbarao & Kenkare, 1977a) hexokinase, and there is some evidence to indicate that these groups are involved in the binding of substrate (Subbarao & Kenkare, 1977b). Carboxyl groups have been reported to be involved in the action of yeast hexokinase (Pho et al., 1974), and from X-ray crystallographic data Anderson et al. (1978) have observed what seems to be an interaction between an aspartyl residue and the 4- and 6-hydroxyl groups of the sugar substrate. There is evidence for the involvement of histidyl residues in the active site of the wheat germ enzyme (Higgins & Easterby, 1974), but this residue has been found not to be involved in either the binding of substrate or the catalysis of yeast hexokinase (Grouselle et al., 1973).

In this study, we have made use of pH kinetics to gain information about the groups responsible for binding and catalysis of substrate molecules by bovine brain hexokinase. We report evidence for a single ionizable group involved in the catalysis of the reaction and in the binding of ATP and a second residue that functions in the binding of the carbohydrate substrate. In addition, we offer observations on an anomalous loss of activity below pH 8 similar to the decrease reported in a recent paper by Viola & Cleland (1978) on the pH profile of yeast hexokinase. Colowick has suggested that this inhibition may be caused by the trivalent metal ion Al^{3+}.[1] Our results indicate that this ion may indeed be responsible for the pH-dependent activity loss. Kinetic evidence indicates that this inhibition is nonlinear competitive with ATP and may be due to the formation of a strongly inhibitory aluminum–ATP complex, as well as interactions of the metal with the enzyme alone.

Experimental Procedures

Materials. Glucose-6-phosphate dehydrogenase was purchased from Boehringer Mannheim. Buffers used in the experiments were from Calbiochem. ATP and $NADP^+$ were products of Sigma, and glucose was from Pfhanstiehl. Distilled deionized water was used in the preparation of all reagents. All other chemicals were of the highest purity available commercially.

Methods. Bovine brain hexokinase was prepared by the method of Redkar & Kenkare (1972) and had a specific ac-

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[1] Dr. S. P. Colowick, personal communication.

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tivity of 62 units/mg. A unit is defined as 1 μmol of glucose-6-P produced per min at 28 °C in a total reaction volume of 3 mL under standard assay conditions. The standard assay contained 10 mM glucose, 5 mM ATP, 50 mM Tris-HCl (pH 7.6), 0.33 mM NADP, 7 mM $MgCl_2$, and 1.7 units of glucose-6-P dehydrogenase in a total volume of 3.0 mL.

The purified enzyme was stored as previously described (Redkar & Kenkare, 1972) and was desalted before each experiment on a Bio-Gel P-10 column which had been equilibrated with 20 mM Hepes, pH 7.6, containing 5 mM β-mercaptoethanol. The enzyme was found to be stable for at least 4 h after desalting. For initial rate studies, the enzyme was diluted into 1 mg/mL bovine serum albumin.

Kinetic assays were carried out in 1.0-mL volumes in 1-cm cuvettes by using a Cary 118C spectrophotometer with a water-jacketed cell compartment. All assays were carried out at 28 °C unless otherwise indicated. Assays were initiated by the addition of 0.003–0.02 unit of hexokinase.

The buffers used for the profile were acetate, 2-(*N*-morpholino)ethanesulfonate (Mes), piperazine-*N*,*N*′-bis(2-ethanesulfonate) (Pipes), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (Hepes), 2-(cyclohexylamino)ethanesulfonate (Ches), and 3-(cyclohexylamino)propanesulfonate (Caps). Each buffer was used within 1 pH unit of its p*K* value. The buffers had no effects upon activity when the enzyme was assayed in their presence at pH 7.6. Buffer concentrations were maintained at 10 mM in each experiment and adjusted to an ionic strength equivalent to 10 mM salt by addition of KCl.

Reaction rates were determined by measuring the conversion of $NADP^+$ to NADPH in the glucose-6-phosphate dehydrogenase–hexokinase coupled assay. The coupling enzyme was dialyzed before use to remove ammonium sulfate. To verify that the rates measured were not limited by the coupling enzyme, various concentrations of hexokinase were added to assays containing a constant amount of glucose-6-phosphate dehydrogenase at the different pH values used. The plot of velocity vs. hexokinase concentration was linear. The amount of couple varied from 0.75 unit/assay at pHs above 7.0 to 8.0 unit/assay at pH 4.5. Each assay mixture contained 10 mM buffer, 0.4 mM $NADP^+$, and 2.5 mM Mg^{2+} in excess of the concentration of ATP. In assays where ATP concentration was varied, glucose concentration was held at saturating levels (2.2 mM). When glucose was the varied substrate, $MgATP^{2-}$ concentration was saturating (10 mM). With this method, breaks in the $-\log V_1$ profile will indicate groups ionizing in the ternary complex, and breaks in the $-\log (V_1/K_m)$ profiles will indicate groups ionizing from the EA complex where A is the saturating substrate. Other conditions were as stated in the figure legends.

For the pH studies in the presence of dimethyl sulfoxide (Me_2SO), only cationic buffers were used. At pH values below 5.5, Mes buffer (p*K* 6.5 at 20 °C) was used in a concentration so as to maintain at least 1.2 mM of the buffer in the neutral form.

Atomic absorption analysis of ATP for aluminum was performed by the Ames Laboratory of the U.S. Department of Energy, Ames, IA. Contaminating aluminum was removed from ATP by extraction with 8-hydroxyquinoline and chloroform.[2]

Initial rate data were analyzed and fit to specific models by the weighted least-squares method, assuming equal variance of velocities by a computer program written in the OMNITAB language (Siano et al., 1975). The value of α was set equal to zero.

[2] Unpublished experiments.

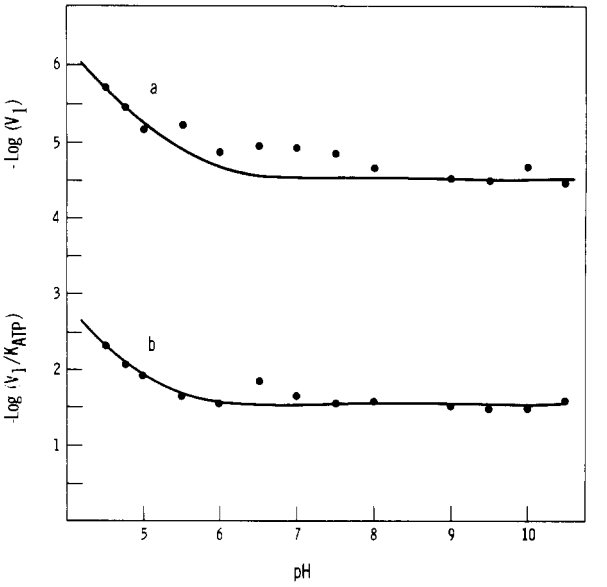

FIGURE 1: (a) Plot of $-\log V_1$ vs. pH with 2.2 mM glucose and $MgATP^{2-}$ concentration varied from 0.25 to 2.0 mM. Individual points represent computer fits of initial rate data. The p*K*, determined by computer fit to eq 2, is 5.66 ± 0.10. (b) Plot of $-\log (V_1/K_m)$ vs. pH. The conditions are the same as in (a). The p*K* is 5.20 ± 0.11. Experiments were carried out at 28 °C. Other conditions are as stated under Experimental Procedures. The curves through the data are the computer-determined fits, assuming one ionizing group with the p*K*'s given.

The data from the pH experiments were plotted according to the procedure of Dixon (1953) and were fitted by an unweighted nonlinear least-squares regression to eq 1, where *f*

$$\log f = \log C - \log \left[1 + \frac{(H^+)^n}{K_a^n} \right] \quad (1)$$

is $V_{1(app)}$ or $V_{1(app)}/K_{s(app)}$ at the pH studied, *C* is V_1 or V_1/K_s, *n* represents the number of groups involved in a dissociation and was varied to obtain the best fit to the experimental data, and K_a is the apparent dissociation constant. For ease in computing, eq 1 was rearranged by combining the log terms on the right side of the equation and multiplying the term within the log function by the quantity K_a/K_a (eq 2).

$$\log f = \log \left[\frac{K_a^n C}{K_a^n + (H^+)^n} \right] \quad (2)$$

Results

Studies of the variation of kinetic parameters with pH may provide information on the functional enzyme groups associated with binding and catalysis. Because information of this type is lacking in the case of brain hexokinase, experiments were undertaken in an attempt to gain insight into the mode of substrate interaction with this enzyme.

Figure 1a,b shows Dixon plots for the pH-dependent variation of the maximal velocity (V_1) and maximal velocity/K_m (V_1/K_{ATP}) for varied ATP concentration at a constant glucose concentration. As the pH was lowered below 6, a limiting slope of −1 was obtained, indicating that the protonation of one group on the enzyme was responsible for the loss of activity. Computer fitting of the data yielded p*K*'s of 5.66 ± 0.10 for the $-\log V_1$ profile and 5.20 ± 0.11 for the $-\log (V_1/K_{ATP})$ profile. No decrease in velocity was seen at basic pH values.

Between the pH values of 7.5 and 5.5, there is a decrease in activity which appears as a "hump" in the $-\log V_1$ profile

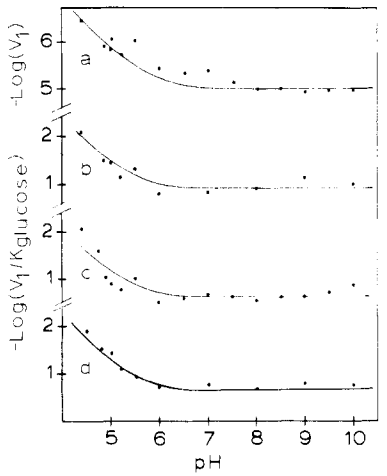

FIGURE 2: (a) Plot of $-\log V_1$ vs. pH. $MgATP^{2-}$ concentration was held constant at 10.0 mM; glucose concentration is varied from 0.02 to 0.1 mM. Individual points represent computer fits of initial rate data. A computer fit of the data to eq 2 yielded a pK of 5.85 ± 0.05. (b) Plot of $-\log (V_1/K_m)$ vs. pH. The conditions are the same as in Figure 2, line a. The profile was taken at 35 °C. (c and d) Plots of $-\log (V_1/K_m)$ vs. pH. The conditions are as above. Line c was determined at 28 °C and line d at 20 °C. Other conditions are as stated under Experimental Procedures. The curves through the data were determined by nonlinear fitting to eq 2, assuming one group was responsible for the activity loss.

(curve a of Figure 1). This loss of velocity is similar to that seen by Viola & Cleland (1978) in their study of the pH kinetics of yeast hexokinase. They found that citrate activated the enzyme in this pH region and speculated that this phenomenon may be related to the citrate activation of yeast hexokinase below pH 7 reported by Kosow & Rose (1971). In the fitting of the data, the values constituting the hump were omitted from the data file. When the experiments were repeated with aluminum-free ATP, profiles superimposable on the theoretical curves generated in Figure 1 were obtained. In accord with the profiles in Figure 1, the data gave the best fit to eq 2 when one group was assumed to dissociate.

The pH profiles generated with glucose as the varied substrate are shown in Figure 2. Curve a of this graph shows the $-\log V_1$ profile at 28 °C. As was the case with varied ATP concentration, as the pH was lowered, the slope of the line asymptotically approached a slope of −1 within the pH range studied. Computer fitting of the data gave a pK of 5.85 ± 0.05. In the pH range from 7.5 to 5.5, a loss in activity resulting in a hump was again observed. These values were omitted from the computer fit. Curve c of Figure 2 displays the $-\log (V_1/K_{glucose})$ profile at 28 °C. The curve was fitted by assuming one group was involved in the protonation. The computer-determined value of the pK is 5.47 ± 0.14. These experiments were also repeated with aluminum-free ATP and showed no significant variation from the theoretical curves shown in Figure 2. The best fit was obtained when $n = 1$.

For investigation of the identity of the groups being protonated, the variation of pK with temperature was determined for each of the profiles. For the $-\log V_1$ profile with the varied ATP concentration, the determined pK's were 5.96 ± 0.06 at 20 °C, 5.66 ± 0.10 at 28 °C, and 5.57 ± 0.08 at 35 °C. The value for ΔH_{ion}, as estimated from the slope of the Arrhenius plot, is 10.1 kcal/mol. The values of the pK's in the $-\log (V_1/K_{ATP})$ profiles when ATP concentration was varied were 5.34 ± 0.11, 5.20 ± 0.12, and 5.06 ± 0.09 at 20, 28, and 35 °C, respectively. The ΔH_{ion} value for this group is 7.7 kcal/mol. Both of these values (7.7 and 10.1 kcal/mol) are much too large to account for the energy required for a simple carboxyl ionization, but they are consistent with the involvement of a histidyl residue ($\Delta H_{ion} \simeq 7$ kcal/mol).

Table I: Effects of Me_2SO on the pK_a of Hexokinase[a]

varied substrate	kinetic parameter	pK in absence of Me_2SO	pK in presence of Me_2SO
$MgATP^{2-}$	V_1	5.66 ± 0.10	5.71 ± 0.06
	V_1/K_{ATP}	5.20 ± 0.12	5.18 ± 0.08
glucose	V_1	5.85 ± 0.05	5.81 ± 0.09
	$V_1/K_{glucose}$	5.47 ± 0.15	5.76 ± 0.12

[a] Computer-determined pK's for varied ATP and glucose concentrations in the absence and presence of Me_2SO. In the varied ATP concentration experiment, glucose concentration was held at 2.2 mM, and $MgATP^{2-}$ concentration was varied from 0.25 to 2.0 mM. When glucose concentration was varied (from 0.02 to 0.1 mM), $MgATP^{2-}$ concentration was held at 10.0 mM. Me_2SO was added to a final concentration of 25% (v/v). All assays were carried out at 28 °C.

The pK's at the various temperatures for the $-\log V_1$ profile with varied glucose concentrations are 5.96 ± 0.10 at 20 °C, 5.85 ± 0.05 at 28 °C, and 5.71 ± 0.05 at 35 °C. As expected, the pK's decreased with increasing temperature. The ΔH_{ion} value, 6.86 kcal/mol, again indicates the possible involvement of a histidyl residue in the catalytic process.

Curves b, c, and d of Figure 2 display the $-\log (V_1/K_{glucose})$ profiles at 35, 28, and 20 °C, respectively. The determined pK's at these three temperatures are 5.45 ± 0.15, 5.47 ± 0.14, and 5.53 ± 0.07 at 35, 28, and 20 °C, respectively. The value for ΔH_{ion}, at only 2.24 kcal/mol, is much less than that required for the ionization of a histidyl residue, and is only a little higher than the ±1.5 kcal/mol required to ionize a simple carboxyl group. This is clearly a different group from that involved in catalysis or in the binding of ATP.

Additional evidence on the nature of the ionizing groups was obtained by observing the change in kinetic parameters with respect to pH in the presence of organic solvent. Dimethyl sulfoxide (Me_2SO) was added to the reaction mixture to give a final concentration of 25% (v/v), and the pH profiles were generated for both varied ATP and glucose. The buffers used in these experiments were of the cationic type. Since dissociation of a proton from this type of buffer does not increase the net quantity of charged species in solution, lowering the dielectric constant of the system by inclusion of an organic solvent will not effect the pK of the buffer. If buffers of the neutral acid type were used, the shift in the equilibrium of the system toward uncharged species caused by the lowering of the dielectric constant would produce an apparent shift in the pK of the buffer toward more basic pH values. This same effect is reflected by the ionizable groups within the enzyme. If the experiment is done in a cationic buffer, the pK of a histidyl group on the enzyme will not be changed by inclusion of an organic solvent in the system, while the pK of a neutral acid group will be raised. Similarly, if the experiment is performed in a neutral acid buffer, the pK of histidyl residues will become more acidic, and the pK of neutral acid groups will not vary (Findlay et al., 1962; Inagami & Sturtevant, 1960).

Table I shows the results of the Me_2SO experiments. While the pKs for the kinetic parameters did not vary with varied ATP concentration or with the log V_1 profile with varied glucose concentration, the pK for the log $(V_1/K_{glucose})$ profile shifted about 0.3 pH units toward the basic end of the pH range. Although this change is somewhat small, it tends to support the view that this group is a carboxyl. The data for the other kinetic parameters are in harmony with the ionizing group(s) being one or more histidyl residues.

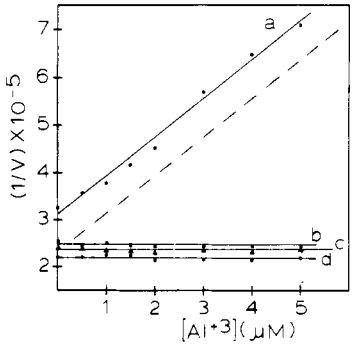

FIGURE 3: Plot of 1/velocity (V) vs. [aluminum] at pHs 7 and 8. Glucose concentration was held at 2.2 mM and $MgATP^{2-}$ concentration at 0.25 mM. The buffer at both pH values was 10 mM Hepes. A 10-fold concentration of aluminum was preincubated with 2.5 mM ATP for 30 min before 0.1 mL of this solution was added to complete the 1.0-mL assay mixture. The aluminum concentrations on the abscissa are the concentrations of aluminum added in the assay. (a) $1/V$ vs. [aluminum] at pH 7. The broken line indicates the inhibition if the aluminum present in the ATP is included in calculating the final concentration of aluminum in solution. (b) $1/V$ vs. [aluminum] at pH 7. The conditions are as in line a, but 1 mM citrate was included in the assay. (c) $1/V$ vs. [aluminum] at pH 8, plus 1 mM citrate. (d) $1/V$ vs. [aluminum] at pH 8 minus citrate.

In 1971, Kosow & Rose (1971) reported the activation of yeast hexokinase by citrate and other ions at pH values below 7. This activation was reduced if reagents were passed through a Chelex column before use. More recently, Viola & Cleland (1978) have shown that a pH-dependent loss of activity by this same enzyme can be restored by the inclusion of citrate in the reaction mixture. Colowick has indicated that this inhibition may be due to aluminum ions present with the ATP in the reaction mixture and can be reversed by citrate, EDTA, and phosphate.[1]

Atomic absorption analysis of the ATP used in these experiments (product no. A-2383, lot no. 96C-7170, by Sigma) indicated that aluminum is indeed present in this preparation at a concentration of 190 ppm. This is equivalent to a concentration of 1.08 μM aluminum in a 0.25 mM solution of ATP. Our first attempt at demonstrating aluminum inhibition with hexokinase indicated approximately 15 μM aluminum was required to produce 50% inhibition of the enzyme, much more than present in the ATP. However, when we preincubated the aluminum with the stock ATP solution for 30 min, we observed significant inhibition of the enzyme with micromolar concentrations of aluminum.[1]

Figure 3 shows the results of an experiment in which the added aluminum was preincubated with 2.5 mM MgATP before addition of the ATP to the reaction mixture. Curve a of Figure 3 indicates that at pH 7, and when preincubated with ATP, aluminum inhibits brain hexokinase at micromolar concentrations. If one takes into account the aluminum already contained in the ATP (190 ppm), the concentrations expressed on the abscissa are all increased by 1 μM, and the inhibition line is offset one unit to the right (broken line). The intercept of the broken line (the apparent $1/V_{max}$ of the line) is close to that for the velocity of hexokinase at pH 8 (line d). The concentration of aluminum required to cause 50% inhibition of the enzyme is then approximately 2.5 μM. Curve b of this figure demonstrates the effect of aluminum on hexokinase when 1 mM citrate has been included in the system. At the concentrations investigated, aluminum had no inhibitory effect. Curves c and d of Figure 3 indicate the effects of aluminum and citrate on the enzyme at pH 8. In curve d, all conditions except pH are identical with those in curve a. Curve c indicates the assay system contains 1 mM citrate. Curves

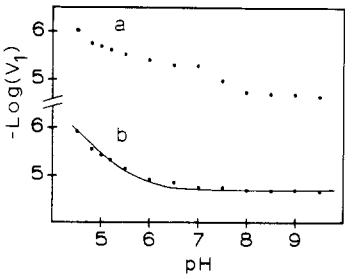

FIGURE 4: (a) Plot of $-\log V_1$ vs. pH. The conditions are as in Figure 1. Glucose concentration was held at 2.2 mM, and ATP concentration was varied from 0.25 to 2.0 mM. Aluminum nitrate was added to the ATP to increase the contamination to 400 ppm and was preincubated with 50 mM ATP before addition to the assay mix. (b) Plot of $-\log V_1$ vs. pH. The conditions were identical with line a, except that 1 mM citrate was included in the assay.

c and d show that at pH 8 aluminum is unable to inhibit the enzyme, and citrate does not activate it.

For determination of the effect of aluminum over the range of the pH profile, the effect of pH on the kinetic parameters of brain hexokinase was redetermined in the presence of added aluminum, and in the presence of added aluminum and citrate. Figure 4 shows the results of this experiment. Curve a of this figure indicates the effect of increasing the aluminum content of the ATP to 400 ppm. The added aluminum was preincubated for 30 min with 50 mM ATP before addition of the nucleotide to the reaction mixture. A comparison of this profile to curve a of Figure 1 shows that doubling the aluminum contamination in the ATP causes a 0.3 log unit change in the profile in the pH range between 7.5 and 5.0 and makes estimation of the pK almost impossible.

Curve b of Figure 4 was generated by using conditions identical with those in curve a except for the inclusion of 1 mM citrate in the assay. All evidence of the hump has been removed, and the typical profile of the Dixon plot is observed. Computer fitting of these data gave a pK of 5.71 $\pm$ 0.10, which agrees well with the value initially determined in the absence of citrate, 5.66 $\pm$ 0.10 (Figure 1, curve a).

In an effort to determine the mode of action of aluminum on hexokinase, we attempted to reverse the aluminum inhibition by the addition of Mg^{2+} to the reaction mixture (Figure 5). The Mg^{2+} concentration was varied from 1 to 10 mM in the absence and presence of added aluminum. The added Mg^{2+} did not have the ability to lessen the aluminum inhibition.

Figures 6 and 7 show double-reciprocal plots of velocity vs. varied ATP and glucose concentrations, respectively, at different, fixed levels of $Al(NO_3)_3$. The data show that aluminum is a competitive inhibitor of ATP and a noncompetitive inhibitor of glucose. The secondary plots (see insert of Figure 6) for slopes in the $1/V$ vs. 1/ATP plots and for slopes and intercepts in the $1/V$ vs. 1/glucose plots (data not shown), vs. $Al(NO_3)_3$, are nonlinear, concave downward.

Discussion

The results of studies of pH changes on enzymatic activity are often difficult to interpret. Several types of data must be compared to draw valid conclusions about the identity of ionizing groups involved in the enzymatic process. To this end, three types of observations were compiled in this study, the pK's of the ionizing groups affecting activity, the changes in these pK's with changing temperature, and the effect of organic solvent on the pK values.

The Dixon plots generated from the data gave relatively simple profiles with limiting slopes approaching -1 on the acid

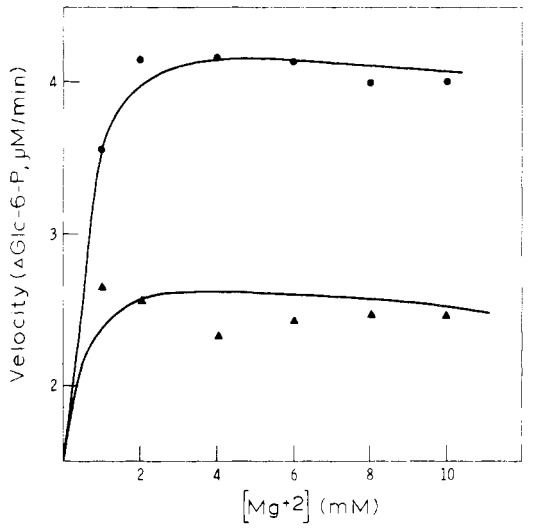

FIGURE 5: Profile showing the effect of increasing Mg^{2+} concentrations on the inhibition caused by 15 μM Al^{3+}. Assays contained 2.2 mM glucose and 2 mM $MgATP^{2-}$ and were done at pH 7 in 10 mM Hepes buffer. Magnesium concentration was varied from 1.0 to 10.0 mM in excess of the concentration of ATP. (▲) 15 μM aluminum nitrate added; (●) no added aluminum.

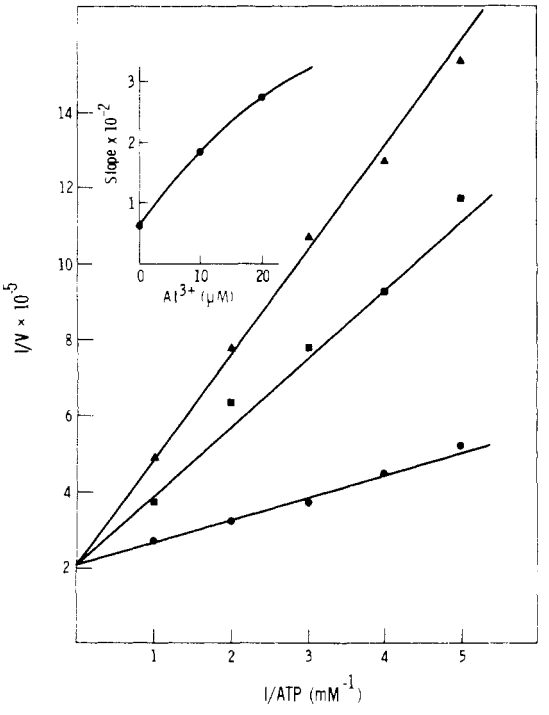

FIGURE 6: Plot of the reciprocal of the initial velocity vs. the reciprocal of the molar concentration of $MgATP^{2-}$ in the absence (●) and presence of 10.0 (■) and 20.0 μM (▲) $Al(NO_3)_3$. Glucose concentration was held at 0.03 mM, and $MgATP^{2-}$ concentration was varied from 0.2 to 1.0 mM. The assays were done at 28 °C in 10 mM Hepes buffer, pH 7.0.

side of the pH scale. No variation in activity was observed at basic pH values. Computer analysis of the data gave the best fit to the theoretical curve when the ionization of one group was assumed to be responsible for the loss of activity in each profile. These groups can be broken down into two types, cationic groups with large ΔH_{ion} values, and a neutral acid group with little dependence upon temperature for ionization. Table II gives a summary of the results obtained in these investigations.

Three of the profiles, those for varied ATP concentration and the -log V_1 profile for varied glucose concentration, were

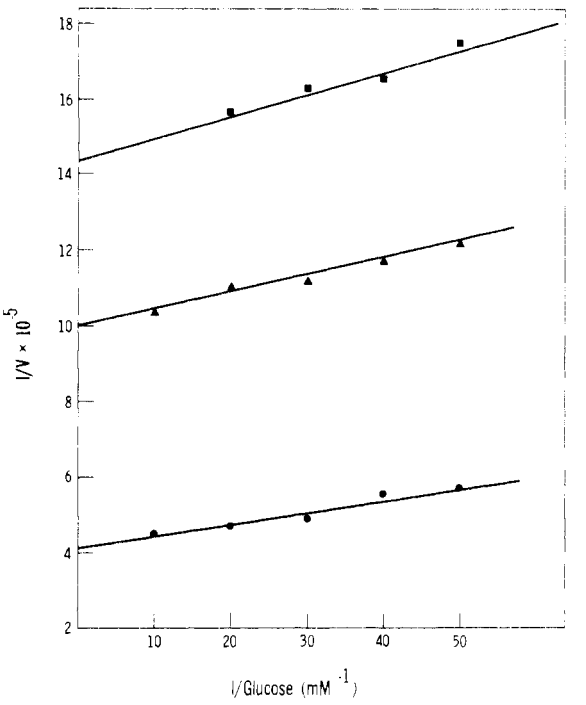

FIGURE 7: Plot of the reciprocal of the initial velocity vs. the reciprocal of the molar concentration of glucose in the absence (●) and presence of 15 (▲) and 30 μM (■) $Al(NO_3)_3$. $MgATP^{2-}$ concentration was held at 0.31 mM while glucose concentration was varied from 0.02 to 0.10 mM. Assays were carried out at 28 °C in 10 mM Hepes buffer, pH 7.0.

Table II: pK's of Catalytically Important Groups in Brain Hexokinase

varied substrate	kinetic parameter	pK_a	ΔH_{ion} (kcal/mol)
$MgATP^{2-}$	V_1	5.66 ± 0.10	10.1
	V_1/K_{ATP}	5.20 ± 0.12	7.7
glucose	V_1	5.85 ± 0.05	6.86
	$V_1/K_{glucose}$	5.47 ± 0.14	2.24

caused by a cationic group, and a neutral acid residue was responsible for the change in parameters in the -log ($V_1/K_{glucose}$) plot. These results can most simply be interpreted in terms of two ionizing groups on the enzyme. The first is involved in the binding of MgATP to the enzyme and with the breakdown of the ternary complex (E·Glc·MgATP) to products. This residue has a pK around 5.7 in the ternary complex and 5.2 in the free enzyme. The high ΔH_{ion} value and the observation that organic solvent does not shift the pK to more basic pH values lend credence to the suggestion that a histidyl residue acts in this capacity. The 0.5 pH unit variation between the pK's could be caused by conformational changes that occur in the enzyme upon binding of the substrate. Wilson (1978) has demonstrated such changes in conformation by studies of the rate of inactivation of rat brain hexokinase by chymotrypsin and glutaraldehyde in the presence and absence of substrate, and McDonald et al. (1979) and Pickover et al. (1979) have shown similar changes in conformation with yeast hexokinase and phosphoglycerate kinase and have speculated that this is a general phenomenon in kinase reactions. Alternatively, the variation in pK could indicate separate histidyl residues are involved in the binding of ATP and the catalysis of the reaction. One or both of these residues may be remote from the active site of the enzyme and function by maintaining the active conformation of the enzyme.

The second ionizable group implicated by these studies participates in the binding of glucose to the enzyme. This

group appears, by its low ΔH_{ion} value, to be of the neutral acid type, and this conclusion is reinforced by the shift of its pK 0.3 pH unit toward the basic end of the pH scale in the presence of organic solvent. This residue is most likely aspartate or glutamate and may function by hydrogen bonding with a hydroxyl group of the sugar substrate. Although the pK for this group is approximately 1.5 pH units higher than that of free aspartate or free glutamate, hydrophobic residues in close proximity could easily perturb its pK by this much.

The effect of aluminum ions on brain hexokinase is difficult to explain and is complicated by the characteristics of aluminum in solution. Evidence shows that, at both acid and basic pH values, aluminum forms polymeric complexes with water and hydroxides (Akitt et al., 1972; Mesmer & Baes, 1971). This could explain why aluminum only inhibited the enzyme in the pH range 7.5–5.5. Outside of this range, the aluminum may assume a polymeric form that is not inhibitory. The inclusion of 1 mM citrate in the reaction reversed the aluminum inhibition, presumably by complexing the aluminum ions. A comparison of the pK values determined in the absence and presence of 1 mM citrate (5.66 ± 0.10 and 5.71 ± 0.10) shows that the aluminum inhibition did not affect the analysis of the data as long as the values between pH 7.5 and 5.5 were omitted from the computer fit.

The site of action of aluminum is not clear. The metal could form an inhibitory complex with ATP (a strong complex since Mg^{2+} was not able to reverse the inhibition), or it could bind directly to the enzyme. Since the inhibition by aluminum is potentiated when it is preincubated with ATP, an AlATP complex is likely to be the inhibiting species. This complex must have a high affinity for the enzyme, however, because it is present at approximately 0.5% of the concentration of ATP. The high affinity of hexokinase for CrATP (Donenberg & Cleland, 1975) suggests that this is likely.

The kinetic experiments presented in Figures 6 and 7 lend credence to an $AlATP^{1-}$ complex being responsible for the inhibition of hexokinase as it is a competitive inhibitor of ATP and a noncompetitive inhibitor of glucose. The nonlinear secondary plot, however, indicates that this is not inhibition of a simple type.

Brain hexokinase is thought to operate by using a rapid equilibrium random mechanism (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974). The generalized equation for competitive inhibition in this mechanism is expressed in eq 3, where A in this case represents ATP concentration, B

$$\frac{1}{V} = \frac{1}{V_1}\left[\frac{K_a}{A}\left(1 + \frac{I^n}{K_i}\right) + \frac{K_b}{B} + \frac{K_{ia}K_b}{AB}\left(1 + \frac{I^n}{K_{ii}}\right)\right] + \frac{1}{V_1} \quad (3)$$

is glucose concentration, and I is the inhibitor concentration, in this case aluminum. In linear competitive inhibition, the exponent of I is 1, and the replots are linear. If the inhibitor binds multiply to the enzyme, the exponent, n, becomes 2 or higher, yielding replots that are concave upward. If the stoichiometry of inhibitor binding is less than 1, n will be less than 1, and the secondary plots will be nonlinear concave downward as is seen in this case. The kinetic data presented in Figures 6 and 7 were fitted to eq 3 by the OMNITAB program (Siano et al., 1975) with a variety of n values, and the exponent on the inhibitor term was found to be around 0.75.

This effect could be explained in several different ways. One could be that Al^{3+} ions form a complex with more than one enzyme molecule. If, for example, two molecules of enzyme complex with one Al^{3+}, an $I^{1/2}$ term would be introduced into the rate equation. More complicated expressions would arise if the complex formed with a different stoichiometry and could yield exponents of I varying from 0 to 1. If such a complex is formed, it may be possible to identify it by determining the molecular weight of brain hexokinase in the presence of Al^{3+}. A large change in the weight of the enzyme would indicate complex formation and might allow a determination of the binding stoichiometry.

A second possibility could be that the inhibitor molecule undergoes a concentration-dependent reaction that modifies it into a noninhibiting form. This is most easily visualized by imagining that the inhibitor undergoes a dimerization reaction (eq 4). If this happens, the added inhibitor (I_0) will be

$$2I \rightleftharpoons I_2 \quad (4)$$

$$I_0 = I + 2I_2 \quad (5)$$

$$I = \frac{-K + (K^2 + 8KI_0)^{1/2}}{4} \quad (6)$$

partitioned between I and I_2 (eq 5), and the concentration of I would be expressed as in eq 6 where K is the dissociation constant of the dimer. If I is inhibiting and I_2 is not, the effect of the dimerization would be to reduce the apparent inhibition caused by an added amount of inhibitor. The formation of such complexes of aluminum is well documented. Akitt et al. (1972) have shown that when dissolved in water $AlCl_3 \cdot 6H_2O$ partitions between the two forms $Al(H_2O)_3^{3+}$ and $Al_2(OH)_2(H_2O)_8^{4+}$. As the pH of the solution is raised, higher order complexes of aluminum form, such as $Al_{13}O_4(OH)_{24}(H_2O)_{12}^{7+}$ and $Al_8(OH)_{20}(H_2O)_x^{4+}$ (Akitt et al., 1972). Other investigators have found species such as $Al_2(OH)_2^{4+}$ and $Al_3(OH)_4^{5+}$ present in solution at acidic pH and more complex forms such as $Al_{14}(OH)_{34}^{8+}$ in alkaline solution (Mesmer & Baes, 1971).

The data presented here support the idea that aluminum is responsible for inhibition of hexokinase below a pH of approximately 8.0. The inhibition is potent and can be reversed by citrate. Above pH 8.0, no inhibition by $Al(NO_3)_3$ is observed, but this could be due to the characteristic of aluminum ions to form polymers at basic pH values.

A paper by Womack & Colowick (1979) has appeared since preparation of the current study for publication. Their findings suggest that aluminum is a proton-dependent inhibitor of yeast hexokinase and acts by complexation of ATP to form an inhibiting species. They also show that, while aluminum will inhibit rat brain hexokinase, it has little effect on rat muscle hexokinase or rabbit phosphofructokinase, and it was not an inhibitor of yeast glucokinase from *Saccharomyces cerevisiae*.

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Resonance Energy Transfer between Catalytic Sites of Bovine Liver Uridine Diphosphoglucose Dehydrogenase†

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ABSTRACT: The catalytic-site thiol groups of three of the six identical subunits of bovine liver uridine diphosphoglucose dehydrogenase (EC 1.1.1.22) were alkylated with a fluorescent substituent that could serve as a donor in an energy-transfer donor–acceptor pair, and the catalytic-site thiol groups on the other three subunits were alkylated with a fluorescent substituent that could serve as an acceptor. The half-of-the-sites reactivity of the enzyme permitted introduction of the donor moiety at only three catalytic sites by reaction with 5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonic acid; doubly labeled enzyme was prepared by subsequent reaction with 5-(iodoacetamido)fluorescein, 5-(iodoacetamido)eosin, or 4-[*N*-(iodoacetoxy)ethyl-*N*-methylamino]-7-nitrobenz-2,1,3-oxadiazole. The specificity of labeling of the catalytic-site thiol groups was demonstrated by peptide mapping of tryptic digests of doubly derivatized enzyme. The magnitude of donor quenching was used to determine energy-transfer efficiencies. The equivalence of transfer efficiencies for the doubly labeled enzyme prepared by successive treatment with 5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonic acid and 5-(iodoacetamido)fluorescein measured by either donor quenching or sensitized fluorescence confirmed that energy transfer was responsible for the observed donor quenching. Theoretical models based on the known hexagonal-planar arrangement of the enzyme subunits and on the half-of-the-sites reactivity behavior of the enzyme are developed to permit calculation of the transfer efficiency as a function of the relative locations of the catalytic sites on the surfaces of the subunits. Comparison of the resulting maps of transfer efficiency vs. surface position with the observed transfer efficiencies allows designation of certain surface regions as likely locations of the catalytic sites. These results suggest that the catalytic sites of bovine liver uridine diphosphoglucose dehydrogenase are probably neither on those subunit surfaces that line the central cavity of the array nor in the intersubunit bonding domains. The preferred catalytic-site locations would be either the alternate poles of consecutive subunits or the outer peripheral regions of the hexagonal ensemble.

Bovine liver uridine diphosphoglucose dehydrogenase (UDPGDH;[1] EC 1.1.1.22) contains six identical 52 000-dalton subunits per native enzyme molecule (Gainey et al., 1972; Uram et al., 1972). As shown by electron microscopy, the six subunits of the hexamer are associated to form a hexagonal array (Franzen et al., 1980a). One of the 10–12 thiol groups per subunit is located in the catalytic site (Gainey et al., 1972; Uram, 1971) and is involved in covalent linkage with the partially and fully oxidized substrates during each enzyme turnover (Ridley & Kirkwood, 1973; Ridley et al., 1975). Furthermore, the enzyme shows half-of-the-sites behavior with respect to both substrate binding (Franzen et al., 1973; Gainey & Phelps, 1974) and reaction of the catalytic-site thiol group with modifying reagents (Franzen et al., 1976, 1978, 1980a).

The differential reactivities of the catalytic-site thiol groups provide a means for preparing altered enzyme molecules in which half of the catalytic sites are covalently bound to one particular substituent and the other half are bound to a dif-

† From the Department of Biological Sciences, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received May 12, 1980.* This work was performed in partial fulfillment of the Ph.D. degree requirements of P.S.M. at the University of Pittsburgh. It was supported by grants from the National Science Foundation (PCM 7708456) and the National Institutes of Health (AM 15332). This work was reported in part at the 2nd Biophysical Discussions meeting, Airlie, VA, May 1980 (Franzen et al., 1980c).

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[1] Abbreviations used: UDPGDH, uridine diphosphoglucose dehydrogenase; BUP, *p*-(bromoacetamido)phenyl uridyl pyrophosphate; IAEDANS, 5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonic acid; IAE, 5-(iodoacetamido)eosin; IAF, 5-(iodoacetamido)fluorescein; IANBD, 4-[*N*-(iodoacetoxy)ethyl-*N*-methylamino]-7-nitrobenz-2,1,3-oxadiazole; TPCK, N^{α}-tosylmethylalanine chloromethyl ketone.

0006-2960/80/0419-6080$01.00/0