S-Adenosylhomocysteinase from Rat Liver

EVIDENCE FOR STRUCTURALLY IDENTICAL AND CATALYTICALLY EQUIVALENT SUBUNITS

Tomoharu Gomi, Yoshihisa Ishiguro, and Motoji Fujioka

From the Department of Biochemistry, Toyama Medical and Pharmaceutical University Faculty of Medicine, Sugitani, Toyama 930-01, Japan

Lines of evidence are presented which indicate that rat liver S-adenosylhomocysteinase consists of four identical or nearly identical subunits. Cross-linking of the enzyme with dimethyl suberimidate followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis yields four distinct protein bands with molecular weights 47,000, 93,000, 145,000, and 190,000. The molecular weight of the largest protein is in excellent agreement with that of the native enzyme. Carboxypeptidase A liberates 4 mol of COOH-terminal tyrosine/mol of enzyme, and the number of arginine-containing peptides in a trypic digest of the enzyme is one-fourth of that arginine residues present in the enzyme. The enzyme reversibly binds 4 mol of the substrate adenosine in a noninteracting manner, and the binding is associated with the reduction of 3.2 mol of enzyme-bound NAD*. However, in the presence of dithiothreitol, the same compound causes a time-dependent irreversible loss of enzyme activity concomitant with the formation of 3.6 mol of enzyme-bound NADH/mol of enzyme. Studies with adenine-labeled adenosine shows that radioactivity corresponding to 3.8 mol of substrate is tightly bound to the inactivated enzyme. Since the inactivation is apparently the consequence of reaction of dithiothreitol with an enzyme-bound intermediate as revealed by the kinetics of inactivation, these results support the conclusion that the four subunits of rat liver S-adenosylhomocysteinase are functionally equivalent.

S-Adenosylhomocysteinase (EC 3.3.1.1) catalyzes the reversible hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine. The enzyme has been purified to homogeneity from a variety of sources including various mammalian tissues (1-6), yellow lupin seed (7), and Alcaligenes faecalis (8) and shown to be an oligomeric protein. While the plant and bacterial enzymes have dimeric and hexameric structures, respectively, it is generally accepted that mammalian S-adenosylhomocysteinas are tetromers (1-4, 6).

Previous work from this laboratory has shown that S-adenosylhomocysteinase purified from rat liver has a molecular weight of 188,000-190,000 and contains 4 mol of tightly bound NAD*/mol of enzyme. The enzyme shows a single protein band with Mr = 47,000 on SDS-polyacrylamide gel electrophoresis (6). The equilibrium dialysis study with [2,8-3H]adenine indicates that the enzyme binds radioactivity corresponding to 4 mol of adenosine in a noninteracting manner (9). Since adenosine bound to the enzyme undergoes partial reactions including oxidation of the 3'-hydroxyl by the enzyme-bound NAD* (3, 6), this observation may be interpreted as each subunit of the enzyme being able to bind the ligand and to catalyze the reaction independent of each other. While these results suggest that the rat liver enzyme has structurally identical and catalytically equivalent subunits, data showing the presence of structurally and/or functionally nonequivalent subunits are reported for S-adenosylhomocysteinases from other sources. Døskeland and Ueland (10) have shown that S-adenosylhomocysteinases from bovine liver, bovine adrenal cortex, and mouse liver contain two types of subunits separable on SDS-polyacrylamide gel electrophoresis. Ables et al. (11) have reported that the calf liver enzyme has two classes of adenosine-binding sites residing on two nonequivalent pairs of subunits, and only one pair participates in catalysis. Half-site reactivity is also noted by Chiang et al. (12) with the beef liver enzyme. S-Adenosylhomocysteinase from yellow lupin seed, a dimer of apparently identical subunits (7), is reported to bind 1 molecule of adenosine tightly (13). These reports prompted us to examine the molecular and catalytic properties of rat liver S-adenosylhomocysteinase in more detail, since chemical evidence for identical subunits is not available, and it is possible to observe noninteracting binding of radioactivity in the equilibrium binding study if two subunits bind adenosine in a nonproductive manner with an association constant similar to the overall equilibrium constant (which is a function of the association constant and equilibrium constants between enzyme species carrying reaction intermediates) found when adenosine is bound to the catalytically active subunits. In this paper, data are presented indicating that rat liver S-adenosylhomocysteinase has four identical subunits which are catalytically independent.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from the sources cited: adenosine, S-adenosyl-L-homocysteine, calf intestinal mucosa adenosine deaminase, yeast alcohol dehydrogenase, cross-linked hemocyanin, and diisopropylphosphate-carboxypeptidase A (Sigma); dimethyl suberimidate dichlorohydrochloride (Wako Pure Chemicals, Osaka); carboxypeptidase Y (Oriental Yeast Co., Tokyo); 1-(p-toluenesulfonyl)amino-2-phephylthethyl chloride (Merek Chemicals, Osaka). Other chemicals were of the highest purity available from local sources. S-Adenosylhomocysteine was purified to homogeneity from rat liver by the method of Fujioka and Takata (6), and its concentration was determined by using a molecular weight of 188,000. Protein was determined by the method of Lowry et al. (14).

Enzyme Assay—The S-adenosylhomocysteinase activity in the direction of synthesis was determined by the rate of formation of [3H]adenosylhomocysteine from [2,8-3H]adenosine and homocysteine as
described previously (6). The assay in the direction of hydrolysis was performed in an assay mixture containing 50 \( \mu \)M S-adenosyl-L-homocysteine and 1.0 IU of adenosine deaminase in 2.0 ml of 50 mM potassium phosphate, pH 6.9. The decrease of absorbance at 265 nm due to the conversion of the product adenosine to inosine was followed (6).

**Cross-linking of S-Adenosylhomocysteinase**—Cross-linking of S-adenosylhomocysteinase with dimethyl suberimidate was carried out essentially as described by Davies and Stark (15). The protein concentration was kept below 0.25 mg/ml in order to minimize intermolecular cross-linking. After incubation of the enzyme with the reagent at room temperature, the cross-linking pattern was determined by SDS-polyacrylamide gel electrophoresis (15).

**COOH-terminal Amino Acid Analysis**—The COOH-terminal amino acid residue of S-adenosylhomocysteinase was determined by digestion with carboxypeptidase A (16) and Y (17).

Prior to digestion, S-adenosylhomocysteinase was denatured by incubating with 1% SDS for 30 min at 50°C. The SDS-denatured enzyme (6.14 mg) was then incubated at 30°C with 0.05 mg of carboxypeptidase A in 1.0 ml of 0.1 M N-ethylmorpholine/acetic acid, pH 8.5, containing 20 \( \mu \)M norleucine as the internal standard. At appropriate times, aliquots (0.2 ml) were removed from the reaction mixture, acidified to pH 2 with 4 N HCl, and the insoluble matter formed was discarded by centrifugation. The supernatant from each aliquot was then applied to a column (0.55 x 3 cm) of Dowex 50-X8, H" form. After washing the column with 10 ml of water, amino acids were eluted with 5 ml of 5 M NH4OH. Identification and quantitation of amino acids released by the peptidase was made on an LKB amino acid analyzer, model 4400. Digestion with carboxypeptidase Y was carried out in the same manner except that 0.1 M pyridine/acetic acid, pH 6.5, was used as the incubation buffer. The controls lacking carboxypeptidase and S-adenosylhomocysteinase were run simultaneously.

**Peptide Mapping**—S-adenosylhomocysteinase was carboxymethylated with iodoacetate according to the method of Hirs (18). After dialysis against water and lyophilization, the reduced and alkylated enzyme was suspended in 0.2 ml of 0.1 M NH4HCO3, pH 7.9, and a freshly prepared solution of trypsin was added to a final enzyme to substrate ratio of 1:100 (by weight). After incubation for 3 h at 37°C, a second addition of trypsin was made, and the mixture was incubated for an additional 3 h. The sample after lyophilization was then applied to a column (0.55 x 3 cm) of Dowex 50-X8, H" form. Ascending chromatography was carried out in chloroform/methylene/ NH4OH (2:2:1, by volume). The plate was dried at 85°C for 20 min. Electrophoresis was then performed in the perpendicular direction at 4°C in pyridine/acetic acid/water (1:10:289, by volume) for 30 min at 800 V. After drying, the plate was sprayed with 0.02% fluorescamine in acetone, and peptides were located by viewing them under ultraviolet light. Arginine-containing peptides were visualized by the Sakaguchi stain (19).

**Determination of Enzyme-bound NAD* and NADH**—The NAD* content of S-adenosylhomocysteinase was determined on a perchloric acid extract by the reaction with ethanol and alcohol dehydrogenase as described previously (6). Determination of the enzyme-bound NADH formed by the reaction of S-adenosylhomocysteinase with adenosine was carried out by incubating the ethanolic KOH extract with saccharopine dehydrogenase (NAD*, 1-lysine-forming) in the presence of excess lysine and \( \alpha \)-ketoglutarate (9).

**RESULTS**

**Cross-linking of S-Adenosylhomocysteinase**—By comparison of the molecular weights of the native and denatured enzymes, we have reported that rat liver S-adenosylhomocysteinase is composed of four polypeptide chains with \( M_r = 47,000 \) (6). In contrast, based on their molecular weight determinations, Kajander and Raina proposed a pentameric structure for the enzyme (5). In order to settle the problem, we carried out cross-linking experiments with dimethyl suberimidate. S-Adenosylhomocysteinase (0.25 mg/ml) was incubated with dimethyl suberimidate and then subjected to SDS-polyacrylamide gel electrophoresis as described by Davies and Stark (15). As shown in Fig. 1, four distinct bands can be seen on the electropherogram. Calibration with molecular weight standards (cross-linked hemocyanin) showed that these bands had molecular weights of 47,000, 93,000, 145,000, and 190,000. That the enzyme monomer represents a single species was confirmed by SDS-polyacrylamide gel electrophoresis in the discontinuous system of Laemmli (20). A single protein band was observed even when the protein load was low and the distance of migration long (results not shown).

The molecular weight of the protein with the slowest mobility seen in the cross-linking experiment is in excellent agreement with that of the native S-adenosylhomocysteinase determined by sedimentation equilibrium and molecular exclusion chromatography (6). Thus, the results establish that rat liver S-adenosylhomocysteinase consists of four subunits with an identical or nearly identical molecular weight.

**End Group Analysis**—Attempts at identifying the NH2-terminal residue uniformly failed. No release of derivatized NH2-terminal amino acids was detected by using dinitrofluorobenzene, phenylisothiocyanate, or dimethylaminobenzene isothiocyanate under conditions which were effective in control experiments with known proteins. Therefore, we tentatively conclude that the NH2-termini of rat liver S-adenosylhomocysteinase are in some way blocked.

Carboxypeptidase A caused a rapid release of tyrosine from S-adenosylhomocysteinase. When incubated at a ratio of sample to enzyme of 200:1 and 30°C, the release was complete within 60 min. No release of other amino acids was observed.
over a period of 4 h. Comparison with the internal standard norleucine showed that 4.04 mol of tyrosine were maximally released per mol of enzyme, consistent with the tetrameric structure.

Digestion of S-adenosylhomocysteinase with carboxypeptidase Y showed release of tyrosine followed by arginine. Under the conditions described under "Experimental Procedures," the amounts of tyrosine released at 60, 120, and 180 min were 2.48, 3.24, and 3.68 mol of enzyme, respectively. The corresponding values for arginine were 1.02, 1.76, and 2.52 mol/mmol of enzyme. Leucine, serine, valine, and alanine were each released at essentially the same rate, but at a rate slower than those of tyrosine and arginine.

Peptide Mapping—Evidence for the presence of identical subunits could be best obtained by peptide mapping of tryptic peptides. Rat liver S-adenosylhomocysteinase contains 56 arginine and 116 lysine residues/mol of enzyme (6). Even if the enzyme contains identical subunits, the abundance of the residues will produce too many peptides for accurate determination of their number by staining with ninhydrin of fluorescamine. In view of a relatively low abundance of arginine residues and the specificity of the Sakaguchi reagent for the amino acid, we determined the number of arginine-containing peptides by staining the peptide map with the Sakaguchi reagent (Fig. 2). Although some spots occurred in clusters, 14 Sakaguchi-positive spots were clearly distinguished by the difference in color intensity and hue. The result indicates that rat liver S-adenosylhomocysteinase has identical or nearly identical subunits.

Reduction of Enzyme-bound NAD+ by Adenosine—Addition of adenosine to S-adenosylhomocysteinase results in the appearance of a peak at 327 nm which is due to the formation of bound NADH (3, 6, 21). It has been shown that 4 mol of adenosine/mol of enzyme are required for the maximal spectral change, and a molar absorptivity of $5.5 \times 10^3$ (M subunit)$^{-1}$ cm$^{-1}$ is calculated (6). Since this value is about one-half of the molar absorptivity of free NADH at 340 nm, the possibility may be considered that only two of the four subunits participate in catalysis even though each can bind adenosine. To examine the possibility, we determined the amount of NADH formed as a function of adenosine concentration. As depicted in Fig. 3, the appearance of NADH and the disappearance of NAD$^+$ closely paralleled with the increase in absorbance, indicating that the spectral change actually reflects NADH formation. Maximally 3.2 mol of NADH are formed per tetrameric enzyme at saturating concentrations of adenosine, showing that the possibility considered above is not the case. The spectrophotometric data can be fitted to Equation 1,

$$K_d = \frac{(\Delta E_0/\Delta \alpha - 1)(L_0 - \Delta \alpha/\Delta \alpha)}{(0.225 \text{ mg/ml})}$$

which is derived under the assumptions that adenosine binds to the subunits independently and the spectral change is proportional to the amount of adenosine bound. In this equation, $K_d$ is the apparent dissociation constant for adenosine, $\Delta$ the difference absorbance at 327 nm, and $\Delta \alpha$ the difference molar absorptivity (in terms of subunits). $E_0$ and $L_0$ represent the total concentrations of enzyme subunit and adenosine, respectively. A least squares analysis yielded values of $0.64 \pm 0.09$ M$^{-1}$ and $(3.7 \pm 0.03) \times 10^3$ (M subunit)$^{-1}$ cm$^{-1}$ for $K_d$ and $\Delta \alpha$, respectively. The value of $K_d$ obtained in this experiment is in good agreement with that found in the equilibrium dialysis study (9). A value of $4.63 \times 10^3$ M$^{-1}$ cm$^{-1}$ was obtained for the molar absorptivity of bound NADH.

The spectral change produced by adenosine was reversible. The original enzyme spectrum was regenerated by gel filtration chromatography on Sephadex G-25. The enzyme after gel filtration was fully catalytically active. Thus, the failure to obtain complete reduction of NAD$^+$ is most likely due to the existence of enzyme species having the oxidized coenzyme in the equilibrium mixture of various enzyme-ligand complexes.

Inactivation of S-Adenosylhomocysteinase by Adenosine—It has been reported that the substrate adenosine inactivates S-adenosylhomocysteinase irreversibly, and the inactivation is associated with tight binding of adenine (5, 12, 22). In order to examine relevance of this phenomenon to the normal catalytic turnover of the enzyme, we incubated rat liver S-adenosylhomocysteinase with adenosine under a variety of conditions and found that inactivation is seen only in the presence of thiols such as dithiothreitol, 2-mercaptoethanol, and cysteamine. The inactivation was observed in phosphate and Tris/HCl buffers. As shown in Fig. 4, the enzyme was inactivated by adenosine following pseudo first order kinetics in the presence of dithiothreitol. Adenosine or dithiothreitol alone was without effect. While the data shown in the figure are those obtained by measuring the residual enzyme activity in the direction of S-adenosylhomocysteine hydrolysis,
same inactivation kinetics were observed when the enzyme assay was conducted in the synthetic direction. A plot of the apparent first order rate constants for inactivation obtained at different concentrations of adenosine and a constant high level of dithiothreitol (10 mM) against the adenosine concentration revealed a saturation behavior (Fig. 5A). By contrast, the rate of inactivation showed a linear dependence on the dithiothreitol concentration (Fig. 5B). These results suggest that the inactivation is due to the reaction of dithiothreitol with the enzyme-substrate complex. Consistent with a high affinity of adenosine for the enzyme, the curve depicted in Fig. 5A is not a hyperbola. Fit of the data to Equation 1 (where being used in place of $\Delta A$) yielded a $K_d$ of 0.61 ± 0.40 $\mu$M, a value comparable to that obtained in the absence of the thiol (cf. Fig. 3 and Ref. 9).

The inactivation was not reversed by gel filtration or by extensive dialysis using 50 mM potassium phosphate, pH 7.2. Incubation of S-adenosylhomocysteinase with [2,8-3H]adenosine together with dithiothreitol resulted in tight binding of

**FIG. 4.** Time course of inactivation of S-adenosylhomocysteinase by adenosine. S-Adenosylhomocysteinase (0.1 mg) was incubated in 0.1 ml of 50 mM potassium phosphate, pH 7.2, at 25 °C with the compounds indicated. □, no addition; ●, 1 mM adenosine; ○, 10 mM dithiothreitol; ●, 1 mM adenosine plus 10 mM dithiothreitol. At times indicated, aliquots (10 µl) were removed and assayed for enzyme activity in the direction of hydrolysis as described under "Experimental Procedures."

**FIG. 5.** Dependence of the pseudo first order rate constant for inactivation on the adenosine (A) and dithiothreitol (DTT) (B) concentrations. S-Adenosylhomocysteinase was incubated with varying concentrations of adenosine in the presence of 10 mM dithiothreitol in A and with varying concentrations of dithiothreitol in the presence of 1 mM adenosine in B. The solid line in A is from the calculated data.

**Fig. 6.** Relationship between loss of enzyme activity and the number of ligands bound to the enzyme. S-Adenosylhomocysteinase (1.1 mg) was incubated with 0.1 mM [2,8-3H]adenosine (2.9 × 10^9 dpm/nmol) and 10 mM dithiothreitol in 1.5 ml of 50 mM potassium phosphate, pH 7.2, at 25 °C. At time intervals, aliquots were removed and passed through columns of Sephadex G-25. The void volume fractions were assayed for enzyme activity, protein, and fixed radioactivity. The line is drawn by a least squares linear regression.

**DISCUSSION**

The results described in this report indicate that rat liver S-adenosylhomocysteinase consists of four structurally identical subunits. The presence of one type of subunit as revealed by discontinuous SDS-polyacrylamide gel electrophoresis and the result of the cross-linking experiment show that the enzyme is composed of four subunits with the same molecular weight. A tryptic peptide map shows 14 arginine-containing peptides. Since this value is exactly one-fourth of the number of arginine residues present in the enzyme (6), the result indicates that all subunits are identical or nearly identical. Consistent with this finding, carboxypeptidase A releases only tyrosine in an amount equivalent to the number of subunits. The presence of a penultimate arginine residue as revealed by carboxypeptidase Y digestion explains why carboxypeptidase A releases only tyrosine. Arginine at the C terminus is not hydrolyzed by the peptidase (16).

Abeles et al. (11) have shown that subunits of the tetrameric calf liver S-adenosylhomocysteinase behave differently in the reaction with the inactivator 2'-deoxyadenosine. Although 2 molecules of 2'-deoxyadenosine are bound to the enzyme and appear to be converted to adenine, 2 molecules of the enzyme-bound NAD+ are reduced and 2 molecules of 2'-deoxyadenosine undergo proton abstraction at C'-2 during the reaction. They have also reported that only two subunits participate in
normal catalysis. Upon incubation with adenine-labeled adenine, radioactivity corresponding to 0.5 mol/mol of subunit becomes tightly bound to the enzyme without affecting the catalytic activity. However, with rat liver S-adenosylhomocysteine titration with adenine shows that the binding of adenine to the enzyme results in the reduction of 80% of enzyme-bound NAD$^+$ (Fig. 3), excluding the possibility of “half-site reactivity.” Apparently, there is no cooperativity in the formation of NADH. Gel filtration or dialysis of the complex regenerates the free enzyme, indicating that the ligand is reversibly bound to the enzyme. The data of Fig. 3 together with the result of the equilibrium binding study with $[^3H]adenosine that the enzyme binds radioactivity corresponding to 4 mol of ligand/mol of enzyme in a noninteracting manner (9) strongly suggest that each subunit of the enzyme independently binds adenine and catalyzes the reaction. It should be noted that the enzyme shows no kinetic cooperativity toward any of the substrates (6).

Irreversible loss of enzyme activity by adenine has been reported for S-adenosylhomocysteinases from human placenta (21), bovine liver (12), and rat liver (5). The results with the rat liver enzyme described herein, however, show that the simultaneous presence of a thiol is necessary for the inactivation to occur. Inactivation by adenine reported earlier might be due to the presence of dithiothreitol in the reaction mixtures. Lack of inactivation by adenine in the absence of a thiol is also noted by Borchardt et al. with the calf liver enzyme (23). The kinetics of inactivation of rat liver S-adenosylhomocysteine by adenine plus dithiothreitol which exhibits a saturation behavior with respect to the adenine concentration and a linear relationship with respect to the dithiothreitol concentration and the fact that the $K_d$ for adenine is low and comparable to that found in the absence of the thiol suggest that dithiothreitol reacts with one of the enzyme-bound intermediates, and this reaction results in a change in the activity of the enzyme. The possibility that inactivation is due to reduction of a protein disulfide whose integrity is essential for catalysis and which becomes accessible to the thiol upon interaction with adenine can be excluded from the fact that the number of free sulfhydryl groups is unchanged after inactivation. The first order loss of enzyme activity and the linear relationship between the extent of inactivation and the presence of adenine indicate that all subunits are equivalent in the inactivation reaction. In contrast to the reversible enzyme-ligand complex formed in the absence of thiol, the inactivated enzyme has all of the coenzyme in the reduced form. This observation indicates that dithiothreitol reacts with one of the enzyme-bound intermediates with the $3'$ hydroxyl oxidized. Irreversible reaction of the thiol would pull the equilibrium to result in complete reduction of NAD$^+$. Since, as discussed above, the inactivation apparently results from the reaction of the thiol with a normal reaction intermediate, the results obtained with adenine as the inactivator are consistent with the idea that all four subunits of rat liver S-adenosylhomocysteine are functionally equivalent in normal catalysis.

Data in support of catalytic equivalence of subunits are seen in a recent study with the substrate analogue 5'-fluorosulfonylbenzoyladenine. This compound inactivates rat liver S-adenosylhomocysteine in a pseudo first order fashion showing characteristics of active site-directed reagent (rate saturation, competition with adenine and adenosine, etc.) (24). Although the reagent is not covalently bound to the enzyme, the loss of activity is linearly related to the disappearance of two sulfhydryl groups/subunit. Treatment of the inactivated enzyme with dithiothreitol results in recovery of enzyme activity as well as regeneration of the sulfhydryl residues. These results suggest that the inactivation is due to the reagent-mediated dithiole formation. Since the reagent apparently binds at the nucleoside-binding site, it would be difficult to reconcile these data with the existence of catalytically nonequivalent subunits.

REFERENCES