kallikrein generated. The direct esterolytic activity of PKA toward BAEE was also found to be inhibited by antithrombin III and by C1-esterase inhibitor.

The two-stage assay has been used to measure PKA in therapeutic products where its presence has been associated with hypotensive reactions (Alving et al., 1978). Because this assay depends on the conversion of PK to kallikrein, it permits an accurate prediction of the activity in vivo, i.e., the generation of kallikrein with the subsequent liberation of bradykinin. PKA levels measured in this manner were found to correlate well with hypotensive activity in an animal model (M. Fournel, unpublished experiments).

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A Kinetic Method for Determining Dissociation Constants for Metal Complexes of Adenosine 5′-Triphosphate and Adenosine 5′-Diphosphate†

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ABSTRACT: A general kinetic method is described for determining the dissociation constants of metal–ATP complexes that act as inhibitory substrate analogues for any enzyme that utilizes $MgATP^{2-}$. The usefulness of the procedure is illustrated by the results obtained from studies of the inhibition of hexokinase by lanthanide–ATP (LnATP) complexes. At relatively low concentrations of Mg^{2+}, these complexes act as linear competitive inhibitors with respect to $MgATP^{2-}$. In the presence of higher, fixed concentrations of Mg^{2+}, however, double reciprocal plots of the inhibition by LnATP vs. MgATP are nonlinear, and the data can be used to determine the ratio of the dissociation constants for the LnATP and MgATP complexes. As values are available for the dissociation constant of MgATP under a variety of conditions, that for any LnATP complex can be calculated. The dissociation constant for EuATP at pH 8.0 is 0.16 μM, while that for GdATP is 0.91 μM at pH 6.0, 0.087 μM at pH 7.95, and 1 μM at pH 8.65. Between pH 6 and 8, the ratio of the dissociation constants for GdATP and $MgATP^{2-}$ remains constant, and thus, within this range of pH, the lanthanide species involved must be Gd^{3+} and $GdATP^{-}$. The method can also be applied to the determination of dissociation constants for inhibitory metal–ADP complexes if $MgADP^{-}$ is used as the variable substrate.

A number of methods have been used to evaluate the dissociation constants for the complexes formed by nucleotides and metal ions (O'Sullivan & Smithers, 1979). These procedures, which involve equilibrium measurements, have been used successfully to determine dissociation constants for the $MgATP^{2-}$ and $MgADP^{-}$ complexes where the values lie within a suitable range (Adolfsen & Moudrianakis, 1978). However, for complexes whose dissociation constants are less than 1 μM, most of these methods are no longer sensitive enough. For example, with the 8-hydroxyquinoline method, Ellis & Mor-

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rison (1974) were only just able to estimate a dissociation constant for EuADP of 1 μM, but were not able to get a reliable value for EuATP. With the electron paramagnetic resonance (EPR) method, in which the level of free Mn^{2+} displaced from its complex by competition with the metal ion of interest is measured, Valentine & Cottam (1973) estimated the dissociation constant of GdATP as 0.1 μM at pH 6. This is the lower limit of the method, however, and it cannot be used at high pH values. There is thus a need for a method that can be used over a wide range of pH and other conditions, and yet still be sensitive enough to measure dissociation constants that are well below 1 μM.

Studies on the inhibition of yeast hexokinase by lanthanide–ATP (LnATP) complexes have shown that, at relatively low concentrations of free Mg^{2+}, the complexes act as linear competitive inhibitors with respect to MgATP. However, at higher concentrations of free Mg^{2+}, the reciprocal plots vs. MgATP in the presence of the inhibitor are not linear, but are concave down. Such a result is due to the effect of free Mg^{2+} in decreasing the concentration of LnATP and increasing the concentration of the substrate, MgATP. Analysis of the inhibition pattern provides a simple and accurate kinetic method for determining the dissociation constant of $LnATP^-$ relative to that of $MgATP^{2-}$. This paper describes the theory for the analysis and reports values of the dissociation constants for GdATP and EuATP. The value for AlATP is given in the following paper (Viola et al., 1980).

Theory

When two metal ions such as Mg^{2+} and Ln undergo reaction with ATP, the dissociation constants for the individual reactions can be expressed as

$$K_{MgATP} = [Mg^{2+}][ATP]/[MgATP] \quad (1)$$

$$K_d = [Ln][ATP]/[LnATP] \quad (2)$$

The charges on ATP, Ln, and the metal–ATP complexes have not been specified, since they may vary with pH and the identity of the metal ion. The magnitude of the values for the apparent dissociation constants for MgATP and LnATP will also be a function of these factors, as well as of temperature and ionic strength. When Mg^{2+}, Ln, and ATP are present in solution, the equilibrium of the overall reaction

$$Mg^{2+} + LnATP \rightleftharpoons Ln + MgATP \quad (3)$$

will depend on the ratio of the values for K_d and K_{MgATP}. Equation 3 predicts that as the concentration of Mg^{2+} is increased there will be a reduction in the concentration of LnATP and an increase in the concentration of MgATP. Thus, for any enzyme-catalyzed reaction that utilizes MgATP as a substrate and is inhibited by LnATP, elevation of the Mg^{2+} concentration must lead to a lowering of the inhibition at fixed concentrations of total ATP and Ln. The degree of reduction in inhibition will be greater at low original MgATP levels, and minimal at high original MgATP levels.

In the derivation of the following theory, it will be assumed that the dissociation constants for MgATP and LnATP are such that, even when the fixed concentration of Mg^{2+} is relatively low, the addition of equimolar amounts of Mg^{2+} or Ln^{3+} and ATP results in virtually complete conversion of ATP to its corresponding metal complex. Therefore, the concentration of free ATP is considered negligibly small.[1] The concentrations of added MgATP and LnATP are denoted by S and I, respectively. It follows from eq 3 that with increasing concentrations of free Mg^{2+}, and provided some LnATP has been added, the actual level of LnATP in solution will be less than I, and the level of MgATP will be greater than S. The conservation equation for Ln is

$$I = [LnATP] + [Ln] \quad (4)$$

while that for total ATP is

$$ATP_t = [MgATP] + [LnATP] = S + I \quad (5)$$

If $[MgATP] = x$

$$[LnATP] = S + I - x \quad (6)$$

$$[Ln] = I - [LnATP] = x - S \quad (7)$$

Then from eq 1 and 2, it follows that

$$[ATP] = \frac{[LnATP]K_d}{[Ln]} = \frac{xK_{MgATP}}{[Mg^{2+}]} = \frac{x}{R} \quad (8)$$

where $R = [Mg^{2+}]/K_{MgATP}$. Substituting for [LnATP] and [Ln] in eq 8 gives

$$(S + I - x)K_d/(x - S) = x/R \quad (9)$$

When MgATP is the variable substrate for, and LnATP is a competitive inhibitor of, a reaction such as that catalyzed by hexokinase, the rate equation (eq 10) can be obtained by

$$v = \frac{Vx}{K\left[1 + \frac{S + I - x}{K_i}\right] + x} \quad (10)$$

substituting into the standard equation for competitive inhibition x for [MgATP] and $(S + I - x)$ for LnATP. In eq 10, V represents the apparent maximum velocity, K is the apparent Michaelis constant, and K_i is the apparent inhibition constant for LnATP under the experimental conditions. When this equation is solved for x and substituted into eq 9, the resulting quadratic equation in v can be solved to give as the rate equation for the system

$$v = \frac{V(b + 2c/K + (b^2 - 4ac)^{1/2})}{2(aK + b + c/K)} \quad (11)$$

where

$$a = (1 + I/K_i)(1 + (S + I)/K_i) + K_dR/K_i \quad (12)$$

$$b = S(1 + (S + I)/K_i) - K_dR(1 - (S + I)/K_i) \quad (13)$$

$$c = -K_dR(S + I) \quad (14)$$

Reciprocal plots vs. $1/S$ at different I levels will intersect on the $1/v$ axis, and the initial slopes will be those expected for a linear competitive inhibition pattern. The reciprocal plot for $I = 0$ is linear, but when I is present, the plots will be concave downward and will reach a constant value as S goes to zero because the true level of MgATP present cannot drop below that generated by reaction of LnATP with Mg^{2+} by eq 3 (see Figure 1).

Equation 10 can be rearranged to eq 15 to allow calculation

$$x = \frac{vK(1 + (I + S)/K_i)}{V - v(1 - K/K_i)} \quad (15)$$

of the true concentration of MgATP in the presence of LnATP

[1] This makes very unlikely any $Ln(ATP)_2$ complexes, while the low levels of LnATP added reduce the likelihood of forming Ln_2ATP complexes. If Ln_2ATP complexes form, the experimental points for the higher inhibition levels used would lie well above their expected positions on graphs such as Figures 1b, 2, or 3, since eq 3 would now be $Mg^{2+} + Ln_2ATP \rightleftharpoons 2Ln + MgATP$; this reaction would proceed to the right less readily at high Ln_2ATP than at low levels.

Table I: Dissociation and Kinetic Constants for GdATP as an Inhibitor vs. MgATP for Hexokinase at Several pH Values[a]

		dissociation constants			kinetic constants[b]	
pH	free $[Mg^{2+}]$ (mM)	K_d (μM)	K_{MgATP} (μM)	ratio	K (μM)	K_i (μM)
6.0	10.0	0.91 ± 0.44	159	0.0057	380 ± 50	123 ± 15
7.95	4.0	0.087 ± 0.019	17.5	0.0050	212 ± 8	53 ± 3
8.65	2.0	1.06 ± 0.54	22.4	0.047	224 ± 34	42 ± 8
8.85	1.0	0.93 ± 0.34	15.6	0.059	97 ± 18	13 ± 2

[a] Values were obtained under the experimental conditions described under Experimental Procedure. Reaction mixtures at pH 7.95 and 8.65 contained 20 mM ammonium sulfate, which elevates K. [b] K and K_i denote the apparent Michaelis constant for MgATP and the apparent inhibition constant for GdATP.

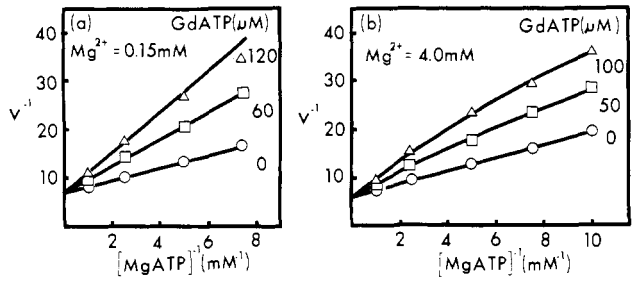

FIGURE 1: Competitive inhibition of hexokinase by GdATP at pH 7.95 in the presence of (a) 0.15 mM free Mg^{2+} and (b) 4.0 mM free Mg^{2+}. The experimental conditions were as described under Experimental Procedure with Taps as the buffer, except that reaction mixtures also contained 20 mM ammonium sulfate. The data of a were fitted to eq 18, while those of b were fitted to eq 11.

and high concentrations of Mg^{2+}. In this equation, v is the initial velocity at low enough [MgATP] for the curvature of the reciprocal plot to be pronounced and in the presence of sufficient LnATP to cause a sizable drop in velocity below the uninhibited rate. Values of K and V can be obtained by analysis of the data in the absence of inhibitor, while that for K_i can be determined from the initial slopes of the double reciprocal plots of the inhibition data.[2] Substitution of x from eq 15 into eq 9 gives

$$K_d = \frac{x(x - s)}{R(S + I - x)} \tag{16}$$

This equation can be used to calculate a preliminary estimate of K_d for use in analysis of the inhibition data by an iterative least-squares method. The computer program for this purpose is available (see paragraph at end of paper regarding supplementary material). It computes K_{MgATP} from the pH, ionic strength, and monovalent cation concentration according to the equation of Adolfsen & Moudrianakis (1978) and then calculates an exact value of K_d, as well as values for K, K_i, and V and standard errors of the estimates. The same general procedure can be used to calculate dissociation constants from data obtained with an enzyme for which metal–ADP complexes act as competitive inhibitors with respect to MgADP, except that the appropriate parameters given by Adolfsen & Moudrianakis (1978) for MgADP must be used in place of those for MgATP.

Experimental Procedure

Materials. Triphosphopyridine nucleotide (TPN) was from P-L Biochemicals. ATP, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (Taps), 3-(cyclohexylamino)-propanesulfonic acid (Chaps), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), crystalline yeast hexokinase (type C-302), and crystalline glucose-6-phosphate dehydrogenase were from Sigma. $EuCl_3 \cdot 6H_2O$ and $Gd(NO_3)_3 \cdot 5H_2O$ were from Ventron Alpha.

Estimation of Reactant Concentrations. Solutions of $MgAc_2$, $EuCl_3$, and $Gd(NO_3)_3$ were standardized by passing measured amounts through a column of Dowex 50-H^+ and titrating the effluent acid with standard alkali. Concentrations of ATP were determined enzymatically by using hexokinase and glucose-6-phosphate dehydrogenase in the presence of glucose and TPN.

Enzyme Assays. Reaction rates were determined at 25 °C by following the formation of TPNH at 340 nm in cuvettes with a 1.0-cm light path. Reaction mixtures contained, in 3.0 mL, buffer (0.05 M of the appropriate pH), glucose (2.5 mM), TPN (0.4 mM), glucose-6-phosphate dehydrogenase (10 units), and hexokinase as well as the indicated concentrations of free Mg^{2+} (the concentration of magnesium that was present over and above that required to form the metal–ATP complexes), MgATP, and lanthanide–ATP. The metal–nucleotide complexes were formed by the addition of equimolar amounts of Na_2ATP and metal ion. Hexokinase was diluted in 0.05 M K-Pipes buffer (pH 7.0), while a stock solution of glucose-6-phosphate dehydrogenase (200 U/mL) was prepared in 40 mM K-Taps (pH 8.0) containing 0.6 mM dithiothreitol. Some stock solutions of the dehydrogenase also contained ammonium sulfate, which gave rise to a final concentration in reaction mixtures of 20 mM.

Results

Studies with GdATP. GdATP is unable to act as a substrate for hexokinase, but does function as an inhibitor of the enzyme. At pH 7.95 and with free Mg^{2+} at the relatively low, fixed concentration of 0.15 mM, the inhibition is linear competitive with respect to MgATP (Figure 1a).

However, when the concentration of free Mg^{2+} is raised to 4.0 mM, the reciprocal plots are no longer linear (Figure 1b). Instead, the lines in the presence of GdATP exhibit a distinct curvature as predicted under Theory. The data of Figure 1b were fitted to eq 11 using the computer program deposited as supplementary material, and the results are listed in Table I. The values for K_{MgATP} in μM were calculated from the equation of Adolfsen & Moudrianakis (1978):

$$K_{MgATP} = 7.14(10^{3.1\mu})(1 + 10^7[H^+] + 17[Na^+]) \tag{17}$$

where $[H^+]$ and $[Na^+]$ are the molar concentrations of hydrogen ions and monovalent cations, respectively, and μ is ionic strength. When the data of Figure 1a were fitted to the equation for linear competitive inhibition

$$v = VA/[K(1 + I/K_{is}) + A] \tag{18}$$

the Michaelis constant for MgATP and the inhibition constant

[2] In practice, it is easier to run the inhibition pattern at 150 μM free Mg^{2+}, which should not be high enough to cause nonlinearity of the reciprocal plots. The resulting pattern can then be analyzed in the usual way to give K and K_i.

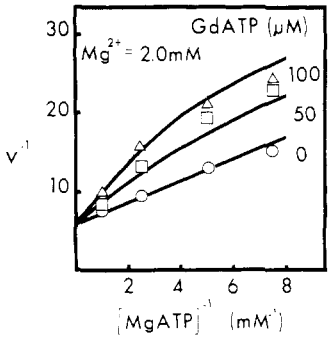

FIGURE 2: Competitive inhibition of hexokinase by GdATP at pH 8.65 in the presence of 2.0 mM free Mg^{2+}. The experimental conditions were the same as those described in the legend to Figure 1. The data were fitted to eq 11.

for GdATP were found to be 0.25 ± 0.02 and 0.12 ± 0.01 mM, respectively.

The dissociation constant for GdATP was determined at several other pH values (Table I), and it should be noted that, while the dissociation constants for both MgATP and GdATP are higher at pH 6.0 than at pH 7.95 because of the protonation of ATP^{4-} to $HATP^{3-}$, the ratio of the values remains constant. Thus, it may be concluded that between these two pH values the ionic species involved are Gd^{3+} and $GdATP^-$. Above pH 8.0, the inhibition data are not well described by eq 11 (see Figure 2), and the value obtained for the dissociation constant of GdATP is considerably elevated (Table I). Such a result suggests that, at these higher pH values, Gd^{3+} undergoes hydrolysis to $Gd(OH)^{2+}$, which does not react with ATP to form a complex that is an inhibitory as $GdATP^-$. Furthermore, the much lower than expected inhibition by 100 μM GdATP (based on the degree of inhibition by 50 μM GdATP) suggests that $Gd(OH)^{2+}$ is undergoing polymerization instead of remaining a mononuclear complex. These conclusions are confirmed by the finding that at pH 10 there is no inhibition of hexokinase by GdATP over the same range of concentrations as used for Figure 1.

Studies with EuATP. No hexokinase activity could be observed when Mg^{2+} was replaced by Eu^{3+}. At pH 8.0, this lanthanide ion formed an inhibitory EuATP complex which behaved like GdATP in causing competitive inhibition of the reaction (Figure 3). The data of Figure 3 fitted well to eq 11, giving $V = 0.158 \pm 0.006$, $K = 63 \pm 7$ μM, $K_i = 18 \pm 2$ μM, and $K_d = 0.16 \pm 0.04$ μM. K_{MgATP} was 12 μM under the conditions of the experiment, so the ratio of K_d and K_{MgATP} is 0.013.

Discussion

The present study has shown that a kinetic procedure can be used to determine dissociation constants for LnATP complexes that cannot be determined readily by thermodynamic methods. The procedure is based on the effect that a high level of free Mg^{2+} has in shifting the equilibrium of the reaction $Mg^{2+} + LnATP^- \rightleftharpoons Ln^{3+} + MgATP^{2-}$ to the right as the level of $MgATP^{2-}$ added is decreased. Since the level of competitive inhibition is thus decreasing, while the level of variable substrate is not decreasing as fast as expected, the result is curved reciprocal plots similar to those seen in Figures 1 and 3. Such data have been fitted to the rate equation that describes these patterns, and values were obtained for the dissociation constants of the GdATP (Table I) and EuATP complexes. The value of 0.91 μM for GdATP at pH 6.0 may be compared with an estimated apparent value of 0.1 μM reported by Valentine & Cottam (1973) at the same pH in the presence of 0.1 M sodium cacodylate.

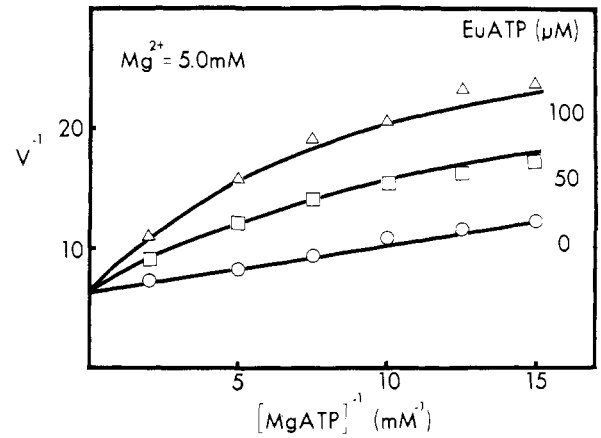

FIGURE 3: Competitive inhibition of hexokinase by EuATP at pH 8.0 in the presence of 5.0 mM free Mg^{2+}. The experimental conditions were as described under Experimental Procedure, using Taps as the buffer. The data were fitted to eq 11.

A major advantage of the procedure is its versatility. For a LnATP complex with a suitable inhibition constant, there should be no problem in determining a dissociation constant that falls within the range of 0.01 to 10 μM. If the dissociation constant is too low, it would not be possible to produce nonlinear competitive inhibition patterns, but an upper limit could be placed on the value for the dissociation constant. In the present work hexokinase has proved to be a very convenient enzyme, but if the competitive inhibition given by a complex were either too strong or too weak than any other enzyme using MgATP as substrate could be used, provided that the degree of inhibition and Michaelis constant for MgATP were suitable. It is, of course, essential that the LnATP complex whose dissociation constant is being measured have negligible ability to act as a substrate; this point should be checked carefully by running the reaction in the absence of Mg^{2+}, since, if the complex were to act as a poor substrate, the inhibition pattern would be similar to that predicted by eq 11. It is also important to establish that the activity of any coupling enzyme used to measure enzyme activity is not limited through its inhibition by the complex under investigation. The proposed procedure can be extended to the determination of dissociation constants for any metal–ATP complex that gives rise to competitive inhibition with respect to MgATP. It should also permit determination of dissociation constants for inhibitory metal–ADP complexes by using $MgADP^-$ as the variable substrate with an appropriate enzyme.

As evidenced by the results obtained with GdATP (Table I), the method can be applied over the full pH range for which the enzyme being used is active. As it is the ratio of the dissociation constants for MgATP and the inhibitory metal–ATP complex that is determined by the procedure, it is easy to detect if the metal ion or the metal–ATP complex undergoes hydrolysis from the variation of this ratio with pH. Such hydrolysis does not occur with Gd^{3+} and GdATP from pH 6 to 8, but does occur at higher pH values (Table I) and is undoubtedly accompanied by the formation of polymeric species. The formation of polymers is manifested by a higher reaction velocity at low MgATP and high inhibitor concentration than is predicted from the results obtained at low inhibitor concentrations (Figure 2). The proposed method is thus capable of characterizing the ionization states of a metal ion and/or its metal–ligand complexes when the metal ion undergoes hydrolysis at concentrations which may be micromolar or even submicromolar. Since this is the concentration range over which these molecules function as inhibitors, the

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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