Rabbit Muscle Phosphofructokinase. 2. Inactivation by the Affinity Label 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine†

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ABSTRACT: The reaction of the fluorescent affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine with rabbit skeletal muscle phosphofructokinase results in an inactivation of the enzyme and in the covalent incorporation of up to one label/monomer. The substrates, MgATP and fructose 6-phosphate, each protect against inactivation of the enzyme, but neither diminishes the extent of covalent incorporation of the label, indicating that the inactivation is not the result of covalent incorporation of the label. Dithiothreitol reactivates the inactivated enzyme but does not reduce the extent of incorporation of the label. A determination of the number of free sulfhydryl groups on the enzyme as a function of the extent of inactivation by the reagent suggests that the inactivation is associated with the loss of two free sulfhydryl groups per phosphofructokinase monomer. The inactivation reaction appears to involve the reversible formation of an enzyme–reagent complex (K_d = 1.11 mM) prior to the conversion of the complex to inactive enzyme (k_1 = 0.98 min^{-1}). In view of the protection afforded by either substrate and the evidence suggesting the formation of an enzyme–reagent complex prior to inactivation, it would appear that the inactivation results from a reagent-mediated formation of a disulfide bond between two cysteinyl residues in close proximity, possibly in or near the catalytic site of the enzyme. The site of covalent attachment of the label appears to be the binding site specific for the activating adenine nucleotides cAMP, AMP, and ADP. The extent of covalent incorporation of the label at this site is diminished in the presence of cAMP, and phosphofructokinase modified at this site by this affinity label is no longer subject to activation by cAMP.

The smallest enzymatically active species of rabbit skeletal muscle phosphofructokinase (EC 2.7.1.11) is a tetramer comprised of four identical or nearly identical monomers of M_r 80 000 (Paetkau & Lardy, 1967; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973; Lad et al., 1973). Each monomer contains at least two adenine nucleotide binding sites in addition to the MgATP binding site at the active site of the enzyme. These are an allosteric inhibitory binding site for ATP (Lardy & Parks, 1956; Lowry & Passonneau, 1966; Hofer & Pette, 1968; Kemp, 1969; Wolfman et al., 1978; Pettigrew & Frieden, 1979) and an allosteric binding site specific for the activating adenine nucleotides cAMP,[1] AMP, and ADP (Passonneau & Lowry, 1962; Lowry & Passonneau, 1966; Kemp & Krebs, 1967). The adenine nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine has been shown to specifically label the activating adenine nucleotide binding site (Pettigrew & Frieden, 1978), and the covalent incorporation of approximately two or more SO_2BzAdo residues/tetramer leads to an activation of phosphofructokinase that is indistinguishable from the activation produced by a saturating concentration of cAMP (Ogilvie, 1983).

A fluorescent adenine nucleotide analogue, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine, has been synthesized and employed as an affinity label for rabbit muscle pyruvate kinase by Likos & Colman (1981). Although 5′-FSO_2BzAdo and 5′-FSO_2Bz-ϵ-Ado differ only by the presence of an etheno bridge between N-1 and 6-NH_2 in the latter reagent, these investigators observed that the modifications produced in pyruvate kinase by these two affinity labels were quite different. In the present study, the reaction of 5′-FSO_2Bz-ϵ-Ado with phosphofructokinase has been investigated. As reported herein, the effects of the modifications produced by 5′-FSO_2Bz-ϵ-Ado on the kinetic and regulatory properties of the enzyme differ markedly from those produced by modification of the enzyme with 5′-FSO_2BzAdo.

Materials and Methods

Materials. All enzymes, substrates, nucleotides, 1,N^6-ethenoadenosine, 1,N^6-ethenoadenosine cyclic 3′,5′-monophosphate, dithiothreitol, and 5′-FSO_2BzAdo were purchased from Sigma Chemical Co. Hexamethylphosphoric triamide and *p*-(fluorosulfonyl)benzoyl chloride were purchased from Aldrich Chemical Co. 5′-FSO_2Bz-ϵ-Ado was synthesized and purified by two recrystallizations as described by Likos & Colman (1981). The twice-recrystallized 5′-FSO_2Bz-ϵ-Ado migrated as a single component (R_f 0.52) when chromatographed on 250-μm thin-layer silica gel G plates (Analtech Inc.) with methyl ethyl ketone–acetone–water (65:20:15).

Rabbit muscle phosphofructokinase (type III, lot 30F-9720) purchased from Sigma was used throughout this study. The enzyme, obtained as a crystalline suspension in ammonium sulfate solution, was isolated by centrifugation, dissolved in 1.0 mL of 0.1 M potassium phosphate–1 mM EDTA–0.1 mM dithiothreitol, pH 7.0, and chromatographed at 4 °C on a Sephadex G-25 fine column (1 cm × 30 cm) equilibrated with the same pH 7.0 buffer. Enzyme concentrations were determined spectrophotometrically with E_{279} = 1.02 mg^{-1} mL (Parmeggiani et al., 1966).

Enzyme Assay Procedure. Phosphofructokinase activity was assayed in a 3.0-mL assay mixture at 25 °C as previously described (Ogilvie, 1983). The standard assay mixture contained 40 mM imidazole hydrochloride, pH 6.95, 50 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.053 mM NADH, 0.133 mM fructose 6-phosphate, 0.15 mM ATP,

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[1] Abbreviations: 5′-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine; SO_2BzAdo, 5′-(*p*-sulfonylbenzoyl)adenosine group; 5′-FSO_2Bz-ϵ-Ado, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; SO_2Bz-ϵ-Ado, 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine group; PFK, phosphofructokinase; cAMP, adenosine cyclic 3′,5′-monophosphate; ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate; F-6-P, fructose 6-phosphate; F-1,6-P_2, fructose 1,6-diphosphate; 3′,5′-cyclic-ϵAMP, 1,N^6-ethenoadenosine cyclic 3′,5′-monophosphate; EDTA, ethylenediaminetetraacetic acid.

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0.9 unit/mL α-glycerophosphate dehydrogenase, 2.9 units/mL triosephosphate isomerase, and 1.3 units/mL aldolase.

Chemical Modification of Phosphofructokinase. Stock solutions of approximately 5 mM FSO_2Bz-ε-Ado were prepared in absolute ethanol, and the concentration was determined spectrophotometrically in 10% (v/v) ethanol–90 mM potassium phosphate–0.9 mM EDTA, pH 7.0, by employing an extinction coefficient at 275 nm of 6211 M^{-1} cm^{-1} (Likos & Colman, 1981). These stock solutions were stored at −20 °C.

Chemical modification of phosphofructokinase at 22 °C was initiated by slowly adding with stirring 0.15 mL of an ethanolic solution of 5′-FSO_2Bz-ε-Ado to 0.85 mL of 0.1 M potassium phosphate–1 mM EDTA–0.05 mM dithiothreitol, pH 7.0, containing the phosphofructokinase. The final concentrations of 5′-FSO_2Bz-ε-Ado and enzyme and the time of reaction employed in these modification reactions varied depending upon the type of experiment, and these values are given in the legends to the figures and in the footnotes to the tables. Although these are designated as modifications at pH 7.0, the pH of the reaction mixture as determined with a glass electrode was 7.3.

In those experiments in which the extent of modification of the enzyme was to be determined, the reaction was stopped and the enzyme separated from the unreacted and noncovalently bound 5′-FSO_2Bz-ε-Ado by chromatographing the 1.0-mL reaction mixture on a Sephadex G-25 fine column (1 cm × 30 cm) at 4 °C with 0.1 M potassium phosphate–1 mM EDTA–0.1 mM dithiothreitol, pH 7.0, to elute the column. The column eluate was monitored at 280 nm, and 1.0-mL fractions were collected. The three fractions with the greatest protein concentrations were combined, and the enzymatic properties and absorbance at 279 nm of the combined fractions were determined. The extent of covalent incorporation of the reagent was then measured by adding 0.05 mL of 6 N NaOH to 2.95 mL of the combined fraction to bring the pH to 12 and following the fluorescence intensity at 405 nm as a function of time in a Hitachi Perkin-Elmer MPF-44A spectrofluorometer with an exciting wavelength of 310 nm as described by Likos & Colman (1981). Native enzyme in the same buffer and at the same concentration was used as the blank, and solutions of 5′-FSO_2Bz-ε-Ado were used as standards. The stoichiometry of the modification was calculated from the concentration of bound reagent thus calculated and the protein concentration as determined from the absorbance at 279 nm corrected for the minor contribution of the incorporated reagent to the absorbance at this wavelength. The extent of modification of the enzyme by 5′-FSO_2BzAdo was calculated as previously described (Ogilvie, 1983).

Determination of Number of Free Sulfhydryl Groups in Phosphofructokinase as a Function of Extent of Inactivation by 5′-FSO_2Bz-ε-Ado. The inactivation reactions were carried out in 1.0-mL reaction volumes containing approximately 0.8 mg of phosphofructokinase, and the extent of inactivation was controlled by varying the initial concentration of 5′-FSO_2Bz-ε-Ado (45–460 μM) and the time of reaction (8–60 min). The reaction was stopped, and the unreacted and noncovalently bound 5′-FSO_2Bz-ε-Ado and the dithiothreitol and K^+ were removed by chromatography at 4 °C on a Sephadex G-25 fine column (1 cm × 30 cm) that had been equilibrated with 25 mM glycylglycine–25 mM sodium phosphate–1 mM EDTA, pH 7.2, and that had been purged with N_2. The three 1-mL fractions containing the highest concentration of protein were combined, and the enzymatic activity and protein concentration in the combined fraction were determined spectrophotometrically. A 2.6-mL aliquot of the combined fraction was mixed with 0.3 mL of 20% sodium dodecyl sulfate and 0.1 mL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid), and the free sulfhydryl group concentration was determined spectrophotometrically at 412 nm by employing an extinction coefficient of 13 600 M^{-1} cm^{-1} (Ellman, 1959) for thionitrobenzoate. A value of 13.92 ± 0.13 –SH groups per M_r 80 000 monomer was calculated for native phosphofructokinase from five different preparations of the enzyme.

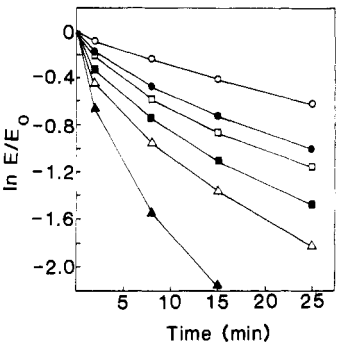


FIGURE 1: Inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado. Phosphofructokinase (40 μg/mL) was incubated at 22 °C with 5′-FSO_2Bz-ε-Ado in 85 mM potassium phosphate–0.85 mM EDTA–0.04 mM dithiothreitol, pH 7.0, containing 15% (v/v) ethanol, and 10-μL aliquots were removed at the times indicated, and their enzymatic activity was determined by the standard assay procedure given under Materials and Methods. The concentrations of 5′-FSO_2Bz-ε-Ado used were 0.051 (○), 0.101 (●), 0.152 (□), 0.203 (■), 0.339 (△), and 0.678 mM (▲).

Results

Inactivation of Phosphofructokinase by 5′-[p-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. As shown in Figure 1, rabbit muscle phosphofructokinase becomes inactivated when incubated at 22 °C with excess 5′-FSO_2Bz-ε-Ado in 15% (v/v) ethanol–85 mM potassium phosphate–0.85 mM EDTA–40 μM dithiothreitol buffer, pH 7.0. Although 5′-FSO_2Bz-ε-Ado is in large excess over the protein in all of these experiments, the nonlinearity of the ln (E/E_0) vs. time plots in Figure 1 indicated that the inactivation reaction is not a simple pseudo-first-order reaction as observed for the inactivation of pyruvate kinase by 5′-FSO_2Bz-ε-Ado (Likos & Colman, 1981), nor does it appear to be biphasic as observed for the inactivation of pyruvate kinase by 5′-[p-(fluorosulfonyl)benzoyl]guanosine (Tomich et al., 1981). The nonlinearity of these plots does not appear to be the result of the depletion of 5′-FSO_2Bz-ε-Ado by reaction with the 40 μM dithiothreitol that is present since plots of data obtained in 2.5 μM dithiothreitol were superimposable on those obtained at 40 μM. On the basis of the rate constants that have been reported for the spontaneous hydrolysis of fluorosulfonyl derivatives (Tomich et al., 1981), it also appears unlikely that the nonlinearity could be the result of the depletion of 5′-FSO_2Bz-ε-Ado by spontaneous hydrolysis. Furthermore, if the reaction between the enzyme and 0.1 mM 5′-FSO_2Bz-ε-Ado is allowed to proceed for 25 min and then a second aliquot of the reagent is added to bring the total concentration to 0.2 mM, the apparent rate constant calculated from the data obtained between 25 and 30 min is twice that calculated for the same time period if the second aliquot is not added but still significantly less than the apparent rate constant calculated from the initial rate of reaction during the first 2 min of reaction. Hence, the nonlinearity would appear to be the result of a change in the reactivity of the protein with time and not reagent depletion.

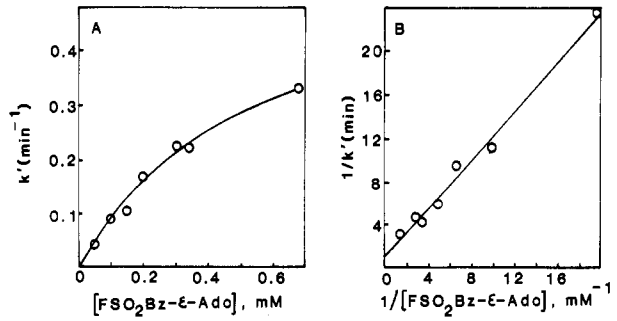


FIGURE 2: (A) Dependence of pseudo-first-order rate constants on concentrations of 5′-FSO_2Bz-ε-Ado. The pseudo-first-order rate constants were calculated from the data in Figure 1 for the rate of inactivation during the initial 2 min of reaction. (B) Double-reciprocal plot of data in (A).

Pseudo-first-order rate constants for the rate of inactivation during the first 2 min of the reaction were calculated from the data of Figure 1, and these are plotted as a function of 5′-FSO_2Bz-ε-Ado concentration in Figure 2A. The hyperbolic shape of this curve suggests that 5′-FSO_2Bz-ε-Ado binds reversibly to phosphofructokinase prior to inactivating the enzyme, i.e.

$$\text{PFK} + 5'\text{-FSO}_2\text{Bz-}\epsilon\text{-Ado} \overset{K_d}{\rightleftharpoons} \text{PFK}\cdot 5'\text{-FSO}_2\text{Bz-}\epsilon\text{-Ado} \xrightarrow{k_1} \text{PFK}'$$

where PFK′ is inactivated enzyme, K_d is the dissociation constant for the enzyme–affinity label complex, and k_1 is the rate constant for inactivation. A similar mechanism has been proposed for the inactivation of rabbit muscle pyruvate kinase by 5′-FSO_2Bz-ε-Ado (Likos & Colman, 1981) and for the inactivation of yeast pyruvate kinase by 5′-FSO_2BzAdo (Likos et al., 1980). From the double-reciprocal plot of these data shown in Figure 2B, values of K_d = 1.11 mM and k_1 = 0.98 min^{-1} were estimated.

Effect of Ligands on Inactivation of Phosphofructokinase by 5′-FSO_2Bz-ε-Ado. As shown in Figure 3, the rate of inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado is markedly decreased by several ligands that serve as substrates and/or allosteric effectors of the enzyme. The two substrates for the enzyme, F-6-P and the MgATP chelate, are each very effective at protecting the enzyme against inactivation by 5′-FSO_2Bz-ε-Ado, suggesting that the binding of either of these ligands to the active site of the enzyme blocks the binding and subsequent reaction of 5′-FSO_2Bz-ε-Ado at the active site or that their binding at the active site induces a conformational change in the enzyme that decreases the binding and subsequent reaction of 5′-FSO_2Bz-ε-Ado at some site other than the active site. The data depicted in Figure 3 also indicate that cAMP, which is an activator of the enzyme and binds to the allosteric site specific for the activating adenine nucleotides, is essentially as effective as either of the two substrates of the enzyme in preventing inactivation of the enzyme by 5′-FSO_2Bz-ε-Ado. Free ATP is much less effective at blocking the inactivation than is the MgATP chelate. That the increased effectiveness of ATP in the presence of $MgCl_2$ is due to chelate formation is supported by the observation that $MgCl_2$ alone is without effect on the rate of inactivation by 5′-FSO_2Bz-ε-Ado. On the other hand, the effectiveness of F-6-P in blocking the inactivation is independent of the concentration of Mg^{2+}, suggesting that Mg^{2+} does not enhance the binding of F-6-P.

Extent and Nature of Covalent Modification of Phosphofructokinase Produced by 5′-FSO_2Bz-ε-Ado. In order to determine the relationship between the extent of inactivation of the enzyme and the extent of covalent labeling of phosphofructokinase by 5′-FSO_2Bz-ε-Ado, samples of phosphofructokinase (0.6–0.9 mg/mL) were reacted with 0.35 mM 5′-FSO_2Bz-ε-Ado for 60 min at 22 °C in the absence and presence of protecting ligands. The 1-mL reaction mixtures were then chromatographed on a Sephadex G-25 fine column (1 cm × 30 cm) at 4 °C to remove any unreacted or noncovalently bound affinity label, and the extent of inactivation, the concentration of covalently bound SO_2Bz-ε-Ado groups, and the protein concentration in the chromatographed solutions were determined as described under Materials and Methods. As can be seen in Table I, modification in the absence of a protecting ligand resulted in an 80% inactivation of the enzyme

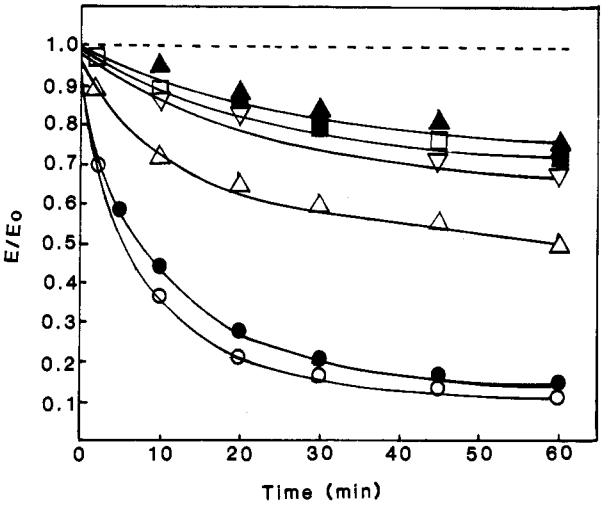


FIGURE 3: Effect of ligands on inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado. Phosphofructokinase (50 μg/mL) was incubated at 22 °C with 0.35 mM 5′-FSO_2Bz-ε-Ado, and 10-μL aliquots were removed and assayed at the times indicated as described in Figure 1. The reaction conditions were the same as those of Figure 1 except for the presence of the following ligands: 1.5 mM ATP (△), 1.5 mM ATP + 5.0 mM $MgCl_2$ (▲), 0.5 mM F-6-P (□), 0.5 mM F-6-P + 5.0 mM $MgCl_2$ (■), 0.2 mM cAMP (▽), 5.0 mM $MgCl_2$ (●), and no ligand (○). The dashed line (---) is the control in which the enzyme was incubated under identical conditions in the absence of 5′-FSO_2Bz-ε-Ado.

Table I: Effect of Ligands on Inactivation and Extent of Covalent Modification of Phosphofructokinase by 5′-FSO_2Bz-ε-Ado

protecting ligand	enzymatic activity relative to native PFK (%)	extent of modification (SO_2Bz-ε-Ado/tetramer)
none[a]	20	1.66
1.5 mM ATP + 5 mM $MgCl_2$[a]	82	1.60
0.5 mM F-6-P[a]	68	1.69
1.0 mM cAMP[a]	78	0.79
0.106 mM 5′-FSO_2BzAdo[b]	115	0.61 (3.34 SO_2BzAdo/tetramer)
none[c]	0.2	4.1

[a] Phosphofructokinase (0.6–0.9 mg/mL) was incubated with 0.35 mM 5′-FSO_2Bz-ε-Ado for 1 h at 22 °C in 85 mM potassium phosphate–0.85 mM EDTA–85 μM dithiothreitol, pH 7.0, containing 15% (v/v) ethanol and protecting ligands at the concentrations indicated. The reaction mixture was then chromatographed on a G-25 column, and the enzymatic activity and extent of covalent modification were determined as described under Materials and Methods. [b] Phosphofructokinase was reacted with 0.106 mM 5′-FSO_2BzAdo for 1 h prior to the 1-h incubation with 0.35 mM 5′-FSO_2Bz-ε-Ado. [c] Conditions same as footnote *a* except 0.47 mM 5′-FSO_2Bz-ε-Ado and an 18-h incubation were used.

Table II: Effect of Dithiothreitol on Enzymatic Activity and Extent of Covalent Modification of Phosphofructokinase Modified by 5′-FSO_2Bz-ε-Ado[a]

enzyme preparation	enzymatic activity (μmol of F-1,6-P_2 min^{-1} mg^{-1})	inactivation (%)	extent of modification (SO_2Bz-ε-Ado/tetramer)
native PFK	30.0	0	0
modified PFK before reduction with dithiothreitol	6.4	79	1.55
modified PFK after reduction with dithiothreitol	31.8	0	1.44

[a] Phosphofructokinase (0.88 mg/mL) was modified with 0.34 mM 5′-FSO_2Bz-ε-Ado for 1 h as described in Table I, footnote *a*. Half the reaction mixture (1 mL) was chromatographed on a G-25 column to isolate the modified enzyme. Dithiothreitol was added to the other half of the reaction mixture (final concentration of dithiothreitol 20 mM), and the solution was incubated at 22 °C for 2.5 h and then chromatographed on a G-25 column to isolate the reduced modified enzyme.

and the covalent incorporation of 1.66 SO_2Bz-ε-Ado groups/tetrameric phosphofructokinase. The two substrates, MgATP and F-6-P, each markedly reduced the extent of inactivation of the enzyme, but neither significantly altered the number of SO_2Bz-ε-Ado groups covalently bound to the enzyme. On the other hand, the adenine nucleotide activator, cAMP, significantly reduced both the extent of inactivation and the extent of covalent modification of the enzyme by 5′-FSO_2Bz-ε-Ado. An even greater protection against inactivation and covalent incorporation of SO_2Bz-ε-Ado groups was afforded by preincubating the enzyme with 5′-FSO_2BzAdo for 1 h prior to the addition of 5′-FSO_2Bz-ε-Ado. As reported in the preceding paper (Ogilvie, 1983), 5′-FSO_2BzAdo is an affinity label specific for the cAMP binding site, and the covalent attachment of approximately two or more SO_2BzAdo groups/tetramer leads to a slight stimulation of phosphofructokinase activity, similar to that observed in Table I, when assayed at 0.15 mM ATP in the standard assay procedure. It is of interest to note that the total number of covalently bound SO_2BzAdo plus SO_2Bz-ε-Ado/tetramer is 3.95, or one/monomer. The last entry in Table I shows that a longer reaction time with 5′-FSO_2Bz-ε-Ado leads to the incorporation of four SO_2Bz-ε-Ado groups/tetramer, which is the same as the number of cAMP binding sites (Kemp & Krebs, 1967), and to total inactivation of the enzyme. Two conclusions can be drawn from the data presented in Table I. As there does not appear to be any correlation between the extent of inactivation of the enzyme by 5′-FSO_2Bz-ε-Ado and the number of covalently attached SO_2Bz-ε-Ado groups, the inactivation does not result from the permanent covalent attachment of the affinity label. Furthermore, since the extent of covalent labeling of the enzyme is decreased by cAMP and by prelabeling the cAMP binding sites with 5′-FSO_2BzAdo and since the maximum number of sites covalently labeled with 5′-FSO_2Bz-ε-Ado is one/monomer, it would appear that the permanent covalent attachment of 5′-FSO_2Bz-ε-Ado occurs specifically at the cAMP binding site.

Reactivation by Dithiothreitol of Phosphofructokinase Inactivated by 5′-FSO_2Bz-ε-Ado. Since the extent of inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado did not correlate with the extent of covalent incorporation of the affinity label, the possibility that the inactivation resulted from a 5′-FSO_2Bz-ε-Ado-mediated oxidation of cysteinyl residues to disulfides was investigated. As shown in Figure 4, modified phosphofructokinase, inactivated to the extent of 92% by 2-h incubation with 0.34 mM FSO_2Bz-ε-Ado, was largely reactivated when incubated for an additional 2 h in 20 mM dithiothreitol. Furthermore, as can be seen from the data presented in Table II, total reactivation of 5′-FSO_2Bz-ε-Ado-inactivated phosphofructokinase by dithiothreitol does not lead to a significant reduction in the number of covalently bound SO_2Bz-ε-Ado groups, confirming the earlier conclusion that inactivation of the enzyme by 5′-FSO_2Bz-ε-Ado does not result from the permanent covalent attachment of the affinity label to the protein. Hence, a possible mechanism for the inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado would appear to involve disulfide bond formation between cysteinyl residues in the protein. The inactivation of rabbit muscle pyruvate kinase by 5′-FSO_2BzAdo and by 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine has also been attributed to disulfide bond formation between cysteinyl residues (Tomich et al., 1981; Annamalai & Colman, 1981).

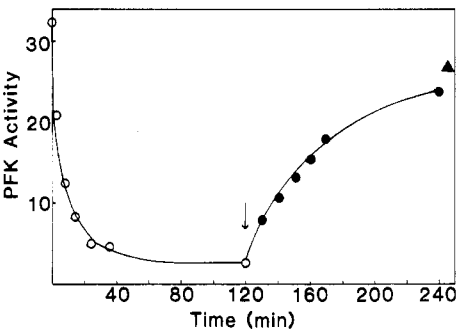


FIGURE 4: Time course for inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado and reactivation of inactivated enzyme by dithiothreitol. Phosphofructokinase (41 μg/mL) was incubated at 22 °C with 0.34 mM 5′-FSO_2Bz-ε-Ado in 85 mM potassium phosphate–0.85 mM EDTA–2.5 μM dithiothreitol, pH 7.0, containing 15% (v/v) ethanol, 10-μL aliquots were removed at the times indicated, and enzymatic activity (μmol of F-1,6-P_2 formed min^{-1} mg^{-1}, was determined as described under Materials and Methods. At 120 min (indicated by the arrow), dithiothreitol was added to a final concentration of 20 mM, and the incubation and assay of 10-μL aliquots were continued. The filled triangle (▲) near the right border represents the enzymatic activity observed 22 h after addition of the dithiothreitol.

Additional support for the involvement of disulfide bond formation in the inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado was obtained by determining the number of free sulfhydryl groups on the enzyme as a function of the extent of inactivation of the enzyme by 5′-FSO_2Bz-ε-Ado. As indicated in Figure 5, the extent of inactivation appears to correlate with the loss of approximately two free sulfhydryl groups/monomer, suggesting that inactivation results from the reagent-mediated formation of one disulfide bond/monomer.

Some Regulatory Kinetic Properties of Phosphofructokinase Modified by 5′-FSO_2Bz-ε-Ado. As shown in Table III, native enzyme is 99% inhibited when assayed at 1.35 mM ATP, and this inhibition by high concentrations of ATP is largely relieved if cAMP is also present in the assay. Modified enzyme containing about two SO_2Bz-ε-Ado groups/tetramer is 92% reactivated by dithiothreitol, and the reactivated enzyme is fully sensitive to inhibition by high concentrations of ATP; however, cAMP relieves only 38% of the inhibition by ATP. Modified enzyme containing four groups/tetramer is also reactivated by dithiothreitol but to a somewhat lesser extent; the reactivated enzyme is still very sensitive to inhibition by ATP, but cAMP is not capable of relieving any significant

Table III: Catalytic Activity and Regulatory Kinetic Properties of Native Phosphofructokinase and Modified Phosphofructokinase after Reactivation by Dithiothreitol

enzyme preparation	enzymatic activity relative to native PFK (%)	inhibition by 1.35 mM ATP (%)	inhibition by 1.35 mM ATP + 1.3 mM cAMP (%)	ATP inhibition relieved by cAMP (%)
native PFK	100	99	14	86
reactivated modified PFK (2.13 SO_2Bz-ε-Ado/tetramer)[a]	92	98	61	38
reactivated modified PFK (4.1 SO_2Bz-ε-Ado/tetramer)[b]	64	94	90	4

[a] Phosphofructokinase (0.8 mg/mL) was modified with 0.47 mM 5′-FSO_2Bz-ε-Ado in the absence of protecting ligands for 1 h as described in the footnotes of Table I. The reaction mixture was then made 20 mM in dithiothreitol and incubated 3 h at 22 °C before chromatography on a G-25 column. [b] Same as footnote *a* except modification reaction time was 18 h.

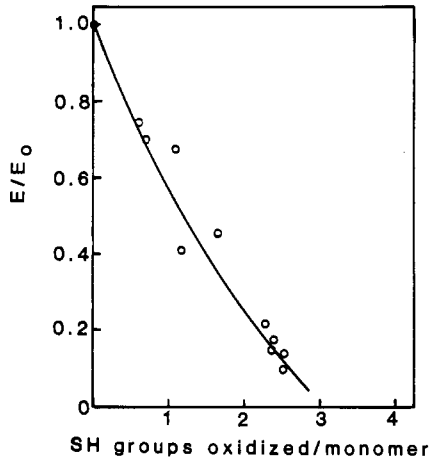


FIGURE 5: Decrease of free sulfhydryl groups in phosphofructokinase as a function of extent of inactivation by 5′-FSO_2Bz-ε-Ado. Phosphofructokinase was inactivated to various degrees by 5′-FSO_2Bz-ε-Ado, and the residual enzymatic activity (E/E_0) and the decrease in the number of free sulfhydryl groups/monomer were determined as described under Materials and Methods.

fraction of the ATP inhibition. These data further support the hypothesis that SO_2Bz-ε-Ado is covalently bound at the cAMP binding site. The covalently bound label does not activate the enzyme, i.e., prevent inhibition by high concentrations of ATP, but it does prevent the binding of cAMP and, hence, activation of the enzyme by cAMP. The dithiothreitol-reactivated enzyme containing four SO_2Bz-ε-Ado groups/tetramer is less stable than is native enzyme or enzyme modified by 5′-FSO_2BzAdo, and it loses 32% of its activity upon storage at 4 °C for 18 h. The inability to achieve total reactivation by dithiothreitol of this modified enzyme may well be a reflection of the increased lability of the enzyme.

The inability of the reactivated modified enzyme to be activated by cAMP is due to the presence of the bound SO_2Bz-ε-Ado group and not the reduction by dithiothreitol. Enzyme modified in the presence of either substrate as a protecting ligand (see Table I) retains most of its catalytic activity and sensitivity to inhibition by ATP but displays a diminished ability to undergo activation by cAMP (only 40% of the inhibition by 1.35 mM ATP is relieved by 1.3 mM cAMP when modified to the extent of 1.66 SO_2Bz-ε-Ado groups/tetramer). On the other hand, enzyme modified in the presence of cAMP as protecting ligand retains most of its catalytic activity, full sensitivity to inhibition by ATP, and full sensitivity to activation by cAMP.

As indicated by the data in Table IV, 3′,5′-cyclic-εAMP even at relatively high concentrations does not activate ATP-inhibited phosphofructokinase. Hence, the inability of covalently bound SO_2Bz-ε-Ado to activate the enzyme is not due to the fact that it is covalently attached but rather to the

Table IV: Effect of cAMP and 3′,5′-Cyclic-εAMP on Inhibition of Phosphofructokinase by ATP

additions to standard assay[a]	enzymatic activity (μmol of F-1,6-P_2 min^{-1} mg^{-1})	inhibition (%)
none	33.47	
ATP (1.2 mM)	0.9	97
ATP (1.2 mM) + cAMP (67 μM)	28.42	15
ATP (1.2 mM) + 3′,5′-cyclic-εAMP (1.33 mM)	1.33	96
ATP (1.2 mM) + cAMP (67 μM) + 3′,5′-cyclic-εAMP (1.33 mM)	24.04	28

[a] Standard assay mixture contains 0.15 mM ATP (see Materials and Methods).

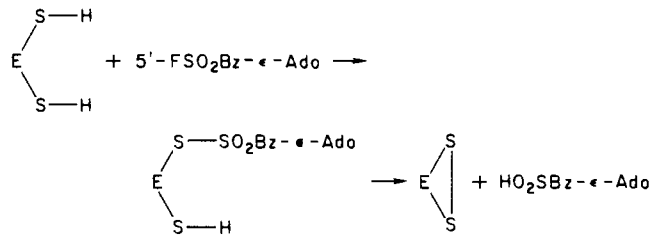
presence of the etheno group. Furthermore, as shown in Table IV, the presence of 3′,5′-cyclic-εAMP in the assay decreases somewhat the effectiveness of cAMP in overcoming the inhibition by ATP, suggesting that 3′,5′-cyclic-εAMP and cAMP compete for the same binding site.

Discussion

The adenine nucleotide analogue, 5′-FSO_2BzAdo, is an affinity label specific for the activating adenine nucleotide binding site on phosphofructokinase, i.e. that site which binds cAMP, AMP, and ADP, and the covalent attachment of the label to this site leads to activation of the enzyme (Pettigrew & Frieden, 1978; Ogilvie, 1983). The results of the present study indicate that the fluorescent nucleotide analogue 5′-FSO_2Bz-ε-Ado is also an affinity label specific for the activating nucleotide binding site of phosphofructokinase; however, the most striking feature of the reaction of 5′-FSO_2Bz-ε-Ado with the enzyme is the rapid inactivation of the enzyme that occurs as the result of a reaction, which appears to be distinct from the reaction that leads to covalent modification of the activating adenine nucleotide binding site. The dependence of the observed pseudo-first-order rate constant for inactivation on the concentration of 5′-FSO_2Bz-ε-Ado suggests that the reagent binds reversibly to the enzyme at this site before irreversibly inactivating the enzyme. Estimated values for the dissociation constant of the enzyme–5′-FSO_2Bz-ε-Ado complex and for the first-order rate constant for the conversion of this complex to inactive enzyme are K_d = 1.11 mM and k_1 = 0.98 min^{-1}. The rate of inactivation of the enzyme is decreased by either MgATP or F-6-P, the two substrates for the enzyme, as well as by cAMP, an allosteric activator of the enzyme.

The other interesting aspect of the inactivation of phosphofructokinase by 5′-FSO_2Bz-ε-Ado is the reactivation of the inactivated enzyme by dithiothreitol. Since (a) dithiothreitol

reactivates the inactive enzyme without altering the number of SO_2Bz-ε-Ado groups covalently bound to the enzyme and (b) the extent of inactivation of the enzyme appears to correlate with the loss of two sulfhydryl groups/monomer and not with the extent of permanent covalent incorporation of SO_2Bz-ε-Ado, a possible mechanism for the inactivation reaction would appear to be disulfide formation between two cysteinyl residues in close proximity at some site on the enzyme, i.e.



where the first step is the formation of a thiol sulfonate and the second step is displacement of a sulfinic acid moiety by the sulfhydryl group of the second cysteine, forming inactive enzyme with a disulfide bond. The latter reaction, the reaction of a thiol sulfonate with a thiol, has been employed to synthesize organic disulfides (Parsons et al., 1965). A similar mechanism has been proposed for the inactivation of rabbit muscle pyruvate kinase by 5′-[*p*-(fluorosulfonyl)benzoyl]-guanosine (Tomich et al., 1981) and by 5′-FSO_2BzAdo (Annamalai & Colman, 1981). Reactivation of the inactive enzyme by dithiothreitol would involve simple reduction of the disulfide bond.

Kemp & Forest (1968) have investigated the reactivity of the 15–16 cysteinyl residues of phosphofructokinase with the nonspecific reagent 5,5′-dithiobis(2-nitrobenzoic acid) and have found that there are two cysteinyl residues/monomer that are very reactive with this reagent and both are protected from reaction by either adenine nucleotides or F-6-P. Furthermore, reaction of these two sulfhydryl groups with the reagent results in the loss of more than 90% of the enzyme activity. The fact that the ligands that protect these two cysteinyl residues against modification by 5,5′-dithiobis(2-nitrobenzoic acid) are the same ligands that protect against inactivation by 5′-FSO_2Bz-ε-Ado and the fact that modification by either reagent results in more than 90% inactivation of the enzyme suggest that these two reagents may modify the same two essential cysteinyl residues. Furthermore, the observations indicating that εATP is a good substrate for phosphofructokinase (Secrist et al., 1972), that FSO_2Bz-ε-Ado, an analogue of ε-ATP, binds reversibly to the enzyme before irreversibly inactivating the enzyme, and that either substrate of the enzyme protects against modification of these cysteinyl residues and inactivation of the enzyme suggest the possibility that these two essential cysteines may be located in or near the active site of the enzyme. It is interesting to note that another kinase, rabbit muscle pyruvate kinase, may also contain two essential active site sulfhydryl groups (Tomich et al., 1981).

In addition to the reaction that leads to inactivation of the enzyme, 5′-FSO_2Bz-ε-Ado also appears to react with the binding site that is specific for the activating adenine nucleotides cAMP, AMP, and ADP. The nature of the modification that occurs at this site is the irreversible covalent attachment of one SO_2Bz-ε-Ado group/site. The reaction that leads to inactivation of the enzyme and the reaction that leads to covalent attachment of the SO_2Bz-ε-Ado group at the activating adenine nucleotide binding site appear to be distinct reactions since the substrates, F-6-P and MgATP, are each very effective at protecting the enzyme against the inactivation reaction but neither affects the extent of covalent modification of the activating adenine nucleotide binding site.

The adenine nucleotide analogues, 5′-FSO_2BzAdo and 5′-FSO_2Bz-ε-Ado, differ only by the presence of a two-carbon etheno bridge between N-1 and 6-NH_2 in the latter, yet the modifications they produce in phosphofructokinase and the effects of these modifications on the regulatory and enzymatic properties of the enzyme differ dramatically. Modification by 5′-FSO_2Bz-ε-Ado leads to an inactivation of the enzyme, which appears to correlate with the loss of two free sulfhydryl groups/monomer, and to the covalent attachment of an SO_2Bz-ε-Ado group at the activating adenine nucleotide binding site. Modification by 5′-FSO_2BzAdo leads only to the covalent attachment of a SO_2BzAdo group at the activating nucleotide binding site. Although both reagents modify the activating adenine nucleotide binding site with groups that are structurally quite similar, only the modified enzyme containing SO_2BzAdo is activated and no longer subject to inhibition by 1.2 mM ATP. The modified enzyme containing the SO_2Bz-ε-Ado group at this site is still fully sensitive to inhibition by 1.2 mM ATP, hence not activated, and, furthermore, cannot be activated by cAMP. Thus, the presence of the etheno group does not prevent 5′-FSO_2Bz-ε-Ado from specifically labeling the allosteric site, which implies that it binds to this site noncovalently before reacting, but it does prevent the bound label from inducing or stabilizing the precise conformational change that leads to activation of the enzyme. Hence, the specificity of the allosteric activating adenine nucleotide binding site reflects not only specificity in binding but also the ability of the ligand once bound to bring about the precise conformational change required for activation. A similar conclusion has been reached by Gottschalk & Kemp (1981), who found that adenosine diphosphoribose (K_d = 5.1 μM) and cAMP (K_d = 1.3 μM) bind competitively to phosphofructokinase but only cAMP activates the enzyme.

The inability of 5′-FSO_2Bz-ε-Ado to induce or stabilize the same conformation as that induced by cAMP and 5′-FSO_2BzAdo may also explain why this reagent inactivates the enzyme whereas 5′-FSO_2BzAdo does not. The data indicate that prelabeling the cAMP binding site with 5′-FSO_2BzAdo not only activates the enzyme but also totally protects the enzyme against inactivation by 5′-FSO_2Bz-ε-Ado. Hence, in the modification of the enzyme by 5′-FSO_2BzAdo, the binding and covalent attachment of the reagent at the cAMP binding site could also protect the enzyme against inactivation by 5′-FSO_2BzAdo just as it protects against inactivation of the enzyme by 5′-FSO_2Bz-ε-Ado. In the modification of the enzyme by 5′-FSO_2Bz-ε-Ado, the binding and covalent attachment of this reagent to the cAMP binding site does not produce the same conformational change as cAMP or 5′-FSO_2BzAdo; thus the conformational change produced may not be totally effective in blocking the inactivation of the enzyme by the reagent. This would also provide a plausible explanation for the nonlinearity observed in the first-order plots for the inactivation of the enzyme by 5′-FSO_2Bz-ε-Ado. This nonlinearity, which appeared to be due to a gradual change in the reactivity of the enzyme with time of reaction rather than to reagent depletion, could be a reflection of a decreased reactivity of the enzyme with the reagent at the site of inactivation as a consequence of covalent modification of the enzyme by the reagent at the cAMP binding site. Consistent with this hypothesis is an observation we have made indicating that, after reactivation with dithiothreitol, the affinity-labeled enzyme containing covalently bound SO_2Bz-ε-Ado groups at the cAMP binding site can be slowly inactivated again with either

FSO_2Bz-ϵ-Ado or FSO_2BzAdo and that this inactivation can again be reversed with dithiothreitol.

Craig & Hammes (1980) have reported that another adenine nucleotide analogue containing an etheno group, 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine, also specifically labels the cAMP binding sites of rabbit muscle phosphofructokinase. An inactivation of only 23% was observed with this reagent; however, the authors quenched the reaction with 0.5 mM dithiothreitol and then dialyzed the modified enzyme against a buffer containing 1 mM dithiothreitol, a procedure that may have partially reactivated the modified enzyme. Whether or not the covalent attachment of this affinity label to the cAMP binding sites relieved the ATP inhibition of the enzyme at pH 7.0 was not stated by the authors.

The two affinity-labeling analogues 5′-FSO_2BzAdo and 5′-FSO_2Bz-ϵ-Ado also differ in their interaction with pyruvate kinase (Likos & Colman, 1981). Just how many enzymes will display a difference in their interaction with these two affinity-labeling analogues is not known, but with those that do, utilization of both analogues could potentially be very useful in the elucidation of site–site interactions, the steric requirements for binding and induction of conformational changes, and the identity of essential and nonessential amino acid residues at the binding sites. Experiments are now in progress in this laboratory to determine the locations of the critical sulfhydryl groups whose oxidation appears to correlate with the inactivation of phosphofructokinase and to identify the amino acid residue or residues at the cAMP binding site that undergo covalent modification by these two affinity-labeling analogues.

Acknowledgments

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Registry No. PFK, 9001-80-3; 5′-FSO_2Bz-ϵ-Ado, 76021-83-5; F-6-P, 643-13-0; 5′-FSO_2BzAdo, 57454-44-1; cAMP, 60-92-4; MgATP, 1476-84-2; 3′,5′-cyclic-ϵAMP, 38806-37-0; dithiothreitol, 3483-12-3.

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