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Inactivation of Rat Liver *S*-Adenosylhomocysteinase by Iodoacetamide†

Tomoharu Gomi and Motoji Fujioka*

ABSTRACT: *S*-Adenosylhomocysteinase (EC 3.3.1.1) from rat liver is inactivated by iodoacetamide following pseudo-first-order reaction kinetics. The apparent first-order rate constant for inactivation is proportional to the concentration of the modifier, and a value of 7.55 M^{-1} min^{-1} is obtained for the second-order rate constant at pH 9.06 and 25 °C. Amino acid analysis of the modified enzyme shows the formation of *S*-(carboxymethyl)cysteine. No peaks corresponding to N^{ϵ}-(carboxymethyl)- and N^{ϵ},N^{ϵ}-bis(carboxymethyl)lysines, *N*-(carboxymethyl)histidines, *S*-(carboxymethyl)homocysteine, homoserine, and homoserine lactone are detected. Glycolic acid is also not found in the acid hydrolysate of the modified enzyme, indicating the absence of modification at carboxyl residues. These results and the finding that the number of residues modified as determined by the incorporation of iodo[1-^{14}C]acetamide is equal to the number of cysteine residues lost by modification establish the site of modification as cysteine residues. Kinetics of inactivation and incorporation of the label from iodo[1-^{14}C]acetamide show that two among three modifiable residues per enzyme subunit are essential for activity and the modification of either results in complete inactivation. The inactivation by iodoacetamide does not involve alteration in the molecular size of enzyme nor release of the bound NAD^+. The modified enzyme still retains the capacity to bind adenosine and to oxidize it as evidenced by the reduction of enzyme-bound NAD^+ but does not catalyze the exchange of the 4′ proton with solvent. Thus, it is suggested that the inability of the modified enzyme to catalyze the overall reaction is due to the failure to abstract the 4′ proton in the catalytic cycle.

S-Adenosylhomocysteinase (EC 3.3.1.1), which catalyzes the cleavage and synthesis of the thioether bond of *S*-adenosyl-L-homocysteine, has been purified to homogeneity from a variety of sources (Ueland & Døskeland, 1977; Guranowski & Pawełkiewicz, 1977; Richards et al., 1978; Palmer & Abeles, 1979; Schatz et al., 1979; Chabannes et al., 1979; Kajander & Raina, 1981; Fujioka & Takata, 1981). The enzyme isolated from rat liver is a tetramer consisting of apparently identical subunits with M_r 47 000 (Fujioka & Takata, 1981). Like *S*-adenosylhomocysteinases from beef liver (Richards et al., 1978; Palmer & Abeles, 1976, 1979) and human placenta (Hershfield et al., 1979), the rat liver enzyme contains 1 mol of tightly bound NAD^+/mol of subunit, which is essential for activity.

The pathway of *S*-adenosylhomocysteinase-catalyzed reaction has been studied in detail by Palmer & Abeles (1979), who showed that *S*-adenosylhomocysteine and adenosine are first oxidized to 3′-keto derivatives by the enzyme-bound NAD^+,[1] followed by a series of reactions to yield 3′-keto-4′,5′-dehydroadenosine. Addition of water or L-homocysteine to this central intermediate and subsequent reactions that are the reversal of the reaction paths result in the formation of product. Although the reaction pathway is fairly well understood, little is known about the active site residues that participate in catalysis or binding of substrates. The only type of residue that appears to be essential for activity is cysteine. *S*-Adenosylhomocysteinases from yellow lupin seeds (Guranowski & Pawełkiewicz, 1977) and rat brain (Schatz et al., 1979) are shown to be inhibited by *p*-(chloromercuri)benzoate and *N*-ethylmaleimide. Preliminary studies in this laboratory have shown that the rat liver enzyme is also inhibited by a variety of sulfhydryl reagents including *p*-(chloromercuri)-benzoate, 5,5′-dithiobis(2-nitrobenzoate), iodoacetate, iodoacetamide, 4-(iodoacetamido)salicylate, and *N*-ethylmaleimide. Complete inhibition of the enzyme activity could be achieved by each of these reagents. The present paper describes the results of chemical modification studies with iodoacetamide and shows that the rat liver *S*-adenosylhomocysteinase contains

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[1] Abbreviations: NAD^+, nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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two sulfhydryl residues per subunit that are essential for catalysis, the alkylation of either of which results in total inactivation.

Experimental Procedures

Materials. Adenosine, *S*-adenosyl-L-homocysteine, calf intestinal mucosa adenosine deaminase (EC 3.5.4.4) (Type III), and yeast alcohol dehydrogenase (EC 1.1.1.1) were obtained from Sigma Chemical Co. Iodoacetamide (Wako Pure Chemicals, Osaka) was recrystallized from hot chloroform. Iodo[1-^{14}C]acetamide (53 mCi/mmol) and tritiated water (5 Ci/mL) were purchased from Amersham/Searle, and [2,8-$^3H_2$]adenosine (34.4 Ci/mmol) was from New England Nuclear. Other chemicals were of the highest grade available from commercial sources. Saccharopine dehydrogenase (L-lysine forming) (EC 1.5.1.7) was prepared from bakers' yeast by the method of Ogawa & Fujioka (1978). *S*-Adenosylhomocysteinase was purified to homogeneity from rat liver as described previously (Fujioka & Takata, 1981). The experiments described below were carried out with the homogeneous enzyme preparation.

Reaction of S-Adenosylhomocysteinase with Iodoacetamide. The solution of iodoacetamide in water was prepared before each experiment. The reaction of *S*-adenosylhomocysteinase with iodoacetamide was carried out in the dark in 0.1 M Tris-HCl buffer at 25 °C. The extent of modification was determined by measuring the residual enzyme activity. An aliquot (≤10 μL) of the reaction mixture was pipetted into an assay mixture (final volume 2.0 mL) containing 50 μM *S*-adenosyl-L-homocysteine and 1.0 IU of calf intestinal mucosa adenosine deaminase in 20 mM potassium phosphate buffer, pH 6.8, and the decrease of absorbance at 265 nm due to the conversion of product adenosine to inosine was followed. This assay is rather sensitive ($\epsilon = 8.1 \times 10^3$ M^{-1} cm^{-1}) and is linear with respect to time within the absorbance change of at least 0.05. Although the substrate *S*-adenosylhomocysteine contains groups capable of reacting with iodoacetamide (amino, thioether, and carboxyl groups), the reagent did not interfere with the assay at concentrations carried over to the assay mixture (≤50 μM). This is shown by an identical activity of the native enzyme in assay mixtures with and without 50 μM iodoacetamide. (The assay mixture with iodoacetamide was preincubated for 5 min in the absence of enzyme. The assay described above is complete in less than 2–3 min). Furthermore, the aliquot of reaction mixture treated with excess dithiothreitol to destroy iodoacetamide showed an identical activity with the untreated aliquot.

Amino Acid Analysis. *S*-Adenosylhomocysteinase (~1 mg) that had been inactivated completely by the reaction with 5 mM iodoacetamide at pH 9.06 was dialyzed extensively against distilled water in the dark. The sample was then lyophilized and hydrolyzed in 0.2 mL of 6 N HCl in an evacuated sealed tube at 110 °C for 22 h. Amino acid analysis was performed on an LKB 4400 amino acid analyzer. The sample treated similarly in the absence of iodoacetamide served as the control.

Incorporation of Radioactive Iodoacetamide into S-Adenosylhomocysteinase. *S*-Adenosylhomocysteinase (5.15 nmol) was incubated with 2.5 μmol of iodo[1-^{14}C]acetamide (1.0×10^6 dpm/μmol) in 0.5 mL of 0.1 M Tris-HCl buffer, pH 9.2. At appropriate times, aliquots were removed for measurements of the radioactivity fixed. The radioactivity incorporated into the protein was determined by the filter paper disk method of Bollum (1968). An aliquot (25 μL) of the reaction mixture was placed on a Toyo Roshi No. 51 filter paper disk (2.5 cm in diameter), and the disk was immediately immersed into 10% trichloroacetic acid. It was then washed 5 times with 50-mL portions of 10% trichloroacetic acid, twice with 50-mL portions of ethanol, and finally with 50 mL of ethyl ether. The radioactivity was determined in an scintillation counter with a toluene scintillation liquid.

Equilibrium Dialysis. The binding of adenosine to the native and iodoacetamide-inactivated enzyme was measured by the equilibrium dialysis method. [2,8-$^3H_2$]Adenosine used as the ligand was purified by paper chromatography just prior to use. Twenty microliters of [^{3}H]adenosine (100 μCi/mL) was diluted with an appropriate amount of unlabeled adenosine, and the mixture was applied to a sheet of DEAE-cellulose paper (Whatman DE-81) (4 × 19 cm). After ascending chromatography in 5 mM K_2HPO_4, the spot corresponding to adenosine was cut out, and [^{3}H]adenosine was eluted from the paper with 1.5 mL of 20 mM potassium phosphate buffer, pH 8.0. The concentration of adenosine was determined from the absorbance at 260 nm ($\epsilon = 1.51 \times 10^4$ M^{-1} cm^{-1}). The equilibrium dialysis was performed in an apparatus containing eight sets of dialysis cells. Each cell was separated into two chambers by a cellulose membrane (Visking 20/32 tubing) pretreated with 5% Na_2CO_3/10 mM EDTA at 100 °C for 5 min. The native or inactivated enzyme in 20 mM potassium phosphate buffer, pH 8.0 (0.2 mL), was placed on one side of the membrane, and an equal volume of the solution containing [2,8-$^3H_2$]adenosine (4.4×10^6 dpm/μmol) ranging from 1 to 120 μM in the same buffer was placed on the other side. The two solutions were allowed to equlibrate with gentle rocking for 5 h at 25 °C. During this period, the native enzyme lost no activity. Samples (0.15 mL) from each side were transferred to vials containing 0.35 mL of water, 5 mL of a scintillation fluid [toluene (667 mL), Triton X-100 (333 mL), 2,5-diphenyloxazole (5 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.4 g)] was added, and the radioactivity was measured in a liquid scintillation spectrometer. The amount of adenosine bound by the enzyme was calculated from the difference in radioactivity between two sides of the dialysis cell and the specific radioactivity of [^{3}H]adenosine.

Synthesis of [4′-^{3}H]Adenosine. [4′-^{3}H]Adenosine was prepared by incubating adenosine with *S*-adenosylhomocysteinase in ^3H_2O (0.5 Ci/mL) as described by Palmer & Abeles (1979). The product after separation from ^3H_2O was purified by paper chromatography as described above. [4′-^{3}H]Adenosine thus prepared had a specific activity of 1.2×10^7 dpm/μmol. When used in the exchange assay, it was diluted with unlabeled adenosine to a specific activity of 2.7×10^5 dpm/μmol.

Determination of Enzyme-Bound NAD$^+$ and NADH. The NAD$^+$ content of the enzyme was determined on a perchloric acid extract as described previously (Fujioka & Takata, 1981). The enzyme-bound NADH formed by the reaction of *S*-adenosylhomocysteinase with adenosine was determined as follows. A reaction mixture containing the enzyme and adenosine was added with 0.5 volume of ethanolic KOH (1 N) with vigorous shaking (Klingenberg, 1974). After being allowed to stand for 30 min at room temperature, the mixture was chilled on ice and the pH was adjusted to 8 with 0.5 M Tris-HCl buffer, pH 8.0, and 2 N HCl. Any precipitate formed was discarded by centrifugation. α-Ketoglutarate and L-lysine were then added to final concentrations of 2 and 10 mM, respectively. The NADH concentration was calculated from the change in absorbance at 340 nm after the addition of excess saccharopine dehydrogenase (L-lysine forming), which catalyzes the reductive condensation of α-ketoglutarate

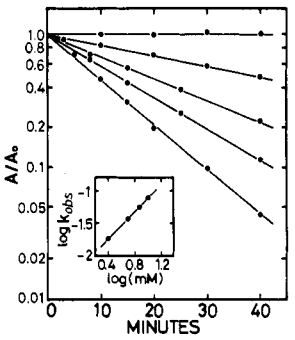

FIGURE 1: Time course of inactivation of *S*-adenosylhomocysteinase by iodoacetamide. The enzyme (2.1 nmol of subunit) was incubated with iodoacetamide in 0.1 mL of Tris-HCl buffer, pH 9.06, at 25 °C. The concentrations of iodoacetamide from top to bottom were 0, 2.5, 5.0, 7.5, and 10.0 mM. The activity measurements were made as described under Experimental Procedures. The activity corresponding to 100% was 0.84 μmol of adenosine formed min^{-1} (mg of protein)$^{-1}$.

and lysine to yield saccharopine in the presence of NADH (Nakatani et al., 1972).

Determination of Sulfhydryl Residues. The number of sulfhydryl residues remaining after iodoacetamide inactivation was determined by the method of Ellman (1959). After separation of excess iodoacetamide by gel filtration on a Sephadex G-25 column (1.8 × 12 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, the sulfhydryl content was determined with 5,5′-dithiobis(2-nitrobenzoate) in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.2% sodium dodecyl sulfate.

Other Analytical Procedures. Protein was determined by the method of Lowry et al. (1951). A subunit molecular weight of 47 000 for *S*-adenosylhomocysteinase (Fujioka & Takata, 1981) was used in all calculations. Radioactivity measurements were made in an Aloka liquid scintillation spectrometer, Model LSC 903. Spectrophotometric determinations and recordings of absorption spectra were made in a Hitachi 320 spectrophotometer.

Results

Inactivation by Iodoacetamide. Incubation of *S*-adenosylhomocysteinase with iodoacetamide in 0.1 M Tris-HCl buffer, pH 9.06, resulted in a time-dependent loss of enzyme activity. As shown in Figure 1, the inactivation followed pseudo-first-order reaction kinetics, the rate of which was linearly related to the reagent concentration. The plot of log (apparent first-order rate constant) vs. log [iodoacetamide] yielded a straight line with a slope of 1.02. The second-order rate constant for inactivation was calculated to be 7.55 M^{-1} min^{-1} at 25 °C from the same data. *S*-Adenosylhomocysteinase was also inactivated by iodoacetate. However, under the same conditions, iodoacetate was about 11 times less active than iodoacetamide. Apparently, a negative charge on the reagent makes it difficult to gain access to the residues being attacked.

Identification of Amino Acid Residue Modified by Iodoacetamide. The amino acid analysis of *S*-adenosylhomocysteinase that had been inactivated completely by iodoacetamide revealed a new peak absent in the native enzyme. The peak, 2.7 mol/mol of subunit in amount, was eluted just ahead of aspartic acid at the position expected for *S*-(carboxymethyl)cysteine. In addition to the cysteine residue, halo acids and their amides are known to alkylate lysine, histidine, methionine, and carboxyl residues in protein (Grundlach et al., 1959). The alkylation of lysine, histidine, and methionine, however, appears to be absent, since no peaks corresponding

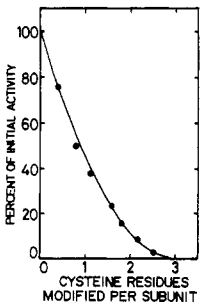

FIGURE 2: Correlation between residual enzyme activity and number of residues modified. The number of residues modified was determined by incorporation of iodo[1-^{14}C]acetamide. The solid line was calculated from eq 1 with $n = 3$ and $i = 2$. The experimental conditions are given under Experimental Procedures.

to N^{ϵ}-(carboxymethyl)- and N^{ϵ},N^{ϵ}-bis(carboxymethyl)lysines, *N*-(carboxymethyl)histidines, *S*-(carboxymethyl)homocysteine, homoserine, and homoserine lactone were detected in amino acid analysis even with a sample 10 times the amount that is appropriate for measuring total amino acid composition. Also, the total numbers of lysine, histidine, and methionine residues did not vary by modification.

Naider & Bohak (1972) have shown that the methionine residue is readily regenerated from its carboxamidomethyl derivative in peptide and protein by treatment with various sulfur nucleophiles. When *S*-adenosylhomocysteinase that had been inactivated with iodo[1-^{14}C]acetamide was incubated with 0.12 M 2-mercaptoethanol for 24 h at pH 8.6 and 30 °C (Naider & Bohak, 1972), no radioactivity was released from the enzyme. The enzyme activity was also not recovered by this treatment. These observations further exclude the possibility of methionine modification.

The reaction of iodoacetamide with the carboxyl group yields the carboxamidomethyl ester. The resulting ester is labile to acid and is hydrolyzed to the parent acid, glycolic acid, and ammonia. This precludes the use of conventional amino acid analysis to detect the modified residue (Takahashi et al., 1967). To test the possibility that the inactivation is due to the modification of glutamic or aspartic acid residue, we examined the presence of glycolic acid in acid hydrolysate of the modified enzyme. *S*-Adenosylhomocysteinase was inactivated with iodo[^{14}C]acetamide, and an acid hydrolysate was prepared as for amino acid analysis (see Experimental Procedures). After evaporation of the HCl, the residue was dissolved in 0.2 M citrate buffer, pH 2.2, and put on a column of Dowex 50-X8, H^+ form. No radioactivity was eluted with water, indicating the absence of glycolic acid.

Relationship between Inactivation and Number of Residues Modified. The relationship between loss of enzyme activity and number of residues modified was examined by incorporation of the label into the protein from iodo[1-^{14}C]acetamide. Since iodoacetamide is introduced into the protein in an acid-stable manner (see above), we have adopted the method of Bollum (1968), which uses trichloroacetic acid, to remove the unreacted reagent. *S*-Adenosylhomocysteinase was incubated with 5 mM iodo[1-^{14}C]acetamide at pH 9.2, and at appropriate times, aliquots were removed for measurements of enzyme activity and radioactivity fixed. Figure 2 shows a plot of residual activity against the number of residues modified. As the figure shows, the modification of three residues per subunit resulted in complete inactivation. So that the total number of residues modifiable by iodoacetamide could be obtained, incubation was continued for a prolonged time after the enzyme activity had been completely lost. No further incorporation of the label was observed, however, indicating

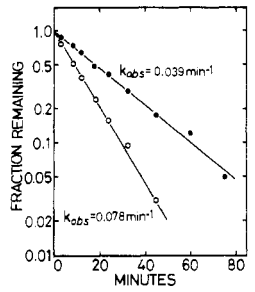

FIGURE 3: Residual enzyme activity (○) and fraction of modifiable residues remaining (●) as a function of time. The number of residues modified was determined by incorporation of iodo[1-^{14}C]acetamide as described under Experimental Procedures. The number of modifiable residues is taken as 3.

that only three residues can be modified under these conditions. Separate experiments showed that the iodoacetamide-inactivated enzyme contained 4.70 sulfhydryl groups per subunit titratable with 5,5′-dithiobis(2-nitrobenzoate) under denaturing conditions. Since 7.63 sulfhydryls are found in the native subunit, the result indicates that three sulfhydryls are lost by iodoacetamide treatment. The agreement of this value with that obtained by incorporation of the radiolabel and the number of (carboxymethyl)cysteine residues in amino acid analysis and the results presented in the previous section firmly establish that only cysteine residues are modified by iodoacetamide.

Although extrapolation of the initial portion of the curve in Figure 2 to zero enzyme activity shows that the modification of two residues is required for inactivation, this method of determining the number of essential residues is usually not valid (Tsou, 1962; Horiike & McCormick, 1979). For determination of the number of essential residues, kinetics of inactivation and modification were examined. Figure 3 shows plots of residual activity and fraction of modifiable residues remaining as a function of time. Both plots followed pseudo-first-order kinetics, the rate of inactivation being twice that of modification. The pseudo-first-order kinetics observed for modification indicates that all three modifiable residues are equally reactive toward the reagent. Therefore, the fact that the rate constant for inactivation is exactly twice that for modification is consistent with the contention that two residues are essential for activity and the modification of either results in total inactivation (Ray & Koshland, 1961). When all of n modifiable residues are equally reactive but only i residues are essential for activity, the number of residues modified at a given stage of modification, m, is related to the residual activity by (Tsou, 1962)

$$m = n[1 - (A/A_0)^{1/i}] \quad (1)$$

The solid line in Figure 2 calculated from eq 1 with $n = 3$ and $i = 2$ shows a satisfactory fit to the experimental data.

Lack of Effect of Modification on NAD$^+$ Content and Molecular Size of Enzyme. Rat liver *S*-adenosylhomocysteinase contains 1 mol of tightly bound NAD$^+$/mol of subunit, which is essential for catalytic activity (Fujioka & Takata, 1981). Although the NAD$^+$ is not removed by gel filtration or dialysis against neutral buffer, it is not covalently bound. Therefore, the possibility may be considered that the modification of cysteine residues results in weakening of the binding force and eventually in dissociation of the coenzyme. This possibility was examined by measurement of the NAD$^+$ content after modification. Reaction of a neutralized perchloric acid extract of the inactivated enzyme with ethanol and alcohol dehydrogenase yielded a compound that was indistinguishable spectrophotometrically from NADH. Calculation from the molar absorbance at 340 nm gave a value of 0.97 mol of NADH/mol of subunit.

Table I: Effect of Adenosine and Adenine on Inactivation[a]

addition to reaction mixture	residual activity (%)
none	45
adenosine (2.5 μM)	52
adenosine (5.0 μM)	59
adenosine (10.0 μM)	72
adenosine (25.0 μM)	96
adenine (0.25 mM)	66
adenine (1.0 mM)	71
adenine (2.0 mM)	70

[a] *S*-Adenosylhomocysteinase (1.72 nmol of enzyme subunit) was preincubated with the compounds at the concentration indicated in 0.09 mL of 0.1 M Tris-HCl buffer, pH 9.06. Iodoacetamide (0.5 μmol, 10 μL) was then added, and the mixture was incubated for 20 min at 25 °C. The residual activity represents the percentage of activity obtained by comparison with that of the uninhibited control.

The iodoacetamide-inactivated enzyme was eluted from a Sephadex G-200 column as a single, symmetrical peak, at the position expected for the native enzyme. The recovery of protein after chromatography was 92% (data not shown).

Thus, the results described above clearly indicate that inactivation by iodoacetamide does not involve release of the bound NAD$^+$ or dissociation of the enzyme into subunits or aggregation to a higher molecular weight substance.

Effect of Adenosine and Adenine on Inactivation. Incubation of *S*-adenosylhomocysteinase with iodoacetamide in the presence of adenosine or adenine, a competitive inhibitor with respect to adenosine or *S*-adenosylhomocysteine, retarded the rate of inactivation (Table I). The concentrations of adenosine used in the experiments of Table I are less than or comparable to that of the enzyme (17.2 μM enzyme monomer). When [adenosine] < [enzyme monomer], the degree of inactivation was very close to that calculated on the assumption that only the free enzyme reacts with the reagent. Indeed, at a high concentration of adenosine (25 μM), an almost full protection was obtained. In contrast to adenosine, adenine afforded only a partial protection even at a concentration as high as 2 mM (about 14 times the K_i value determined kinetically). The partial protection observed with adenine implies that the cysteines are not located within the binding site for the adenine portion of substrates. The finding that adenosine affords complete protection rather suggests that they are in the vicinity of the binding site for the ribose moiety.

Properties of Modified Enzyme. Although *S*-adenosylhomocysteinase is protected against inactivation by the substrate adenosine, the enzyme rendered inactive in catalyzing the overall reaction by iodoacetamide still retained the capacity to bind adenosine as shown by equilibrium dialysis studies. A Scatchard plot of the binding data yielded a straight line with approximately one binding site per subunit and an apparent K_d of 13.5 μM (Figure 4B). The value of K_d for the native, unmodified enzyme determined under the same conditions was 0.8 μM (Figure 4A).

It has been proposed that adenosine is oxidized to the 3′-keto derivative by the enzyme-bound NAD$^+$, followed by the removal of a proton from the C′-4 position to form an α-ketocarbanion that is converted to 3′-keto-4′,5′-dehydroadenosine (Palmer & Abeles, 1979). Since the iodoacetamide-inactivated enzyme stilll binds adenosine, the effect of modification on these catalytic events was examined. As with the native enzyme, the addition of adenosine to the modified, inactivated enzyme caused a concentration-dependent increase in absor-

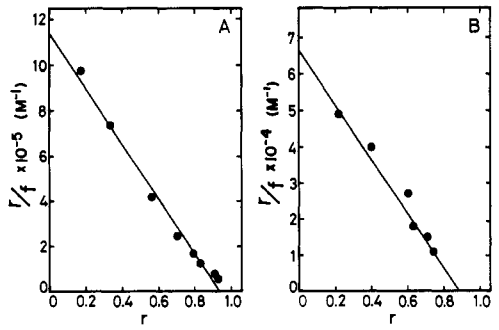

FIGURE 4: Equilibrium dialysis of native (A) and iodoacetamide-modified (B) enzymes. The experimental conditions are described under Experimental Procedures. The concentrations of native and modified enzymes were (in terms of subunit) 10.1 and 8.4 μM, respectively. r represents the number of moles of adenosine bound per mole of enzyme subunit and f the concentration of free adenosine. The lines were drawn by a least-squares linear regression. The iodoacetamide-modified enzyme was prepared by incubating *S*-adenosylhomocysteinase with 5 mM iodoacetamide at pH 9.2 and 25 °C for 120 min, followed by gel filtration over a Sephadex G-25 column (1.8 × 12 cm) to remove excess reagent.

Table II: Contents of NAD^+ and NADH after Addition of Adenosine[a]

	mol/mol of enzyme subunit		
	NAD^+	NADH	NAD^+ + NADH
native enzyme	0.16	0.68	0.84
iodoacetamide-modified enzyme[b]	0.49	0.33	0.82

[a] The native enzyme (13.5 nmol of enzyme subunit) and iodoacetamide-modified enzyme (12.8 nmol of enzyme subunit) were incubated with 0.19 mM adenosine in 2.67 mL of 20 mM potassium phosphate buffer, pH 8.0, at 25 °C. After 60 min, aliquots of the reaction mixture were determined for NAD^+ and NADH as described under Experimental Procedures. All determinations were made in duplicate or triplicate, and the average values are given in the table. [b] The iodoacetamide-modified enzyme was prepared as described in Figure 4.

bance at around 330 nm due to the formation of NADH (Palmer & Abeles, 1976, 1979; Fujioka & Takata, 1981). The maximum change in absorbance obtained by extrapolation to infinite adenosine concentration was 2.1×10^3 (M subunit)$^{-1}$ cm^{-1}, a value about 50% of that found with the native enzyme. Direct measurement of NADH after the addition of a saturating concentration of adenosine shows that this value accounts well for the amount of NADH formed (Table II). Thus, a decreased absorbance obtained with the modified enzyme is due to the formation of less NADH rather than to a change in the local environment of the coenzyme site upon modification.

The ability of the modified enzyme to catalyze the exchange of the 4′ proton with solvent was tested with [4′-^{3}H]adenosine as a substrate. Essentially no radioactivity was removed from the substrate by incubation with the modified enzyme even when the amount of the enzyme was increased 10-fold over that of the native enzyme (Figure 5).

The results described above indicate that the modification of essential cysteine residues does not prevent the binding of substrate and suggest that the inability of the modified enzyme to catalyze the overall reaction is due to the loss of ability to abstract the 4′ proton in the catalytic cycle.

Discussion

Iodoacetamide inactivates *S*-adenosylhomocysteinase rather rapidly at high pH in a pseudo-first-order fashion. The data

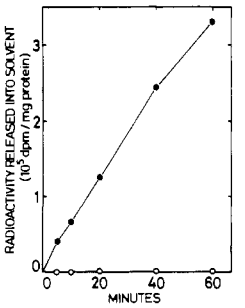

FIGURE 5: Exchange of C′-4 proton of adenosine. The native enzyme (●) (2.86 μg) or iodoacetamide-modified enzyme (○) (26.7 μg) was incubated with [4′-^{3}H]adenosine (0.39 mM, 2.7×10^5 dpm/μmol) in 1.1 mL of 20 mM potassium phosphate buffer, pH 8.0, at 25 °C. At the times indicated, a 0.2-mL aliquot was removed from the reaction mixture and added to a test tube containing 10 μL of 1.0 M $HClO_4$ (0 °C). The mixture was then transferred to a 0.55 × 2.4 cm column of Dowex 50-X8, 200–400 mesh, H^+ form, and the test tube and column were washed with 3 mL of water. The radioactivity of the eluate was determined in a scintillation liquid containing Triton X-100. The modified enzyme was prepared as described in Figure 4.

presented in this paper rule out the possibility of modification of lysine, histidine, methionine, and carboxyl residues and support the conclusion that inactivation is due to the alkylation of cysteine residues.

Incorporation of the label from iodo[^{14}C]acetamide into the enzyme and the number of sulfhydryl groups lost by modificatioin clearly show that three out of eight cysteine residues present per subunit (Fujioka & Takata, 1981) are susceptible to modification at pH 9.2. These three residues are equally reactive toward iodoacetamide as shown by the kinetics of incorporatioin of the label (Figure 3). The pseudo-first-order loss of enzyme activity shows the inactivation proceeds in an all-or-none fashion. When modification of more than one residues is required for complete inactivation, i.e., partially active enzyme species are present, a semilog plot of residual activity vs. time would show a curvature that is either concave up or concave down (Ray & Koshland, 1961). Therefore, under the circumstances, the fact that the rate constant for inactivation is exactly twice that for modification (Figure 3) indicates that two residues are involved in enzyme activity and the modification of either of which results in complete inactivation. If three residues are essential (in the sense that the modificatioin of any one abolishes the enzyme activity), the value of the inactivation rate constant will be 3 times that of modification, and the presence of only one essential residue will give an identical rate constant. The reaction order of unity with respect to iodoacetamide (Figure 1, inset) shows that 1 mol of the reagent reacts with a residue but does not mean that one residue is essential (Ray & Koshland, 1961).

The modification of essential cysteine residues does not result in alteration in the molecular size of enzyme or in release of the bound NAD^+. Therefore, it is concluded that these residues are involved in the catalytic function of the enzyme. The complete protection against inactivation afforded by adenosine strongly suggests that these residues are located at or near the active site, but their modification does not prevent the binding of substrate as shown by the equilibrium dialysis study. As shown by Palmer & Abeles (1979), adenosine when bound to *S*-adenosylhomocysteinase undergoes a series of reactions ultimately to form 3′-keto-4′,5′-dehydroadenosine. During this conversion, the bound NAD^+ is reduced to NADH. Measurement of NAD^+ and NADH contents of the native enzyme that has been incubated with an excess of adenosine shows that about 81% of the coenzyme is reduced (Table II). However, when an aliquot of the incubation

mixture is diluted into an assay mixture containing *S*-adenosylhomocysteine and adenosine deaminase, the enzyme is almost fully active in catalyzing the overall reaction.[2] Since the bound NAD^+ is required to initiate catalysis, these findings indicate that various intermediates remain bound to the enzyme, and an equilibrium is established between free adenosine, free enzyme, and various enzyme species carrying intermediates. In this situation, when [2,8-$^3H_2$]adenosine is used as the ligand, a Scatchard plot would yield a straight line that intersects the abscissa at a point corresponding to the number of binding sites, but the K_d is a function of equilibrium constants of various steps. Therefore, from apparent K_d values affinities of the ligand to the native and modified enzyme cannot be compared. (With the modified enzyme, the number of intermediates might also be different.)

The iodoacetamide-modified enzyme apparently catalyzes the oxidation of the 3′-hydroxyl of adenosine as seen by the reduction of enzyme-bound NAD^+, but less NADH is produced in the modified enzyme than in the native enzyme (Table II). It is quite possible that introduction of a substituent to the cysteine residue or abolition of certain catalytic step(s) results in alteration in the equilibrium positions of various intermediates.

The failure of the modified enzyme to catalyze the 4′-proton exchange (Figure 5) suggests that the essential sulfhydryls are involved in some way in the proton abstraction reaction. However, it is unlikely that these residues act as base catalysts in view of their high p*K* values. The rate constant for inactivation increased progressively with increasing pH between pH 7 and pH 10 (data not shown). Since monohalo acids and their amides react only with the unprotonated form of a residue (Smythe, 1936; Hagen, 1956), the result shows that essential residues have high p*K* values. There is no example, to our knowledge, in which a sulfhydryl group acts as a base catalyst. It is also difficult to imagine how two residues participate in a single catalytic event. One possibility for the role of the cysteine residues is that each is located close enough to a functional base group critical for the proton abstraction and the modification of one of these abolishes the function of the latter by steric reason or by a local conformational change.

[2] It has been shown that *S*-adenosylhomocysteinases from human placenta, human lymphoblast (Hershfield, 1979; Hershfield et al., 1979), and beef liver (Chiang et al., 1981) are subject to irreversible inactivation by adenosine. In contrast, the rat liver *S*-adenosylhomocysteinase undergoes a very slow inactivation by adenosine; only 9% inactivation is observed by incubation with 1 mM adenosine for 6 h at pH 7.2 and 25 °C. Under the conditions of Figure 4 and Table II, no appreciable loss in enzyme activity was noted.

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