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Use of pH Studies to Elucidate the Chemical Mechanism of Yeast Hexokinase†

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ABSTRACT: The pH variation of the kinetic parameters of the reaction catalyzed by yeast hexokinase was examined for the forward and reverse reactions. In the forward direction, the kinetic parameters showed a decrease on the acid side below pH 7. The V_{max} and V/K profile for MgATP had a "hollow" in the profiles, which was eliminated by the addition of citrate. The V/K profile for 2,5-anhydro-D-mannitol also gave a hollow in the profile, which was eliminated by the addition of citrate; however, the V/K profile for 1,5-anhydro-D-mannitol did not have a hollow and was not activated by citrate. The V/K profile for glucose did not show this hollow and subsequent citrate activation, but reflects several groups whose protonation caused loss of activity. At least some of these groups are important for substrate binding, but not for catalysis, and cannot be protonated in the enzyme–glucose complex. The binding of metal–ATP is pH independent in this region, with the V/K profile for MgATP in the presence of citrate reflecting the catalytic pK of 6.2. Since the pK of MgATP (5.3) is not seen in the V/K_{MgATP} profile, it appears that yeast hexokinase can utilize both MgATP and MgHATP equally well. In the reverse reaction, deprotonation of a group with p$K \approx$ 6.8 caused loss of activity. The temperature independence of this pK, and of the pK seen in the V/K_{MgATP} profile in the forward direction in the presence of citrate, suggests the presence of a carboxylate group on the enzyme which must be deprotonated for yeast hexokinase to catalyze the transfer of the γ-phosphate of ATP to sugars and protonated to catalyze the reverse reaction. This was confirmed by the effects of organic solvent perturbations on the pK, indicating a catalytic group on the enzyme of the neutral acid type.

The long-standing controversy concerning the kinetic mechanism of yeast hexokinase (EC 2.7.1.1) has apparently been resolved in favor of a random kinetic mechanism (Rudolph and Fromm, 1971; Danenberg and Cleland, 1975). Work is now proceeding in several labs to determine the individual steps in the chemical mechanism, the groups on the enzyme which are responsible for the binding of substrates, and the groups which participate in the catalytic steps. Chemical-modification studies have been carried out by several labs in an attempt to determine the amino acid residues essential for activity. Histidyl residues have been shown to have no role in either binding or catalysis (Grouselle et al., 1973), while evidence has been presented (Pho et al., 1974) to show the involvement of carboxyl groups at the active site. Later work led to the isolation of a modified glutamyl residue which was proposed to have a catalytic function (Pho et al., 1977). The modification of a single tyrosyl residue caused the loss of activity (Coffe and Pudles, 1977), but this was postulated to be a result of enzyme dissociation and conformational changes at the active site. Early modification studies (Fasella and Hammes, 1963) concluded that sulfhydryl groups had no role in the catalytic process; however, more recent work (Otieno et al., 1977) has shown the presence of a single thiol group at the active site whose derivatization leads to the loss of activity.

X-ray crystallographic studies of the yeast hexokinase complex with substrates at 2.7-Å resolution (Steitz et al., 1977) concluded that protein side chains were interacting with the 3- and 4-hydroxyl groups of the sugar substrate. When the resolution was refined to 2.1-Å (Anderson et al., 1978), there appeared to be an aspartyl residue interacting with the 4- and 6-hydroxyl groups.

Relatively little use has been made of the diagnostic power of steady-state kinetics in determining the groups on the enzyme responsible for substrate binding and catalysis. Some preliminary work on yeast hexokinase (Bohnensack and Hofmann, 1969), over a limited pH range, concluded that the group responsible for a decrease in activity in the V_{max} profile with a pK = 6.8 was probably a histidyl residue. This conclusion was later disputed by the modification studies mentioned above.

We have undertaken a more extensive kinetic study of the reaction catalyzed by yeast hexokinase and report evidence

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obtained from an examination of the V_{max} and V/K profiles for both substrates in the forward and reverse reactions and from the pH variation of K_i for competitive inhibitors.

Materials and Methods

Yeast hexokinase (type C-302), glucose-6-phosphate dehydrogenase, α-glycerophosphate dehydrogenase, glycerokinase, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma. Glucose dehydrogenase and mutarotase were purchased from Boehringer. 6-Deoxy-D-glucose was purchased from Sefochem Fine Chemicals. Adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) was purchased from P-L Biochemicals. 2,5-Anhydro-D-mannose was synthesized by nitrous acid treatment of glucosamine, and 2,5-anhydro-D-mannitol by subsequent reduction with borohydride at pH 7 (Horton and Philips, 1973). 1,5-Anhydro-D-mannitol was prepared by treating D-mannitol with HCl (Fletcher, 1963). CrATP was prepared by the procedure of DePamphilis and Cleland (1973). All other materials were of the highest purity commercially available.

Kinetic Assays. Kinetic studies were run in 3.0-mL total volume in 1-cm cuvettes by measuring absorbance changes at 340 nm with a Gilford optical-density converter and a 10-mV recorder. Temperature was maintained at ±0.1 °C of the stated values with thermospacers and a circulating water bath. Assays were run at 25 °C, unless otherwise specified.

All reactions were initiated by the addition of 0.4–1.0 unit of hexokinase per assay. Dilutions of the stock enzyme solution were made with 10 mM buffer containing 1 mg/mL bovine serum albumin. For the profiles run in neutral acid buffers, acetate buffers were used below pH 5.5, a mixed acetate–cacodylate buffer was used to cover the range from pH 4.5 to 8.0, and a cacodylate–borate buffer system was used at high pH. For the profiles run in cationic acid buffers, 2-(*N*-morpholino)ethanesulfonate (Mes), piperazine-*N*,*N*′-bis(2-ethanesulfonate) (Pipes), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (Hepes), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate (Taps), 2-(cyclohexylamino)ethanesulfonate (Ches), and 3-(cyclohexylamino)propanesulfonate (Caps) were used as buffers within 1 pH unit of their p*K* values.

The forward reaction rates with glucose as a substrate were measured using a glucose-6-phosphate dehydrogenase couple. The reaction mixture contained 50 mM buffer, 0.4–0.6 mM NADP, 1 mM citrate, and 5–10 units of glucose-6-phosphate dehydrogenase. For experiments run in the absence of added citrate, glucose-6-phosphate dehydrogenase was dialyzed to remove the citrate present in the lyophilized powder. Magnesium(II) was added as the acetate salt and was maintained at 1 mM excess over the total concentration of ATP and citrate. Substrate levels were as stated for each experiment. When 1,5-anhydro-D-mannitol and 2,5-anhydro-D-mannitol were used as substrates, the forward reaction rate was measured using the pyruvate kinase–lactate dehydrogenase couple to follow the production of ADP. The reaction mixture contained 50 mM buffer, 0.4–0.6 mM phosphoenolpyruvate, 0.4 mM NADH, and 25 units each of pyruvate kinase and lactate dehydrogenase. The reverse reaction rates were measured by coupling the production of ATP with glycerokinase and dihydroxyacetone. The appearance of dihydroxyacetone phosphate was monitored with α-glycerophosphate dehydrogenase by following the disappearance of NADH. The reaction mixture contained 50 mM buffer, 10 mM dihydroxyacetone, 0.4 mM NADH, 8 units of glycerokinase, and 5 units of α-glycerophosphate dehydrogenase. Magnesium ions were maintained at 5 mM excess over the ADP concentration. Alternately, the reverse reaction rate was monitored by coupling the production of glucose with glucose dehydrogenase. Since the dehydrogenase is specific for the β-anomer of glucose, mutorotase was added to the assay mixture to catalyze the interconversion between α- and β-D-glucose. The reaction mixture contained 50 mM buffer, 0.4–0.6 mM NADP, 6 units of glucose dehydrogenase, and 25 units of mutarotase.

For all coupled assays, the level of the coupling enzymes was varied to verify that the rates being measured were totally limited by the levels of yeast hexokinase and its substrates and were independent of the levels of coupling enzymes present in the assay. The rates obtained by the different coupled assays used in measuring either the forward or reverse reactions of yeast hexokinase were identical with each other, within experimental error, for the reaction measured in a given direction.

For the variable-temperature studies, pH measurements were made by a pH meter calibrated at the temperature of the experiment. For the pH studies run in the presence of organic solvents, 25% *N*,*N*′-dimethylformamide was added after the pH of the assay mixture was determined.

Data Processing. All computer fits to the data were performed using Fortran programs which assume equal variance for the velocities or the logarithm of the fitted parameter (Cleland, 1967). Reciprocal plots at each pH, at saturating levels of the fixed substrate, were fitted to eq 1, where v is the experimetally determined velocity, V is the maximum velocity, A is the substrate concentration, and K is the Michaelis constant. Data from the experiments with 6-deoxy-D-glucose and CrATP as competitive inhibitors were fitted to eq 2, where K_{is} is the slope inhibition constant. The pH profile data were fitted to eq 3 or 4, depending on whether activity was lost on the acid or basic side of the profile, and y is either V, V/K, or $1/K_{is}$. The temperature-dependence data were fitted to eq 5.

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

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{2}$$

$$\log y = \log\left(\frac{C}{1 + [H^+]/K_a}\right) \tag{3}$$

$$\log y = \log\left(\frac{C}{1 + K_a/[H^+]}\right) \tag{4}$$

$$pK = \frac{\Delta H_{ion}}{2.303RT} \tag{5}$$

Results

Forward Reaction. The pH variation of the maximum velocity of yeast hexokinase with glucose and MgATP as substrates is shown in Figure 1A. The maximum velocity decreased below pH 8 with a slope of 1, indicative of the protonation of a single group causing loss of activity. However, below pH 6.5, the profile leveled out before continuing to decrease. The V/K profile for MgATP (Figure 2A, solid circles) showed a similar shape. After breaking at pH 7 with a slope >1, the profile leveled out below pH 6 and remained unchanged to below pH 4.5. The V/K profile for glucose (Figure 1B) did not show this "hollow" in the profile but began to decrease very sharply below pH 6.5. The data were fitted to an equation assuming a variable number of ionizable groups with identical p*K* values. The best fit to the data was obtained when five groups were assumed, indicating that at least several groups are being protonated in a concerted fashion to cause a loss of activity.

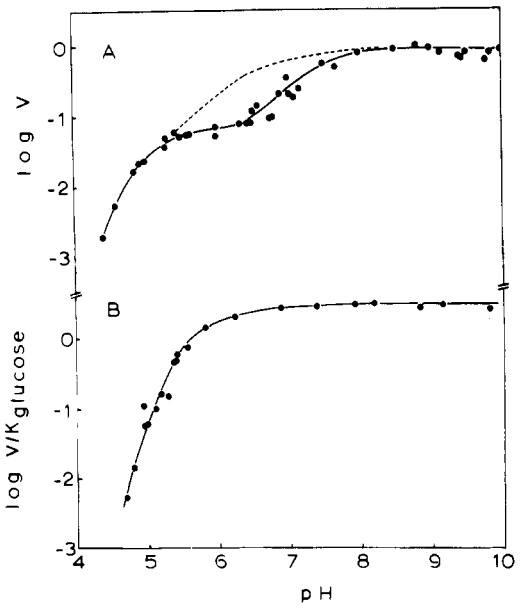

FIGURE 1: (A) V_{max} profile of yeast hexokinase. Individual data points represent computer fits of velocities at saturating (20 mM) glucose and varying MgATP to eq 1. The curve through the data is hand drawn. (B) V/K profile for glucose. Individual data points were determined from computer fits of velocities at saturating (3 mM) MgATP and varying glucose to eq 1. The curve through the data is a computer fit assuming five groups with an identical pK value of 5.05 ± 0.01.

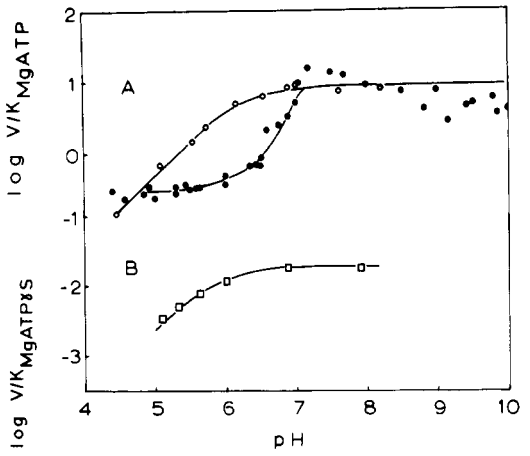

FIGURE 2: (a) V/K profile for MgATP. Individual data points were determined under the same conditions as Figure 1A: (●) citrate = 0, the curve through the data is hand drawn; (○) citrate = 1.0 mM, the curve through the data is a computer fit to eq 3 with a pK value of 6.15 ± 0.03. (B) V/K profile for MgATPγS: (□) citrate = 1.0 mM. Conditions are the same as for curve A. The curve through the data is a computer fit to eq 3, with a pK value of 6.0 ± 0.1.

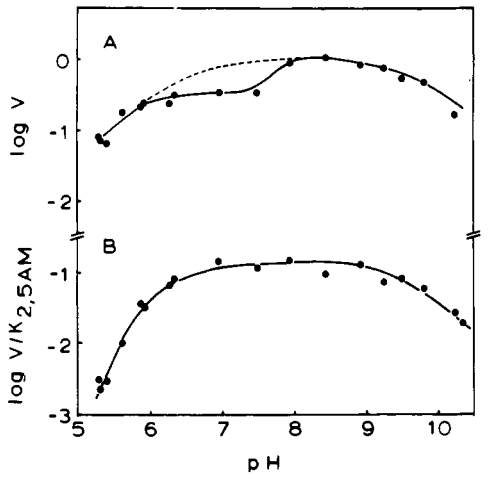

FIGURE 3: (A) V_{max} profile for 2,5-anhydro-D-mannitol. Assays were run at 5 mM MgATP with 2,5-anhydro-D-mannitol varied from 0.5 to 5 times K_M. The solid line through the data is hand drawn, while the dashed line represents a computer fit to eq 3 and 4, with pK values of 6.28 and 9.89. (B) V/K profile for 2,5-anhydro-D-mannitol. Substrate concentrations were identical to A. The curve through the data points is a computer fit to eq 4 on the basic side (pK = 9.49) and to an equation assuming four groups with identical pK values on the acid side (pK = 5.57).

The temperature dependence of the V_{max} and V/K_{Glc} profiles was examined to determine the identity of the group or groups whose protonation caused loss of activity. Profiles were run at 15, 25, and 35 °C, and both the V_{max} and V/K_{Glc} profiles had decreases in pK with increasing temperature. The V_{max} profile was fitted to eq 3 and gave values of 6.87 ± 0.01, 6.61 ± 0.04, and 6.44 ± 0.07, respectively, at increasing temperatures. The computer fit to the temperature dependence of the V/K profile for glucose gave values of 6.59 ± 0.05, 6.15 ± 0.04, and 6.05 ± 0.04, respectively. The data were fitted to eq 5 by the least-squares method to determine ΔH_{ion}. For the V_{max} profile, ΔH_{ion} = 8.8 ± 0.7 kcal/mol, while the V/K_{Glc} profile gave ΔH_{ion} = 11.1 ± 3.6 kcal/mol. These values of ΔH_{ion} would rule out simple ionization of a carboxyl group (ΔH_{ion} = ±1.5 kcal/mol) as being solely responsible for the decrease in activity at low pH.

Shill and Neet (1975) have shown that yeast hexokinase gives nonlinear burst kinetics below pH 7.5, in the region where hollows were observed in the V_{max} and V/K_{MgATP} profiles. To determine if the hollows observed in these profiles are an artifact of inaccurate initial velocity tangents drawn to the nonlinear time courses, the pH profiles were determined with substrates which did not show burst kinetics. 2,5-Anhydro-D-mannitol is a good substrate for yeast hexokinase (V_{max} = 150% of glucose) and 1,5-anhydro-D-mannitol is a poor substrate (V_{max} = 6%), but both give linear time courses across the entire pH range (Viola and Cleland, 1978, unpublished results). The V_{max} and V/K profiles for 2,5-anhydro-D-mannitol are shown in Figure 3. These profiles decrease on both the acid and basic sides, with the computer fit to eq 3 and 4 giving values of pK_a = 6.28 and pK_b = 9.89 for the V_{max} profile, and values of pK_a = 6.79 and pK_b = 9.49 for the V/K profile for 2,5-anhydro-D-mannitol. The V_{max} profile showed a hollow below pH 8 similar to that observed in the V_{max} profile with saturating glucose, while the $V/K_{2,5\text{-AM}}$ profile was the same shape as V/K for glucose, with no hollow and a sharp break to a slope greater than one below pH 6. The V/K profile for 1,5-anhydro-D-mannitol (Figure 4B) had this same general shape, while the V_{max} profile (Figure 4A) did not show the pronounced hollow observed in the other V_{max} profiles.

Kosow and Rose (1960) reported the activation of yeast hexokinase by citrate and other anions below pH 7, but no activation was seen at pH 8.5. The pH dependence of this activation was examined to see what effect it would have on the general shape of the profiles. Figure 5 shows the effect of increasing levels of citrate on the V_{max} profile with saturating glucose and MgATP. Even at very low levels of citrate (0.1 mM), the hollow observed in the profile had been essentially removed to give a smooth curve with a single break. The same results were obtained when the V_{max} profile with saturating 2,5-anhydro-D-mannitol was rerun in the presence of citrate. However, the V_{max} profile with saturating 1,5-anhydro-D-mannitol, which did not show a hollow in the absence of citrate, was not activated by citrate at any pH value across the profile. The V/K profile for MgATP in the presence of saturating glucose was also activated by citrate below pH 7 (Figure 2A,

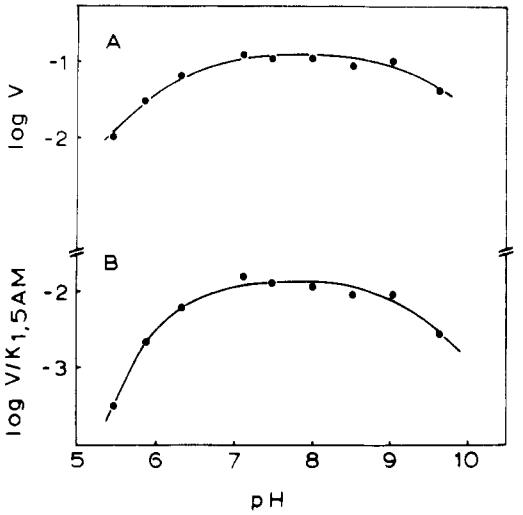

FIGURE 4: (A) V_{max} profile for 1,5-anhydro-D-mannitol. Assays were run at 5 mM MgATP with 1,5-anhydro-D-mannitol varied from 0.5 to 5 times K_M. The curve through the data points is a computer fit to eq 3 and 4, with pK values of 6.46 and 9.36. (B) V/K profile for 1,5-anhydro-D-mannitol. Substrate concentrations were identical to A. The curve through the data points is a computer fit to eq 4 on the basic side (pK = 9.02) and to an equation assuming four groups with identical pK values on the acid side (pK = 5.66).

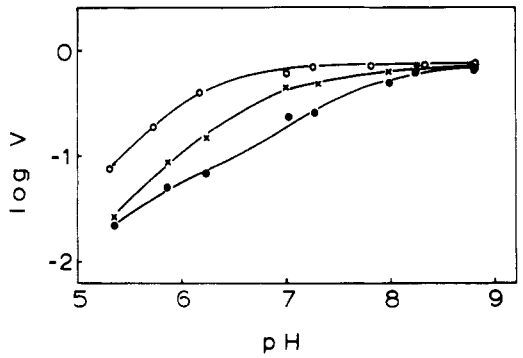

FIGURE 5: pH dependence of citrate activation. Assays were run at 5 mM MgATP and 20 mM glucose: (●) citrate = 0; (×) citrate = 0.1 mM; (○) citrate = 1.0 mM. The curves through the data are hand drawn with the exception of the computer fit to the data at 1.0 mM citrate.

open circles) with the effect of removing the hollow and plateau seen in the absence of citrate. The V/K profile for MgATPγS in the presence of citrate showed the same general shape and pK value (Figure 2B). As expected, the V/K profile for MgATP with saturating 1,5-anhydro-D-mannitol, in the absence of citrate, gave the same shape as the citrate-activated profile with saturating glucose present.

The temperature dependence of the V_{max} and V/K_{MgATP} profiles was reexamined in the presence of citrate. Removing the anomalous shapes from these profiles should simplify the determination of the pK values of the groups responsible for the loss of activity. The pK values determined from the V_{max} profile still showed a temperature dependence, with the data fitted to eq 3, giving values of 6.85 ± 0.01 at 9 °C, 6.53 ± 0.01 at 17 °C, 6.23 ± 0.02 at 25 °C, and 6.11 ± 0.01 at 33 °C. These pK values were fitted to eq 5, giving ΔH_{ion} = 12.3 ± 1.3 kcal/mol. This value is larger than would be expected for the simple ionization of a single group below pH 7 and is probably due to an associated change in protein conformation which accompanies protonation. On the other hand, the V/K profile for MgATP in the presence of citrate gave a pK value which was temperature independent, with a value of 6.19 ± 0.04 at

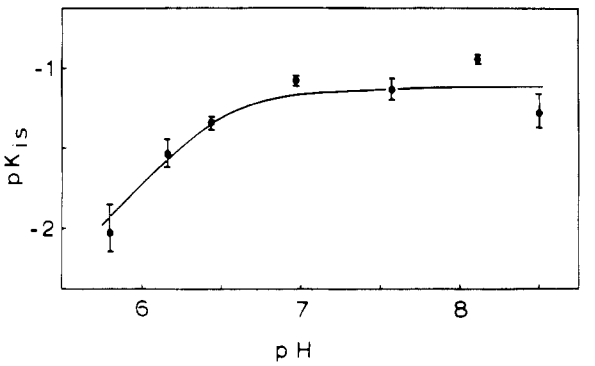

FIGURE 6: pH variation of K_i for 6-deoxy-D-glucose. Assays were run at 2 mM MgATP, with glucose varied from 0.5 to 5 times K_M. Each point represents a computer fit of a competitive inhibition pattern to eq 2. The curve through the data is a computer fit to eq 3, with $y = 1/K_{is}$, giving a pK value of 6.57 ± 0.07.

TABLE I: Effect of DMF on the pK of the V/K_{MgATP} Profile.[a]

buffer type	pK values −DMF	pK values +DMF
neutral	6.15 ± 0.03	6.08 ± 0.02
cationic	6.15 ± 0.03	6.62 ± 0.02

[a] The assay mixture contained 50 mM buffer, 2 mM glucose, 1 mM citrate, 0.4 mM NADP, 0.06–0.60 mM ATP, 2 mM excess Mg(II), and 5 units of glucose-6-phosphate dehydrogenase. The reaction was initiated by addition of 0.4 unit of hexokinase. In the experiments with organic solvent present, DMF was added to the assay mixture to a final concentration of 25% (v/v). The pK values were obtained from computer fits of the data to eq 3.

10 °C and 6.15 ± 0.03 at 25 °C, and appears to reflect the protonation of a single group on the enzyme.

To distinguish between groups on the enzyme which function in substrate binding, but do not play a role in the catalytic steps, the pH dependence of K_i was examined for 6-deoxy-D-glucose, a competitive inhibitor of glucose, and CrATP, a competitive inhibitor of MgATP. The pH profile of the K_i of 6-deoxy-D-glucose is shown in Figure 6. The break in the profile on the acid side corresponds to the break seen in the V/K_{Glc} profile, although the limiting slope attained in the K_i profile does not appear to be as great as that seen in the V/K_{Glc} profile. This indicates that at least some of the groups whose protonation caused loss of activity in the V/K_{Glc} profile play a role in substrate binding but not in catalysis. It was not possible to extend the K_i profile to lower pH values because of the limited availability of 6-deoxy-D-glucose and the large K_i values below pH 6. In contrast to the results observed in the V/K_{sugar} profiles, the K_i of CrATP was pH independent in the region from pH 5 to 7. At pH 5, the competitive inhibition pattern was fitted to eq 2, giving a value of K_{is} = 1.5 μM. At pH 7.0, K_{is} was determined to be 4.0 μM, indicating that the single, temperature-independent pK seen in the V/K profile for MgATP is that of a catalytic group on the enzyme.

Further evidence on the nature of the catalytic group was obtained by examining the effect of increasing the hydrophobicity of the solvent, by the addition of organic solvents, on the pK values. The results of adding 25% DMF to the V/K_{MgATP} profile run in the presence of citrate in both neutral and cationic acid buffers are shown in Table I.

Reverse Reaction. The pH profiles of the reverse reaction catalyzed by yeast hexokinase were examined to further characterize the catalytic group on the enzyme. Ionization of a group with a pK value of 8.1 causes loss of activity in the V_{max}

profile (Figure 7A). The V/K profile for glucose 6-phosphate (Figure 7B) was fit with pK = 6.8, while the V/K profile for MgADP (Figure 7C) gave a pK of 8.2. The temperature dependence of the pK values seen in the V_{max} and V/K_{G6P} profiles was examined from 15 to 35 °C, and showed no variation over this temperature range ($\Delta H_{ion} < 1$ kcal/mol). The V_{max} profile was fitted with pK values of 8.03, 8.13, and 8.07, and the V/K_{G6P} profile with pK values of 6.76, 6.86, and 6.73 at 15, 25 and 35 °C, respectively.

Discussion

The pH variation of the kinetic parameters for the forward reaction, in the absence of citrate, gave complex profiles which could not be readily analyzed in terms of protonation of single groups on the enzyme resulting in the loss of activity. While all three kinetic parameters decreased below pH 7, only the V/K profile for glucose gave a smooth curve with a single break which was amenable to computer analysis (Figure 1B). The asymptote on the acid side reaches a limiting slope of about 5, indicating a cooperative protonation of several groups, probably associated with a change in enzyme conformation to cause activity loss.

Activation by citrate had the effect of removing the hollow in the V_{max} and V/K profile for MgATP. The limiting value of the asymptote to the acid side of the V_{max} profile still had a slope >1, although not as high as that seen in the V/K_{Glc} profile. Formation of an enzyme-sugar complex must prevent protonation of some of the groups seen in the free enzyme. These are probably groups which have a role in the binding of glucose to the enzyme, although it is known that glucose binding causes changes in the enzyme conformation (Anderson and Steitz, 1975) which may affect these groups. The V/K_{MgATP} profile in the presence of citrate is well fit by an equation assuming protonation of a single group on the enzyme with a pK = 6.2. When the V/K_{MgATP} profile was run using MgATPγS as the variable substrate, the shape and pK obtained from the profile were virtually identical to that observed with MgATP (Figure 2B). Thus, the pK observed in the profile could not be that due to the protonation of MgATP or MgATPγS (pK values of 5.3 and <4.2, respectively) or of the free ATP or ATPγS (pK values of 6.7 and 5.3) (Jaffe and Cohn, 1978) but must be due to the protonation of a group on the enzyme. The failure of the V/K_{MgATP} profile to show the pK of the nucleotide implies that yeast hexokinase can utilize both MgATP and MgHATP equally well as substrates.[1] The generality of this hypothesis remains to be tested with other kinases.

The V_{max} profile with 2,5-anhydro-D-mannitol as substrate (Figure 3A) shows the same general shape as that run with saturating glucose; however, in the V_{max} profile with 1,5-anhydro-D-mannitol as substrate, no hollow was observed (Figure 4A). With good substrates for yeast hexokinase, there is a slow step other than the chemical reaction which limits the rate and causes the hollow observed in the profiles. With poor substrates where the rate of the chemical reaction has been slowed, there has been a change in the step which limits the rate, and the hollow is no longer seen.

The pH variation of K_i for competitive inhibitors of the reaction catalyzed by yeast hexokinase was examined, because the combination of inhibitor with enzyme is at equilibrium in the steady state, and, therefore, the true pK of the free enzyme is observed. These profiles also serve to distinguish between groups on the enzyme which play a role in substrate binding and groups which are necessary for catalysis. The pH profile for 6-deoxy-D-glucose, a competitive inhibitor of glucose, showed a pK = 6.6, indicating the presence of a group or groups on the enzyme with this pK which are responsible for the binding of glucose to the free enzyme. The pH independence of the profile with CrATP, a competitive inhibitor of MgATP, suggests that there are no groups on the free enzyme which are protonated in this region to cause diminished binding of MgATP. The absence of a binding pK in the CrATP profile indicates that the single pK observed in the V/K_{MgATP} profile must be due to the protonation of a catalytic group.

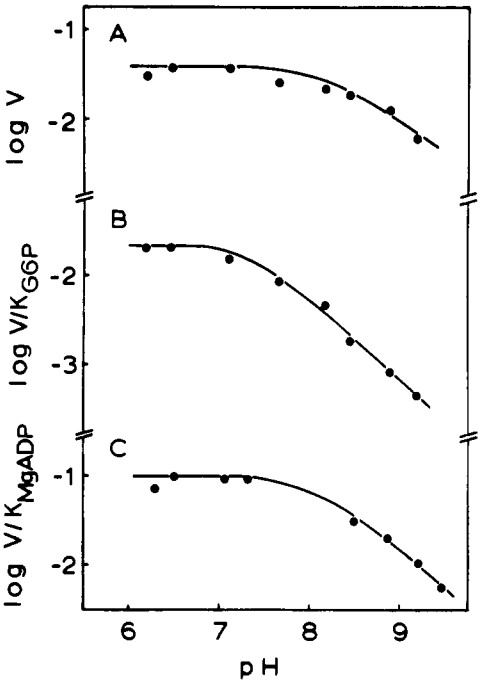

FIGURE 7: pH profiles of the reverse reaction. The curves through the data points are computer fits to eq 4. (A) V_{max} profile. The individual data points represent computer fits of velocities to eq 1, at 5 mM MgADP and varying levels of glucose 6-phosphate. The profile was fit with a pK value of 8.13 ± 0.08. (B) V/K profile for glucose 6-phosphate. The data points were determined under the same conditions as curve A. The profile was fit with a pK value of 7.03 ± 0.05. (C) V/K profile for MgADP. The individual data points represent computer fits to velocities at 50–100 mM glucose 6-phosphate and varying levels of MgADP. The profile was fit with a pK value of 8.17 ± 0.03.

The reverse reaction profiles are pH dependent with a single break from a slope of 0 to −1 in the region from pH 6.8 to 8.2. The V/K profile for glucose 6-phosphate was fitted to a pK = 6.8 (Figure 7B), almost identical to the pK = 6.6 observed in the 6-deoxy-D-glucose profile in the forward direction, suggesting that the same group which must be deprotonated in the forward direction must be protonated in the reverse direction. The V_{max} and V/K_{MgADP} profiles were fit with pK values displaced about 1.5 pH units above the pK of the V/K_{G6P} profile (Figures 7A and 7C). The V_{max} and V/K profiles all showed decreases on the acid side below pH 6 (not shown in Figure 7), which may be a result of the protonation of groups on the enzyme responsible for substrate binding, similar to that observed in the profiles in the forward direction. Both the glucose dehydrogenase–mutarotase and glycerokinase–α-glycerophosphate dehydrogenase couples gave loss of activity

[1] At the lowest pH value (4.4), under conditions where total magnesium ion was maintained at 1 mM excess over total citrate and ATP, only 10% of the ATP is present as $MgATP^{2-}$, while 75% is $MgHATP^{-}$ (the other major form being 15% of $HATP^{3-}$). If only $MgATP^{2-}$ were active as a substrate for yeast hexokinase, the profile should have dropped 1.0 unit below the asymptote at this pH, as opposed to an expected drop of 0.07 unit if $MgATP^{2-}$ and $MgHATP^{-}$ are equally active, and the only inactive species are the free nucleotides.

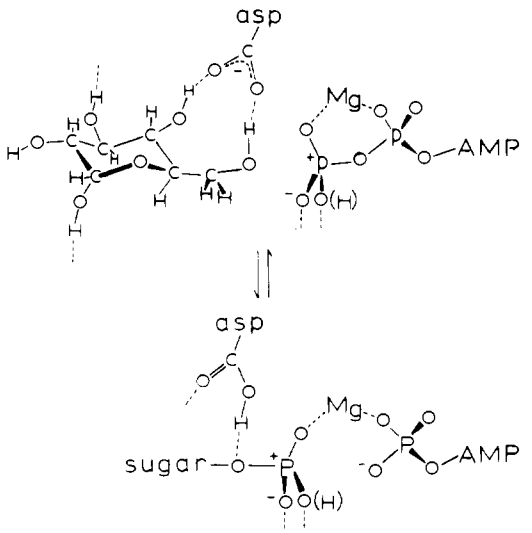

FIGURE 8: Diagram of the proposed transphosphorylation mechanism of yeast hexokinase.

below pH 6, causing long lags in the time course and making this pH region difficult to interpret for the profiles of the reverse reaction.

The temperature dependence of V_{max} and V/K for glucose, observed both in the presence and absence of citrate, is probably due to protonation of several groups on the enzyme, along with an associated change in enzyme conformation. It does not appear likely that these effects will be separated in the pH profiles and be of diagnostic importance. However, the V/K profile for MgATP in the presence of citrate shows a clean break from a slope of 0 to 1, and the pK determined from a fit to this profile is temperature independent. The same shape and pK, and presumably the same temperature independence, were observed for the V/K_{MgATP} profile in the presence of saturating 1,5-anhydro-D-mannitol. This suggests that the group which must be deprotonated in the forward direction is a carboxylate group.

The elevated pK value of the carboxylate group on yeast hexokinase over that observed for simple carboxylic acids is not unexpected, given the deep cleft on the enzyme in which the active site is located (Anderson and Steitz, 1975). Limited accessibility to solvent would tend to make ionization less favorable, thus increasing the pK. Similar results have been observed with other enzymes; for example, fumarase has been shown to have a carboxylate at the active site with a pK value of about 5.9 (Brandt et al., 1963). Binding of either substrate to the enzyme further limits solvent accessibility, with the pK elevated to 6.4 in the presence of malate and to 7.0 in the presence of fumarate.

Recent evidence from X-ray crystallographic work (Anderson et al., 1978) has revealed the presence of a group on the enzyme, possibly an aspartyl residue, which is interacting with the 6-hydroxyl group of glucose in the enzyme–glucose complex. This is probably the carboxylate seen in the pH profiles, functioning in the forward direction as a general base to remove the proton from the 6-hydroxyl group to facilitate nucleophilic attack on the γ-phosphate of ATP. In the reverse reaction, this catalytic group functions as a general acid, donating a proton to the ester oxygen of glucose 6-phosphate to facilitate the oxygen–phosphorus bond-breaking step. This was further verified by the temperature independence of the pK values seen in the reverse direction in the V_{max} and V/K profiles for glucose 6-phosphate, suggesting that the same carboxylate group must be deprotonated in the reverse direction.

Further evidence for the identity of this catalytic group comes from the effect of organic solvents on the V/K profile for MgATP in the presence of citrate. When the profile was run in either neutral or cationic acid buffers, in the absence of organic solvents, the data were fit with a pK value of 6.15 ± 0.03. When organic solvents are added to increase the hydrophobicity, the pK of a neutral acid is elevated because the charge separation involved in the ionization of the neutral acid is suppressed. The same effect is seen on the pK values of neutral acid buffers. When the buffer and catalytic group are of the same acid type, in this case both neutral acids, this "frame-shift" in pH causes no net change in the observed pK, if the two pK values are affected to the same extent by organic solvents. Addition of 25% DMF caused a slight decrease in the pK value observed with neutral acid buffers. This is probably due to a slight difference in the degree of solvation between the deprotonated form of the buffer and the catalytic group on the enzyme, as compared to the protonated forms. However, when the buffer used is a cationic acid where charge separation is not involved in the ionization, only the pK of the catalytic group is elevated. Addition of 25% DMF to the profile run in cationic acid buffers caused an increase in the pK of about 0.5 pH unit, confirming the identity of the catalytic group as being of the neutral acid type.

These results indicate that the chemical mechanism of yeast hexokinase is similar to that of bovine liver fructokinase (Raushel and Cleland, 1977), with a carboxylate group of the enzyme functioning to accept a proton from the sugar hydroxyl in the forward direction and donating a proton in the reverse reaction. Detailed kinetic analysis has not been performed on a sufficient number of kinases to determine whether this mechanism is general for all kinases.

The details of the transphosphorylation reaction are presented in Figure 8. The stereochemistry of the magnesium(II) complex of ATP was obtained from studies on the substitution of inert tetramminecobalt(III) complex of ATP, the Λ isomer of which is a substrate for hexokinase (Cornelius and Cleland, 1978). The diagram notes that both the unprotonated and protonated forms of MgATP are apparently utilized as substrates. The sugar conformation, hydrogen-bonding interactions, and the tentative identification of the catalytic carboxylate group as aspartate were obtained from X-ray studies of the enzyme–glucose complex (Anderson et al., 1978); however, the relative orientation of the two substrates has not been established. Subsequent to the transphosphorylation, Mg(II) presumably migrates from the position shown to the α- and β-phosphates of ADP before products are released; the inability to undergo this shift probably causes the slow rates of the overall reaction observed with CrATP (Danenberg and Cleland, 1975).

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Kinetic Studies on Electron Transfer and Interaction between Nitrogenase Components from *Azotobacter vinelandii*†

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ABSTRACT: Kinetic properties of electron transfer by nitrogenase of *Azotobacter vinelandii* are dependent on the concentration of the two components of nitrogenase. An excess of the MoFe protein inhibits electron transfer in a distinctive manner, and the inhibition is reversed by increasing levels of reductant. The saturation curve for Fe protein is hyperbolic, indicating that only one Fe protein molecule per MoFe protein is required for full activity in ATP hydrolysis and electron transfer. These results can be interpreted on the basis of a complex between the Fe protein and the MoFe protein that dissociates rapidly during turnover. Both 2:1 and 1:1 complexes (Fe-MoFe) are active. Dithionite appears to be a relatively poor reductant for nitrogenase from *Azotobacter vinelandii,* whereas azotobacter flavodoxin is much better. In the presence of the flavodoxin it is possible to increase the specific activity of the Fe protein more than 50% relative to its activity with dithionite alone as a reductant; specific activities greater than 3000 nmol of C_2H_4 formed min^{-1} (mg of Fe protein)$^{-1}$ have been observed.

Nitrogenase consists of two proteins, the larger containing molybdenum and iron (the MoFe protein) and the smaller containing iron (the Fe protein). These are readily separated and purified independently (Winter and Burris, 1976). The easy separation and recombination of the two proteins without irreversible loss of activity allow one to vary the ratio of the nitrogenase components in reaction mixtures. The nitrogenase of *Azotobacter vinelandii* also can be isolated as a particulate complex that is relatively resistant to oxygen denaturation and contains a set ratio (for a given preparation) of the two nitrogenase components (Winter and Burris, 1976). A thorough understanding of the nature of the interactions between the two proteins would eliminate many of the uncertainties inherent in studying a two-protein component system.

The interaction between the two proteins has been studied extensively (Shah et al., 1975; Emerich and Burris, 1976; Davis et al., 1975; Thorneley et al., 1975; Thorneley, 1975; Bui and Mortenson, 1969; Ljones and Burris, 1972a; Bergersen and Turner, 1973; Silverstein and Bulen, 1970) by steady-state kinetics, rapid-reaction kinetics, sedimentation velocity, and gel-filtration experiments. The dilution effect (decreasing the specific activity when the concentration of the nitrogenase is decreased at a constant ratio of the two proteins) serves as a useful probe (Thorneley et al., 1975; Silverstein and Bulen, 1970). Although this approach can provide an estimate of the association constant between the two proteins (Thorneley et al., 1975), it is a relatively insensitive measure of the stoichiometry of the complex. Other experiments have involved the titration of one component with the other (Emerich and Burris, 1976; Bergersen and Turner, 1973). These results, however, must be interpreted with caution, because when the MoFe protein is present in sufficient excess it causes inhibition. The inhibition by excess MoFe protein has been used as evidence of the nature of the active complex (Ljones and Burris, 1972a). Sedimentation velocity studies have indicated that a 1:1 complex (Fe-MoFe) is formed (Thorneley et al., 1975), whereas both kinetic and gel-filtration experiments with tight-binding but inactive heterologous crosses (the MoFe protein from one organism plus the Fe protein from another) of the nitrogenase proteins have indicated that a 2:1 complex

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