Articles

Characterization of the Inhibition of Rabbit Muscle Adenylate Kinase by Fluoride and Beryllium Ions†

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ABSTRACT: In the presence of NaF, $BeCl_2$, ADP, and Mg^{2+}, rabbit muscle adenylate kinase was strongly inhibited. Molecules responsible for the inhibition were identified after subjecting the inhibited enzyme to filtration–centrifugation through small Sephadex G-50 columns. Full inhibition was correlated with the entrapment of 2 mol of ADP, 1 mol of beryllium, and 1 mol of magnesium per mole of enzyme; when an excess of AMP was present together with ADP, only 1 mol of bound ADP was detected in the desalted inhibited enzyme, due to the release of a loosely bound AMP. The fluorometal species identified in the ADP–fluoroberyllate entrapped complex were BeF^+, BeF_2, and BeF_3^-. When inhibited adenylate kinase was diluted in a medium deprived of the inhibitory species, the enzyme activity was progressively recovered, and a k_{off} value of 0.23 min^{-1} for the release of the inhibitor complex was calculated. The possibility that the inhibitory nucleotide–fluoroberyllate complex behaves as a transition-state analog is discussed.

Inhibition or activation of a number of nucleotide binding proteins by millimolar concentrations of fluoride has been reported for many years (Hewitt & Nicholas, 1963). Only recently has the role of contaminating aluminum or beryllium ions been demonstrated in this process (Sternweis & Gilman, 1982). Al^{3+} and Be^{2+} are known to bind up to six and four fluoride anions, respectively, generating stable fluorometal complexes (Goldstein, 1964; Mesmer & Baes, 1969). In solution these complexes combine with nucleotides, as revealed by NMR (Issartel et al., 1991a). The fluorometal complexes behave as phosphate analogs. In the case of transducin, a G protein involved in visual excitation, they combine with GDP in the nucleotide binding site of the protein, and the resulting GDP–fluorometal complex mimicks GTP, thus promoting the activation of transducin (Bigay et al., 1985). Likewise, fluorometals have been found to affect the activities of mitochondrial ATPase (Lunardi et al., 1988), tubulin (Carlier et al., 1988), actin (Combeau & Carlier, 1988, 1989), and myosin (Maruta et al., 1991).

The present study deals with the effect of the fluoroberyllate complex on adenylate kinase, a low molecular mass phosphotransferase that catalyzes the transfer of a phosphoryl group from ATP to AMP in the presence of Mg^{2+} according to the reaction MgATP + AMP ⇔ MgADP + ADP (Noda, 1958; Kuby et al., 1962; Hamada & Kuby, 1978). The kinetics of inhibition of adenylate kinase in the presence of fluoride and beryllium has been studied, and the correlation of inhibition with the binding of nucleotide–fluorometal has been established.

MATERIALS AND METHODS

Chemicals. All reagents used were of the purest grade commercially available. Adenylate kinase, pyruvate kinase, lactate dehydrogenase, AMP, ADP, ATP, GDP, and IDP were from Boehringer (Mannheim). Ap_5A and d-ADP[1] were from Sigma. Beryllium chloride was obtained from Fluka, and ultrapure sodium fluoride was from Riedel de Haen. NaF solutions were made and stored in plastic containers. All reactions were performed in plastic tubes.

Adenylate Kinase Preparation. Before use, rabbit muscle adenylate kinase (380 units/mg) was further purified to apparent homogeneity. Fifteen milligrams of the enzyme (ammonium sulfate suspension) was resuspended in 3 mL of buffer A (20 mM Tris-HCl, 100 mM NaCl, 5 mM DDT, 1 mM EDTA, and 55% ammonium sulfate, final pH 7.5). The turbid solution was centrifuged for 15 min at 15000*g* (+4 °C). The supernatant was loaded on a Sephacryl S-100 HR (Pharmacia) column (100 × 2.5 cm) equilibrated with buffer A, except the ammonium sulfate was omitted. The fractions corresponding to the main peak of absorbancy at 280 nm were pooled, and ammonium sulfate was added to reach 85% saturation. As shown by analysis of the enzyme by HPLC (C_4 column, 7 μm, 1000 Å), the Sephacryl chromatography efficiently removed five minor contaminants representing about 25% of the total protein content. The specific activity of the purified protein was about 500 units/mg, and its molecular mass analyzed by ion spray mass spectrometry was 21 680 ± 2 Da. This mass value agrees with that predicted from the enzyme amino acid sequence (Kuby et al., 1984), but does not suggest that the N-terminal acetyl-methionyl residue is oxidized as reported by the same authors.

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[1] Abbreviations: Ap_5A, bis(5′-adenosyl) pentaphosphate; d-ADP, 2′-deoxyadenosine 5′-diphosphate; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; LDH, lactate dehydrogenase; PEP, phosphoenolpyruvate; pF, colog of the free fluoride concentration; PK, pyruvate kinase.

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We have tested the effectiveness of our adenylate kinase preparation by its ability to bind the high-affinity ligand [^{3}H]-Ap_5A. [^{3}H]Ap_5A was synthesized by a procedure adapted from the procedure of Ng and Orgel (1987). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was used as the coupling agent between [^{3}H]ADP and ATP. [^{3}H]Ap_5A was purified by a two-step HPLC procedure, and its purity was checked by thin-layer chromatography on silica gel plates in dioxane/2-propanol/20% NH_4OH/H_2O (40:20:50:30). Commercial Ap_2A, Ap_3A, Ap_4A, Ap_5A, and Ap_6A were used as references. From the [^{3}H]ADP radioactive specific activity and the [^{3}H]Ap_5A radioactivity and UV absorbancy was calculated a molar extinction coefficient of 27 300 M^{-1} cm^{-1} at 256 nm for [^{3}H]Ap_5A, a value in good agreement with that reported in the literature (Holler et al., 1983). For an accurate measurement of binding of [^{3}H]Ap_5A to adenylate kinase, the equilibrium dialysis method was found to give more reproducible results than centrifugation–filtration on short columns of Sephadex G-50.

The equilibrium binding measurements were conducted at +4 °C in a Dianorm apparatus equipped with microcells (2 × 250 μL) and Spectrapor 2 (Spectrum Medical Industries) membranes (M_r cutoff 12000–14000). One half-cell was filled with 180 μL of the desalted enzyme solution. The other half-cell was filled with 180 μL of 100 mM KCl, 50 mM Tris Cl, and 2 mM $MgCl_2$ (pH 7.5) (KTMg medium) containing [^{3}H]-Ap_5A. The same operation was conducted with increasing concentrations of [^{3}H]Ap_5A. Dialysis lasted for 20 h at +4 °C under continuous rotation at 12 rpm. Preliminary assays showed that dialysis equilibration was totally achieved after this period of time and that less than 5% of the enzyme activity was lost. Furthermore, it was verified that no protein escaped through the dialysis membrane. Using our adenylate kinase preparation, the K_d for [^{3}H]Ap_5A was found to be 32 nM and the amount of bound [^{3}H]Ap_5A (mol/mol of enzyme) was 0.75 ± 0.05 mol (three assays). As adenylate kinase binds Ap_5A in a 1:1 stoichiometry (Feldhaus et al., 1975; Reinstein et al., 1990), it was concluded that only 75% of our adenylate kinase preparation was functional. Therefore, the experimental binding data obtained for the various ligands tested in the course of this work were corrected appropriately.

Kinetic Analysis. For kinetic analysis, a coupled colorimetric assay of the forward reaction catalyzed by adenylate kinase was used. The assay medium consisted of 2 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.5), 80 mM KCl, 200 μM NADH, 400 μM PEP, 80 mM KCl, and 10 units of lactate dehydrogenase (LDH) and pyruvate kinase (PK) (Berghäuser, 1975). Where not specified, AMP and ATP concentrations were 150 and 200 μM, respectively. The final volume was 1 mL. It was checked to ensure that the coupling enzymes, PK and LDH, were devoid of contaminating adenylate kinase activity and that beryllium and fluoride ions, *per se*, had no effect on the regenerating system under our experimental conditions. The amount of adenylate kinase added to initiate the reaction was between 50 and 100 ng per reaction. The decrease in the absorbance at 340 nm due to the consumption of NADH was recorded using a Uvikon 930 (Kontron) spectrophotometer. The rates of ATP hydrolysis were calculated by extrapolating the slope to $t = 0$. To test the effect of fluoroberyllate on adenylate kinase, the enzyme was preincubated with nucleotide(s) and fluoride and beryllium ions, and then the inhibited preparation was diluted 100-fold prior to the assay of the remaining activity.

Binding Measurements. The reversible binding of radiolabeled nucleotides to purified adenylate kinase was performed as follows. A sample of the stock solution of adenylate kinase in ammonium sulfate was pelleted by centrifugation for 15 min at 15000*g* at 4 °C. The supernatant was discarded. The pellet was dissolved in KTMg medium and then subjected to desalting by centrifugation–filtration, using 1-mL tuberculin syringes filled with Sephadex G-50 (fine), and inserted in conical centrigue tubes as described by Penefsky (1977). These small G-50 Sephadex columns were equilibrated in KTMg medium. Samples of desalted adenylate kinase adjusted to a final concentration of about 20 μM were incubated for 5 min at 25 °C with radiolabeled nucleotides in the presence or absence of NaF and $BeCl_2$ and then subjected to centrifugation–filtration through G-50 Sephadex columns. About 60% of the protein was recovered by centrifugation at 450*g* for 2 min.

Part of this material was used for the determinations of protein content by the method of Bradford (1976) and of bound radioactivity by scintillation counting. For the analysis of bound fluorometal and nucleotides, another fraction of the eluate was subjected to total digestion by trypsin (1:10 (w/w)) for 30 min at 37 °C. Trypsin digestion was preferred to protein precipitation by perchloric acid or heat treatment at 95 °C. These two methods of extraction of protein-bound nucleotides were tested in preliminary experiments. It was found that adenylate kinase was resistant to heat denaturation and that the KOH-neutralized perchloric extract, even after removal of the precipitate in the cold, often interfered with the fluorescence assay of beryllium (see below). Magnesium in the tryptic digest was analyzed by flame atomic absorption spectrometry using a Perkin-Elmer Model 2380 spectrometer (Paschen & Fuchs, 1971), and beryllium was measured by fluorescence (Petidier et al., 1985). The nature (mono-, di-, or triphosphate) of the nucleotide bound to the inhibited adenylate kinase was determined by HPLC of the tryptic digest. It was checked that the 30-min incubation period at 37 °C used for adenylate kinase digestion by trypsin did not modify the ATP content of the samples. The ODS-2 column (15 × 0.46 cm, 3 μm; Société Française de Chromatographie, Eragny, France) used for HPLC was perfused at a flow rate of 500-μL/min with a buffer containing 50 mM KH_2PO_4 and 2 mM tetrabutylammonium (pH 6.0). The nucleotides were eluted by a 0–40% linear gradient of 60% acetonitrile in 2 mM tetrabutylammonium and 50 mM KH_2PO_4 (pH 6.0) in the following order: AMP at 8.8 min, ADP at 12.7 min, and ATP at 16.5 min. With an amount of injected nucleotide ranging between 100 and 500 pmol, which corresponded to routine conditions, the average nucleotide recovery calculated with reference to external standards was 85%.

RESULTS

The Simultaneous Presence of ADP, Mg^{2+}, NaF, and $BeCl_2$ with Adenylate Kinase Results in Inhibition of the Enzyme. The activity of rabbit muscle adenylate kinase was inhibited by more than 90% after a 5-min preincubation with ADP, $MgCl_2$, $BeCl_2$, and NaF present together at appropriate concentrations (Table I, row 1). Under similar conditions, *Escherichia coli* adenylate kinase was also inhibited, but far less effectively than rabbit muscle adenylate kinase (less than 20%; not shown). This might be attributed to the differences which exist in the amino acid sequences of the *E. coli* and the rabbit muscle enzymes, despite the global homology which exists between the two proteins (Schulz et al., 1986). All of the experiments described here were therefore carried out with the rabbit muscle enzyme.

$AlCl_3$ was found to be as efficient as $BeCl_2$ for eliciting inhibition, but no inhibition was observed when either $BeCl_2$,

Table I: Effect of $BeCl_2$, NaF, Mg^{2+}, and ADP on the Activity of Rabbit Muscle Adenylate Kinase[a]

experiment	NaF	$BeCl_2$	Mg^{2+}	ADP	inhibition (%)
1	+	+	+	+	92 ± 5
2	+	+	+	–	<1
3	+	+	–	+	<1
4	+	–	+	+	<1
5	–	+	+	+	4 ± 2

[a] The rate of ATP hydrolysis was recorded after a 5-min preincubation of 1 μM adenylate kinase at 25 °C with or without 50 μM ADP, and/or 20 μM $BeCl_2$, and/or 5 mM NaF, and/or 2 mM $MgCl_2$. The percentage of ATPase inhibition was calculated by reference to the same enzyme preincubated with ADP alone. Average results (±standard deviation) are from three experiments.

Table II: Effect of the Nature of the Nucleotide on the Inhibition of Adenylate Kinase by Fluoride and Beryllium[a]

experiment	ADP	d-ADP	AMP	ATP	inhibition (%)
1	+	–	–	–	94 ± 5
2	–	–	+	–	<1
3	–	–	–	+	<1
4	–	+	–	–	<1
5	–	+	+	–	92 ● 5
6	–	+	–	+	<1

[a] The rate of ATP hydrolysis was recorded after a 5-min preincubation of 1 μM adenylate kinase at 25 °C with 20 μM $BeCl_2$, 5 mM NaF, 2 mM $MgCl_2$, and 50 μM concentrations of the indicated nucleotide(s). The percentage of ATPase inhibition was calculated by reference to the same enzyme preincubated with ADP alone.

ADP, or Mg^{2+} was omitted in the preincubation medium (Table I, rows 1-4) or when ADP was replaced by AMP or ATP. These observations recall the previously described inhibition of mitochondrial H^+-ATPase by fluoride in the presence of $BeCl_2$ or $AlCl_3$ and ADP, i.e., via the formation of nucleotide–fluorometal complexes (Lunardi et al., 1988; Dupuis et al., 1989; Issartel et al., 1991b).

It should be noted that, in the absence of NaF and in the presence of a low concentration of $BeCl_2$ (20 μM), adenylate kinase was slightly inhibited (Table I, row 5). With increasing concentrations of $BeCl_2$, inhibition became significant and reached a maximal value of 20% at 500 μM $BeCl_2$ (data not shown). The mechanism by which high concentrations of $BeCl_2$, in the absence of NaF, inhibit adenylate kinase activity, possibly by the formation of beryllium–nucleotide complexes (Issartel et al., 1991a), was not further explored.

For Inhibition of Adenylate Kinase by Fluoroberyllate To Occur, ADP Can Be Replaced by Other Nucleotide Diphosphates, Provided That AMP Is Present. When, in the preincubation medium, ADP was replaced by AMP or ATP, no inhibition was observed (Table II, rows 1-3). No inhibition occurred when ADP was replaced by other nucleoside diphosphates such as d-ADP (Table II, row 4), GDP, or IDP, whose affinity for adenylate kinase is much lower than that of ADP (Noda, 1973). Interestingly, inhibition reappeared when AMP together with d-ADP (Table II, row 5), GDP, or IDP (not shown) was added, while d-ADP supplemented with ATP could not elicit inhibition (Table II, row 6).

Inhibition of Adenylate Kinase by Incubation with NaF and $BeCl_2$ in the Presence of ADP and $MgCl_2$ Is Correlated with Strong Binding of Nucleotide by the Enzyme. After a 5-min preincubation with $BeCl_2$, NaF, $MgCl_2$, and increasing concentrations of [^{3}H]ADP, adenylate kinase (20 μM) was freed of unbound ligands by filtration–centrifugation through G-50 Sephadex (see Materials and Methods). The eluted enzyme material was used for the determination of protein concentration and bound radioactivity. A significant amount

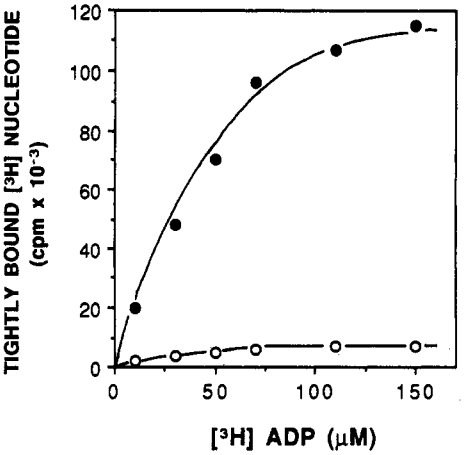

FIGURE 1: Effect of $BeCl_2$ on the retention of [^{3}H]ADP by adenylate kinase. Adenylate kinase (20 μM) was preincubated for 5 min in 100 mM KCl and 50 mM Tris-HCl (pH 7.5) in the presence of 5 mM NaF, 2 mM $MgCl_2$, 80 μM $BeCl_2$, and increasing concentrations of [^{3}H]ADP (●). A parallel incubation was carried out in the absence of $BeCl_2$ (○). Following incubation, unbound ligands were removed by filtration–centrifugation through Sephadex G-50 columns preequilibrated in KTMg buffer. The amount of recovered radioactivity was expressed as the number of counts per 10 μg of eluted adenylate kinase and plotted versus the concentration of [^{3}H]ADP present in the incubation medium.

of bound radioactivity was detected, which reached a plateau at 100 μM [^{3}H]ADP (Figure 1). When the same experiment was conducted in the absence of $BeCl_2$, NaF, or $MgCl_2$, virtually no enzyme-bound radioactivity was eluted (Figure 1). Thus, strong binding of nucleotide by adenylate kinase occurred only when NaF, $BeCl_2$, and $MgCl_2$ were all present in the preincubation medium, i.e., under the same conditions as those leading to the loss of activity of the enzyme (Table I).

The relationship between the extent of inhibition of the adenylate kinase activity and the amount of entrapped nucleotide was assessed by varying the concentration of [^{3}H]-ADP in the preincubation medium containing adenylate kinase, NaF, $BeCl_2$, and $MgCl_2$. After 5 min, the preincubation medium was either loaded on the top of a G-50 Sephadex column and centrifuged to measure its nucleotide content or diluted in a photometric cuvette containing the assay medium for the determination of enzyme activity. As shown in Figure 2, the extent of inhibition of the adenylate kinase activity was linearly related to the amount of enzyme-bound radioactive nucleotide. By extrapolation to 100% inhibition, an amount of 1.8 mol of radioactive nucleotide bound per mole of inhibited enzyme could be calculated. The same value was found when an excess of [^{3}H]ATP (500 μM) was added to the preincubation medium (not shown). On the other hand, full inhibition was obtained with only 0.9 mol of radioactive nucleotide bound per mole of enzyme when an excess of [^{3}H]AMP (500 μM) was present in the preincubation medium together with ADP (Figure 2) or d-ADP (not shown). Thus, depending on the experimental conditions, one or two nucleotides are entrapped in the presence of NaF, $BeCl_2$, and $MgCl_2$ in fully inhibited G-50 desalted adenylate kinase.

Kinetics of Inhibition of Adenylate Kinase by NaF, $BeCl_2$, $MgCl_2$, and ADP. As shown in Figure 3A, adenylate kinase that had been inhibited by preincubation with NaF, $BeCl_2$, $MgCl_2$, and ADP, i.e., adenylate kinase with two nucleotides entrapped, could progressively recover its activity upon dilution in a buffer devoid of NaF and $BeCl_2$. The recovery of the activity corresponded to a first-order reaction (Figure 3B) with a k_{off} rate of 0.23 ± 0.02 min^{-1}, corresponding to a $t_{1/2}$

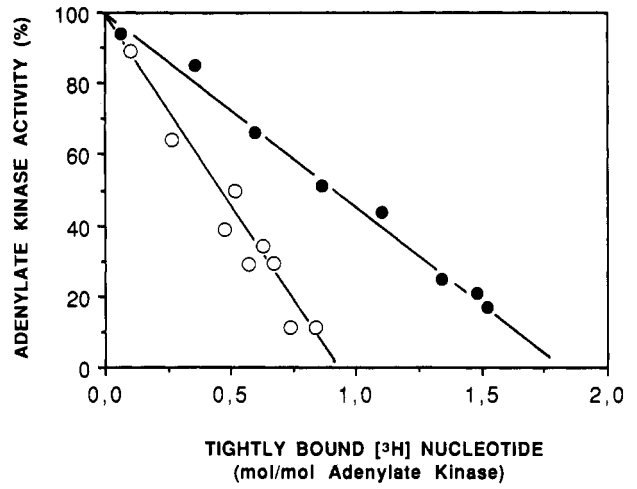

FIGURE 2: Correlation between the amount of bound nucleotide and the extent of inhibition of adenylate kinase by fluoroberyllate. Adenylate kinase was incubated at a concentration of 20 μM at 25 °C in KTMg medium containing 5 mM NaF, 80 μM $BeCl_2$, and increasing concentrations of [^{3}H]ADP up to 500 μM (●). After 5 min, the enzyme sample was divided into two fractions. One was loaded on the top of a G-50 column (1 mL) equilibrated with the KTMg buffer and centrifuged as described in Materials and Methods to measure the amount of bound nucleotide. The other fraction was diluted to 10 nM enzyme in a 1-mL cuvette containing the assay medium. Similar conditions to those were used for a second experiment, except that excess [^{3}H]AMP (500 μM) was included in the inhibitory medium (○). The specific radioactivity of the [^{3}H]-AMP solution was adjusted to the same value as that of the [^{3}H]-ADP solution.

of about 3 min. The same k_{off} value was found when an excess of AMP was present in the preincubation medium together with ADP or when ADP was replaced by d-ADP plus AMP (not shown). This k_{off} rate value is in the same range as that reported for the dissociation of BeF_2OH from transducin (Antonny & Chabre, 1992), but is much higher than that calculated for the mitochondrial H^+-ATPase, whose inhibition by fluorometals was quasi-irreversible (Lunardi et al., 1988).

Characterization of the Inhibitory Complex. The curve depicting the inhibition of adenylate kinase by NaF, $BeCl_2$, and $MgCl_2$ as a function of the concentration of free fluoride in solution was bell-shaped, with maximum inhibition occurring at pF values ranging between 2 and 3 (Figure 4). This inhibition curve was compared to the theoretical distribution curves calculated for various fluoroberyllate species in solution as a function of fluoride concentration (Martin, 1988; Issartel et al., 1991b). Clearly, the fluoroberyllate complexes BeF^+, BeF_2, and BeF_3^-, but not Be^{2+} or BeF_4^{2+}, are candidates as inhibitory species for adenylate kinase.

The mono-, di-, or triphosphate nature of the entrapped nucleotide recovered after G-50 centrifugation–filtration was analyzed as follows. Adenylate kinase inhibited by NaF, $BeCl_2$, $MgCl_2$, and [^{3}H]ADP was subjected to centrifugation–filtration through G-50 Sephadex equilibrated with 100 mM KCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 2 mM ADP, and 500 μM bis(5′-adenosyl) pentaphosphate (Ap_5A), a potent inhibitor of adenylate kinase (Lienhard & Secemski, 1973), in order to block any consumption of [^{3}H]ADP by some uninhibited enzyme. The eluted protein was digested by trypsin. The nucleotides released in the digest were separated by HPLC and analyzed for radioactivity, as described in Materials and Methods. More than 90% of the radioactivity was recovered in the form of [^{3}H]ADP (Table III, row 1). A similar result was obtained when the inhibitory medium was supplemented with an excess (500 μM) of [H^3]AMP or [H^3]-ATP (Table III, rows 2 and 3). When EDTA, ADP, and Ap_5A were not added in the G-50 desalting buffer, adenylate kinase recovered a substantial catalytic activity before tryptic denaturation was completed, leading to the presence of [^{3}H]-AMP, [^{3}H]ADP, and [^{3}H]ATP (Table III, row 4).

The amounts of beryllium and magnesium ions bound to the inhibited enzyme were determined as described in Materials and Methods. In the presence of ADP, fluoride, and beryllium in the inhibition medium, the fully inhibited enzyme was shown to contain ADP, beryllium, and magnesium in a ratio of 2:1:1 (Table IV, row 1). The additional presence of ATP in the inhibition medium did not modify this stoichiometry (Table IV, row 2). However, a 1:1:1 stoichiometry was obtained when a high concentration of AMP (500 μM) was present together with ADP in the inhibitory medium (Table IV, row 3). Therefore, whatever the number of ADP entrapped in the inhibited adenylate kinase, i.e., one or two, only 1 mol of beryllium and 1 mol of magnesium are present per mole of G-50 desalted, inhibited enzyme.

DISCUSSION

Adenylate kinase can be added to a list of nucleotide binding enzymes, including transducin (Bigay et al., 1987), tubulin (Carlier et al., 1988), mitochondrial ATPase (Lunardi et al., 1988), actin (Combeau & Carlier, 1988, 1989), and myosin (Maruta et al., 1991), in which the nucleotide binding site is believed to bind a nucleoside diphosphate fluorometal complex mimicking the natural nucleoside triphosphate and preventing any nucleotide exchange reaction at the nucleotide binding site. As for the mitochondrial F_1-ATPase (Issartel et al., 1991b), the fluorometal species BeF^+, BeF_2, and BeF_3^- are candidates for binding to the catalytic site of adenylate kinase. Mg^{2+} was demonstrated to be present in the nucleotide–fluorometal inhibitory complex. Interestingly, Mg^{2+} is considered a necessary component for the correct placement of the phosphate chain in the enzyme, in particular the orientation of the β–γ phosphate bond (Ray et al., 1988). On the other hand, in contrast with the other enzymes which catalyze nucleoside triphosphatase reactions wherein the product P_i is mimicked by fluorometals, adenylate kinase transfers a phosphate group from ATP bound at one site to AMP bound to another site of the enzyme.

Inhibition of Adenylate Kinase by ADP–Fluoroberyllate Depends on the Binding of ADP–Fluoroberyllate to the ATP Site of the Enzyme and of ADP or AMP to the AMP Site. Inhibition of adenylate kinase in the presence of fluoroberyllate developed in parallel with the strong binding of ADP, with full inhibition corresponding to the tight binding of 2 mol of ADP per mole of enzyme. However, when an excess of AMP with respect to ADP was present with fluoroberyllate, full inhibition corresponded to the tight binding of only 1 mol of ADP per mole of enzyme. In both cases, 1 mol of beryllium and 1 mol of magnesium per mole of enzyme were titrated in the inhibited complex, and the relief of inhibition upon dilution followed the same time course, with the recovery of half-activity in about 3 min. These experiments suggested that the fluoroberyllate and tightly bound ADP that are present in the inhibited adenylate kinase are both engaged in the same nucleotide binding site of the enzyme, in the form of an ADP–fluoroberyllate complex.

In the course of catalysis, the ATP binding site of adenylate kinase is able to recognize and bind Mg-ADP or Mg-ATP, whereas the AMP binding site specifically binds free AMP or free ADP (Noda, 1958, 1973; Kuby et al., 1962; Hamada & Kuby, 1978). It should be stressed that the AMP site

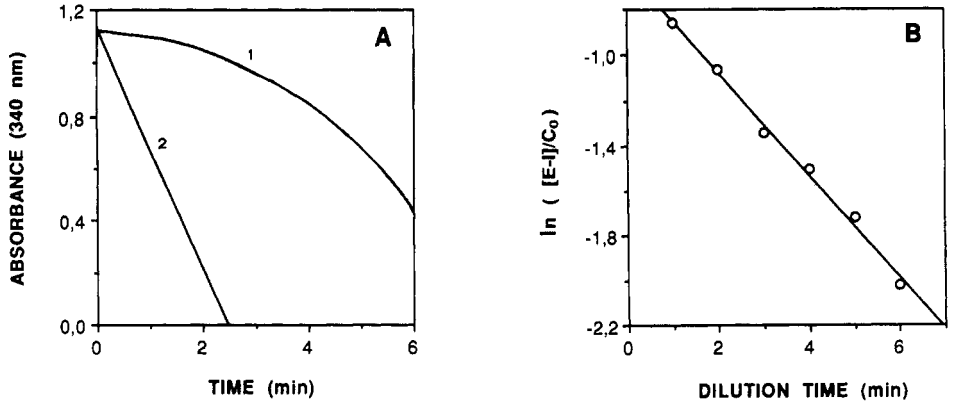

FIGURE 3: Kinetics of inhibition of adenylase kinase by ADP, NaF, $BeCl_2$, and $MgCl_2$. Panel A: (1) Adenylate kinase (1 μM) was preincubated for 5 min at 25 °C in KTMg medium in the presence of 5 mM NaF, 20 μM $BeCl_2$, and 50 μM ADP. Then, 990 μL of the assay medium (see Materials and Methods) was added to 10 μL of the inhibited enzyme. The activity of the enzyme was immediately monitored by the decrease in the absorbance at 340 nm. (2) The same conditions as above were used except that $BeCl_2$ was omitted from the preincubation medium. Panel B: Adenylate kinase (C_0 = 1 μM) was inhibited by incubation for 5 min at 25 °C in KTMg medium supplemented with 5 mM NaF, 20 μM $BeCl_2$, and 50 μM ADP. Then, at time t = 0, 990 μL of the solution for the coupled colorimetric assay without AMP and ATP was added to 10 μL of the inhibited enzyme, leading to the dilution of the inhibitory complex (it was checked that, at these final concentrations, ADP, $BeCl_2$, and NaF did not elicit inhibition of adenylate kinase). At various times (t = 1, 2, 3, 4, 5, and 6 min), called "dilution times", AMP and ATP were added and the decrease in absorbance at 340 nm was immediately monitored. For each dilution time t, the initial slope of the curve allowed the calculation of the concentration of inhibited enzyme ($[E\text{-}I]_t$) by comparison with an uninhibited sample (with no NaF and $BeCl_2$ in the preincubation medium). Then, ln ($[E\text{-}I]_t/C_0$) was plotted against the dilution time. As the plot was a straight line, it was concluded that the release of the ADP–fluorometal from adenylate kinase follows first-order kinetics ($d[E\text{-}I]/[E\text{-}I] = -k_{off}dt$). The k_{off} value was calculated from the slope of the plot.

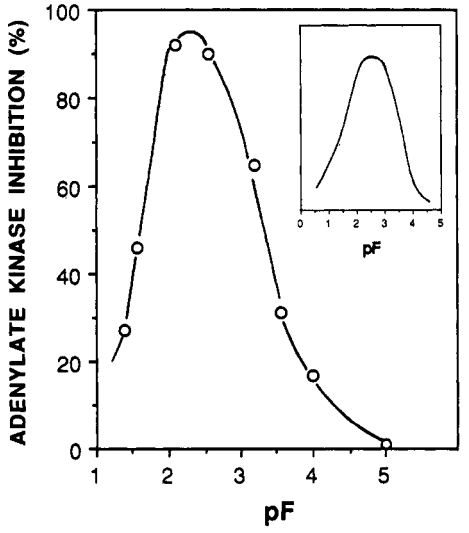

FIGURE 4: NaF-dependent inhibition of adenylate kinase in the presence of ADP and $BeCl_2$. Adenylate kinase (1 μM) was preincubated at 25 °C in KTMg medium with 50 μM ADP, 20 μM $BeCl_2$, and NaF concentrations ranging from 0.01 to 30 mM, with a final volume of 1 mL. After 5 min, 10 μL was withdrawn to measure the enzyme activity as described in Materials and Methods. The percentage of inhibition was calculated using an enzyme sample incubated with ADP alone as reference. Insert: Distribution curve for total concentrations of fluoroberyllate complexes containing one, two, and three fluorides.

binds nothing but free AMP or free ADP, whereas the specificity of the ATP site regarding the nature of the nucleotide base is broader (Noda, 1973). The view that the ADP–fluoroberyllate complex is entrapped in the ATP binding site of the enzyme is supported by the structural similarity of ATP and the ADP–fluoroberyllate complex and also by the fact that Mg^{2+} is required for the strong binding of the inhibitory ADP–fluoroberyllate complex, as it is for the binding of the true substrate, MgATP, in the ATP binding site.

The stoichiometry of nucleotides and fluoroberyllate entrapped in the inhibited adenylate kinase depends on the conditions of incubation of the enzyme with the nucleotides. The data can be interpreted as follows. In the presence of

Table III: Identification of the Nature (Mono-, Di-, or Triphosphate) of the Nucleotide Engaged in the Tightly Bound Nucleotide–Fluoroberyllate Inhibitory Complex[a]

experiment	bound [^{3}H]AMP[b]	bound [^{3}H]ADP[b]	bound [^{3}H]ATP[b]
1	700 ± 100	18000 ± 1000	850 ± 100
2	450 ± 100	9600 ± 1000	350 ● 100
3	900 ± 100	19100 ± 1000	950 ± 100
4	6500 ± 500	8200 ± 1000	5500 ± 500

[a] In experiments 1–4, adenylate kinase (20 μM) was incubated at 25 °C in KTMg medium supplemented with 5 mM NaF, 80 μM $BeCl_2$, and 100 μM [^{3}H]ADP. In experiments 2 and 3, excess [^{3}H]AMP (500 μM) and excess [^{3}H]ATP (500 μM) were added, respectively. After 5 min, the four enzyme samples were subjected to centrifugation–filtration, using 1-mL G-50 columns preequilibrated with 100 mM KCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 2 mM ADP, and 500 μM Ap_5A for experiments 1–3, and with 100 mM KCl and 50 mM Tris-HCl (pH 7.5) for experiment 4. The eluted adenylate kinase was digested by trypsin, and the nucleotides present in the digest were analyzed by HPLC (see Materials and Methods). The specific radioactivities of the [^{3}H]AMP and [^{3}H]ATP solutions were adjusted to the same value as the specific radioactivity of the [^{3}H]ADP solution. [b] Radioactivity is expressed as cpm per 10 μg of adenylate kinase.

ADP and fluoroberyllate, adenylate kinase is expected to bind one ADP as an ADP–fluoroberyllate complex in its ATP binding site and a second ADP in its AMP binding site. Excess ATP does not modify this stoichiometry, probably because the ATP binding site of the enzyme is already occupied by the strongly bound ADP–fluoroberyllate complex. On the other hand, in the presence of excess AMP, the amount of bound ADP is decreased by about one-half, and the added AMP is not recovered in a bound form after subjecting adenylate kinase to filtration through G-50 Sephadex. A possible explanation is that ADP still binds tightly to the ATP site, but AMP competes efficiently against ADP for binding to the AMP site, and the binding of AMP in this site, contrary to that of ADP, is not tight enough to survive the G-50 filtration step. To solve this dilemma, a complementary experiment was carried out, using a preincubation medium containing adenylate kinase, Mg^{2+}, fluoride, beryllium, and d-ADP instead of ADP. In contrast to the strong inhibition developed in the presence of ADP, no inhibition occurred when ADP was

Table IV: Stoichiometries of Tightly Bound ADP, Beryllium, and Magnesium on Inhibited Adenylate Kinase[a]

experiment	ADP/AK	Be/AK	Mg^{2+}/AK
1	1.8	0.8	0.9
2	1.7	0.9	0.8
3	0.9	1.1	0.8

[a] Experiment 1: Adenylate kinase (AK) (20 μM) was incubated at 25 °C in KTMg medium supplemented with 5 mM NaF, 80 μM $BeCl_2$, and 100 μM [^{3}H]ADP, i.e., under conditions resulting in full inhibition of the enzyme. After 5 min, the enzyme was loaded on a 1-mL G-50 column preequilibrated with KTMg medium. A 20-μL aliquot of the eluted material was analyzed for its protein content. The remaining adenylate kinase sample was digested by trypsin, and its nucleotide, beryllium, and magnesium contents were analyzed as described in Materials and Methods. Experiment 2: Same experimental conditions as in 1, except that excess [^{3}H]ATP (500 μM) was added in the inhibition medium. Experiment 3: Same experimental conditions as in 1, except that excess [^{3}H]AMP (500 μM) was added in the inhibition medium. The specific radioactivities of the [^{3}H]AMP and [^{3}H]ATP solutions were adjusted to the same value as the specific radioactivity of the [^{3}H]ADP solution.

replaced by d-ADP, probably because d-ADP, contrary to ADP, does not bind to the highly specific AMP site (Noda, 1973). Addition of AMP in the preincubation medium, together with d-ADP, was found to block the enzyme activity. Thus, it is clear that in conditions where d-ADP replaced ADP both the binding of d-ADP to the ATP site of the enzyme and the binding of AMP in the AMP site are required for inhibition to occur, even though the binding of AMP is rather loose, as evidenced by the fact that no enzyme-bound AMP was recovered after a G-50 filtration step. In summary, adenylate kinase inhibition by fluoride and beryllium ions develops only when the two nucleotide binding sites of the enzyme are occupied, whatever the nature of the couple of bound nucleotides.

Mechanism of Adenylate Kinase Inhibition by Fluoride and Beryllium Ions. At least two explanations, which are not necessarily exclusive of each other, can be proposed for the inhibition of adenylate kinase by nucleotides and magnesium, beryllium, and fluoride ions. (1) The strong electronegativity of fluoride might contribute to the establishment of tight hydrogen bonds between the fluoride atoms of the ADP–fluorometal complex and the amino acid residues present in the ATP binding site of the enzyme. (2) The nucleotide–fluoroberyllate complex might be a transition-state analog which blocks catalysis. Such analogs, approaching the modified structure of the substrate in the transition state, are expected to be very potent enzyme inhibitors, with affinites far exceeding that of the substrate (Wolfenden, 1972). This mechanism of inhibition has been suggested to explain the inhibition of mitochondrial F_1 (Dupuis et al., 1989) and myosin subfragment 1 (Phan & Reisler, 1992; Werber et al., 1992) ATPase activities and the stabilization of F-actin and microtubules (Combeau & Carlier, 1989) by fluorometal species. In the case of adenylate kinase, it has been shown by a stereochemical study of thiophosphoryl group transfer that the γ-phosphate of ATP is transferred directly to AMP, and not *via* a covalent phosphoryl enzyme intermediate (Richard & Frey, 1978) (Figure 5A–C). Therefore, a transition-state analog for adenylate kinase should resemble ADP interacting with AMP *via* a pentavalent phosphate. As a matter of fact, we have shown that a closely related complex is expected to form in the catalytic site of the enzyme when inhibition by beryllium and fluoride develops in the presence of both ADP and AMP (Figure 5D), with the restriction that fluoroberyllate complexes are in a tetrahedral, and not pentahedral, configuration.

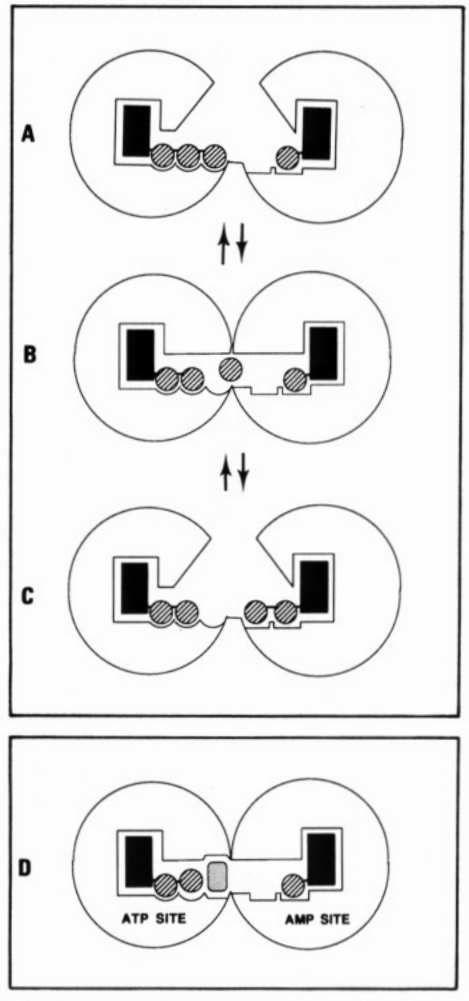

FIGURE 5: Schematic representation of adenylate kinase structure with bound ligands. Adenylate kinase nucleotide binding site structures in the course of the catalysis (A–C) and in the presence of the ADP–fluoroberyllate–AMP inhibitory complex (D). B attempts to represent the structure of the enzyme at the transition state. Black rectangle: adenine moiety. Hatched circle: phosphate group. Shaded rectangle: fluoroberyllate complex.

When AMP was omitted in the preincubation medium and ADP was the only nucleotide present, fluoroberyllate was entrapped just as efficiently by the enzyme, but in this case with two molecules of ADP. The structural similarity between the entrapped ADP–BeFx–ADP inhibitory complex and the bisubstrate analog bis(5′-adenosyl) pentaphosphate (Ap_5A) (Lienhard & Secemski, 1973) inclines us to ask the question of whether such a nucleotide–fluoroberyllate–nucleotide complex might behave as a bisubstrate analog. As shown here, the K_d value of adenylate kinase for Ap_5A is 32 nM, denoting high affinity. Among the effects that may contribute to such a high affinity for a bisubstrate analog are the special features of binding to specific regions of the enzyme active site; as mentioned by Reinstein et al. (1990), the binding of one functional part of the bisubstrate analog increases the local concentration of the other counterpart. Such a possibility might be considered if the ADP–BeFx–ADP complex and also the ADP–BeFx–AMP complex were shown to preexist in solution. With the present experimental data, especially the slow dissociation of the inhibitory complex from the inhibited adenylate kinase as expected for a transition-state analog (Jencks, 1975), the transition-state analog hypothesis appears to us more realistic.

The finding that the release of AMP from the AMP–BeFx–ADP adenylate kinase bound complex does not modify the

tightness of the binding of the remaining fluorometal nucleotide, namely, ADP–BeFx, remains puzzling. It suggests that the modified conformation of the inhibited enzyme, which might be the conformation of a first transition state, relaxes very slowly, even when the AMP moiety of the inhibitory complex is removed. In other words, in the presence of fluoroberyllate, the occupancy of both nucleotide binding sites of adenylate kinase is necessary for the enzyme to acquire a frozen conformation, which is adjusted to the conformation of the inhibitory complex; after it has been completed, this conformational change remains quite stable, independent of the events which may occur at the level of the AMP site.

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