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Affinity Labeling of Rabbit Muscle Pyruvate Kinase by a New Fluorescent Nucleotide Alkylating Agent 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine†

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ABSTRACT: This paper describes the synthesis and characterization of a new fluorescent nucleotide analogue, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine, which is capable of reacting covalently with nucleophilic groups in proteins. This nucleotide analogue, with a fluorescence emission maximum at 412 nm, functions as an active site directed irreversible inhibitor of rabbit muscle pyruvate kinase. The inactivation follows pseudo-first-order kinetics at pH 8.0. A plot of the rate constant for inactivation vs. the analogue concentration yields hyperbolic kinetics, indicative of reversible binding of the analogue prior to covalent reaction. Significant protection is afforded by phosphoenolpyruvate, while MgATP, MgADP, and Mg alone decrease the rate of inactivation but not to the same extent as does phosphoenolpyruvate. The metal-free nucleotides ADP or ATP as well as pyruvate have no effect on the rate of inactivation. The incorporation of 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine into pyruvate kinase was measured from the fluorescence of modified enzyme. A total of 2 mol of reagent is incorporated/mol of enzyme subunit when the enzyme is 100% inactivated. However, if the modified enzyme is treated with dithiothreitol or β-mercaptoethylamine, only 1 mol of reagent is found incorporated/enzyme subunit. Concomitant with this decrease in incorporation upon treatment of the modified enzyme with thiols is a reactivation that is proportional to the loss of incorporated reagent. Thus, there are two sites of modification per enzyme subunit: reaction of 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine at one site leads to the formation of inactive enzyme which can be reactivated by the addition of dithiothreitol or β-mercaptoethylamine, whereas reaction at the second site has no effect on enzyme activity and under the conditions used is not reversible by dithiothreitol. The reagent incorporated cannot be attributed to reaction at tyrosine or lysine residues. The reversibility by thiols of modification at the site responsible for inactivation suggests that the amino acid residue modified may be a cysteine. These studies indicate that 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine may provide a means of introducing a covalently bound fluorescent probe at nucleotide sites in other proteins.

Affinity labeling using nucleotide analogues with reactive functional groups has proved to be an effective approach to exploring the nucleotide binding sites of proteins (Colman et al., 1977; Hampton et al., 1977; Gulyaev et al., 1976). Among the most generally applicable of the nucleotide alkylating agents is 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, which labels specifically nucleotide binding sites of glutamate dehydrogenase (Pal et al., 1975), rabbit muscle and yeast pyruvate kinases (Wyatt & Colman, 1977; Likos et al., 1980), phosphofructokinase (Mansour & Colman, 1978; Pettigrew & Frieden, 1978; Weng et al., 1980), mitochondrial F_1 ATPase[1] (Esch & Allison, 1978; DiPietro et al., 1979), chloroplast ATPase (DeBenedetti & Jagendorf, 1979), cAMP-dependent protein kinase (Zoller & Taylor, 1979; Hixson & Krebs, 1979), malate dehydrogenase (Roy & Colman, 1979), glutamine synthetase (Foster & Kingdon, 1980), and an ADP receptor protein of platelets (Bennett et al., 1978; Mills et al., 1980). Fluorescent analogues of natural biochemical compounds as well as fluorescent labeling agents have been valuable in probing the environment of binding sites in proteins and in elucidating distances between defined site markers on proteins by energy transfer (Brand & Witholt, 1967; Horton & Koshland, 1967; Stryer, 1978). A fluorescent nucleotide alkylating agent should thus provide an effective tool for introducing into a defined nucleotide binding site a covalent fluorescent probe which can then be used to examine the properties of that site and its interactions with other sites of the protein. In order to interpret this type of information, it is necessary first to characterize the reaction of the analogue with a particular protein. This paper describes the synthesis and structure determination of the new fluorescent nucleotide alkylating agent, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. Rabbit muscle pyruvate kinase has been shown to utilize 1,N^6-ethenoadenosine diphosphate as a substitute for ADP in its catalytic reaction (Secrist et al., 1972; Barrio et

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[1] Abbreviations used: 5′-*p*-FSO_2BzεAdo, 5′-[*p*-(fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine; CBS-Lys, N^{ϵ}-(4-carboxybenzenesulfonyl)lysine; CBS-Tyr, *O*-(4-carboxybenzenesulfonyl)tyrosine; 5′-*p*-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; 3′-*p*-FSO_2BzAdo, 3′-[*p*-(fluorosulfonyl)benzoyl]adenosine; ATPase, adenosine 5′-triphosphatase; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide, NADH, reduced NAD; Tris, tris(hydroxymethyl)aminomethane; AMP, adenosine 5′-phosphate.

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al., 1973), and the covalent reaction of pyruvate kinase with 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine is here explored. A preliminary account of this work has been presented (Colman, 1980).

Experimental Procedures

Materials. Rabbit muscle pyruvate kinase was purchased from Boehringer-Mannheim as an ammonium sulfate suspension. The enzyme (5–25 mg/mL) was dialyzed at 4 °C against 10 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM EDTA and was stored at 4 °C. The specific activity was 250–330 units/mg. The enzyme concentration was determined by using $E_{280}^{1\%} = 5.4$ (Bücher & Pfleiderer, 1955) and a molecular weight of 57 000 per subunit (Cottam et al., 1969). The nucleotides ADP and ATP as well as phosphoenolpyruvate, pyruvate, dithiothreitol, and β-mercaptoethylamine were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Determination of Enzyme Activity. Enzyme activity was measured by means of a coupled assay with lactate dehydrogenase being used to convert to lactate the pyruvate generated by pyruvate kinase from phosphoenolpyruvate and ADP. A Gilford Model 240 spectophotometer equipped with a recorder with an expanded scale (0–0.1 absorbance, full scale) was used to monitor at 340 nm the conversion of NADH to NAD concomitant with the reduction of pyruvate to lactate. Enzyme activity was measured 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.

Synthesis of 5′-[p-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. Ethenoadenosine hydrochloride was prepared by reaction of chloroacetaldehyde with adenosine according to the procedure of Secrist et al. (1972). To 100 mg (0.31 mmol) of ethenoadenosine dissolved in 0.7 mL of hexamethylphosphoramide with warming to 50 °C was added 95 mg (0.43 mmol) of *p*-(fluorosulfonyl)benzoyl chloride in small amounts over a 5-min period with continual stirring. After reacting 30 min at room temperature, an additional 50 mg (0.23 mmol) of *p*-(fluorosulfonyl)benzoyl chloride were slowly added. The reaction was monitored by thin-layer chromatography on 200-μm silica gel plates (EM Reagents) with methyl ethyl ketone–acetone–water (65:20:15) as the solvent. After 2.5 h total the reaction was judged to be complete as indicated by the disappearance of the dark blue fluorescent spot due to ethenoadenosine. The reaction mixture was extracted twice with 2 mL of petroleum ether. To the resulting oil was slowly added 3 mL of a 1:1 mixture of diethyl ether and ethyl acetate which resulted in the formation of a fine white precipitate. The product was collected by filtration and dried under vacuum. Typical yields were 140–150 mg of material. Analysis of the material by thin-layer chromatography on 200-μm silica gel plates with methyl ethyl ketone–acetone–water (65:20:15) as solvent indicated two yellowish fluorescent compounds. The majority of the material migrated with an average R_f equal to 0.56 and was subsequently identified as 5′-*p*-FSO_2BzεAdo[1] (see Results). The minor component had an average R_f of 0.76 and is presumably the 3′ derivative or the 3′,5′-disubstituted compound. In other preparations a small amount of unreacted ethenoadenosine ($R_f = 0.29$) was also found. The product is conveniently purified by preparative thin-layer chromatography on 500-μm silica gel plates (Analtech Inc.) with methyl ethyl ketone–acetone–water (65:20:15) as solvent. Approximately 25 mg of impure material was applied. After development, the 5′-*p*-FSO_2BzεAdo was extracted with 95% ethanol. Alternatively, the product can be crystallized from hexamethylphosphoramide in the following manner. A total of 100 mg of material are dissolved with warming in 1 mL of hexamethylphosphoramide followed by addition of ~1 mL of a 1:1 mixture of diethyl ether and ethyl acetate to the cloud point. After standing overnight at 40 °C, the white precipitate was collected by centrifugation and the material dried under vacuum. After two such crystallizations, the material was pure as judged by thin-layer chromatography and high-pressure liquid chromatography. High-pressure liquid chromatography was performed on a Varian Model 5000 liquid chromatography employing Varian MCH-10 resin (4 × 300 mm) at 30 °C. The solvent used for elution was 50% methanol–50% water. The flow rate was 2 mL/min. The 5′-*p*-FSO_2BzεAdo exhibited a retention time of 6.4 min under these conditions and was well separated from ethenoadenosine (1.6 min).

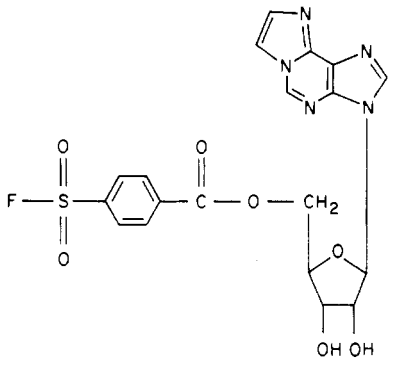

FIGURE 1: Structure of 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine.

Elemental analysis was determined of the material purified by crystallization. Anal. Calcd for $C_{25}H_{35}N_8O_8FSPCl$: C, 43.32; H, 5.05; N, 16.17; Cl 5.13; F, 2.74. Found: C, 43.24; H, 5.52; N, 16.13; Cl, 4.60; F, 2.57. The fluoride analysis, measured on a Beckman pH meter equipped with a fluoride-specific electrode (Orion), was determined on material that had been hydrolyzed at pH 13 for 2 h on a boiling water bath. The elemental analysis is consistent with the hydrochloride salt of the structure shown in Figure 1 and indicates that the material as isolated contained 1 mol of hexamethylphosphoramide/mol of compound. The molecular weight is taken as 692.5.

Reaction of 5′-p-FSO_2BzεAdo with Rabbit Muscle Pyruvate Kinase. Pyruvate kinase (0.48–2.46 mg/mL) was incubated with 5′-*p*-FSO_2BzεAdo at 30 °C in 20 mM potassium barbital, pH 8.0, containing 200 mM KCl and 15% dimethylformamide. During the course of the reaction 2-μL aliquots were withdrawn at given time intervals, diluted with 200 μL of 10 mM potassium phosphate, pH 7.5, and the activity of 2 μL of the diluted enzyme was determined as described above. Solutions of 5′-*p*-FSO_2BzεAdo were prepared by dissolving the reagent in 100% dimethylformamide, and concentrations were determined from the absorbance at 275 nm ($\epsilon_{275} = 7632\ M^{-1}\ cm^{-1}$, in ethanol). A control consisted of incubating enzyme under identical conditions (i.e., containing 15% dimethylformamide) but in the absence of 5′-*p*-FSO_2BzεAdo. The rate of reaction of pyruvate kinase with 5′-*p*-FSO_2BzεAdo 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, calculated from a least-squares analysis of the data for each experiment, were corrected for any loss in activity of the control.

Incorporation of 5′-p-FSO_2BzεAdo by Rabbit Muscle Py-

ruvate Kinase. For determination of the stoichiometry of the reaction of pyruvate kinase with 5′-*p*-FSO_2BzεAdo, the enzyme (1.3–2.3 mg/mL) was incubated with the reagent at 30 °C under the conditions described above. At a given extent of inactivation, either solid urea and dithiothreitol was added simultaneously or in other experiments, urea was added alone. The amounts of dithiothreitol and urea that were added were calculated on the basis of the volume of the reaction mixture; thus, the concentrations of urea (6 M) and dithiothreitol (20 mM) do not reflect the increase in volume due to the added urea. The reaction mixture (0.85–1.2 mg of protein) was immediately applied to a Sephadex G-25 column (0.9 × 21.5 cm) equilibrated with 50 mM potassium phosphate, pH 7.0, containing 6 M urea. This procedure completely separated the modified enzyme from the unreacted 5′-*p*-FSO_2BzεAdo. Protein concentration of modified enzyme was determined by using the Bio-Rad Protein Assay which is based on the method of Bradford (1976). Standard solutions were prepared by dilution of native pyruvate kinase with 50 mM potassium phosphate, pH 7.0, containing 6 M urea.

The amount of 5′-*p*-FSO_2BzεAdo bound to the protein was determined from the fluorescence intensity of the modified pyruvate kinase by using a thermostated Hitachi Perkin-Elmer MPF-3 spectrofluorometer. The fluorescence intensity of the modified enzyme was measured at 405 nm with an exciting wavelength of 310 nm. A blank consisted of unmodified pyruvate kinase at the same protein concentration and in the same buffer as the modified enzyme. The fluorescence of the modified enzyme, corrected for its blank, was compared to the fluorescence of 5′-*p*-FSO_2BzεAdo standards measured under identical conditions and corrected for buffer fluorescence and/or scattering. All measurements were made at 25 °C.

Alternatively, the incorporation of reagent was also measured by raising the pH of 2 mL of the modified enzyme to 13 with 5 μL of 12.5 N NaOH. Upon such treatment, a rapid increase in fluorescence intensity was observed followed by a slow loss of fluorescence (see Results). Extrapolation of the data to zero time was used as the maximum value for the increase in fluorescence and was compared to the extrapolated values for 5′-*p*-FSO_2BzεAdo standard solutions treated in an identical manner. This procedure proved to be more sensitive and gave results comparable to the procedure described above.

Reactivation of Modified Pyruvate Kinase by Dithiothreitol and β-Mercaptoethylamine. For the reactivation of modified enzyme, stock solutions of dithiothreitol or β-mercaptoethylamine in 20 mM potassium barbital, pH 8.0, containing 200 mM KCl were prepared just before use. At a given extent of inactivation of pyruvate kinase with 5′-*p*-FSO_2BzεAdo, dithiothreitol or β-mercaptoethylamine was added to a final concentration of 100 mM to both the experimental and the control, and enzyme activity was determined as described above. The reactivation was followed for 20 min. After 20 min of reaction with dithiothreitol or β-mercaptoethylamine, solid urea was added, the sample was applied to Sephadex G-25 equilibrated with 50 mM potassium phosphate, pH 7.0, containing 6 M urea, and the incorporation was determined as described above.

Identification of the Reactive Amino Acid Residues. For identification of the amino acid residues that reacted with 5′-*p*-FSO_2BzεAdo, pyruvate kinase was inactivated to an extent of 80% with 0.8 mM 5′-*p*-FSO_2BzεAdo. The reaction was stopped by the simultaneous addition of urea and dithiothreitol as described above. The modified enzyme was dialyzed extensively against water for 24 h and then taken to dryness under vacuum. After hydrolysis with 6 N HCl for 20 h at 107 °C, the sample was analyzed either by application to a Beckman Model 120C amino acid analyzer or thin-layer electrophoresis. The standard two-column procedure used on the Model 120C amino acid analyzer was converted to a one-column run using the 55-cm column (AA15 ion-exchange resin) and the following buffer change schedule: buffer was 0.2 N sodium citrate, pH 3.25, initially; at 100 min, buffer was changed to 0.2 N sodium citrate, pH 4.23; finally, buffer was changed to 0.2 N sodium citrate, pH 6.4, containing 0.8 N NaCl from 130 to 300 min. The column temperature was 55.5 °C. The elution times of the acidic and neutral amino acids with the exception of tyrosine and phenylalanine were unaffected by this modification (e.g., glutamate was eluted at 67.5 min and leucine at 158 min). Tyrosine eluted at 171 min, phenylalanine at 176 min, histidine at 201 min, lysine at 209 min, ammonia at 227 min, and arginine at 289 min. The amino acid derivatives N^{ϵ}-(4-carboxybenzenesulfonyl)-lysine and *O*-(4-carboxybenzenesulfonyl)tyrosine, which are the expected products for acid-hydrolyzed enzyme modified at either lysine or tyrosine by 5′-*p*-FSO_2BzεAdo, eluted at 168 and 182 min, respectively. The synthesis of these derivatives has been previously described (Esch & Allison, 1978; Likos et al., 1980).

Thin-layer electrophoresis was performed by using cellulose thin-layer plates (Eastman) with pyridine acetate, pH 6.4 (pyridine–acetic acid–water, 25:1:250), for 90 min at 400 V/20 cm. After the plate was dried, a strip was sprayed with ninhydrin and compared to an electrophoretogram of amino acid standards plus CBS-Lys and CBS-Tyr. The region of the electrophoretogram corresponding to CBS-Lys and CBS-Tyr was scraped off, eluted with water, dried, dissolved in 0.2 N sodium citrate, pH 2.2, and applied to the amino acid analyzer. A standard solution of amino acids containing known amounts of CBS-Lys and CBS-Tyr was treated in an identical manner, and a recovery of ~57% for both CBS-Lys and CBS-Tyr was estimated.

Since cysteine and possibly serine appeared to be likely candidates for modification by 5′-*p*-FSO_2BzεAdo yielding the thiosulfonate derivative of cysteine and the serine sulfonate ester, respectively, modified pyruvate kinase was reacted with 1.8 M β-mercaptoethylamine in 8 M urea, pH 8, for 5 h at 40 °C (Gold, 1965). After adjustment of the pH to 3, the samples were dialyzed against 1 mM HCl and then hydrolyzed with 6 N HCl at 107 °C for 17 h. The hydrolyzed samples were then applied to the 15-cm column (AA15 ion-exchange resin) of the amino acid analyzer and eluted with 0.2 N sodium citrate, pH 6.4. The column temperature was 55.5 °C. Under these conditions histidine eluted at 30.5 min, lysine at 71 min, ammonia at 102 min, and arginine at 158 min. The expected products after reaction of β-mercaptoethylamine with the modified protein followed by acid hydrolysis are the mixed disulfide cysteine–cysteamine for initial reaction of 5′-*p*-FSO_2BzεAdo with cysteine (Parsons et al., 1965) and aminoethylcysteine for initial reaction at a serine residue (Gold, 1965). These derivatives eluted at 114 and 77 min, respectively. There was sufficient resolution between lysine and aminoethylcysteine that at a ratio of 750 nmol of lysine to 10 nmol of aminoethylcysteine, the aminoethylcysteine could be quantitated. The mixed disulfide, cysteine–cysteamine, was synthesized according to the procedure of Purdie (1971).

Results

Characterization of 5′-[p-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. The effect of pH on the UV spectrum of 5′-*p*-FSO_2BzεAdo is shown in Figure 2A. Spectrophotometric titration of 5′-*p*-FSO_2BzεAdo indicated that the change in absorbance as a function of pH was governed by a p*K* of

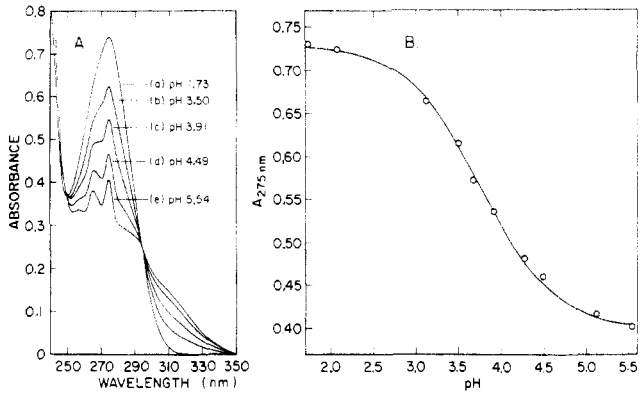

FIGURE 2: Effect of pH on the ultraviolet absorption spectrum of 5′-*p*-FSO_2BzεAdo. (A) Spectra of 5′-*p*-FSO_2BzεAdo (6.47×10^{-5} M) were measured in 0.8 M sodium formate (pH 1.73–3.50) or 0.8 M sodium acetate (pH 3.67–5.54). (B) The points indicated for absorbance at 275 nm at each given pH are experimental, and the curve is a theoretical titration curve calculated for a p*K* of 3.75.

3.75 (Figure 2B). The extinction coefficient at 275 nm was determined to be 6211 M^{-1} cm^{-1} in aqueous buffer, pH 5.5, which compares closely to the extinction for ethenoadenosine. In ethanol (95%) the extinction coefficient is 7632 M^{-1} cm^{-1}. The compound exhibits a fluorescence emission maximum at 412 nm (310-nm excitation) and a maximum at 308 nm in the excitation spectrum. Both the UV and fluorescence spectra are similar to those of ethenoadenosine (Secrist et al., 1972). The quantum yield of 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine in 25 mM potassium phosphate buffer, pH 7.0, was determined to be 0.008 relative to quinine sulfate in 0.1 N H_2SO_4 for which a quantum yield of 0.7 has been determined (Scott et al., 1970).

The NMR spectra were obtained in dimethyl-d_6 sulfoxide by using either a Bruker HFX-90 spectrometer (^{13}C spectrum) or a Perkin-Elmer R-12 (60-MHz) spectrometer (^1H spectrum). The proton NMR spectrum of 5′-*p*-FSO_2BzεAdo had peaks centered at δ = 4.75 (m, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$ of ribose), 6.25 (d, $H_{1'}$ of ribose), 8.13 (d, 1, C_8-H), 8.33 (s, 4, aromatic), 8.55 (d, 1, C_7-H), 8.95 (s, 1, C_2-H), and 9.67 (s, 1, C_5-H). The numbering of the ethenoadenosine ring is in accordance with Secrist et al. (1972). The spectrum was consistent with the structure shown in Figure 1. The proton-decoupled ^{13}C NMR spectrum of the compound had signals arising from the ribose moiety centered at 65.2 ($C_{5'}$), 70.0 ($C_{2'}$), 73.3 ($C_{3'}$), 81.7 ($C_{4'}$), and 88.4 ($C_{1'}$) ppm. For comparison, ethenoadenosine had peaks centered at 60.7 ($C_{5'}$), 70.0 ($C_{2'}$), 74.4 ($C_{3'}$), 85.7 ($C_{4'}$), and 88.1 ($C_{1'}$) ppm. The differences in the chemical shifts of ribose $C_{5'}$ and $C_{4'}$ of 5′-*p*-FSO_2BzεAdo relative to those in ethenoadenosine parallel the differences seen between 5′-AMP and adenosine (Schleich et al., 1975), suggesting that the *p*-(fluorosulfonyl)benzoyl substituent is indeed at the 5′ position of the ribose ring. The remainder of the ^{13}C NMR spectrum of 5′-*p*-FSO_2BzεAdo had peaks centered at 114.3, 119.7, 123.4, 128.7, 130.7, 135.8, 137.2, 141.8, and 163.7 ppm. For comparison, ethenoadenosine had peaks centered at 114.0, 119.2, 123.0, 137.1, and 142.2 ppm. The 163.7-ppm signal is due to the carbonyl carbon, and the remaining signals that are not found in the ethenoadenosine spectrum presumably are due to the aromatic carbons.

Further evidence supporting the assignment of the (fluorosulfonyl)benzoyl moiety on the 5′ position of the ribose ring was obtained by comparison of the average R_f values of 5′-*p*-FSO_2BzAdo, 3′-*p*-FSO_2BzAdo, and 5′-*p*-FSO_2BzεAdo when they were chromatographed on silica gel and borate-treated silica gel plates with methyl ethyl ketone–acetone–water

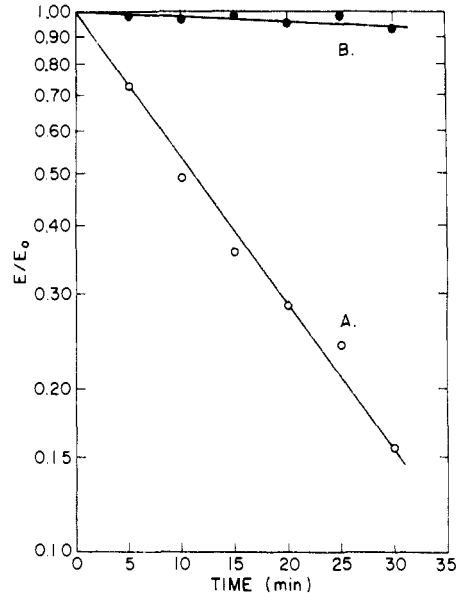

FIGURE 3: Reaction of 5′-*p*-FSO_2BzεAdo with rabbit muscle pyruvate kinase. (A) Pyruvate kinase (0.48 mg/mL) was incubated with 5′-*p*-FSO_2BzεAdo (4.38 mM) at 30 °C in 20 mM potassium barbital buffer, pH 8.0, containing 200 mM KCl and 15% dimethylformamide. At the indicated times a 5-μL aliquot was withdrawn and assayed for activity as described under Experimental Procedures. (B) As a control, pyruvate kinase was incubated under identical conditions in the absence of 5′-*p*-FSO_2BzεAdo.

(75:70:5) as solvent. On silica gel the following R_f values were found: 5′-*p*-FSO_2BzAdo, 0.49; 3′-*p*-FSO_2BzAdo, 0.57; 5′-*p*-FSO_2BzεAdo, 0.36. The borate-treated plate was prepared by evenly spraying a 20 cm × 20 cm × 200 μm silica gel 60 plate (Brinkman) with 40 mL of a 2.5% (w/v) boric acid–ethanol solution. On this borate-treated silica gel plate the following R_f values were found: 5′-*p*-FSO_2BzAdo, 0.15; 3′-*p*-FSO_2BzAdo, 0.60; 5′-*p*-FSO_2BzεAdo, 0.11. Thus, as expected for those compounds containing the cis 2′-OH–3′-OH structural element, both 5′-*p*-FSO_2BzAdo and 5′-*p*-FSO_2BzεAdo were retarded on the borate-treated plate relative to 3′-p-FSO_2BzAdo. All of the analytical and spectral data are thus consistent with the structure of 5′-*p*-(fluorosulfonyl)benzoyl-1,N^6-ethenoadenosine shown in Figure 1.

Inactivation of Muscle Pyruvate Kinase by 5′-[p-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. Muscle pyruvate kinase is inactivated upon incubation with 4.38 mM 5′-*p*-FSO_2BzεAdo at 30 °C in 20 mM potassium barbital, pH 8.0 (Figure 3, line A). As a control, enzyme was incubated under identical conditions but without added reagent (Figure 3, line B). All rate constants are corrected for the small loss activity of the control. Thus, under these conditions the pseudo-first-order rate constant for inactivation is 0.06 min^{-1}.

The dependence of the observed rate constant for inactivation on the 5′-*p*-FSO_2BzεAdo concentration (Figure 4) indicated that the analogue reversibly binds to the enzyme prior to irreversible inactivation, thus suggesting the minimal kinetic mechanism

$$E + 5'\text{-}p\text{-}FSO_2Bz\epsilon Ado \overset{K}{\rightleftharpoons} [E\cdot 5'\text{-}p\text{-}FSO_2Bz\epsilon Ado] \xrightarrow{k} E' + F^- + H^+$$

where E′ is the modified and inactivated enzyme species. A value of K = 1.67 mM was estimated for the enzyme–reagent complex with k = 0.078 min^{-1}.

The effects of added substrates on the rate of inactivation of pyruvate kinase by 5′-*p*-FSO_2BzεAdo are shown in Table I. All ligands are present in concentrations high relative to

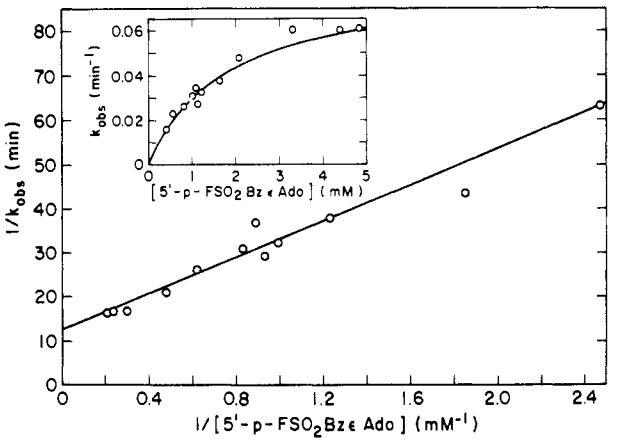

FIGURE 4: Dependence of pseudo-first-order rate constants for inactivation upon concentration of 5′-*p*-FSO_2BzεAdo. Pyruvate kinase (0.48 mg/mL) was incubated with varying concentrations of 5′-*p*-FSO_2BzεAdo at 30 °C in 20 mM potassium barbital buffer, pH 8.0, containing 200 mM KCl and 15% dimethylformamide. The rate constants were calculated as described under Experimental Procedures. The 5′-*p*-FSOBzεAdo concentrations were determined by measurement of the 275-nm absorbance.

Table I: Effect of Substrates on the Inactivation of Pyruvate Kinase by 5′-*p*-FSO_2 BzεAdo[a]

additions to reaction mixture	k_{obsd} (min^{-1})
none	0.035
phosphoenolpyruvate (1 mM)	0.004
pyruvate (10 mM)	0.032
ADP (10 mM)	0.041
ATP (10 mM)	0.034
Mg^{2+} (10 mM)	0.016
ADP (10 mM) + Mg^{2+} (5 mM)	0.013
ADP (10 mM) + Mg^{2+} (10 mM)	0.010
ADP (10 mM) + Mg^{2+} (20 mM)	0.007
ATP (10 mM) + Mg^{2+} (5 mM)	0.035
ATP (10 mM) + Mg^{2+} (10 mM)	0.016
ATP (10 mM) + Mg^{2+} (20 mM)	0.011

[a] Pyruvate kinase (0.48 mg/mL) was incubated with 5′-*p*-FSO_2-BzεAdo (1.08 mM) at 30 °C in 20 mM potassium barbital, pH 8.0, containing 200 mM KCl and 15% dimethylformamide.

their K_m values. Significant protection is provided by phosphoenolpyruvate. In contrast, pyruvate as well as the metal-free nucleotides, ADP and ATP, has no effect on the rate of inactivation, while free Mg^{2+} at 10 mM affords an ~2-fold reduction in the rate of inactivation. ADP plus Mg^{2+} protect the enzyme, although not as well as does phosphoenolpyruvate. At the concentrations of total ADP and Mg^{2+} of 10 mM and 5 mM, respectively, the concentration of MgADP is 4.43 mM while the level of free Mg^{2+} is only 0.57 mM. It is evident that the protection observed under these conditions must result from the binding of MgADP since the protective effect of free Mg^{2+} requires a much higher concentration (10 mM). At a constant total concentration of 10 mM ADP, as the total Mg^{2+} concentration is raised to 10 mM (7.66 mM MgADP; 2.34 mM free Mg^{2+}) and 20 mM (9.37 mM MgADP; 10.6 mM free Mg^{2+}), it is not clear whether the MgADP complex is solely responsible for protection or whether MgADP and the free Mg^{2+} are protecting in concert. In the case of ATP plus Mg^{2+} it is apparent that at 5 mM total Mg^{2+} (4.91 mM MgATP; 0.09 mM free Mg^{2+}) there is no protection by the MgATP complex, although at 10 mM total Mg^{2+} (9.05 mM MgATP; 0.95 mM free Mg^{2+}) a significant decrease in the rate of inactivation is observed. It seems likely that this protection is due to MgATP and that at the lowest total Mg^{2+} concentration the amount of MgATP is below its binding

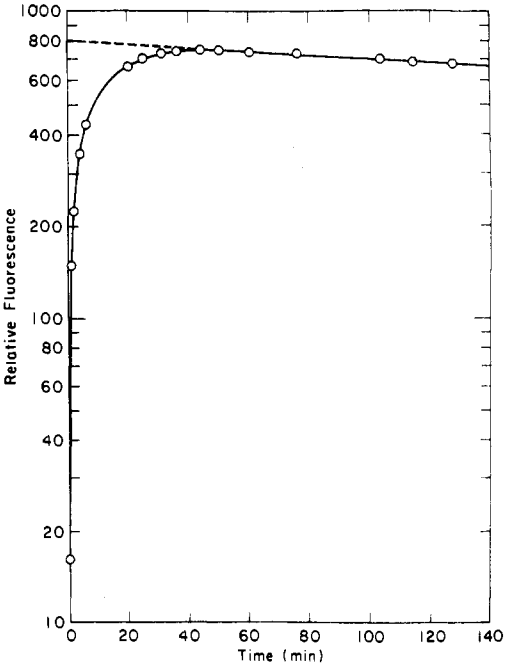

FIGURE 5: Time course for increase in fluorescence of modified pyruvate kinase upon base hydrolysis. The increase in the selective fluorescence of modified pyruvate kinase (4.5 μM) in 50 mM potassium phosphate buffer, pH 7.0, containing 6 M urea was measured as a function of time upon raising of the pH to 12.5 with NaOH. The value obtained upon extrapolation of the data to zero time was compared to values obtained for standard solutions of 5′-*p*-FSO_2BzεAdo treated in a identical manner.

constant. The further reduction in the inactivation rate observed when the total Mg^{2+} concentration is raised to 20 mM (9.90 mM MgATP; 10.1 mM free Mg^{2+}) is presumably attributable to the enhanced level of free metal ion.

Stoichiometry of Reaction of Muscle Pyruvate Kinase with 5′-p-FSO₂BzεAdo. The stoichiometry of the reaction indicates that the reagent reacts covalently and in a limited manner with pyruvate kinase. The incorporation of 5′-*p*-FSO_2BzεAdo per mole of subunit was determined as described under Experimental Procedures. The amount of reagent covalently bound to the protein was determined either by comparison of the fluorescence of the modified enzyme, after correction for intrinsic protein fluorescence, to 5′-*p*-FSO_2BzεAdo standards or by treating the modified protein with NaOH and comparing the fluorescence to 5′-*p*-FSO_2BzεAdo standards treated in an identical manner. Upon raising of the pH of the modified pyruvate kinase to 13, a rapid increase in fluorescence was observed (Figure 5) followed by a slow decrease. Since the low quantum yield of 5′-*p*-FSO_2BzεAdo is presumably due to interaction between the ethenoadenosine and the *p*-(fluorosulfonyl)benzoyl moieties, the increase in fluorescence can be attributed to hydrolysis of the ester linkage between these two groups which decreases the ring interactions. The slow loss of fluorescence is due to hydrolysis of ethenoadenosine itself, since ethenoadenosine when treated with base loses fluorescence at the same rate. The extrapolated fluorescence of base-treated 5′-*p*-FSO_2BzεAdo standards was used to determine the amount of ethenoadenosine produced from base-hydrolyzed modified pyruvate kinase.[2] Both procedures for

[2] The fluorescence value obtained upon extrapolation to zero time was typically 15–20% less than that obtained for ethenoadenosine at the same concentration. For this reason the extrapolated fluorescence of base-treated 5′-*p*-FSO_2BzεAdo standards were used to determine the amount of ethenoadenosine produced from base-hydrolyzed modified pyruvate kinase.

Table II: Effect of Addition of Dithiothreitol or β-Mercaptoethylamine on Inactivated Pyruvate Kinase and the Extent of Incorporation

(1) before addition		(2) after addition		difference: (1) − (2)		incorpn calcd for 100% inactivated and totally reactivated enzyme[e]
inactn (%)[a]	incorpn (mol of 5′-*p*-SO_2BzεAdo/mol of subunit)[b]	inactn (%)	incorpn (mol of 5′-*p*-SO_2BzεAdo/mol of subunit)	act. regained (%)	Δincorpn (mol of 5′-*p*-SO_2BzεAdo lost/mol of subunit)	
21	0.41	5[c]	0.27[c]	16	0.14	0.88
50	0.99	6[c]	0.59[c]	44	0.40	0.91
55	1.08	5[d]	0.58[d]	50	0.50	1.00
66	1.30	6[c]	0.74[c]	60	0.56	0.93
						av: 0.93

[a] Pyruvate kinase (2.3 mg/mL) was incubated with 5′-*p*-FSO_2BzεAdo (0.81 mM) at 30 °C in 20 mm potassium barbital, pH 8.0, containing 200 mM KCl and 15% dimethylformamide. The extent of inactivation was measured 1 min prior to addition of dithiothreitol or β-mercaptoethylamine. [b] Determined from line B of Figure 7. [c,d] Dithiothreitol (*c*) or β-mercaptoethylamine (*d*) was added to both the reaction mixture and the control at a given extent of inactivation to a final concentration of 100 mM. The regain in activity was followed, and the percent inactivation at the end of 20 min was measured. Solid urea was then added to the reaction mixture, the sample applied to a G-25 column, and the incorporation determined, as described under Experimental Procedures. [e] Calculated from (100% act. regained/actual act. regained) × Δincorpn.

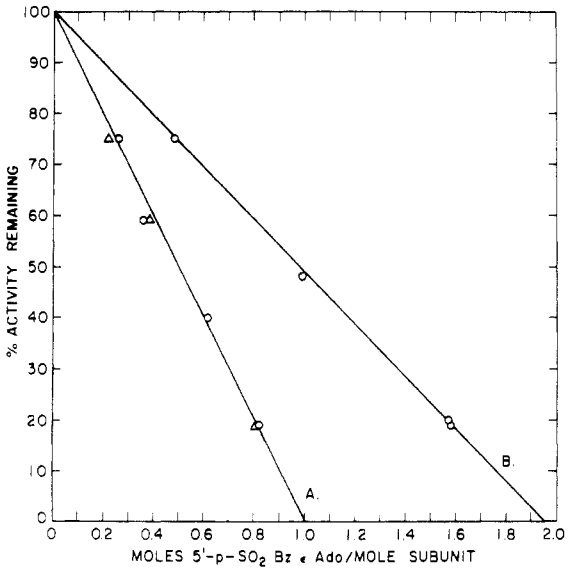

FIGURE 6: Incorporation of 5′-*p*-SO_2BzεAdo per mole of pyruvate kinase subunit as a function off inactivation. Pyruvate kinase (1.3–2.3 mg/mL) was incubated with 5′-*p*-FSO_2BzεAdo as described in Figure 3. The reaction was stopped at differing extents of inactivation either by the simultaneous addition of dithiothreitol and urea (line A) or by addition of urea alone (line B), followed by immediate removal of the unreacted reagent by gel filtration on Sephadex G-25. The incorporation was determined as described under Experimental Procedures by measuring the fluorescence of the modified enzyme either before (○) or after treatment with base (△). The extent of measured incorporation is plotted vs. the percent of activity remaining after correction for any minor loss of activity in the control.

determining the amount of reagent bound gave comparable results (Figure 6, line A).

When 20 mM dithiothreitol and urea were added to the reaction mixture simultaneously and the extent of incorporation was determined as a function of percent activity remaining, a linear relationship was found (Figure 6, line A). Extrapolation of the data to complete inactivation indicated that 1 mol of reagent had reacted/mol of enzyme subunit. If urea alone was added and the incorporation determined, the plot of percent activity remaining vs. extent of incorporation was linear; however, extrapolation to complete inactivation indicated that ~2 mol of reagent had reacted/mol of enzyme subunit (Figure 6, line B). It thus appears that the addition of dithiothreitol caused a decrease of one in the measurable groups incorporated per mole of enzyme subunit.

When dithiothreitol alone was added to the reaction mixture at differing extents of inactivation, immediate reactivation of the enzyme to ~95% of its original activity was observed as shown in Table II. Dithiothreitol had no effect on the control. No further changes in enzyme activity were observed after 20 min. β-Mercaptoethylamine reacted with the modified enzyme in analogous manner. After a 20-min incubation of the modified enzyme with dithiothreitol or β-mercaptoethylamine, urea was added, the sample was applied to Sephadex G-25, and the incorporation was determined as described under Experimental Procedures. The results, shown in Table II, indicate that both dithiothreitol and β-mercaptoethylamine decreased the extent of incorporation. In fact, if the extent of incorporation is compared to the percent activity remaining at the time of addition of dithiothreitol or β-mercaptoethylamine to the reaction mixture, the data parallel those seen for simultaneous addition of urea and dithiothreitol (Figure 6, line A). The amount of activity regained also parallels the loss of incorporated reagent [Table II, difference: (1) − (2)]. Extrapolation of the data to 100% regain in activity for totally inactivated enzyme indicated that 0.93 mol of reagent would be lost/subunit. These results suggest that there are two sites of modification per subunit for reaction with 5′-*p*-FSO_2BzεAdo. Reaction at one of these sites leads to a loss of enzyme activity which can be reversed by dithiothreitol or β-mercaptoethylamine, whereas reaction at the second site has no effect on enzyme activity and is not reversed by dithiothreitol or β-mercaptoethylamine under these conditions.

Identification of Reactive Amino Acid Residue. For determination of the nature of the amino acid residue modified upon reaction with 5′-*p*-FSO_2BzεAdo, pyruvate kinase was inactivated to an extent of 80%, followed by addition of dithiothreitol and urea. After acid hydrolysis of the modified enzyme and analysis by thin-layer electrophoresis or by direct application of the hydrolysate to the amino acid analyzer as described under Experimental Procedures, no CBS-Tyr was detected and only 0.12–0.14 mol of CBS-Lys/mol of subunit was found. [It is not expected that dithiothreitol would reverse the modification of a lysyl (CBS-Lys) or tyrosyl (CBS-Tyr) residue.] Approximately 0.8 mol of 5′-*p*-SO_2BzεAdo/mol of subunit is incorporated by pyruvate kinase treated in this manner (Figure 6, line A). Thus, the amount of derivatized lysine found was not stoichiometric with the extent of inactivation.

The reactivation of the modified enzyme by dithiothreitol or β-mercaptoethylamine and the concomitant stoichiometric loss of incorporated reagent suggested modification of a residue other than lysine or tyrosine, with cysteine or serine the most likely candidates. If reaction of 5′-*p*-FSO_2BzεAdo occurred

at a cysteine residue, a thiolsulfonate would be formed, whereas modification of serine would yield the alkyl sulfonate ester. Since both derivatives were expected to be unstable under the conditions required for acid hydrolysis of the protein, inactivated pyruvate kinase was reacted with β-mercaptoethylamine in order to convert the modified amino acid residue to a more stable derivative. The expected products of this reaction after acid hydrolysis are the mixed disulfide, cysteine–cysteamine, from the cysteine thiosulfonate (Parsons et al., 1965) and aminoethylcysteine for initial reaction of 5′-*p*-$FSO_2BzεAdo$ at serine sulfonate residue (Gold, 1965). Pyruvate kinase, which was 80% inactivated and was not treated with dithiothreitol and therefore had a measured incorporation of 1.58 mol of reagent/mol of subunit, was reacted with 1.8 M β-mercaptoethylamine, pH 8.0, in 8 M urea as described under Experimental Procedures. The acid hydrolysate was then applied to the short column of the amino acid analyzer and eluted as described under Experimental Procedures. Neither the mixed disulfide, cysteine–cysteamine, nor aminoethylcysteine was observed. Enzyme, which was inactivated to an extent of 55% followed by reactivation with 100 mM β-mercaptoethylamine, had a measured incorporation of 0.58 mol of reagent/mol of subunit (Table II, line 3). Acid hydrolysis and amino acid analysis of this modified enzyme and a control unmodified enzyme revealed cysteine–cysteamine disulfide in both samples in identical amounts (0.5 mol of mixed disulfide/mol of subunit). Aminoethylcysteine was not observed in either modified or control pyruvate kinase. Finally, enzyme that was inactivated to an extent of 80% but treated with dithiothreitol prior to reaction with β-mercaptoethylamine was also found to have approximately equal amounts of mixed disulfide per mole of subunit for both modified and control enzyme and a complete absence of aminoethylcysteine. The occurrence of mixed disulfide in the unmodified control enzyme as well as the modified pyruvate kinase may have resulted from reaction of a cystinyl residue with β-mercaptoethylamine or of cysteine with oxidized β-mercaptoethylamine.

Discussion

The fluorescent nucleotide analogue, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine reacts with muscle pyruvate kinase in a limited and specific manner, exhibiting characteristics expected for an affinity label. Thus, in the absence of thiols, 2 mol of reagent is incorporated/mol of enzyme subunit; various degrees of protection against inactivation are offered by substrates, and the rate of inactivation as a function of 5′-*p*-$FSO_2BzεAdo$ concentration follows saturation kinetics.

The most striking feature of the reaction of 5′-*p*-$FSO_2BzεAdo$ with muscle pyruvate kinase is that the inactivation of the enzyme and incorporation of 1 mol of reagent/mol of subunit are reversed by the addition of dithiothreitol or β-mercaptoethylamine. Since the amount of activity regained upon addition of thiols parallels the loss in incorporated reagent, the simplest model is that modification at only one site is responsible for the loss in enzyme activity, whereas modification to the extent of 1 mol of reagent/mol of subunit at the second site has no effect. This conclusion is consistent with the linear time course of inactivation that is observed. These results may be compared with those obtained for reaction of pyruvate kinase with 5′-*p*-FSO_2BzAdo (Wyatt & Colman, 1977) for which a biphasic inactivation was observed suggesting two sites of modification, one of which yields partially active enzyme and the second of which leads to complete inactivation. The comparison indicates that the analogues of adenosine and ethenoadenosine differ in their interaction with the enzyme. From the linearity of the plot of percent activity remaining vs. extent of incorporation of 5′-(sulfonylbenzoyl)ethenoadenosine determined in the absence of added dithiothreitol or β-mercaptoethylamine, it can be inferred that the modification at the two sites must either be occurring at random but at equal rates or the reactions may be sequential, with modification at the second nonessential site occurring only after reaction at the essential site. In accordance with the latter model, reaction at the second site must occur at a rate equal to or faster than that for reaction at the first site. It is possible that the two sites which react with 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine in the rabbit muscle pyruvate kinase are related to the two nucleotide binding sites indicated in the 2.6-Å resolution electron density map of cat muscle pyruvate kinase, only one of which appears to be at the active site (Stammers &Muirhead, 1975; Stuart et al., 1979).

The protection experiments suggest that 5′-*p*-$FSO_2BzεAdo$ reacts at the binding site involved in the catalytic function, thus accounting for the decrease in the rate of inactivation afforded by MgADP and possibly MgATP. The most significant protection is produced by phosphoenolpyruvate. Together with the lack of protection by pyruvate and the partial protection by Mg^{2+}, the results indicate that the site of modification may be near the phosphate binding region for phosphoenolpyruvate and the metal binding site. These two sites might be expected to be close to each other since it has been suggested that Mg^{2+} mediates the phosphoryl transfer (Mildvan, 1974).

The identity of the critical modified amino acid residue obtained upon reaction of 5′-*p*-$FSO_2BzεAdo$ with pyruvate kinase was of particular interest, since 5′-*p*-FSO_2BzAdo and 5′-*p*-$FSO_2BzεAdo$ appear to react in a different manner as judged by the kinetics of inactivation and recent evidence indicates that one residue of pyruvate kinase modified by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (Wyatt & Colman, 1977) is a tyrosine (A. E. Annamalai and R. F. Colman, unpublished experiments). Cysteine (Flashner et al., 1972) and histidine (Dann & Britton, 1974) have been implicated as being near the phosphoenolpyruvate binding site rather than the nucleotide site. The identity of the amino acid residues which react with 5′-*p*-$FSO_2BzεAdo$, however, could only be inferred. The less than stoichiometric amount of CBS-Lys found and the absence of CBS-Tyr indicate that these two residues are not the primary sites of modification. The small amount of CBS-Lys (0.12–0.14 mol/mol of subunit) that was found may be responsible for the lack of total reactivation upon addition of dithiothreitol or β-mercaptoethylamine. There also appeared to be the possibility that a serine could be the site of modification since the sulfonate ester can undergo thiolysis by β-mercaptoethylamine or dithiothreitol to form enzyme-bound thioether with displacement of the sulfonic acid (Gold, 1965). However, if serine were the site of modification, it could not be an essential residue for either catalysis or binding of substrates since treatment with the sulfhydryl compounds causes restoration of 95% of the original activity. Furthermore, the failure to observe aminoethylcysteine in the acid hydrolysates of modified enzyme treated with β-mercaptoethylamine excludes the possibility of reaction with serine. The reactivation of the modified enzyme by dithiothreitol suggests that a cysteine within the active center reacts to form a thiosulfonate upon reaction with 5′-*p*-$FSO_2BzεAdo$ and that the dithiothreitol treatment leads to regeneration of the unmodified cysteine. A similar sequence of reactions has been proposed for the inactivation of papain (Whitaker & Pirez-Villasenor,

1968) in which the thiolsulfonate formed during the reaction between the reactive cysteine and phenylmethanesulfonyl fluoride was decomposed by dithiothreitol, leading to reactivation of the enzyme. The lack of significant levels of cysteine–cysteamine in the acid hydrolysates of modified enzyme after exposure to β-mercaptoethylamine may be due to reaction of the cysteamine–cysteinyl residue with additional β-mercaptoethylamine to regenerate an unmodified cysteinyl residue and β-mercaptoethylamine disulfide; this interpretation is consistent with the measured reactivation of modified enzyme by β-mercaptoethylamine. The results thus indicate that in contrast to the reaction of 5′-*p*-FSO_2BzAdo with pyruvate kinase, the primary site of modification is not tyrosine and tentatively suggest that the residues modified by 5′-*p*-FSO_2BzϵAdo are cysteine.

The reactivation by dithiothreitol of pyruvate kinase which had been inactivated by 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine may have important implications for studies previously reported on the modification of several enzymes by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine. In a number of cases, dithiothreitol or mercaptoethanol was used to stop the reaction between 5′-FSO_2BzAdo and the enzyme (Pettigrew & Frieden, 1978; Mansour & Colman, 1978; Esch & Allison, 1978; Zoller & Taylor, 1979). The results of the present study indicate the need to ascertain the effect of added thiol on the activity and extent of reagent incorporation for these other modified enzymes.

The use of 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine offers a convenient means of introducing a covalent fluorescent probe into a nucleotide binding site of an enzyme, thus permitting the determination of the distance between sites by energy transfer measurements and the effects of ligand binding on the nucleotide site for allosteric enzymes. Additionally, the low quantum yield of 5′-*p*-FSO_2BzϵAdo as compared to ethenoadenosine suggests that the former, in free solution, exists preferentially in a folded conformation, i.e., one in which the purine and benzoyl moieties are stacked. The possibility exists that when covalently linked to the native form of an enzyme, the reagent may be bound in an open conformation which could be indicated by an enhancement of fluorescence. The fluorescence measurements may thus provide several types of information on the environment of nucleotide binding sites. In the case of rabbit muscle pyruvate kinase as here described, 5′-FSO_2BzϵAdo labels two sites, and therefore interpretation of the fluorescence characteristics of the modified enzyme would not be straightforward. Experiments designed to label separately each of the nucleotide binding sites of pyruvate kinase with the fluorescent probe are now in progress. After this work had been completed, a paper by Craig & Hammes (1980) appeared reporting on the use of 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine in studying an allosteric nucleotide site of phosphofructokinase. The compound described by Craig and Hammes has excitation and emission maxima at 356 and 490 nm, respectively, which contrast with the excitation and emission maxima of 308 and 412 nm, respectively, for 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. Thus, the two fluorescent nucleotide alkylating agents would be expected to be complementary in probing the nucleotide sites of proteins.

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Isotope-Exchange Evidence for an Ordered Mechanism for Rat-Liver Glucokinase, a Monomeric Cooperative Enzyme†

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ABSTRACT: The order of addition of substrates and release of products in the reaction catalyzed by rat-liver glucokinase has been studied by measurements of isotope exchange. Experiments at chemical equilibrium showed some degree of randomness, but steady-state experiments showed a predominantly ordered process with glucose binding first and glucose 6-phosphate released last. Experiments to trap binary complexes in the steady state demonstrated the existence of complexes of the enzyme with glucose and with glucose 6-phosphate but gave no evidence for the occurrence of corresponding complexes with ATP or ADP. Flux ratios measured in both the forward and reverse reactions provided a more rigorous and quantitative confirmation of these characteristics of the reaction. These observations support the interpretation of glucokinase cooperativity in terms of a "mnemonical" mechanism and conflict with an alternative interpretation in terms of a random addition of substrates.

Mammals have evolved diverse mechanisms for controlling the rate of phosphorylation of glucose by ATP,[1] in accordance with the differing metabolic functions of different tissues. In brain and muscle, for example, glucose is phosphorylated to provide for the energetic needs of the tissues, so that the rate ought to depend on the demand for glucose 6-phosphate, not on the supply of glucose: in these tissues, therefore, the predominant isoenzymes of hexokinase are saturated at low glucose concentrations but are highly susceptible to inhibition by glucose 6-phosphate (Weil-Malherbe & Bone, 1951; Crane & Sols, 1954; Hanson & Fromm, 1965; Copley & Fromm, 1967; Bachelard et al., 1971). In the liver, however, the purpose of glucose phosphorylation is not primarily to provide the liver with energy but to regulate the concentration of glucose in the blood, so the rate should depend on the supply of glucose, not on the demand for glucose 6-phosphate. It is not surprising, therefore, that glucokinase (also known as hexokinase type IV), the principal isoenzyme in hepatocytes, is insensitive to glucose 6-phosphate but responds cooperatively to glucose, with a maximum sensitivity to glucose at ~2.5 mM, lower than but comparable with the normal concentration of 5 mM in blood (Niemeyer et al., 1975; Storer & Cornish-Bowden, 1976b). This behavior has been observed not only with the purified enzyme but also with isolated rat hepatocytes and with cell-free extracts (Bontemps et al., 1978).

There is considerable evidence that glucokinase is a monomeric enzyme under all conditions that have been studied, including those that exist under normal assay conditions (Holroyde et al., 1976; Cárdenas et al., 1978, 1979), with only a single binding site for glucose on each molecule (Connolly & Trayer, 1979). This is a highly unusual property for an enzyme with pronounced positive cooperativity and shows that the cooperativity of glucokinase cannot be explained in terms of the quasi-equilibrium models that have dominated ideas about cooperativity for 15 years (Monod et al., 1965; Koshland et al., 1966). Instead, the phenomenon must be purely kinetic in origin.

Storer & Cornish-Bowden (1977) proposed a model for glucokinase based on the "mnemonical" mechanism derived by Ricard et al. (1974) from the concept of "enzyme memory" introduced by Rabin (1967). Rather similar ideas have also been developed by Shill & Neet (1975). In its simplest form the mnemonical mechanism requires glucokinase to exist with two forms of free enzyme that bind glucose with different affinities to give the same enzyme–glucose complex. If this complex is capable of reacting fast enough at high ATP concentrations to prevent equilibration between the two forms of free enzyme and the enzyme–glucose complex, then apparent cooperativity of glucose binding is generated by the changes in the relative proportions of the two forms of free enzyme that occur as the glucose concentration increases. At low ATP concentrations, on the other hand, equilibration of glucose binding cannot be prevented, because the enzyme–glucose complex cannot be removed fast enough; no cooperativity with respect to glucose occurs therefore at very low ATP concentrations. A further postulate of the mechanism is that ATP does not bind to the free enzyme. Thus ATP participates in one step only of the mechanism and therefore no deviation from Michaelis–Menten kinetics with respect to ATP occurs at any glucose concentration. Cárdenas et al. (1979) have interpreted their very similar data for glucokinase in terms of a somewhat more complex model: this resembles the mnemonical model in postulating the existence of distinct forms of free enzyme, but it also includes an alternative order of binding of substrates with ATP capable of binding first. In

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[1] Abbreviations used: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Glc, glucose; G6P, glucose 6-phosphate; enzymes, hexokinase, ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1; glucokinase, also known as hexokinase type IV or type D, ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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