was from Boehringer Mannheim, and NADH was from PL Biochemicals. Compounds 1-4 (series **a**, **c**, and **d**) and **4b** were synthesized as described in the preceding paper of this series.[1] Syntheses of the AMP analogue of **1a** (n = 8)[4] and of the AMP analogue of **1b** (n = 8)[2] were reported previously. The stock solutions of each ATP derivative contained an equimolar amount of $MgSO_4$.

The enzyme-catalyzed reactions were followed spectrophotometrically as described previously.[3] The system for kinetic determinations with all four adenylate kinases consisted of 1 mL of 0.1 M Tris-HCl (pH 7.6) containing $MgSO_4$ (2 mM), KCl (0.12 M), PEP cyclohexylammonium salt (0.31 mM), NADH (0.38 mM), pyruvate kinase (8.6 units), lactate dehydrogenase (8.6 units), and AMP (0.25 mM). Initial velocities were proportional to the amount of adenylate kinase activity added and independent of the levels of the two secondary enzymes.

Substrate constants were determined from Lineweaver-Burk double-reciprocal plots of initial velocity vs. substrate level, all of which were linear. Five or more levels of substrate in the range of 0.5-4.0 × K_M were studied in the above assay system. Compounds were tested initially for substrate activity at concentrations of ca. 0.1 and 1.0 mM. Controls lacking ATP or ATP derivatives were employed in every case because traces of ADP and/or ATP (commonly present in preparations of adenylate kinases) initiated, after a lag period dependent on the level of AK activity employed, a slow but rapidly accelerating conversion of the AMP to ADP. The last component added in the studies of substrate activity was the adenylate kinase preparation.

Studies of reversible inhibition employed levels of the ATP derivatives in the range of 1-4 × K_i and five or more levels of ATP in the range of 0.5-4.0 × K_M of ATP. Inhibition constants (K_i) were derived from replots of inhibitor level vs. slopes of the Lineweaver-Burk plots.

Studies of the rates of inactivation of adenylate kinases were carried out in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.12 M KCl, 2 mM $MgSO_4$, the magnesium complex of the ATP derivative, MgATP (when required), and 10-100 times the level of adenylate kinase activity employed in kinetic studies. At various time intervals, enzyme activity was measured by the addition of appropriate aliquots of the mixture to the assay mixture described above containing 1 mM ATP. The enzyme activity in control solutions lacking the ATP derivative was monitored for the same period of time (6-8 h, 22-23 °C). The *E. coli* enzyme activity in the control solutions remained essentially unchanged during this period; the activity of the rat AK preparations varied by ±5%.

Acknowledgment. This work was supported by U.S. Public Health Service Research Grant CA-11196 from the National Cancer Institute and grants to the Institute for Cancer Research (U.S. Public Health Service Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

Use of Adenine Nucleotide Derivatives to Assess the Potential of Exo-Active-Site-Directed Reagents as Species- or Isozyme-Specific Enzyme Inactivators. 5.[1] Interactions of Adenosine 5'-Triphosphate Derivatives with Rat Pyruvate Kinases, *Escherichia coli* Thymidine Kinase, and Yeast and Rat Hexokinases

Alexander Hampton,* Arvind D. Patel, Ram R. Chawla, Francis Kappler, and Ton T. Hai

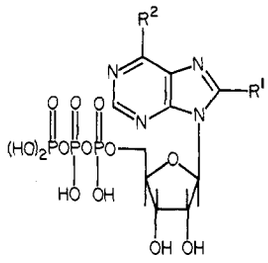
The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111.
Received December 21, 1981

Adenosine 5'-triphosphate (ATP) derivatives of the types N^6-R-ATP [R = $(CH_2)_nNHCOCH_2I$, $(CH_2)_nNHCO(CH_2)_mNHCOCH_2I$, or $(CH_2)_nCON(Me)(CH_2)_mN(Me)CO(CH_2)_nNHCOCH_2I$], N^6-Me-N^6-R-ATP [R = $(CH_2)_nN(Me)CO(CH_2)_mNHCOCH_2I$], and 8-R-ATP [R = $NH(CH_2)_nNHCOCH_2I$] with 5-19 spacer atoms between N^6 or C-8 and iodine have been evaluated as potential exo-ATP-site-directed reagents for phosphokinases. Substrate and inhibitor properties indicated that the compounds possessed affinity for the ATP sites of the muscle (M), kidney (K), and liver (L) isozymes of rat pyruvate kinase (PK), of *E. coli* thymidine kinase (TK), and of yeast hexokinase (HK) and rat HK I, II, and III isozymes. Tests for time-dependent loss of enzyme activity (inactivation) were performed under conditions in which a large proportion of each phosphokinase was present as an enzyme-inhibitor complex. No ATP-site-directed inactivations resulted when the M, L, or K isozymes of PK were exposed for 8 h, 22 °C, to 5 mM levels of 18 ATP derivatives or 6 analogous ADP derivatives or when yeast HK or rat HK I, II, or III was exposed for 6 h, 22 °C, to 5 mM levels of 28 ATP derivatives. *Escherichia coli* TK was inactivated by 6 of 25 ATP derivatives tested at 10 mM, 6 h, 0 °C; inactivation was slowed by MgATP in the case of N^6-CH_3-N^6-R-ATP [R = $(CH_2)_4N(CH_3)CO(CH_2)_5NHCOCH_2I$]. Only 1% of 298 enzyme-inhibitor combinations exhibited ATP-site-directed inactivation, signifying that few suitably positioned and sufficiently reactive nucleophilic groups were present near the enzymic ATP sites. Studies have now shown that exo-active-site-directed reagents can act as isozyme- or species-selective enzyme inhibitors. The present survey indicates that in many cases such reagents may be difficult of access when data are not available regarding structural or physicochemical features of the target enzyme adjacent to its catalytic site.

In order to obtain evidence regarding the utility of exo-active-site-directed reagents[2] as species- or isozyme-specific inhibitors, we have synthesized a series of ATP derivatives **1a-4a** with substituents bearing an iodoacetyl group[3,4] and have studied them as potential ATP-site-directed inactivators of various ATP-utilizing phosphokinases. Factors involved in the selection of the enzymes

(1) For paper 4, see Hampton, A.; Picker, D.; Nealy, K. A.; Maeda, M. *J. Med. Chem.*, preceding paper in this issue.
(2) Baker, B. R. "Design of Active-Site-Directed Irreversible Enzyme Inhibitors"; Wiley, New York, 1967.
(3) Hampton, A.; Slotin, L. A.; Chawla, R. R. *J. Med. Chem.* **1976**, *19*, 1279.
(4) Hampton, A.; Patel, A. P.; Maeda, M.; Hai, T. T.; Cheng, C.-D.; Kang, J. B.; Kappler, F.; Abo, M.; Preston, R. K. *J. Med. Chem.*, first paper in a series of three in this issue.

0022-2623/82/1825-0386$01.25/0



	series	R^3
1, R^1 = H; R^2 = $NH(CH_2)_nNHR^3$ (n = 2–8)	a	$COCH_2I$
2, R^1 = H; R^2 = $NH(CH_2)_nCON(Me)(CH_2)_mN(Me)CO(CH_2)_nNHR^3$ (n, m = 3 or 4)	b	$COCH_3$
3, R^1 = H; R^2 = $NR^4(CH_2)_nNR^4CO(CH_2)_mNHR^3$ (R^4 = H or Me) (R^4 = H: n = 3, m = 5 or 6; n = 4, m = 5; n = 6, m = 1–6. R^4 = CH_3: n = 4, m = 3, 5, or 6; n = 6, m = 5 or 6)	c	H
4, R^1 = $NH(CH_2)_nNHR^3$; R^2 = NH_2 (n = 2, 4, 6, or 8)	d	Cbz

that were studied and of the types of substituents attached to ATP have been discussed earlier.[4,5] Previous reports have described interactions of **1a** (n = 5-8) with variants of mammalian muscle adenylate kinase (AK),[3] interactions of ADP derivatives corresponding to **1a** (n = 2–8) with the muscle, liver, and kidney isozymes of pyruvate kinase (PK),[5] and interactions of **1a–4a** with *E. coli* AK and three isozymes of AK from rat liver and muscle.[1] The present report describes studies in which additional members of the above series of ATP derivatives and some of their ADP counterparts were examined as potential ATP-site-directed inactivators of rat muscle, liver, and kidney PK. Also reported here are substrate and reversible inhibitor properties of **1a–4a** that were determined in order to assess the affinity of these compounds for the ATP sites of *E. coli* thymidine kinase (TK), yeast hexokinase (HK), and the rat isozymes HK I, HK II, and HK III. Finally, the present report describes studies of the ability of **1a–4a** to bring about ATP-site-directed inactivation of *E. coli* TK or of the above four hexokinases.

Studies with Rat Pyruvate Kinases. That most, if not all, of the N^6-substituted ATP derivatives **1a** and **2a** can bind to the ATP sites of rat pyruvate kinases is suggested by the substrate activity (velocity 7–54% that of ADP at 0.2 mM) of the ADP analogues of **1a** (n = 2–8) with the M, K, and L isozymes[5] and by the substrate activity of the ADP analogue of **2d** (n = 4, m = 3) which has a relatively long N^6 substituent yet has a K_M value one-seventh that of ADP with the M isozyme.[4] That the N^6,N^6-disubstituted ATP derivatives **3a** also probably possess significant affinity for the ATP sites is indicated by the finding that attachment of $CH_3CON(CH_3)(CH_2)_4$ to N^6 of N^6-Me-ADP permits moderate substrate activity (rel V_{max} = 18–21% that of ADP) to persist with the M, K, and L rat isozymes with K_M values which are, respectively, 7-, 3-, and 1.5-fold that of ADP.[4] On this basis, 18 N^6-substituted and N^6,N^6-disubstituted ATP derivatives were tested as their 1:1 complexes with Mg^{2+} for ability at a 5 mM level to effect time-dependent inhibition of the M, K, and L isozymes during 8 h at 22 °C. The pH and the concentrations of free Mg^{2+} and K^+ that were employed in these tests have been reported to produce near-optimal activity of all three isozymes.[6] The mixtures contained sufficient fructose 1,6-diphosphate to fully activate the K and L forms.[6] The following ATP derivatives were tested: **1a** (n = 2–8); **2a** (n = m = 3); **3a**, R^4 = H (n = 3, m = 5 or 6; n = 6, m = 1–4); and **3a**, R^4 = Me (n = 4, m = 5 or 6; n = 6, m = 5 or 6). None of these compounds effected significant time-dependent inhibition of any isozyme. Since in the case of rabbit muscle PK it is known that the affinity of MgADP (K_i = 0.8 mM) for the ADP site is ca. 2.5 times greater than that of MgATP (K_i = 2.1 mM),[7] the six ATP derivatives **3a** (R^4 = H: n = 3, m = 5 or 6; n = 6, m = 1–4) were converted to the corresponding ADP derivatives by the action of yeast hexokinase as used previously[5] for the synthesis of ADP analogues of **1a** (n = 2–8). None of these MgADP derivatives, at a level of 5 mM, caused time-dependent inhibition. Paper electrophoretic analysis at pH 3.5 indicated that the above ADP and ATP derivatives remained stable during the tests for time-dependent isozyme inhibition. Phosphoenolpyruvate, the second substrate of PK, was not included during these tests because its binding site overlaps that of ATP and because kinetic analysis indicates that it does not promote binding of ADP to the rat M, K, and L forms.[6]

Studies with *E. coli* Thymidine Kinase (TK). Twenty-four N^6-substituted and N^6,N^6-disubstituted ATP derivatives (Table I) were tested as substrates of partially purified *E. coli* TK at concentrations of their 1:1 Mg complexes that were 2-fold higher than the K_M (1.4 mM)[8] of ATP under the assay conditions used. All of these ATP derivatives acted as phosphate donors in the enzyme-catalyzed reaction and produced initial velocities that ranged between 13 and 71% of the velocity produced by an equal concentration of MgATP. In the presence of a presumably near-saturating level (5.05 mM) of one of the MgATP derivatives (**1b**, n = 6), V_{max} was 56% that produced by MgATP, and the K_M of thymidine was unchanged.

Inhibition of TK-catalyzed formation of TMP by 1.4-mM levels of 26 ATP derivatives **1a–3a** varied from 0 to 39% in the presence of a 4-fold higher level of MgATP (Table I). Affinities of some of the ATP derivatives for the enzyme were comparable to that of ATP (K_M = 1.4 mM). Two of the ATP derivatives activated rather than inhibited the reaction (Table I), possibly because of interaction at the postulated ATP activation site of *E. coli* TK.[8] *E. coli* TK was exposed for 6 h at 0 °C in the assay medium lacking TdR to 10 mM levels of 25 MgATP derivatives, **1a–3a**. The ATP derivatives, by virtue of their pronounced substrate activities, presumably act as inhibitors competitive toward ATP, and the inhibition data of Table I would then indicate that under these conditions at least 75% of them would convert half or more of the TK to a TK–inhibitor complex. The ATP derivatives were unaffected by the TK preparation, as judged by paper chromatographic and electrophoretic analysis and an analysis for retention of covalently bound iodine described previously.[1] Progressive loss of enzyme activity was brought about by **2a** (n = m = 3), **2a** (n = 4, m = 3), **3a** (R^4 = H, n = 3, m = 5), **3a** (R^4 = H, n = 3, m = 6), **3a** (R^4 = Me, n = 4, m = 3), and **3a** (R^4 = Me, n = 4, m = 5) (Table I). A saturating level (10 mM) of MgATP slowed the rate of loss of activity only in the case of **3a** (R^4 = Me, n = 4, m = 5) (Figure 1). The inactivation by **3a** (R^4 = Me, n = 4, m = 5) followed pseudo-first-order kinetics with $t_{1/2}$ = 3.5 h. The ATP derivative **3b** (R^4 = Me, n = 4, m

(5) Hampton, A.; Kappler, F.; Maeda, M.; Patel, A. D. *J. Med. Chem.* **1978**, *21*, 1137.

(6) Ibsen, K. H.; Trippett, P. *Arch. Biochem. Biophys.* **1973**, *156*, 730.

(7) Ainsworth, S.; Macfarlane, N. *Biochem. J.* **1973**, *131*, 223.

(8) Okazaki, R.; Kornberg, A. *J. Biol. Chem.* **1964**, *239*, 275.

Table I. Substrate and Inhibitor Properties of N^6-Substituted Derivatives of ATP with *E. coli* Thymidine Kinase (TK)

ATP deriv	R^4	n	m	substrate act.: initial velocity,[a] rel %	initial inhibn of TK by 1.4 mM ATP deriv,[b] %	loss of TK act. after 6 h, 0 °C, with 10 mM ATP deriv, %
ATP				100		
1a		2		35	3	0
1a		3		32	6	0
1a		4		68	39	0
1a		5		13	26	0
1a		6[c]			0	0
1a		7		38	10	0
1a		8[d]			19	0
2a		3	3[c]	>14[e]	8	44
2a		3	4[c]	14	12	0
2a		4	3[c]	>34[e]	5	51
2a		4	4	34	9	0
3a	H	3	5[c]	>39[e]	13	74
3a	H	3	6[c]	>25[e]	28	58
3a	H	4	5	71	−11[f]	0
3a	H	6	1	63	2	0
3a	H	6	2	53	1	0
3a	H	6	3	36	0	0
3a	H	6	4[c]	39	12	0
3a	H	6	5[c]	37	8	0
3a	H	6	6[c]	41	12	0
3a	Me	4	3[c]	>33[e]	0	60
3a	Me	4	5[c]	>13[e]	11	53
3a	Me	4	6	55	1	0
3a	Me	6	5	49	5	0
3a	Me	6	6	57	16	0
3b	Me	4	5[c]	41	−27[f]	0

[a] Velocity of 2.8 mM MgATP derivative relative to that of 2.8 mM MgATP in the assay system given under Experimental Section. [b] Inhibitions were determined in the presence of 5.7 mM ATP. See Experimental Section for other conditions. [c] The solution of TK in the mixture used in the tests for time-dependent inhibition was stored at 0 °C for 1 h prior to addition of the ATP derivative. [d] Due to its limited solubility, this compound was tested for initial inhibition at 1.07 mM and for time-dependent loss of TK activity at 7.5 mM. [e] The value represents a minimum of substrate activity because partial loss of TK activity had occurred in the 0.5 h prior to assay. [f] Activation.

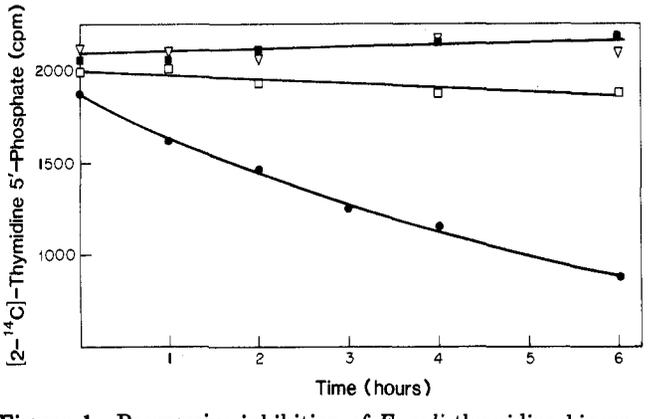


Figure 1. Progressive inhibition of *E. coli* thymidine kinase mediated by **3a** (R^4 = Me, n = 4, m = 5): (●) 10 mM inhibitor present in the assay system under conditions given under Experimental Section; (□) 10 mM inhibitor and 10 mM MgATP present; (▽) no additions to the assay system; (■) 10 mM MgATP present.

Table II. Substrate Activity of ATP Derivatives with Yeast Hexokinase and Rat Hexokinases I–III

ATP deriv	R^4	n	m	initial velocity relative to that of ATP[a] yeast	HK I	HK II	HK III
ATP				1.0	1.0	1.0	1.0
1a		2		0.75	0.58	0.34	0.5
1a		5		0.75	0.92	0.60	1.31
1a		8		1.82	1.79	1.26	2.81
1b		8			1.12	0.60	1.25
2a		3	3	0.94	0.63	0.43	1.02
3a	H	3	5	1.06	0.79	0.57	1.04
3a	H	6	1	0.52	0.92	0.63	2.06
3a	H	6	3	1.00	1.30	0.91	2.02
3a	H	6	5	1.02	1.25	0.63	1.37
3a	Me	4	3	1.20	0.88	0.35	0.93
3a	Me	6	6	0.94	1.66	0.85	2.06
4a		8		0.54	1.16	0.82	1.81

[a] ATP and its derivatives were added as their 1:1 Mg complexes and were present initially at 5.0 mM in the assay system given under Experimental Section.

= 5) did not inactivate the TK (Table I), suggesting that the effect involves alkylation of the enzyme.

Studies with Yeast and Rat Hexokinases. The ATP derivatives **1a–4a** were good substrates of all four hexokinases as judged by initial reaction velocities produced by 5 mM solutions (5.5–38 × K_M of ATP) in the presence of a saturating but not inhibitory level of glucose. Representative velocities are listed in Table II and are seen to be frequently higher than observed with 5 mM MgATP. The velocities usually followed the order HK III > HK I or yeast > HK II. Substrate constants were determined for two ATP derivatives with long and bulky substituents, namely, **2d** (n = 4, m = 3) and **3d** (R^4 = Me, n = 4, m = 5). The K_M values (Table III) indicate that the N^6-substituents of **1a–3a** probably interfere little with binding to the ATP sites and may sometimes assist binding, as suggested by the K_M of **2d** (n = 4, m = 3) relative to the K_M of ATP with HK I, II, and III.

The tests for inactivation of all four hexokinases were carried out at 22 °C for 6 h in an assay system which contained a saturating but not inhibitory level of glucose. Glucose was included because inhibition by the bisubstrate analogue P^1-(adenosine-5′),P^2-(glucose-6) tetraphosphate and other evidence indicate that glucose enhances the

Table III. Substrate Constants of ATP Derivatives with Yeast and Rat Hexokinases

enzyme	compd	K_M,[a] mM	V_{max},[b] rel %
yeast HK	2d, $n = 4$, $m = 3$	0.32	75
	3d, R^4 = Me, $n = 4$, $m = 5$	0.30	75
	ATP	0.13	100
rat HK I	2d, $n = 4$, $m = 3$	0.18	45
	ATP	0.44	100
rat HK II	2d, $n = 4$, $m = 3$	0.27	35
	ATP	0.71	100
rat HK III	2d, $n = 4$, $m = 3$	0.10	23
	ATP	0.91	100

[a] K_M = substrate concentration for half-maximal velocity. [b] Maximal velocity.

affinity of ATP for yeast HK[9] and for HK I–III.[10] The enhancement factor appears to be ~40 in the case of yeast HK.[9] The level of hexokinase activity was adjusted so that less than 5% of each ATP derivative became converted to the corresponding ADP derivative during the tests for HK inactivation. The ATP derivatives were otherwise stable during the tests, as indicated by the methods of analysis described above in the studies with TK. A total of 28 MgATP derivatives were tested at a level (5 mM) that was 5.5–38 times higher than the K_M values of MgATP. The substrate properties of the ATP derivatives indicate that this level is sufficient to convert major fractions of each HK to a dissociable HK–inhibitor complex. Time-dependent inhibition in excess of 10% was effected only by compounds of structure **1a** in which n was 5–8 (Table IV). Activity was restored by addition of HK but not by addition of the secondary assay enzyme, glucose-6-phosphate dehydrogenase. Replacement of iodine in **1a** ($n = 8$) by hydrogen to give **1b** ($n = 8$) abolished the progressive loss of activity of the four hexokinases, suggesting that the effect involves alkylation of the enzymes. The rates of inactivation by 5 mM levels of **1a** ($n = 5$–8) were not slowed by the presence of a saturating level (10 mM) of MgATP, and the effects thus appear not to be ATP site directed. This view is consistent with the observation that a 5 mM level of the AMP analogue of **1a** ($n = 8$)[3] inactivated HK II more rapidly (67% in 3 h) than **1a** ($n = 8$) itself, although the AMP derivative presumably binds more weakly than **1a** ($n = 8$) to the ATP site.

Design of Exo-Active-Site-Directed Reagents and Their Potential as Species- or Isozyme-Selective Enzyme Inactivators. It is clear that, from a practical viewpoint, the question of the potential of exo-active-site-directed reagents as species- or isozyme-selective enzyme inactivators is bound up with the question of how readily such inactivators can be designed. In the present studies of their design, properties of bridging substituents that could be expected to favor exo-active-site alkylation of an enzyme were considered to be (1) flexibility and lack of bulk, (2) sufficient spacer atoms (5–19 were employed) to permit alkylation of groups not directly involved in catalysis and, hence, more likely to differ in microenvironment or location from isozyme to isozyme, (3) the presence of a powerful electrophile at the distal end of the substrate substituent to maximize the frequency of exo-alkylation, and (4) a substituent with a mixed polar–nonpolar character more similar to that of the enzyme than of the predominantly polar substrate residue (ATP) or other molecules in the aqueous environment. Substrate and inhibitor studies described in this and our previous papers provided evidence that many of the N^6- or 8-substituted ATP derivatives, **1a–4a**, had affinity for the ATP sites of rabbit, pig, and carp muscle AK,[3] of rat AK II and AK III,[1] of the rat M, K, and L isozymes of PK[5], of yeast HK and rat HK I–III, and of *E. coli* TK. On this basis, we examined for time-dependent loss of catalytic activity a total of 298 enzyme–inhibitor combinations involving these enzymes. The inhibitor concentrations used in these tests were high enough to ensure that a large and usually major fraction of each enzyme was present as an enzyme–inhibitor complex. The results of these studies are summarized in Table V. Only three instances were encountered of a time-dependent loss of activity that appeared to be ATP site directed. Two of these exhibited species selectivity[3] and isozyme selectivity,[5] respectively, while the third instance, which involved *E. coli* TK, has not yet been evaluated for possible selectivity.

The low frequency of ATP-site-directed inactivations implies that regions of the enzymes adjacent to their ATP binding sites possess relatively few nucleophilic groups that satisfy both the steric and the reactivity requirements necessary for them to become alkylated by the iodoacetyl groups of the inhibitors. It might be supposed that exo-ATP-site alkylations did occur in additional dehydrogenase,[13] but that no inactivation was detected because the ATP moiety was able to freely dissociate from the ATP site due to a relatively large number of spacer atoms and to poor affinity of the substituent for those regions of the enzyme between the ATP site and the alkylated group. Contrary to this supposition, however, the inactivations of AK, PK, and TK observed in this work (see Table VI) all proceeded to within at least 80–90% of completion despite the presence of 9–14 spacer atoms and of substituents which appeared to contribute little to the binding of the inhibitor to the enzyme. Another possibility is that the low incidence of ATP-site-directed inactivations found in the present survey could to a limited extent result from similarity in amino acid composition between the enzyme variants that were studied. Amino acid analyses have been reported for all the variants studied with the exception of rat HK III, *E. coli* AK, and the rat PK isozymes. Comparative aspects of the amino acid compositions are set out in Table VII where data for pig muscle, kidney, and liver PK isozymes are included to illustrate differences to be expected among the corresponding rat PK isozymes. Table VII reveals extensive differences in amino acid composition: for all the mammalian isozymes except rat AK II, the number of amino acid residues present per mole of enzyme is nonidentical in the case of more than half the amino acids present, and the average differences per amino acid vary from 9 to 22%. A third possibility is that the low incidence of ATP-site-directed inactivations may be associated with frequent positioning of the iodoacetyl group in predominantly lipophilic regions of the enzyme where relatively few nucleophilic groups are present. Were this the case, the substituents attached to ATP would be expected to tend usually to enhance affinity for the test enzymes because the combined energy of van der Waals and hydrophobic forces between one methylene in a substituent and an enzymic methylene can increase affinity by as much as 10-fold.[2] The substrate and inhibitor properties of the ATP derivatives indicate that, to the contrary, enhanced affinities were encountered less frequently than reduced affinities and that the degree of enhancement usually was considerably less than 10-fold even when the substituent possessed considerable nonpolar

(9) Danenberg, P. V.; Danenberg, K. D. *Biochim. Biophys. Acta* 1977, *480*, 351.
(10) Hampton, A.; Hai, T. T., unpublished results.

Table IV. Time-Dependent Inhibition of Yeast Hexokinase and Rat Hexokinases I–III by N^6- and 8-Substituted ATP Derivatives[a]

ATP derivative	R^4	n	m	loss of HK act. after 6 h, 22 °C, % yeast	HK I	HK II	HK III
ATP[a]				0	0	0	0
ATP[a] + iodoacetamide[b]				0	5	7	0
1a		2		0	0	0	0
1a		3		0	0	0	0
1a		4		0	8	0	0
1a		5		0	8	14	0
1a		6		23	23	35	33
1a		7		22	20	30	20
1a		8		30	35	41	47
1b		8		0	0	−12[c]	0
2a		3	3	0	0	0	0
2a		3	4	0	0	0	0
2a		4	3	0	0	0	0
2a		4	4	0	0	0	0
3a	H	3	5	0	0	8	0
3a	H	3	6	0	0	0	10
3a	H	4	5	0	0	5	0
3a	H	6	1	−10[c]	0	0	0
3a	H	6	2	0	0	0	0
3a	H	6	3	0	0	0	0
3a	H	6	4	0	0	0	0
3a	H	6	5	0	0	0	0
3a	H	6	6	0	0	0	10
3a	Me	4	3	0	0	0	0
3a	Me	4	5	−9[c]	0	0	0
3a	Me	4	6	0			
3a	Me	6	6	0	0	0	8
4a		2			0	0	0
4a		4		0	0	0	0
4a		6		0	0	0	0
4a		8		0	8	8	0

[a] All compounds were tested at 5.0 mM under the conditions given under Experimental Section and were added as their 1:1 Mg complexes. [b] 5.0 mM. [c] The HK activity increased during the test period.

Table V. Evaluation of ATP and ADP Derivatives as Potential Exo-ATP-Site-Directed Enzyme Reagents

enzyme[a]	no. of enzyme-reagent combinations tested[b]	no. of exo-ATP-site inactivations obsd
AK (pig, rabbit, carp muscle)	18	1[c]
AK II, III (rat)	50	0[d]
PK (rat; M, K, L)	54	0[e]
	18 (ADP derivs)	0[e]
	21 (ADP derivs)	1[f]
TK (*E. coli*)	25	1[e]
HK (yeast, rat HK I–III)	112	0[e]

[a] AK = adenylate kinase; PK = pyruvate kinase; TK = thymidine kinase; HK = hexokinase; M, K, L = muscle, liver, kidney, respectively. [b] Reagents tested are ATP derivatives unless noted as ADP derivatives. [c] Reference 3. [d] Reference 1. [e] This report. [f] Reference 5.

character. Thus, only 3 of 25 compounds had greater affinity for *E. coli* TK than ATP itself, and the maximum increase in affinity was 2-fold, assuming that all inhibitions were competitive with respect to ATP (Table I). The affinities of representative members of the present series of ATP derivatives for rat AK II and AK III were 3–62 times less than that of ATP,[1] and the affinities of **1a** (n = 5–8) for rabbit, pig, and carp muscle AK were only 1–2 times higher than that of ATP.[3] The affinity of the ADP analogue of **1a** (n = 8) for rat liver PK was 100-fold less than that of ADP,[5] and N^6-methyl-N^6-(4-acetamidobutyl)-ADP appeared from inhibition studies to bind 1.5–7 times less tightly than ADP to the rat liver, kidney, and muscle PK isozymes.[4] Finally, compound **2d** (n = 4, m = 3), which has a large and relatively nonpolar substituent containing 12 methylenes, 2 methyls, and a carbobenzyloxy group, bound ca. 9 times more strongly to HK III than did ATP but only two times more strongly to HK I and II and half as strongly to yeast HK than did ATP (Table III).

In addition to the instance involving adenylate kinases reported in the course of the present studies,[3] at least three other well-documented examples of species-selective exo-active-site alkylation of enzymes have been reported. These have involved inactivation of lactate dehydrogenase,[11] thymidylate synthetase,[12] and alcohol dehydrogenase,[13] respectively. In the last instance, design of selective inhibitors was considerably aided by the availability of detailed information from X-ray crystallography of the tertiary structure of one of the dehydrogenases. The existing examples of species- or isozyme-selective enzyme inactivation thus serve to substantiate the hypothesis of Baker[2] that exo-active-site-directed reagents can exert selective inhibitory effects. It is clear that such effects are of potential value in the design

(11) Baker, B. R.; Patel, R. P. *J. Pharm. Sci.* **1964**, *53*, 714.
(12) Barfknecht, R. L.; Huet-Rose, R. A.; Kampf, A.; Mertes, M. P. *J. Am. Chem. Soc.* **1976**, *98*, 5041.
(13) Chen, W.-S.; Bohlken, D. P.; Plapp, B. V. *J. Med. Chem.* **1981**, *24*, 190.
(14) Criss, W. E.; Pradhan, T. K.; Morris, H. P. *Cancer Res.* **1974**, *34*, 3062.
(15) Tomasselli, A. G.; Noda, L. H. *Eur. J. Biochem.* **1980**, *103*, 481.
(16) Berglund, L.; Ljungstrom, O.; Engstrom, L. *J. Biol. Chem.* **1977**, *252*, 6108.
(17) Holroyde, M. J.; Trayer, I. P. *FEBS Lett.* **1976**, *62*, 215.
(18) Wright, C. L.; Warsy, A. S.; Holroyde, M. J.; Trayer, I. P. *Biochem. J.* **1978**, *175*, 125.
(19) Colowick, S. P *Enzymes, 3rd Ed.*, **1973**, *9*, 1.

Table VI. Lengths and Enzyme Affinities of Substituents of ATP Derivatives That Function as Exo-ATP-Site-Directed Enzyme Reagents

enzyme inactivated	reagent used	no. of spacer atoms from N^6 to I	affinity rel to normal substrate[a]	extent of inactivation, %
AK (rabbit muscle)[b]	1a (n = 6)	9	0.28[c]	>90[b]
PK (rat liver)[d]	1a (n = 8)	11	0.011[c]	>90[d]
TK (*E. coli*)	3a (R^4 = Me; n = 4; m = 5)	14	0.56[e]	>80

[a] Affinity is expressed as K_M (ATP or ADP)/K_i of inhibitor. [b] Reference 3. [c] K_i is assumed equal in value to the apparent dissociation constant, K. [d] Reference 5. [e] The K_i value was calculated to be 2.5 mM from the inhibition listed in Table I on the assumption that inhibition is competitive with respect to ATP.

Table VII. Differences in Amino Acid Composition among Adenylate Kinases, Pyruvate Kinases, and Hexokinases

enzyme	fraction of amino acid residues differing in no. per enzyme molecule	av % differences in the no. of each amino acid residue per enzyme molecule
rat AK II[a]	6/16[b]	9[b]
rat AK III[a]	9/16[b]	17[b]
pig muscle AK[c]	10/18[d]	9[d]
pig kidney PK[e]	12/18[f]	10[f]
pig liver PK[e]	15/18[f]	22[f]
rat HK I[g]	14/16[h]	16[h]
rat HK III[i]	ND	ND
yeast HK (P-II)[j]	16/16[h]	28[h]

[a] Data from ref 14. [b] Comparison is made with the composition of rat muscle AK. [c] Data from ref 15. [d] Comparison is made with rabbit muscle AK. [e] Data from ref 16. [f] Comparison is made with pig muscle PK. [g] Data from ref 17. [h] Comparison is made with rat HK II. [i] The amino acid composition of HK III is not yet determined.[18] [j] Data from ref 19.

of chemotherapeutic agents. The findings in the present survey imply that exo-active-site-directed reagents may in many instances not be easy of access when little information is at hand regarding structural or physicochemical properties of regions of the target enzyme adjacent to its catalytic site and when, under these circumstances, substrate substituents are employed as probes for nucleophilic groups situated in those regions.

Experimental Section

Studies with *E. coli* Thymidine Kinase. *E. coli* B cells (harvested in late log phase) were obtained from Miles Laboratories, Inc. [2-^{14}C]Thymidine (57 mCi/mmol) was obtained from New England Nuclear, Inc. The thymidine kinase preparation was identical with one described previously.[20] Compound **3b** (R^4 = Me, n = 4, m = 5) was prepared by acetylation of the previously described ω-amino ATP derivative **3c** (R^4 = Me, n = 4, m = 5)[4] with *N*-acetoxysuccinimide under conditions used to convert **1c–3c** to **1a–3a**.[4] The compound was purified by anion-exchange chromatography on DEAE-cellulose and precipitation as its tetrasodium salt by procedures previously described;[4] the product was homogeneous as indicated by paper chromatography and paper electrophoresis in systems previously used for compounds **3a**[4] and by its UV absorption spectrum in water λ_{max} 277 nm, ε 20 300).

Studies of time-dependent loss of TK activity mediated by **1a–3a** were carried out at 0 °C in 250 μL of 71 mM Tris-HCl buffer (pH 7.8) containing the ATP derivative (10 mM), $MgCl_2$ (10 mM), $MnCl_2$ (5 mM), bovine serum albumin (1 mg), and 5 μL of the TK preparation. Mixtures lacking inhibitor or, when appropriate, containing 10 mM ATP were run as controls; the TK activity of these remained essentially constant for 7 h. In some experiments the activity of the controls increased as much as 10% during the first hour. For this reason, in some experiments (see Table I) the mixture was stored at 0 °C for 1 h prior to addition of the ATP derivative. At intervals, aliquots (10 μL) of the mixture were diluted to 70 μL to give a mixture containing 71 mM Tris-HCl buffer (pH 7.8), ATP (5.7 mM), ATP analogue (1.42 mM), $MgCl_2$ (2.85 mM), $MnCl_2$ (0.71 mM), thymidine (0.083 mM; 7.6 mCi/mmol), and bovine serum albumin (1.14 μg/μL). The aliquot taken at zero time provided the data on initial inhibition given in Table I. For the assay, each mixture was rocked in a water bath at 37 °C for 10 min and then immersed in boiling water for 2 min and cooled in ice. Denatured protein was removed by centrifugation and 20 μL of the supernatant was applied, together with 0.1 μmol of carrier TMP (thymidine 5′-phosphate), on Whatman No. 3MM paper. Electrophoresis was carried out in 0.05 M triethylammonium bicarbonate buffer, pH 7.6, at a gradient of 27 V/cm for 1 h. After the paper was dried, the TMP spot was cut out, immersed in a toluene–phosphor mixture, and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 2425. The radioactivity was corrected for the blank value found for unincubated reaction mixtures; in all reactions, the [^{14}C]TMP present in the 20-μL aliquot generated no less than 1000 cpm.

The substrate activity of **1a–3a** reported in Table I was obtained in the course of the above studies of time-dependent TK inhibition. Thus, 0.5 h after addition of the inhibitor, 20 μL of the test solution was diluted to 70 μL to give a 2.8 mM solution of the inhibitor in an assay mixture that was the same as described above except that it lacked ATP and contained twice the level of $MnCl_2$ (1.42 mM). The mixture was kept at 37 °C for 15 min, and the amount of ^{14}C-labeled TMP formed was compared with the amount formed from 2.8 mM ATP under the same conditions. The reaction rates remained linear for 20 min. The K_M of thymidine was 36 μM in the presence of 5.05 mM **1b** (n = 6); a value of 33 ± 3 μM was obtained, as reported previously[20] in the presence of 5.7 mM ATP and 0.71 mM $MnCl_2$ in the above assay mixture. Reaction rates were proportional to enzyme level for both 5.0 mM **1b** (n = 6) and for 5.7 mM ATP.

Studies with Rat Pyruvate Kinases. The partial purification and assay of the muscle, kidney, and liver isozymes of rat pyruvate kinase were carried out as described previously.[5] Lactate dehydrogenase, fructose 1,6-diphosphate, and phosphoenolpyruvate were obtained from Sigma Chemical Co., and reduced nicotinamide adenine dinucleotide was obtained from P-L Biochemicals, Inc. Studies of time-dependent inhibition by ATP derivatives were performed at 22 °C in 0.2 mL of 0.05 M triethanolamine buffer (pH 7.0) containing $MgCl_2$ (5 mM), KCl (67 mM), reduced nicotinamide adenine dinucleotide (0.4 mM), fructose 1,6-diphosphate (10 μM), lactate dehydrogenase (1 μg), MgATP derivative (5 mM), and pyruvate kinase which was present either at 10-fold (for the M isozyme) or 2-fold (L and K isozymes) the normal assay level. At various time intervals, aliquots (10 μL for the M isozyme; 20 μL for the L and K isozymes) were assayed in the system previously described[5] in the presence of 0.373 mM ADP. Mixtures lacking the ATP or ADP analogues were utilized as controls. Inactivation mixtures and their controls were maintained and assayed at 22 °C. In most experiments, the activity of the controls increased approximately 5% (L and K) or 20% (M) during the first hour and then remained constant; the L and K isozymes lost no activity during a total of 8 h, and the M isozyme lost none during 24 h.

ADP derivatives were prepared from the ATP derivatives **3a** (R^4 = H; n = 3, m = 5 or 6; n = 6, m = 1–4) (3–4 μmol) by

(20) Hampton, A.; Kappler, F.; Chawla, R. R. *J. Med. Chem.* **1979**, *22*, 1524.

treatment with yeast hexokinase (7 units) at 22 °C for 2 h in 0.2 mL of 0.05 M triethanolamine buffer (pH 7.0) containing $MgCl_2$ (5 mM) and glucose (10 mM).[5] Conversions were quantitative as judged by paper electrophoresis run at pH 3.5.[5] Suitable portions were added to the above mixture used for studies of time-dependent inhibition of the pyruvate kinases so as to give 5 mM solutions of the MgADP derivatives, after which pyruvate kinase activity was monitored at various time intervals as detailed above.

Studies with Hexokinases. NADP was obtained from P-L laboratories, and glucose-6-phosphate dehydrogenase (Type 1) and yeast HK were from Boehringer Mannheim. The glucose-6-phosphate dehydrogenase was collected by centrifugation before incorporating it into assay media. The yeast HK was subjected to isoelectric focusing at pH 4–6 at 8 °C in the presence of 0.5 M glucose by the method detailed below. The recovery of activity was 32%. Of this, 0.7% was in a zone of pI = 5.05 and 99.3% in a zone of pI = 4.72. These components presumably are the P-I and P-II yeast HK isozymes with reported pI values of 5.3 and 5.0, respectively.[21] In the present kinetic studies, yeast HK was used without prior electrofocusing.

The HK-catalyzed reactions were followed at 22 °C by measuring the rate of change of absorbancy at 340 nm in a Varian Model 635 spectrophotometer. The normal assay mixture comprised 1 mL of 0.05 M Tris-HCl (pH 7.6) containing 6.6 mM $MgCl_2$, 10 mM α-D-glucose, 0.93 mM NADP, 5 mM ATP, and 0.35 unit of glucose-6-phosphate dehydrogenase.

Hexokinase was extracted from liver and muscle of an adult male Sprague–Dawley rat by described procedures.[22,23] After solid glucose was added to give a level of 0.1 M, the preparations were stable at 4 °C for at least 6 weeks. A portion (15 mL) of the liver extract was concentrated to 3 mL by ultrafiltration in an Amicon B15 membrane unit, applied as a zone in a preformed Sephadex slab, and subjected to electrofocusing at 8 °C in an LKB Model 2117-501 apparatus using an ampholine mixture (1:1) of pH ranges 4–6 and 5–8 in the presence of 10 mM, pH 7.0 phosphate buffer, 100 mM glucose, 0.5 mM dithiothreitol, 1 mM EDTA, and 56% (v/v) glycerol. In the absence of glycerol, no activity was recovered. The pH gradient was determined with a Thomas No. 4094-L15 combination glass electrode. Major, well-separated zones of HK I (pI = 6.25) and HK III (pI = 5.2) and a minor zone of HK II (pI = 5.7) were obtained. Purified HK I from rat brain was reported to have a pI value of 6.35.[24] The K_M values for ATP (Table III) agreed with reported values[24] for HK I (0.44 mM), HK II (0.78 mM), and HK III (0.98 mM). As expected, the order of relative pI values (I > II > III) was opposite to the reported order of relative electrophoretic mobility toward the anode (III > II > I).[26] The recovery of HK activity after electrofocusing was 25–30%. Zones containing HK I or III were centrifuged through glass wool and dialyzed at 4 °C against 0.02 M triethanolamine buffer, pH 7.2, containing 0.1 M KCl, 0.1 M glucose, 1.0 mM EDTA, and glycerol (5%, v/v). The dialysis caused no loss of activity. The HK I and HK III preparations were stable at 4 °C for at least 6 days.

The muscle isozyme (HK II) preparation used in the present studies was obtained by dialysis of the muscle extract described above under the conditions used for dialysis of the HK I and HK III preparations. When the muscle extract was subjected to electrofocusing under the conditions given above, HK II appeared as an apparently homogeneous zone of activity of pI = 5.7; the only other activity, presumably HK I, was a zone of pI = 6.2, which contained 3% as much activity as the HK II zone. When the electrofocused HK II was dialyzed as described above, all activity was lost within 16 h. When dithiothreitol (0.5 mM) was present during this dialysis, all the HK II activity was retained.

The substrate constants of Table III were determined from double-reciprocal plots of velocity vs. substrate level, all of which were linear. Initial velocities were linear and proportional to the concentration of the HK and independent of the level of glucose-6-phosphate dehydrogenase in the assay system.

The studies of time-dependent HK inhibition (Table IV) were carried out at 22 °C in the assay system described above from which ATP was omitted. Since the HK activity sometimes increased by as much as 50% during the initial 2 h, the mixture was stored for 2 h at 22 °C before addition of the MgATP derivative as the final component. The rate of change of absorbance at 340 nm was measured at intervals of 0.5–1 h for 6 h. The level of HK was adjusted so that during this period less than 5% of the ATP derivative underwent conversion to the corresponding ADP derivative. Studies of time-dependent inhibition by the AMP and ADP analogues of **1a** (n = 8) and the ADP analogue of **1b** (n = 8) were carried out in the presence of 5 mM ATP.

N^6-(8-Acetamido-n-octyl)adenosine 5′-Triphosphate (1b, n = 8). Triethylammonium N^6-(8-acetamido-n-octyl)adenosine 5′-phosphate[5] was converted to **1b** (n = 8) by the general method described for the preparation of the N-(Cbz)-ATP derivatives **1d**, **2d**, and **3d**.[4] Compound **1b** (n = 8), obtained as its tetrasodium salt in 66% yield, was homogeneous on paper electrophoretograms run at pH 3.5 (mobility = 1.72 relative to AMP = 1) and on paper chromatograms developed in 1-butanol–acetic acid–water (5:2:3, v/v) (R_f 0.64): UV λ_{max} (H_2O) 268 nm (ε 17 400). Anal. ($C_{20}H_{31}N_6O_{14}P_3Na_4 \cdot 3H_2O \cdot CH_3OH$) C, H, N, P.

Acknowledgment. This work was supported by U.S. Public Health Service Research Grant CA-11196 from the National Cancer Institute and grants to the Institute for Cancer Research (U.S. Public Health Service Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

(21) Hoggett, J. G.; Kellett, G. L. *Eur. J. Biochem.* **1976**, *66*, 65.
(22) Holroyde, M. J.; Allen, M. B.; Storer, A. C.; Warsy, A. S.; Chesher, J. M. E.; Trayer, I. P.; Cornish-Bowden, A.; Walker, D. G. *Biochem. J.* **1976**, *153*, 363.
(23) Holroyde, M. J.; Trayer, I. P. *Fed. Eur. Biol. Soc. Lett.* **1976**, *62*, 215.
(24) Chou, A. C.; Wilson, J. E. *Arch. Biochem. Biophys.* **1972**, *151*, 48.
(25) Grossbard, L.; Schimke, R. T. *J. Biol. Chem.* **1966**, *241*, 3546.
(26) Katzen, H. M.; Schimke, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **1965**, *54*, 1218.