Inactivation of Rabbit, Pig, and Carp Adenylate Kinases by N^6-*o*- and *p*-Fluorobenzoyladenosine 5′-Triphosphates†

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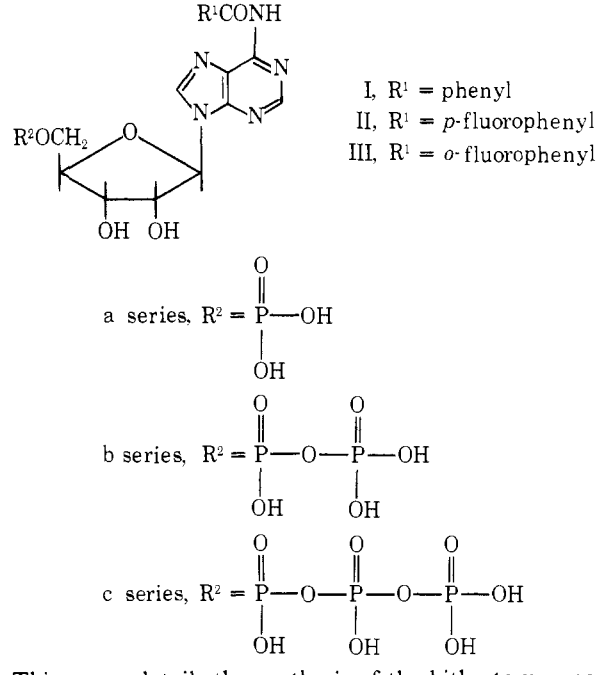
ABSTRACT: N^6-*o*- and *p*-fluorobenzoyladenosine 5′-triphosphates (IIIc and IIc, respectively) have been synthesized as potential adenosine 5′-triphosphate (ATP) site-directed reagents for enzymes. IIc and IIIc were substrates of yeast hexokinase; neither they nor the corresponding ADP derivatives inactivated yeast hexokinase or rabbit pyruvate kinase. IIc rapidly inactivated rabbit and carp muscle adenylate kinases; the effect is probably ATP site directed because N^6-benzoyl-ATP did not inactivate and was a substrate (V_{max} = 28 and 10%, respectively, that of ATP), and because ATP retarded the inactivation. The inactivations followed pseudo-first-order kinetics; in the presence of 2.64 m*M* ATP at 0° the half-life of the rabbit kinase was 210 min with 50 μM IIc and the half-life of the carp kinase was 130 min with 100 μM IIc. Adenylate kinase of pig muscle was inactivated by IIc in a manner similar to the rabbit and carp enzymes except that the rate of inactivation exhibited an inflexion. IIIc inactivated rabbit, pig, and carp adenylate kinases by pseudo-first-order kinetics; the rate constants for inactivation at 0° were 9.1×10^{-3}, 1.3×10^{-3}, and 1.9×10^{-3} min^{-1} and the apparent dissociation constants (K) of the IIIc–enzyme complexes were 710, 970, and 720 μM, respectively. From the substrate properties of IIIc alone and in admixture with ATP its dissociation constants (K_i) from the ATP sites of the enzymes were found to be 500, 770, and 845 μM, respectively. The similarity between the K and K_i values, together with marked retardation of the inactivations by ATP, indicates that IIIc is an ATP-site-directed reagent for the three adenylate kinases.

Recent work has shown that in some enzyme–ATP complexes there is room near the 6-amino group of ATP to accommodate small or medium-sized N^6 substituents. Thus, 1,N^6-etheno-ATP is a substrate of AMP kinase, pyruvate kinase, hexokinase, and phosphofructokinase (Secrist et al., 1972), while N^6,N^6-dimethyl-ADP is a substrate of pyruvate kinase (Hohnadel and Cooper, 1973) and a variety of N^6-alkyl and N^6-acyl derivatives of ATP are good substrates of yeast hexokinase (Gabbai and Posternak, 1971). These findings prompted us to prepare N^6-*o*- and *p*-fluorobenzoyl-ATP (IIIc and IIc) as candidate ATP-site-directed reagents. The fluorobenzoyl substituent[1] was selected for the following reasons: (1) the relatively small size of fluorine should minimize steric hindrance to reaction with enzymic nucleophilic groups; (2) fluorine has a considerably greater tendency than the other halogens to undergo nucleophilic displacement from halogenonitrobenzenes (Bunnett and Zahler, 1951; Miller, 1968) and nonactivated halobenzenes (Boswell et al., 1974) and does so under surprisingly mild conditions; (3) the parent fluorobenzamides are stable in physiological buffers; (4) in addition to the ortho and para carbons the benzamido carbonyl groups of IIc and IIIc also can readily be attacked by a nucleophilic group of an enzyme, thus doubling the probability that IIc or IIIc might form a covalent bond at the ATP site of any given enzyme.



This paper details the synthesis of the hitherto unreported N^6-benzoyl derivatives of AMP, ADP, and ATP (structures Ia, Ib, and Ic, respectively) of their *p*-fluoro derivatives IIa, IIb, and IIc, and of their *o*-fluoro derivatives IIIa, IIIb, and IIIc. The substrate and inhibitor properties of these analogues with yeast hexokinase, rabbit pyruvate kinase, and rabbit, pig, and carp muscle AMP kinases are described. IIc and IIIc cause irreversible inhibition of the three AMP kinases, and evidence is presented which suggests that they react covalently at the ATP sites. The inactivation of pig AMP kinase is currently of additional in-

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[1] This substituent was used for the same purpose in earlier studies with 5′-acylaminomethyl derivatives of adenine nucleotides (Kappler and Hampton, 1975; A. Hampton and F. Kappler, unpublished findings).

terest because the amino acid sequence of this enzyme is known (Heil et al., 1974) and an x-ray crystallographic analysis at 3 Å of its structure has been reported (Schulz et al., 1974).

Experimental Section

Synthesis of ADP and ATP Derivatives. Pyridine was distilled from *p*-toluenesulfonyl chloride and then from calcium hydride and stored over potassium hydroxide. *N,N*-Dimethylformamide was distilled from calcium hydride and stored over molecular sieves. Dioxane was distilled from phosphorus pentoxide. Tri-*n*-butylamine, triethylamine, diphenyl phosphorochloridate, benzoyl chloride, and *o*- and *p*-fluorobenzoyl chlorides were distilled before use. AMP was purchased from P-L Biochemicals. Tri-*n*-butylammonium pyrophosphate was prepared at room temperature according to the method of Moffatt and Khorana (1961) and stored at 5°. Paper chromatography (Table I) was carried out by the descending technique on Whatman No. 1 paper in (A) isobutyric acid–1 *M* NH_4OH (60:40) and (B) 1-propanol–water (7:3). Electrophoresis was carried out on Whatman No. 1 paper at pH 3.5 in 0.035 *M* citric acid–0.0148 *M* sodium citrate (1:1). Ultraviolet spectra were obtained on a Cary Model 15 spectrophotometer and ^1H nuclear magnetic resonance (^1H NMR) spectra with a Varian XL-100-15 instrument and are recorded as parts per million downfield from an external standard (concentric capillary) of $SiMe_4$. Evaporations were carried out in vacuo at bath temperatures below 30°. Elemental analyses were performed by Midwest Microlab, Ltd., Indianapolis, Ind., and Galbraith Laboratories Inc., Knoxville, Tenn. Phosphate analyses of nucleoside di- and triphosphates were performed by the method of Lowry and Lopez (1946) after treatment of approximately 1 μmol of these compounds for 60 min at 22° in 1 ml of Tris buffer (pH 10.4) containing 0.02 mg of alkaline phosphatase of calf intestinal mucosa (Type VII, Sigma Chemical Co.).

N^6-Benzoyladenosine 5′-Phosphate (Ia). The procedures are modifications of those used by Ralph and Khorana (1961) for the preparation of *N*-benzoyldeoxyadenosine 5′-phosphate. That the benzoyl group is located at N^6 follows from the demonstration by Anzai and Matsui (1973) that both benzoyl groups of *N,N*-dibenzoyladenosine are located at N^6. An aqueous solution of AMP (1.44 mmol) was percolated through a column of Dowex 50 ion-exchange resin (pyridinium form, 20 ml). The eluate and aqueous washings were combined and evaporated in vacuo and the residue was dried by repeated evaporation from it of pyridine (5 × 30 ml). To the residual oil was added pyridine (30 ml) and benzoyl chloride (3.75 ml, 30 mmol) and the light yellow solution was stirred under anhydrous conditions in the dark. After 1.5 hr at room temperature it was poured into cold (<5°) water–chloroform (1:1, 200 ml) and the mixture was kept at 0° for 15 min. The aqueous layer was washed with chloroform (2 × 30 ml) and the chloroform solutions were combined and evaporated in vacuo. A solution of the residue in pyridine–water (2:1, 60 ml) was cooled in an ice bath. Aqueous 2 *N* NaOH (50 ml) at 0° was added and the mixture was stirred in an ice bath for 9 min. Excess of pyridinium Dowex-50 was added to remove sodium ions and the resin was removed by filtration and washed with water. The eluates were concentrated in vacuo to a gum which was extracted with anhydrous ether (5 × 50 ml). The residue was treated with anhydrous methanol (10 ml) and triethylamine (0.5 ml, 3.6 mmol) and volatiles were removed in vacuo. The residue was dissolved in methanol (10 ml) with gentle warming and 3 ml of 1 *M* NaI in acetone was added. An excess of acetone was added and the precipitate thereby obtained was dried over P_2O_5 in vacuo at room temperature to give Ia as a white amorphous powder (725 mg, 90% yield): uv λ_{max} (pH 7) 281 nm (ε 20300); ^1H NMR (100 MHz, Me_2SO-d_6) 9.13 (s, 2, H-2 and H-8), 8.46 (d, 2, J = 6 Hz, *o*-Ph), 7.91 (m, 3, *m*- and *p*-Ph), 6.50 (d, 1, J = 5 Hz, H-1′), 5.40–4.60 (broad m, H-2′, H-3′, H-4′, H-5′, H-5″, and H_2O). Anal. Calcd for $C_{17}H_{16}N_5O_8PNa_2 \cdot 3.5\ H_2O$: C, 36.57; H, 4.15; N, 12.54; P, 5.55. Found: C, 36.73; H, 3.92; N, 13.03; P, 5.30.

Table I: Paper Chromatography and Electrophoresis.

Compd	Electrophoretic Mobility,[a] pH 3.5	R_f in System A	R_f in System B
Ia	1.26	0.71	0.31
Ib	1.79	0.58	0.21
Ic	1.85	0.49	0.19
IIa	1.26	0.73	0.33
IIb	1.71	0.61	0.22
IIc	1.78	0.50	0.17
IIIa	1.25	0.73	0.34
IIIb	1.72	0.58	0.22
IIIc	1.80	0.50	0.18
AMP	1.00	0.57	0.20

[a] Values are relative to those of AMP.

N^6-Benzoyladenosine 5′-Triphosphate (Ic). Ia (0.18 mmol) was converted to Ic by the method of Michelson (1964) except that the final purification was performed by descending chromatography on 6 sheets of Whatman 3MM paper (each 23 cm wide) in solvent A. The band at R_f 0.49 was eluted with water; the yield of Ic determined spectrophotometrically was 64%. The solution was concentrated to dryness and treated with methanol (5 ml) and triethylamine (0.2 ml). The mixture was warmed to effect dissolution and the solution was evaporated to dryness and treated with methanol (1 ml) and 1 *M* NaI in acetone (0.3 ml). Excess of acetone was added and the precipitate was collected and dried over P_2O_5 in vacuo at room temperature to give Ic (86 mg) as a white amorphous powder which was homogeneous in the systems of Table I. The uv spectrum in water (pH 7) showed a maximum at 281 nm (ε 19400, calculated for a trihydrate of the tetrasodium salt). The action of alkaline phosphatase liberated 3.05 times more inorganic phosphate from Ic than from an equivalent amount of AMP.

N^6-Benzoyladenosine 5′-Diphosphate (Ib). Compound Ic (20 mg, 0.027 mmol) was incubated at 37° for 24 hr in a solution containing 0.523 ml of 0.1 *M* Tris-HCl (pH 7.6), 0.067 ml of 0.1 *M* $MgCl_2$, 0.4 ml of 10% glucose solution, and 10 μl of diluted yeast hexokinase solution (20 μl of commercial hexokinase preparation + 980 μl of Tris buffer + 10 mg of glucose). The solution was applied across 46 cm of Whatman 3MM paper which was then developed in solvent A. The band at R_f 0.58 was eluted with water and the extract was lyopholized. The sodium salt of Ib was isolated in the manner described above for Ic and obtained as a white amorphous powder (16 mg, 92% yield) which was homogeneous in the systems of Table I. The uv spectrum in water (pH 7) showed a maximum at 281 nm (ε 19400, calculated for a trihydrate of the trisodium salt). Alkaline phosphatase liberated 1.94 times more inorganic phosphate from Ib than from an equivalent amount of AMP.

N^6-p-Fluorobenzoyladenosine 5'-Monophosphate (IIa). This was prepared from AMP and *p*-fluorobenzoyl chloride by the same procedures as described above for the synthesis of Ia except that treatment of the reaction products with NaOH–pyridine–water was allowed to proceed for only 4 min. The yield of IIa was 84%: uv_{max} (pH 7) 282 nm (ϵ 20100); ^1H NMR (100 MHz, Me_2SO-d_6) 9.12 (s, 2, H-2 and H-8), 8.53 (broad m, 2, *o*-Ph), 7.71 (broad m, 2, *m*-Ph), 6.47 (broad s, 1, H-1'), 5.45–4.44 (broad m, H-2', H-3', H-4', H-5', H-5'', and H_2O).

Anal. Calcd for $C_{17}H_{15}N_5O_8FPNa_2 \cdot 3H_2O$: C, 35.98; H, 3.73; N, 12.35; P, 5.46; F, 3.35. Found: C, 35.83; H, 3.81; N, 12.09; P, 5.40; F, 3.57.

N^6-p-Fluorobenzoyladenosine 5'-Triphosphate (IIc). Compound IIa (100 mg, 0.16 mmol) was converted to IIc in an identical manner as conversion of Ia to Ic. The yield was 60.7 mg (50%) and the product was homogeneous in the systems of Table I; uv λ_{max} (pH 7) 282 nm (ϵ 20900; calculated for a trihydrate of the tetrasodium salt). The product produced 2.92 times as much inorganic phosphate as an equivalent amount of AMP after treatment with alkaline phosphatase.

N^6-p-Fluorobenzoyladenosine 5'-Diphosphate (IIb). This was prepared from IIc (24 mg) by the method used for the synthesis of Ib. The sodium salt (82% yield) was a white amorphous powder which was homogeneous in the systems of Table I; uv λ_{max} (pH 7) 282 nm (ϵ 20540; calculated for a trihydrate of the trisodium salt). Alkaline phosphatase liberated 2.04 times more inorganic phosphate from IIb than from an equivalent amount of AMP.

N^6-o-Fluorobenzoyladenosine 5'-Phosphate (IIIa). The procedures were the same as those for IIa. The reaction of *o*-fluorobenzoyl chloride with AMP (1.44 mmol) gave the disodium salt of IIIa as a white amorphous powder (671 mg, 79% yield; dried over P_2O_5 in vacuo at 25°): uv max (pH 7) 282 nm (ϵ 19800); ^1H NMR (100 MHz, Me_2SO-d_6) δ 9.12 (s, 2, H-2 and H-8), 8.20 (broad m, 1, *o*-Ph), 7.60 (broad m, 1, *p*-Ph), 7.0–7.3 (broad m, 2, *m*-Ph), 6.47 (broad s, 1, H-1'), 5.45–4.44 (broad m, H-2', H-3', H-4', H-5', H-5'', and H_2O). Anal. Calcd for $C_{17}H_{15}N_5O_8FPNa_2 \cdot 4.5H_2O$: C, 34.35; H, 4.07; N, 11.78; P, 5.21; F, 3.20. Found C, 34.52; H, 3.47; N, 11.59; P, 5.14; F, 3.20.

N^6-o-Fluorobenzoyladenosine 5'-Triphosphate (IIIc). The procedure was based on the method of Hoard and Ott (1965) for the synthesis of deoxynucleoside 5'-triphosphates. The sodium salt of IIIc (yield 69%) was white amorphous powder which was homogeneous in the systems of Table I. The uv spectrum in water (pH 7) showed a maximum at 282 nm (ϵ 20300, calculated for a trihydrate of the tetrasodium salt). The action of alkaline phosphatase liberated 2.89 times more inorganic phosphate from IIIc than from an equivalent amount of AMP.

N^6-o-Fluorobenzoyladenosine 5'-Diphosphate (IIIb). This was prepared from IIIc as described for the preparation of Ib. The sodium salt, which was obtained in 90% yield, was a white powder which showed only a single component in the systems of Table I; the uv spectrum in water (pH 7) showed a maximum at 282 nm (ϵ 19700, calculated for a trihydrate of the trisodium salt). Alkaline phosphatase liberated 2.06 times more inorganic phosphate from IIIb than from an equivalent amount of AMP.

Enzyme Kinetic Studies. Pig and rabbit muscle adenylate kinase, rabbit muscle pyruvate kinase, and yeast hexokinase were purchased from Boehringer while carp muscle myokinase was a gift from Dr. Mildred Cohn. All assays were carried out at 22° by measuring the rate of change of optical density at 340 nm for a period of 5 min in a Cary Model 15 spectrophotometer using 1-cm cells containing a final volume of 1 ml. Initial velocities were in every case linear and proportional to the concentration of primary enzyme and independent of the level of secondary enzymes in the assay systems. Each substrate study employed four or more concentrations of substrate and each study of reversible inhibition employed, in addition, two or more levels of inhibitor. Substrate constants were determined from Lineweaver-Burk plots, all of which were linear, and enzyme-inhibitor dissociation constants (K_i values) were obtained from replots of inhibitor concentration vs. slope in cases where such replots were linear. When the replots were nonlinear, apparent K_i values were determined from the slopes given by zero inhibitor and the lowest inhibitor level studied.

For studies of substrate and reversible inhibitor kinetics each adenylate kinase was employed in 1 ml of 0.1 *M* Tris-HCl (pH 7.6) containing $MgSO_4$ (0.92 m*M*), KCl (0.11 *M*), phosphoenolpyruvate cyclohexylammonium salt (0.31 m*M*), NADH (0.38 m*M*), AMP (0.39 m*M*; included in the studies of ATP and ATP analogues), ATP (0.24 m*M*; included when studying AMP and AMP analogues), pyruvate kinase (10 μg), and lactate dehydrogenase (10 μg). In studies of the AMP derivatives as inhibitors a stock solution of ATP (5 mg), P-enolpyruvate (5 mg), NADH (10 mg), 0.1 *M* Tris-HCl (pH 7.6, 1.42 ml), 4 *M* KCl (1 ml), 32 m*M* $MgSO_4$ (1 ml), 35 μl pyruvate kinase (10 mg/ml), 35 μl of LDH (10 mg/ml), and the adenylate kinase (10 μl, 1.82 μg) was made daily and stored at 22° for 1 hr to permit stabilization of the absorbancy at 340 nm; 100 μl of this solution was added to 0.1 *M* Tris-HCl after which the AMP derivative and/or AMP were added to bring the volume to 1 ml. For studies of reversible inhibition by the ATP derivative IIc, ATP was omitted from the above stock solution which then required no storage period prior to use. ATP was added to the assay mixture 1 hr prior to addition of IIc after which the reaction was initiated by the addition of AMP (3.9 m*M*, 100 μl). The K_i values of Ic and IIIc were obtained by adding each together with an equimolar amount of ATP to the same assay mixture and the reaction was then started with the same final level (0.39 m*M*) of AMP.

Studies of the rate of inactivation of rabbit and carp adenylate kinase by IIc were carried out in solutions which included ATP (2.64 m*M*) and all the assay components except AMP at levels such that addition of 400 μl to 500 μl of Tris buffer and 100 μl of 3.9 m*M* AMP gave the assay concentrations listed above except that the final concentration of Mg^{2+} was 4.5 m*M* and that of ATP was 1.06 m*M*. Studies with pig adenylate kinase and IIc employed higher levels of ATP and proportionately more Mg^{2+}. Studies of inactivation by IIIc were carried out similarly except that ATP was absent during inactivation and that Mg^{2+} and ATP were employed at their standard assay levels. Mixtures lacking IIc or IIIc were utilized as controls to monitor denaturation of the enzymes. Inactivation mixtures and their controls were maintained at 0° and assayed at 22°.

Pyruvate kinase was studied in 1 ml of 0.1 *M* Tris-HCl (pH 7.6) containing $MgCl_2$ (10 m*M*), KCl (0.1 *M*), phosphoenolpyruvate cyclohexylammonium salt (0.066 m*M*), NADH (0.15 m*M*), and lactate dehydrogenase (12 μg).

Hexokinase was studied in 1 ml of 0.1 *M* Tris-HCl (pH 7.6) containing $MgCl_2$ (6 m*M*), α(D)-glucose (0.2 *M*),

Table II: Substrate and Inhibition Constants of the N^6-Substituted Adenine Nucleotides.

Enzyme	Compd	Substrate Constants: Protein per Assay (μg)	K_m (μM)	V_{max} (rel. %)	Inhibition Constants: Type[p]	K_i[q] (μM)
Adenylate kinase (rabbit muscle)	AMP	0.052	320	100[a]		500[b]
	Ia	1×10^3		0	L, NC	160[c]
	IIa	1×10^3		0	L, NC	290[d]
	IIIa	1×10^3		0	L, NC	330[u]
	ATP	0.052	330	100[e]		330[t]
	Ic	0.49	1250	28		995[s]
	IIc	1.40		0	NL, C	[f]
	IIIc	0.052	500	58		500[s]
Adenylate kinase (pig muscle)	AMP	0.052	250	100[g]		
	Ia	1×10^3		0	L, NC	225[c]
	IIa	1×10^3		0	L, NC	315[d]
	IIIa	1×10^3		0	L, NC	360[u]
	ATP	0.052	180	100[h]		180[r]
	Ic	0.49	1000	31		
	IIc	1.40		0	NL, NC	[f]
	IIIc	0.104	385	47		770[s]
Adenylate kinase (carp muscle)	AMP	0.28	570	100[i]		
	Ia	50.0		0	L, NC	155[j]
	IIa	50.0		0	L, NC	3300[k]
	IIIa	1×10^3		0	L, NC	410[u]
	ATP	0.24	800	100[i]		
	Ic	0.60	690	10		
	IIc	1.00		0	NL, C	[f]
	IIIc	0.24	625	12		845[s]
Pyruvate kinase (rabbit)	ADP	0.10	312	100[l]		
	Ib	10.0	625	0.5	L, C	630[m]
	IIb	200.0		0	NL, C	53[n]
	IIIb	10.0	600	0.5		
Hexokinase (yeast)	ATP	0.1	101	100[o]		
	Ic	0.2	46	56		
	IIc	0.2	115	70		
	IIIc	0.2	85	53		

[a] V_{max} was 135 μmol per min per mg of protein. [b] Value from Noda (1962). [c] The levels of inhibitor were 89 and 178 μM. [d] The levels of inhibitor were 146 and 367 μM. [e] V_{max} was 122 μmol per min per mg of protein. [f] The levels of IIc were 50 and 100 μM. K_i values could not be assigned because of concomitant enzyme inactivation. [g] V_{max} was 115 μmol per min per mg of protein. [h] V_{max} was 100 μmol per min per mg of protein. [i] V_{max} was 144 μmol per min per mg of protein. [j] Ia was employed at 96 and 240 μM. [k] IIa was employed at 1.11 and 2.22 mM. [l] V_{max} was 119 μmol per min per mg of protein. [m] 190 and 380 μM levels of Ib were used. [n] IIb used at 57 and 113 μM. [o] V_{max} was 106 μmol per min per mg of protein. [p] L = linear, NL = nonlinear, C = competitive, NC = noncompetitive. [q] The calculation of apparent K_i values for NL cases is described in the Experimental Section. [r] The assignment of this value is explained in the Discussion. [s] Determined from the V_{max} of an equimolar mixture of the ATP derivative with ATP by the method of Webb (1963). [t] Callaghan and Weber (1959) showed that $K_i = K_m$. [u] IIIa was employed at 185 and 370 μM.

NADPH (0.78 mM), and glucose-6-phosphate dehydrogenase (35 μg).

Results

Hexokinase. The ATP derivatives Ic, IIc, and IIIc were good substrates of the enzyme and gave linear Burk–Lineweaver plots; the K_m and V_{max} values obtained therefrom are listed in Table II. IIc and IIIc displayed no tendency to inactivate the enzyme during the rate determinations. Further studies of possible inactivation were not undertaken. The effectiveness of these triphosphates as substrates was utilized for the preparation of the corresponding ADP derivatives Ib, IIb, and IIIb (described in the Experimental Section).

Pyruvate Kinase. N^6-Benzoyl-ADP (Ib) was a substrate and a linear competitive inhibitor; the dissociation constant and the Michaelis constant (Table II) were the same within experimental error. N^6-*o*-Fluorobenzoyl-ADP had similar substrate properties to Ib, but N^6-*p*-fluorobenzoyl-ADP (IIb) had no detectable substrate properties with high levels of enzyme and displayed a nonlinear competitive inhibition in which the slope given by the higher level of IIb was ca. 20% less than required for linearity. Compound IIc was not inhibitory at a level of 100 μM. In the presence of all the assay components except ADP, the enzyme was not inactivated when exposed at 0° for 5 hr to IIc (100 μM) or for 6 hr to IIb (260 μM) or IIIb (3 mM).

Adenylate Kinase (Rabbit Muscle). The AMP derivatives Ia, IIa, and IIIa showed no substrate activity and were noncompetitive inhibitors (Table II). N^6-Benzoyl-ATP and its *o*-fluoro derivative were good substrates whereas the *p*-fluoro derivative IIc showed no substrate activity in the presence of three times more enzyme. In examining these ATP derivatives as possible substrates of the three adenylate kinases of the present work, it was necessary to employ less than 1.5 μg of each kinase per assay in order to delay for approximately 10 min the onset of a sharply accelerating conversion of the AMP to ATP initiated by traces of ATP and/or ADP present in the adenylate kinase preparations.

The dissociation constant (K_i) of IIIc for its binding to the ATP site of the enzyme is given in Table II and was determined from the equation of Webb (1963) which relates the K_i values of two substrates to their individual V_{max} values and to the V_{max} of an equimolar mixture of the two. Determination of the V_{max} of an equimolar mixture of ATP and IIIc is shown in Figure 1.

Compound IIc behaved as a nonlinear competitive inhibi-

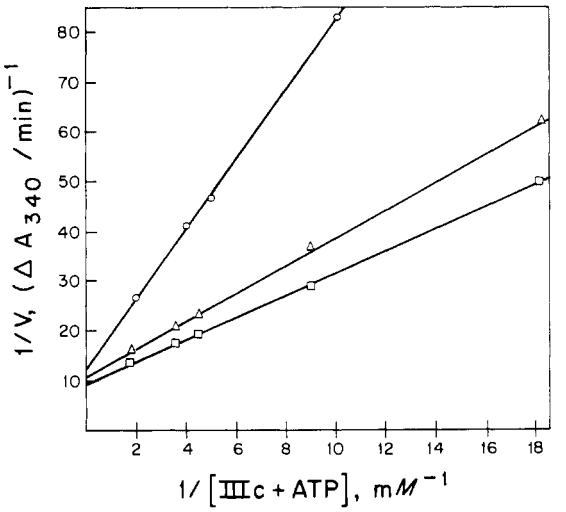

FIGURE 1: Substrate activity of equimolar mixtures of ATP and its N^6-*o*-fluorobenzoyl derivative IIIc with rabbit (□), pig (△), and carp (○) adenylate kinases. Amounts of enzyme per assay were 0.052 μg (pig and rabbit) and 0.24 μg (carp).

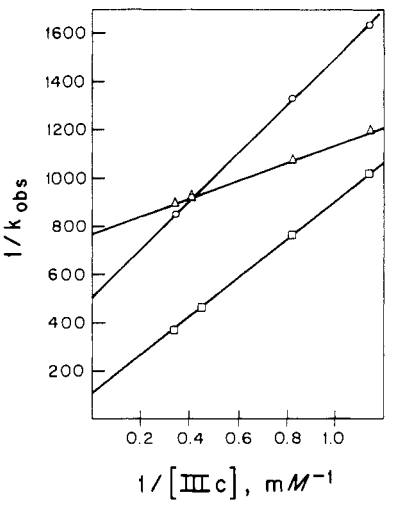

FIGURE 2: Inactivation of rabbit (□), pig (△), and carp (○) adenylate kinases by N^6-*o*-fluorobenzoyl-ATP (IIIc); k_{obsd} is the apparent first-order rate constant for inactivation by a given level of IIIc.

tor with respect to ATP. Other data (see below) show that under the conditions used (IIc, 50 and 100 μ*M*; ATP, 125 μ*M* to 1.5 m*M*) the observed reaction velocities, though giving linear double-reciprocal plots, must to varying degrees be diminished by partial inactivation of the enzyme. No attempt was made to make a more precise study of reversible inhibition because the levels of ATP required to virtually suppress inactivation are five- to tenfold higher than the Michaelis constant of ATP and measurements of inhibition would consequently diminish in accuracy.

When the enzyme was exposed at 0° in the assay buffer (pH 7.6) to 50 μ*M* IIc, 33% of the activity was lost within 3 min, and after 100 min 50% of the activity was lost at which time 11% denaturation of the control had taken place. With 100 μ*M* IIc, 75% of the activity was lost within 5 min. When the pH was reduced to 6.6 with the object of showing the initial rapid rate of inactivation to a measurable value, denaturation became too rapid. In the presence of 2.64 m*M* ATP the process was slowed sufficiently to permit study of all stages of the progressive loss of activity; for example, 50 μ*M* IIc effected 33% inactivation after 110 min at 0° but did so in less than 3 min in the absence of ATP. The rate of inactivation at 0° by 25 and 50 μ*M* IIc in the presence of 2.64 m*M* ATP was studied over a period of 5 hr. Control mixtures which lacked IIc showed a slow linear decrease in activity and after 5 hr 91–95% of the initial activity was still present. The logarithm of the residual enzyme activity bore a linear relationship to the time of exposure of the enzyme, indicating that the inactivation follows first-order kinetics. The half-life of the enzyme was 350 min with 25 μ*M* IIc and 210 min with 50 μ*M* IIc under these conditions.

Compound IIIc also progressively inactivated the enzyme; it was not necessary, as in the case of IIc, to add ATP in order to study the kinetics. The inactivation followed first-order kinetics at 0° during at least 400 min; the apparent first-order rate constants (k_{obsd}) for inactivation by four levels (0.88, 1.22, 2.22, and 2.93 m*M*) of IIIc were calculated from the elapsed times for 50% inactivation. The reciprocals of the k_{obsd} values were plotted against the reciprocal of the IIIc concentration (Figure 2). From the equation of Gold and Fahrney (1964) given in the Discussion it follows that the slope of this plot is K/k_2 and the intercept on the vertical axis is $1/k_2$ from which k_2 (the pseudo-first-order rate constant for formation of a bond between IIIc and the enzyme) was found to be 9.1×10^{-3} min^{-1} and K (defined in the Discussion) was 710 μ*M*.

Compounds Ic, IIc, and IIIc were stable in 0.1 *M* Tris-HCl (pH 7.6) at 0° for at least 3 months. In addition, the compounds were stable for at least 24 hr at 25° in the presence of all the foregoing assay components (excluding adenylate kinase and AMP) as shown by paper electrophoresis and paper chromatography in solvent A followed by elution of appropriate areas with water and determination of the uv spectra of the eluate at appropriate pH values.

No inactivation of the enzyme was detected upon treatment of it for 6 hr at 0° with 1.1 m*M* *p*-fluorobenzamide, 1.1 m*M* IIa, or 3 m*M* IIIa in the presence of all the assay components except AMP and ATP. No inactivation was observed upon similar treatment of the enzyme for 6.5 hr with Ic (1.3 m*M*). In this case enzyme activity was assayed after addition only of AMP since Ic is an efficient substitute for ATP. No inactivation of the assay components was observed when they were treated at 0° for 6 hr with 100 μ*M* IIc or 2.2 m*M* IIIc in the absence of AMP, ATP, and adenylate kinase.

Adenylate Kinase (*Carp Muscle*). The substrate and reversible inhibitor properties of Ia, IIa, IIIa, Ic, IIc, and IIIc (Table II) were qualitatively the same as for the adenylate kinase from rabbit muscle. Determination of the K_i value of IIIc is shown in Figure 1. In the absence of ATP IIc inactivated the enzyme too rapidly to permit determination of the initial rate of inactivation. In the presence of 2.64 m*M* ATP inactivation proceeded at a convenient rate when 100 or 200 μ*M* IIc was employed, and the relationship between the logarithm of the residual enzyme activity and the time of treatment of the enzyme was linear for both levels of IIc. The half-life of the carp enzyme at 0° was found to be 130 and 110 min, respectively. In the absence of IIc the enzyme lost 1% of its activity during 6 hr at 0°.

IIIc inactivated the carp enzyme at 0° at a similar rate as the rabbit enzyme and by first-order kinetics. During the total time (6 hr) of the rate determination, control mixtures lacking IIIc lost 1–2% of their activity. From the slope and intercept of the plot shown in Figure 2 k_2 was found to be 1.9×10^{-3} min^{-1} and K to be 720 μ*M*. The velocity of in-

activation was reduced by ATP in a quantitatively similar manner as for the rabbit enzyme.

Adenylate Kinase (Pig Muscle). The substrate and inhibitor properties of compounds Ia, IIa, IIIa, Ic, and IIIc (Table II) resembled those observed with the corresponding rabbit and carp muscle enzymes. The K_i value of IIIc was calculated from the plot shown in Figure 1. The reversible-type inhibition by IIc was noncompetitive with respect to ATP at similar ATP levels (0.2–1.0 mM) as those employed in the case of the rabbit and carp AMP kinases. The enzyme lost all activity when treated with 400 μM IIc for 10 min at 0°; ATP was then added to a level of 16 mM but failed to restore any enzyme activity after 5 hr. Figure 3 shows the time course of enzyme inactivation by 100 μM IIc and illustrates a substantial reduction in the rate of inactivation brought about by prior addition of 3 mM ATP. In the presence of 3 mM ATP the rate of inactivation exhibited an inflexion after about 60 min either when residual enzyme activity was plotted against time, as in Figure 3, or when the logarithm of residual enzyme activity was plotted against time.

IIIc inactivated the enzyme at 0° by first-order kinetics. During the total time (6 hr) of the inactivation studies control mixtures lacking IIIc lost 4–6% of their activity in linear fashion. Inactivation by IIIc was corrected for these losses in activity. The plot in Figure 2 gave $k_2 = 1.3 \times 10^{-3}$ min^{-1} and $K = 970$ μM. ATP reduced the rate of inactivation to a similar degree as it did in the case of the rabbit enzyme.

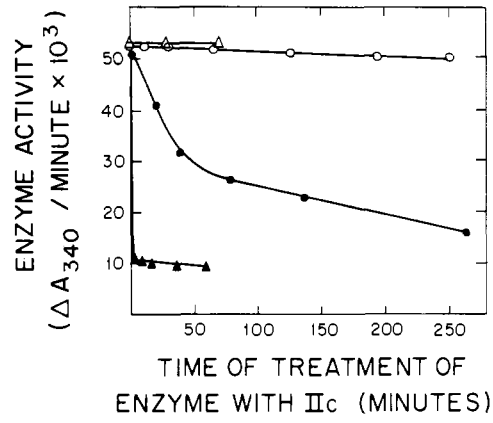

FIGURE 3: Rate of inactivation of pig muscle AMP kinase at 0° by 100 μM IIc. Conditions used: IIc present, ATP absent (▲); IIc and ATP absent (△); IIc and 3 mM ATP present (●); IIc absent, 3 mM ATP present (○).

Discussion

The effectiveness of N^6-benzoyl-ATP and the N^6-*o*- and *p*-fluorobenzoyl-ATP derivatives as substrates of yeast hexokinase (Table II) is not surprising because other ATP derivatives bearing comparably large N^6 substituents (e.g., butyryl) are also good substrates of this enzyme (Gabbai and Posternak, 1971). That N^6-benzoyl-ADP and N^6-*o*-fluorobenzoyl-ADP are substrates of muscle pyruvate kinase is consistent with the absence of a specific interaction between this enzyme and the adenine ring of ADP which is replaceable by guanine, cytosine, or uracil with little loss of substrate activity (Plowman and Krall, 1965); furthermore, N^6,N^6-dimethyl-ADP (Hohnadel and Cooper, 1973) and 1,N^6-etheno-ADP (Secrist et al., 1972) are also substrates. However, N^6-*p*-fluorobenzoyl-ADP showed no substrate activity in the presence of 20-fold more pyruvate kinase than was used to obtain the substrate constants of N^6-benzoyl-ADP. N^6-Benzoyl-ADP and its *p*-fluoro derivative were competitive inhibitors of pyruvate kinase with respect to ADP and their respective K_i values (Table II) reveal that introduction of the fluorine atom in the para position enhances binding to the enzyme approximately 12-fold. This suggests that the lack of substrate properties of N^6-*p*-fluorobenzoyl-ADP might result from some type of dipole–dipole binding between the *p*-fluorobenzoyl group and pyruvate kinase rather than from lack of room in the complex between N^6-*p*-fluorobenzoyl-ADP and the enzyme to accommodate the fluorine.

N^6-*p*-Fluorobenzoyl-ATP (IIc) was a powerful inhibitor of rabbit muscle AMP kinase. In the absence of ATP a rapid and progressive loss of enzyme activity occurred, e.g., a concentration of IIc which was 15% that of the ATP–enzyme dissociation constant caused 33% reduction in enzyme activity in less than 3 min at 0°. The effect was concluded to be exerted exclusively on the AMP kinase and to be mediated by N^6-*p*-fluorophenyl-ATP itself because this compound did not inhibit either of the two additional enzymes (pyruvate kinase and lactic dehydrogenase) present in the coupled assay system nor could it be demonstrated to react with any other components of that system; moreover, progressive loss of AMP kinase activity occurred at a comparable rate upon exposure of AMP kinase to the inhibitor in a simple buffer solution. N^6-Benzoyl-ATP did not inactivate rabbit AMP kinase at a 100-fold higher concentration and a 400-fold longer time than required by its *p*-fluoro derivative to produce significant inactivation and hence the effect almost certainly involves covalent bond formation between N^6-*p*-fluorobenzoyl-ATP and a nucleophilic group of the enzyme. The leaving group in this reaction was not identified, and it could either be fluoride ion, in view of the pronounced electrophilic character of fluoroaromatics described in the introduction, or it could be ATP or ADP, since during the chemical synthesis of N^6-*p*-fluorobenzoyl-AMP and its *o*-fluoro isomer it was noted that introduction of the fluorine markedly promotes N^6-debenzoylation by hydroxyl ions. Various lines of evidence suggest that formation of the enzyme–inhibitor bond is preceded by reversible adsorption of N^6-*p*-fluorobenzoyl-ATP at the enzymic ATP binding site: (a) the marked effectiveness of N^6-benzoyl-ATP as a substrate indicates that its *p*-fluoro derivative, which possesses little extra bulk (van der Waals radii: F, 1.35; H, 1.00), probably also adsorbs to the ATP site; (b) ATP retards the inactivation (illustrated in Figure 3 for pig adenylate kinase); (c) substrate specificity data and magnetic resonance studies (Price et al., 1973) show that the AMP and ATP sites of rabbit AMP kinase are not equivalent and the inability of N^6-*p*-fluorobenzoyl-AMP to inactivate the enzyme establishes that the irreversible inhibition caused by the corresponding ATP derivative IIc is not AMP site directed.

The rapid inactivation of carp adenylate kinase by IIc also appears to be ATP site directed on the basis of the lines of evidence listed above for the rabbit enzyme. In the case of the pig enzyme the evidence for ATP-site-directed inactivation is somewhat weaker insofar as IIc behaved as a noncompetitive reversible inhibitor under the conditions used, and the time course of inactivation (Figure 3) showed an inflexion which precluded determination of the order of the reaction, at least over the period during which inactivation exceeded 20%. The inflexion may indicate differences of re-

activity toward IIc among the ATP sites in the pig enzyme preparation.

N^6-*o*-Fluorobenzoyl-ATP (IIIc) was an efficient phosphoryl donor for rabbit, pig, and carp AMP kinases (Table II). This enabled determination of K_i values for dissociation of IIIc from the ATP site of these enzymes [the rabbit and pig enzymes are believed to possess one ATP site (Price et al., 1973)]. For this purpose the procedure of Webb (1963) was employed which from Michaelis–Menton assumptions enables determination of the K_i value of an analogue substrate from a knowledge of the K_i value of the normal substrate together with the individual V_{max} values of the two substrates and of an equimolar mixture of the two. Figure 1 shows the V_{max} determination for equimolar mixtures of IIIc and ATP. To calculate the K_i of IIIc in the case of the rabbit enzyme the K_i of MgATP was taken to be equal to the K_m (330 μM) of ATP as indicated by the work of Callaghan and Weber (1959). The K_i value of MgATP for the pig enzyme was taken as 180 μM, i.e., four times higher than the K_i (45 μM) of Mn^{2+}ATP (Price et al., 1973) because in the parallel case of creatine kinase, which is mechanistically similar to AMP kinase (Mildvan, 1970), the K_i of MgADP is approximately four times higher than the K_i of Mn^{2+}ADP (O'Sullivan and Cohn, 1966). In the case of carp AMP kinase, no direct or indirect evidence for the K_i of MgATP is available, and for the purpose of calculating the K_i of IIIc, the value was assumed to be equal to the K_m of MgATP by analogy with the rabbit and pig enzymes.

Inactivation of the three AMP kinases by IIIc followed saturation kinetics (Figure 2) indicating that formation of a bond between IIIc and the enzymes is preceded by reversible binding of IIIc to each enzyme, as shown in eq 1. A kinetic expression (Gold and Fahrney, 1964) for such two-step inactivations

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E{-}I \text{ (inactive)} \qquad (1)$$

is given in:

$$1/k = 1/k_2 + (K/k_2)(1/[I]) \qquad (2)$$

in which k is the observed first-order rate constant for inactivation, k_2 is the pseudo-first-order rate constant for formation of a bond between inhibitor and enzyme, K is $(k_{-1} + k_2)/k_1$, and [I] is the level of inhibitor. Since in most instances it may be expected that k_{-1} will be much larger than k_2, it follows that K will usually be numerically almost identical with the enzyme–IIIc dissociation constant K_i. The values of K for the rabbit, pig, and carp enzymes were found from the plots of Figure 2 to be 710, 970, and 720 μM, respectively, and the K_i values for dissociation of IIIc from the ATP sites of these enzymes were found, from the studies of their substrate properties described above, to be 500, 770, and 845 μM, respectively. The two sets of values are thus in reasonable agreement. This agreement between K and K_i would appear to constitute good evidence that the site to which IIIc is bound prior to its inactivation of these enzymes is the same as the site to which it is bound prior to its catalytic conversion to IIIb. In harmony with this view, ATP markedly slowed the inactivations of the three enzymes by IIIc, e.g., the half-life for inactivation of rabbit AMP kinase at 0° by 2.2 mM IIIc was five times longer in the presence of 0.55 mM ATP.

The pseudo-first-order rate constants for inactivation of the three AMP kinases have values between 1.3×10^{-3} and 9.1×10^{-3} min^{-1}, suggesting the possibility that IIIc might react with the same amino acid residue at the ATP site of each enzyme. Steric considerations show that IIc could react with the same amino acid residue(s) as does IIIc. The present findings do not exclude the possibility that IIc or IIIc may react with more than one amino acid residue of these adenylate kinases. Inactivation by the present group of site-directed reagents is not a characteristic of all kinases which utilize adenine nucleotides. Thus yeast hexokinase was apparently not inactivated by IIc or IIIc, although searching tests could not be made owing to the excellent substrate properties of IIc and IIIc and to the instability of the enzyme in the absence of glucose; in addition, rabbit pyruvate kinase was not inactivated when treated for long periods with high levels of IIb, IIc, IIIb, or IIIc.

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