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Affinity Labeling of Rabbit Muscle Pyruvate Kinase by 5′-*p*-Fluorosulfonylbenzoyladenosine†

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ABSTRACT: Rabbit muscle pyruvate kinase is irreversibly inactivated upon incubation with the adenine nucleotide analogue, 5′-*p*-fluorosulfonylbenzoyladenosine. A plot of the time dependence of the logarithm of the enzymatic activity at a given time divided by the initial enzymatic activity (log E/E_0) reveals a biphasic rate of inactivation, which is consistent with a rapid reaction to form partially active enzyme having 54% of the original activity, followed by a slower reaction to yield totally inert enzyme. In addition to the pyruvate kinase activity of the enzyme, modification with 5′-*p*-fluorosulfonylbenzoyladenosine also disrupts its ability to catalyze the decarboxylation of oxaloacetate and the ATP-dependent enolization of pyruvate. In correspondence with the time dependence of inactivation, the rate of incorporation of 5′-*p*-[^{14}C]fluorosulfonylbenzoyladenosine is also biphasic. Two moles of reagent per mole of enzyme subunit are bound when the enzyme is completely inactive. The pseudo-first-order rate constant for the rapid rate is linearly dependent on reagent concentration, whereas the constant for the slow rate exhibits saturation kinetics, suggesting that the reagent binds reversibly to the second site prior to modification. The adenosine moiety is essential for the effectiveness of 5′-*p*-fluorosulfonylbenzoyladenosine, since *p*-fluorosulfonylbenzoic acid does not inactivate pyruvate kinase at a significant rate. Thus, the reaction of 5′-*p*-fluorosulfonylbenzoyladenosine with pyruvate kinase exhibits several of the characteristics of affinity labeling of the enzyme. Protection against inactivation by 5′-*p*-fluorosulfonylbenzoyladenosine is provided by the addition to the incubation mixture of phosphoenolpyruvate, Mg-ADP or Mg^{2+}. In contrast, the addition of pyruvate, Mg-ATP, or ADP and ATP alone has no effect on the rate of inactivation. These observations are consistent with the postulate that the 5′-*p*-fluorosulfonylbenzoyladenosine specifically labels amino acid residues in the binding region for Mg^{2+} and the phosphoryl group of phosphoenolpyruvate which is transferred during the catalytic reaction. The rate of inactivation increases with increasing pH, and k_1 depends on the unprotonated form of an amino acid residue with pK = 8.5. On the basis of the pH dependence of the reaction of pyruvate kinase with 5′-*p*-fluorosulfonylbenzoyladenosine and the elimination of cysteine residues as possible sites of reaction, it is postulated that lysyl or tyrosyl residues are the most probable candidates for the critical amino acids.

Although adenine nucleotides participate in a large number of biochemical reactions as substrates or allosteric regulators, in most cases little is known concerning the chemical nature of the association of the purine nucleotide with the enzyme. One approach to this problem is through the use of specific nucleotide analogues which mimic the normal substrate or effector but have alkylating functional groups at definite positions of the purine or ribose ring. A few reports have appeared in which purine derivatives have been used to modify enzymes which have purine nucleotide binding sites (Schaffer and Odin, 1966; Hampton and Nomura, 1967; Hulla and Fasold, 1972; Anderson et al., 1973; Faust et al., 1974). However, several of these purine nucleotide analogues have bulky groups in a region of the molecule that may be critical for binding (e.g., 6-(purine 5′-ribonucleotide)-5-(2-nitrobenzoic acid)thioether (Hulla and Fasold, 1972)); this steric factor may prevent binding or alter the position of binding of the nucleotide analogue to the enzyme. Other compounds lack the critical ribose phosphate moiety [e.g., 9-(*p*-bromoacetamidobenzoyl)adenine (Schaffer and Odin, 1966)); it might be anticipated that the ribose phosphate moiety would be an important constituent of any general nucleotide affinity label, since several allosteric enzymes are known to respond to nucleotides while being insensitive to the corresponding purines. Photoaffinity labeling has received much attention, as exemplified by studies of diazomalonyl-cAMP (Guthrow et al., 1973), 8-azidoadenosine 3′,5′-monophosphate (Pomerantz et al., 1975), and 8-azido-ATP (Haley and Hoffman, 1974). Although they exhibit broad reactivity, the application of these photolabile analogues has certain inherent difficulties, since upon irradiation the label frequently tends to react with the solvent as well as with any amino acid adjacent to the compound. Quantitative reaction with any particular amino acid is rarely accomplished, making it difficult to ascertain which residues are actually involved in binding the purine nucleotide.

The reagent used in the present paper is 5′-*p*-fluorosulfonylbenzoyladenosine (shown in Figure 1), which might

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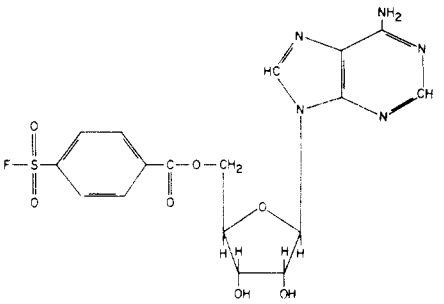


FIGURE 1: Structure of 5′-*p*-fluorosulfonylbenzoyladenosine.

reasonably be considered as an analogue of ADP or ATP. In addition to the adenine and ribose moieties, it has a carbonyl group adjacent to the 5′ position which is structurally similar to the first phosphoryl group of the naturally occurring purine nucleotides. If the molecule is arranged in an extended conformation, the sulfonyl fluoride moiety may be located in a position analogous to the γ-phosphoryl group of ATP. In addition to its structural similarity to naturally occurring adenosine nucleotides, in the sulfonyl fluoride substituent it has a sulfonating group of broad specificity capable of reacting with lysine, tyrosine, histidine, and serine (Paulos and Price, 1974). This reagent has previously been used as a probe in the study of the regulatory site of glutamate dehydrogenase (Pal et al., 1975a). It is here shown to be effective in chemically modifying a kinase.

Considerable information is presently available concerning the geometry of the active site and the arrangement of substrates within this region for pyruvate kinase (Sloan and Mildvan, 1976; Gupta et al., 1976; Mildvan et al., 1976a,b; Melamud and Mildvan, 1975; James and Cohn, 1974). By the use of specific paramagnetic probes and nuclear magnetic resonance, the conformation, affinity, and rate of dissociation of the enzyme-bound substrates have been determined. Furthermore, the distances between certain key atoms have been measured for the various substrates which form the catalytically active species. In addition, preliminary results have been obtained from x-ray crystallographic studies of cat muscle pyruvate kinase (Stammers and Muirhead, 1975). Chemical-modification studies have been conducted using trinitrobenzenesulfonate (Hollenberg et al., 1971), and 5,5′-dithiobis(2-nitrobenzoic acid) (Flashner et al., 1972) which suggest that lysyl and cysteinyl residues participate in the nucleotide binding site. Inactivation by diethyl pyrocarbonate has implicated histidyl residues in the activity of the enzyme obtained from either rabbit muscle or yeast (Dann and Britton, 1974; Bornmann and Hess, 1974). However, much remains to be learned concerning the amino acid environment of the nucleotide binding region. In this paper, the 5′-*p*-fluorosulfonylbenzoyladenosine apparently functions as an affinity label of the active site of rabbit muscle pyruvate kinase.[1]

Experimental Procedure

Materials. Rabbit muscle pyruvate kinase was purchased from Boehringer-Mannheim as either a crystalline suspension in ammonium sulfate or dissolved in 50% glycerol. The enzyme with a specific activity of about 200 enzyme units/mg was dialyzed at 4 °C against 0.05 M potassium phosphate buffer, pH 7.5, concentrated to give a final concentration of approximately 20 mg/mL and was stored in aliquots at −85 °C. This preparation, which exhibited only one band on polyacrylamide disc gel electrophoresis using 7% acrylamide at pH 8.9 as described by Davis (1964), was used without further purification in all the studies described in this paper. The enzyme concentration was determined using $E_{280\ nm}^{1\%} = 5.40$ (Boyer, 1962) and a molecular weight of 57 000 per subunit (Cottam et al., 1969) was used in all calculations involving pyruvate kinase.

The nucleotides ADP and ATP as well as phosphoenolpyruvate and pyruvate were purchased from Sigma Chemicals. The [8-^{14}C]adenosine was obtained from New England Nuclear Corp. All other chemicals were reagent grade.

Preparation of 5′-p-Fluorosulfonylbenzoyladenosine. The 5′-*p*-fluorosulfonylbenzoyladenosine, shown in Figure 1, was prepared by reaction of adenosine with 5′-*p*-fluorosulfonylbenzoyl chloride either by the precedure of Pal et al. (1975a) or by the following modified procedure. Adenosine (1.13 g, 4.2 mmol) was dissolved in 10 mL of hexamethylphosphoric triamide by warming to 50 °C in a water bath. Upon cooling, *p*-fluorosulfonylbenzoyl chloride (1.32 g, 6 mmol) was added and the mixture was allowed to stand at room temperature overnight (18 h). The reaction mixture was extracted with 30 mL of petroleum ether. After separation of the two layers, the product was precipitated from the lower layer by the slow addition of 40 mL of ethyl acetate–diethyl ether (1:1). The product was collected by filtration and air dried. This procedure, which should be conducted under a hood, yields between 1.6 and 2.0 g of product. A sample was dried at room temperature under vacuum for analysis. The elemental analysis of the compound revealed the presence of 1 mole of hexamethylphosphoric triamide per mol of 5′-*p*-fluorosulfonylbenzoyladenosine, thus giving a molecular weight of 632.7. This product chromatographed together with the reagent prepared by Pal et al. (1975a) using EM silica gel F-254 thin-layer chromatography plates and a solvent system consisting either of methyl ethyl ketone–acetone–water (65:20:15), $R_f = 0.76$, or methanol–chloroform (15:85), $R_f = 0.56$. The ultraviolet absorption spectrum exhibits maxima at the same wavelengths reported previously: at 232 nm ($\epsilon = 1.88 \times 10^4$ cm^{-1}M^{-1}) and 259 nm ($\epsilon = 1.35 \times 10^4$ cm^{-1}M^{-1}). Although the 5′-FSO_2BzAdo[2] prepared by either procedure inactivates pyruvate kinase in the same fashion, i.e., the rate constants determined from the inactivation profile are identical, there are several differences between the products. First, the 5′-FSO_2BzAdo prepared by the procedure of Pal et al. (1975a) contains 1 mol of dimethylformamide per mol of compound, whereas the reagent prepared by the above procedure contains 1 mol of hexamethylphosphoric triamide per mol of 5′-FSO_2BzAdo. Second, the extinction coefficients for the compound at 232 and 259 nm are different; the values previously reported for the compound containing dimethylformamide were 2.17×10^4 cm^{-1}M^{-1} and 1.58×10^4 cm^{-1}M^{-1}, respectively. These differences have been consistent for several different preparations of the reagent using both procedures. Third, the reagent prepared previously has a melting point (159–160 °C) which is 10 °C higher than the one prepared by the modified procedure above.

Radioactive 5′-*p*-fluorosulfonylbenzoyladenosine was prepared by the addition of 100 μCi of [8-^{14}C]adenosine to the nonlabeled adenosine (5 mmol). Reaction with *p*-fluorosul-

[1] A preliminary version of this study was presented at the 67th Annual Meeting of the American Society of Biological Chemists in San Francisco, Calif., June, 1976.

[2] Abbreviations used are: 5′-FSO_2BzAdo, 5′-*p*-fluorosulfonylbenzoyladenosine; 5′-SO_2BzAdo, 5′-*p*-sulfonylbenzoyladenosine; Tris-Cl, 2-amino-2-hydroxymethyl-1,3-propanediol chloride; PEP, phosphoenolpyruvate; AMP and ADP, adenosine mono- and diphosphates.

fonylbenzoyl chloride (6 mmol) was conducted as described.

Determination of Enzyme Activity. The predominant assay procedure used in this study was the spectrophotometric assay described by Pon and Bondar (1967) which measures directly and continuously the disappearance of phosphoenolpyruvate at 230 nm. A Gilford, Model 240, spectrophotometer equipped with an expanded scale recorder (0–0.1 absorbance full scale) was used. The enzymatic activity was determined at 30 °C in 0.05 M Tris-Cl buffer, pH 7.5, containing 3 mM ADP, 0.3 mM PEP, 7.2 mM $MgSO_4$, and 72 mM KCl. The reaction was carried out in a 10-mm quartz cell fitted with a 9-mm quartz insert giving an effective path length of 1 mm. The total volume of the assay solution was 1.0 mL. In the determinations of Michaelis constants for ADP and PEP, all concentrations were maintained constant at these levels except for the one variable substrate.

For certain experiments, as indicated, the activity of pyruvate kinase was measured titrimetrically by the rate of addition of 5 mM hydrochloric acid required to maintain the pH constant. A Radiometer pH stat was used, following the procedure outlined by Melchior (1965). The pH-stat assay allowed for the use of higher substrate concentrations. In a typical assay, the reaction mixture (total volume = 4 mL) contained 15 mM ADP, 4 mM PEP, 1.6 mM $MgSO_4$, and 0.05 M KCl at pH 7.5. The total volume of acid added did not exceed 0.25 mL.

The oxaloacetate decarboxylase activity of pyruvate kinase (Creighton and Rose, 1976a) was determined according to the procedure of Kosicki (1968). The assay depends upon the decrease in absorbance at 260 nm of the enol form of the equilibrated tautomeric mixture of the oxaloacetate–Mn^{2+} complex.

Pyruvate kinase is known to catalyze the enolization of pyruvate (Rose, 1960). This activity was measured by a procedure similar to that described by Flashner et al. (1973). An aliquot of enzyme, which had been dialyzed against 0.05 M Tris-Cl buffer, pH 7.5, was incubated with [3-^{3}H]pyruvate in 0.05 M Tris-Cl buffer, pH 7.5 containing 8 mM $MgCl_2$, 0.1 M KCl, and 60 mM cold pyruvate and either 2.7 mM ATP for the ATP-dependent enolization or 0.1 M potassium phosphate for the inorganic phosphate-dependent reaction. The total volume of the incubation mixture was 0.15 mL. The reaction was carried out at 37 °C for 30 min, at which time a 0.1-mL aliquot of the incubation mixture was transferred to a 0.75 × 4 cm Dowex-1-chloride column. The column was washed with 3 mL of water and allowed to run dry. The water collected from the column contained all of the ^3H_2O exchange during the enolization. The radioactivity in 1 mL of the wash was measured in a Packard Tri-carb liquid scintillation counter, Model 3330.

Reaction of 5′-p-Fluorosulfonylbenzoyladenosine with Pyruvate Kinase. Pyruvate kinase (1 mg/mL) was incubated with 5′-FSO_2BzAdo at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.20 M KCl and 15% dimethylformamide. The 15% dimethylformamide was necessary to maintain the solubility of 5′-FSO_2BzAdo and had no effect on the enzyme activity or stability. During the course of the reaction, aliquots were withdrawn at given time intervals and assayed by one of the procedures described above. Since the 5′-FSO_2BzAdo was diluted approximately 500-fold in the assay solution, and since relatively high concentrations of PEP, Mg^{2+}, and ADP were present in the assay solution (all of which protect against inactivation by the reagent), it was considered that no appreciable additional reaction between pyruvate kinase and 5′-FSO_2BzAdo took place during the 1–2 min required to conduct the assay. Indeed, the individual assays were linear and seemed to reflect the residual enzymatic activity at the time of withdrawal of aliquots from the reaction mixture. The rate of reaction of pyruvate kinase with 5′-FSO_2BzAdo was assessed from a semilogarithmic plot of E/E_0 as a function of time, where E and E_0 represent the enzymatic activities of a given time and at zero time, respectively. In experiments involving the addition of protecting agents to the reaction mixture, the concentration of KCl was adjusted to maintain a constant ionic strength (μ = 0.2).

In order to measure the incorporation of 5′-FSO_2BzAdo by the enzyme, radioactive 5′-FSO_2BzAdo was incubated with pyruvate kinase at 30 °C under conditions similar to those given above. During the course of the reaction, 0.5-mL aliquots were withdrawn at specified times and immediately dialyzed against 0.05 M sodium phosphate buffer, pH 7.4, at 4 °C for 12 to 16 h. (The rapid decrease in the temperature by 26 °C, as well as the dialysis to remove the 5′-FSO_2BzAdo and the added presence of phosphate ion which has been found to react with fluorosulfonylbenzoyladenosine (Pal et al., 1976), all combine to effectively prevent appreciable further reaction of pyruvate kinase with 5′-FSO_2BzAdo. This conclusion is supported by the observation that partially active enzyme samples assayed after 2 h of dialysis under these conditions still exhibited essentially the same percentage residual activity as they had when first removed from the reaction mixture.) For the incorporation experiments, the protein was then dialyzed for at least 24 h against 0.05 M sodium phosphate buffer, pH 7.4, containing 14.2 mM mercaptoethanol and 0.5% sodium dodecyl sulfate in order to ensure removal of all noncovalently bound reagent. Final dialysis to remove the detergent and mercaptoethanol was carried out against 0.05 M sodium phosphate buffer, pH 7.4, for 12 h. The protein concentration was determined by the Lowry method (1951). The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter, Model 3330.

Titration of Sulfhydryl Groups of Pyruvate Kinase as a Function of Inactivation. Pyruvate kinase was inactivated with 5′-FSO_2BzAdo as described above. Concomitantly, aliquots were withdrawn at definite time intervals, mixed with excess 5,5′-dithiobis(2-nitrobenzoate) and the number of sulfhydryl groups was determined from the absorbance at 412 nm, using $\epsilon = 1.36 \times 10^4$ (Ellman, 1958). A 0.1-mL aliquot of the reaction mixture was added to a cuvette with a 10-mm path length containing 0.7 mL of 0.1 M Tris-Cl buffer, pH 7.7, and 0.1 mL of 20% sodium dodecyl sulfate. Finally, 0.1 mL of a 10 mM solution of 5,5′-dithiobis(2-nitrobenzoate) was added, and the absorbance was read at 412 nm.

Results

Inactivation of Pyruvate Kinase by 5′-p-Fluorosulfonylbenzoyladenosine. Rabbit muscle pyruvate kinase is inactivated upon incubation with 5′-*p*-fluorosulfonylbenzoyladenosine at 30 °C in 0.02 M potassium barbital buffer, pH 7.4. In contrast, enzyme incubated under similar conditions in the absence of the reagent does not lose activity. A plot of log E/E_0 vs. time (Figure 2) reveals a biphasic rate of inactivation which can be described by a rapid reaction to form partially active enzyme (with 54% residual activity = F_1) followed by a slower reaction to yield totally inert enzyme. The slower phase has been observed to follow the same rate at least as far as a residual activity of 10%. This model has been described by Ray and Koshland (1961), according to which the pseudo-first-order rate constant for the slow reaction (k_2) can be deter-

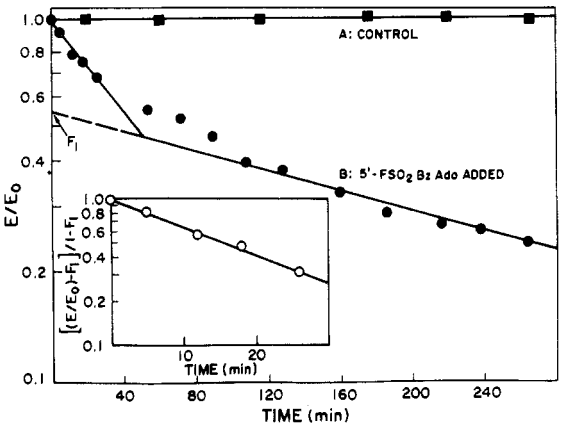


FIGURE 2: Reaction of 5′-*p*-fluorosulfonylbenzoyladenosine with pyruvate kinase. Pyruvate kinase (1.0 mg/mL) was incubated with 5′-*p*-FSO_2BzAdo (2.1 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.2 M KCl and 15% dimethylformamide. At each indicated time, a 2-μL aliquot was withdrawn and assayed for pyruvate kinase activity as described under Experimental Procedure by the decrease in absorbance at 230 nm. Insert: Determination of the pseudo-first-order rate constant for the rapid phase of the inactivation curve in accordance with the model purposed by Ray and Koshland (1961), where F_1 represents the activity of the partially active intermediate ($F_1 = 0.54$).

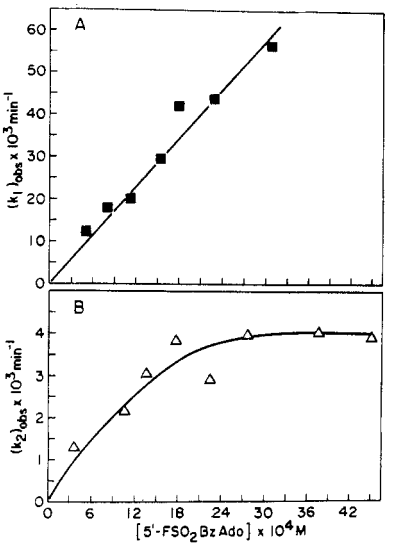


FIGURE 3: Dependence of the pseudo-first-order rate constants on the concentration of 5′-*p*-fluorosulfonylbenzoyladenosine. Pyruvate kinase (1 mg/mL) was incubated with several concentrations of 5′-FSO_2BzAdo at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.2 M KCl and 15% dimethylformamide. The rate constants for (A) the fast (k_1) and (B) the slow (k_2) reactions were determined as described in Figure 2.

TABLE I: Michaelis Constants for Substrates for Unreacted and Modified Pyruvate Kinase.[a]

Incubation Mixture	Time (min)	Residual Act. (%)	Michaelis Constants PEP (μM)	Michaelis Constants ADP (mM)
Control	0	100	86	1.2
	120	95	94	1.2
5′-FSO_2-BzAdo added	30	60	125	1.6
	60	43	125	1.4
	120	26	167	1.4

[a] Pyruvate kinase was incubated at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.2 M KCl and either 15% dimethylformamide alone (control) or 15% dimethylformamide and 2.5 mM 5′-FSO_2BzAdo. After removal of excess reagent, the Michaelis constants were measured as described under Experimental Procedure. In the determination of the K_M for PEP and ADP, the potassium salt of PEP was used. Under similar conditions, native enzyme which was not exposed to the incubation treatment exhibited K_M values of 70 μM for PEP and 0.68 mM for ADP.

mined directly from the second phase of the graph, whereas the pseudo-first-order rate constant for the fast reaction (k_1) can be estimated from a plot of log $[(E/E_0) - F_1]/(1 - F_1)$ vs. time (Figure 2, insert). At 2.1 mM 5′-FSO_2BzAdo, determination of the pseudo-first-order rate constants by this procedure gives a value for the fast rate ($k_1 = 4.52 \times 10^{-2}$ min^{-1}) that is approximately ten times faster than the second rate ($k_2 = 0.36 \times 10^{-2}$ min^{-1}).

Because the spectrophotometric assay for pyruvate kinase uses relatively low substrate concentrations (i.e., 0.8 mM PEP and 3 mM ADP), one possible explanation for the biphasic nature of the inactivation curve might be that reaction with 5′-FSO_2BzAdo produced an enzyme species which was intrinsically active but which exhibited a decreased affinity for substrates. In order to test this hypothesis, pyruvate kinase was incubated either with 2.5 mM 5′-FSO_2BzAdo in 15% dimethylformamide or with 15% dimethylformamide alone at 30 °C as described under Experimental Procedure. Aliquots were removed at the specified time intervals and dialyzed for 2 h at 4 °C against two changes of 0.05 M potassium phosphate buffer, pH 7.4, to remove the excess reagent. The Michaelis constants for phosphoenolpyruvate and ADP were measured using these various aliquots, with the results recorded in Table I. Although the residual activity was reduced to 26% during the time of incubation with the reagent, no significant change was noted in the K_M for ADP and at most a twofold increase was observed in the K_M for PEP. This change is much too small to account for the loss of activity illustrated by Figure 2. Furthermore, the effect of 5′-FSO_2BzAdo on the activity of the enzyme could also be monitored using a titrimetric assay in which the concentrations of substrates were considerably higher (i.e., 4 mM PEP and 15 mM ADP). Results similar to those shown in Figure 2 were obtained. Thus, it appears that reaction of 5′-*p*-fluorosulfonylbenzoyladenosine with pyruvate kinase produces an initial decrease in the maximum velocity of the enzyme, followed by a total inactivation.

In the case of the fast reaction, the pseudo-first-order rate constant is linearly dependent on the concentration of 5′-FSO_2BzAdo (Figure 3A), indicating that there is no rapidly reversible binding of the reagent to the first site prior to inactivation. A second-order rate constant of 19.8 min^{-1} M^{-1} may be calculated for this rapid reaction. In contrast, for the slow reaction the plot of the pseudo-first-order rate constant vs. reagent concentration is not linear, but, rather, reveals saturation kinetics (Figure 3B). It appears that 5′-FSO_2BzAdo does bind reversibly to the second site prior to the irreversible inactivation. This type of kinetics may be described in terms of the following relationship:

$$E + R \overset{K_R}{\rightleftharpoons} [ER] \xrightarrow{k_2} X \qquad (1)$$

where [ER] is the concentration of the complex of enzyme and 5′-FSO_2BzAdo, X is the inactive form of the enzyme, and K_R is the dissociation constant for the ER complex. The observed rate constant for inactivation ($(k_2)_{obsd}$) can be expressed as:

TABLE II: Effect of 5′-*p*-Fluorosulfonylbenzoyladenosine Modification of Pyruvate Kinase on Detritiation of Pyruvate Activated by ATP or by P_i.[a]

Enzyme Sample Time (min)	Residual Pyruvate Kinase Act. (%)	ATP-Dependent Enolization cpm[b]	ATP-Dependent Enolization % Residual Act.	P_i-Dependent Enolization cpm[c]	P_i-Dependent Enolization % Residual Act.
0	100	5555	100	1322	100
20	46	2928	52	1054	79
60	13	701	13	848	64

[a] Pyruvate kinase (1 mg/mL) was incubated with 5′-FSO_2BzAdo (2.5 mM) at 30 °C in 0.02 M potassium phosphate buffer, pH 7.7, containing 0.2 M KCl and 15% dimethylformamide. Aliquots were assayed for pyruvate kinase activity by the $\Delta A_{230\,nm}$ method. In addition, 0.5-mL aliquots were removed at designated times and dialyzed for 2 h against two changes of 0.05 M Tris-Cl buffer, pH 7.5. These samples (0.5 mL) were then used to determine the enolization activity, as described under Experimental Procedures, of modified pyruvate kinase. [b] Values are corrected for 923 cpm exchanged by incubating [3-^{3}H]pyruvate under same conditions in the absence of enzyme. [c] Values are corrected for 1127 cpm exchanged by incubating [3-^{3}H]pyruvate under same conditions in the absence of enzyme.

$$(k_2)_{obsd} = \frac{k_2}{1 + (K_R/[R])} \quad (2)$$

A value of K_R = 0.84 mM may be estimated for the reversible enzyme–reagent complex, with $k_2 = 4.2 \times 10^{-3}$ min^{-1}. The K_R value for the enzyme–5′-FSO_2BzAdo complex compares with the K_M of 0.68 mM for ADP. The different reagent concentration dependence of the two phases of the reaction supports a model of two discrete loci for reaction of 5′-FSO_2BzAdo with pyruvate kinase.

Recently, Creighton and Rose (1976a,b) reported that oxaloacetate decarboxylase and pyruvate kinase activities were attributable to the same enzyme. Both activities require the presence of a divalent cation. In order to ascertain whether or not 5′-FSO_2BzAdo had any effect on the oxaloacetate decarboxylase function, the enzyme was incubated with the reagent under conditions known to inactivate pyruvate kinase, and the oxaloacetate decarboxylase activity was determined according to the procedure outlined by Kosicki (1968). Thus, the enzyme (1 mg/mL) was incubated with 5′-FSO_2BzAdo (2.5 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.2 M KCl and 15% dimethylformamide, and aliquots were assayed for residual activity at defined time intervals. The time dependence of loss of oxaloacetate decarboxylase activity was biphasic and was very similar to that obtained for pyruvate kinase inactivation. The remaining activity at 2 h was 25%, which compared quite well with the residual pyruvate kinase activity obtained in the absence of substrates (Table III). These results suggest that 5′-FSO_2BzAdo exerts a similar effect on the pyruvate kinase and the oxaloacetate decarboxylase activities of this enzyme.

Another reaction catalyzed by pyruvate kinase is the enolization of pyruvate (Rose, 1960). This reaction requires, in addition to pyruvate, a divalent metal ion (Mn^{2+} in this case), plus either inorganic phosphate or ATP. In order to compare the effect of 5′-FSO_2BzAdo on the pyruvate kinase and pyruvate enolization activities, enzyme (1 mg/mL) was incubated with reagent (2.5 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.7. Two-microliter aliquots were withdrawn at various time intervals and assayed by the $\Delta A_{230\,nm}$ method, to follow the loss in pyruvate kinase activity. Concomitantly,

TABLE III: Effect of Substrates on the Inactivation of Pyruvate Kinase by 5′-*p*-Fluorosulfonylbenzoyladenosine.[a]

Additions to Reaction Mixture	Residual Act. (%)
None	26
PEP (1 mM)	66
ATP (10 mM)	27
ADP (10 mM)	24
Pyruvate (10 mM)	27
$MgSO_4$ (10 mM)	65
$MgSO_4$ (10 mM) + PEP (1 mM)	67
$MgSO_4$ (10 mM) + ADP (10 mM)	66
$MgSO_4$ (10 mM) + ATP (10 mM)	22
$MgSO_4$ (20 mM) + ATP (10 mM)	65

[a] Pyruvate kinase (1 mg/mL) was incubated with 5′-FSO_2BzAdo (2.5 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 15% dimethylformamide. The ionic strength was adjusted to 0.2 by the addition of KCl. Aliquots were assayed for pyruvate kinase activity and the residual activity at 2 h is reported.

0.5-mL samples were removed at various times and dialyzed for 2 h against two changes of 0.05 M Tris-Cl buffer, pH 7.5. Aliquots of the dialyzed enzyme (0.25 mL) were incubated with [3-^{3}H]pyruvate in the presence of either inorganic phosphate or ATP, as described under Experimental Procedure. The results, recorded in Table II, indicate a close parallel between the loss of the ability of the enzyme to catalyze the ATP-dependent enolization of pyruvate, and the ability to promote the pyruvate kinase reaction. When the pyruvate kinase activity falls to 13% of its original value, the ATP-dependent detritiation activity is also only 13% of that exhibited by unreacted enzyme. In contrast, at the same level of pyruvate kinase activity (13%), the inorganic phosphate promoted enolization of pyruvate still retains 64% of its original activity. It appears that the pyruvate enolization reaction is not identical when promoted by inorganic phosphate and ATP and that the amino acid residues required must differ at least by those attacked by 5′-FSO_2BzAdo.

In order to evaluate the contribution of the adenosine moiety to the effectiveness of 5′-*p*-fluorosulfonylbenzoyladenosine in inactivating pyruvate kinase, the ability of *p*-fluorosulfonylbenzoic acid to produce inactivation was tested. The enzyme (1 mg/mL) was incubated with *p*-fluorosulfonylbenzoic acid (1.63 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.2 M KCl and 15% dimethylformamide. After 2 h of incubation time, the residual pyruvate kinase activity was 93% of control, whereas, under similar conditions, but with 5′-FSO_2BzAdo as reagent, the residual activity at 2 h was 35% of control. Thsse data indicate the importance of the adenosine moiety in determining the effectiveness of 5′-FSO_2BzAdo.

Effect of Substrates and Coenzymes on Reaction with 5′-FSO_2BzAdo. Table III records the effect on residual activity at 2 h of adding various ligands to the incubation mixture of enzyme and 2.5 mM 5′-FSO_2BzAdo. This time period is sufficiently long to reflect inactivation resulting from both the rapid and slow phases of the reaction. Significant protection against loss of activity is afforded by the substrate PEP alone, or by Mg^{2+} either alone or added together with ADP or PEP. The protection provided by magnesium and PEP or ADP is no greater than that produced by the metal ion alone. The presence of pyruvate, ADP, and ATP alone does not appreciably affect the extent of inactivation. It is interesting to note that, in contrast to ADP and Mg^{2+}, the simultaneous addition of equimolar concentrations of ATP and Mg^{2+} does not protect

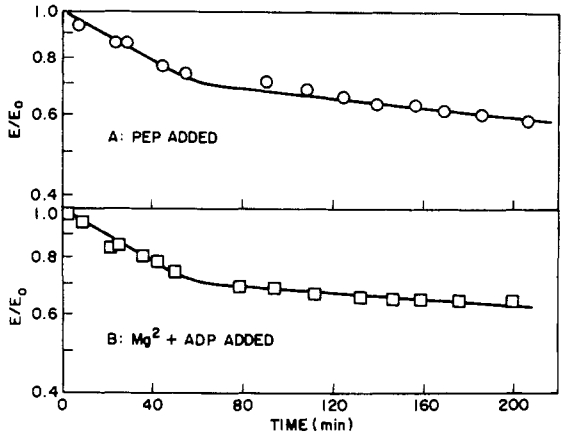


FIGURE 4: Protection against the inactivation of pyruvate kinase with 5′-*p*-fluorosulfonylbenzoyladenosine. (A) Pyruvate kinase (1 mg/mL) was incubated with 5′-FSO_2BzAdo (2.55 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.18 M KCl, 15% dimethylformamide, and 5 mM PEP ($k_i = 1.08 \times 10^{-2}$ min^{-1}). (B) The enzyme (1 mg/mL) was incubated with 5′-FSO_2BzAdo (2.55 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.1 M KCl, 15% dimethylformamide, and 20 mM $MgSO_4$ + 20 mM ADP ($k_i = 1.23 \times 10^{-2}$ min^{-1}). Pyruvate kinase activity was measured spectrophotometrically at the indicated times.

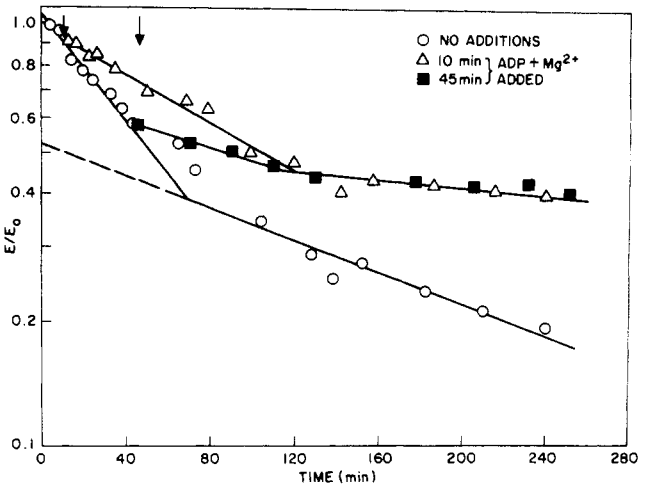


FIGURE 5: Effect on the rate of inactivation by 5′-FSO_2BzAdo of the addition at various times of Mg-ADP. Pyruvate kinase (1 mg/mL) was incubated with 5′-FSO_2BzAdo (2.3 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.2 M KCl and 15% dimethylformamide, and the time course of inactivation was followed (⊙). The reaction mixture was divided into three equal parts. At 10 min (△) Mg-ADP was added to one solution to give a final concentration of 20 mM. At 45 min (■) Mg-ADP was added to another solution to yield the same final concentration. In all three cases, 2 μL was removed at the indicated times and assayed for pyruvate kinase activity by following the decrease in absorbance at 230 nm.

against inactivation. It may well be that the metal ion in the Mg–ATP chelate occupies a different site than it does in Mg-ADP or when present as free Mg^{2+}. It should be noted that when magnesium is added in concentrations in excess of ATP significant protection is again observed. This pattern of protecting ligands suggests that reaction of 5′-FSO_2BzAdo may take place in the general region of the binding site for metal ion and the phosphoryl group of PEP which is transferred to ADP in the catalytic step.

Potassium ions were present in all the experiments listed in Table III in order to maintain constant ionic strength. Since potassium ion is a known activator of pyruvate kinase (Boyer, 1962), it was necessary to substitute for K^+ another monovalent cation in order to test specifically the effect of that activator on the course of reaction with 5′-FSO_2BzAdo. Tetramethylammonium ion, which is known not to activate the enzyme (Kayne and Suelter, 1965), was used for this purpose. Pyruvate kinase was inactivated by incubating the enzyme with 2.5 mM 5′-FSO_2BzAdo in 0.02 M barbital buffer, pH 7.4, containing either 0.2 M KCl and 15% dimethylformamide or 0.2 M tetramethylammonium chloride and 15% dimethylformamide. The pseudo-first-order rate constants obtained under these conditions were identical, implying that the monovalent cation binding site was not the locus of attack of the 5′-FSO_2BzAdo.

Since oxaloacetate is a known substrate for one of the secondary reactions catalyzed by pyruvate kinase (Creighton and Rose, 1976a), its effect as a protectant against the loss of pyruvate kinase activity was examined. Enzyme, incubated with 5′-FSO_2BzAdo under the same conditions as in Table III (line 1) except for the addition of 0.01 M oxaloacetate, exhibited the same time dependence for inactivation observed when no substrates were present. Thus, oxaloacetate does not protect against the inactivation caused by the reaction of the enzyme with 5′-FSO_2BzAdo.

The substrates PEP or Mg-ADP reduce the rate constant for the rapid phase of the reaction approximately fivefold as compared to that observed for the enzyme alone (Figure 4); however, total protection against the effect of 5′-FSO_2BzAdo was never attained, even at concentrations of 25 mM PEP and 50 mM Mg-ADP. One possible explanation is that these results reflect a selective protection whereby the substrates completely prevent reaction at one site, but do not affect reaction at both sites. Alternatively, there might be reduced reaction at both sites. In order to ascertain whether the ligands influence both phases of the reaction, an experiment was conducted in which the protective substrates Mg^{2+} and ADP were added either at 10 or at 45 min following the initiation of the reaction. As shown in Figure 5, the effect of the protectants is immediate; that is, the rate of inactivation was significantly reduced following the addition of Mg-ADP at 10 min and was similar to that shown in Figure 4. At 45 min in the absence of substrates, the reaction at the first site should be almost complete; therefore, if the protection afforded by Mg-ADP is primarily directed against reaction at the first site, little effect on the second rate of inactivation should have been observed when Mg-ADP was added at 45 min. However, Figure 5 shows that the rate of the second reaction was reduced. From the results of this experiment, it can be concluded that the protection afforded by Mg-ADP is directed against both sites attacked by 5′-FSO_2BzAdo.

Stoichiometry of Reaction of Pyruvate Kinase with 5′-p-Fluorosulfonylbenzoyladenosine. The 5′-fluorosulfonylbenzoyl analogue of adenosine reacts irreversibly and in a limited fashion with rabbit muscle pyruvate kinase. The stoichiometry of this reaction was determined as described under Experimental Procedure by the measurement of the incorporation of 5′-SO_2BzAdo labeled with ^{14}C at the C-8 position of the adenosine. A plot of the moles of 5′-SO_2BzAdo incorporated as a function of time is biphasic (Figure 6), in correspondence with the time dependence of inactivation (Figure 2). A comparison of the residual activity and incorporation of 5′-SO_2BzAdo at a given time allows the generation of a plot of percent inactivation vs. moles of 5′-SO_2BzAdo incorporated per mole of subunit, which is linear as far as 20% residual enzymatic activity (Figure 7). An extrapolated value of 2.1 mol of 5′-SO_2BzAdo per mole of enzyme subunit at 100% inacti-

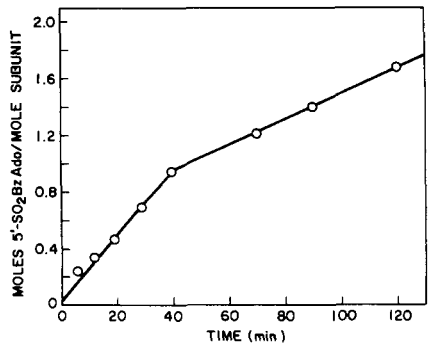


FIGURE 6: Incorporation of radioactive 5′-*p*-fluorosulfonylbenzoyladenosine per subunit of pyruvate kinase as a function of time. Pyruvate kinase was incubated with 5′-[^{14}C]FSO_2BzAdo (2.5 mM) at 30 °C in 0.02 M potassium barbital buffer, pH 7.4, containing 0.2 M KCl and 15% dimethylformamide. At each indicated time, about 0.5-mL aliquots were withdrawn and quickly dialyzed against 0.05 M sodium phosphate buffer, pH 7.4, at 4 °C. This sample was further dialyzed against 0.05 M sodium phosphate buffer, pH 7.4, containing 14.2 mM mercaptoethanol and 0.5% sodium dodecyl sulfate, followed by final dialysis against 0.05 M sodium phosphate buffer, pH 7.4. The residual activity was determined by assaying 2-μL aliquots during the course of inactivation at various times as described in Figure 2, and yielded rates of inactivation similar to those shown in Figure 2. The protein concentrations were determined by the Lowry method.

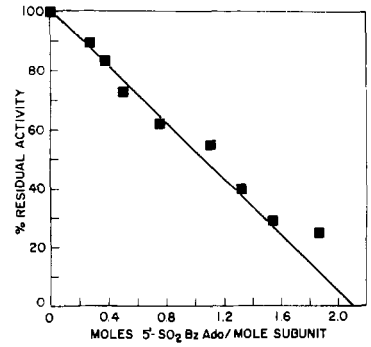


FIGURE 7: Incorporation of radioactive 5′-*p*-fluorosulfonylbenzoyladenosine per subunit of pyruvate kinase as a function of inactivation. The conditions for this experiment are the same as those found in the legend of Figure 6.

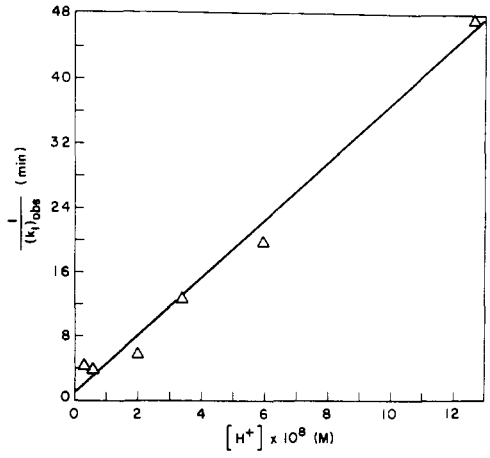


FIGURE 8: Determination of the pK of the reactive group of pyruvate kinase. Pyruvate kinase (1 mg/mL) was incubated with 5′-FSO_2BzAdo (2.5 mM) at 30 °C in 0.02 M potassium barbital buffer, at various hydrogen ion concentrations indicated above, containing 0.2 M KCl and 15% dimethylformamide. The pseudo-first-order rate constants were determined for the fast rate, using the correction $F_1 = 0.54$. From the plot of the reciprocal of $(k_1)_{obsd}$ vs. the hydrogen ion concentration, a pK of 8.5 was estimated.

vation was estimated from this graph, although some deviations may occur in the direction of greater incorporation for enzyme inactivated more extensively than 80%.

Similar incorporation experiments were conducted in the presence of the protectants 25 mM PEP and 20 mM Mg-ADP. Incorporation studies performed in the presence of 20 mM Mg-ADP revealed a value of 0.5 mol of 5′-SO_2BzAdo bound per mole of enzyme subunit concomitant with a 30% loss in activity after 2-h reaction. This value is consistent with the expected incorporation for this extent of inactivation when compared to the unprotected case. It seems likely that Mg^{2+} and ADP decrease the rate of reaction at the same residues which are attacked in the absence of substrates.

In contrast, in the presence of 25 mM PEP, a value of 2 mol of 5′-SO_2BzAdo per mol of enzyme subunit was obtained concomitant with a 25% loss in activity in 2 h. This compares to approximately 1.6 mol of reagent being incorporated per mol of enzyme subunit at 74% inactivation in the unprotected case under similar conditions in 2 h. The implication is that PEP changes the specificity of the enzyme for reaction with 5′-FSO_2BzAdo and causes different groups to react with the nucleotide derivative.

Determination of pK for the Reactive Group. There is a significant effect of pH on both rates of pyruvate kinase by 1.94 mM 5′-FSBA. The rate at pH 8.5 is approximately ten times greater than at pH 6.9, suggesting that the reaction involves the basic form of an ionizable group of the enzyme. The observed rate constant for the rapid reaction can be described by the following equation:

$$(k_1)_{obsd} = \frac{k_1}{1 + ([H^+]/K)} \quad (3)$$

where $(k_1)_{obsd}$ is the rate constant observed at a particular hydrogen ion concentration, k_1 is the maximum rate constant which is independent of pH, and K is the dissociation constant for an ionizable enzyme functional group. In reciprocal form, the equation becomes:

$$\frac{1}{(k_1)_{obsd}} = \left(\frac{1}{k_1}\right) + \left(\frac{1}{k_1 K}\right)[H^+] \quad (4)$$

A plot of the reciprocal of $(k_1)_{obsd}$ vs. the hydrogen ion concentration is linear, as shown in Figure 8, and reveals a pK of 8.5 for the reactive group. The three residues which are most likely to exhibit pK's in this range are cysteine, tyrosine, and lysine.

Cysteine was tested as a possible site of modification by measuring the free sulfhydryl content of pyruvate kinase by titration of aliquots with 5,5′-dithiobis(2-nitrobenzoate) during the course of incubation of the enzyme with 2.5 mM 5′-FSO_2BzAdo. During the time period examined, the enzyme lost 75% of its activity, while the free sulfhydryl content remained constant. Cysteine is therefore excluded as the possible site of attack by 5′-FSO_2BzAdo.

Discussion

The reaction of pyruvate kinase with 5′-*p*-fluorosulfonylbenzoyladenosine exhibits many of the characteristics expected for an affinity label. The extent of the reaction is limited; i.e., under the conditions described, only 2 mol of 5′-SO_2BzAdo was incorporated per mol of enzyme subunit. The adenosine portion of the structure is essential for the reaction of the compound with pyruvate kinase, since *p*-fluorosulfonylbenzoic acid does not inactivate the enzyme. Furthermore, the second rate of inactivation does demonstrate saturation kinetics. Fi-

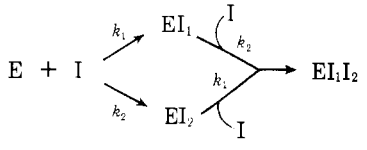
nally, the inclusion of certain substrates, either alone or in combination, during the reaction between pyruvate kinase and 5′-FSO_2BzAdo decreases the rate of inactivation of the enzyme.

When pyruvate kinase is incubated with 5′-FSO_2BzAdo, the kinetics of inactivation are biphasic. The inactivation profile is characterized by a rapid loss in activity to approximately 54% of its original level, followed by a much slower inactivation, the difference in rates being approximately tenfold. This type of biphasic kinetics might be expected if the reaction of 5′-FSO_2BzAdo with the enzyme subunit were taking place at two discrete sites, with only the less reactive site being absolutely essential for catalysis. Alternatively, this biphasic behavior might be expected if the initial reaction of 5′-FSO_2BzAdo with two subunits altered the accessibility of remaining subunits to reaction with the reagent. If this latter case were true then one might expect to observe in partially active enzyme species altered kinetic parameters, such as the Michaelis constant for one or both of the substrates. As illustrated in Table I, the K_M for both phosphoenolpyruvate and ADP were not altered to any appreciable extent. More important, this latter model would also predict the incorporation of only 1 mol of SO_2BzAdo per mol of enzyme subunit. In contrast, it has been found that 2 mol of reagent are bound per enzyme subunit, supporting the model which predicts the reaction of 5′-FSO_2BzAdo with two discrete loci on the enzyme subunit. Furthermore, this proposition is supported by the difference in the dependence of the two rates on the concentration of 5′-FSO_2BzAdo. The rapid rate is linearly dependent on reagent concentration, whereas the slower rate exhibits saturation kinetics. This observation implies that the reaction at the first site does not, while reaction at the second site does involve prior binding of the compound to the enzyme.

The relationship between these two reacting sites per subunit may be examined from two points of view. First, it may be considered that the two sites react in a fixed sequence; i.e., reaction at the first site occurs more rapidly and exposes or makes more accessible the second site for (slower) reaction with the reagent. This type of event could be represented by:

$$E + I \xrightarrow{k_1} EI_1 + I \xrightarrow{k_2} EI_1I_2 \qquad (5)$$

where E is the free enzyme, I the 5′-FSO_2BzAdo, EI_1, the partially active intermediate, and EI_1I_2 the totally inactive species with both sites modified. Second, it might be considered that the two sites are independent of one another and that the reaction of 5′-FSO_2BzAdo takes place simultaneously but at different rates. This could be represented as:



(6)

where EI_1 is the enzyme modified at the first site only and EI_2 is the enzyme modified at the second site only. A distinction between these two models depends on the detection of significant amounts of EI_2, which would be difficult to accomplish or exclude experimentally in view of the large differential between k_1 and k_2. However, both models suggest that the site attacked in the more rapid reaction is probably not an essential amino acid residue, since the species modified in this manner retains partially its enzymatic activity. It is more probable that the second modification is an essential one, since it does give rise to totally inactive enzyme. This two-site model may relate to the 6 Å resolution electron density map of cat muscle pyruvate kinase analyzed by Stammers and Muirhead (1975), which indicates the existence of two nucleotide binding sites, one of which is at the active site, with the other being about 25 Å away from the first. It is possible that the binding of the first mole of 5′-FSO_2BzAdo is at the site removed from the active site causing a conformational change which decreases the enzyme's ability to catalyze the phosphoryl transfer but does not totally prevent the reaction.

The function of the enzyme sites or regions which react with 5′-FSO_2BzAdo may be elucidated by considering the pattern of protection against modification afforded by the substrates. The extent of inactivation was markedly decreased under conditions where phosphoenolpyruvate, Mg^{2+}, and Mg-ADP were included as part of the reaction mixture; however, no protection was noted in the presence of pyruvate, ADP, and ATP alone. In the case of the Mg-ADP, the protection was no better than if only the metal ion alone was included, suggesting that the primary protection was due to the metal ion. From these results, it seems that the 5′-FSO_2BzAdo is reacting with amino acid residues located in the region of the metal ion binding site and the phosphoryl transfer site. This phosphoryl transfer site would normally be occupied by phosphoenolpyruvate and be very close to the group liganded to the Mg^{2+}, on the assumption that the metal ion mediates the phosphoryl transfer as suggested by Mildvan (1974). The designation of the metal-phosphoryl transfer region as the site of attack by 5′-FSO_2BzAdo is consistent with the loss of oxaloacetate decarboxylase activity, since both reactions require the presence of a divalent metal cation. Thus, one might anticipate a disruption of the decarboxylation if the binding of metal ion is impaired. It has also been observed that neither pyruvate nor oxaloacetate protect the enzyme against loss of activity. Although pyruvate is a common product in the oxaloacetate decarboxylase and pyruvate kinase reactions, oxaloacetate and phosphoenolpyruvate must occupy sites on the enzyme which are distinguishable. The carboxyl group which is lost during the decarboxylation reaction is not isosteric with the phosphoryl group transferred during the pyruvate kinase reaction; i.e., the carboxyl group of oxaloacetate would not overlap with the region occupied by the phosphoryl moiety of phosphoenolpyruvate. Thus, the lack of protection by oxaloacetate is not unexpected.

In contrast, it is rather more surprising that the simultaneous addition of equimolar concentrations of ATP and Mg^{2+} does not protect against inactivation. It may well be that the metal ion in the Mg-ATP chelate occupies a different site than does Mg-ADP or Mg^{2+} alone. This postulate is supported by the recent studies of Mildvan and co-workers (Melamud and Mildvan, 1975; Mildvan et al., 1976a,b; Sloan and Mildvan, 1976) in which it is proposed that pyruvate kinase requires two divalent metal ions per active site. One metal ion is associated directly with the enzyme, while the other is chelated to the phosphates of ATP. This interpretation suggests that 5′-FSO_2BzAdo is reacting with amino acid residues involved in the binding of Mg^{2+} to the enzyme and that Mg-ATP does not protect because this metal ion is located at a different position within the active site. It should also be noted that when the magnesium is added in concentrations in excess of ATP significant protection is again observed, indicating that Mg-ATP does not hinder the binding of the metal ion or the protection it affords against inactivation. In contrast, the metal in Mg-ADP must occupy the same site as does free Mg^{2+}, since Mg-ADP protects at least as well as does metal ion alone.

The effects of chemical modification on the ability of enzyme to catalyze the detritiation of pyruvate may also help to elucidate the site of attack by 5′-FSO_2BzAdo. Relatively little decrease was observed in the inorganic phosphate stimulated detritiation reaction, implying that the enzyme is still able to bind pyruvate. In contrast, the ATP-dependent enzymatic detritiation reaction is lost proportional to the decrease in pyruvate kinase activity. This result may be considered from two viewpoints. First, that the 5′-FSO_2BzAdo prevents the binding of the metal nucleotide or, second, that the modified enzyme cannot bind the metal ion directly. Mildvan has previously demonstrated that both the enzyme-bound metal ion and the nucleotide-chelated metal ion are necessary for the enolization of pyruvate (Mildvan et al., 1976a); therefore, if either interaction is perturbed or prevented, a loss in detritiation activity would result. This result, coupled with the protection studies discussed earlier, suggests that the enzyme-bound metal ion is the one affected by reaction of pyruvate kinase with 5′-FSO_2BzAdo. This data also implies that distinct mechanisms exist for the enolization of pyruvate as promoted by either inorganic phosphate or ATP, since the corresponding losses in detritiation activity are not noted.

Complete protection of pyruvate kinase by PEP or Mg-ADP has not been achieved. This observation might be explained by assuming that the first site modified by 5′-FSO_2BzAdo is not directly at the binding site, but rather is located in the general region of the metal-binding site; the presence of substrates may then alter the conformation of the protein to make the reactive group less accessible but not prevent totally the reaction. It is more difficult to test whether the substrates protect completely against the second reaction. When Mg-ADP is added at various times during the course of inactivation, it is apparent that the protective effect of the ligands is directed against both phases of the inactivation. However, under the protecting conditions, the reaction of 5′-FSO_2BzAdo with the second site on the enzyme becomes so slow that it is comparable to the rate of decomposition of the reagent (half-life = 8 h under the conditions present in the reaction mixture). Kinetically, one cannot distinguish between partial and total protection against reaction at the second site. Thus, the possibility of partial protection against reaction at the first site and total protection against reaction at the second site has not been excluded.

The stoichiometry of binding of reagent in the presence of various ligands might provide a clue as to whether they protect against one or both sites. Using 5′-[^{14}C]FSO_2BzAdo, it has been established that 2 mol of reagent is bound covalently to the subunit of pyruvate kinase, even after treatment with 0.5% sodium dodecyl sulfate and 6 M urea. When the enzyme reacted in the presence of Mg-ADP, the incorporation of reagent was as expected for protection against both sites; i.e., 0.5 mol of SO_2BzAdo was bound to the enzyme concomitant with a 30% loss in activity. However, when the enzyme was protected with phosphoenolpyruvate, the incorporation was higher than one would predict when correlated with activity loss; i.e., 2 mol of SO_2BzAdo per mol of subunit was incorporated concomitant with a 25% loss in activity. Upon binding to the enzyme, phosphoenolpyruvate may induce a conformational change exposing groups normally not available for modification. Studies are in progress of fingerprint patterns of the tryptic peptides of pyruvate kinase labeled with and without phosphoenolpyruvate in order to assess this postulate.

An identification of the nature of the amino acids of pyruvate kinase which react with 5′-FSO_2BzAdo is in order. The pH dependence of the reaction of 5′-*p*-fluorosulfonylbenzoyladenosine with pyruvate kinase has revealed a pK of 8.5 for the reactive group on the enzyme associated with the rapid rate of inactivation. These data suggest lysine, cysteine, or tyrosine as the amino acid residue modified by the reagent. Cysteine has been excluded as the site of reaction with 5′-FSO_2BzAdo, although it has been shown previously to be essential to catalytic activity (Hollenberg et al., 1971). When the sulfhydryl content of pyruvate kinase was estimated as a function of inactivation, it remained unchanged. Lysine has previously been shown to be essential to catalytic activity (Flashner et al., 1972). At present, the actual amino acid residues modified by reaction of 5′-FSO_2BzAdo have not yet been identified; however, lysine and tyrosine must be considered as the most likely candidates.

The reagent 5′-*p*-fluorosulfonylbenzoyladenosine has been used effectively and specifically in the modification of the region associated with the substrate binding site of pyruvate kinase and a regulatory site of glutamate dehydrogenase (Pal et al., 1975a). In determining whether or not this reagent is suitable for use in other enzyme systems involving purine nucleotides, two important factors must first be considered which are related to the limited solubility of the reagent in aqueous solutions. First, the enzyme should be stable to the inclusion of solvents having lower dielectric constants, such as dimethylformamide or ethanol, since the addition of these solvents is necessary for reagent solubility. Second, the K_M for the purine nucleotide should be less than 1 mM. This reduces the necessity of adding high concentrations of reagent, with the attendent relatively large volumes of solvent, and would therefore increase the probability of reaction between 5′-FSO_2BzAdo and the enzyme. A case in point is yeast hexokinase. This enzyme is not stable in dimethylformamide, even at the level of 1%, although it does retain activity in 3% ethanol. Under saturating conditions of reagent in 3% ethanol, there were indications that 5′-FSO_2BzAdo was reacting with hexokinase; however, the half-life of the reaction was approximately 6 h. The reagent half-life under similar conditions is on the order of 8 h, thereby making this reaction unfeasible. Furthermore, the concentration of reagent obtainable under these conditions was 0.30–0.55 mM, which compares to a K_M for nucleotide of 1.5 mM. Therefore, the results obtained with hexokinase are as one might predict; that is, little, if any, inactivation was noted because of the unfavorable K_M for the nucleotide and the lack of stability of hexokinase in solvents of lower dielectric constant.

From this paper and the work previously reported by Pal et al. (1975a,b) on glutamate dehydrogenase, it appears that 5′-FSO_2BzAdo may be useful in the affinity labeling of the regulatory and active sites of enzymes. Within the constraints imposed by the limitations of solubility and stability of the reagent, 5′-*p*-fluorosulfonylbenzoyladenosine thus may have broad applicability in the labeling of adenine nucleotide sites in proteins.

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