Modification of Two Essential Cysteines in Rabbit Muscle Pyruvate Kinase by the Guanine Nucleotide Analogue 5′-[*p*-(Fluorosulfonyl)benzoyl]guanosine†

John M. Tomich, Cristina Marti,‡ and Roberta F. Colman*

ABSTRACT: Reaction of rabbit muscle pyruvate kinase with the affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (5′-FSBG), at pH 7.65 and 7.93, leads to a loss in enzyme activity. The inactivation is characterized by a biphasic kinetic profile, with the initial phase accounting for approximately 55% of the reduction in enzymatic activity. For both the rapid and slow phases, at pH 7.93, the inactivation rate constants are linearly proportional to the reagent concentration (from 0.48 to 3.0 mM), yielding second-order rate constants of 195 min^{-1} M^{-1} and 19 min^{-1} M^{-1}, respectively. The effect of ligands was tested on the two phases of inactivation. For both, a decrease in the inactivation rate was produced by Mg^{2+} alone, but the best protection was provided by Mg^{2+} plus either ADP or GDP, suggesting that the reaction occurs in the region of the metal–nucleotide binding site. Modified pyruvate kinase is completely reactivated by incubation with 20 mM dithiothreitol, indicating the involvement of cysteine in the inactivation process. Reaction with [5′-^{3}H]-5′-FSBG leads to the incorporation of up to 1.3 mol of radioactive reagent per mol of enzyme subunit; however, identical radiolabel incorporation is observed before or after dithiothreitol reactivation of modified enzyme. This result implies that the labeled amino acid residue, measured by means of incorporation, is not directly involved in the inactivation process. In contrast, inactivation was found to correlate well with the loss of two free sulfhydryl groups per enzyme subunit and the restoration of activity to correlate with the regeneration of two free sulfhydryls after treatment of modified enzyme with dithiothreitol. It is proposed that inactivation of pyruvate kinase by 5′-FSBG proceeds by formation of thiol sulfonate followed by a rapid displacement of the sulfinic acid moiety by a second cysteine to yield a disulfide. A negative cooperativity in the interaction of pyruvate kinase subunits with 5′-[*p*-(fluorosulfonyl)-benzoyl]guanosine might best account for the biphasic inactivation kinetics.

The involvement of one or more sulfhydryl groups in the catalytic function of rabbit skeletal muscle pyruvate kinase has been extensively documented. This localization has been accomplished principally through the use of the general thiol modifying reagents *p*-(chloromercuri)benzoate (Mildvan & Leigh, 1964; Mildvan & Cohn 1966), iodoacetamide (Jacobson & Black, 1971), 5,5′-dithiobis(2-nitrobenzoate) (Flashner et al., 1972, 1973), and 5′-chloro-4-oxopentanoic acid (Bloxham & Chalkley, 1976; Chalkley & Bloxham, 1976; Bloxham et al., 1978). In all cases, varying degrees of protection against inactivation (by some or all of the constituents of the catalytic reaction) have created some ambiguity as to the number and location of the modified sulfhydryls.

Previous reports from this laboratory, employing several nucleotide affinity probes, have suggested that inactivation is due to the modification of different amino acid residues depending on the affinity reagent employed. Inactivation of rabbit muscle pyruvate kinase by 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine was described by Wyatt & Colman (1977). Inactivation in that case appears to be attributable in part to reaction with a single tyrosine residue and in part to reaction with cysteine (Annamalai & Colman, 1981). In yeast pyruvate kinase, 5′-FSBA[1] inactivation is caused by reaction with both lysine and tyrosine residues (Likos et al., 1980). The fluorescent affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine, on the other hand, appeared to inactivate rabbit muscle pyruvate kinase through reaction with a single sulfhydryl group (Likos & Colman, 1981).

In this paper, we report on the inactivation of rabbit muscle pyruvate kinase by the nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine previously developed in this laboratory (Pal et al., 1978). 5′-FSBG has been shown to cause loss of GTP inhibition by covalent reaction in the allosteric site of glutamate dehydrogenase (Pal & Colman, 1979). Because of the broad nucleotide specificity of rabbit muscle pyruvate kinase, which uses GDP in addition to ADP as coenzyme (Plowman & Krall, 1965), it was thought that reaction with this affinity probe would aid in the mapping of essential amino acid residues within the active site of pyruvate kinase. Evidence is here presented that reaction of rabbit muscle pyruvate kinase with 5′-FSBG results in a loss of activity which is directly correlated with the modification of two cysteine residues.

Experimental Procedures

Materials. Rabbit skeletal muscle pyruvate kinase was purchased from Boehringer Mannheim Corp. as an ammonium sulfate suspension. The enzyme (8–16 mg/mL) was dialyzed overnight at 4 °C against 10 mM potassium phosphate buffer, pH 7.4, containing 1.0 mM dithiothreitol. After excess DTT was removed by dialysis, with three changes of dialysate, at 4 °C against the above phosphate buffer containing 0.2 mM EDTA, any insoluble material was removed by centrifugation for 20 min at 15000 rpm. The enzyme concentration was determined by using $E_{280}^{0.1\%} = 0.54$ (Bucher & Pfleiderer, 1955)

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received March 12, 1981; revised manuscript received July 14, 1981.* This work was supported by National Science Foundation Grant PCM-80 21572.

‡ Present address: Departamento de Farmacologia, Instituto de Ciencias Medico-Biologicas, Universidad de Concepcion, Concepcion, Chile.

[1] Abbreviations used: 5′-FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine; 5′-FSBG, 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Tris, tris(hydroxymethyl)aminomethane.

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and a molecular weight of 57 000 per subunit (Cottam et al., 1969). The specific activity was 160–220 units/mg. Lactate dehydrogenase from hog muscle was also purchased from Boehringer Mannheim Corp. as a solution (10 mg/mL) in 50% glycerol and was used without further purification. The nucleotides GDP, GTP, ADP, ATP, and NADH as well as phosphoenolpyruvate, pyruvate, dithiothreitol, and 5,5′-dithiobis(2-nitrobenzoate) were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Determination of Enzyme Activity. Pyruvate kinase activity was measured spectrophotometrically at 340 nm by means of a coupled assay with lactate dehydrogenase, as previously outlined (Likos & Colman, 1981). The enzyme activity was monitored at 30 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM KCl, 10 mM $MgSO_4$, 0.5 mM phosphoenolpyruvate, 3 mM ADP, 0.25 mM NADH, and lactate dehydrogenase at a concentration of 0.1 mg/mL. The volume of the assay solution was 1 mL.

Determination of Enzyme Sulfhydryl Groups with 5,5′-Dithiobis(2-nitrobenzoate). Aliquots of pyruvate kinase (0.2 mL of 1 mg/mL in the appropriate reaction buffers) were added to 0.6 mL of 200 mM Tris-HCl buffer, pH 8.0, in a 1.0-mL quartz cuvette. Sodium dodecyl sulfate (0.1 mL of 10% w/v) was added to denature the protein. This solution was balanced at 412 nm against protein-free buffer blank identical with the test solution in a Cary Model 219 dual-beam spectrophotometer. A freshly prepared 10 mM DTNB solution (0.1 mL) was added to each cuvette, and the concentration of free enzyme SH groups was calculated from the change in absorbance and a molar extinction coefficient for thionitrobenzoate at 412 nm of 13 600 (Ellman, 1958).

Preparation of 5′-[p-(Fluorosulfonyl)benzoyl]guanosine. The compound 5′-[p-(fluorosulfonyl)benzoyl]guanosine (5′-FSBG) was synthesized by reaction of guanosine hydrochloride with p-(fluorosulfonyl)benzoyl chloride as previously reported (Pal et al., 1978). Radioactive 5′-[p-(fluorosulfonyl)-benzoyl]guanosine was prepared by the addition of 50 μCi of [5′-^{3}H]guanosine (obtained from New England Nuclear Corp.) to 1.0 g of nonradioactive guanosine. The synthesis of radioactive guanosine hydrochloride and 5′-[p-(fluorosulfonyl)benzoyl]guanosine was conducted as previously described (Pal et al., 1978).

Reaction of 5′-FSBG with Rabbit Muscle Pyruvate Kinase. Pyruvate kinase (0.8–1.1 mg/mL) was incubated with 5′-FSBG at 30 °C in 20 mM barbital, pH 7.65–8.0, containing either 200 mM NaCl or 200 mM KCl, 3.5 mM potassium phosphate, and 15% dimethylformamide. During the course of the reaction 2-μL aliquots were withdrawn at given time intervals, diluted 100-fold with 10 mM potassium phosphate, pH 7.5, and assayed for catalytic activity by using 2 μL of the diluted enzyme. Solutions of 5′-FSBG were prepared by dissolving the reagent in redistilled dimethylformamide and determining the concentration of the solution from the absorbance at 275 nm (ϵ_{275} = 7632 M^{-1} cm^{-1} in ethanol). A control consisted of incubating the enzyme under identical conditions (i.e., containing 15% dimethylformamide) but in the absence of 5′-FSBG. The rate of reaction of pyruvate kinase with the nucleotide analogue was determined from a semilogarithmic plot of E/E_0 as a function of time, where E represents the enzymatic activities at a given time for the experimental or control reaction and E_0 represents the initial activity. All rate constants were calculated from least-squares analysis of the data for each experiment.

The kinetics of inactivation were also followed in the presence of various substrates and modifiers. These compounds or ions were either preincubated with the enzyme at 30 °C for a minimum of 10 min prior to addition of 1.66 mM 5′-FSBG or were added 10.5 min after the initiation of inactivation by 5′-FSBG, depending on which phase of the inactivation process was being examined.

Determination of the Spontaneous Decomposition Rate of 5′-FSBG. A stock solution of 5′-FSBG was prepared by dissolving the reagent in redistilled dimethylformamide as described earlier. Aliquots of the stock solution were added to 30 °C to barbital buffers, at various pH values, that contained 200 mM NaCl, 3.5 mM potassium phosphate, and 15% dimethylformamide. The decomposition of 5′-FSBG was monitored by measuring, at specified times, the loss of fluoride with a standardized Orion combination fluoride electrode. The decomposition rate was determined from a semilogarithmic plot of $[5'\text{-FSBG}]_t/[5'\text{-FSBG}]_0$ as a function of time.

Incorporation of [5′-^{3}H]-5′-FSBG by Rabbit Muscle Pyruvate Kinase. For determination of the stoichiometry of the reaction of pyruvate kinase with [^{3}H]-5′-FSBG, the enzyme (1 mg/mL) was incubated at 30 °C under the identical conditions outlined above. At given time periods in the reaction, 0.5-mL aliquots were removed and treated in one of two ways: excess reagent was removed immediately by utilizing the gel filtration column–centrifugation technique (Penefsky, 1979) or was removed by the same method following pretreatment with 20 mM dithiothreitol for 30 min at 30 °C. Sephadex G-50–80 equilibrated in 10 mM sodium phosphate, pH 7.4, was packed in 5-mL plastic syringes and centrifuged in a bench top clinical centrifuge at 4 °C for 10 min at the lowest speed followed by 2 min at three-fourths maximal speed. All aliquots were applied dropwise, and the columns were recentrifuged exactly as described above. The eluant was collected and reapplied to a second Sephadex column. The second eluant was then analyzed for protein content (Bradford, 1976) and radioactivity, using ACS scintillation cocktail (Amersham) in a Packard Tri-Carb liquid scintillation counter.

Reactivation of Modified Pyruvate Kinase by Dithiothreitol. For the reactivation of modified enzyme, a stock solution of dithiothreitol in 10 mM sodium phosphate buffer, pH 7.5, was prepared just before use. At various times of inactivation (15, 30, 45, 60, 80, 120, and 180 min), aliquots were removed and incubated at 30 °C with 20 mM dithiothreitol. The increase in specific activity of the enzyme was then followed by the lactate dehydrogenase coupled assay as previously described.

In addition to following the increase in enzymatic activity, the change in sulfhydryl concentration was also monitored. At particular periods of inactivation, dithiothreitol (20 mM final concentration) was added to aliquots of the modified enzyme and allowed to stand for 30 min at 30 °C. Excess DTT and 5′-FSBG were then removed by a two-step procedure employing first the gel filtration–centrifugation technique previously described followed by dialysis for a total of 3 h against 2 L of 10 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA; two changes of buffer were made during the 3-h period. The number of sulfhydryl groups per enzyme subunit was then determined by reaction with DTNB as previously outlined.

Results

Inactivation of Rabbit Muscle Pyruvate Kinase by 5′-[p-(Fluorosulfonyl)benzoyl]guanosine. Rabbit muscle pyruvate kinase (1 mg/mL was inactivated upon incubation with 1.66 mM 5′-FSBG at 30 °C in 20 mM sodium barbital buffer at pH 7.65 (Figure 1A) and 7.93 (Figure 1B). A plot of log E/E_0 vs. time revealed a similar inactivation profile at both pH values; after a rapid initial decrease in activity, a second slower

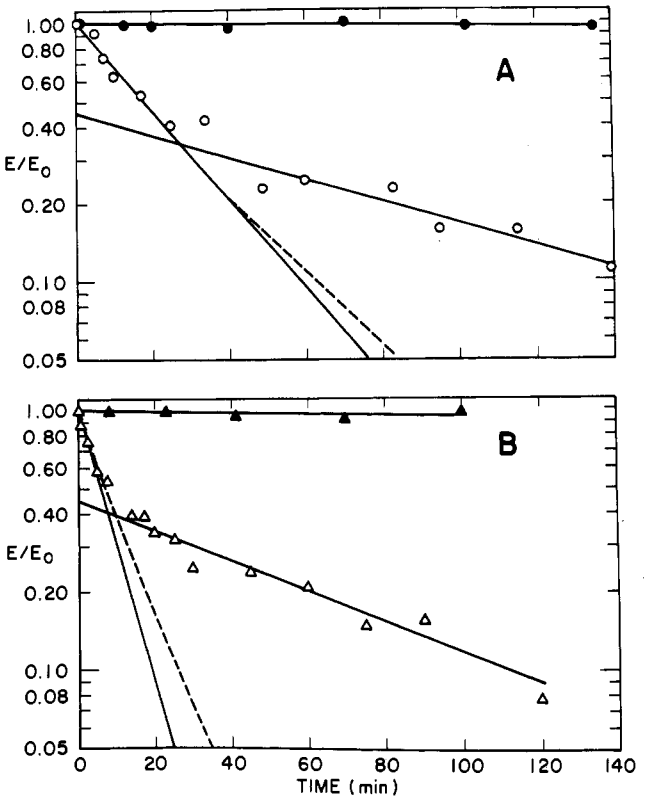


FIGURE 1: Inactivation of pyruvate kinase by 5′-FSBG at pH 7.65 and 7.93. Rabbit muscle pyruvate kinase (1.0 mg/mL) was incubated with 1.66 mM 5′-FSBG (open symbols) at 30 °C in 20 mM sodium barbital buffer containing 200 mM NaCl, 3.5 mM potassium phosphate, and 15% dimethylformamide at pH 7.65 (A) and pH 7.93 (B). In each case, the enzyme was also incubated under identical conditions except for the omission of 5′-FSBG (closed symbols) as a control. At the times indicated, aliquots were withdrawn and assayed for enzymatic activity as described under Experimental Procedures. E and E_0 represent activities at the given time and at zero time, respectively. The expected effect of decomposition of 5′-FSBG on the initial rapid rate of inactivation is shown by the broken lines.

rate was established. It is known that sulfonyl fluoride derivatives can hydrolyze spontaneously (Likos et al., 1980), and it seemed possible that the apparent biphasic kinetics could be attributable to a time-dependent depletion of 5′-FSBG. The theoretical curves (Figure 1, broken lines) corresponding to a decrease in the initial rate of inactivation due exclusively to reagent decomposition were calculated by (Purdie & Heggie, 1970; Ashni et al., 1972)

$$\ln E/E_0 = -\frac{k_{\text{inact}}[5'\text{-FSBG}]_0}{k'}[1 - e^{-k't}] \qquad (1)$$

where k_{intact} is the observed rapid initial rate constant for enzyme inactivation and k' is the rate constant for 5′-FSBG decomposition. The rate constant, k', was determined by incubating 5′-FSBG under conditions identical with those used for reaction with pyruvate kinase except for the absence of protein. The spontaneous release of fluoride ion was measured as described under Experimental Procedures, yielding a value for k' of 0.0031 min^{-1} at pH 7.65 and 0.0077 min^{-1} at pH 7.93. The effect of reagent decomposition is obviously too small to account for the biphasic nature of the inactivation curves.

The dependence of the rates of inactivation of pyruvate kinase on 5′-FSBG concentration is shown in Figure 2A. A biphasic profile was obtained at each concentration, with a common intersection point observed at approximately 45% of original enzymatic activity for the extrapolation of the slower rates back to zero time (broken lines). This result suggests that at each concentration inactivation proceeds by a two-step

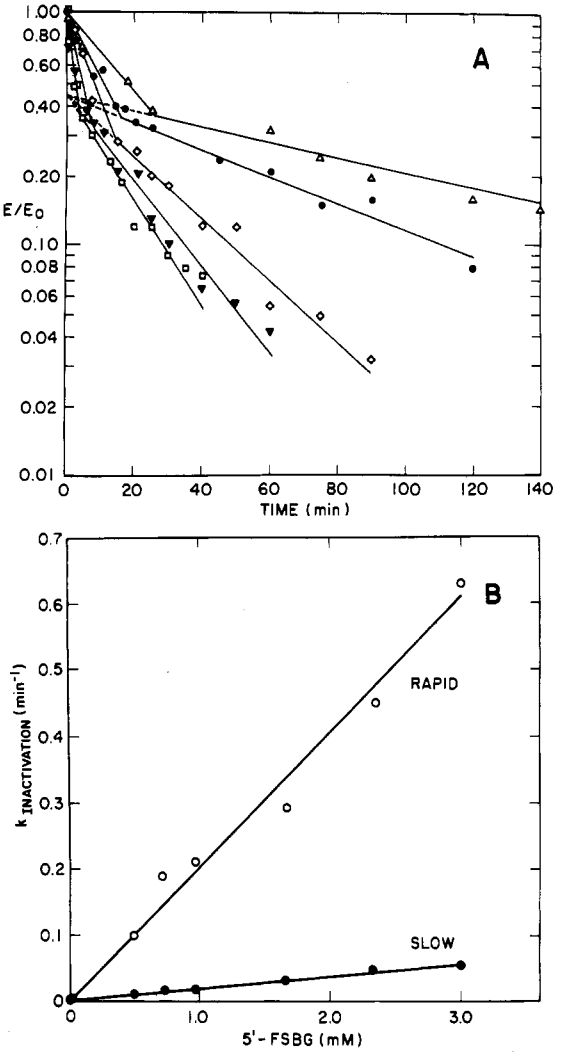


FIGURE 2: Determination of the second-order rate constants for the rapid and slow inactivation phases at pH 7.93. (A) Pyruvate kinase (1.0 mg/mL) was incubated with varying concentrations of 5′-FSBG: 0.48 (△), 0.95 (●), 1.66 (◊), 2.38 (▼), and 3.0 mM (□) under the same conditions described in Figure 1B. The extrapolation of the slow phase back to zero time is denoted by the dashed lines. (For clarity of presentation, points have been left off the lines drawn for the rapid reaction phase.) (B) The dependence of the corrected rapid inactivation rate constant (○) as well as the slow inactivation rate constant (●) on the concentration of 5′-FSBG has been plotted. The slopes of the resulting lines yield their respective second-order inactivation rate constants.

mechanism: inactivation during the first phase leads to a loss of approximately 55% of the original activity, while further decrease in activity is due to a second slower process. After the rapid initial apparent rate of inactivation is corrected for the contribution resulting from the second phase (Ray & Koshland, 1961), plots of k_{inact} vs. 5′-FSBG concentration were generated (Figure 2B). For each phase, the inactivation rate constants were directly proportional to the reagent concentration over the range tested (0.48–3.0 mM). Higher concentrations of 5′-FSBG were not examined because of the limited solubility of the reagent in aqueous solutions. The linear nature of these functions gives no positive indication of reversible binding of 5′-FSBG prior to irreversible modification for either event; however, this observation is also consistent with a dissociation constant for the enzyme–reagent complex which is higher than the 5′-FSBG concentrations tested. The second-order rate constants were determined for both the rapid and slow phases and were found to be 195 min^{-1} M^{-1} and 19 min^{-1} M^{-1}, respectively.

Table I: Effect of Ligands on Apparent Initial Rate of Inactivation by 5′-FSBG at pH 7.65[a]

additions to reaction mixture	k_{inact} (ligand added)/ k_{inact} (no addition)
none	1.00
pyruvate (10 mM)	0.88
phosphenolpyruvate (1 mM)	0.69
ADP (10 mM)	0.67
ATP (10 mM)	0.69
Mg^{2+} (1 mM)	1.00
Mg^{2+} (10 mM)	0.24
ADP (10 mM) + Mg^{2+} (10 mM)	0.09
ADP (10 mM) + Mg^{2+} (20 mM)	0.05
ATP (10 mM) + Mg^{2+} (10 mM)	0.35
ATP (10 mM) + Mg^{2+} (20 mM)	0.26

[a] Pyruvate kinase (1.0 mg/mL) was incubated with 5′-FSBG (2.5 mM) at 30 °C in 20 mM barbital buffer, pH 7.65, containing 200 mM KCl and 15% dimethylformamide. The approximate constants were determined as described in the text.

Effect of Substrates and Ions on the Reaction of Pyruvate Kinase with 5′-FSBG. In an attempt to evaluate the site(s) of attack of 5′-FSBG on pyruvate kinase, the effect of various ligands on the inactivation reactions was tested. pH 7.65 was chosen for the initial experiments to minimize any effects on the observed rates of inactivation due to reagent depletion. At this pH, a half-life of 225 min was calculated for the rate of decomposition for 5′-FSBG; this choice of experimental conditions allowed us to assess the protection experiments over an extended period of time. The apparent initial inactivation rate includes contributions from both phases of the reaction (although the initial phase predominates due to the difference in the second-order rate constants). The ratio, therefore, of the measured initial rate constant in the presence of added ligands to the measured initial rate constant in the absence of ligands (Table I) gives an indication as to which substrates, ions, or coenzymes influence the overall reaction. Little decrease in the inactivation rate constant was caused by the reaction product, pyruvate, and only slightly better protection was afforded by the substrate, phosphoenolpyruvate. ADP and ATP in the absence of Mg^{2+} failed to contribute significantly to a reduction in the rate of inactivation. No detectable decrease in the rate of inactivation was caused by including 1 mM Mg^{2+} in the incubation mixture, but addition of 10 mM Mg^{2+} resulted in a 4-fold decrease in the rate. The concentration of Mg^{2+} required to produce an observable effect on the rate constant is not unreasonable in view of the report of Kwan et al. (1975) that 1 mM Mg^{2+} is required for half-maximal activation at pH 7.5, although under different buffer conditions.

The greatest degree of protection was proferred by 10 mM ADP in the presence of Mg^{2+} (10 and 20 mM). Protection might be ascribed to binding of the Mg–ADP complex, of free Mg^{2+}, or of both. At the total concentrations of 10 mM each of ADP and Mg^{2+}, the concentration of Mg–ADP is 7.76 mM[2] while the level of free Mg^{2+} is 2.34 mM. It appears that the protection observed under these conditions results primarily from the binding of the Mg–ADP complex. When the Mg^{2+} concentration is raised to 20 mM, with the ADP concentration being held constant, the total concentration of the Mg–ADP complex becomes 9.4 mM, while the free Mg^{2+} level rises to 10.6 mM. In this case, free Mg^{2+} may also contribute to the observed protection against 5′-FSBG. ATP (10 mM) in the presence of 10 mM total Mg^{2+} provides about a 3-fold reduction in the rate of inactivation. The concentration of Mg–ATP under these conditions is 9.05 mM[2] while free Mg^{2+} is only 0.95 mM. As shown in Table I (line 6), 1.0 mM Mg^{2+} did not provide protection; therefore, it seems that the 3-fold decrease seen upon the addition of ATP (10 mM) and Mg^{2+} (10 mM) must be due to the binding of the metal–nucleotide complex. Increasing the amount of total Mg^{2+} to 20 mM, while the total ATP concentration is maintained at 10 mM, resulted in a further decrease in the inactivation rate constant which is presumably attributable to enhanced levels of free metal ion (9.90 mM Mg–ATP; 10.1 mM free Mg^{2+}); in fact, the degree of protection provided by 10 mM ATP and 20 mM Mg^{2+} was no greater than that afforded by the corresponding concentration of free Mg^{2+} alone. The results in Table I are consistent with the postulate that the locus of modification of 5′-FSBG is within the Mg–ADP binding region.

Table II: Effect of Ligands on Two Phases of Inactivation Reaction at pH 7.93[a]

additions to reaction mixture	k, rapid phase (min^{-1})	k, slow phase (min^{-1})
none	0.290	0.032
phosphoenolpyruvate (1 mM)	0.12	0.018
Mg^{2+} (10 mM)	0.087	0.017
Mg^{2+} (20 mM) + ATP (10 mM)	0.100	0.012
Mg^{2+} (20 mM) + ADP (10 mM)	0.052	0.0045
Mg^{2+} (20 mM) + GTP (10 mM)	0.22	0.022
Mg^{2+} (20 mM) + GDP (10 mM)	0.072	0.014

[a] Pyruvate kinase (1.0 mg/mL) was incubated with 1.55 mM 5′-FSBG at 30 °C in 20 mM sodium barbital, pH 7.93, containing 200 mM NaCl and 15% dimethylformamide as described in Figure 1. Various ligands were added either prior to or 10.5 min after the addition of 5′-FSBG. The values for k for the slow phase were obtained directly from the slope of the line obtained after the ligand was added at 10.5 min. The rate constant for the rapid phase was calculated by assuming that even in the presence of ligands, this phase produced a partially active enzyme species with 45% residual activity. The true k for the rapid phase was therefore calculated by correcting the apparent rate of the first phase for the contribution of the measured inactivation rate of the second phase in the presence of ligands.

Activation of pyruvate kinase has been shown to possess an absolute requirement for monovalent cations, with potassium being the best activator (Kayne, 1973); the potential of the other cations of this group to activate pyruvate kinase varies widely. In addition to testing the effects of those ligands described in Table I, the effects of K^+ and Na^+ cations on both the rate of inactivation by 5′-FSBG and the degree of protection afforded by phosphoenolpyruvate were examined. Inactivation and protection experiments were performed in which either NaCl or KCl (200 mM) was included in the barbital buffer. No difference was detected, indicating that potassium was not required for reaction of 5′-FSBG. The ability to interchange the monovalent cations was exploited in later experiments, in which sodium dodecyl sulfate was added to denature the enzyme prior to the determination of the number of unmodified enzymatic sulfhydryl groups.

In an attempt to differentiate the two distinct phases of the inactivation of pyruvate kinase by 5′-FSBG, those ligands which appreciably reduced the apparent inactivation rate of the initial phase, at pH 7.65, were examined in more detail at pH 7.93. Table II summarizes the calculated k_{inact} values for both phases in the prescence of certain critical ligands. Raising the pH from 7.65 to 7.93 had a profound influence upon the extent to which metal–nucleotides protect. Free magnesium and phosphoenolpyruvate apeared to protect to

[2] The concentrations of free Mg^{2+} and Mg–ADP and Mg–ATP complexes were calculated from the total Mg^{2+}, ADP, and ATP concentrations by use of the association constants given in Bock (1960).

Table III: Effect of Varying the Concentration of Mg^{2+}–Nucleotide Complex on k (Rapid Phase)[a]

additions to reaction mixture	$[Mg^{2+}]$ (mM)	[Mg-nucleotide][b] (mM)	k_{obsd}, rapid reaction (min^{-1})	K_d (mM)
none			0.33	
Mg^{2+} (8 mM) + ADP (10 mM)	1.38	6.62	0.16	6.23
Mg^{2+} (10 mM) + ADP (12 mM)	1.64	8.36	0.13	5.43
Mg^{2+} (13 mM) + ADP (15 mM)	2.0	11.0	0.104	5.04
Mg^{2+} (20 mM) + ADP (22 mM)	2.7	17.3	0.081	5.63
Mg^{2+} (20 mM) + ADP (10 mM)	10.6	9.4	0.059	2.05
Mg^{2+} (5.5 mM) + GDP (7.5 mM)	1.34	4.16	0.094	1.65
Mg^{2+} (5 mM) + GDP (15 mM)	0.46	4.54	0.130	2.95
Mg^{2+} (8 mM) + GDP (10 mM)	1.77	6.23	0.120	3.56
Mg^{2+} (13 mM) + GDP (15 mM)	2.5	10.5	0.075	3.09
Mg^{2+} (20 mM) + GDP (10 mM)	10.86	9.14	0.083	3.07
Mg^{2+} (30 mM) + GDP (20 mM)	12.8	17.4	0.050	3.10

[a] Pyruvate kinase (1.0 mg/mL) was incubated with 1.66 mM 5′-FSBG at 30 °C in 20 mM sodium barbital, pH 7.93, containing 200 mM NaCl and 15% dimethylformamide as described in Figure 1. Varying concentrations of metal-nucleotides were added prior to the addition of 5′-FSBG. The rate constant for the rapid phase was calculated as described in Table II. [b] The effective Mg-nucleotide concentrations were calculated by using 1.4×10^3 M^{-1} as the association constant for Mg^{2+} with ADP (Bock, 1960) and an estimated value of 9.30×10^2 M^{-1} for the association constant for Mg^{2+} with GDP. This estimate was calculated from the ratio of association constants for Mn–ADP and Mn–GDP, 1.65×10^4 M^{-1} and 1.10×10^4 M^{-1}, respectively, at pH 8.03 (Colman, 1972) and the known association constant for Mg–ADP stated above.

about the same extent. Mg^{2+} plus ATP and Mg^{2+} plus GDP were no better than free Mg^{2+}. Inclusion of GTP together with Mg^{2+} in the reaction mixture, however, significantly reduced the degree of protection to a level below that of free metal alone.

Since free metal appeared to be one of the critical ligands responsible for the lowered rates of inactivation, dependence of k_{obsd} for the rapid reaction was subsequently monitored as a function of free metal concentration. Mg^{2+} conferred only partial protection at saturating level, reaching a limit of about 0.09 min^{-1} for the pseudo-first-order rate constant. The Mg^{2+} concentration dependence of the rate constant for the rapid phase yielded a K_d for the enzyme–Mg^{2+} complex of approximately 320 μM, which is in good agreement with values previously reported for this enzyme (Suelter & Melander, 1963; Mildvan & Cohn, 1965).

In a similar series of experiments, increasing concentrations of metal–nucleotide complex were also tested for the concentration-dependent changes in rate constant, with the results recorded in Table III. These experiments were designed so that the levels of free Mg^{2+} were close to saturating. The concentrations of complex were calculated from the total concentrations of metal and nucleotide and the known association constants for the metal–nucleotide complexes. The observed rates of inactivation for the rapid phase, k_{obsd}, were determined at each metal–nucleotide complex concentration after the contribution of the slow phase was corrected for, as described earlier. The dissociation constants were calculated by

$$1 + \frac{[S]}{K_d} = \frac{k}{k_{obsd}} \quad (2)$$

where [S] equals the concentration of metal–nucleotide complex and k and k_{obsd} are the corrected pseudo-first-order rate of inactivation for the rapid phase in the absence and presence of protectants. The rate constant in the absence of ligands was chosen as the correct rate for the calculation of the dissociation constants (as opposed to the rate constant measured at saturating Mg^{2+} concentrations) since plots of $1/k_{obsd}$ vs. [Mg^{2+}–nucleotide] for both GDP and ADP are linear and extrapolate at zero metal–nucleotide complex to values of k_{obsd} close to those characteristic of the absence of ligands.

As the concentration of Mg–ADP complex was raised, a progressive decrease in the observed rate constant was noted over the concentration range 6–17 mM Mg–ADP complex and 1.4–2.7 mM free Mg^{2+}, allowing the calculation of a mean K_d for enzyme–Mg–ADP of 5.6 ± 0.49 mM. At the lowest concentrations of Mg^{2+} and ADP, it is clear that the k_{obsd} is greater than would be expected for the calculated concentration of free metal alone. It thus appears that free magnesium binds more weakly in the presence of metal–nucleotide complex. When the free Mg^{2+} concentration is greatly increased (Table III, line 6 as compared to line 3 or 4), there is a further decrease in k_{obsd}. This could be attributed to an effect of free Mg^{2+} in tightening the binding of Mg–ADP complex (K_d = 2.05 mM). Alternatively, the decrease in k_{obsd} could be the result of an additive effect of metal–ADP complex and free Mg^{2+} (binding with a perturbed K_d in the millimolar range).

Inclusion of varying concentrations of GDP with Mg^{2+} in the reaction mixture also appears to provide increased protection with increasing metal–nucleotide concentrations, yielding a mean value of 2.9 ± 0.65 mM for the dissociation constant of the enzyme–Mg–GDP complex (Table III). The relatively constant value of K_d over a wide range of free Mg^{2+} concentration suggests either that free Mg^{2+} does not influence the binding of Mg–GDP to pyruvate kinase or that Mg–GDP decreases the affinity of the enzyme for free Mg^{2+} such that binding of free metal is not observed in the concentration range tested.

Reactivation of Modified Pyruvate Kinase by Dithiothreitol. In the reaction of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine with rabbit muscle pyruvate kinase (Likos & Colman, 1981), it was observed that the addition of 100 mM dithiothreitol rapidly restored enzyme activity with the concomitant loss of 1 mol of bound ligand per mol of enzyme subunit. After modifying rabbit muscle pyruvate kinase with 0.78 mM 5′-FSBG at pH 7.95 for various time periods, we performed a similar experiment. At 15, 30, 45, 60, 80, 120, and 150 min of inactivation by 5′-FSBG, aliquots were removed and treated with dithiothreitol, and the change in enzyme activity was monitored. Reactivation to levels comparable to the original enzyme activity occurred after a 30-min treatment with DTT in the samples which had been inactivated for 15 and 30 min with 5′-FSBG. Unmodified enzyme was also incubated in the presence of 20 mM dithiothreitol, as a control, and no detectable increase in activity could be measured in that case. Between 45 and 80 min of inactivation, reactivation proceeded at a somewhat slower rate but with a return to approximately 90% of the original activity after a 30-min incubation with

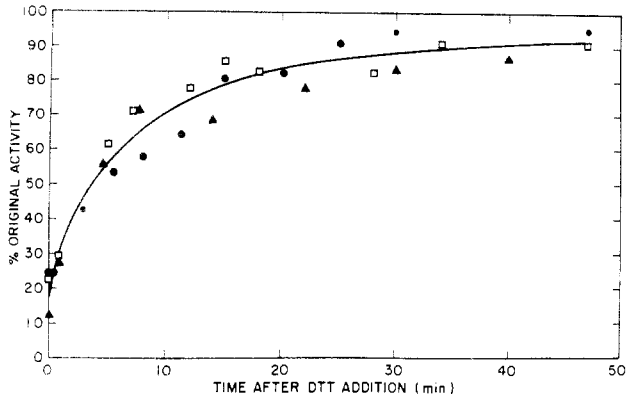


FIGURE 3: Reactivation of modified enzyme by dithiothreitol. After different periods of inactivation at pH 7.93 [45 (□), 60 (●), and 80 min (▲)] by 0.8 mM 5′-FSBG, 20 mM dithiothreitol was added to the incubation mixtures, and aliquots were removed at various times to measure the increase in enzymatic activity. Unmodified enzyme was also incubated in the presence of 20 mM DTT, as a control. No detectable increase in activity could be measured.

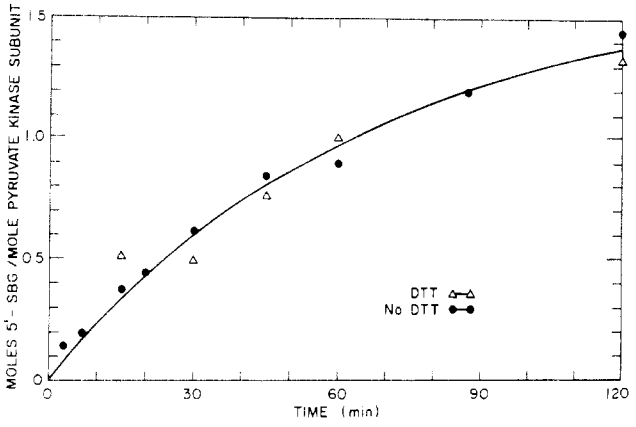


FIGURE 4: Time-dependent incorporation of [5′-^{3}H]-5′-SBG per mole of rabbit muscle pyruvate kinase subunit in the absence and presence of dithiothreitol. Pyruvate kinase (1.0 mg/mL) was incubated with 0.8 mM [5′-^{3}H]-5′-FSBG at 30 °C in 20 mM sodium barbital buffer, pH 8.02, containing 200 mM NaCl, 3.5 mM potassium phosphate, and 15% dimethylformamide. At the indicated times, 0.5-mL samples were removed and either immediately centrifuged (●) over a Sephadex G-50 column at 4 °C as described under Experimental Procedures or treated with 20 mM dithiothreitol (△) at 30 °C for 30 min prior to application to the column.

DTT (Figure 3). Increasingly greater periods of inactivation, such as 120 and 150 min, further decreased the rate of reactivation and apparently reduced the maximum possible regain, so that a 30-min incubation with DTT resulted in an enzyme which possessed about 80% of the original activity. The restoration of activity of modified enzyme through the addition of dithiothreitol strongly suggests the involvement of cysteinyl residue(s) in the inactivation process.

Stoichiometry of the Reaction of Pyruvate Kinase with [5′-^{3}H]-5′-FSBG in the Absence and Presence of DTT. The stoichiometry of the reaction indicated that at pH 8, [5′-^{3}H]-5′-FSBG reacted covalently and in a limited manner with rabbit muscle pyruvate kinase (Figure 4). The incorporation of [5′-^{3}H]-5′-SBG per mole of subunit was determined as described under Experimental Procedures. The amount of reagent covalently bound varied insignificantly whether or not 20 mM DTT was added at the times indicated. If the moles of [^{3}H]SBG are plotted as a function of the residual activity (E/E_0), no simple relationship is seen. Furthermore, the observation of the same radioactive incorporation after reactivation by DTT strongly implies that the tritium-labeled amino acid is not essential for catalytic activity. Similar results were also obtained with [5′-^{3}H]-5′-FSBG at pH 7.65.

Table IV: Relationship between Catalytic Activity and SH Groups Modified before and after Treatment with Dithiothreitol

time of incubation with 5′-FSBG (min)	before addition of DTT[a]		after reactivation by DTT[b]	
	original activity (%)	SH groups reacted	original activity (%)	SH groups reacted
0	100	0.00	100	0.00
15	45	1.10	100	0.04
20	30	1.30	100	0.00
30	24	1.60	100	0.00
60	14	2.00	95	0.15

[a] Pyruvate kinase (1.0 mg/mL) was incubated with 5′-FSBG (0.8 mM) at 30 °C in 20 mM sodium barbital, pH 7.95, containing 200 mM NaCl and 15% dimethylformamide. Activity and sulfhydryl groups were measured at the indicated times as described under Experimental Procedures. [b] Pyruvate kinase (1.0 mg/mL) was incubated with 5′-FSBG as described above. At the indicated times of inactivation, dithiothreitol (20 mM final concentration) was added to aliquots of the modified enzyme. After 30 min, samples were assayed for activity. Free sulfhydryls were determined after removal of excess reducing agent, as described under Experimental Procedures.

Titration of Sulfhydryls during Inactivation and Reactivation. At various times during both the inactivation and reactivation processes, the number of free sulfhydryl groups in pyruvate kinase was determined by reaction with 5,5′-dithiobis(2-nitrobenzoate) as described under Experimental Procedures. The data from such an experiment are shown in Table IV. The DTT pretreated enzyme control exhibited 8.53 ± 0.15 SH groups per mol of pyruvate kinase subunit, which compares well with values previously observed for pyruvate kinase (Anderson & Randall, 1975; Flashner et al., 1972). During the inactivation process, a limited number of sulfhydryl groups reacted in the presence of 0.78 mM 5′-FSBG at pH 7.95. When the sulfhydryls of the modified enzyme were assayed after treatment with and removal of excess dithiothreitol, concomitant with reactivation of the enzyme, the number of reacted sulfhydryl groups was decreased so that the total number of free cysteine residues became essentially that of the native enzyme.

During incubation of pyruvate kinase with 5′-FSBG, a linear relationship was found between the decrease in residual activity and the increase in the number of sulfhydryl groups reacted (Figure 5). Extrapolation of the data to complete inactivation indicated that 2 mol of cysteine had reacted per mol of enzyme subunit. Thus there seems to be a correlation between, on one hand, loss of activity and a loss of about two SH groups, and on the other hand, a regain of activity and a restoration of about two free SH groups.

Effect of Mg^{2+} and GDP on the Loss of Free Sulfhydryl Groups during Inactivation. The loss of free sulfhydryls was also titrated at a higher concentration of 5′-FSBG (1.66 mM at pH 7.93). Under these conditions, three sulfhydryls were modified after 100 min of inactivation (Figure 6A). Inclusion of 13 mM Mg^{2+} and 15 mM GDP in the inactivation medium (prior to the introduction of 5′-FSBG) altered the extent to which the sulfhydryls reacted with the affinity label. It appeared that an average value of 2.4 sulfhydryl groups had reacted after 90 min of inactivation. As was seen in Table III, the addition of Mg^{2+} and GDP also decreased the rate of inactivation. In Figure 6B, the loss of sulfhydryl (depicted in Figure 6A) was plotted vs. the appropriate residual activity measured at the corresponding time. When the inactivation was carried out with 1.66 mM 5′-FSBG at pH 7.93 in the

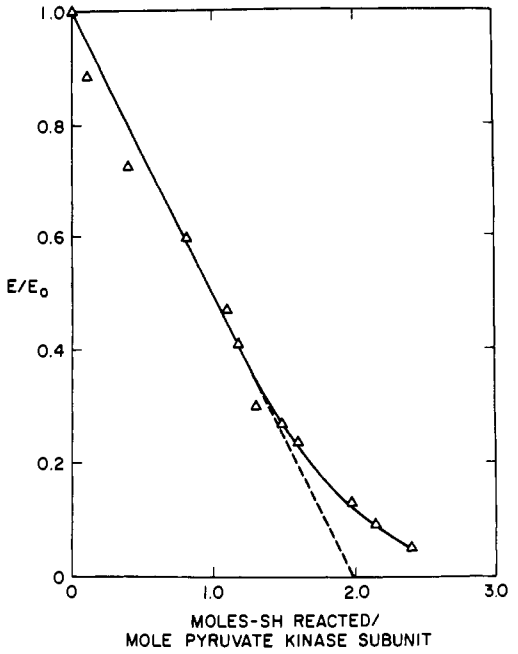


FIGURE 5: Loss of free sulfhydryl groups as a function of inactivation. Pyruvate kinase (1 mg/mL) was incubated with 0.8 mM 5′-FSBG at pH 7.95 under conditions similar to those outlined in Figure 1B. The inactivation (E/E_0) was followed by monitoring the enzyme activity as described under Experimental Procedures. The loss of free SH groups was determined by the DTNB assay also described under Experimental Procedures. The dashed line designates the extrapolation to zero residual activity of the linear portion of the curve.

absence of ligands, extrapolation to zero activity yielded a value of 2.3 modified sulfhydryls. This is in reasonable agreement with the value of 1.98 sulfhydryls modified per enzyme subunit as seen in Figure 5. More importantly, the data points for sulfhydryls vs. E/E_0 obtained under protected conditions (i.e., with Mg^{2+} and GDP added) fall on the same line established for the modified enzyme in the absence of ligands (Figure 6B). This result suggests that protection by metal and nucleotide prevents inactivation by reducing the rate of reaction of active site sulfhydryl groups with 5′-FSBG.

Discussion

The experiments presented here demonstrate that rabbit muscle pyruvate kinase is inactivated by the purine nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine by a specific and limited mode of action. 5′-FSBG has previously been proposed as an affinity label of GTP binding sites in proteins, such as the allosteric site of glutamate dehydrogenase (Pal & Colman, 1979). However, this is the first detailed report of a specific inactivation of a kinase by 5′-FSBG.

Fluorosulfonyl derivatives have been shown to react with the amino acids tyrosine, histidine, lysine, and serine (Paulos & Price, 1974). Likos & Colman (1981) inferred that a cysteinyl residue was modified during the inactivation of pyruvate kinase by 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenonadenosine (5′-FSBεA). This inference was based primarily on studies which showed that reactivation of modified enzyme occurred upon incubation with dithiothreitol. In the present study, more direct evidence has been obtained for the modification of cysteine residues by a fluorosulfonyl derivative.

Apparently contradictory results obtained from two types of experiments suggest, on one hand, that incorporation of radioactive 5′-SBG is not decreased to any measurable extent upon the addition of dithiothreitol while, on the other hand, inclusion of dithiothreitol with modified enzyme results in complete restoration of enzymatic activity. The critical group

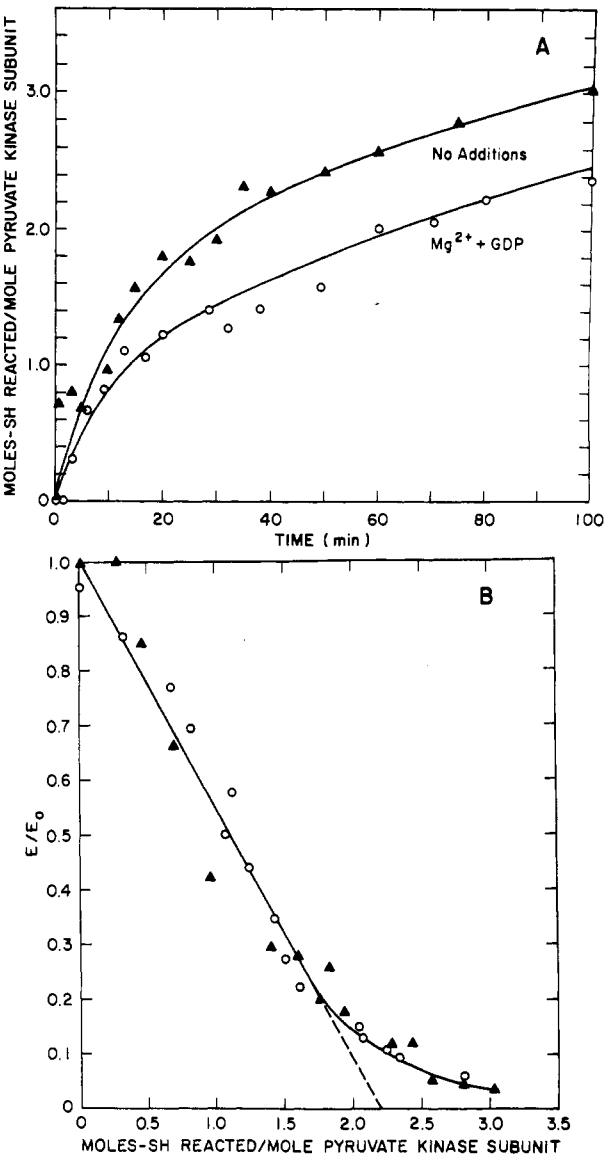


FIGURE 6: Effect of Mg^{2+} and GDP on the reactivity of 5′-FSBG toward sulfhydryl groups. (A) Pyruvate kinase (1.0 mg/mL) was incubated with 1.66 mM 5′-FSBG under conditions identical with those described in Figure 1B. The moles of SH groups reacted were followed with (○) and without (▲) the addition of Mg^{2+} (13 mM) and GDP (15 mM) to the inactivation medium. The DTNB assay described under Experimental Procedures was used. (B) This secondary plot depicts the residual enzymatic activity as a function of the loss in free sulfhydryl groups, shown in (A), in the presence (○) and absence (▲) of Mg^{2+} (13 mM) and GDP (15 mM).

involved cannot be the one which remains covalently labeled by 5′-FSBG because there is no direct correlation between incorporation and inactivation. In contrast, there is a good correlation with respect to activity loss and the loss of two free sulfhydryl groups upon incubation with 5′-FSBG. Addition of dithiothreitol, which is known to react with both thiol sulfonates and disulfides to regenerate free sulfhydryls (Parsons et al., 1965), results in both the regeneration of two free sulfhydryls and the regain of activity. The decrease in both the rate of inactivation and the number of sulfhydryls modified upon incubation of pyruvate kinase with 5′-FSBG in the presence of the protectants Mg^{2+} and GDP suggests that inactivation is decreased to the same extent that the modification of sulfhydryls is decreased (Figure 6B).

In a number of cases, dithiothreitol or mercaptoethanol has been used to quench the reaction between a fluorosulfonyl nucleotide analogue and an enzyme (Pettigrew & Frieden,

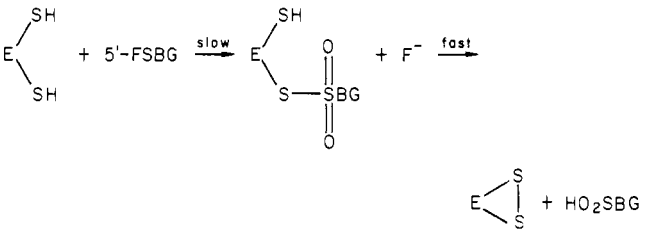
1978; Mansour & Colman, 1978; Esch & Allison, 1978; Zoller & Taylor, 1979; Craig & Hammes, 1980; Hixson & Krebs, 1981). The results of the present study suggest the need to ascertain the effect of added thiol on the activity, extent of reagent incorporation and/or free sulfhydryls regenerated for these modified enzymes.

The reaction of pyruvate kinase with 5′-FSBG at pH 7.65 and 7.93 is characterized by a biphasic rate of inactivation which cannot be accounted for through reagent depletion or decomposition. The initial phase of the inactivation (referred to as the rapid phase) seems to account for approximately 55% of the loss in enzyme activity whereas the second or slow phase is responsible for the remaining 45% of activity. Several models might account for the kinetics observed. One possible explantation is that 5′-FSBG reacts with two entirely separate sulfhydryls groups at two different rates to form thiol sulfonates. This model predicts that one should observe a decrease in free sulfhydryl content with a concomitant increase in incorporation of radiolabel, both of which should correlate with activity loss. Addition of dithiothreitol would displace the thiol sulfonate and therefore cause loss of radioactivity. These expectations, however, are contrary to fact, thus eliminating this model. A second model involves an initial reaction at a single cysteine subunit to form a thiol sulfonate, which is followed by reaction of that thiol sulfonate with an adjacent cysteine to yield a sulfinic acid and a disulfide in each subunit (Parsons et al., 1965). This model would be consistent with the results in the sense that the final product, one disulfide per subunit, would not contain the elements of the reagent and therefore would not be measurable by incorporation of radioactivity. This model is also consistent with the results of Flashner et al. (1972) using DTNB.

With the assumption that inactivation of pyruvate kinase by 5′-FSBG is the result of the formation of one disulfide per subunit, what reaction sequence could account for the observed biphasic kinetics? One explantation worth consideration is that reaction of 5′-FSBG with the first sulfhydryl occurs relatively rapidly, with formation of a partially active thiol sulfonate–enzyme. The second slower reaction which leads to formation of the disulfide through displacement of the sulfinic acid would then completely inactivate the enzyme. This scheme would predict a relative accumulation of the thiol sulfonate intermediate. This scheme would also predict that if the second cysteine is topographically close to the first sulfhydryl group, formation of the thiol sulfonate would inevitably lead to the formation of the disulfide. This conjecture is not substantiated in that it is possible, through the use of protecting ligands, to limit reaction at the second site once the first site has been modified (Table II). The independent nature of the two reaction sites suggests that the two rates observed during the inactivation process are not due, respectively, to esterification and to disulfide formation. However, the most convincing evidence against this proposed mechanism comes from the failure of dithiothreitol to decrease, at any time during the reaction sequence of 5′-FSBG with the enzyme, the radioactive incorporation of 5′-SBG, thus arguing strongly against the accumulation of any measurable amount of thiol sulfonate intermediate.

A more reasonable reaction mechanism would have the reaction producing the thiol sulfonate as the rate-limiting step, with disulfide formation occurring extremely rapidly, in accordance with Scheme I. This two-step mechanism accords with the observations regarding radioactive incorporation before and after dithiothreitol treatment, but how can it account for the biphasic kinetics?

Scheme I



Under native conditions, pyruvate kinase exists as a tetramer containing four similar subunits (Cottam et al., 1969). Although muscle pyruvate kinase is not generally considered as a regulatory enzyme, it has been found to display positive cooperative binding of the amino acid effector phenylalanine (Carminatti et al., 1971; Kayne & Price, 1973). This cooperative binding, leading to a reversible inhibition of the enzyme, provides strong evidence for the possibility of effector-mediated subunit interactions. Also, Bloxham & Chalkley (1976) reported a positive cooperative effect on the reaction of 5-chloro-4-oxopentanoic acid with rabbit muscle pyruvate kinase. Examples exist of enzyme subunit interactions being exhibited as positive cooperativity for the binding of some ligands and negative cooperativity in the binding of other ligands (Koshland & Levitzki, 1974). A negative cooperativity in the interaction of pyruvate kinase subunits with 5′-FSBG might best account for the results presented in this paper. Reaction of 5′-FSBG with the first two subunits of pyruvate kinase might occur relatively rapidly by the mechanism presented in Scheme I; however, once two subunits were modified, reaction with the second two subunits would proceed more slowly. In the case of reaction with all subunits, the rate-limiting step would be the formation of the thiol sulfonate ester followed by a faster reaction of this intermediate to yield a disulfide. Thus, when the enzyme loses 50% of its original activity, half of the subunits would contain the disulfide linkage, and the other two subunits would be unmodified. This hypothesis is consistent with the experiments involving incorporation of radioactive reagent as well as with the experiments examining the effects of ligands on the rates of inactivation by 5′-FSBG. Protection by ligands against both phases of the reaction has been observed. The pattern of protection is qualitatively the same for both phases (i.e., those ligands which protect well in the first phase do likewise with the second).

Besides indicating the cooperative nature of the 5′-FSBG–pyruvate kinase interaction, protection experiments provide insight into the relative spatial assignment of the sulfhydryls within the active site. 5′-FSBG appears to inactivate the enzyme through modification of two cysteines located at or near the metal–nucleotide binding sites. Phosphoenolpyruvate, K^+, Na^+, pyruvate, and free nucleotide binding sites do not appear to overlap those residues modified by 5′-FSBG, as indicated by their inability to decrease appreciably the rate of inactivation. The evidence also suggests the existence of two distinct sites for free divalent metal and metal–ADP complex since augmentative effects on protection are observed upon addition of both Mg^{2+} and Mg–ADP. Mildvan et al. (1976) have proposed two sites on the basis of a requirement for activity of free divalent metal in addition to metal–ADP complex.

In the present study, free Mg^{2+} has been found to produce a partial protection against inactivation by 5′-FSBG, reaching a limiting rate constant which is about 3-fold lower than that observed in the absence of ligands; this result suggests that protection by Mg^{2+} is an indirect effect of the binding of the free metal at a site outside the catalytic center. The K_d of 320

μM for the enzyme–Mg^{2+} complex determined from this partial protection against inactivation by 5′-FSBG, is consistent with values in the literature, as previously described under Results.

Pyruvate kinase has been shown to utilize both ADP and GDP as the nucleoside diphosphate substrates (Tietz & Ochoa, 1958; Strominger, 1955). From the present study, the greatest protection against inactivation by 5′-FSBG is afforded by Mg–GDP and Mg–ADP. Protection is complete and can be used to calculate binding constants not too different from those reported in the literature. The calculated K_d for Mg–GDP with pyruvate kinase at all concentrations of free metal ion is 2.9 mM, compared with a published value for K_m at pH 8.0 of 0.76 mM (Plowman & Krall, 1965). Addition of ADP plus Mg^{2+} to the reaction mixture yields two values for K_d, one at low concentrations of Mg^{2+} and the other at high concentrations. High concentrations of Mg^{2+} have been reported to tighten the binding of the metal–nucleotide complex (Gupta et al., 1976). Such an effect would explain the change in K_d observed upon the addition of high concentrations of Mg^{2+} (Table III) and indicates that the K_d of 2.05 mM obtained at a high Mg^{2+} concentration is the value most comparable to the 0.60 mM given at high Mg^{2+} concentrations by Rao et al. (1979). The approximate 3.5-fold difference between these dissociation constants might reflect differences in the experimental conditions. The similarity in both the degree of protection conveyed as well as the magnitude of the dissociation constants strongly implies that the Mg–ADP and Mg–GDP sites are overlapping if not identical under the conditions employed in the present study. The protection afforded by Mg–GDP and Mg–ADP indicates that 5′-FSBG reacts at or near the metal–nucleoside diphosphate binding site. (It should also be noted, however, that as in the case of any chemical modification study, the possibility must be also considered that reaction occurs at a site distinct from the active site and that protection by the metal–nucleotide complex could be an indirect result of a conformational change caused by the ligands.) In any case, the Mg–nucleoside diphosphate and Mg–nucleoside triphosphate sites do not appear to be identical since the Mg–nucleoside diphosphate complexes are much more effective in decreasing the rate of inactivation by 5′-FSBG.

As outlined under Results, there appears to be an interaction between the sites for free metal and metal–nucleotide complex. Mg–nucleoside di- and triphosphates seem to weaken the binding of free Mg^{2+} to the enzyme, yet when the free Mg^{2+} concentration is raised high enough, an apparent strenthening of the binding of Mg–ADP is observed. This response, however, is not seen in the presence of Mg–GDP.

Pyruvate kinase reacts in a limited manner with each of the nucleotide affinity labels: 5′-[(fluorosulfonyl)benzoyl]-adenosine (5′-FSBA), 5′-[(fluorosulfonyl)benzoyl]guanosine (5′-FSBG) and 5′-[(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBϵA). However, the exact mode of interaction seems to vary with the compound. Plowman & Krall (1965) determined that ADP and GDP behave quite differently with regard to the pH dependence of their respective K_m and V_{max} values. These observations suggest that although muscle pyruvate kinase can interchange these purine nucleotides, their exact mode and sites of binding are pH dependent and may be distinct for each of these nucleotides. It is therefore not surprising that 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) differs greatly with respect to the site of modification of pyruvate kinase (Wyatt & Colman, 1977). 5′-FSBA appears to inactivate the enzyme by modification of two discrete types of amino acid residues (Annamalai & Colman, 1981), while inactivation by 5′-FSBG leads to modification of two sulfhydryl groups, at least one of which is critical for activity. Inactivation of pyruvate kinase of 5′-FSBG seems to resemble more closely that observed for this enzyme by 5′-[*p*-(fluorosulfonyl)benzoyl]1,N^6-ethenoadenosine (Likos & Colman, 1981). Although no biphasic kinetics were observed in that case, a single critical cysteine residue appeared to be modified.

As seen above, the nature of the purine moiety appears to direct the nature of the reaction of these sulfonyl fluoride compounds with pyruvate kinase, observations consistent with the behavior of these compounds as affinity labels of this enzyme. Wyatt & Colman (1977) investigated the reactivity toward pyruvate kinase of *p*-(fluorosulfonyl)benzoic acid, which lacks a purine nucleotide substituent; little effect on the enzyme activity was observed under conditions where 5′-FSBA was shown to inactivate rapidly. In the present study, protection against inactivation by various ligands provides further evidence for the specificity of the reaction of 5′-FSBG with pyruvate kinase. The lack of a saturation effect in the dependence of inactivation rate constants on reagent concentration might tend to refute the notion of 5′-FSBG acting as a true affinity label. However, because of the necessarily limited reagent concentration range employed, it is not possible to ascertain definitively whether or not reversible binding occurs prior to the irreversible modification.

When compared with previously reported sulfhydryl modifying reagents, use of 5′-FSBG provides far greater insight into the number and location of critical sulfhydryls within the active site of muscle pyruvate kinase. In most cases, use of nonspecific reagents such as methyl methanethiosulfonate (Bloxham et al., 1978), iodoacetamide (Jacobson & Black, 1971), DTNB (Flashner et al., 1972), and *p*-(chloromercuri)benzoate (Mildvan & Leigh, 1964; Mildvan & Cohn, 1966) resulted in multiple cysteinyl modifications. A critical sulfhydryl was generally identified through differences between the number of groups modified with and without the addition of protectants. Every reagent seemed to elicit a slightly different protection pattern by ligands of the enzyme, with no consistent discernible pattern. The variety of protection patterns can best be reconciled if more than one cysteine residue is included in the active site, as indicated in this report. Experiments designed to ascertain the relationship and proximity between the two active-site sulfhydryls affected by 5′-FSBG and other amino acid residues in the region of the nucleotide binding site of pyruvate kinase are in progress in this laboratory.

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