Granot, J., Kondo, H., Armstrong, R. N., Mildvan, A. S., & Kaiser, E. T. (1979) *Biochemistry 18*, 2339.
Granot, J., Mildvan, A. S., Hyama, K., Kondo, H., & Kaiser, E. T. (1980) *J. Biol. Chem. 255*, 4569.
Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5175.
Hartl, F. T., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 2347.
Hixson, C. S., & Krebs, E. G. (1979) *J. Biol. Chem. 254*, 7509.
Hoppe, J., Marutzky, R., Freist, W., & Wagner, K. G. (1977) *Eur. J. Biochem. 80*, 369.
Hoppe, J., Freist, W., Marutzky, R., & Shaltiel, S. (1978) *Eur. J. Biochem. 90*, 427.
Kupfer, A., Gani, V., Jiminez, J., & Shaltiel, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3073.
Leonard, N. J., Sprecker, M. A., & Morrice, A. G. (1976) *J. Am. Chem. Soc. 98*, 3987.
Leonard, N. J., Scopes, D. I. C., Van Der Lijn, P., & Barrio, J. R. (1978) *Biochemistry 17*, 3677.
Lowry, D. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 3743.
Nelson, N. C., & Taylor, S. S. (1983) *J. Biol. Chem. 258*, 10981.
Perrin, F. (1926) *J. Phys. Radium 7*, 390.
Perrin, F. (1929) *Ann. Phys. (Paris) 12*, 169.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691.
Rosevear, P. R., Bramson, H. N., O'Brian, C., Kaiser, E. T., & Mildvan, A. S. (1983) *Biochemistry 22*, 3439.
Roskoski, R., Jr. (1983) *Methods Enzymol. 99*, 3.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Schmidt, M. J., Truex, L. L., Leonard, N. J., Scopes, D. I. C., & Barrio, J. R. (1978) *J. Cyclic Nucleotide Res. 4*, 201.
Scopes, D. I. C., Barrio, J. R., & Leonard, N. J. (1977) *Science (Washington, D.C.) 195*, 296.
Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. L., Demaille, J. C., Fischer, E. H., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 848.
Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H., & Titani, K. (1983) *Biochemistry 22*, 3702.
Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J. 159*, 409.
Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A., & Titani, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2544.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Witt, J. J., & Roskoski, R., Jr. (1975) *Biochemistry 14*, 4503.
Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 8363.
Zoller, M. J., Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 10837.

5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine-Mediated Inactivation of *S*-Adenosylhomocysteinase†

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ABSTRACT: Rat liver *S*-adenosylhomocysteinase (EC 3.3.1.1) is inactivated by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine following pseudo-first-order kinetics. A plot of the apparent first-order rate constant for inactivation vs. the 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine concentration exhibits a hyperbolic curve indicative of the formation of a reversible enzyme–reagent complex prior to the inactivation. Values of 71.0 ± 7.7 μM and 0.14 ± 0.01 min^{-1} are estimated for K_d and k, respectively, at pH 8.25 and 25 °C. The substrate adenosine and a competitive inhibitor adenine completely protect the enzyme against inactivation. Values of dissociation constant for these ligands calculated from the protection experiments agree well with those obtained by other means, indicating that 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine competes with these ligands for the same binding site. The inactivation is not reversed by dialysis against phosphate buffer or tris(hydroxymethyl)aminomethane hydrochloride buffer, but a full enzyme activity is regained by treatment with dithiothreitol. The inactivation is not accompanied by covalent attachment of the reagent but is correlated with the loss of two sulfhydryl groups per enzyme subunit. Thus, the inactivation appears to result from a reagent-mediated formation of a disulfide between two cysteine residues in close proximity. The 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine-modified enzyme which is not capable of catalyzing the overall reaction can still catalyze the partial reactions such as the oxidation of the 3′-hydroxyl and the abstraction of the 4′-proton of adenosine.

S-Adenosylhomocysteinase (EC 3.3.1.1), which catalyzes the reversible hydrolysis of *S*-adenosylhomocysteine to adenosine and homocysteine, has been purified to apparent homogeneity from a variety of sources. The enzymes from mammalian sources are tetramers consisting of subunits with molecular weights of 45 000–48 000 (Schatz et al., 1979; Palmer & Abeles, 1979; Fujioka & Takata, 1981; Døskeland & Ueland, 1982) and, as far as examined, contain 4 mol of tightly bound NAD per mol of enzyme (Richards et al., 1978; Palmer & Abeles, 1979; Fujioka & Takata, 1981; Ueland, 1982a). The enzyme-bound NAD is essential for catalysis, and a mechanism of action of *S*-adenosylhomocysteinase is proposed that involves the oxidation/reduction of substrates by the coenzyme in the catalytic cycle (Palmer & Abeles, 1976, 1979).

Amino acid residues involved in the catalytic activity of rat liver *S*-adenosylhomocysteinase have been investigated in this laboratory. A histidine (Gomi & Fujioka, 1983), arginine

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0006-2960/84/0423-4357$01.50/0

(Takata & Fujioka, 1983), and two cysteine residues (Gomi & Fujioka, 1982) per enzyme subunit have been shown to be essential by means of chemical modification with diethyl pyrocarbonate, phenylglyoxal, and iodoacetamide, respectively. Although the protection afforded by the substrate against inactivation by these reagents suggests that they occur at or near the active site, no definitive conclusion can be drawn by the use of group-specific reagents. To clarify the point and also to obtain information on the topography of residues with respect to the bound substrate, it is desirable to use substrate analogues that covalently modify the active site residues. As one such reagent, we used 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine (FSBA)[1] in the present study. FSBA may be regarded as an analogue of ADP or ATP and has been used as an affinity label for a number of kinases, synthetases, and dehydrogenases (Colman, 1983). Although the substrates of *S*-adenosylhomocysteinase are not nucleotides, the compound was chosen because ADP and ATP are reported to be competitive inhibitors with respect to adenosine and *S*-adenosylhomocysteine (Ueland & Saebø, 1979) and the enzyme is known to interact reversibly or irreversibly with a number of *S*-adenosylhomocysteine analogues modified in the amino acid portion as well as with base- and sugar-modified analogues (Chiang et al., 1977; Ueland, 1982b). The results described herein show that FSBA rapidly inactivates *S*-adenosylhomocysteinase from rat liver, and this inactivation is correlated with the loss of two sulfhydryl groups per enzyme subunit.

Experimental Procedures

Materials. Adenosine, *S*-adenosyl-L-homocysteine, FSBA hydrochloride, and calf intestinal mucosa adenosine deaminase (EC 3.5.4.4) (type III) were purchased from Sigma Chemical Co., and adenine was from Kohjin Co., Tokyo. *p*-(Fluorosulfonyl)benzoyl chloride and *p*-(fluorosulfonyl)benzoic acid were products of Aldrich Chemical Co. [2-^{3}H]Adenosine (24 Ci/mmol) and titrated water (5 Ci/mL) were obtained from Amersham, and [2,8-^{3}H]adenosine (34.4 Ci/mmol) was from New England Nuclear. Other chemicals were of the highest purity available from commercial sources.

S-Adenosylhomocysteinase was purified to homogeneity from rat liver as described previously, and its concentration was determined by using a subunit molecular weight of 47 000 (Fujioka & Takata, 1981). Protein determination was made by the method of Lowry et al. (1951).

Radioactive FSBA was synthesized according to Wyatt & Colman (1977) with [2-^{3}H]adenosine. After recrystallization from dimethylformamide (Pal et al., 1975), the product was further purified by preparative thin-layer chromatography on silica gel 60 (PLC plate 13792, Merck) with chloroform/methanol (85:15 v/v) as the solvent (Boettcher & Meister, 1980). The purified preparation had a specific radioactivity of 5.2×10^5 cpm/μmol.

Reaction of S-Adenosylhomocysteinase with 5′-[p-(Fluorosulfonyl)benzoyl]adenosine. The reaction of *S*-adenosylhomocysteinase with FSBA was carried out at 25 °C in 20 mM sodium barbital/0.2 M KCl (pH 8.25), containing 10% dimethylformamide (v/v). The reaction was started by the addition of FSBA in dimethylformamide. Inclusion of dimethylformamide at a level of 10% was required to maintain the solubility of FSBA over the incubation period and had no effect on the activity of *S*-adenosylhomocysteinase. The extent of inactivation was monitored by measuring the residual enzyme activity in the direction of *S*-adenosylhomocysteine hydrolysis. An aliquot (10 μL) of the reaction mixture was added to 1.99 mL of 50 mM potassium phosphate (pH 6.9), containing 0.1 μmol of *S*-adenosylhomocysteine and 1.0 IU of adenosine deaminase, and the resulting decrease of absorbance at 265 nm due to the conversion of product adenosine to inosine was followed (Gomi & Fujioka, 1982). The reagent carried over to the assay mixture (<2.5 μM) did not interfere with the assay. With 2.5 μM FSBA, no detectable inactivation of *S*-adenosylhomocysteinase occurred in 50 mM potassium phosphate (pH 6.9) over a period of 30 min. The assay described above is complete within 3 min.

Determination of Sulfhydryl Groups. *S*-Adenosylhomocysteinase was incubated with FSBA as described above, and at intervals, aliquots of the reaction mixture were removed and freed from the reagent by the column gel centrifugation technique as described by Penefsky (1979). The eluates were assayed for protein, enzyme activity, and sulfhydryl content. The number of total sulfhydryl groups was determined by the reaction with DTNB. An aliquot (0.2 mL) of each eluate was added to 1.75 mL of 0.2 M Tris-HCl (pH 8.0), containing 0.51 M $(NH_4)_2SO_4$ and 0.23% sodium dodecyl sulfate, in a quartz cuvette. The reaction mixture minus enzyme was used as a blank. After the base line was recorded, 0.05 mL of 10 mM DTNB in 0.1 M Tris-HCl (pH 8.0) (freshly prepared) was added to each cuvette, and the change in absorbance at 412 nm was determined. The number of sulfhydryl groups was calculated by using a value of 13600 M^{-1} cm^{-1} as the molar absorptivity of thionitrobenzoate (Ellman, 1959).

Equilibrium Dialysis. The binding of adenosine to the FSBA-inactivated enzyme was determined by the equilibrium dialysis method. The equilibrium dialysis was carried out at 20 °C in 20 mM potassium phosphate (pH 8.0) with 24 μM FSBA-inactivated enzyme and 1–200 μM [2,8-^{3}H]adenosine. The inactivated enzyme was prepared by incubation with 0.1 mM FSBA, followed by gel filtration through Sephadex G-50 equilibrated and eluted with 50 mM potassium phosphate (pH 8.0). Details of the procedure are described previously (Gomi & Fujioka, 1982).

Synthesis of [4′-^{3}H]Adenosine. [4′-^{3}H]Adenosine was prepared by incubating adenosine with *S*-adenosylhomocysteinase in [^{3}H]H_2O as described by Palmer & Abeles (1979) and purified by chromatography on DEAE-cellulose paper (Gomi & Fujioka, 1982).

Other Analytical Procedures. Spectrophotometric determinations and recordings of absorption spectra were made with a Hitachi Model 320 spectrophotometer. Radioactivity was determined in an Aloka liquid scintillation spectrometer, Model LSC 903.

Results

Inactivation of S-Adenosylhomocysteinase by 5′-[p-(Fluorosulfonyl)benzoyl]adenosine. When incubated with 0.44 mM FSBA at 25 °C in 20 mM sodium barbital/0.2 M KCl (pH 8.25), containing 10% dimethylformamide, *S*-adenosylhomocysteinase was inactivated following pseudo-first-order kinetics. Whereas FSBA is reported to be somewhat unstable above pH 7.6 (Colman, 1983), under these conditions, the semilogarithmic plot of residual enzyme activity vs. time was linear for at least 30 min (not shown). The enzyme activity fell to 3.6% of the initial value in 30 min, and no measurable activity remained at 60 min. The enzyme incubated under the same conditions but in the absence of FSBA lost no activity. The inactivation was not reserved by dilution or by dialysis against 50 mM potassium phosphate buffer or Tris-HCl buffer between pH 7 and pH 8.

[1] Abbreviations: FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

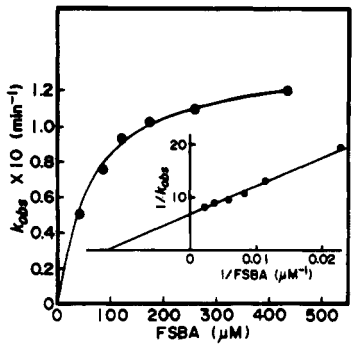

FIGURE 1: Dependence of the pseudo-first-order rate constant for inactivation on the FSBA concentration. *S*-Adenosylhomocysteinase (10.3 μM subunit) was incubated with various concentrations of FSBA as described under Experimental Procedures. Pseudo-first-order rate constants (k_{obsd}) were determined from semilogarithmic plots of residual activity vs. time. Solid lines are drawn from the calculated data.

The rate of inactivation as a function of FSBA concentration revealed a saturation kinetics (Figure 1), consistent with the idea that FSBA rapidly forms a reversible complex with the enzyme prior to the irreversible inactivation (eq 1), where

$$E + FSBA \underset{}{\overset{K_i}{\rightleftharpoons}} E\text{–}FSBA \xrightarrow{k} X \qquad (1)$$

E–FSBA is the reversible complex of enzyme with FSBA, X is the inactive form of the enzyme, K_i is the dissociation constant for FSBA from the reversible complex, and k is the first-order rate constant for inactivation. Under the circumstances, the observed rate constant for inactivation (k_{obsd}) is expressed as (Kitz & Wilson, 1962)

$$k_{obsd} = \frac{k}{1 + K_i/[FSBA]} \qquad (2)$$

Fit of the data of Figure 1 to eq 2 by a least-squares method yielded values of 71.0 ± 7.7 μM and 0.14 ± 0.01 min^{-1} for K_i and k, respectively.

Effect of Ligands on Inactivation. To show that FSBA binds and reacts at the active site, the effect of added adenosine and adenine on the rate of inactivation was studied. Adenine is a linear competitive inhibitor with respect to adenosine and *S*-adenosylhomocysteine and thus binds reversibly at the binding site for the nucleoside substrates. If FSBA is an active site directed reagent, it should compete with these ligands for the same binding site. For such competition, the observed rate of inactivation is described by

$$k_{obsd} = \frac{k}{1 + \frac{K_i}{[FSBA]}\left(1 + \frac{[L]}{K_d}\right)} \qquad (3)$$

where K_i and k are as defined earlier, L represents a ligand, and K_d is its dissociation constant from the *E*–ligand complex. *S*-Adenosylhomocysteinase (7 μM subunit) was incubated with 67 μM FSBA in the presence of adenine (0–250 μM). The rate of inactivation showed a progressive decrease with increasing concentration of adenine, and as predicted from eq 3, a plot of $1/k_{obsd}$ vs. the adenine concentration was a straight line (not shown). Direct fit of the experimental data to eq 3 by a least-squares method gave values of 70.5 ± 1.7 and 47.3 ± 2.3 μM for K_i and K_d, respectively. The value of K_d for adenine is in excellent agreement with that obtained by steady-state kinetic analysis with adenine as a competitive inhibitor of *S*-adenosylhomocysteine. A value of 47.6 ± 2.4 μM was found by the latter method in 50 mM potassium phosphate (pH 8.27) at 25 °C.

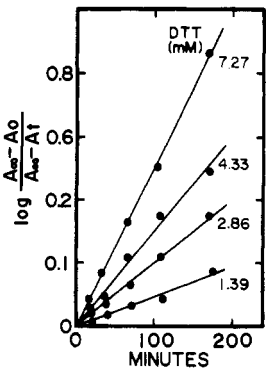

FIGURE 2: Reactivation of the FSBA-inactivated *S*-adenosylhomocysteinase with dithiothreitol (DTT). The enzyme (22.8 μM subunit) was incubated with 0.1 mM FSBA as described under Experimental Procedures. At 60 min, when the residual activity was 3% of the initial value, the reaction mixture was divided into aliquots, and each aliquot was incubated under nitrogen with DTT at the concentration indicated. The DTT concentration was corrected for a small decrease due to the reaction with unreacted FSBA on the assumption that 1 mol of FSBA had reacted with 1 mol of enzyme subunit. Lines are drawn by a least-squares linear regression.

S-Adenosylhomocysteinase binds adenosine tightly; an equilibrium binding study gave a K_d value[2] of about 0.8 μM at pH 8.0 (Gomi & Fujioka, 1982). As expected from the high affinity, the addition of adenosine in 5-fold molar excess over enzyme subunit showed an almost complete protection. Because of a low K_d, the amount of adenosine to be added to give fractional saturation should be less than or comparable to the enzyme concentration, and therefore, it is not possible to use the same approach as above to determine its K_d as a protector. However, the apparent first-order rate constants for inactivation were very close to those calculated under the assumption that adenosine and FSBA compete for the same site with K_d = 0.8 μM and K_i = 70.5 μM. In the presence of 10 and 20 μM adenosine, the observed rates were 0.016 and 0.008 min^{-1}, while the calculated values were 0.018 and 0.007 min^{-1}.

Reactivation of Inactivated Enzyme by Dithiothreitol. Upon incubation with 10 mM dithiothreitol at pH 8.3, the FSBA-inactivated enzyme (residual activity < 0.2%) showed recovery of enzyme activity. A recovery greater than 95% of the original activity was obtained within 10 h at 25 °C. Under the assumption that the original activity is regained after prolonged incubation, plots were made of log $[(A_\infty - A_0)/(A_\infty - A_t)]$ vs. time at several concentrations of dithiothreitol. (A_∞ and A_0 represent the original activity and the activity before reactivation, and A_t is the activity at time t.) As shown in Figure 2, all plots gave straight lines, the slopes of which were directly proportional to the concentration of the thiol. This indicates that the reactivation proceeds by a bimolecular reaction between the inactivated enzyme and the thiol. The second-order rate constant for reactivation was calculated to be 1.59 ± 0.01 min^{-1} M^{-1}.

Relationship between Inactivation and Number of Sulfhydryl Groups Disappeared. The results described above strongly suggest that the inactivation of *S*-adenosylhomocysteinase by FSBA involves the modification of cysteine residue(s). To test this possibility, the number of enzyme

[2] Because of the occurrence of partial reactions, the K_d does not represent the dissociation constant for adenosine from the *E*–adenosine complex. It is a function of the dissociation constant and equilibrium constants between enzyme species carrying the reaction intermediates.

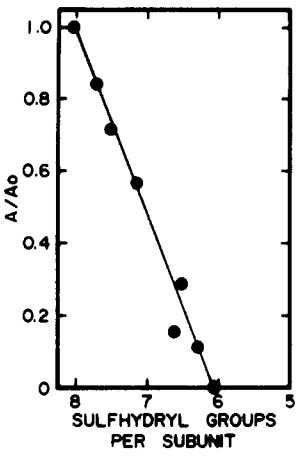

FIGURE 3: Correlation between the residual enzyme activity and the number of sulfhydryl groups disappeared. The experimental details are described under Experimental Procedures. The line is drawn by a least-squares linear regression.

sulfhydryl groups was determined as a function of inactivation. As shown in Figure 3, the extent of inactivation was linearly related to the disappearance of free sulfhydryl groups. Complete inactivation was accompanied by the loss of 1.90 ± 0.07 sulfhydryl groups per enzyme monomer. A separate experiment showed that the dithiothreitol-reactivated enzyme contained 7.7 sulfhydryls per subunit. The native subunit possesses 8 sulfhydryl groups (Fujioka & Takata, 1981).

The stoichiometry of the reaction of *S*-adenosylhomocysteinase with FSBA was examined with 5′-[*p*-(fluorosulfonyl)benzoyl][2-^{3}H]adenosine. The enzyme was inactivated by incubation with 0.1 mM [^{3}H]FSBA as described under Experimental Procedures, and the radioactivity associated with the enzyme was determined immediately after gel column centrifugation (Penefsky, 1979). However, no appreciable radioactivity above the background level was found in the protein fraction.

Properties of 5′-[p-(Fluorosulfonyl)benzoyl]adenosine-Inactivated Enzyme. Since the inactivation of *S*-adenosylhomocysteinase by FSBA is apparently not a result of covalent attachment of the reagent, the ability of the inactivated enzyme to bind substrate and to catalyze the partial reactions was determined. (The term "inactivated enzyme" refers to the enzyme that is not capable of catalyzing the overall reaction.) The equilibrium binding study with [^{3}H]adenosine as described under Experimental Procedures showed that the inactivated enzyme retained the capacity to bind the ligand. A Scatchard plot of the binding data yielded a straight line which extrapolated to 0.82 ± 0.03 mol of adenosine bound/mol of subunit (data not shown). The K_d value[2] was calculated to be 12.05 ± 0.75 μM. The comparative value for the native enzyme is 0.8 μM (Gomi & Fujioka, 1982).

Adenosine when bound to *S*-adenosylhomocysteinase undergoes a series of reactions ultimately to form 3′-keto-4′,5′-dehydroadenosine (Palmer & Abeles, 1979). The reaction is thought to be initiated by the oxidation of the 3′-hydroxyl by the enzyme-bound NAD, followed by the removal of the C′-4 proton. As with the native enzyme, the addition of adenosine to the FSBA-inactivated enzyme showed a concentration-dependent increase in absorbance at 327 nm, which is due to the reduction of the bound NAD (Palmer & Abeles, 1976, 1979; Gomi & Fujioka, 1982). The maximum change in absorbance obtained by extrapolation to infinite adenosine concentration was (7.79 ± 0.14) × 10^2 (M subunit)$^{-1}$ cm^{-1}, a value about 19% of that found with the native enzyme. The difference in molar absorptivity between the native and inactivated enzymes may be explained most simply by a shift of equilibrium among enzyme species carrying various reaction intermediates as a result of modification (Gomi & Fujioka, 1982).

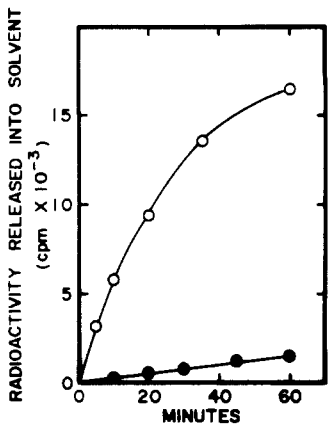

FIGURE 4: Exchange of the C′-4 proton of adenosine. The native (○) or FSBA-inactivated enzyme (●) (36 μg) was incubated with 0.42 mM [4′-^{3}H]adenosine (2.5 × 10^5 cpm/μmol) in 1.2 mL of 20 mM potassium phosphate (pH 8.0) at 25 °C. At times indicated, a 0.2-mL aliquot was removed from the reaction mixture and added to a test tube containing 10 μL of 1.0 M $HClO_4$. The mixture was then transferred to a 0.55 × 2.5 cm column of Dowex 50-X8, H^+ form, and the test tube and the column were washed with 3 mL of water. The radioactivity of the eluate was determined in a toluene scintillation liquid containing Triton X-100. The inactivated enzyme was prepared as described under Experimental Procedures.

The ability of the inactivated enzyme to carry out the exchange of the 4′-proton with solvent was tested with [4′-^{3}H]adenosine. The inactivated enzyme catalyzed the exchange reaction at a rate about 9% of that with the native enzyme (Figure 4).

Discussion

The adenine nucleotide analogue FSBA rapidly inactivates *S*-adenosylhomocysteinase in a pseudo-first-order fashion. The rate of inactivation as a function of FSBA concentration shows a saturation behavior, suggesting that the reagent binds reversibly to the enzyme before the irreversible inactivation. A rather low value of about 71 μM is estimated for the dissociation constant for FSBA from the reversible complex. This value is about 90 and 12 times greater than the values of K_d for adenosine and K_m for *S*-adenosylhomocysteine, but only 1.5-fold larger than the K_d for adenine. The importance of adenosine moiety in effecting the inactivation is seen by the lack of effect of *p*-(fluorosulfonyl)benzoic acid. The compound at 1 mM causes essentially no inactivation over a 60-min incubation period. The substrate adenosine and a competitive inhibitor adenine at high concentrations completely protect the enzyme against inactivation. Furthermore, the values of the dissociation constant for these ligand determined as protectors agree very well with those obtained by other means, indicating that FSBA competes with the ligands for the same binding site.

The argument presented above implies that FSBA is an active site directed reagent for *S*-adenosylhomocysteinase. However, incubation of the enzyme with radioactive FSBA does not lead to appreciable incorporation of the label, showing that the inactivation is not the result of covalent attachment of the reagent. The inactivation is linearly related to the loss of two sulfhydryl groups per monomer, and the inactivated enzyme is fully reactivated by dithiothreitol in a biomolecular process. The reactivation is accompanied by the regeneration of two sulfhydryl residues. Therefore, a probable mechanism

for the inactivation would be disulfide formation between two adjacent cysteine residues on the enzyme. FSBA first reacts in a rate-limiting step with an enzyme sulfhydryl group to form a thiosulfonate, which then undergoes a rapid attack by an adjacent cysteine residue to yield a sulfinic acid and a disulfide. Thiosulfonates are known to react readily with thiols to form disulfides (Parsons et al., 1965). A similar mechanism is proposed for the inactivation of rabbit muscle pyruvate kinase (Tomich et al., 1981; Annamalai & Colman, 1981), of myosin subfragment 1 (Togashi & Reisler, 1982), and of rabbit muscle phosphofructokinase (Ogilvie, 1983) by *p*-(fluorosulfonyl)-benzoyl derivative of nucleoside.

The equilibrium binding study indicates that the FSBA-inactivated *S*-adenosylhomocysteinase binds the substrate adenosine in a ratio of approximately 1 mol/mol of enzyme monomer, with an apparent K_d of 12 μM. As evidenced by the appearance of an absorption band near 330 nm on addition of adenosine, the inactivated enzyme retains the capacity to oxidize the 3′-hydroxyl of substrate (Palmer & Abeles, 1976, 1979). A slow but significant exchange of the 4′-proton of adenosine with solvent is also observed with the modified enzyme. The chemical mechanism proposed for the *S*-adenosylhomocysteinase-catalyzed reaction involves the oxidation of 3′-hydroxyl of adenosine or *S*-adenosylhomocysteine, followed by the abstraction of the 4′-proton. The resulting α-keto carbanion eliminates water or homocysteine to form 3′-keto-4′,5′-dehydroadenosine. Addition of homocysteine or water to this intermediate and the reversal of the pathway result in the formation of product. Thus, the failure of the FSBA-treated enzyme to catalyze the overall reaction would be due to the abolition of a step beyond the 4′-proton abstraction.

A previous investigation with the nonspecific reagent iodoacetamide has indicated that rat liver *S*-adenosylhomocysteinase has two cysteine residues per subunit, the alkylation of either of which results in total inactivation (Gomi & Fujioka, 1982). It is also shown that the inactive, carboxamidomethylated enzyme binds adenosine and catalyzes the oxidation of the 3′-hydroxyl but not the 4′-proton exchange reaction. Since adenosine completely protects the enzyme against inactivation, it is suggested that the cysteines are located at or near the nucleoside-binding site. The identity of these residues with the ones unraveled in the present study remains to be clarified.

Abeles et al. (1982) have recently reported that *S*-adenosylhomocysteinase from beef liver has two classes of adenosine-binding sites, residing on nonequivalent pairs of subunits, and only one pair participates in catalysis. This conclusion is based on the observations that, upon incubation with radioactive adenosine, an amount of radioactivity corresponding to 0.5 mol of adenosine/mol of subunit becomes tightly bound to the enzyme, and this radioactivity cannot be removed by gel filtration or even by incubation with unlabeled adenosine or adenosine plus homocysteine. Also, the enzyme with tightly bound adenosine is fully catalytically active. With rat liver *S*-adenosylhomocysteinase, however, our data show that each subunit is catalytically active. The equilibrium dialysis study with [^{3}H]adenosine indicates that the rat liver enzyme binds an amount of radioactivity corresponding to 4 equiv of adenosine in a noninteracting manner (Gomi & Fujioka, 1982). (Under the conditions, the partial reactions occur, and the free enzyme and enzyme forms with bound intermediates are in equilibrium.) An isotope trapping experiment such as the one described by Rose (1980) shows that almost all of the enzyme-bound radioactive adenosine is converted to *S*-adenosylhomocysteine in the presence of excess unlabeled adenosine and homocysteine, and no radioactivity remained on the enzyme after the conversion.[3] The results of the present investigation with FSBA are also consistent, with each subunit of rat liver *S*-adenosylcysteinase being functionally equivalent. The extent of inactivation is linearly related to the loss of two sulfhydryl groups per subunit with possible formation of a disulfide, and as seen by the pseudo-first-order loss of enzyme activity, there appears to be no interaction between subunits in the reaction with FSBA. It would be difficult to interpret these findings in terms of nonequivalent subunits.

In summary, the present work strongly suggests that rat liver *S*-adenosylhomocysteinase possesses two cysteine residues in close proximity at the active site region remote from the binding site for the adenine portion of substrates, and the integrity of these residues is crucial for catalysis.

Registry No. FSBA, 57454-44-1; adenosine, 58-61-7; adenine, 73-24-5; *S*-adenosylhomocysteinase, 9025-54-1.

References

Abeles, R. H., Fish, S., & Lapinskas, B. (1982) *Biochemistry 21*, 5557–5562.

Annamalai, A. L., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 10276–10283.

Boettcher, B. R., & Meister, A. (1980) *J. Biol. Chem. 255*, 7129–7133.

Chiang, P. K., Richards, H. H., & Cantoni, G. L. (1977) *Mol. Pharmacol. 13*, 939–947.

Colman, R. F. (1983) *Annu. Rev. Biochem. 52*, 67–91.

Døskeland, S. O., & Ueland, P. M. (1982) *Biochim. Biophys. Acta 708*, 185–193.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Fujioka, M., & Takata, Y. (1981) *J. Biol. Chem. 256*, 1631–1635.

Gomi, T., & Fujioka, M. (1982) *Biochemistry 21*, 4171–4176.

Gomi, T., & Fujioka, M. (1983) *Biochemistry 22*, 137–143.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Ogilvie, J. W. (1983) *Biochemistry 22*, 5915–5921.

Pal, P. K., Wechter, W. J., & Colman, R. F. (1975) *J. Biol. Chem. 250*, 8140–8147.

Palmer, J. L., & Abeles, R. H. (1976) *J. Biol. Chem. 251*, 5817–5819.

Palmer, J. L., & Abeles, R. H. (1979) *J. Biol. Chem. 254*, 1217–1226.

Parsons, T. F., Buckman, J. D., Pearson, D. E., & Field, L. (1965) *J. Org. Chem. 30*, 1923–1926.

Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.

Richards, H. H., Chiang, P. K., & Cantoni, G. L. (1978) *J. Biol. Chem. 253*, 4476–4480.

Rose, I. A. (1980) *Methods Enzymol. 64*, 47–59.

Schatz, R. A., Vunnam, C. R., & Sellinger, O. Z. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 143–154, Elsevier/North-Holland, New York.

Takata, Y., & Fujioka, M. (1983) *J. Biol. Chem. 258*, 7374–7378.

Togashi, C. T., & Reisler, E. (1982) *J. Biol. Chem. 257*, 10112–10118.

[3] T. Gomi and M. Fujioka, unpublished result.

Tomich, J. M., Marti, C., & Colman, R. F. (1981) *Biochemistry 20*, 6711–6720.
Ueland, P. M. (1982a) *Int. J. Biochem. 14*, 207–213.
Ueland, P. M. (1982b) *Pharmacol. Rev. 34*, 223–253.
Ueland, P. M., & Saebø, J. (1979) *Biochemistry 18*, 4130–4135.
Wyatt, J. L., & Colman, R. F. (1977) *Biochemistry 16*, 1333–1342.

Characterization of Sarcoplasmic Reticulum Adenosinetriphosphatase Purified by Selective Column Adsorption†

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ABSTRACT: Preparations of sarcoplasmic reticulum ATPase made by conventional procedures, with over 85% of the protein consisting of one band in sodium dodecyl sulfate gel electrophoresis, were solubilized in Triton X-100 and separated on an Affi-Gel blue column. All the ATPase activity was eluted in a single fraction containing about 60% of the applied protein. This purified fraction required combination with about 1 mol of fluoresceinyl 5-isothiocyanate (FITC) for inactivation, whereas the original preparation was inactivated by reaction with about 0.6 mol of FITC/mol. The inactive protein retained on the column had an amino acid composition like that of the active protein. The separation on the Affi-Gel blue column provides a convenient procedure for preparation of more active ATPase. The rate of inactivation of the ATPase solubilized in detergent-containing solutions was measured at different protein concentrations. The $t_{1/2}$ for inactivation was proportional to the square root of the protein concentration. Results are consistent with inactivation proceeding through a small fraction of monomeric ATPase present.

The Ca^{2+}- and Mg^{2+}-dependent sarcoplasmic reticulum (SR)[1] is composed of a single polypeptide chain with a M_r in the range of 100 000–120 000 (Martonosi & Halpin, 1971; Louis & Shooter, 1972; Thorley-Lawson & Green, 1973; Rizzolo et al., 1976; Dean & Tanford, 1978; MacLennan et al., 1978). The enzyme couples ATP hydrolysis to active transport of Ca^{2+} into the SR luminal space and is reported to comprise up to 90% of the membrane protein of the SR (Meissner, 1975; Fleischer & McIntyre, 1982). Numerous studies, utilizing various approaches, have shown that in the SR membrane an oligomeric form of the ATPase is the prevailing species [de Meis (1981) and references cited therein] although the monomeric enzyme is known to possess ATPase activity (Dean & Tanford, 1978; Kosk-Kosicka et al., 1983; Moller et al., 1980). The functional significance of the native ATPase oligomer is still a matter of controversy.

Recently, it was found that the SR ATPase is completely inactivated by binding 4.2 nmol of FITC/mg of protein (Pick & Karlish, 1980; Pick & Bassilian, 1980; Pick, 1981a,b). From these studies, it was suggested that the SR ATPase shows half-sites reactivity, i.e., that, due to strong interactions between the two subunits in an ATPase dimer, only one subunit is labeled with FITC at any given time. This conclusion was recently criticized by Mitchinson et al. (1982), who found that 0.89 mol of FITC was needed for complete inactivation of 1 mol of detergent-purified ATPase and concluded that a 1:1 stoichiometry exists between active ATPase and the inactivating agent.

Other studies from this laboratory indicated a possible functional role of enzyme dimers (McIntosh & Boyer, 1983). For further investigations, it appeared advantageous to attempt to obtain fully active enzyme through the aid of affinity chromatography. Suitability for affinity chromatography of enzyme dissolved by nonionic detergent was explored. We found, unexpectedly, that an inactive portion of the ATPase preparation could be selectively retained on a column of Affi-Gel blue. Such columns have been found to retain proteins with nucleotide binding sites (Stellwegen, 1977). This paper describes a simple procedure for the removal of inactive protein from SR ATPase preparation and presents studies with this preparation on the FITC inactivation and on the concentration dependency of inactivation of the preparation that give information about the dimeric state of the enzyme dissolved in detergent.

Materials and Methods

SR vesicles were isolated from rabbit skeletal muscle according to Champeil et al. (1978) and were stored at liquid nitrogen temperature in a medium containing 0.3 M sucrose, 0.1 M KCl, and 10 mM Hepes, pH 7.4. The specific ATPase activity of the SR vesicles was 1.3–1.5 μmol of P_i (mg of protein)$^{-1}$ min^{-1} and was determined at 25 °C in a medium containing 0.1 M KCl, 5 mM Mg^{2+}, 5 mM oxalate, 1 mM ATP, and 0.1 mM Ca^{2+} in 20 mM Mops buffer, pH 7.0. The basal ATPase activity (Ca^{2+} independent) was 25–40 times smaller than the Ca^{2+}-dependent activity.

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[1] Abbreviations: FITC, fluoresceinyl 5-isothiocyanate (isomer I); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; SR, sarcoplasmic reticulum; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-4362$01.50/0