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Lanthanide–Adenosine 5′-Triphosphate Complexes: Determination of Their Dissociation Constants and Mechanism of Action as Inhibitors of Yeast Hexokinase†

John F. Morrison and W. W. Cleland*

ABSTRACT: A kinetic procedure has been used to determine values for the dissociation constants of the complexes that ATP forms with lanthanum, 13 lanthanides, and scandium (Ln). The data indicate that the strength of the interaction increases as the ionic radius of the Ln(III) ion decreases and is much greater than that with Mg(II) and Mn(II). The LnATP complexes act as inhibitors of the reaction catalyzed by hexokinase, and the inhibition increases with the decreasing size of the Ln ion. While the ATP complexes of La, Ce, Pr, and Nd act as classical inhibitors, the nucleotide complexes formed with the other lanthanide ions behave as slow-binding inhibitors. The slow-binding inhibition with each LnATP complex conforms to a mechanism which involves rapid formation of an enzyme–glucose–LnATP complex which then undergoes a slow, reversible isomerization reaction. The reasons for the thermodynamic and kinetic behavior of the LnATP complexes are discussed.

Several years ago, it was suggested that Ln(III) ions[1] may function as good activators of phosphotransferases (Morrison & Heyde, 1972). It was pointed out that, while the electronic properties and ionic sizes of Ln ions were similar to those of the bivalent metal ions that function as activators, they possess a higher charge density. Subsequent investigations showed that, rather than functioning as activators, Ln ions acted as potent inhibitors of creatine kinase (Ellis & Morrison, 1974; Williams & Morrison, 1979) and several other phosphotransferases including hexokinase.

Ln ions undergo nonenzymic interactions with nucleotides to form Ln–nucleotide complexes (Ellis & Morrison, 1974; Morrison & Cleland, 1980), and it has been demonstrated that such complexes act as inhibitory analogues of MgATP. Thus,

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[1] To facilitate discussion, lanthanum will be regarded as a member of the lanthanide series, and the abbreviation Ln will be used for the entire group of trivalent ions.

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EuADP behaves as a tight-binding inhibitor of creatine kinase (Williams & Morrison, 1979) while GdATP inhibits the hexokinase reaction (Morrison & Cleland, 1980; Viola et al., 1980). For studies of this type, it is important to have values for the dissociation constants of the metal–nucleotide complexes so that the distribution of Mg and Ln between free and bound forms can be determined. Values have been obtain for several Mg–nucleotide complexes (O'Sullivan & Smithers, 1979), but up until recently, no satisfactory procedure was available to estimate dissociation constants for Ln–nucleotide complexes [cf. Ellis & Morrison (1974)]. The kinetic procedure developed by Morrison & Cleland (1980) opened the way for such determinations. This method has now been used to determine dissociation constants at pH 8.0 for the complexes that ATP forms with lanthanum, the 13 available lanthanides, and scandium. The data show that the strength of the complex increases with decreasing ionic radius of the lanthanide ion.

Investigations have also been made of the inhibition of hexokinase at pH 8.0 by each of the LnATP complexes.[2] The results indicate that the binding of the complexes to the enzyme–glucose complex increases with the decreasing ionic radius of the lanthanide ion and that with the smaller ions the inhibition is of the slow-binding type (Morrison, 1982).

Theory and Data Analysis

Analysis of Kinetic Data. Since it has been established that GdATP functions as a linear competitive inhibitor with respect to MgATP at pH 8.0 (Morrison & Cleland, 1980), it was assumed that all LnATP complexes act as inhibitory analogues of MgATP. Dissociation constants (K_i) for the reaction with the enzyme–glucose complex of LnATP complexes which function as classical inhibitors were determined from plots of $1/v$ against inhibitor concentration. Such data were fitted to eq 1 to yield an apparent inhibition constant [$K_{i(app)}$] from

$$v_{inhib} = v_{uninhib}/[1 + I/K_{i(app)}] \quad (1)$$

which the true dissociation constant (K_i) was calculated by using the relationship

$$K_i = K_{i(app)}/(1 + A/K_a) \quad (2)$$

where K_a is the Michaelis constant for MgATP and A is the fixed concentration of MgATP.

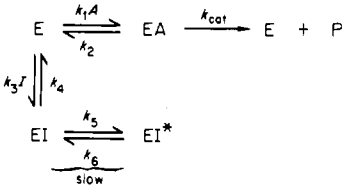
Kinetic parameters associated with the inhibition of hexokinase by LnATP complexes that act as slow-binding inhibitors were determined by analysis of progress curve data. Each experiment consisted of seven to nine progress curves which were obtained in the absence, as well as in the presence, of increasing concentrations of inhibitor (I). From each progress curve, 15–20 data points were taken, with the points being chosen so that the increment in the absorbance at 340 nm between each time value was virtually the same. The progress curve data were fitted to eq 3 to yield values for the initial

$$P = v_s t + (v_0 - v_s)(1 - e^{-k't})/k' + d \quad (3)$$

velocity (v_0), the steady-state velocity (v_s), the apparent first order rate constant (k'), and the displacement of the curve on the vertical ordinate (d).

With each of the LnATP complexes that function as slow-binding inhibitors, the initial velocity, the steady-state velocity, and the apparent first-order rate constant vary as a function of the LnATP concentration. Thus, the inhibition can be described by Scheme I, which assumes that each LnATP

Scheme I



complex interacts rapidly with the hexokinase–glucose complex (E) to form a ternary complex (EI) which then undergoes a slow, reversible isomerization to form an EI* complex. The dissociation constant associated with EI formation is denoted by K_i while the overall inhibition constant, K_i^*, is given by the relationship

$$K_i^* = \frac{K_i k_6}{k_5 + k_6} \quad (4)$$

where k_5 and k_6 represent forward and reverse isomerization constants, respectively. Since the concentrations of the LnATP complexes required to cause inhibition of the hexokinase reaction were considerably in excess of the enzyme concentration, it was not necessary to allow for depletion of the inhibitor concentration as a result of the formation of an enzyme–inhibitor complex. Under these circumstances, values for K_i and K_i^* can be obtained by fitting initial and steady-state velocity data, respectively, to eq 1 and 2 while the value for k_6 can be determined from the relationship

$$k_6 = \frac{v_s k'}{v_0} \quad (5)$$

The ratio of k_5/k_6 is given by the relationship

$$\frac{k_5}{k_6} = \frac{K_i}{K_i^*} - 1 \quad (6)$$

which allows calculation of the value for k_5. Alternatively, for Scheme I the relationship between k' and the concentration of inhibitor is given by the expression

$$k' = k_6\left\{\frac{1 + I/[K_i^*(1 + A/K_a)]}{1 + I/[K_i(1 + A/K_a)]}\right\} \quad (7)$$

which by replacement of K_i^* from eq 4 can also be written as

$$k' = k_6 + k_5\left(\frac{I/K_i}{1 + A/K_a + I/K_i}\right) \quad (8)$$

Equations 7 and 8 predict a hyperbolic variation of k' with I, and thus a fit of the data to eq 9 will yield a limiting value,

$$k' = k_6\left(\frac{1 + I/K_{in}}{1 + I/K_{id}}\right) \quad (9)$$

as I tends to infinity, of k_6K_{id}/K_{in} which is equal to $k_5 + k_6$. Knowing the value of k_6 from eq 5, we can calculate the value for k_5.

In the aforementioned analyses, each value of v_0, v_s, and k' obtained from a single progress curve is treated as an independently determined value. That is, the values are considered and analyzed in the same way as initial velocity data from steady-state kinetic experiments. It is also possible to make an overall fit of several progress curves with different inhibitor concentrations to the equation obtained by substituting eq 8 into eq 3. Before such overall fits are made, the displacement value, determined from the fit to eq 3 of each progress curve

[2] The charge on LnATP complexes at pH 8.0 will not be specified in the absence of information about pK_a values for the ionization of coordinated water in such complexes.

of the set, must be subtracted from the product coordinates. From the resulting values for K_i, k_5, and k_6, values for K_i^* can be calculated by using eq 4. For comparative purposes, overall fits of progress curve data at different inhibitor concentrations were made also for the case in which EI* does not exist to the equation obtained by substituting eq 10 into eq 3. In eq 10,

$$k' = k_4\left[1 + \frac{I}{K_i(1 + A/K_a)}\right] \quad (10)$$

$K_i = k_3/k_4$ where k_3 and k_4 represent, respectively, the rates of formation and dissociation of the EI complex, which are slow in this mechanism.

The fit of data to eq 1–3 and 9 was performed by using computer programs of Cleland (1979) while the fitting of slow-binding inhibition data was performed by using the STEPIT computer program. This program was obtained from the Quantum Chemistry Program Exchange at Indiana University. Standard errors (SE) of quotients were calculated by using the expression

$$SE(X/Y) = (X/Y)\{[SE(X)/X]^2 + [SE(Y)/Y]^2\}^{1/2} \quad (11)$$

Weighted mean values were determined by using the relationships of eq 12:

$$\text{weighted mean} = \frac{\sum x_i w_i}{\sum w_i} \qquad SE(\text{mean}) = \frac{1}{(\sum w_i)^{1/2}} \quad (12)$$

where $w_i = 1/[SE(X_i)]^2$.

Data which appeared to yield parallel lines for plots of $1/v$ against 1/[glucose] at different inhibitor concentrations were fitted to both eq 13 and eq 14 by using the computer programs of Cleland (1979):

$$v = \frac{VA}{K_a + A(1 + I/K_{ii})} \quad (13)$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (14)$$

Determination of Dissociation Constants (K_d) for LnATP Complexes. K_d values for LnATP complexes were determined by the kinetic procedure described previously by Morrison & Cleland (1980). The basis of the method is the effect that a relatively high concentration of free Mg^{2+} will have in shifting to the right the equilibrium of the reaction

$$Mg^{2+} + LnATP \rightleftharpoons Ln^{3+} + MgATP^{2-} \quad (15)$$

as the concentration of added $MgATP^{2-}$ is decreased. Such a shift will result in a decrease in the inhibition by LnATP because of the reduction in its concentration and the corresponding increase in the concentration of $MgATP^{2-}$. The shift in the equilibrium was monitored by measuring the change in either the initial or the steady-state velocity that occurs as the nominal fixed concentration of $MgATP^{2-}$ is varied in the presence of a LnATP inhibitor and a relatively high concentration of Mg^{2+}. With LnATP complexes that behave as competitive inhibitors with respect to MgATP, the variation in velocity is described by

$$v = \frac{V[b + 2c/K + (b^2 - 4ac)^{1/2}]}{2(aK + b + c/K)} \quad (16)$$

where $a = (1 + I/K_i)[1 + (S + I)/K_i] + K_dR/K_i$, $b = S[1 + (S + I)/K_i] - K_dR[1 - (S + I)/K_i]$, and $c = -K_dR(S + I)$. In eq 16, S and I represent the added concentrations of $MgATP^{2-}$ and LnATP, respectively, R equals the free Mg^{2+} concentration divided by the dissociation constant for MgATP, K_i represents the dissociation constant for the release of LnATP from the enzyme–glucose–LnATP complex, K_d denotes the dissociation constant for the LnATP complex, and V represents the apparent maximum velocity of the reaction. The dissociation constant for MgATP (K_{MgATP}) under the chosen experimental conditions was calculated in micromolar by using the expression given by Adolfsen & Moudrianakis (1978):

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

where $[H^+]$ and $[Na^+]$ are the molar concentrations of hydrogen ions and monovalent cations, respectively, and μ is the ionic strength.

Initial or steady-state velocity data were fitted to eq 16 by using the computer program described previously (Morrison & Cleland, 1980) to obtain values for V, K, K_i, and K_d.

Experimental Procedures

Materials. Crystalline yeast hexokinase (type C-302), glucose-6-phosphate dehydrogenase from bakers' yeast (type VII), 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid (Taps), 2-(*N*-morpholino)ethanesulfonic acid (Mes), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), and glucose were supplied by Sigma. ATP was obtained from Boehringer-Mannheim and triphosphopyridine nucleotide (TPN) from P-L Biochemicals. Lanthanide salts were products of Ventron Alpha. $Mg(OAc)_2$ was purchased from Fisher and dithiothreitol from Calbiochem. Before use, the suspension of glucose-6-phosphate dehydrogenase in 3.2 M ammonium sulfate was centrifuged and the crystalline material dissolved in 0.05 M K-Pipes buffer (pH 7.0) containing 1.0 mM dithiothreitol. The solution (80 units/mL) was stored at 4 °C.

Estimation of Reactant Concentrations. Solutions of $Mg(OAc)_2$ and lanthanide salts were standardized by adding aliquots to a column of Dowex-50 (H^+ form) which was then washed with water. The effluents were titrated with standard alkali. The concentration of ATP was determined enzymically by using hexokinase and glucose-6-phosphate dehydrogenase in the presence of TPN and excess glucose.

Determination of Enzyme Activity. Reaction rates were determined at 25 °C in a Cary 118 spectrophotometer by following the formation of TPNH at 340 nm in cuvettes of 1-cm light path. Reaction mixtures contained, in a total volume of 3.0 mL, 0.05 M K-Taps buffer (pH 8.0), 2.5 mM glucose, 0.2 mM TPN, 4 units of glucose-6-phosphate dehydrogenase, hexokinase, and variable concentrations of free Mg^{2+}, $MgATP^{2-}$, and LnATP. The metal–nucleotide complexes were formed by the addition of equimolar amounts of metal ion and Na_2ATP. The LnATP complexes were prepared just prior to use. Free Mg^{2+} is the concentration of metal ion in excess of that required to form $MgATP^{2-}$.

Determination of Dissociation Constants. Dissociation constants for LnATP complexes were determined at pH 8.0 so as to avoid the complications that arise at higher pH values because of the hydrolysis of Ln(III) ions and the formation of polynuclear complexes. In addition, at this pH, ATP can be considered to exist solely as ATP^{4-}. The procedure has been described by Morrison & Cleland (1980) and relies on the reduction of the inhibition of the hexokinase reaction that will occur in the presence of relatively high concentrations of free Mg^{2+} (see Theory and Data Analysis). The concentration of free Mg^{2+} in reaction mixtures was increased as the dissociation constant for the LnATP complex decreased.

Results

Dissociation Constants for LnATP Complexes. The values obtained for the dissociation of LnATP complexes at pH 8.0

Table I: Dissociation Constants for LnATP Complexes and Kinetic Parameters Associated with Their Inhibition of Hexokinase at pH 8.0

Ln of LnATP complex	ionic radius (Å)	dissociation constant[a] (μM)	K_i[b] (μM)	k_s (min^{-1})	k_6 (min^{-1})	K_i^* (μM)	k_s/k_6
La	1.03	0.33 ± 0.06	410 ± 23				
Ce	1.01	0.35 ± 0.06	340 ± 16				
Pr	0.99	0.31 ± 0.07	210 ± 8				
Nd	0.98	0.29 ± 0.05	160 ± 10				
Sm	0.96	0.22 ± 0.01	95 ± 10	0.05 ± 0.05	0.10 ± 0.003	63 ± 22	0.5 ± 0.5
Eu	0.95	0.16 ± 0.04	54 ± 5	0.10 ± 0.02	0.05 ± 0.003	18 ± 3	2.0 ± 0.4
Gd	0.94	0.087 ± 0.005	42 ± 4	0.10 ± 0.03	0.10 ± 0.002	21 ± 4	1.0 ± 0.3
Tb	0.92	0.094 ± 0.024	11 ± 1	0.12 ± 0.03	0.06 ± 0.003	3.7 ± 0.7	2.0 ± 0.5
Dy	0.91	0.049 ± 0.009	5.1 ± 0.3	0.16 ± 0.03	0.10 ± 0.01	2.0 ± 0.3	1.6 ± 0.3
Ho	0.90	0.099 ± 0.010	2.1 ± 0.1	0.23 ± 0.09	0.31 ± 0.01	1.2 ± 0.2	0.7 ± 0.3
Er	0.89	0.086 ± 0.019	1.4 ± 0.2	1.7 ± 0.3	0.66 ± 0.02	0.39 ± 0.08	2.6 ± 0.5
Tm	0.88	0.038 ± 0.010	1.3 ± 0.9	5.0 ± 0.9	0.46 ± 0.01	0.10 ± 0.02	11 ± 2
Yb	0.87	0.024 ± 0.006	0.8 ± 0.1	7.0 ± 1.0	0.28 ± 0.01	0.029 ± 0.005	25 ± 4
Lu	0.86	0.044 ± 0.011	0.9 ± 0.2	13 ± 2	0.21 ± 0.01	0.014 ± 0.003	62 ± 10

[a] Values were determined as described under Experimental Procedures. [b] Values of K_i, K_i^*, k_s, and k_6 were obtained by the stepwise procedure described under Theory and Data Analysis. The Michaelis constant for $MgATP^{2-}$ was taken to be 113 ± 1 μM with free Mg^{2+} at a concentration of 0.15 mM.

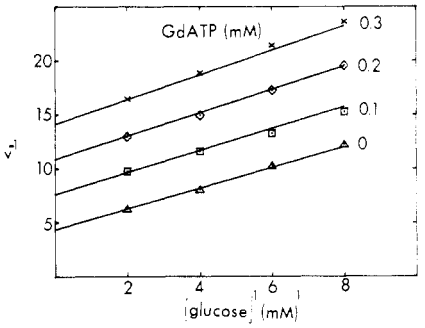


FIGURE 1: Inhibition of hexokinase by GdATP with respect to glucose concentration at pH 8.0. $MgATP^{2-}$ concentration was held constant at 0.2 mM. The lines were drawn by using parameter values obtained from a fit of the data to eq 14.

are listed in Table I. The table also contains values for the ionic radii of the lanthanides as given by Shannon (1976) for ions with a coordination number of 6 [cf. Martin & Richardson (1979)]. It is apparent that the strength of the interaction between Ln and ATP generally increases as the ionic radius of the lanthanide decreases and that the difference between the most tightly bound and the most weakly bound species is in the vicinity of 10-fold. The progression is not smooth, but all the values are considerably lower than that of 14.3 μM for $MgATP^{2-}$ (O'Sullivan & Smithers, 1979) and more comparable to that of 0.32 μM for AlATP at pH 7.0 (Viola et al., 1980).

Inhibition of Hexokinase by LnATP Complexes. It has been demonstrated previously that GdATP acts as a linear competitive inhibitor with respect to $MgATP^{2-}$ of the reaction catalyzed by yeast hexokinase at pH 8.0 (Morrison & Cleland, 1980). When the inhibition is studied in relationship to glucose, the effect of GdATP on the intercepts of a double-reciprocal plot is marked while that on the slopes is negligibly small (Figure 1). Since it is known that the hexokinase reaction conforms to a random mechanism (Purich et al., 1973), it can be concluded that GdATP binds much more strongly with the enzyme–glucose complex than with the free enzyme. In this respect, the behavior of GdATP is similar to that of CrATP (Danenberg & Cleland, 1975). The dissociation constant for the reaction of GdATP with the enzyme–glucose complex was calculated to be 48 ± 3 μM. This value may be compared with that of 42 ± 4 μM for the same interaction as determined from the competitive inhibition by GdATP with respect to $MgATP^{2-}$ in the presence of a saturating concentration of glucose. The value obtained for the interaction of GdATP with free enzyme by fitting the data to eq 14 was not well determined at 560 ± 260 μM.

On the basis of results obtained with GdATP and EuATP (Morrison & Cleland, 1980; Ellis & Morrison, 1974), it was assumed that LnATP complexes would act as competitive inhibitors of hexokinase with respect to $MgATP^{2+}$. Therefore, the kinetic parameters associated with the inhibition were determined by varying the LnATP concentration in the presence of a saturating concentration of glucose and a nonsaturating concentration of $MgATP^{2-}$. Apparent dissociation constants for the combination with the enzyme–glucose complex of LnATP complexes that act as classical inhibitors were determined from plots of $1/v$ against LnATP concentration. When LnATP complexes gave rise to slow-binding inhibition, progress curve data were analyzed, as described under Theory and Data Analysis, to obtain values for the various kinetic parameters. Since periods of up to 15 min were required to obtain progress curve data, it was important to ensure that enzyme inactivation did not occur over the time of data collection. Tests in the presence of 2 μM LuATP, which is a potent slow-binding inhibitor, showed that both the initial and the steady-state velocities varied as a linear function of enzyme concentration.

In earlier investigations of the inhibition of hexokinase by a limited number of LnATP complexes, no attempt was made to reduce the enzyme concentration to lower levels in order to determine if a particular complex could be observed to act either as a classical or as a slow-binding inhibitor (Viola et al., 1980). Such an approach has been used in the present investigation. The data in Table I indicate that the ATP complexes of all lanthanides except Nd, Pr, Ce, and La can act as slow-binding inhibitors. Thus, the ATP complexes of Eu, Gd, and Tb can be made to behave as slow-binding inhibitors when the catalytic rate of the reaction is slowed down sufficiently (Viola et al., 1980; Morrison, 1982). Since both the initial and steady-state rates vary as a function of inhibitor concentration for each LnATP complex, and yield values for K_i and K_i^*, respectively, the mechanism of inhibition is in accord with the model illustrated in Scheme I. A typical set of progress curves for a slow-binding inhibitor is illustrated with LuATP (Figure 2). The variation in the initial and steady-state rates as a function of TmATP concentration is shown in Figure 3, and the difference in the magnitudes of the values for K_i and K_i^* is well illustrated by the slopes of the two lines (cf. Table I).

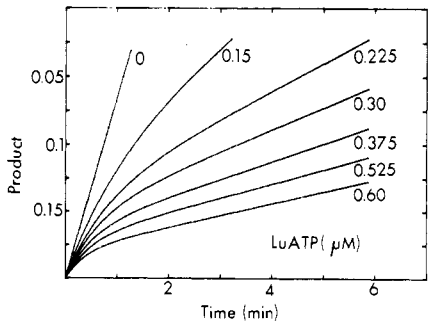


FIGURE 2: Slow-binding inhibition of hexokinase by LuATP at pH 8.0. The concentrations of glucose and $MgATP^{2-}$ were held constant at 2.5 and 0.2 mM, respectively.

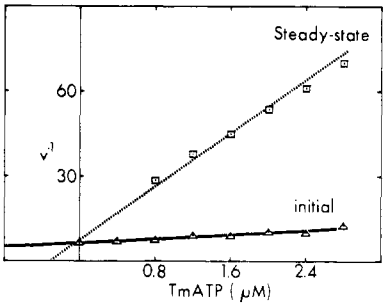


FIGURE 3: Variation of the initial and steady-state velocities of the hexokinase reaction as a function of the concentration of TmATP at pH 8.0. The concentrations of glucose and $MgATP^{2-}$ were as given in the legend to Figure 2.

Inspection of the values for K_i (Table I) shows that the initial binding of LnATP complexes to the enzyme–glucose complex increases as the ionic radius of the lanthanide ion decreases. With Sm through Lu, the binding is further enhanced as a result of the ternary complex undergoing an isomerization reaction. The ratio k_5/k_6 gives a measure of the degree of curvature in progress curves (cf. Figure 2). It is apparent that the enhancement is marked only with the nucleotide complexes involving Tm, Yb, and Lu which must be considered as potent inhibitors of hexokinase. The variation in values for the reverse isomerization constant (k_6) is relatively small so that it is the increasing rate of the forward isomerization constant (k_5) that is primarily responsible for the enhanced overall binding of these three LnATP complexes.

The values reported in Table I for k_5 were determined, as outlined under Theory and Data Analysis, by calculations that did not involve the use of values for the first-order rate constant, k'. However, the value of this parameter can be used to obtain k_5 by fitting data for the variation of k' as a function of inhibitor concentration to eq 9. The results of such analyses showed that this procedure was not as satisfactory as that utilized to obtain the data of Table I. Reasonable results were obtained only with the more inhibitory LnATP complexes. A comparison of k_5 values for TmATP, YbATP, and LuATP as determined by each of the two procedures is given in Table II. It has been pointed out by Duggleby et al. (1982) that systematic errors that arise because of displacement of time (t) values or drift in the absorbance will not affect the value of k'. But it does appear that the relationship between k' and the inhibitor concentration must be well-defined.

To demonstrate the reversibility of the inhibition and to determine the effect of glucose on this reversal, the enzyme was preincubated with LuATP in the presence and absence of glucose. An aliquot of the enzyme solution was then added to a reaction mixture which contained saturating concentrations of glucose and $MgATP^{2-}$. The dilution was such that the final concentration of LnATP was negligibly small. The results (Figure 4) show clearly that recovery of enzyme activity was immediate when no glucose was present in the incubation mixtures and time dependent when it was present. Analysis of the data for the slow recovery of hexokinase activity by fitting to eq 3 yielded a first-order rate constant of 0.28 min^{-1} as compared with a directly determined value of 0.21 min^{-1} (Table I).

Table II: Comparison of Values for k_5 As Determined by Two Different Procedures[a]

LnATP complex	k_5 (min^{-1}) procedure 1	k_5 (min^{-1}) procedure 2
TmATP	8.5 ± 6.2	5.0 ± 0.9
YbATP	4.8 ± 1.0	7.0 ± 1.0
LuATP	14.8 ± 3.3	13.0 ± 2.0

[a] Procedure 1 involved the fitting of data for the variation of k' as a function of the LnATP concentration to eq 9. For procedure 2, initial and steady-state velocity data were fitted to eq 1 and 2 to obtain values for K_i and K_i^*, respectively. These values were substituted into eq 6 to yield a value for k_5/k_6 from which a value for k_6 was obtained by using the weighted mean value for k_6 as determined from the relationship given in eq 5.

Table III: Effect of pH on the Binding of LuATP to the Hexokinase–Glucose Complex[a]

pH	inhibition constant K_i (μM)	inhibition constant K_i^* (μM)	app K_m for $MgATP^{2-}$ (mM)
8.0	0.91 ± 0.15	0.015 ± 0.001	0.02 ± 0.01
7.0	1.9 ± 0.4	0.33 ± 0.07	0.16 ± 0.04
6.0	10.8 ± 0.6		0.86 ± 0.05

[a] Values for K_i and K_i^* were obtained by fitting initial and steady-state velocity data, respectively, to eq 1 and 2. Free Mg^{2+} was held constant at a concentration of 0.15 mM and $MgATP^{2-}$ at a concentration of 0.4 mM.

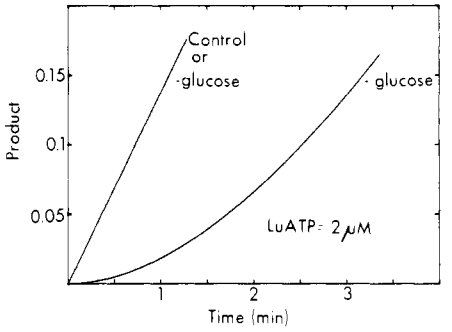


FIGURE 4: Recovery of hexokinase activity after preincubation with LuATP in the absence or presence of glucose. The enzyme was preincubated with 2 μM LuATP ± glucose at pH 8.0 for 15 min at 25 °C. An aliquot (10 μL) of the solution was then added to 3.0 mL of reaction mixture containing 2.5 mM glucose and 2.0 mM $MgATP^{2-}$. The coupled assay described under Experimental Procedures was used to monitor recovery of enzyme activity.

Effect of pH on the Inhibition of Hexokinase by LnATP. To gain an indication of the effect of pH on the binding of LuATP to the hexokinase–glucose complex, inhibition experiments were performed at pH values of 6.0, 7.0, and 8.0. At each pH value, the concentrations of free Mg^{2+} and $MgATP^{2-}$ were held constant at 0.15 and 0.2 mM, respectively, while the concentration of LuATP was varied. Apparent K_m values for $MgATP^{2-}$ were determined at each pH and used to calculate true K_i values from the apparent values obtained from Dixon plots. For these experiments, the solution of ATP had been allowed to stand overnight with citrylaminoethylcellulose to reduce the content of aluminum which as AlATP

Table IV: Kinetic Parameters Associated with the Inhibition of Hexokinase by LnATP Complexes As Determined from Overall Fits of Progress Curve Data[a]

Ln of LnATP complex	K_i (μM)	k_5 (min^{-1})	k_6 (min^{-1})	K_i* (μM)	k_5/k_6
Sm	130 ± 8	0.59 ± 0.10	0.43 ± 0.04	55 ± 8	1.4 ± 0.3
Eu	60 ± 3	0.33 ± 0.05	0.22 ± 0.02	24 ± 3	1.5 ± 0.3
Gd	59 ± 2	0.36 ± 0.04	0.25 ± 0.02	24 ± 3	1.4 ± 0.3
Tb	17 ± 1	0.50 ± 0.10	0.36 ± 0.05	7.0 ± 1.4	1.4 ± 0.3
Dy	6.9 ± 0.2	0.39 ± 0.04	0.31 ± 0.03	3.1 ± 0.4	1.3 ± 0.3
Ho	2.3 ± 0.2	0.92 ± 0.25	0.83 ± 0.14	1.1 ± 0.3	1.1 ± 0.3
Er	2.0 ± 0.1	3.3 ± 0.3	0.78 ± 0.03	0.38 ± 0.04	4.2 ± 0.4
Tm	1.8 ± 0.1	7.9 ± 0.8	0.48 ± 0.01	0.10 ± 0.01	17 ± 2
Yb	1.2 ± 0.1	11.7 ± 1.1	0.31 ± 0.01	0.031 ± 0.004	38 ± 4
Lu	1.5 ± 0.2	19.6 ± 2.8	0.21 ± 0.01	0.016 ± 0.003	93 ± 14

[a] Data for progress curves obtained at several different concentrations of each inhibitor were fitted to the equation obtained by substituting eq 4 and 7 into eq 3. Values for K_i* were calculated by using the relationship given in eq 4.

behaves as a slow-binding inhibitor (Womack & Colowick, 1979; Viola et al., 1980). The results in Table III show that the binding of LuATP decreases with pH and that at pH 6.0 no slow-binding inhibition is observed.

Comparative Analysis of Slow-Binding Inhibition Data for LnATP Complexes. An overall fit of the set of progress curves obtained with each of the slow-binding LnATP inhibitors yielded the data listed in Table IV. It should be noted that there is generally good agreement between the results in Tables I and IV except for the magnitude of the values for k_5 and k_6 as obtained with the LnATP complexes that bind more weakly. However, the ratios of these isomerization constants are not significantly different.

Overall fits of the slow-binding inhibition data were also made to eq 3 and 10, which describe a mechanism of inhibition in which formation of EI in Scheme I is slow and EI* does not form. For this mechanism [see Cha (1975) and Morrison (1982)], the slow development of inhibition would appear simply to be due to the slow interaction of the inhibitor with the enzyme. Convergence was obtained with the sets of data for each slow-binding LnATP inhibitor, but the analysis of the variance indicated that the fit was poorer in each case. The least difference in the variance values was observed with LuATP. This is as expected; for the greater the difference in magnitude is between K_i and K_i*, the lower is the steady-state concentration of EI (Scheme I). Under these same circumstances, the form of eq 7 would degenerate to that of eq 10.

It should be noted that no systematic investigations have yet been undertaken to determine the best procedure for analyzing slow-binding inhibition data. No particular attention been paid to the number and location of the data points that should be taken from a progress curve nor to the weighting factors that should apply to each progress curve of a set.

Studies with ScATP. The ATP complex of scandium also functions as a slow-binding inhibitor of the hexokinase reaction at pH 8.0. Analysis of initial and steady-state velocity data yielded K_i and K_i* values of 1.6 ± 0.1 and 0.13 ± 0.01 μM, respectively. The dissociation constant for ScATP at the same pH was estimated to be 0.4–0.5 μM.

Discussion

The present results establish that all the Ln ions interact strongly with ATP. Although the strength of interaction is greater for the ions of smaller ionic radius, the overall variation in the values for the dissociation constant is not more than 10-fold (Table I). As these values are very much lower than those of 14 μM for $MgATP^{2-}$ and 10 μM for $MnATP^{2-}$ complexes (O'Sullivan & Smithers, 1979), the question arises as to why this is so. Mn(II) is bound to the α-, β-, and γ-phosphoryl groups of ATP with metal–phosphorus distances of 3.2, 3.1, and 3.1 Å, respectively (Sloan & Mildvan, 1976). In addition, there is significant interaction between the metal ion and the N-7 nitrogen of the adenine ring (Sloan & Mildvan, 1976; Glassman et al., 1971). By contrast, with LnATP complexes, the corresponding metal–phosphorus distances are 5.1, 3.5, and 3.3 Å, and there is no interaction of Ln with the adenine ring (Tanswell et al., 1975). Thus, the more stable LnATP complexes have fewer interactions and a weaker link to the α-phosphoryl group. These effects must be more than compensated for by the higher charge density of the Ln(III) ions. The decrease in the dissociation constants of the LnATP complexes as the size of the Ln ion decreases is consistent with the bonding being primarily ionic. Tanswell et al. (1975) postulated, however, that two oxygens of the γ-phosphate, and possibly the α–β bridge oxygen, were in the inner coordination sphere, as opposed to the situation with other ATP complexes where only a single oxygen from each phosphate is thought to be coordinated. Such a coordination scheme for LnATP might explain why these complexes are not substrates for phosphotransferases, while $Co^{III}(NH_3)_4ATP$ and CrATP are.

The kinetic data for the inhibition of hexokinase by LnATP complexes confirm and extend the findings of Viola et al. (1980). Thus, the ATP complexes of Ln ions with large ionic radii act as classical inhibitors while those with smaller ionic radii act as slow-binding inhibitors (Table I). The nucleotide complexes of lanthanide ions such as Eu, Gd, and Tb can behave as either classical or slow-binding inhibitors depending on the concentration of enzyme used to study the reaction. The slow-binding inhibition data are both qualitatively and quantitatively in accord with the inhibition mechanism described in Scheme I. They are less well described by a mechanism that involves slow interaction between enzyme and inhibitor and formation of only one EI complex [mechanism A of Morrison (1982); Cha, 1975] and are not in accord with the model discussed by Duggleby et al. (1982) in which two forms of enzyme are in slow equilibrium, with one combining with inhibitor and the other combining with substrate. If such a mechanism were to apply with the hexokinase reaction, the same values for the forward (k_5) and reverse (k_6) isomerization rates would be obtained irrespective of the identity of the Ln ion.

The overall binding of LnATP complexes depends both on the strength of the initial interaction with the enzyme–glucose complex and on the isomerization constant (k_5/k_6) associated with the conformational change that the ternary complex undergoes (Table I). The strength of the initial interaction and the magnitude of the isomerization constant are greatest with Ln of small ionic radius.

On the basis of the limited number of investigations that have been undertaken so far, it appears that slow-binding inhibition may occur either because the inhibitor is a so-called transition-state analogue[3] or because it binds at the active site of the enzyme in a manner different from that of the substrate. The studies by Schloss et al. (1980) on aconitase and by Frieden et al. (1980) on adenosine deaminase and adenylate deaminase draw attention to the structural similarities between the compounds that act as slow-binding inhibitors and the postulated intermediates that would form on the surface of the enzyme. On the other hand, methotrexate, which is an analogue of dihydrofolate that strongly inhibits dihydrofolate reductase, is bound at the active site of the enzyme upside down relative to that of the substrate (Bolin et al., 1982). It might be considered that, as bound to the enzyme following the isomerization step, the slow-binding LnATP complexes are present on the enzyme as transition-state analogues. Indeed, they behave as expected for a transition-state analogue in that they bind more strongly to the enzyme than does the substrate. If the Michaelis constant for $MgATP^{2-}$ of about 100 μM is regarded as approximating its dissociation constant for combination with the hexokinase–glucose complex, then LuATP binds 5000-fold more strongly to the same complex. In this connection, it is of interest that LnATP complexes, which probably possess a single negative charge, and $MgHATP^{-}$ (Viola & Cleland, 1978) are bound more strongly to the enzyme than $MgATP^{2-}$.

It is also possible that the increased charge density of the trivalent Ln ion, which enhances complex formation with ATP, is responsible for different or fortuitous additional bonding when the LnATP complex is on the enzyme. The fact that most $M^{III}ATP$ complexes act as very effective, slow-binding inhibitors of hexokinase (Womack & Colowick, 1979; Viola et al., 1980) tends to suggest that a fortuitous interaction between M(III) and a negatively charged group on the enzyme may well be the feature that all the metal–nucleotide complexes have in common.

It appears that LnATP complexes are bidentate chelates (Tanswell et al., 1975), and in this respect, they are structurally similar to the β–γ bidentate isomer of $Cr^{III}ATP$ which is a substrate which also acts as a slow-binding inhibitor, binding to the hexokinase–glucose complex with a dissociation constant of 69 nM (Danenberg & Cleland 1975; Dunaway-Mariano & Cleland, 1980) and to the similarly exchange-inert $Co^{III}(NH_3)_4ATP$ complex which is also a substrate. However, the structures of the enzyme–LnATP and enzyme–CrATP complexes may well be different, since CrATP can form only an outer-sphere complex with any negatively charged group on the enzyme surface, while LnATP could form an inner-sphere complex. The answer to the question as to why LnATP complexes do not function as substrates in the way that $MgATP^{2-}$, $MgHATP^{-}$, and the Cr(III) and Co(III) complexes do must come from the results of X-ray crystallography. A knowledge of the three-dimensional structure of the hexokinase–glucose–LuATP complex should go a long way toward providing an answer to the question. The enzyme complex must contain glucose since it is apparent that the slow conformational change associated with slow-binding inhibition does not occur in the absence of glucose (Figure 4).

Registry No. Hexokinase, 9001-51-8.

[3] Most transition-state analogues should be called intermediate-state analogues, since they are analogues of actual intermediates in the reaction, such as tetrahedral adducts or carbanions, rather than of transition states. It would be difficult to prepare a good analogue of an actual transition-state structure, since such a structure involves extended bond lengths and often unusual bond angles.

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