Inactivation of *Escherichia coli* Glycerol Kinase by 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine: Protection by the Hydrolyzed Reagent†

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ABSTRACT: Incubation of *Escherichia coli* glycerol kinase (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSO_2BzAdo) at pH 8.0 and 25 °C results in the loss of enzyme activity, which is not restored by the addition of β-mercaptoethanol or dithiothreitol. The FSO_2BzAdo concentration dependence of the inactivation kinetics is described by a mechanism that includes the equilibrium binding of the reagent to the enzyme prior to a first-order inactivation reaction in addition to effects of reagent hydrolysis. The hydrolysis of the reagent has two effects on the observed kinetics. The first effect is deviation from pseudo-first-order kinetic behavior due to depletion of the reagent. The second effect is the novel protection of the enzyme from inactivation due to binding of the sulfonate hydrolysis product. The rate constant for the hydrolysis reaction, determined independently from the kinetics of F^- release, is 0.021 min^{-1} under these conditions. Determinations of the reaction stoichiometry with ^3H-labeled FSO_2BzAdo show that the inactivation is associated with the covalent incorporation of 1.08 mol of reagent/mol of enzyme subunit. Ligand protection experiments show that ATP, AMP, dAMP, NADH, 5′-adenylyl imidodiphosphate, and the sulfonate hydrolysis product of FSO_2BzAdo provide protection from inactivation. The protection obtained with ATP is not dependent on Mg^{2+}. Less protection is obtained with glycerol, GMP, etheno-AMP, and cAMP. No protection is obtained with CMP, UMP, TMP, etheno-CMP, GTP, or fructose 1,6-bisphosphate. The results are consistent with modification by FSO_2BzAdo of a single adenine nucleotide binding site per enzyme subunit.

Glycerol kinase (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) catalyzes the MgATP-dependent phosphorylation of glycerol to yield *sn*-glycerol 3-phosphate (Lin, 1976). In *Escherichia coli*, the role of this enzyme is mobilization of glycerol to serve as a carbon source. The *E. coli* enzyme is a tetramer composed of subunits with M_r's of 55 000 (Thorner & Paulus, 1971). This enzyme is of interest from at least two viewpoints. First, it shows broad specificity for phosphorylation of glycerol analogues and may be useful and important for the introduction of chirality in organic synthesis (Crans & Whitesides, 1985). Second, it catalyzes the rate-limiting step in the utilization of glycerol by *E. coli* (Zwaig et al., 1970) and is a regulatory enzyme whose activity is modulated by several ligands. It is subject to inhibition by Fru-1,6-P_2[1] (Zwaig & Lin, 1966). This regulation is of particular interest because it displays behavior that is characteristic of a V system (Monod et al., 1965) and is thus representative of a little-studied type of regulatory behavior. It has been shown that alterations in dimer–tetramer assembly are involved in regulation by Fru-1,6-P_2 (deRiel & Paulus, 1978). Recently, it was shown that glycerol kinase is also regulated by protein–protein interactions with enzyme III^{glc} of the phosphotransferase system (Novotny et al., 1985).

At pH 7.0, the steady-state kinetics of *E. coli* glycerol kinase display regulatory behavior with respect to the substrate MgATP (Thorner & Paulus, 1973; Pettigrew, 1986). In particular, double-reciprocal plots of the data are concave downward, indicating either negative homotropic interactions or two classes of MgATP binding sites. It was recently shown (Pettigrew, 1986) that AMP displays complex inhibition behavior with respect to MgATP. In addition, AMP, cAMP, ATP, and MgATP protect the enzyme from inactivation due to modification of sulfhydryl groups (Pettigrew, 1986). These observations suggest that there may be an adenine nucleotide binding site on glycerol kinase that is involved in the AMP inhibition in addition to the adenine nucleotide binding site for the substrate ATP. One approach to studying such nucleotide binding sites is affinity labeling. In the case of adenine nucleotides, the affinity label FSO_2BzAdo has proven to be useful (Colman, 1983). Results presented below show that this affinity label can be used to modify one adenine nucleotide binding site per subunit of *E. coli* glycerol kinase, resulting in inactivation of the enzyme.

Materials and Methods

Materials. All chemicals and enzymes were purchased from Sigma Chemical Co., unless indicated otherwise. Radioactively labeled FSO_2BzAdo was synthesized as described by Wyatt and Colman (1977) with [2,8-^3H]adenosine, which was purchased from ICN Pharmaceuticals, Inc. Hexamethylphosphoric triamide, adenosine, *p*-(fluorosulfonyl)benzoyl chloride, propane-1,2-diol, and propane-1,3-diol were purchased from Aldrich Chemical Co. Dimethylformamide was a product of Pierce Chemical Co. Silica gel plates with

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[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; εAMP, 1,N^6-ethenoadenosine 5′-monophosphate; εCMP, 3,N^4-ethenocytidine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; Fru-1,6-P_2, fructose 1,6-bisphosphate; FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine; SO_3BzAdo^-, 5′-(*p*-sulfonatobenzoyl)adenosine.

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fluorescent indicator were purchased from Kodak. Glycerol kinase was purified from cells of *E. coli* carrying a cloned copy of the gene on plasmid pCJ102 as described (Pettigrew, 1986).

Methods. Glycerol kinase activity was determined by the previously described ADP-coupled assay (Pettigrew, 1986). Reactions were initiated by the addition of enzyme to cuvettes that were maintained at a temperature of 25 °C. One unit of glycerol kinase activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of product in 1 min under these conditions.

In studies of the inactivation of glycerol kinase by FSO_2BzAdo, enzyme was incubated at a concentration of 0.05 mg/mL in 0.1 M triethanolamine buffer at pH 8.0 and 25 °C. The modification reactions were initiated by the addition of FSO_2BzAdo dissolved in dimethylformamide. The final concentration of dimethylformamide in the reaction was 5% (v/v). Aliquots of 0.01 mL were removed from the reaction and diluted 100-fold in the assay for determination of the specific activity at the times indicated in the figure legends and tables. The time courses of the inactivations were simulated by the program KINSIM (Barshop et al., 1983) with the mechanism that is described under Results and Discussion.

The rate constant for the hydrolysis of FSO_2BzAdo (Colman, 1983) was determined from the rate of F^- release on an Orion Model 501 Digital Ionalyzer with an Orion F^--specific ion electrode. Reactions were initiated by the addition of FSO_2BzAdo to a final concentration of 1 mM to 0.1 M triethanolamine buffer at pH 8.0. The reaction vessel was immersed in a water bath maintained at a temperature of 25 °C. The final concentration of dimethylformamide was 5% (v/v). The voltage from the Digital Ionalyzer was continuously recorded, and the concentration of F^- was determined from a linear ($r^2 = 0.999$) calibration curve that was prepared with solutions from 10^{-5} to 10^{-3} M NaF (Rowe & Hyman, 1983). The rate constant for hydrolysis was determined from a linear ($r^2 = 0.996$) plot of log ($[FSO_2BzAdo]_t/[FSO_2BzAdo]_0$) vs. time.

For the ligand protection experiments, glycerol kinase was incubated at 0.05 mg/mL in 0.1 M triethanolamine buffer at pH 8.0 and 25 °C, with other additions as indicated. Modification reactions were initiated by the addition of FSO_2BzAdo to a final concentration of 0.7 mM. After 0.5 h, a 0.01-mL aliquot was diluted 100-fold into the assay described above for determination of the remaining enzyme activity. The remaining enzyme activities are expressed as percentages of that of a control to which no additions were made, i.e., no ligands or FSO_2BzAdo. Most determinations were performed in triplicate, and the standard deviation is given with the results. A "*t*" test of the significance between two sample means (Hodgson, 1959) was used to compare the activity remaining in incubations with ligands to the activity remaining in the incubation with no added ligands, i.e., FSO_2BzAdo alone. If the determination was performed less than 3 times, the result of each of the determinations is given. Separate control experiments showed that none of the ligands alone affects the activity of glycerol kinase. Furthermore, at the concentrations obtained after the 100-fold dilution into the assay, none of the ligands affect the coupled assay system.

For ligand protection studies, SO_3BzAdo^- was prepared by overnight hydrolysis of FSO_2BzAdo in 0.1 M triethanolamine buffer at pH 8 with 5% (v/v) dimethylformamide. Thin-layer chromatography of the reaction product with a solvent system of methanol–chloroform (15:85) (Wyatt & Colman, 1977) showed a single compound that remained at the origin, with no material that comigrated with FSO_2BzAdo (R_f 0.18) or adenosine (R_f 0.07). The observation that the hydrolysis product remains at the origin in this chromatography system is consistent with the expected sulfonic acid product.

In determinations of the stoichiometry of FSO_2BzAdo incorporation, glycerol kinase was incubated with 1.84 mM [^{3}H]FSO_2BzAdo in 0.1 mM triethanolamine buffer at pH 8.0 and 25 °C containing 5% (v/v) dimethylformamide for 2 h. β-Mercaptoethanol was then added to a final concentration of 0.14 M. The specific activity of the modified enzyme was determined. The modified enzyme was dialyzed exhaustively against 0.1 M triethanolamine buffer at pH 7.0. After dialysis, the solution was clarified by centrifugation. The concentration of the modified enzyme was determined from measurements of the absorbance at 280 and 259 nm and solution of simultaneous equations. The extinction coefficient of the native enzyme at 280 nm is 1.4 $(mg/mL)^{-1}$ cm^{-1} (Thorner & Paulus, 1973), while the extinction coefficient of FSO_2BzAdo at 259 nm is 1.35×10^4 M^{-1} cm^{-1} (Wyatt & Colman, 1977). These extinction coefficients were used in empirical determinations of the extinction coefficient of the native enzyme at 259 nm and the extinction coefficient of FSO_2BzAdo at 280 nm. The values that were obtained are 0.7 $(mg/mL)^{-1}$ cm^{-1} and 0.47×10^4 M^{-1} cm^{-1}, respectively. The radioactivity of the modified enzyme was determined by evaporating 0.5 mL of the dialyzed solution to dryness in a glass scintillation vial. The residue was dissolved in 0.1 mL of distilled water, and 10 mL of Liquiscint (National Diagnostics) was added for scintillation counting. The specific radioactivity of the ^{3}H-labeled FSO_2BzAdo (1.3×10^5 cpm/μmol) was determined by the same protocol for evaporation and scintillation counting. The concentration of the ^{3}H-labeled FSO_2BzAdo was determined from the absorbance at 259 nm of a suitable dilution with the extinction coefficient described above. The molar concentration of the enzyme was calculated on the basis of a subunit M_r of 55 000 (Thorner & Paulus, 1971).

The homogeneity of the GMP used in these studies was assessed by high-performance liquid chromatography. Samples (100 nmol) were applied to an Altex ODS column (4.6 × 150 mm) and eluted isocratically with 5 mM *tert*-butyl ammonium bisulfate–5 mM K_2HPO_4, pH 5.0, at a flow rate of 2.0 mL/min. Elution profiles were monitored at 254 nm and showed that the GMP contained no other nucleotides at detectable levels (<0.1%).

Results and Discussion

Inactivation of Glycerol Kinase by FSO_2BzAdo. Incubation of glycerol kinase with FSO_2BzAdo results in a time-dependent loss of enzyme activity. Results obtained at pH 8.0 with three concentrations of FSO_2BzAdo are presented in Figure 1. In controls that contain no addition or 2 mM phenylmethanesulfonyl fluoride, no activity is lost. The solid lines that are drawn through the data points for the inactivation were simulated as described under Materials and Methods with the following mechanism:

$$FSO_2BzAdo + H_2O \xrightarrow{k_1} SO_3BzAdo^- + F^- \quad (1)$$

$$E + SO_3BzAdo^- \overset{K_2}{\rightleftharpoons} E{\cdot}SO_3BzAdo \quad (2)$$

$$E + FSO_2BzAdo \overset{K_3}{\rightleftharpoons} E{\cdot}FSO_2BzAdo \xrightarrow{k_4} ESO_2BzAdo + F^- \quad (3)$$

In this mechanism, k_1 and k_4 are first-order rate constants while K_2 and K_3 are dissociation constants. Values of these constants that were used for the simulations are given in the legend to Figure 1. The kinetics of inactivation and its de-

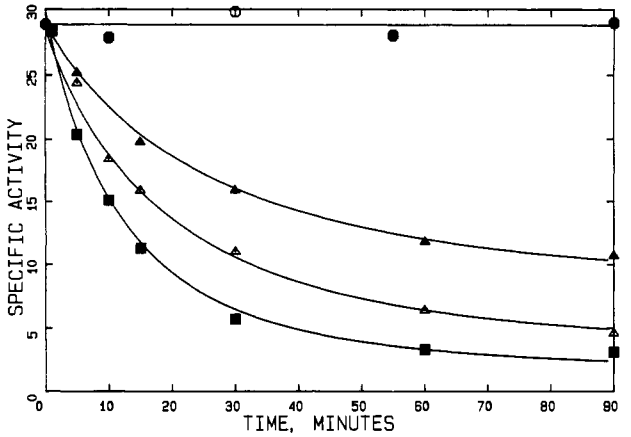

FIGURE 1: Inactivation of glycerol kinase by sulfonyl fluorides. Glycerol kinase was incubated at 25 °C at a final concentration of 0.05 mg/mL in 0.1 M triethanolamine buffer at pH 8.0 with 5% dimethylformamide. The modification reaction was initiated by the addition of sulfonyl fluoride to the concentrations indicated below. At the indicated times, 0.01 mL was removed for determination of the specific activity by the coupled assay described under Materials and Methods. For the lower three curves, the points are the experimental data, while the solid lines were calculated according to eq 1–3 with the following parameter values: k_1 = 0.021 min^{-1}; K_2 = 0.75 mM; K_3 = 2 mM; k_4 = 0.19 ± 0.03 min^{-1}. Concentrations were as follows: 0 mM sulfonyl fluoride (●); 2 mM phenylmethanesulfonyl fluoride (○); 0.28 mM FSO$_2$BzAdo (▲); 0.7 mM FSO$_2$BzAdo (△); 1.36 mM FSO$_2$BzAdo (■).

pendence on FSO$_2$BzAdo concentration are well described by this mechanism. The kinetic behavior with respect to FSO$_2$BzAdo, given by eq 3, is that expected for an affinity label. That is, the reagent binds to the enzyme in an equilibrium reaction prior to an irreversible first-order inactivation that results in the covalent incorporation of SO$_2$BzAdo. The FSO$_2$BzAdo concentration dependence of the inactivation is accounted for in terms of the equilibrium binding of the reagent to the enzyme, while the first-order rate constant for the inactivation is independent of the reagent concentration.

The mechanism that describes the kinetics of inactivation includes effects due to hydrolysis of the reagent. It has been shown that FSO$_2$BzAdo undergoes hydrolysis to yield SO$_3$BzAdo$^-$ and F$^-$, and the rate of this reaction increases above pH 7.6 (Colman, 1983). For example, a half-time of 32 min is observed for the hydrolysis reaction at pH 8.6 in 20 mM sodium barbital buffer at 25 °C (Likos et al., 1980). The hydrolysis of FSO$_2$BzAdo has two effects on the observed kinetics of inactivation of glycerol kinase. First, it reduces the effective concentration of the reagent; this effect is given by eq 1. The result of this effect is deviation of the kinetics from pseudo-first-order behavior. Analysis of the contribution of this effect to the observed kinetics was facilitated by the independent determination of the rate constant for hydrolysis, k_1, under these conditions. The independently determined value for k_1, 0.021 min^{-1}, which corresponds to a half-time of 33 min, was used in the simulations of the inactivation.

The second effect of FSO$_2$BzAdo hydrolysis is binding of SO$_3$BzAdo$^-$, the sulfonate hydrolysis product, to the enzyme. This is given by eq 2. As a consequence of this binding, the enzyme is protected from complete inactivation. Support for this conclusion is provided by the results of ligand protection studies that are described below. This protection by the hydrolyzed reagent does not appear to have been previously observed in studies with FSO$_2$BzAdo. However, it was suggested by the observation that the inactivation kinetics could not be adequately described by eq 1 and 3, where the rate constant for reagent hydrolysis was independently determined.

Table I: Stoichiometry of FSO$_2$BzAdo Incorporation

expt	sp act. of modified enzyme (units/mg)	protein concn (mg/mL)	radioactivity incorporated: cpm/mg	radioactivity incorporated: mol of SO$_2$BzAdo/mol of subunit
1	1.8	0.38	2500	1.06
2	2.3	0.44	2590	1.10

In simulations using only eq 1 and 3 in which the data at early times are well described, the data at longer times are not fitted because complete inactivation is predicted (not shown). The data in Figure 1 show that the enzyme is not completely inactivated. In the context of the proposed mechanism, complete inactivation is not observed because the hydrolysis product binds to the enzyme and protects it from inactivation while the remaining reagent is hydrolyzed, thus preventing further reaction. This hypothesis is supported by two observations that suggest that the remaining activity reflects that of unmodified enzyme. First, the kinetic properties of enzyme that has been inactivated to the extent of 90% are the same as those of native enzyme with respect to AMP inhibition and apparent ATP activation (Pettigrew, 1986); that is, the same complex behavior and apparent Michaelis constants are observed while the maximum velocity is 10% that of the native enzyme. It might be expected that if the remaining activity reflected enzyme that was 100% modified but only partially inactivated, the binding of AMP or ATP would be changed, and this change would be reflected in the kinetic properties. Second, the partially inactivated enzyme is completely inactivated by a second incubation with FSO$_2$BzAdo. Before the second incubation, the partially inactivated enzyme must be dialyzed to remove the hydrolyzed reagent and dimethylformamide. The removal of the dimethylformamide is necessary because the enzyme loses activity upon incubation at dimethylformamide concentrations greater than 5%.

The activity of modified glycerol kinase is not altered by addition of either 0.1 M dithiothreitol or 0.14 M β-mercaptoethanol. Thus, it appears that the inactivation of glycerol kinase by FSO$_2$BzAdo does not involve the modification of sulfhydryl groups, as is the case for pyruvate kinase (Colman, 1983). This is of interest because adenine nucleotides do protect enzyme sulfhydryl groups from modification (Pettigrew, 1986). Thus, it appears that FSO$_2$BzAdo may ultimately provide information about a second type of amino acid residue in an adenine nucleotide binding site on the enzyme.

Stoichiometry of FSO$_2$BzAdo Incorporation. The stoichiometry of incorporation of ^{3}H-labeled FSO$_2$BzAdo was determined as described under Materials and Methods. Results of two such experiments are summarized in Table I. In these experiments, enzyme that was inactivated to the extent of 90–95% contains an average of 1.08 mol of SO$_2$BzAdo/mol of enzyme subunit. The incorporated radioactivity is not removed by treatment with β-mercaptoethanol or by exhaustive dialysis vs. either 0.1 M triethanolamine buffer at pH 7 or 6 M guanidine hydrochloride in 0.1 M sodium borate–0.01 M EDTA buffer at pH 8.6. This result is consistent with the covalent incorporation of SO$_2$BzAdo at one site per subunit of glycerol kinase.

Ligand Protection of Glycerol Kinase from Inactivation by FSO$_2$BzAdo. Glycerol kinase is protected from inactivation by FSO$_2$BzAdo by the addition of ligands to the incubation. Results of ligand protection experiments are summarized in Table II. The following ligands provide significant protection from the inactivation: ATP, SO$_3$BzAdo$^-$, AMP-PNP, AMP, dAMP, NADH, cAMP, εAMP, GMP, glycerol, and pro-

Table II: Ligand Protection from FSO_2BzAdo Inactivation of Glycerol Kinase

ligand (mM)	remaining activity (% control)[a]	p[b]
none	32 ± 0.6	
ATP (4.3)	69 ± 4.4	<0.01
ATP (4.3) + $MgCl_2$ (5.0)	72 ± 3.9	<0.01
SO_3BzAdo^- (1.4)	62, 66	
AMP-PNP	63 ± 0.1	<0.01
AMP (5.0)	54 ± 0.3	<0.01
dAMP (5.0)	55 ± 1.2	<0.01
cAMP (5.0)	43 ± 1.5	<0.01
ϵAMP (5.0)	48 ± 1.2	<0.01
GMP (5.0)	36 ± 0.1	0.05
GTP (4.9)	33 ± 1.4	0.85
GTP (4.9) + $MgCl_2$ (5.0)	33 ± 0.2	0.75
NADH (5.0)	54 ± 2.1	<0.01
NADH (0.5)	34	
TMP (5.0)	34 ± 0.4	0.25
CMP (5.0)	33 ± 0.7	0.4
ϵCMP (5.0)	30 ± 0.4	0.15
UMP (5.0)	33 ± 0.1	0.4
glycerol (2.0)	45 ± 0.1	<0.01
propane-1,2-diol (10.0)	37 ± 0.2	0.03
propane-1,3-diol (10.0)	32 ± 0.9	>0.9
$MgCl_2$ (5.0)	28 ± 0.7	0.04
NaF (0.5)	34	
Fru-1,6-P_2 (2.0)	34 ± 0.8	0.2

[a] The remaining activity is expressed as a percentage of that of a control to which no additions were made. The standard deviation is given for experiments performed in triplicate. For experiments that were not performed in triplicate, the results of each determination are given. [b] The probability that the difference between the indicated value and that observed for no ligand occurs by chance, as given by the "t" test of significance between two sample means (Hodgman, 1959).

pane-1,2-diol. Interestingly, the protection that is afforded by ATP does not require Mg^{2+}. The similarity in the degree of protection that is obtained with AMP and dAMP suggests that the 2′-hydroxyl group on the ribose portion of AMP plays little role in its binding. The protection that is afforded by SO_3BzAdo^- is consistent with its binding to the enzyme as postulated above in the mechanism that describes the kinetics of the inactivation. The observed degree of protection by SO_3BzAdo^- is in good quantitative agreement with the predictions of this mechanism. That is, simulations according to the mechanism predict that there should be 67% activity remaining with 1.4 mM SO_3BzAdo^- added to the incubation, while the average of the experimentally observed values is 64%.

The following ligands do not provide significant protection from the inactivation: pyrimidine nucleotides, GTP, propane-1,3-diol, sodium fluoride, and fructose 1,6-bisphosphate. Interestingly, magnesium chloride alone appears to significantly increase the rate of the inactivation. Results of these ligand protection studies strongly suggest that the binding site which is modified by FSO_2BzAdo binds purine nucleotides and has a higher affinity for adenine nucleotides than for guanine nucleotides.

The observation that the site binds adenine nucleotides provides an explanation for the protection that is observed with NADH. Glycerol kinase is not bound by Blue Dextran affinity columns.[2] This suggests that it lacks a dinucleotide binding site. Thus, it is likely that the protection by NADH reflects the binding of the adenine ring portion of this dinucleotide. At a concentration of 0.5 mM, NADH provides little protection, thus indicating a relatively low affinity for binding to the enzyme. This observation is of interest in the context of the use of the NADH-coupled assay to determine the activity of the enzyme, suggesting that binding of NADH to the enzyme at the concentration used in the assay (0.3 mM) should be negligible.

Relationships between the site modified by FSO_2BzAdo and the active site of glycerol kinase are not known at this time. The modification results in the complete loss of catalytic activity. Furthermore, protection from inactivation is obtained with active site ligands: glycerol, propane-1,2-diol, and ATP. The relative degree of protection that is provided by glycerol and propane-1,2-diol appears to reflect their relative affinities for binding to the active site. That is, better protection is obtained with glycerol, for which the Michaelis constant is 10 μM (Thorner & Paulus, 1973). The propane-1,2-diol used in these studies acts as an apparent competitive inhibitor with a K_i of 3.6 mM, although it is a racemic mixture (Pettigrew, 1986).

The protection by ATP does not require Mg^{2+}, and MgATP is the true substrate (Hayashi & Lin, 1967). The protection by ATP or MgATP is somewhat unexpected in view of the kinetic mechanism of the enzyme. The kinetic mechanisms of glycerol kinases from *Candida mycoderma* (Janson & Cleland, 1974) and *E. coli* (Thorner & Paulus, 1973) are ordered with glycerol binding before MgATP. It is possible, however, that ATP and/or MgATP binds at the active site in the absence of glycerol and the ordered kinetic mechanism is a consequence of the relative rate constants for substrate addition in what is actually a random kinetic mechanism.

On the other hand, the site modified by FSO_2BzAdo may be different from the active site. One suggestion that this may be the case is provided by the protection obtained with glycerol. The degree of protection is either less than or greater than expected, depending upon which of two cases of the structure of bound FSO_2BzAdo is considered. The (fluorosulfonyl)-benzoyl group of FSO_2BzAdo corresponds structurally to the triphosphate portion of ATP (Colman, 1983). If the FSO_2BzAdo binds in an extended conformation, as ATP does when bound to pyruvate kinase (Sloan & Mildvan, 1976), the reactive portion of the reagent, which corresponds to the γ-phosphate group, is expected to be located in the immediate vicinity of the glycerol hydroxyl group to which this phosphate group is transferred. In this case, it would seem that glycerol should provide about the same degree of protection as obtained with ATP or MgATP, i.e., significantly more protection than is observed. On the other hand, FSO_2BzAdo may not bind in an extended conformation. Jacobson and Colman (1984) have shown that FSO_2BzAdo exists in solution in a conformation in which the purine ring is intramolecularly stacked with the benzoyl moiety. They also observed similar stacking of 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine that is covalently bound to glutamate dehydrogenase and pyruvate kinase. It is thus likely that FSO_2BzAdo is bound to glycerol kinase in a stacked conformation. If, in this case, the purine moiety occupies the same site as it does when ATP is bound, the reactive portion of the FSO_2BzAdo will not be located in the same region as the γ-phosphate group of ATP. Then, it might be expected that glycerol should provide no protection from the inactivation, i.e., significantly less than is observed.

The protection by GMP and AMP also suggests that the site of modification by FSO_2BzAdo may be different from the active site. At a concentration of 5 mM, GMP provides modest protection from inactivation. Determination of the homogeneity of the GMP used in these studies, as described under Materials and Methods, showed that no other nucleotides were present at detectable levels. Thus, the protection that is obtained with GMP does not appear to be due to nucleotide contaminants. On the other hand, no protection is

[2] Unpublished experiments.

observed with 5 mM GTP in the presence or absence of Mg^{2+}. Furthermore, GTP does not serve as a substrate (Hayashi & Lin, 1967),[2] suggesting that guanine nucleotides are not productively bound at the active site. As previously described (Thorner, 1972; Pettigrew, 1986), AMP is an inhibitor of glycerol kinase, displaying complex inhibition behavior. This complex behavior is consistent with binding of AMP at regulatory sites. Thus, the protection by GMP and AMP may reflect binding at sites other than the active site.

FSO_2BzAdo has been used in affinity labeling studies of several kinases. In the cases of pyruvate kinases (Wyatt & Colman, 1977; Likos et al., 1980), cAMP-dependent protein kinase (Zoller & Taylor, 1979; Hixson & Krebs, 1979), cGMP-dependent protein kinase (Hixson & Krebs, 1981), and casein kinase II (Hathaway et al., 1981), FSO_2BzAdo modifies the nucleotide binding region of the active site. In the case of rabbit muscle phosphofructokinase, however, this reagent modifies an adenine nucleotide activator site (Pettigrew & Frieden, 1978). At this juncture, it is not known whether the site on glycerol kinase that is modified by FSO_2BzAdo is the active site or a regulatory site. Studies of the equilibrium binding of adenine nucleotides to native and FSO_2BzAdo-modified glycerol kinases should prove useful in resolving this question.

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Registry No. FSO_2BzAdo, 57454-44-1; ATP, 56-65-5; AMP, 61-19-8; dAMP, 653-63-4; NADH, 58-68-4; AMP-PNP, 25612-73-1; GMP, 85-32-5; cAMP, 60-92-4; ϵAMP, 42578-95-0; $HO_3SBzAdo$, 106252-25-9; EC 2.7.1.30, 9030-66-4; $HOCH_2CH(OH)CH_2OH$, 56-81-5.

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