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Affinity Labeling of the Active Site of *Escherichia coli* Glutamine Synthetase by 5′-*p*-Fluorosulfonylbenzoyladenosine*

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The interaction of *Escherichia coli* glutamine synthetase with the adenosine 5′-triphosphate analogue, 5′-*p*-fluorosulfonylbenzoyladenosine (5′-FSO_2BzAdo), has been studied. This interaction results in the covalent attachment of the 5′-FSO_2BzAdo to the enzyme with concomitant loss of catalytic activity. Although adenine nucleotides interact with glutamine synthetase at three distinct sites—a noncovalent AMP effector site, a regulatory site of covalent adenylylation, and the catalytic ATP/ADP binding site—our studies suggest that reaction with 5′-FSO_2BzAdo occurs only at the active center. When glutamine synthetase was incubated with 5′-FSO_2BzAdo, the decrease in catalytic activity obeyed pseudo-first order kinetics. The plot of the observed rate constant of inactivation *versus* the concentration of 5′-FSO_2BzAdo was hyperbolic, consistent with reversible binding of the analogue to the enzyme prior to covalent attachment. Protection against inactivation was afforded by ATP and ADP; L-glutamate did not protect the enzyme against inactivation, but rather enhanced the rate of inactivation, consistent with the observations of others (Timmons, R. B., Rhee, S. G., Luterman, D. L., and Chock, P. B. (1974) *Biochemistry* 13, 4479-4485) that there is synergism in the binding of the two substrates to the enzyme. The incorporation of approximately 1.09 mol of the 5′-FSO_2BzAdo/mol of glutamine synthetase subunit resulted in the total loss of enzymatic activity. The results suggest that 5′-FSO_2BzAdo occupies the ATP binding site at the active center of glutamine synthetase and binds covalently to an amino acid residue nearby.

The glutamine synthetase of *Escherichia coli* is composed of 12 identical subunits (1) with a molecular weight of approximately 49,000/subunit. On each subunit there exists a regulatory site of covalent adenylylation (2, 3), a catalytically active center, and at least two distinct metal ion binding sites: the n_1 site and the n_2 site (4, 5). Studies by Hunt *et al.* (4) and Hunt and Ginsburg (5) have demonstrated that metal ion bound to the high affinity n_1 site is responsible for maintaining an active conformation of the enzyme, whereas metal ion bound to the n_2 site chelates the nucleotide substrate. The physical relationships of these sites have been reviewed recently by Villafranca and Balakrishnan (6).

Extensive work has been done on the inactivation of glutamine synthetase by the proposed transition state analogue, L-methionine-*SR*-sulfoximine (5-9). This analogue has been shown to form, in the presence of ATP and Me^{2+}, methionine sulfoximine phosphate, in which the sulfoximine nitrogen has been phosphorylated. This phosphorylated derivative remains bound to the enzyme active center (7). Hunt and Ginsburg (5) have shown that the binding of methionine sulfoximine to the enzyme is near the n_1 metal ion site.

To date there have been comparatively few reports on the interaction of glutamine synthetase with analogues of ATP or ADP. Rhee *et al.* (10) have shown that ADP binds competitively with respect to ATP, while other studies (11) have demonstrated strong synergism of binding between L-glutamate and ATP. Pillai *et al.* (12) have reported recently on the interaction of glutamine synthetase and stereoisomers of thiophosphate esters of ATP and ADP, showing that in the reactions catalyzed by the enzyme, metal ions bind to both the α- and β-phosphoryl groups of ATP or ADP. The present study was undertaken to examine the interaction of *E. coli* glutamine synthetase with an analogue of the substrate ATP, 5′-*p*-fluorosulfonylbenzoyladenosine, developed by Pal *et al.* (13). 5′-FSO_2BzAdo[1] has been shown to label covalently either regulatory or catalytic sites of dehydrogenases (13, 14), kinases (15-19), F_1-ATPase (20, 21), and an ADP-binding protein in human platelets (22). The present report deals with studies on the inactivation of glutamine synthetase by 5′-FSO_2BzAdo. Preliminary reports of these studies have been presented (23, 24).

EXPERIMENTAL PROCEDURES

Materials

5′-FSO_2BzAdo was either prepared as described below or purchased from Sigma as the HCl salt. ATP, ADP, AMP, L-glutamate, L-glutamine, 4-morpholinopropanesulfonic acid, and imidazole were also purchased from Sigma. Reagents for polyacrylamide gel electrophoresis, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis molecular weight standards, were purchased from Bio-Rad. Hexamethylphosphoramide, *p*-fluorosulfonylbenzoylchloride, and adenosine were purchased from Aldrich. Cellulose thin layer chromatography sheets and silica gel thin layer chromatography sheets were obtained from Schleicher & Schuell as well as from Eastman. All other chemicals used in this study were reagent grade.

Kilogram quantities of *E. coli* W were supplied by Dr. David Novelli, E. F. Phares, and Mary Long of the Oak Ridge National Laboratory, Oak Ridge, TN, and were grown by the procedure of Phares (25). Bacterial cell paste was shipped to us packed in dry ice and was stored at −20°C until used.

Methods

Preparation of Buffers—All buffers used in this study were prepared and adjusted to the proper pH at room temperature. A Radi-

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[1] The abbreviations used are: 5′-FSO_2BzAdo, 5′-*p*-fluorosulfonylbenzoyladenosine; Me_2SO, dimethyl sulfoxide; EtOH, ethanol.

ometer PHM62 pH-meter (The London Company, Cleveland, OH) equipped with a combination electrode was calibrated for use at room temperature.

Purification of Glutamine Synthetase—The glutamine synthetase used for these studies was purified by the procedure of Woolfolk *et al.* (26), with omission of the final crystallization step. Purity of the enzyme preparation was assessed by polyacrylamide gel electrophoresis by the procedure of Weber and Osborn (27), as well as by NH_2-terminal sequence analysis of the intact protein which had been dialyzed exhaustively against distilled water. The average state of adenylylation of the enzyme preparation was determined both spectrophotometrically (28), and enzymatically according to Stadtman *et al.* (29). The enzyme used in these studies contained an average of 1.9 adenylyl groups/dodecamer.

Assay of Enzymatic Activity—Glutamine synthetase catalyzes several reactions, including those described by Equations 1 and 2.

$$\text{ATP} + \text{NH}_3 + \text{L-Glu} \xrightarrow{\text{Me}^{2+}} \text{ADP} + \text{L-Gln} + \text{P}_i \quad (1)$$

$$\text{NH}_2\text{OH} + \text{L-Gln} \xrightarrow[\text{Me}^{2+}]{\text{ADP, arsenate}} \gamma\text{-glutamylhydroxamate} + \text{NH}_3 \quad (2)$$

Either of these two reactions can form the basis for an enzyme assay. When using the biosynthetic assay described by Equation 1, the rate of production of inorganic phosphate was measured (26). When using the transferase assay described by Equation 2, the rate of production of γ-glutamylhydroxamate was measured (26). Both assays were performed at 37°C. Rhee *et al.* (10) have shown that a single active center catalyzes both of the reactions described above. Only the transferase assay was used to monitor enzymatic activity during the purification of the enzyme.

Protein Concentration—The concentration of purified glutamine synthetase was determined spectrophotometrically as described by Ginsburg *et al.* (30) as well as by the procedure of Lowry *et al.* (31) using bovine serum albumin as the standard.

Preparation of 5′-FSO_2BzAdo—5′-FSO_2BzAdo was prepared as described by Wyatt and Colman (15). Radiolabeled 5′-FSO_2BzAdo was prepared by the addition of [2,8-^{3}H]adenosine to the unlabeled adenosine used in the normal preparation. However, when preparing radiolabeled 5′-FSO_2BzAdo, the amount of each component was reduced 10-fold in order to obtain a higher specific radioactivity in the final product. The specific radioactivity of the product was 1.67×10^9 cpm/mmol. The purity of the analogue preparations was assessed by a combination of chromatographic techniques. We have found that the thin layer chromatographic system described by Wyatt and Colman (15) and Hixson and Krebs (16) apparently results in a small amount of hydrolysis of the analogue during the chromatography, thereby leading to the appearance of contaminating adenosine. As an alternative to thin layer, we have used high performance liquid chromatography on a C-18 reverse-phase column.[2] The concentrations of stock 5′-FSO_2BzAdo solutions were determined by their absorbance at 232 nm (ϵ = 18.8 cm^{-1} mM^{-1}) and at 259 nm (ϵ = 13.5 cm^{-1} nM^{-1}), as described by Wyatt and Colman (15).

Reaction of Glutamine Synthetase and 5′-FSO_2BzAdo—Glutamine synthetase (0.385 mg/ml) was incubated with various concentrations of 5′-FSO_2BzAdo at 4°C in a reaction mixture containing 0.2 M morpholinopropanesulfonic acid buffer (pH 7.3), 5 mM $MgCl_2$, and 10% by volume of a 1:1 (v/v) mixture of Me_2SO and EtOH. The organic solvent was added to ensure solubility of the analogue. To obtain the proper concentrations of 5′-FSO_2BzAdo, dilutions of a stock solution of the analogue (in Me_2SO) were made in the Me_2SO/EtOH mixture described above. The Mg^{2+} was required to maintain an active conformation of the enzyme (32, 33).

To follow the inactivation of the enzyme by 5′-FSO_2BzAdo, 10-μl aliquots were withdrawn at several times of reaction and added to an assay tube containing (in a final volume of 1.0 ml) 0.1 M L-glutamate, 0.05 M NH_4Cl, 0.05 M $MgCl_2$, 0.05 M imidazole (pH 7.0), and 0.0076 M ATP at 37°C. The dilution of the analogue (1:100) plus the presence of the ATP quenched the reaction of the enzyme with the 5′-FSO_2BzAdo. After 5 min, 0.20 ml was withdrawn and added to 1.8 ml of 0.8% ferrous sulfate in 0.015 N H_2SO_4. Color was developed by the addition of ammonium molybdate, and the absorbance at 660 nm was determined in a Beckman Acta CIII dual beam spectrophotometer. The production of inorganic phosphate was shown to be linear during the time of the assay. Residual activity is expressed as the ratio of the absorbance at 660 nm at the indicated time of reaction (E) to the absorbance at 660 nm at zero time (E_0).

Stoichiometry of Inactivation—Glutamine synthetase was incubated with radiolabeled 5′-FSO_2BzAdo as described above, except that the concentration of the enzyme was 1.0 mg/ml. At timed intervals, 1.5-ml portions of the reaction mixture were transferred to tubes containing 0.15 ml of 1.0 M sodium phosphate (pH 7.3). The sodium phosphate was included to react with the excess 5′-FSO_2BzAdo, thereby quenching the reaction of the enzyme with the analogue (17). The samples were immediately gel-filtered through Sephadex G-25 (2.5 × 10 cm), equilibrated with 0.01 M imidazole (pH 7.0), 10^{-4} M $MnCl_2$, and 10% EtOH. The absorbance at 232 nm was monitored, and the fractions containing the protein were pooled and dialyzed against the 0.01 M imidazole (pH 7.0), 10^{-4} M $MnCl_2$. The protein concentration was determined by the method of Lowry *et al.* (31) using bovine serum albumin in 0.01 M imidazole (pH 7.0), 10^{-4} M $MnCl_2$ as the standard.

The incorporation of 5′-FSO_2BzAdo was monitored by two methods. For determination of bound radioactivity, 0.5-ml aliquots of the gel-filtered protein pools were counted in an LKB BetaRack liquid scintillation counter. The incorporation was also measured spectrophotometrically using the equation derived by Shapiro and Stadtman (28) shown below.

$$X = 15.3\left(\frac{A_{260\text{ nm}}}{A_{290\text{ nm}}}\right) - 13.6 \quad (3)$$

where X represents the number of mol of adenosine bound/mol of dodecamer and $A_{260\text{ nm}}$ and $A_{290\text{ nm}}$ are the absorbances at 260 nm and 290 nm, respectively.

RESULTS

Inactivation of Glutamine Synthetase by 5′-FSO_2BzAdo—When glutamine synthetase was incubated with 5′-FSO_2BzAdo as described, the loss of catalytic activity obeyed pseudo-first order kinetics. The plot of the logarithm of residual catalytic activity *versus* the time of reaction with the analogue was linear (Fig. 1) as predicted by Equation 4 for a first order reaction.

$$\log (E/E_0) = -(k_{obs}/2.303) \times t \quad (4)$$

where (E/E_0) is the residual catalytic activity, k_{obs} is the observed first order rate constant of inactivation, and t is the time of reaction with the analogue.

The linear relationship described by Equation 4 allows for the determination of k_{obs} from the data in Fig. 1. The hyperbolic relationship between the value of k_{obs} and the concentration of 5′-FSO_2BzAdo shown in Fig. 2*A* is consistent with the formation of a Michaelis complex between the analogue and the enzyme prior to the covalent attachment of the 5′-FSO_2BzAdo to the enzyme, and the concomitant loss of catalytic activity. This is consistent with the mechanism described by Equation 5 below.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \quad (5)$$

where E is the enzyme, I is the 5′-FSO_2BzAdo, EI is the Michaelis complex of E and I, and EI^* is the inactive, covalently modified enzyme. A steady state treatment of this type of mechanism as described by Hixson and Krebs (16) yields the relationship in Equation 6.

$$(1/k_{obs}) = (K_I/k_2)(1/[I]) + (1/k_2) \quad (6)$$

In this equation, $K_I = k_{-1}/k_1$. Thus, a plot of $(1/k_{obs})$ *versus* (1/5′-FSO_2BzAdo) should be linear with an x intercept value of $-1/K_I$, and a y intercept value of $1/k_2$. Such a plot is shown in Fig. 2*B*. The values for K_I and k_2 are 0.224 mM and 0.232 min^{-1}, respectively.

Effect of Substrates on the Inactivation of Glutamine Synthetase by 5′-FSO_2BzAdo—To determine the effect of sub-

[2] W. Barry Foster, Claudia M. Noyes, and Henry S. Kingdon, manuscript in preparation.

strates on the observed rate of inactivation, reaction mixtures were prepared as described in Table I. As shown in this table, complete protection against inactivation was afforded by 10 mM ATP, 1.0 mM ATP, and 1.0 mM ADP. When 2.27 mM AMP was added to the reaction, the rate of inactivation was decreased. Glutamine was shown to exert little, if any, effect on the inactivation. When 1.0 mM glutamate was added to the reaction, the rate of inactivation was significantly enhanced. To examine this phenomenon further, the studies represented in Fig. 1 were repeated, except that each reaction mixture contained 0.1 M glutamate, with the concentrations of all other components as before. Treatment of these data in the same manner as used to construct Fig. 2*B* earlier yields values of K_I and k_2 of 0.183 mM and 0.375 min^{-1}, respectively. This decrease in the value of K_I suggests that glutamate enhances the binding of the 5'-FSO_2BzAdo to the enzyme as well as increasing the rate of covalent bond formation.

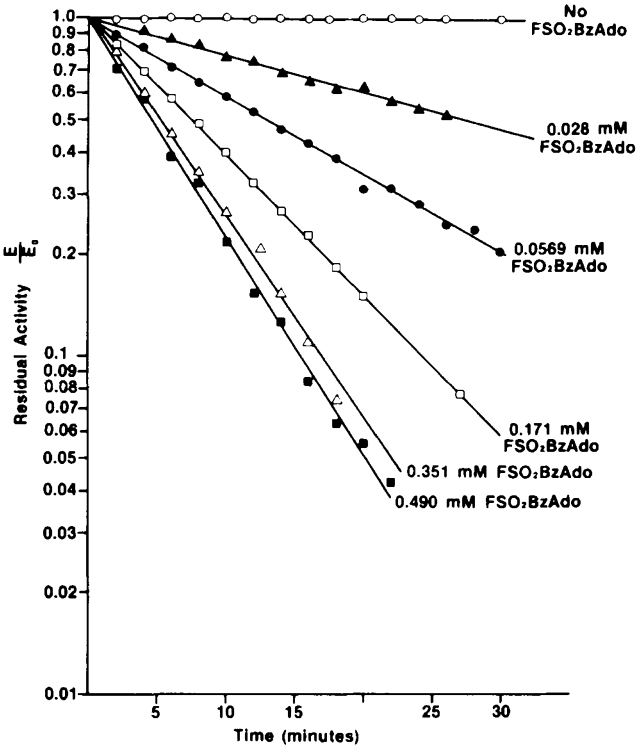


FIG. 1. **Inactivation of glutamine synthetase by 5'-FSO_2BzAdo.** Glutamine synthetase (0.385 mg/ml) was incubated with the indicated concentration of 5'-FSO_2BzAdo at 4°C in 0.2 M morpholinopropanesulfonic acid buffer (pH 7.3) containing 5.0 mM $MgCl_2$ and 10% Me_2SO/EtOH (1:1, v/v). The loss of catalytic activity was monitored as described under "Methods."

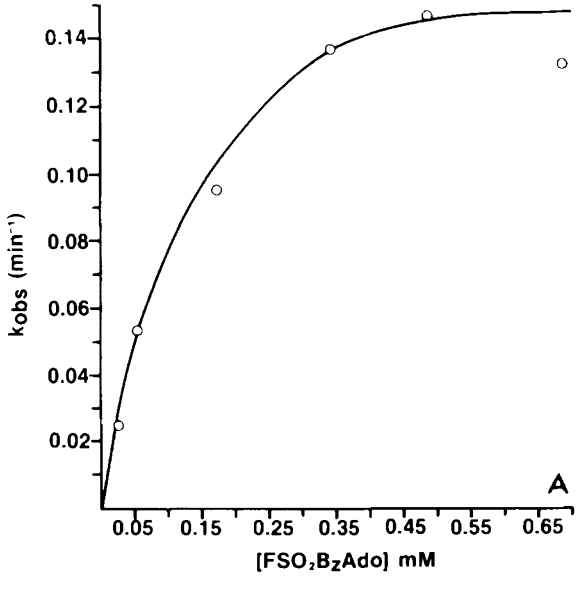
Competitive Binding of ATP and 5'-FSO_2BzAdo—To demonstrate that 5'-FSO_2BzAdo competes with ATP for the substrate binding site at the active center, the competition experiments shown in Fig. 3*A* were performed. These experiments involved the incubation of the enzyme with 5'-FSO_2BzAdo under assay conditions as described in Fig. 3*A*. The analysis of data is in accordance with the mechanism described by Equation 7.

TABLE I

Effect of substrates on the inactivation of glutamine synthetase by 5'-FSO_2 BzAdo

Glutamine synthetase (0.385 mg/ml) was incubated with 0.060 mM 5'-FSO_2BzAdo as described in Fig. 1, except for the indicated addition. The inactivation was followed as described under "Methods." The numbers represent the percentage of residual catalytic activity following incubation with the analogue for 13 min as determined by the biosynthetic assay reaction (Equation 1 in text).

Addition	Residual activity
None	42
10 mM ATP	100
1.0 mM ATP	98
1.0 mM ADP	100
1.0 mM L-glutamate	25
1.0 mM L-glutamine	49
2.27 mM AMP	74



$$\frac{1}{k_{obs}} = \frac{K_I}{k_2}\left(\frac{1}{[I]}\right) + \frac{1}{k_2}$$

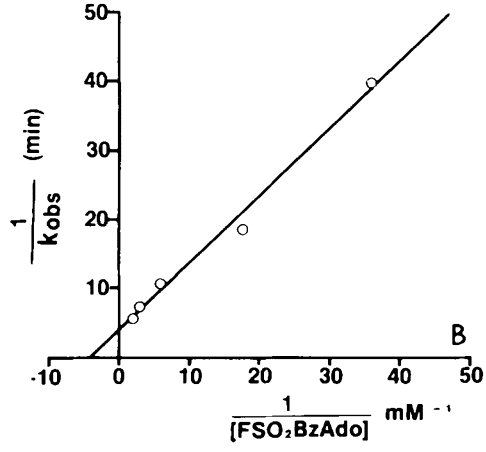


FIG. 2. **Dependence of k_{obs} on 5'-FSO_2BzAdo concentration.** Glutamine synthetase was incubated with 5'-FSO_2BzAdo as described in Fig. 1, and a value for k_{obs} was calculated as described in the text (Equation 4) for each concentration of the analogue used. *A*, k_{obs} is plotted *versus* the corresponding concentration of 5'-FSO_2BzAdo to demonstrate the dependence of k_{obs} on the analogue concentration. Concentrations of 5'-FSO_2BzAdo higher than those used to construct this figure were insoluble under the conditions of the reaction, and formed precipitates prior to the addition of enzyme. *B*, the data from *A* is replotted in double reciprocal form to allow determination of K_I and k_2 according to Equation 6 in the text.

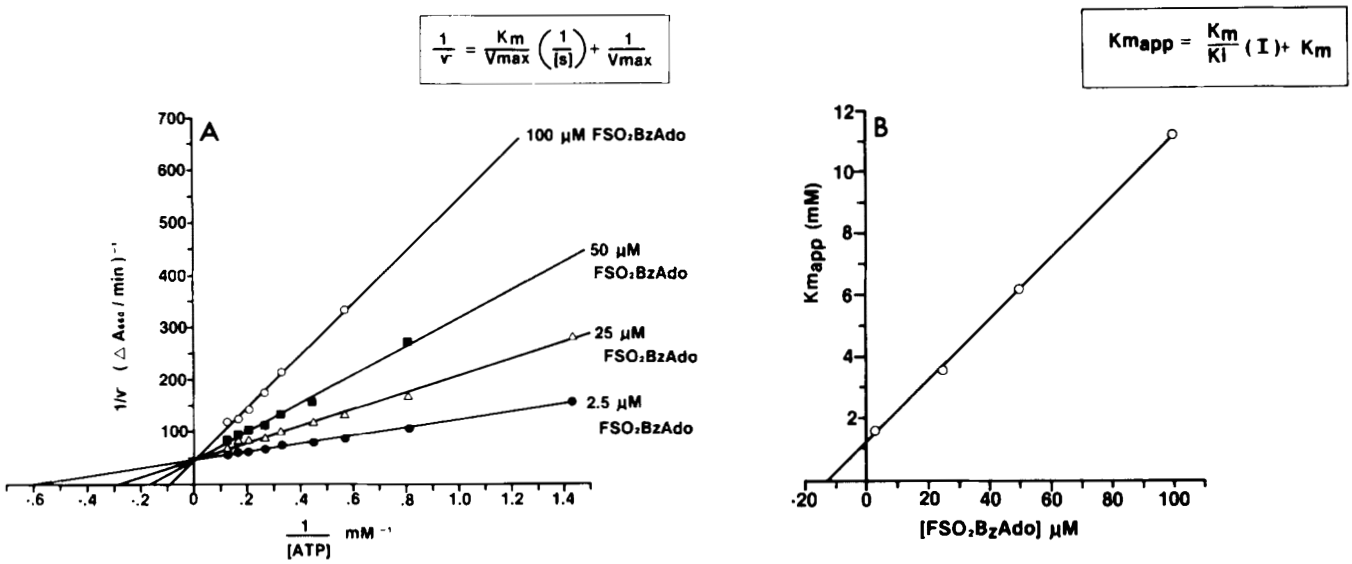


FIG. 3. **Competitive binding of 5'-FSO_2BzAdo and ATP to glutamine synthetase.** The Mg^{2+}-supported biosynthetic activity of glutamine synthetase was measured in the presence of various concentrations of ATP and 5'-FSO_2BzAdo. In addition to the enzyme, each assay tube contained 0.01 M L-glutamate (pH 7.0), 0.05 M NH_4Cl, 0.05 M $MgCl_2$, 0.05 M imidazole (pH 7.0), 10% Me_2SO/EtOH, plus the indicated concentration of ATP and 5'-FSO_2BzAdo. Reactions were carried out at 37°C. *A*, the initial velocity data is presented as a series of double reciprocal plots (according to Equation 8 in the text). The concentration of ATP is indicated in reciprocal form on the *x* axis, and the corresponding concentration of 5'-FSO_2BzAdo is indicated on the graph. *B*, $K_{m,app}$ values obtained from the data in *A* are replotted as described by Segel (35) to determine K_i.

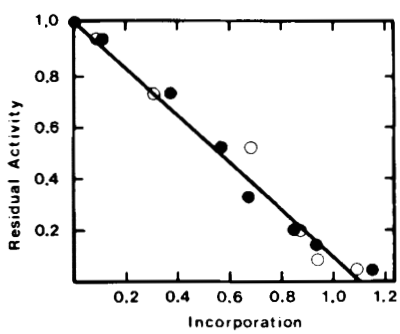


FIG. 4. **The stoichiometry of the inactivation of glutamine synthetase by 5'-FSO_2BzAdo.** Glutamine synthetase (1.0 mg/ml) was incubated with radiolabeled 5'-FSO_2BzAdo and treated as described under "Methods." The gel-filtered samples were analyzed for residual activity in the Mg^{2+}-supported biosynthetic assay (*y* axis), the incorporation of radioactivity (●), and the incorporation of the analogue into the enzyme as determined spectrophotometrically by the procedure described in the text (○). The units of the *x* axis are moles of analogue/mol of subunit.

$$\begin{array}{l} E + S \underset{}{\overset{K_d}{\longleftrightarrow}} ES \xrightarrow{k'_2} E + P \\ + \\ I \\ \downarrow K_i \\ EI \end{array} \qquad (7)$$

Here, E is the enzyme, S is the substrate (ATP), and I is the competitive inhibitor (5'-FSO_2BzAdo). The relationship between the velocity and substrate concentration in the presence of a competitive inhibitor can be described by Equation 8.

$$(1/v) = \left(\frac{K_{m,app}}{V_{max}}\right)\left(\frac{1}{S}\right) + (1/V_{max}) \qquad (8)$$

Thus in the presence of a competitive inhibitor, the apparent K_m, where $K_{m,app} = K_m(1 + ([I]/K_i))$ increases with increasing concentrations of the inhibitor, while there is no apparent effect on the V_{max}. Such is the case with 5'-FSO_2BzAdo and glutamine synthetase as shown in Fig. 3*A*. In order to determine a value of K_i relative to ATP, a replot of $K_{m,app}$ *versus* concentration of the analogue was constructed (Fig. 3*B*). From this graph, $K_i = 0.0125$ mM. The *y* intercept of the graph in Fig. 3*B* represents the value of K_m in the absence of the inhibitor. This value (1.20 mM) agrees closely with the experimentally determined value of K_m (1.13 mM) calculated from a double reciprocal plot of data obtained in the absence of the inhibitor (not shown).

Stoichiometry of Inactivation—The incorporation of 5'-FSO_2BzAdo was determined spectrophotometrically as described under "Methods." The data presented in Fig. 4 demonstrates that there is a direct correlation between the loss of enzymatic activity and the incorporation of the analogue. The incorporation of radioactivity also correlated well with the inactivation. Extrapolation of the data (Fig. 4) indicates that the complete loss of enzymatic activity was concomitant with the covalent incorporation of approximately 1.09 mol of 5'-FSO_2BzAdo/mol of the glutamine synthetase subunit.

DISCUSSION AND CONCLUSION

The inactivation of *E. coli* glutamine synthetase by 5'-FSO_2BzAdo proceeds via the formation of a reversible, noncovalent Michaelis complex between the enzyme and the analogue prior to covalent binding. The formation of this complex is enhanced by L-glutamate. The effect of L-glutamate is significant because the binding of L-glutamate and ATP has been shown by others (11) to involve strong synergism. Thus L-glutamate exerts an effect on the binding of the nucleotide analogue to the enzyme that is similar to its effect on the binding of the nucleotide substrate. The inactivation of glutamine synthetase by 5'-FSO_2BzAdo apparently results from the covalent attachment of the analogue to an amino acid residue at or near the ATP binding site of the active center, as demonstrated by the protection against inactivation afforded by ATP and ADP. Given the structure of 5'-FSO_2BzAdo, it seems likely that the covalent bond formed during the inactivation is located near the site of interaction of the terminal phosphate of ATP with the enzyme. The K_i for 5'-FSO_2BzAdo (0.224 mM) compares favorably with the dissociation constant for ATP (~0.2 mM) as determined by

fluorescence (11) and sedimentation (34) studies. Thus, in the absence of added substrates, the enzyme has approximately the same binding affinity for the analogue as for the substrate ATP. The value of K_i determined in the presence of substrate (0.0125 mM, Fig. 3*B*) is considerably lower than the corresponding value obtained in the absence of substrate (0.224 mM, Fig. 2*B*). Thus, although our data indicate that reaction with 5′-FSO_2BzAdo occurs at the active center of glutamine synthetase, the possibility that covalent modification could occur at some other site should not be overlooked. The resulting loss of activity could then arise from a conformational change in the region of the active center.

The inactivation of glutamine synthetase by 5′-FSO_2BzAdo results from the incorporation of approximately 1 mol of the analogue/mol of subunit. The loss of catalytic activity parallels the incorporation of the analogue. These results further indicate that the reaction between glutamine synthetase and 5′-FSO_2BzAdo occurs at a single site on the enzyme, presumably the ATP binding site.

Based on the results of the present study, 5′-FSO_2BzAdo can be used to probe further the active center of glutamine synthetase and its interaction with nucleotide substrates. Studies are presently underway to identify the amino acid residue modified by 5′-FSO_2BzAdo and to isolate and characterize a tryptic peptide containing this modified residue.

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