Substrate Synergism and the Kinetic Mechanism of Yeast Hexokinase†

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ABSTRACT: Michaelis constants for MgATP with yeast hexokinase vary from 28 μM with D-mannose to above 4 mM for the slow ATPase reaction, with the different values reflecting the degree of synergism in binding of MgATP and the sugar substrate. The best substrates show the greatest synergism, but the correlation is not exact. Similar synergistic binding between MgADP or its methylene analogue and phosphorylated sugars is seen. Product inhibition of MgADP vs. MgATP and vice versa appears noncompetitive at low levels of variable substrate but becomes competitive at high levels. These patterns show that MgATP can combine with E–glucose-6-P (K_i = 4 mM) and MgADP with E–glucose (K_i = 1.6 mM). Isotope partitioning studies with glucose or glucose-6-P have determined the rates of release of these substrates from binary and ternary complexes and, together with reverse isotope exchange studies and the product inhibition studies mentioned above, have shown that the kinetic mechanism is a somewhat random one in which dissociation of sugars from productive ternary complexes is very slow, but release from nonproductive ternary complexes occurs at rates similar to those from binary enzyme–sugar complexes. D-Arabinose-5-P has a K_m of 4.6 mM and a V_{max} 5% that for glucose-6-P, confirming that the high K_m for D-arabinose in the forward direction is caused by the low proportion in the furanose form. The dissociation constant of MgADP in the absence of sugars was determined from the K_i of 5.8 mM for MgADP as a competitive inhibitor vs. MgATP of the slow ATPase reaction.

Yeast hexokinase catalyzes the phosphorylation by MgATP of the 6-hydroxyl of glucose and a number of similar molecules with furanose- or pyranose-like rings and the same stereochemistry at carbons 3–5:

$$\text{MgATP} + \text{D-glucose} \rightleftharpoons \text{D-glucose-6-P} + \text{MgADP} \quad (1)$$

The isotope partitioning study of Rose et al. (1974), in which a complex of labeled glucose and enzyme was mixed with a solution of unlabeled glucose and MgATP, showed that in the forward direction glucose is sticky (that is, dissociates from the enzyme at a rate equal to or less than V_1/E_t) but MgATP is not. ATP analogues which are competitive vs. MgATP, such as ATP 6-glucose or adenosine tetraphosphate 6-glucose (Danenberg & Danenberg, 1977) or one in which glucose replaces ribose (Hohnadel & Cooper, 1973), appear uncompetitive vs. glucose. Such patterns are usually taken to indicate ordered binding, but the fact that CrATP[1] is noncompetitive vs. glucose if initial velocities are measured shows that sugar and nucleotide binding can be random and suggests that the uncompetitive patterns shown by ATP analogues vs. glucose result from a high degree of synergism in binding. Such synergism is seen for the slow ATPase reaction catalyzed by hexokinase, with lyxose lowering the K_m of MgATP from 4 to 0.1 mM (DelaFuente et al., 1970). The fact that the substrate inhibition by MgATP induced by lyxose is competitive vs. glucose, but partial, also supports the concept of random but highly synergistic binding of sugars and nucleotides (Danenberg & Cleland, 1975).

The chemical mechanism of hexokinase has recently been shown by Viola & Cleland (1978) to involve proton transfer during phosphorylation from the 6-hydroxyl group of the substrate to a carboxyl group on the enzyme which has been identified by X-ray studies as an aspartate (Anderson et al., 1978). The failure of Taylor & Cleland (1980) to find an isotope effect with [6-^{18}O]fructose, even when Ca^{2+} or GTP was used in place of Mg^{2+} or ATP to slow down the reaction, shows that phosphoryl transfer is not normally the rate-limiting step with good substrates, and the finding by Wilkinson & Rose (1979) that the equilibrium constant for the phosphorylation on the enzyme is near unity and that the phosphorylation appears to be at equilibrium during the reaction suggests that release of the first product limits this portion of the reaction mechanism.

Despite all of this knowledge of the enzyme and its mechanism, some points about the kinetic mechanism are still uncertain. The degree of synergism in the binding of nucleotides and various substrates has not been clearly defined, nor have the rate-limiting steps in both directions been firmly established. Some of the reported product inhibition patterns seem not to be consistent with the postulated random mechanism. The present work involves (1) determination of the synergism in binding of nucleotides and substrates in various complexes, (2) a demonstration that the product inhibition patterns *are* consistent with the random mechanism, (3) some evidence on the rate-limiting steps in both directions, and (4) an attempt to formulate a kinetic mechanism consistent with all available data.

Materials and Methods

Yeast hexokinase (C-302), glucose-6-P dehydrogenase, α-glycerophosphate dehydrogenase, glycerokinase, pyruvate kinase, lactate dehydrogenase, and α,β-methylene-ADP were from Sigma. Glucose dehydrogenase and mutarotase were from Boehringer. 1-Deoxy-D-fructose was a gift from Dr. William L. Dills, Jr. 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 & Philips, 1973). 1,5-Anhydro-D-mannitol was prepared by treating D-mannitol with HCl (Fletcher, 1963). 2,5-Anhydro-D-glucitol was prepared by the method of Koerner et al. (1977). 1,5-Anhydro-D-glucitol was prepared by the method of Ness et al. (1950), and small amounts of contam-

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[1] Abbreviations: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; CrATP, tetraaquochromium(III) adenosine 5′-triphosphate.

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inating glucose were removed by treatment with glucose oxidase and recrystallization from ethanol. Radiochemicals were from New England Nuclear, and [^{14}C]glucose-6-P was purified by chromatography on Dowex-1-Cl.

Kinetic Assays. Kinetic studies were run in 3.0 mL total volume in 1-cm path-length cuvettes by measuring absorbance changes at 340 nm with a Gilford optical density converter and a 10-mV recorder. The temperature was maintained at ±0.1 °C of the stated values with thermospacers and a circulating water bath. The forward and reverse reaction rates of hexokinase were measured by coupled assays as described by Viola & Cleland (1978).

Reaction mixtures for following the ATPase activity of hexokinase in the presence of ADP contained 1.0–2.5 mM Mg[γ-^{32}P]ATP (3×10^5 cpm/μmol), 0–6 mM MgADP, 5 mM excess $MgCl_2$ (over the total nucleotide concentration), 50 mM Pipes,[1] pH 7.0, and 400 units of yeast hexokinase in 3.0 mL. Aliquots of 0.5 mL were removed at various times and added to 0.5 mL of a solution containing 1.25% NaCl and 1.25% ammonium molybdate in 0.25 N H_2SO_4. One milliliter of 2-methyl-1-propanol was added and the solution vortexed. A 0.5-mL aliquot of the 2-methyl-1-propanol layer was removed and counted to determine the inorganic phosphate formed.

Determination of Hexokinase Concentration. The concentration of yeast hexokinase active sites was determined by a chromium(III) ATP binding study (Danenberg & Cleland, 1975). About 1 mg of hexokinase was incubated with 25 mM Pipes (pH 7), 300 μM [^{14}C]glucose, and 1 mM CrATP in 0.25 mL total volume. The incubation mixture was applied to a Sephadex G-25 column (1 × 26 cm) and eluted with 25 mM Pipes (pH 7). The fractions were counted, and the counts in the protein peak and glucose peak were compared to determine the amount of bound substrate.

Isotope Exchange Studies. In the forward direction, hexokinase was added to a mixture containing 50 mM buffer, 3 mM ATP, 5 mM [^{3}H]ADP (8.8×10^5 cpm/μmol), 13 mM $Mg(OAc)_2$, 10 mM glucose, 1 mM citrate, 1 mM NADP, and 10 units of glucose-6-phosphate dehydrogenase, and the reaction was allowed to proceed until 0.16–0.32 mM glucose 6-phosphate had been formed. For the isotope exchange studies run in the absence of citrate, hexokinase was preincubated in a solution containing 50 mM buffer, 3 mM ATP, 10 mM glucose, 1 mM NADP, and 10 units of glucose-6-phosphate dehydrogenase, and the reaction was initiated by addition of 10 mM $Mg(OAc)_2$ and 3 mM [^{3}H]ADP (8.8×10^5 cpm/μmol) and allowed to proceed as above.

In the reverse direction hexokinase was added to a solution containing 50 mM buffer, 50 mM glucose 6-phosphate, 3 mM ADP, 0.5 mM [^{3}H]ATP (3.0×10^6 cpm/μmol), 8.5 mM $Mg(OAc)_2$, 1 mM NADP, 10 units of glucose dehydrogenase, and 50 units of mutarotase, and the reaction was allowed to proceed until 80 μM glucose had been formed (except 16 μM at pH 8.43). The reactions were terminated by addition of 60% perchloric acid and several drops of CCl_4. The solution was vortexed, filtered to remove denatured protein, and run through a column of Dowex-1-Cl at pH 8. After removal of weakly binding materials (adenine, adenosine, and AMP) with 3 mM HCl, ADP was eluted with 10 mM HCl containing 20 mM NaCl and ATP with 10 mM HCl containing 200 mM NaCl (Cohn & Carter, 1950).

Isotope Partitioning Studies. Isotope partitioning studies were run according to the procedure of Rose et al. (1974). For the forward reaction, 1–2 mg of yeast hexokinase was incubated with 50 mM buffer and various amounts of [^{14}C]glucose in 0.2 mL total volume at pH 8. This solution was delivered by Eppendorf pipet into a rapidly stirring solution containing 50 mM buffer, 20-fold excess of unlabeled glucose, various levels of ATP, and $Mg(OAc)_2$ at 5 mM excess over the ATP concentration. After several seconds the reaction was terminated by addition of 60% $HClO_4$. Several drops of CCl_4 were added, and the reaction mixture was vigorously vortexed to denature the enzyme. The solution was heated for several minutes to completely inactivate the hexokinase, cooled, and neutralized to pH 8 with KOH. The precipitated protein and $KClO_4$ were removed by filtration, and glucose and glucose 6-phosphate were separated by chromatography on a column of Dowex-1-Cl. The background level of glucose 6-phosphate formed from the diluted [^{14}C]glucose pool after the first turnover of the enzyme was corrected for by running a blank with the enzyme incubated with unlabeled glucose and the [^{14}C]glucose present in the rapidly stirring chase solution. Alternately, internal corrections were made for each experiment by the method of Rose et al. (1974).

For the reverse reaction, a solution containing 2.6 mg of yeast hexokinase, 2.0 mM [^{14}C]glucose 6-phosphate (2.5×10^6 cpm/μmol), and 50 mM Pipes, pH 7, in 0.2 mL was added to 10 mL of a rapidly stirring solution containing 50 mM Pipes, pH 7, 50 mM glucose 6-phosphate, and various levels of MgADP. Samples were isolated and counted by the same procedure as was used for the forward reaction partitioning studies.

Data Processing. All fits to the data were performed by using Fortran programs which assume equal variance for the velocities or the logarithm of the fitted parameter (Cleland, 1979). Initial velocity patterns were fitted to eq 2, where v is the experimentally determined velocity, V is the maximum velocity, A and B are the substrate concentrations, K_a and K_b are the respective Michaelis constants, and K_{ia} is the dissociation constant of A. The isotope partitioning data in the forward direction were fitted to eq 3, where P^*_{max} is the maximum labeled product formed at infinite substrate concentration, A and B are the concentrations of glucose and MgATP, K_{ia} is the binary dissociation constant of glucose, and K' is the trapping coefficient for MgATP from the pattern. The data in the reverse direction were fitted to eq 4, with [MgADP] as B. Competitive, noncompetitive, and uncompetitive product inhibition data were fitted to eq 5, 6, and 7, respectively, where K is the apparent Michaelis constant, K_{is} is the slope inhibition constant, and K_{ii} is the intercept inhibition constant.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

$$\log P^* = \log \left[\frac{P^*_{max}}{(1 + K_{ia}/A)(1 + K'/B)} \right] \quad (3)$$

$$P^* = P^*_{max}/(1 + K'/B) \quad (4)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (5)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (6)$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \quad (7)$$

Results

Michaelis Constants for MgATP and MgADP as a Function of Substrate. The substrates for yeast hexokinase show a strong synergism in binding to the enzyme (Danenberg &

Table I: Effect of Various Sugars and Sugar Analogues on K_{MgATP}[a]

substrate	K_{sugar} (mM)	K_{MgATP} (mM)	rel V[b]	rel V/K_{MgATP}[b]
D-mannose	0.09	0.028 ± 0.006	42	96
D-fructose	1.8	0.036 ± 0.003	136	236
D-glucose	0.1	0.063 ± 0.004	(100)	(100)
2-deoxy-D-glucose	0.3	0.087 ± 0.002	96	69
D-glucosamine	1.5	0.101 ± 0.004	70	44
2,5-anhydro-D-mannose	0.56	0.24 ± 0.02	121	32
2,5-anhydro-D-mannitol	4.7	0.3 ± 0.02	168	36
1,5-anhydro-D-glucitol	3.0	0.416 ± 0.005	3	0.49
1,5-anhydro-D-mannitol	8.2	1.52 ± 0.06	10	0.43
1-deoxy-D-fructose	155	2.16 ± 0.05	1.8	0.053
2,5-anhydro-D-glucitol	500	2.22 ± 0.06	22	0.62
D-arabinose	9.6[c]	2.3 ± 0.4	21	0.57
water		4.4 ± 0.3	0.026	3.6×10^{-4}

[a] MgATP was varied at a constant level of sugar 10 times its K_m, except where the values are based on a full initial velocity pattern. Mg^{2+} was 1 mM in excess over ATP, and Hepes, pH 8, was the buffer. ADP production was followed by coupling with pyruvate kinase and lactate dehydrogenase. [b] Values expressed as a percentage of that with glucose as the substrate. [c] The actual K_m value determined for arabinose was 475 mM; the value quoted here is for the β-furanose anomer which is 2% of the anomeric mixture in solution (Angyal & Pickles, 1972); the predominant pyranose will not act as substrate.

Table II: Kinetic Constants for Yeast Hexokinase Substrates[a]

substrate	rel V[b]	K_{sugar} (mM)	$K_{i\,sugar}$ (mM)	K_{MgATP} (mM)	$K_{i\,MgATP}$ (mM)
D-mannose	42	0.091 ± 0.005	0.048 ± 0.015	0.025 ± 0.002	0.013 ± 0.004
D-fructose	136	1.77 ± 0.13	3.2 ± 0.7	0.037 ± 0.006	0.061 ± 0.007
2,5-anhydro-D-mannitol	128	4.7 ± 0.4	61 ± 8	0.14 ± 0.02	1.8 ± 0.2
2,5-anhydro-D-mannose	121	0.56 ± 0.05	7.2 ± 0.9	0.24 ± 0.02	3.1 ± 0.4
1,5-anhydro-D-mannitol	8	8.2 ± 0.8	13.7 ± 1.6	0.92 ± 0.09	1.5 ± 0.2
1-deoxy-D-fructose	1.8	155 ± 9[c]	155 ± 9	2.16 ± 0.05[c]	2.16 ± 0.05
2,5-anhydro-D-glucitol	22	500 ± 40[c]	500 ± 40	2.22 ± 0.06[c]	2.22 ± 0.06
substrate	rel V[b]	$K_{sugar\text{-}P}$ (mM)	$K_{i\,sugar\text{-}P}$ (mM)	K_{MgADP} (mM)	$K_{i\,MgADP}$ (mM)
D-mannose-6-P	95	0.66 ± 0.10	(15 ± 27)	(0.01 ± 0.02)	0.28 ± 0.08
D-fructose-6-P	68	1.3 ± 0.2	15 ± 5	0.12 ± 0.04	1.4 ± 0.3
D-glucose-6-P	100	3.0 ± 0.4	21 ± 6	0.23 ± 0.05	1.6 ± 0.3

[a] Reactions in the forward direction were in Hepes, pH 8, at 25 °C with Mg^{2+} at 1 mM excess over MgATP. The reaction was followed by coupling the production of ADP with pyruvate kinase and lactate dehydrogenase. Reactions in the reverse direction were run at pH 7.5, with Mg^{2+} in 5 mM excess over MgADP. ATP production was coupled with glycerol kinase and glycerophosphate dehydrogenase (Viola & Cleland, 1978). Data fitted to eq 2. [b] Relative to the value for glucose in the forward direction or glucose-6-P in the reverse reaction. [c] The fit was to eq 2 with K_i assumed equal to K_m. The errors were much larger when K_m and K_i were not assumed equal, and the two values were not significantly different in each case.

Cleland, 1975) with the K_m for MgATP reported as 5 mM for the ATPase reaction of hexokinase (Kaji & Colowick, 1965), while a value of <100 μM was obtained in the hexokinase reaction with glucose as the substrate. Sugar substrates with a 10000-fold range in K_m values were examined to determine the effect on the K_m of MgATP (Table I). Initial velocity patterns were run with some of the stronger and weaker binding sugar and sugar analogue substrates, and the K_m and K_i values are given in Table II.

The substrate activity of D-arabinose-5-P was also checked. Phosphorylation of the 5-hydroxyl allows the sugar phosphate to cyclize only into a furanose form, and while the V_{max} in the reverse reaction was only 5% that of glucose-6-P, the K_m of D-arabinose-5-P was 4.6 ± 0.6 mM. In the forward reaction the high K_m (475 mM) of D-arabinose results from the small percentage (2%) in the furanose form.

Product and Dead-End Inhibition Patterns. In a random mechanism MgADP should be a competitive inhibitor against MgATP and glucose 6-phosphate competitive against glucose.[2] A noncompetitive pattern has been reported, however, for MgADP as a product inhibitor against MgATP (Kosow & Rose, 1970), and we also observed what appeared to be a noncompetitive inhibition pattern when low levels of the variable substrate were used. However, when the product

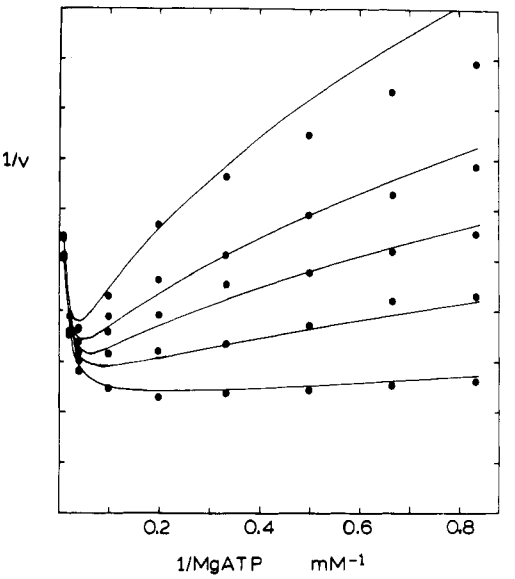

FIGURE 1: Product inhibition of MgADP vs. MgATP at 10 mM glucose, pH 7. The lines are calculated from an equation analogous to eq 14 but with a substrate inhibition term in the denominator, using kinetic constants chosen as described in the text. MgADP levels from bottom to top: 0, 2, 4, 6, and 10 mM.

[2] It has been mistakenly believed [see Table II of Cleland (1963)] that such patterns would appear noncompetitive for a steady-state random mechanism. However, if the mechanism is truly random in the sense that dead-end complexes between unlike substrates can form (E–glucose–MgADP or E–glucose-6-P–MgATP in the present case), these patterns do converge on the vertical axis, although they will normally look noncompetitive at low levels of the variable substrate.

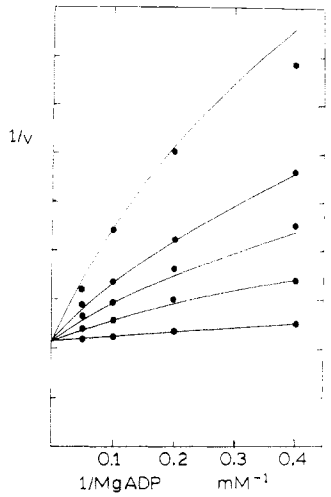

FIGURE 2: Product inhibition of MgATP vs. MgADP at 50 mM glucose-6-P, pH 7. The lines are calculated from eq 14 by using kinetic constants chosen as described in the text. MgATP levels from bottom to top: 0, 0.3, 0.6, 1.0, and 2.0 mM.

inhibition patterns were run at high levels of variable substrate, as can be seen in Figure 1 for MgADP as a product inhibitor against MgATP at 10 mM glucose, pH 7, the reciprocal plots became nonlinear, with the pattern converging and the inhibition by MgADP almost completely overcome by MgATP at 20 mM substrate. Superimposed on this competitive pattern is apparently total substrate inhibition by MgATP at very high levels. Inhibition by glucose-6-P against glucose at 5 mM MgATP, pH 7.5, appeared to be noncompetitive even at the highest levels of glucose used (100 mM). The kinetic parameters from a fit to eq 6 were K_{is} = 80 ± 30 mM and K_{ii} = 200 ± 15 mM.

In the reverse direction product inhibition by MgATP against MgADP at 50 mM glucose-6-P, pH 7.0, showed a nonlinear pattern similar to that seen for MgADP as a product inhibitor in the forward direction (Figure 2). However, no substrate inhibition by MgADP was observed at levels up to 100 mM. Glucose was a competitive inhibitor against glucose-6-P at 0.5 mM MgADP, pH 7.5, with K_{is} = 84 ± 3 μM, and was noncompetitive vs. MgADP at 5 mM glucose-6-P, pH 7.5, with K_{is} = 0.20 ± 0.02 mM and K_{ii} = 0.13 ± 0.01 mM.

α,β-Methylene-ADP (an analogue with the bridge oxygen between the α- and β-phosphate groups replaced by a methylene group) was examined as an inhibitor of the reverse reaction of hexokinase at pH 7 and found to be competitive with MgADP with an apparent K_{is} of 4.1 ± 1.1 mM at 20 mM glucose-6-P. Uncompetitive inhibition was seen when glucose-6-P was the varied substrate (K_{ii} = 5.4 ± 0.2 mM at 0.10 mM MgADP).

The dissociation constant of MgADP in the absence of sugars was determined from the competitive inhibition of MgADP against MgATP in the ATPase reaction catalyzed by hexokinase at pH 7. The data were fitted to eq 5, giving a K_{is} for MgADP of 5.8 ± 0.2 mM and a K_m for MgATP of 7.3 ± 0.7 mM.

Isotope Partitioning Studies. Hexokinase was incubated at pH 8 with varying levels of [^{14}C]glucose and added to a rapidly stirring "chase" solution containing fixed levels of MgATP. This set of experiments was repeated at several MgATP levels, and the concentration of [^{14}C]glucose 6-phosphate formed at various substrate levels was fitted to eq 3. The maximum labeled product formed at infinite substrate concentration (P^*_{max}) was 1.20 ± 0.15 nmol when 1.39 nmol of hexokinase was present per assay. K', the trapping coefficient for MgATP, was 88 ± 12 μM. K_{ia}, the dissociation constant of glucose from the binary enzyme–glucose complex, was 99 ± 16 μM.

Table III: Reverse Reaction–Back-Exchange of MgATP[a]

pH	[citrate] (mM)	inhibition[b] (%)	exchange rate / reaction rate
5.59	0	6.7[c]	0.016
6.11	0	19	0.052
6.92	0	26	0.109
7.31	0	44	0.108
7.41	0	35	0.143
7.81	0	57	0.231
8.19	0	69	0.471
8.22	0	69	0.322
8.40	0	77	0.888
6.41	1.0	26	0.412
6.96	1.0	44	1.01
7.76	1.0	57	1.41
8.43	1.0	74	3.87

[a] Back-exchange studies were run with 50 mM glucose 6-phosphate, 3 mM MgADP, 0.5 mM [^{3}H]MgATP except as noted, 5 mM excess Mg^{2+}, 1 mM NADP, and 50 mM buffer. Reaction rates were measured by coupling the production of glucose with glucose dehydrogenase (Viola & Cleland, 1978). [b] Percent inhibition of the reaction in the presence of 0.5 mM MgATP, compared to the rate in the absence of product. [c] MgATP was 1 mM.

For the reverse reaction at pH 7, the level of MgADP was varied at 2 mM labeled glucose-6-P. From a fit of the data to eq 4, glucose formation at infinite MgADP was 6.2 ± 0.4 nmol, compared to 14.5 nmol of enzyme active sites present, and K', the trapping coefficient for MgADP, was 38 ± 6 mM.

Reverse Isotope Exchange Studies. The exchange of labeled nucleotide product with substrate against the reaction flux was examined in the forward and reverse reactions catalyzed by the enzyme and in the presence and absence of citrate, which activates the forward reaction by removing inhibitory metal ions (mainly Al^{3+}) present in commercial ATP (Womack & Colowick, 1979; Viola et al., 1980). In the forward reaction in the presence of citrate, back-exchange of ADP was not detected at pH 5.4 because of the decreased affinity for MgADP, which gave only 4% inhibition at the 5 mM level used. At pH 6.49 and 7.83, the ratio of isotope exchange from ADP to ATP to the forward reaction rate was 0.014 and 0.029, and 5 mM MgADP gave 49% and 67% inhibition, respectively. For reactions run at pH 6.5 in the absence of citrate, activation by MgADP was seen when the enzyme was preincubated with metal-free nucleotides and the reaction started with Mg^{2+} (90% activation when MgATP was 3 mM and 155% when it was 1 mM), but the ratios of exchange rate to reaction rate (0.020) were essentially the same as was observed in the presence of citrate.

In the reverse reaction, as the pH was increased the ratio of exchange rate to reaction rate also increased until the rate of back-exchange became comparable to the rate of reaction (Table III). When the reverse reaction was run in the presence of citrate, a further enhancement of the exchange rate was observed, and at pH 8.4 the rate of nucleotide exchange was almost 4 times the reverse reaction rate.

Discussion

Synergism in Binding of Nucleotides and Sugars. The dissociation constant of MgATP from hexokinase in the absence of sugars is given by its K_m in the slow ATPase reaction catalyzed by the enzyme. The values of 4–7 mM seen in the present work agree with those reported previously (Kaji & Colowick, 1965; Noat et al., 1969; DelaFuente et al., 1970), and the inhibition constant of MgADP as a competitive in-

hibitor of the ATPase activity has the same value. When various sugar or sugar analogue substrates are present, however, the Michaelis constant of MgATP varies all the way from 28 μM with D-mannose to 2.3 mM with D-arabinose, with almost every possible value in between being represented in Table I.[3] The natural substrates mannose, fructose, and glucose show the highest degree of synergism, while slow substrates lacking a hydroxyl group at C-1 show the least synergism.[4] The effect does not correlate with maximum velocity, since 2,5-anhydro-D-mannitol, the fastest substrate known for the enzyme, only lowers K_{MgATP} to 0.3 mM and fructose, which is a faster substrate than D-mannose, shows somewhat less synergism in binding. A similar variation among substrates in their ability to induce nucleotide binding has been reported for endogenous AlATP (present as a contaminant in commercial ATP) by Viola et al. (1980) and for CrATP by Danenberg & Cleland (1975). The ability of a substrate to induce synergistic binding of MgATP and the trivalent metal–ATP complexes is not always the same, however. Thus 2,5-anhydro-D-glucitol induces very tight binding of CrATP but hardly affects the binding of MgATP, while 2,5-anhydro-D-mannitol shows the opposite pattern. The factors responsible for inducing these conformation changes that lead to tighter binding will probably be clarified only when crystals of a ternary enzyme–nucleotide–sugar complex suitable for X-ray analysis become available.

Synergistic binding of MgADP and phosphorylated substrate is also seen, with the K_m of MgADP reduced by the natural sugar-P substrates to levels similar to that seen for MgATP (Table II). Clearly the binding of glucose-6-P and α,β-methylene-ADP is also highly synergistic, since this inhibitor is uncompetitive vs. glucose-6-P, and thus appears to bind only to the binary E–glucose-6-P complex. (The dissociation constant from free enzyme must be more than an order of magnitude higher than that from E–glucose-6-P or the pattern would appear noncompetitive.)[5] This synergism appears restricted to complexes which fill, or nearly fill, the active site, however. Thus AMP is noncompetitive vs. glucose (Fromm & Zewe, 1962) while ATP analogues such as the one in which glucose replaces ribose (Hohnadel & Cooper, 1973) and ATP 6-glucose are uncompetitive (Danenberg & Danenberg, 1977). Lyxose also induces substrate inhibition by MgATP because of the synergistic binding between the two molecules (Danenberg & Cleland, 1975). As we will discuss below, glucose and MgADP show only a 3- or 4-fold synergism in their binding, while glucose-6-P and MgATP do not show synergistic binding.

Isotope Partitioning Studies. In their original studies at pH 7 on the binding of glucose to hexokinase by the use of this technique, in which the partitioning of a binary E–[^{14}C]glucose complex between product formation and dissociation is measured after dilution with unlabeled glucose and variable MgATP, Rose et al. (1974) found that glucose did not dissociate from E–glucose–MgATP at a detectable rate and was released from E–glucose at 0.3 of V_{max} in the forward direction. This method can also be used to determine the dissociation constant of glucose if the experiments are done at several nonsaturating levels of glucose (the observed amount of radioactive glucose-6-P obeys eq 3 in this case). For the isozyme we have been using, the dissociation constant of glucose at pH 8 was 99 μM, in excellent agreement with the value seen in initial velocity studies as the apparent K_m at low MgATP levels. The maximum amount converted to glucose-6-P at saturating MgATP was not significantly different from the enzyme level, and thus we also find that glucose does not dissociate at an appreciable rate from E–glucose–MgATP. However, the trapping coefficient (K') was 88 μM, while K_{MgATP} for the chemical reaction was 63 μM. The equation which describes the rate of dissociation of glucose from E–glucose (k_2) relative to V_{max} is

$$k_2/(V/E_t) = K'/K_{MgATP} = 1.4 \quad (8)$$

This is a higher value than that found by Rose et al. (1974) at pH 7. Since the maximum velocity in the forward direction at pH 7 is 5.3-fold greater than that in the reverse direction and the ratio at pH 8 is slightly higher (Viola & Cleland, 1978), k_2 is at least 8-fold greater than the reverse V_{max}, and thus glucose release is only partly rate limiting for the reverse reaction at pH 8 with this isozyme.[6]

The isotope partitioning experiment with labeled glucose-6-P demonstrates that there also is little E–glucose-6-P present in the steady state during the forward reaction. In this experiment the ratio of the rate constant for release of glucose-6-P from the ternary complex (k_9) to the net rate constant (k') for reaction of E–glucose-6-P–MgADP and release of the first product (which is MgATP) is

$$k_9/k' = E_t/[P^*_{max}(1 + K_{iq}/Q)] - 1 \quad (9)$$

where Q is glucose-6-P (2.0 mM) and K_{iq} its dissociation constant. Since P^*_{max} (6.2 mmol) was 43% of E_t (14.5 nmol), a value of K_{iq} greater than 2.7 mM makes the right side of eq 9 negative. The observed value of K_{iq} from an initial velocity pattern at pH 7, however, was 8.4 ± 1.8 mM.[7] The

[3] While a K_m is not normally a dissociation constant, it will be for MgATP if (1) MgATP dissociates more rapidly from the enzyme than it reacts to give products and (2) the chemical reaction and release of the first product are slower than release of the second product. Wilkinson & Rose (1979) concluded that the second criterion is met for glucose, and it probably holds for other substrates as well, especially for those with low maximum velocities. The first criterion may not be valid, and the rate constants chosen by Wilkinson & Rose (1979) and by us (see Discussion) suggest that the K_m values will be somewhat larger than the true dissociation constants. The K_ms thus underestimate the true synergism in substrate binding.

[4] For some reason a hydroxyl group at C-1 is necessary for a high maximum velocity with yeast hexokinase, regardless whether a five- or six-membered ring is present, while a hydroxyl at C-2 is not necessary and can be on either side of the ring. Thus 1-deoxy-D-fructose, D-arabinofuranose, 1,5-anhydro-D-glucitol, and 1,5-anhydro-D-mannitol are all slow substrates, while 2-deoxy-D-glucose, 2,5-anhydro-D-mannitol, 2,5-anhydro-D-glucitol, and 2,5-anhydro-D-mannose (which is 60% hydrated in water; Viola & Cleland, 1980) show high maximum velocities. The requirement for a hydroxyl at C-1 is specific for the yeast enzyme; 1,5-anhydro-D-glucitol and 1,5-anhydro-D-mannitol are good substrates for the brain enzyme (Sols & Crane, 1954).

[5] The fact that α,β-methylene-ADP is competitive vs. MgADP and uncompetitive vs. glucose-6-P shows that the K_bA term in the denominator of eq 2 is multiplied by $(1 + I/K_i)$, where A, B, and I are [glucose-6-P], [MgADP], and [inhibitor]. The value of K_i can then be obtained either by dividing the apparent K_{is} from the competitive inhibition by $(1 + K_{ia}/A)$ or by dividing the apparent K_{ii} from the uncompetitive inhibition by $(1 + B/K_b)$. At pH 7, K_{ia} = 8.4 mM and K_b = 0.23 mM, so the value obtained from K_{ii} is 3.8 ± 0.2 mM, while that from K_{is} is 2.9 ± 0.8. Thus, α,β-methylene-ADP binds to E–glucose-6-P with a dissociation constant of 3–4 mM.

[6] Rose et al. (1974) treated hexokinase with trypsin to enhance its binding of glucose, and this treatment presumably caused the slower release of glucose observed in their experiments.

[7] It is probable that K_{iq} is really closer to 7 mM than to 3 mM, since this value gives a better fit to the Haldane relationship at pH 7, and thus the value of P^*_{max} is about twice as high as it should be. Possible explanations for the discrepancy are (1) glucose-6-P binds more tightly in the absence of nucleotides than it does in the steady state, (2) more than one molecule of glucose-6-P binds in the vicinity of the active site, and the extra molecules preferentially enter the active site following the first turnover, and (3) the enzyme concentration was in error.

isotope partitioning experiment thus shows that k_9/k' must be close to zero and that glucose-6-P is released very slowly from the ternary complex. If glucose-6-P is released very slowly from the ternary complex, its rate of release from the binary complex with the enzyme (k_7) is

$$k_7/(V_2/E_t) = K'/K_{MgADP} \quad (10)$$

where V_2/E_t is the turnover number in the reverse reaction, K' is the trapping coefficient for MgADP in the isotope partitioning experiment (38 mM), and K_{MgADP} is the Michaelis constant in the chemical reaction (0.23 mM). Thus k_7 is 165 times V_2/E_t, and since V_1 is 5.3 times V_2 at pH 7, k_7 is 31 times V_1/E_t. It is thus clear that MgADP is normally always released first and that only about 3% of the enzyme will be E–glucose-6-P in the steady state.

The mechanism proposed by Wilkinson & Rose (1979) for yeast hexokinase is fundamentally an ordered one in which the equilibrium constant for interconversion of E–MgATP–glucose and E–MgADP–glucose-6-P is unity, and the interconversion is fast compared to other steps:

$$E \underset{k_2}{\overset{k_1[\text{glucose}]}{\rightleftharpoons}} \text{E–glucose} \underset{k_4}{\overset{k_3[\text{MgATP}]}{\rightleftharpoons}} (\text{E–glucose–MgATP} \overset{\text{fast}}{\underset{K_{eq}=1}{\rightleftharpoons}} \text{E–glucose-6-P–MgADP}) \underset{k_6[\text{MgADP}]}{\overset{k_5}{\rightleftharpoons}} \text{E–glucose-6-P} \underset{k_8[\text{glucose-6-P}]}{\overset{k_7}{\rightleftharpoons}} E \quad (11)$$

These authors assigned values of 2×10^6, 10^6, 2×10^5, and 2×10^6 M^{-1} s^{-1} for k_1, k_3, k_6, and k_8 and values of 60, 200, 400, and 6000 s^{-1} for k_2, k_4, k_5, and k_7 at pH 7.5, respectively.

Since we know that $k_2/(V_1/E_t) = 1.4$, $k_7/(V_2/E_t) = 165$, $V_1 = 5.3V_2$, $K_{MgATP} = 63$ μM, $K_{i\ glucose} = 100$ μM, $K_{glucose} = 100$ μM, $K_{MgADP} = 230$ μM, $K_{i\ glucose\text{-}6\text{-}P} = 6.7$ mM, and $K_{eq} = 1300$ at pH 7, we can evaluate the relative values of the rate constants for the isozyme we have used. If we let k_5 and k_7 have the same values as those of Wilkinson & Rose (1979), then we obtain values of 2.7×10^6, 3.7×10^6, 9×10^5, and 9×10^5 M^{-1} s^{-1} for k_1, k_3, k_6, and k_8 and 270, 84, 400, and 6000 s^{-1} for k_2, k_4, k_5, and k_7. These values are similar to those of Wilkinson & Rose (1979) and differ largely in the ratios of k_2 and k_4, and k_6 and k_8.

If, in an ordered mechanism such as mechanism 11, k_7 is 165 times V_2/E_t, the initial velocity pattern in the back-reaction should nearly be an equilibrium ordered one, and $K_{glucose\text{-}6\text{-}P}$ should be much smaller than $K_{i\ glucose\text{-}6\text{-}P}$. In mechanism 11 with the rate constants we have chosen, $K_{i\ glucose\text{-}6\text{-}P} = 6.7$ mM, while $K_{glucose\text{-}6\text{-}P} = 40$ μM. However, experimentally the Michaelis constant for glucose-6-P, while varying with pH, is never less than 3 mM. An obvious explanation for the discrepancy is that MgADP forms a binary complex with free enzyme, as expected in a random mechanism, but to explain the value seen, MgADP would have to have a dissociation constant similar to its K_m (about 0.23 mM) rather than the value seen as a competitive inhibitor in the ATPase reaction in the absence of sugars (5.8 mM). What is probably happening is that at saturating MgADP the E–glucose complex resulting from release of MgATP after reaction does not dissociate to give free enzyme but rather combines with MgADP. Glucose-6-P thus combines largely not with free enzyme under these conditions but with E–MgADP after glucose release, and a decreased biomolecular rate constant for this addition step leads to a higher apparent K_m for glucose-6-P than that predicted by mechanism 11. The product inhibition studies discussed below provide supporting evidence for this picture.

Analysis of Product Inhibition Patterns. Kosow & Rose (1970) have reported that MgADP was a noncompetitive inhibitor of MgATP, and from the hyperbolic intercept replot, they concluded that the inability of MgADP to inhibit the reaction totally at high concentrations was due to the presence of an alternate pathway for the reaction. In a random mechanism the similar substrate–product pairs (glucose–glucose-6-P and MgATP–MgADP) should compete for the same site on the enzyme and give competitive inhibition patterns. The patterns in Figure 1 and 2 show nonlinear reciprocal plots in the presence of the product inhibitor, with the inhibition appearing noncompetitive at low substrate levels but becoming competitive as the variable substrate is raised far enough. In addition, MgATP shows substrate inhibition which apparently becomes total at infinite MgATP. Since MgADP does not show a similar effect in the same concentration range, the substrate inhibition by MgATP must be a specific effect, but the cause is not clear at this time. The only exception to the expected pattern was the inhibition of glucose-6-P vs. glucose, which appeared noncompetitive even at glucose levels as high as 100 mM. Possibly glucose-6-P combines at another site in addition to the glucose one or is competing for Mg^{2+} to account for the observed K_{ii} value of 200 mM. The inhibition of glucose was competitive vs. glucose-6-P, however.

Mechanism 11 predicts a strictly noncompetitive product inhibition by MgADP vs. MgATP, and vice versa. Since the product inhibition by MgATP becomes competitive at high MgADP, we must assume that MgADP can combine with E–glucose as well as with E–glucose-6-P. However, since MgADP does not show substrate inhibition at high levels, glucose release from E–glucose–MgADP occurs at nearly the same rate as from E–glucose (that is, x is close to unity):

$$E \xleftarrow{k_2} \text{E-glucose} \underset{K_{Ip}}{\rightleftharpoons} \text{E-glucose-MgADP} \xrightarrow{xk_2} \text{E-MgADP} \quad (12)$$

This modification of mechanism 11 correctly predicts the observed pattern in Figure 2, but since a similar analysis of the inhibition pattern in Figure 1 requires that MgATP also combine with E–glucose-6-P, we also add the following to mechanism 11 where y is also close to unity:

$$\begin{array}{ccc} \text{E-glucose-6-P} & \xrightarrow{k_7} & \text{E} \\ {\scriptstyle K_{Ib}}\updownarrow & & \\ \text{E-glucose-6-P-MgATP} & \xrightarrow{yk_7} & \text{E-MgATP} \end{array} \quad (13)$$

The rate equation for this full model now predicts that the data in Figure 2 (with glucose-6-P saturating) will follow

$$v = V(1 + K_{Ip}/P)/[1 + K_{Ip}/P + (K_p/P)(1 + B/K_{ii}) + (K_pK_{Ip}/P^2)(1 + B/K_{Ib})[1 + B/(K_{ia}K_b/K_a)]] \quad (14)$$

where K_{il} is the reciprocal of the sum of reciprocals of K_{Ib} and $K_{ib}K_p/K_{Ip}$, P = [MgADP], B = [MgATP], and K_{ia}, K_a, K_{ib}, K_b, and K_p are the normal kinetic constants for mechanism 11 as defined by Cleland (1963). The reciprocal plot with $B = 0$ is a straight line, but at finite MgATP levels, the reciprocal plots will be 2/1 functions (Cleland, 1963), concave down, as observed. The predicted pattern is competitive, with K_{il} as the observed inhibition constant at high P.

The complexity of this rate equation makes it impractical to fit data to it directly by the least-squares method, so we have used a successive fitting process plus analysis of the least-squares surface for the remaining parameters (Cleland, 1979) to determine the values or possible ranges for the values of the constants in the equation. Fitting of the data in the absence of B, plus those data points in the presence of B at the highest

P level, to eq 5 gave K_p and K_{il} as 0.37 ± 0.11 and 0.072 ± 0.018 mM. Adopting these values for K_p and K_{il}, we constructed a least-squares surface for the data in Figure 2 as a function of K_{Ip} and K_{Ib} (assuming that only one of the B terms in the $1/P^2$ portion of eq 14 would be significant). At each chosen value of K_{Ip} and K_{Ib}, we computed V from the average of values given by solution of eq 14 for each data point and also computed the residual least square. This analysis showed that K_{Ip} was reasonably well determined around 1.6 mM but that K_{Ib} was very poorly defined. While the lowest residual least square was obtained by making K_{Ib} infinite, we can only say that neither K_{Ib} nor $K_{ia}K_b/K_a$ in eq 14 is likely to be less than 2 mM. The insensitivity of the data to these constants results from all of the data being obtained at high MgADP levels.

When MgADP is a product inhibitor vs. MgATP, a similar pattern is seen, except for the substrate inhibition by MgATP. The curved reciprocal plots in the presence of MgADP now confirm that MgATP combines with E–glucose-6-P and further show that release of glucose-6-P is not appreciably decreased.[8] The rate equation for this product inhibition pattern predicted by mechanism 11 as modified by eq 12 and 13 is similar in form to eq 14, except with A and B being interchanged with Q and P in concentration terms and subscripts on kinetic constants. To allow for the observed substrate inhibition, a $(B/K_{IIb})(1 + K_{Ib}/B)$ term must also be added to the denominator, where K_{IIb} is the substrate inhibition constant for MgATP. We again used a successive fitting process for data analysis. The data in the absence of MgADP, plus those in its presence where MgATP was at least 10 mM, were fitted to

$$v = \frac{VB}{K_b(1 + P/K_{il}) + B + B^2/K_{IIb}} \quad (15)$$

which gave K_b = 0.30 ± 0.05 mM, K_{il} = 0.245 ± 0.033 mM, and K_{IIb} = 71 ± 4 mM. A least-squares surface was then constructed as described above as a function of K_{Ib} and K_pK_{iq}/K_q, assuming K_b, K_{il}, and K_{IIb} to have the values given above and K_{Ip} = 1.6 mM. The value of K_pK_{iq}/K_q appeared to be large (probably over 20 mM), and K_{Ib} was at least 4 mM (a value of 6 mM, for example, corresponding at a K_pK_{iq}/K_q value of 10 mM, and a value of 10 mM matching one of 5 mM, although the latter choice of constants nearly doubled the square root of the average residual least square).

These product inhibition patterns thus suggest that there is some synergism in the binding of glucose and MgADP (a decrease from 6 to 1.6 mM in the dissociation constant of MgADP) but little synergism in the binding of glucose-6-P and MgATP, since the dissociation constant of MgATP is not appreciably changed. The picture of the kinetic mechanism which emerges from the isotope partitioning and product inhibition experiments is of a partly random mechanism in which dissociation of sugars from productive ternary complexes is very slow, but dissociation of sugars from nonproductive ternary complexes occurs at rates similar to those from binary E–sugar complexes. In addition, synergistic binding of sugars and nucleotides is much less in nonproductive than in productive ternary complexes.

Reverse Isotope Exchange. In order to analyze the reverse isotope exchange data, we must consider the pH variation of the various steps in mechanism 11, as modified by the addition of the steps shown in eq 12 and 13. The pH studies of Viola & Cleland (1978) show that V_1 and V/K_{MgATP} decrease a factor of 10 per pH unit below a pK of 6.1, while V_2 and V/K_{MgADP} decrease above a pK around 8.2. These pKs are presumably those of the acid–base catalyst on the enzyme which is thought to be an aspartate (Viola & Cleland, 1978; Anderson et al., 1978). Note that the presence of a nucleotide does not perturb the pK but that the presence of glucose perturbs the pK downward from its value of 6.6 in free enzyme (the pK from the pK_i profile for 6-deoxyglucose; Viola & Cleland, 1978), while glucose-6-P perturbs the value upward.

The rate constants which should be pH dependent are those for interconversion of E–glucose–MgATP and E–glucose-6-P–MgADP, which apparently are faster than the rate constants for reactant release (k_4 and k_5 in mechanism 11). As a result, the equilibrium constant for the transphosphorylation step, which is near unity at pH 7.5, should decrease a factor of 10 per pH unit below pH 6 and increase a factor of 10 per pH unit above pH 8.2. In the pH range 6–8, however, k_4, k_5 and the equilibrium constant of the transphosphorylation step should be nearly constant, with changes in $K_{i\,glucose\text{-}6\text{-}P}$ making the Haldane relationship fit the overall K_{eq} for the reaction.[9]

In mechanism 11, as modified by eq 12 and 13, the predicted ratio of the rate of exchange of ADP back into ATP and the rate of the forward reaction is

$$v^*/v = k_6[\text{MgADP}]/[k_7(1 + y[\text{MgATP}]/K_{Ib})(1 + k_5/k_4)] \quad (16)$$

where y is the ratio of dissociation rate constants for glucose-6-P in the presence and absence of MgATP (see eq 13).

When the rate constants we have chosen above are used in eq 16 with [MgATP] = 3 mM, [MgADP] = 5 mM and K_{Ib} = 4 mM

$$v^*/v = 0.13/(1 + 0.75y) \quad (17)$$

If y = 1.0, v^*/v = 0.074, while a value of y of 4.4 matches the experimental value of 0.03 at pH 7.8. The exchange data are thus reasonably consistent with the values predicted by mechanisms 11–13 and the rate constants we have chosen to go with them. The exchange ratio does drop by a factor of 2 from pH 8 to 6 and decreases further at low pH presumably because a larger amount of ADP is present as $HADP^{2-}$ rather than $MgADP^-$. Note that the presence or absence of citrate does not affect the exchange ratio. This is consistent with the proposal that citrate activates the reaction solely by decreasing the inhibitory effect of endogeneous Al^{3+} present in ATP (Womack & Colowick, 1979; Viola et al., 1980).[10] The effect of ADP in activating the reaction in the absence of citrate presumably results from sequestering of Al^{3+} by ADP and the fact that AlADP is not a tight inhibitor of hexokinase.

In the reverse reaction, the exchange ratio for conversion of MgATP back into MgADP is

[8] If we attempt to explain the substrate inhibition by MgATP as the result of combination with E–glucose-6-P in a manner which prevents glucose-6-P release, the reciprocal plots are not concave down in the presence of MgADP as in Figure 1.

[9] Since K_{iq} for the reaction increases a factor of 10 per pH unit above the pK of glucose-6-P, while the pK of the acid–base group on the enzyme is 6.6, $K_{i\,glucose}$ should increase below pH 6.6 and $K_{i\,glucose\text{-}6\text{-}P}$ above pH 6.6, leveling out at pH 8.2. Below pH 6, both glucose and glucose-6-P will be bound more loosely because of a number of groups whose protonation diminishes binding, and below its pK glucose-6-P is bound more loosely because of its protonation.

[10] It is not clear whether citrate actually sequesters the Al^{3+} or forms an AlATP–citrate complex which is not inhibitory. In view of the rapidity with which citrate acts (as long as AlATP has not had a chance to combine with E–glucose and undergo the slow conformation change which locks AlATP on the enzyme), the latter explanation may be the correct one.

$$v^*/v = k_3[\text{MgATP}]/[k_2(1 + x[\text{MgADP}]/K_{\text{Ip}})(1 + k_4/k_5)] \quad (18)$$

where x is the ratio of rate constants for release of glucose in the presence and absence of MgADP (see eq 12). With the rate constants we have adopted and $x = 1$, v^*/v should be about 2.0. The experimental value in the presence of citrate is near 1 at pH 7, becomes almost 4 at pH 8.43, and decreases at low pH. In contrast to the situation in the forward reaction, citrate has a large effect on the reverse isotope exchange ratio, with the ratio being as much as an order of magnitude lower in the absence of citrate. Apparently in the absence of citrate, AlATP present as a contaminant in the ATP is specifically inhibiting the exchange reaction by combining with E–glucose to form an E–glucose–AlATP complex (Viola et al., 1980). If glucose can dissociate from this complex, and AlATP dissociates after glucose-6-P binds, the partitioning of the E–glucose complex toward reverse isotope exchange as opposed to dissociation of glucose is specifically decreased.

References

Anderson, C. H., Stenkamp, R. E., McDonald, R. C., & Steitz, T. A. (1978) *J. Mol. Biol. 123*, 207.

Angyal, S. J., & Pickles, V. A. (1972) *Aust. J. Chem. 25*, 1695.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104.

Cleland, W. W. (1979) *Methods Enzymol 63*, 103.

Cohn, W. E., & Carter, C. E. (1950) *J. Am. Chem. Soc. 72*, 4273.

Danenberg, K. D., & Cleland, W. W. (1975) *Biochemistry 14*, 28.

Danenberg, K. D., & Danenberg, P. V. (1977) *Biochim. Biophys. Acta 480*, 351.

DelaFuente, G., Lagunas, R., & Sols, A. (1970) *Eur. J. Biochem. 16*, 226.

Fletcher, H. G., Jr. (1963) *Methods Carbohydr. Chem. 2*, 196.

Fromm, H. J., & Zewe, V. (1962) *J. Biol. Chem. 237*, 3027.

Hohnadel, D. C., & Cooper, C. (1973) *Eur. J. Biochem. 31*, 180.

Horton, D., & Philips, K. (1973) *Carbohydr. Res. 30*, 367.

Kaji, A., & Colowick, S. P. (1965) *J. Biol. Chem. 240*, 4454.

Koerner, R. A. W., Jr., Voll, R. J., & Younathan, E. S. (1977) *Carbohydr. Res. 59*, 403.

Kosow, D. P., & Rose, I. A. (1970) *J. Biol. Chem. 245*, 198.

Ness, R. K., Fletcher, H. G., Jr., & Hudson, C. S. (1950) *J. Am. Chem. Soc. 72*, 4547.

Noat, G., Ricard, J., Borel, M., & Got, C. (1969) *Eur. J. Biochem. 11*, 106.

Rose, I. A., O'Connell, E. L., Litwin, S., & Bar-Tana, J. (1974) *J. Biol. Chem. 249*, 5163.

Sols, A., & Crane, R. K. (1954) *J. Biol. Chem. 210*, 581.

Taylor, K. B., & Cleland, W. W. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2213.

Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111.

Viola, R. E., & Cleland, W. W. (1980) *Biochemistry 19*, 1861.

Viola, R. E., Morrison, J. F., & Cleland, W. W. (1980) *Biochemistry 19*, 3131.

Wilkinson, K. D., & Rose, I. A. (1979) *J. Biol. Chem. 254*, 12567.

Womack, F. C., & Colowick, S. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5080.

Investigation of Diffusion-Limited Rates of Chymotrypsin Reactions by Viscosity Variation†

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ABSTRACT: The possibility that the rates of acylation of chymotrypsin by certain highly reactive substrates approach the diffusion-controlled limits was investigated by measuring the values of k_{cat}/K_m for three substrates as a function of increasing viscosity with sucrose and ficoll as the viscosogenic reagents. The values of k_{cat}/K_m (pH 8.0, 25 °C) representing the acylation rate constants are the following: *N*-(methoxycarbonyl)-L-tryptophan *p*-nitrophenyl ester, 3.5×10^7 M^{-1} s^{-1}; *N*-acetyl-L-tryptophan methyl ester, 8×10^5 M^{-1} s^{-1}; *N*-acetyl-L-tryptophan *p*-nitroanilide, 300 M^{-1} s^{-1}. The rate constants decrease significantly with increasing viscosity for the first compound, decrease slightly for the second, and are insensitive to this perturbation for the third. The *p*-nitroanilide results taken together with the observation that the high concentrations of sucrose or ficoll used produce insignificant changes in k_{cat} for the ester substrates argue against a general nonspecific perturbation in the enzyme structure effected by these reagents. The values of the association rate constants calculated from these results are 9×10^7 and 1×10^7 M^{-1} s^{-1} for the *p*-nitrophenyl and methyl esters, respectively. The values of k_{cat}/K_m divided by the association rate constants show that the rates of acylation by the *p*-nitrophenyl ester occur at ca. 40% and by the methyl ester at ca. 10% of the diffusion limits. Possibilities involving reorientation of a nonproductively bound substrate within the ES complex or desolvation of part of the active site of the enzyme are considered to account for the lower association rate constant for the methyl as compared to the *p*-nitrophenyl ester.

Classically, the rate-determining steps in enzyme-catalyzed reactions have been considered to be those involving the chemical conversion of the ES to the EP complexes. While this situation undoubtedly obtains for many enzymes, there is increasing evidence that for others substrate association and/or product dissociation processes proceed more slowly than the chemical steps (Cleland, 1975); therefore, these reactions are at least partly diffusion limited. Albery & Knowles (1976) have remarked that such enzymes can be considered to have been perfected from an evolutionary point of view, since further improvements in catalytic efficiency would not be manifested in enhanced rates of reaction.

While an enzyme of substantial molecular weight, accommodating a single set of substrates and catalyzing a chemical

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