information is clearly of more value than a knowledge of what happens when the inhibitory metal ion is present in the millimolar range, as is the case when classical methods are applied. The present procedure appears to be the one of choice for determining dissociation constants of lanthanide complexes with ATP and ADP and of similar complexes with low dissociation constants, and its application to AlATP is described in the following paper (Viola et al., 1980).

Supplementary Material Available

A description of data input and output for a FORTRAN program which makes a least-squares fit to eq 11, along with the program listing, a sample input data deck, and the corresponding output (6 pages). Ordering information is given on any current masthead page.

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Interaction of Metal(III)–Adenosine 5′-Triphosphate Complexes with Yeast Hexokinase†

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ABSTRACT: In the presence of glucose, yeast hexokinase is specifically and strongly inhibited by all $M^{III}ATP$ (M = metal) complexes that do not hydrolyze at neutral pH, as long as the ionic radius of the metal is less than 0.89 Å. K_i values vary from the micromolar range (0.16 μM for AlATP at pH 7, for example) to as low as 13 nM for LuATP. With glucose and fructose, the tightly bound complexes also show reversible, slow binding behavior, but with poor substrates, little or no change in inhibition constant with time is observed. The kinetics of citrate as an activator of the hexokinase reaction are consistent with its reaction with AlATP present as a contaminant in commercial ATP to form Al citrate. The complex of Al(III) with citrate is 5 orders of magnitude more stable than AlATP, whose K_d is 0.7 μM at pH 7. ATP that has been treated with excess EDTA and adsorbed on and eluted from charcoal is free of aluminum, and citrate no longer affects the kinetics of the hexokinase reaction. Glycerokinase is also specifically inhibited by trivalent metal ATP complexes (K_i = 4 μM at pH 7 for AlATP).

Previous studies have shown that yeast hexokinase displays nonlinear kinetics at pH 7, but not at pH 8. A slow interconversion is observed from a faster initial rate to a slower steady-state rate (Shill & Neet, 1971), and this interconversion is prevented by the presence of certain anions (Kosow & Rose, 1971), with citrate being the most efficient. It has been postulated that this slow transient process and the activation by anions are regulatory properties of yeast hexokinase that play a physiological role (Shill & Neet, 1975; Peters & Neet, 1977). However, Womack & Colowick (1979) have recently shown the presence of variable amounts of contaminating trivalent aluminum ion in commercial preparations of ATP, and that yeast hexokinase is inhibited by an aluminum–ATP complex at pH 7 or below (but not at pH 8). They suggest that the activation by citrate results from removal of aluminum from the ATP complex by chelation with citrate.

In this report we will show that those trivalent metals which form stable complexes with ATP at neutral pH are very tight inhibitors of yeast hexokinase, and that at pH 7 most of them show a progressive increase in the degree of inhibition with time similar to that seen with aluminum, and are thus "slow binding" inhibitors by the definition of Williams & Morrison (1979). We confirm the postulate of Womack & Colowick (1979) by showing that ATP which has been freed from Al(III) by treatment with excess EDTA and adsorption on and elution from charcoal shows a linear time course in the hexokinase reaction at pH 7 and no activation by citrate.

Experimental Procedure

Materials. Yeast hexokinase (type C-302; isozyme S-II) and other enzymes and biochemicals were from Sigma (ATP was lot 78C-7110). Lanthanides were from Ventron Alpha, and other metals except $AlCl_3$ and $FeCl_3$ were obtained as free metals or oxides from Aldrich and converted to chlorides with HCl. The concentrations of stock solutions of trivalent metal ions were determined by passage through Dowex-50-H^+ and titration of the eluant and washings. To minimize hydrolysis, solutions of metal ions at pH 3 or below were mixed with Na_2ATP in equimolar quantities, and aliquots of these solutions were added to assay mixtures just prior to the addition of enzyme. Assays were run with the identical levels of substrates and inhibitors at the beginning and end of each set of experiments to verify the stability of the M(III)ATP complexes over the course of the experiments.

Preparation of Metal-Free ATP. Na_2ATP (200 mg) was dissolved in 5 mL of 30 mM EDTA, and the pH was adjusted

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to 6.5 with base. After 30 min, charcoal (acid and ammonia–ethanol washed) was added, and the solution was stirred for 10 min at 0 °C. The suspension was centrifuged, and the concentration of ATP in the supernatant was determined from the absorbance at 260 nm. Addition of charcoal was repeated until the concentration of ATP in the supernatant was less than 5% of the original level. The charcoal suspension was filtered through a Celite pad, which was washed with 2 mM EDTA and then water. Elution with 0.5% ammonia in 50% ethanol and evaporation to dryness gave metal-free ATP.

Kinetic Assays. Spectrophotometric assays were used in which pyridine nucleotide absorbance was followed at 340 nm. With glucose as substrate, the glucose-6-phosphate dehydrogenase couple was used, while, with other substrates, the pyruvate kinase–lactate dehydrogenase couple was employed. Reaction volumes were 3 mL.

Data Analysis. While the AlATP, GdATP, EuATP, and NdATP complexes were shown to be competitive inhibitors and the K_i values were determined by fits to eq 1, most inhibition studies involved determining initial velocities at fixed MgATP and several levels of inhibitor, including zero. Such data were fitted to

$$v = VA/(K(1 + I/K_i) + A) \quad (1)$$

and K_i was calculated from

$$v = v_0/(1 + I/K_{i,app}) \quad (2)$$

$$K_i = K_{i,app}/(1 + A/K) \quad (3)$$

where A is [MgATP] and K is its Michaelis constant under the conditions of the experiment.

Where the time courses of the reactions were very curved, data for product formed as a function of time were fitted to eq 4 to obtain values for initial (v_i) and final steady-state (v_{ss})

$$P = v_{ss}t - (v_{ss} - v_i)(1 - e^{-kt})/k \quad (4)$$

velocities. Both v_i and v_{ss} as a function of inhibitor were then separately analyzed as described above to give K_i values.

The hyperbolic uncompetitive activation of citrate in overcoming the inhibition of AlATP was fitted to

$$v = VA/[K + A(1 + I/K_{in})/(1 + I/K_{id})] \quad (5)$$

where A is [glucose], K is its Michaelis constant, and K_{in} and K_{id} are the activation constants.

Results

Determination of M^{III}ATP Inhibition Constants. The inhibition of yeast hexokinase by AlATP was found to be linear competitive vs. MgATP in the range from 0.1 to 50 μM inhibitor. The time course of the reaction was linear at pH 6, but at pH 7 or 8 there was an initial burst, followed by slowing down to a final steady-state rate. Similar inhibition patterns were seen for NdATP, EuATP, and GdATP, and it was assumed that other M^{III}ATP complexes would similarly be competitive vs. MgATP. The K_i values from these experiments are shown in Table I, along with K_i values for a number of other trivalent metal–ATP complexes as inhibitors of yeast hexokinase. In no case was the time course appreciably nonlinear at pH 6, but for most of the complexes where the K_i was less than 10 μM, a burst was seen at pH 7 or 8. Most complexes gave similar K_i values over the pH range 6–8, except for those where hydrolysis occurs in the neutral pH range (the elevated values at pH 8 with AlATP and at pH 7 with FeATP). By titration of 10 mM AlATP with KOH, a pK of 7.6 was observed (pKs of 3.4 and 4.3 were also seen). Ionization of this group (probably a water coordinated to aluminum) is presumably responsible for the decreased affinity of AlATP for hexokinase at pH 8. In the case of FeATP, an insoluble precipitate formed slowly at pH 7 during the assay, but the complex appeared stable at pH 6.

Table I: K_i Values for the Interaction of M^{III} ATP Complexes with the Hexokinase–Glucose Complex[a]

metal(III)	ionic radius[b] (A)	pH	K_i (μM) initial	K_i (μM) steady state[c]
Al	0.535	6	0.69 ± 0.07	
		7	0.32 ± 0.06	0.16 ± 0.01
		8	52 ± 12	8.0 ± 0.9
Cr	0.615	5.8	0.069[d]	[e]
Fe	0.645	6	2.1 ± 0.3	
		7	42 ± 19	15 ± 3
Sc	0.745	6	8.0 ± 1.2	
		7	14.7 ± 1.6	1.4 ± 0.3
In	0.800	6	0.7	
		7	1.12 ± 0.18	0.43 ± 0.10
Y	0.900	7	14.5 ± 1.0	
lanthanide ions				
Lu	0.861	8	0.84 ± 0.36	0.013 ± 0.001
Yb	0.868	8	0.84 ± 0.22	0.025 ± 0.002
Er	0.890	8	1.16 ± 0.33	0.30 ± 0.02
Tb	0.923	8	10 ± 1	
Gd	0.938	8	32 ± 1[f]	
Eu	0.947	8	36 ± 4	
Nd	0.983	8	105 ± 4	
Ce	1.01	8	149 ± 8	
La	1.032	8	174 ± 8	

[a] Assay conditions: 50–100 mM buffer, 1–2.5 mM glucose, 0.4 mM $NADP^+$, 0.07–0.2 mM MgATP, 0.15–0.27 mM free Mg^{2+}, 20 units of glucose-6-phosphate dehydrogenase, 0.2 unit of yeast hexokinase, and varying levels of M^{III} ATP. The observed inhibition constants were corrected for the level of MgATP present using eq 3 and Michaelis constants measured at the appropriate pH. [b] Effective ionic radius in the 3^+ state for coordination number 6 (Shannon, 1976). The values are higher for the higher coordination numbers that are common for the lanthanide ions, but the trends are the same (for coordination number 9, the values range from 1.032 for lutetium to 1.216 for lanthanum). [c] Where no value is given, the time course of the reaction did not show a burst. [d] The value is for mixed bidentate isomers (Dunaway-Mariano & Cleland, 1980). Unlike the other complexes in this table, CrATP is an inert complex, and two of the four bidentate isomers act as substrates, although product release is much slower than phosphate transfer. [e] The final K_i at pH 7 is at least an order of magnitude less than the initial one (Danenberg & Cleland, 1975). [f] The K_i values determined from the experiments in the previous paper (Morrison & Cleland, 1980) were 123 μM at pH 6, 53 μM at pH 7.95, 42 μM at pH 8.65, and 13 μM at pH 8.85. The ionic strength was not constant in these experiments.

AlATP was also a good inhibitor of glycerokinase, with a K_i of 1.4 ± 0.2 μM at pH 6.6 and 4.0 ± 0.5 μM at pH 7. The time course of the reaction was linear at both pH values.

Inhibition of Hexokinase by Endogenous Al in ATP and Preparation of Aluminum-Free ATP. To verify the proposal made by Womack & Colowick (1979) that metal ions in ATP were inhibiting the reaction catalyzed by yeast hexokinase, stock solutions of ATP were treated by the following methods to remove contaminating metals: (1) a solution was stored overnight over two changes of chelex resin; (2) a solution was extracted by shaking with a 0.01% solution of dithizone in carbon tetrachloride; (3) ammonium bicarbonate was added to a solution of ATP, and the solution was run through a Bio-Gel P-2 column and eluted with 10 mM ammonium bicarbonate. The ATP was collected and lyophilized to remove the ammonium bicarbonate. The buffer and other reactants of the hexokinase coupled assay were treated with chelex or dialyzed to remove contaminating metals, and glucose-6-

phosphate dehydrogenase was dialyzed to remove citrate.

The time course of the hexokinase reaction was followed using ATP samples treated by each method and with an untreated sample of ATP. In each case the time course was nonlinear at pH 7, with an initial burst followed by a decay to a slower rate, as has been previously observed by Shill & Neet (1971). Analysis of the time courses showed that this decay represents a single exponential process, with the rate constant giving a linear double reciprocal plot against sugar concentration (maximum value is 3.15 min^{-1}, and apparent K_m is 0.5 mM with fructose at pH 7 and 1 mM MgATP). Addition of 1 mM citrate to the reaction mixtures with each ATP sample caused activation of the initial rates and no slowing down, as has been observed by Kosow & Rose (1971). The results suggest that either the commercial ATP sample used contained no contaminating metal ions that inhibited hexokinase, or the three methods employed were ineffective in removing significant amounts of these metal ions.

A more stringent purification procedure was devised for preparation of metal-free ATP as described under Experimental Procedure, using treatment with EDTA and purification on charcoal. ATP samples treated by this procedure gave linear time courses with yeast hexokinase at pH 7, and the rates were not affected by the addition of citrate. The effectiveness of this procedure for removing metal ions from ATP was verified by adding $AlCl_3$ to a solution of ATP purified by the EDTA–charcoal method, at a level sufficient to cause 90% inhibition of the hexokinase reaction. Treatment of this solution with the EDTA–charcoal procedure gave a sample that had rates in the hexokinase reaction identical with those of the originally purified ATP solution.

The kinetics of removal of contaminating metal ions during the hexokinase reaction were examined by following the time course of a reaction mixture containing 1 μM AlATP. When the final inhibited rate was attained, various levels of different metal chelators were added. Of those tested, citrate and 8-hydroxyquinoline-5-sulfonic acid gave the best rate of reactivation, with a half-time for activation of 0.3–0.4 min. Reactivation with EDTA was relatively slow, with a half-time of about 1.5 min, and the extent of reactivation was not as complete as with citrate. Addition of either imidazole or glycine gave no activation. Womack & Colowick (1979) also have observed much more rapid reactivation by citrate than by EDTA. In addition, they found 3-phosphoglycerate to be nearly as fast and effective as citrate.

Activation by Citrate. Although the work of Womack & Colowick (1979) and our success in preparing ATP free of aluminum that gave rates not stimulated by citrate suggest that the only role of citrate in the hexokinase reaction is to remove contaminating aluminum from ATP, we did check to see whether citrate had any other effects on the reaction, and whether the kinetics of activation agreed with the proposed model. While some preliminary work by Womack & Colowick (1971) indicated that the susceptibility of hexokinase to inhibitors and activators is not altered by dissociation into monomers, citrate might still alter the state of aggregation of the protein. The enzyme we used was a trypsinized form of yeast hexokinase (isozyme S-II), which does not associate readily. The molecular weight of hexokinase was determined at pH 7 by gel filtration in the presence and absence of metal-free substrates, and with and without added citrate, and in all cases only the monomeric species (mol wt 50 000) was detected. Some results from light scattering experiments (Schulze & Colowick, 1969) have indicated that even the trypsinized form of hexokinase begins to dimerize at very low pH. These results were confirmed by gel filtration experiments at pH 5.5, where the average molecular weight shifted from 50 000 to about 80 000, but citrate did not affect the monomer–dimer equilibrium at this pH.

Table II: Degree of Inhibition by Endogenous Al(III) in ATP with Different Sugar Substrates

sugar	rel V_{max} [a]	inhibited rate (%) [b] initial	steady state	steady state/initial [c]
5-thio-D-glucose	0.020	100	100	1.0
1,5-anhydro-D-glucitol	0.024	100	100	1.0
D-mannosamine	0.024	100	100	1.0
1,5-anhydro-D-mannitol	0.037	100	100	1.0
D-mannose	0.40	93	60	0.58
D-glucosamine	0.40	92	59	0.62
2,5-anhydro-D-mannose	0.63	71	39	0.52
2-deoxy-D-glucose	0.91	84	33	0.39
D-glucose	1.00	74	24	0.31
D-fructose	1.09	90	23	0.25
2,5-anhydro-D-mannitol	1.58	54	43	0.80

[a] Assay conditions: 50 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (Pipes) (pH 7), 3 mM ATP (not treated to remove Al(III)), 4 mM Mg^{2+}, 0.5 mM phosphoenolpyruvate, 0.4 mM NADH, 1 mM citrate, 100 units of lactate dehydrogenase, 60 units of pyruvate kinase, and 0.4 unit of hexokinase. [b] Rates in the absence of citrate as a percentage of the rate in the presence of citrate. The inhibition is by endogenous Al(III) present in the ATP. [c] Ratio of final steady state to initial rates in the absence of citrate.

The effect of citrate chelation on V and $V/K_{glucose}$ was determined by varying glucose at saturating MgATP (3 mM; the ATP was not Al free) and 1 mM Mg^{2+}, pH 7. The addition of citrate at fixed levels up to 0.2 mM caused hyperbolic uncompetitive activation, and the data were fitted to eq 5. For glucose as the varied sugar substrate, K_{id} was 45 $\pm$ 8 μM, and K_{in} was 110 $\pm$ 24 μM when initial velocities were measured. When 2,5-anhydromannitol was the variable substrate, K_{id} was 28 $\pm$ 6 μM and K_{in} was 74 $\pm$ 17 μM. At nonsaturating MgATP (50 μM), the values with glucose were K_{id} = 6.7 $\pm$ 1.2 μM and K_{in} = 19 $\pm$ 4 μM.

Effect of Different Sugars on AlATP Inhibition. The inhibition by endogenous aluminum in ATP was examined at pH 7 in the presence of different sugar substrates to determine if the different levels of synergism observed in the binding of different substrates and MgATP (Danenberg & Cleland, 1975; R. E. Viola and W. W. Cleland, unpublished experiments) are reflected in the inhibition by trivalent metal ions. The results in Table II indicate that little inhibition is seen with the slower substrates for the hexokinase-catalyzed reaction when aluminum is present as a contaminant in ATP. The better sugar substrates, however, show the same nonlinear time course and tighter final inhibition by AlATP as is seen with glucose.

Effects of AlATP on the Reaction Time Course. Assay mixtures were prepared with saturating glucose and sub-K_m levels of MgATP (containing contaminating AlATP). After we added enzyme to initiate the reaction, high levels of MgATP were added at various times after initiation, and the reaction time course was determined. The progress curve was nonlinear when the additions were made shortly after initiation of the reactions (Figure 1A), but if the reaction was allowed to proceed further before addition of higher levels of MgATP, the time course after addition became linear. Finally, as the

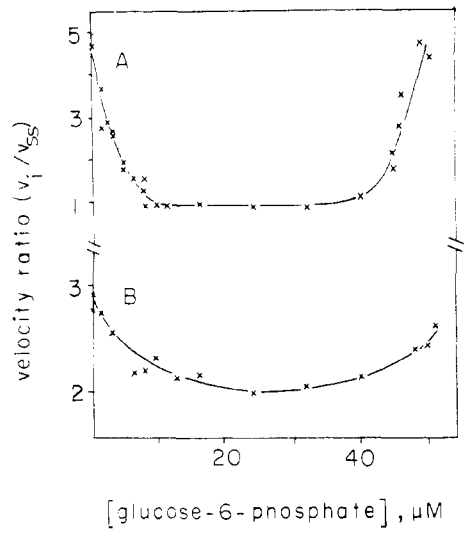

FIGURE 1: Effects of endogenous Al(III) in ATP on the progress curve of the hexokinase reaction. One substrate was originally limiting, so that reaction was slow. After reaction for a measured period of time, an excess of this substrate was added, and the ratio of initial to final steady-state velocities was plotted against the concentration of product formed before addition of high levels of the originally limiting substrate. Assay conditions common to both sets of experiments: 50 mM Pipes, pH 7, 0.6 mM $NADP^+$, 10 units of glucose-6-phosphate dehydrogenase, and 0.2 unit of yeast hexokinase at 15 °C. The ATP used was not free of Al. (A) Assays initially contained 20 mM glucose, 2 mM Mg^{2+}, and 50 μM ATP. At various times 1 mM ATP was added, and the initial and final steady-state velocities after the addition were determined. (B) Assays initially contained 4 mM ATP, 5 mM Mg^{2+}, and 50 μM glucose. At various times 2 mM glucose was added, and the initial and steady-state velocities after the addition were determined.

limiting amount of ATP became depleted before addition of more ATP, the time course after addition once again became nonlinear (Figure 1A). When the experiment was repeated with phosphoenolpyruvate and pyruvate kinase present in the assay mixture to recycle the MgADP formed back to MgATP, the time course after the addition of high levels of MgATP remained linear even after the reaction had proceeded several-fold further than in Figure 1A.

When the experiment was repeated with glucose as the limiting substrate, similar results were observed. In the presence of saturating levels of MgATP containing contaminating AlATP, the time course was nonlinear with limiting glucose. While the time course after addition of high glucose did become more linear as the reaction was allowed to proceed further before addition (Figure 1B), it did not become completely linear as was observed with MgATP limiting.

Determination of the Dissociation Constant of AlATP. The dissociation constant of AlATP was determined by the kinetic procedure described in the preceding paper (Morrison & Cleland, 1980). As illustrated in Figure 2, both the initial and final rates were used for the analysis, and while the K_i value of AlATP decreased from 0.40 ± 0.05 μM for the initial rates to 0.12 ± 0.01 μM for the final rates, the K_d values for the AlATP complex were 0.73 ± 0.22 and 0.62 ± 0.12 μM, respectively.

Discussion

Inhibition by AlATP. The experiments reported here demonstrate that the nonlinear time course of the hexokinase reaction at pH 7 is caused by endogenous Al(III) in commercial ATP, and confirm the proposals of Womack & Colowick (1979) that the sole mechanism of citrate as an activator is removal of aluminum from ATP. We find that when

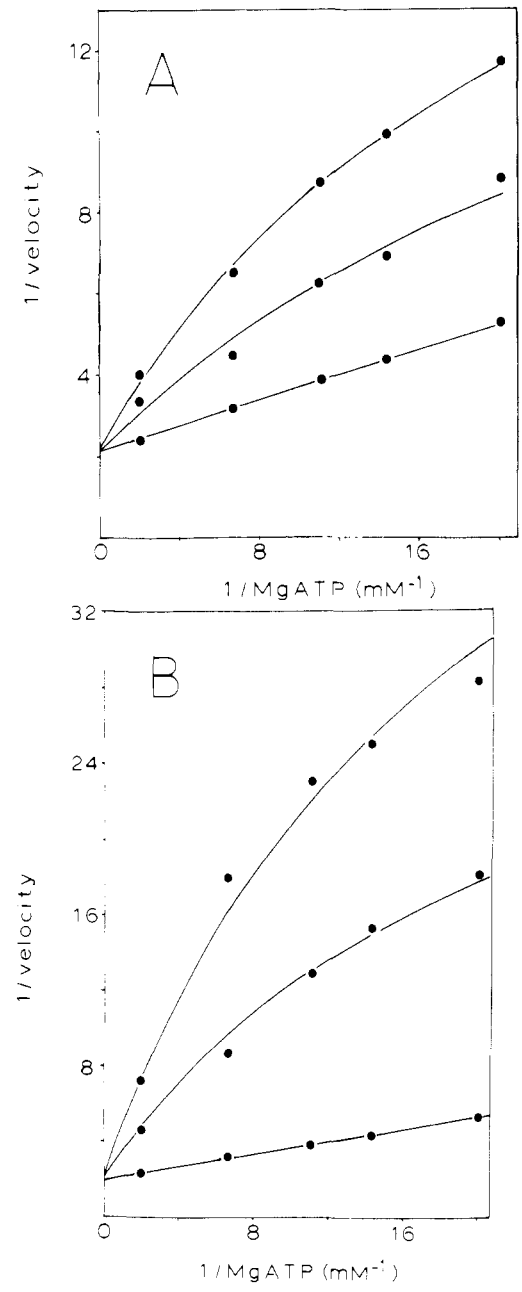

FIGURE 2: Determination of the dissociation constant of the AlATP complex by the kinetic method of Morrison & Cleland (1980). (A) Initial velocities determined from tangents drawn to the initial portion of the nonlinear progress curves. (B) Final velocities determined from tangents to the steady-state portion of the progress curves. Assay conditions: aluminum-free ATP, varied as shown, 100 mM Pipes, pH 6.95, 1 mM glucose, 0.4 mM $NADP^+$, 5 mM Mg^{2+}, 0, 1, and 2 μM AlATP, 25 units of glucose-6-phosphate dehydrogenase (citrate-free), and 0.02 unit of yeast hexokinase. The solid lines in each case are from a fit to the expected rate equation (Morrison & Cleland, 1980). The dissociation constant of MgATP under the conditions of the experiment (0.1 M K^+, μ = 0.09) was 52 μM.

metals are completely removed from ATP by treatment with excess EDTA, followed by adsorption on and elution from charcoal, the time course of the reaction at pH 7 is linear, and citrate has no effect. Citrate also has no effect on the dimerization of the enzyme. It is now simple to explain the sharp decrease in V or V/K_{MgATP} in the absence of citrate as the pH is lowered (Viola & Cleland, 1978), since AlATP has a pK of 7.6, and only below this pH will there appear to be a competitive inhibitor in MgATP (the ionized form of AlATP is a much weaker inhibitor, if it binds at all or indeed if it remains a stable complex above the pK). The rates at pH 5 are not

appreciably affected by the presence of citrate (Viola & Cleland, 1978), so either AlATP has become less inhibitory at this pH or MgATP binds more tightly at low pH than at pH 7. The Michaelis constant of MgATP does in fact drop from about 180 μM at pH 5.5–6.3 to 71 μM at pH 4.9, 24 μM at pH 4.6, and to 7 μM at pH 4.4, so it appears that $MgHATP^-$, which has the same overall charge that a $M^{III}ATP$ complex has at neutral pH, also binds very tightly to hexokinase. From the titration curve of AlATP it appears that the pK of 4.3 corresponds to protonation of the phosphate to give AlHATP and, since this complex has no net charge, it should bind very poorly to hexokinase. Thus, both an increase in affinity of MgATP and loss of it for AlATP cause the lack of observed inhibition at very low pH.

The above model of citrate activation agrees with its kinetics as an activator. When ATP containing endogenous Al(III) is used as the substrate, citrate gives hyperbolic uncompetitive activation vs. glucose. The lack of an effect on $V/K_{glucose}$ (and absence of any citrate effect on the pH profile of V/K_{gluose} (Viola & Cleland, 1978)) is exactly the pattern predicted by a mechanism (either ordered or random) where there is a competitive inhibitor present in substrate B (MgATP here) which combines strongly only with the EA complex, and where an activator eliminates the inhibition by reacting with the inhibitor. The activation should be hyperbolic uncompetitive as observed when A (glucose) is varied, and the K_{id} value from the fit to eq 5 is the level of citrate that will convert half of the AlATP to aluminum citrate under the conditions of the experiment. The K_{in} value is simply K_{id} times the factor $(1 + rK_b/K_i)$, where K_b is the Michaelis constant of MgATP, r is the ratio of AlATP to MgATP, and K_i is the dissociation constant of AlATP from its complex with E–glucose. Our experiments show that r is about 0.008 for the ATP we were using. This falls in the range of 0.0006–0.01 reported by Womack & Colowick (1979) for the aluminum content of commercial ATP samples (under the conditions of our experiments, almost all of the Al(III) present would be chelated by ATP, and almost all of the rest of the ATP would be MgATP).

Inhibition of Hexokinase by Trivalent Metal–ATP Complexes. It is clear from Table I that not only Al but also all other trivalent metals tested show inhibition of hexokinase by their ATP complexes, as long as these complexes do not hydrolyze at neutral pH. Whether hydrolysis occurs in a given case depends on the dissociation constant of the metal–ATP complex, the pK of the aquo complex of the free trivalent metal ion, and the pK of water coordinated to the metal ion in the ATP complex. It may also depend on concentrations (for example, when solubility products are exceeded for insoluble hydroxides, or when polymerization occurs without precipitation). The apparent pK of 7.6 for AlATP may result from ionization of coordinated water, while FeATP undergoes a more complex reaction at pH 7 because an insoluble polymeric hydroxide was observed to form slowly during the reaction.

The lanthanide–ATP complexes show a steady trend downward in K_i as the ionic radius decreases. Apparently 0.89 Å is the critical size above which affinity for the metal–ATP complex is rapidly lost. Ionic radii above 1.0 Å give even less binding than that shown by $MgATP^{2-}$ (about 100 μM). The lanthanide ions do not hydrolyze at as low a pH as the other trivalent ions in Table I, and thus can readily be used at pH values at least up to 8 (see Morrison & Cleland (1980) for the properties of GdATP as a function of pH). Lanthanide–ATP complexes are thus ideal when one wants a well-behaved and high pH stable competitive inhibitor of hexokinase, and by picking the right ion one can adjust the K_i value from 13 nM to over 150 mM.

It is intriguing that so far only yeast and brain hexokinases and yeast glycerokinase show much tighter binding by AlATP and CrATP than by MgATP (slow binding behavior is limited to hexokinase, however, and is not seen with glycerokinase and either AlATP or CrATP). Womack & Colowick (1979) report that muscle hexokinase is much less sensitive to AlATP, and that yeast glucokinase and rabbit phosphofructokinase are not inhibited. Janson & Cleland (1974) and Dunaway-Mariano & Cleland (1980) have shown that pyruvate, creatine, acetate, fructose-6-phosphate, 3-phosphoglycerate, arginine, and adenylate kinases are inhibited by CrATP with dissociation constants in the 10–1000 μM range. The very tight binding of CrATP with hexokinase (69 nM) and glycerokinase (140 nM) is shown only by the β,γ-bidentate isomers, however, with tridentate CrATP showing K_i values of 100–200 μM (Dunaway-Mariano & Cleland, 1980), while the other enzymes show similar K_i values for bidentate and tridentate isomers. Dunaway-Mariano & Cleland (1980) have postulated from these data, and from the fact that β-monodentate CrADP also inhibits hexokinase much more strongly than bidentate CrADP, while this is not true for the other kinases, that hexokinase and glycerokinase are unique among kinases in having an extended configuration for the tripolyphosphate part of ATP. The product of the reaction is thus β-monodentate MgADP, and the α phosphate never becomes coordinated, unlike other kinases where Mg shifts from β,γ to α,β coordination prior to release of MgADP.

The above data suggest that the inhibitory $M^{III}ATP$ complexes are β,γ-bidentate ones and, thus, that some of the differences in binding strength of the complexes in Table I may relate to the different proportions of bidentate isomers present in solution. None of the data, however, explain why $M^{III}ATP$, as opposed to $M^{II}ATP$ complexes, are so tightly bound to hexokinase and glycerokinase. We suggest that the extra positive charge on the trivalent metal is interacting with a negative charge such as that on a nearby carboxyl group to enhance the binding. This interaction is probably a second sphere one (that is, the carboxyl is hydrogen bonded to a water coordinated to the metal), since CrATP, which is an inert complex from which coordinated water cannot rapidly be displaced, behaves the same as the rapidly equilibrating complexes. Presumably, the putative carboxyl comes close enough to interact in this way only during the fast conformation change that follows binding of both sugar and metal–nucleotide (the further conformation change which tightens inhibition is discussed below). We see no value for such an interaction with trivalent metals, and presume that the fact that it is seen only for hexokinase and glycerokinase simply reflects similar properties and, in view of the many other similarities between these two enzymes (Dunaway-Mariano & Cleland, 1980), possibly a common evolutionary history.

One puzzle is why $M^{III}ATP$ complexes other than CrATP are not substrates for hexokinase. While we have not looked for rapid single turnovers on the enzyme of the kind demonstrated by Dunaway-Mariano & Cleland (1980) with CrATP, we have shown that multiple turnovers do not occur at a measurable rate, and it is hard to see why product release should be slow if turnover does occur. (With CrATP, release of the CrADP–glucose 6-phosphate product is slow because the coordination bond from chromium to glucose 6-phosphate cannot break, and, until it does, the enzyme does not sense that the reaction is over and remains in the closed catalytic conformation (Dunaway-Mariano & Cleland, 1980)). Possibly

the extra positive charge on the trivalent metal ion attracts enzyme ligands into the inner coordination sphere that would normally show second sphere interactions, with resulting distortion of the geometry of the active site. Such ligand insertion could of course not occur with $Cr(H_2O)_4ATP$ because of the inert coordination bonds to water. We will probably require X-ray evidence to solve this problem.

Slow Binding Behavior. The first observation of slow binding behavior with hexokinase was the biphasic time course at pH 7 (a burst, followed by a slower steady-state rate), now known to be caused by endogenous Al(III) in ATP. The second observation was the biphasic time course in the presence of MgATP and the inert β,γ-bidentate CrATP complex, which was recognized by Peters & Neet (1976) as being similar to that seen with MgATP alone. This change in rate (with a rate constant of 1 or 2 min^{-1} at 17 °C, pH 7) was ascribed by Danenberg & Cleland (1975) to a conformation change which led to tight binding of CrATP and sugar, and preceded phosphate transfer to form CrADP–glucose 6-phosphate, which was then released from the enzyme very slowly (0.04 min^{-1}). However, Dunaway-Mariano & Cleland (1980) have recently found that phosphate transfer is very rapid, having a rate constant at pH 5.5, 0 °C, of 0.2 s^{-1}, and thus it is clear that the biphasic time course in the presence of CrATP is not related to catalytic activity but represents slow binding behavior on the part of the inhibitor. As can be seen from Table I, other tightly bound $M^{III}ATP$ complexes also show such slow binding behavior at pH 7–8, with the ratio of final to initial K_i varying from about 0.5 for AlATP to 0.015 for LuATP. While there are some exceptions, the more tightly bound complexes seem to show the greatest degree of change, although the rate constant for the change (2–3 min^{-1}) is similar for all of the inhibitors.

The conformation change responsible for the slow binding behavior occurs only in the presence of sugars or sugar analogues, with the degree of change a function of the nature of the sugar. This is true both for CrATP (Danenberg & Cleland, 1975) and for AlATP (Table II). Glucose, fructose, mannose, lyxose, 2-deoxyglucose, and 2,5-anhydroglucitol all showed decreases from initial to steady-state rates of at least an order of magnitude when enzyme was added to reaction mixtures containing MgATP, CrATP, and substrate, while glucosamine and 2,5-anhydromannose gave ratios of final to initial rates of 0.38 and 0.36, 2,5-anhydromannitol showed too little change to be measurable, and 1,5-anhydromannitol and 1,5-anhydroglucitol showed no decrease in rate (Danenberg & Cleland, 1975). A similar pattern is seen in Table II, although the ratios are not as small as for CrATP. Fructose shows the greatest change (0.25), with other good substrates giving values from 0.31 for glucose to 0.58 for mannose. As with CrATP, 2,5-anhydromannose and glucosamine are near the upper end of the scale (0.52 and 0.62), while 2,5-anhydromannitol (the fastest substrate) shows only a small effect (0.80), and the poor substrates, including the 1,5-anhydroalditols, show no effect at all.

Thus, the ability of a molecule to induce the conformation change in the presence of MgATP that leads to catalysis is paralleled by an ability to induce the slow conformation change that tightens the binding of $M^{III}ATP$ complexes. 2,5-Anhydromannitol is a notable exception, especially since 2,5-anhydroglucitol (with nearly the same V_{max} as glucose, although with a much higher K_m) is as effective as glucose in causing the tight binding of CrATP (Danenberg & Cleland, 1975). No reason for this difference is apparent, but it does indicate that the conformation change in the presence of MgATP is not the same as that seen during the slow binding phenomenon. X-ray evidence will probably be required to tell us much about the nature of these conformation changes.

The requirement for a sugar to set off the conformation change that tightens binding of $M^{III}ATP$ complexes explains some previously puzzling observations concerning the time course of the reaction with commercial ATP at pH 7. It has been observed that preincubation of enzyme with ATP and glucose, followed by addition of Mg^{2+}, gave a linear time course with the same rate as the final steady state one when reaction was started with enzyme (Peters & Neet, 1977). Clearly, the AlATP present during the preincubation has had sufficient time to induce fully the conformation change leading to tighter binding. The requirement for glucose to be present during preincubation is strong evidence that the conformation change occurs only in the presence of a sugar. The effect of ADP or glucose 6-phosphate during preincubation in preventing the conformation change is simply explained by the competition between glucose and glucose 6-phosphate or between AlATP and ADP for their respective binding sites. Clearly, neither glucose 6-phosphate, ATP, nor ADP allows the conformation change; it requires a free sugar and a $M^{III}ATP$ complex.

The conformation change during which $M^{III}ATP$ binding is tightened is reversible, since the activity of enzyme inhibited by YbATP can be restored in a time-dependent manner by dilution, and since citrate or other chelators will activate even after preincubation of AlATP, glucose, and enzyme. Activation with EDTA shows a rate constant of about 0.5 min^{-1}, which is about what one calculates if the rate-limiting step is reversal of the conformation change, followed by unimolecular dissociation of AlATP from the enzyme and then further to Al(III) and ATP. The activation by citrate is faster than this by a factor of 5, however. While this possibly could result from bimolecular attack of citrate on the aluminum of the E-glucose–AlATP complex, the fact that the rate constant for reactivation did not appear to depend linearly on citrate concentration suggests that citrate attacks AlATP only after it is released from the enzyme surface. If AlATP normally recombined with the enzyme 4 times faster than it dissociated to Al(III) and ATP (at which point Al(III) could combine with EDTA), the observed rate of reactivation by EDTA would be 0.2 the true rate of release of AlATP from the enzyme, while if citrate reacted rapidly with AlATP to give Al citrate and free ATP, the observed reaction rate would be the AlATP release rate. Thus, it is not only the high stability constant of Al citrate[1] that makes citrate the efficient activator it is, but it is also its ability to react with AlATP in a bimolecular fashion.

The reversibility of the conformation change induced by AlATP and glucose is also seen in the experiments in Figure 1, in which ATP containing endogenous aluminum was used. In these experiments one substrate was originally present at a low level, and the time course of the reaction after the addition of an excess of the originally limiting substrate was characterized by the ratio of initial (v_i) to final steady-state (v_{ss}) velocity. This v_i/v_{ss} ratio serves to detect the degree to

[1] From the data of Pattnik & Pani (1961) on the stability of aluminum citrate and its pKs (3.5 and 6.8) and the pKs of citrate and Al^{3+} (which is converted to $AlOH^{2+}$ with a pK of 5 (Frink & Peech, 1963)), it is possible to estimate a dissociation constant of 8 pM for the reaction Al citrate$^- \rightleftharpoons AlOH^{2+}$ + citrate^{3-}. The aluminum citrate complex will be 60% in the Al citrate^{2-} form at pH 7, but in what form Al(III) exists is not known, since at levels where study is practical, it precipitates as $Al(OH)_3$. The aluminum citrate complex is clearly about 5 orders of magnitude more stable than AlATP, however, at neutral pH.

which the conformation change induced by AlATP and glucose has gone to completion prior to the addition of the originally limiting substrate. In Figure 1A, the time course of the conformation change in the presence of saturating glucose and limiting MgATP can be seen by the drop in the v_i/v_{ss} ratio to 1.0 by the time 20% of the MgATP has reacted. Only after 80% of the MgATP has reacted does the conformation change begin to reverse, and by the time the reaction has reached equilibrium, the replacement of AlATP by MgADP on the enzyme has completely reversed it. In these experiments, the high level of glucose present ensured that the sugar site was always occupied by glucose, and not by glucose 6-phosphate. In Figure 1B a similar phenomenon is seen, but because glucose was present initially at about half K_m levels, no more than $^1/_3$ of the enzyme had glucose on it at any time. The conformation change thus never went to completion, so that a burst was always seen when high glucose was added later. The kinetics seen in Figure 1 are thus fully consistent with the model we have postulated, and in particular show again the requirement for a sugar to allow slow binding behavior by AlATP.

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Reactivity of Sulfhydryl Groups of the Flavoenzyme D-Lactate Dehydrogenase and Effect on Catalytic Activity†

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ABSTRACT: The zinc-dependent flavoenzyme D-lactate dehydrogenase from *Megasphaera elsdenii* possesses about nine sulfhydryl residues and no disulfide bonds per 55000 molecular weight subunit. Four to six of these sulfhydryl residues (depending on the pH) undergo a gradual oxidation with concomitant inactivation which can be prevented by dithiothreitol. A slow loss in the flavin binding ability of the enzyme occurs subsequent to sulfhydryl oxidation and thus precludes full reactivation by dithiothreitol. However, complete reactivation of enzyme retaining its flavin is obtained upon incubation with dithiothreitol. Both the oxidation and inactivation processes can be accelerated by catalytic levels of cupric ions. Approximately the same number of sulfhydryl residues are lost by copper inactivation (about five at pH 7.0) and dithiothreitol restores the starting activity per flavin. EDTA or pretreatment of buffers with Chelex 100 protects the enzyme from inactivation due to sulfhydryl oxidation even though up to two sulfhydryls are oxidized under these conditions. It is suggested that the spontaneous inactivation involves trace metal catalyzed oxidation of sulfhydryl residues. Three of the enzyme sulfhydryl residues react with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) at widely separated rates. The first reacts rapidly, and its modification is associated with a loss of ~95% of the catalytic activity. This thiol can be quantitatively modified by reaction with 1 equiv of DTNB or bromopyruvate, resulting in a 95% loss in catalytic activity. Enzyme samples allowed to spontaneously inactivate to varying degrees show a parallel loss of the first two thiols reactive with DTNB, suggesting that these residues are oxidized to a disulfide. Reaction of these two thiols with DTNB also results in the production of a disulfide enzyme since nearly 2 equiv of TNB is released when enzyme is reacted with 1 equiv of DTNB. *p*-(Chloromercuri)benzoate reacts with about seven of the enzyme thiols with complete inactivation occurring upon modification of four residues. Analysis of several enzyme preparations for their reactive thiol content indicates at most a 10% deviation from the expected value of 1 residue/flavin. Since the activity/flavin ratio has been found to vary up to 30% in several preparations, it is suggested that this cannot be accounted for by sulfhydryl oxidation and is likely to result from the demonstrated presence of modified flavins with differential catalytic activities.

A pyridine nucleotide independent D-lactate dehydrogenase catalyzes the first step in lactate oxidation in the anaerobic bacterium *Megasphaera elsdenii* (formerly known as *Peptostreptococcus elsdenii*) when grown in lactate (Baldwin & Milligan, 1964; Brockman, 1971). This enzyme, first identified by Baldwin & Milligan (1964), was later shown by Brockman & Wood (1975) to function as part of an electron transport chain involving two other enzymes, an electron-transferring flavoprotein (ETF)[1] and a butyryl-CoA dehydrogenase (BD-

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