Chemical Modification of Arginine Residues of Rat Liver
S-Adenosylhomocysteinase*

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Rat liver S-adenosylhomocysteinase (EC 3.3.1.1) is inactivated by phenylglyoxal following pseudo-first order kinetics. The dependence of the apparent first order rate constant for inactivation on the phenylglyoxal concentration shows that the inactivation is second order in reagent. This fact together with the reversibility of inactivation upon removal of excess reagent and the lack of reaction at residues other than arginine as revealed by amino acid analysis and incorporation of phenylglyoxal into the protein indicate that the inactivation is due to the modification of arginine. The substrate adenosine largely but not completely protects the enzyme against inactivation. Although the modification of two arginine residues/subunit is required for complete inactivation, the relationship between loss of enzyme activity and the number of arginine residues modified, and the comparison of the numbers of phenylglyoxal incorporated into the enzyme in the presence and absence of adenosine indicate that one residue which reacts very rapidly with the reagent compared with the other is critical for activity. Although the phenylglyoxal treatment does not result in alteration of the molecular size of the enzyme or dissociation of the bound NAD*, the intrinsic protein fluorescence is largely lost upon modification. The equilibrium binding study shows that the modified enzyme apparently fails to bind adenosine.

S-Adenosylhomocysteinase (EC 3.3.1.1) is an enzyme in the transsulfuration pathway of methionine metabolism. The enzyme hydrolytically cleaves S-adenosylhomocysteine, the product and a potent inhibitor of transmethylation reactions, and provides homocysteine for the synthesis of cysteine and the regeneration of methionine. S-Adenosylhomocysteinase from rat liver is a tetramer consisting of apparently identical subunits with $M_r = 47,000$ (1). The enzyme possesses four tightly bound NAD* as the coenzyme and four apparently noninteracting binding sites for adenosine (1, 2).

While the chemistry of reaction catalyzed by the enzyme is fairly well understood (9), little information is available on the amino acid residues which participate in the binding of the coenzyme and substrates and in catalysis. Recent chemical modification studies with iodoacetamide (2) and diethyl pyrocarbonate (4) have revealed that the rat liver S-adenosylhomocysteinase has essential cysteine and histidine residues for which catalytic functions are implicated. In a continuing effort to elucidate the role of active site residues, we have performed a chemical modification study with an arginine-selective reagent phenylglyoxal. In this report, we present evidence that S-adenosylhomocysteinase has an arginine residue per enzyme subunit whose modification causes the loss of ability to bind the substrate adenosine.

EXPERIMENTAL PROCEDURES

Materials—Adenosine, S-adenosyl-L-homocysteine, phenylglyoxal, and calf intestinal mucosa adenosine deaminase (EC 3.5.4.4) (type III) were obtained from Sigma Chemical Co. (St. Louis, Mo.) and [3H]phenylglyoxal (3.9 $\times 10^4$ cpm/pmol) in 1.0 ml of 50 mM potassium phosphate buffer, pH 7.0, was purchased from Commissariat a l'Energie Atomique, and [2,8-3H]adenosine was from New England Nuclear. Other chemicals were of the highest grade available from local sources. S-Adenosylhomocysteinase was purified from rat liver by the method of Fujioka and Takata (1), and its concentration was determined using a molecular weight of 180,000 (1). Protein was determined by the method of Lowry et al. (5).

Inactivation by Phenylglyoxal—The inactivation of S-adenosylhomocysteinase was carried out by incubating the enzyme with an appropriate amount of phenylglyoxal in 50 mM potassium phosphate or potassium pyrophosphate buffer at 25 °C. The extent of inactivation was monitored by measuring the residual enzyme activity on aliquots (10-20 µl) removed from the reaction mixture. The enzyme assay was carried out in the direction of S-adenosylhomocysteinase hydrolysis in an assay mixture containing 50 µM S-adenosyl-L-homocysteine and 1.0 IU of adenosine deaminase in 2.0 ml of 50 mM potassium phosphate buffer, pH 6.9. The decrease in absorbance at 265 nm due to the conversion of the product adenosine to inosine was followed (1). This assay is rather sensitive ($\Delta A = 8.1 \times 10^{-4}$ M⁻¹ cm⁻¹) and is linear with respect to time and enzyme concentration within the absorbance change of at least 0.05. Although the phenylglyoxal inactivation is reversible on dilution, no deviation from linearity was noted during the time required for assay (=< 2 min).

Incorporation of [7-14C]Phenylglyoxal into S-Adenosylhomocysteinase—S-Adenosylhomocysteinase (58.5 nmol) was incubated with 5 mM [7-14C]phenylglyoxal (3.8 $\times 10^4$ cpn/µmol) in 1.0 ml of 50 mM potassium pyrophosphate buffer, pH 8.5, at 25°C. At intervals, aliquots (0.1 ml) were withdrawn, and the unreacted reagent was removed by gel filtration on Sephadex G-50 fine equilibrated with 50 mM potassium phosphate buffer, pH 8.0. Column centrifugation technique as described by Penefsky (6) was used to minimize the reversal of modification. The eluate was assayed for enzyme activity, protein, and the radioactivity fixed. The measurement of enzyme activity was made immediately after centrifugation.

Amino Acid Analysis—S-Adenosylhomocysteinase was incubated with 5 mM phenylglyoxal in 50 mM potassium pyrophosphate buffer, pH 8.5, until less than 2% of the original activity was detected. The reaction mixture was then diluted by one-third with 1 N HCl to prevent regeneration of free arginine (7) and immediately dialyzed against 0.1 N HCl. After evaporation of the HCl, the residue was hydrolyzed in 6 N HCl for 23 h at 110 °C in a sealed evacuated ampule. Amino acid analysis was performed in an LKB 4400 amino acid analyzer. The enzyme treated similarly but in the absence of phenylglyoxal served as the control.

Determination of Enzyme-bound NAD*—After separation of excess phenylglyoxal by Sephadex G-50 column centrifugation, the inactivated enzyme (5 mg, <2% of the original activity) was treated with perchloric acid to extract the bound NAD*. The supernatant obtained by centrifugation was then neutralized and the NAD* con...
tent was determined spectrophotometrically by the increase in absorbance at 340 nm after addition of ethanol and alcohol dehydrogenase. Details of the procedure are described elsewhere (1).

Equilibrium Dialysis—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% NaCO3/10 mM EDTA at 100°C for 5 min. The native or inactivated enzyme after separation of excess phenylglyoxal by gel filtration in 20 mM potassium phosphate buffer, pH 8.0 (10 μM enzyme subunit, 0.2 ml), was placed on one side of the membrane, and an equal volume of the solution containing [2,8-3H]adenosine (7 × 10^6 cpm/μmol) in the same buffer was placed on the other side. The concentration ranges of [3H]adenosine were 1 to 5 μM for the native enzyme and 5 to 120 μM for the inactivated enzyme, respectively. The two solutions were allowed to equilibrate with gentle rocking for 15 h at 0°C. Samples (0.1 ml) from each side were transferred to vials containing 0.4 ml of water, 5 ml of a scintillation liquid (toluene, 667 ml; Triton X 100, 333 ml; 2,5-diphenyloxazole, 5 g; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.4 g) were added, and the radioactivity was measured in an Aloka liquid scintillation spectrometer, model LSC 903. The amount of adenosine bound to the enzyme was calculated from the difference in radioactivity between two sides of the dialysis cell and the specific radioactivity of [3H]adenosine. 

RESULTS

Kinetics of Inactivation—Incubation of S-adenosylhomocysteinase with phenylglyoxal in 50 mM potassium pyrophosphate buffer at pH 8.5 and 25°C resulted in a pseudo-first order loss of enzyme activity (Fig. 1). After incubation for 50 min with 5 mM phenylglyoxal, less than 2% of the original activity was observed. A plot of the apparent first order rate constants for inactivation obtained at different concentrations of phenylglyoxal against its concentrations was non-linear. However, when a plot was made using the squares of phenylglyoxal concentration a straight line was obtained, indicating that the inactivation is first order with respect to the enzyme but second order with respect to phenylglyoxal (Fig. 2). This observation is consistent with the known stoichiometry of the reaction of phenylglyoxal with arginine in which two molecules of phenylglyoxal react with a single guanido group (7).

The rate of inactivation was dependent on pH. The apparent first order rate constant for inactivation increased progressively with the increase in pH up to pH 10, the highest pH examined (data not shown). The instability of S-adenosylhomocysteinase precluded experiments at higher pH values.

The phenylglyoxal inactivation was reversible. When the enzyme that had been inactivated to 50% of the original activity was freed from the reagent by gel filtration, a slow recovery of enzyme activity was observed. Upon incubation at pH 8.0 and 25°C, the enzyme activity returned to more than 95% of the initial value within 3 h. Under the same conditions, the recovery with an almost completely inactivated enzyme was about 78%. The extent and time course of reactivation were not affected by the presence of NAD + (50 μM) or S-adenosylhomocysteine (50 μM).

Amino Acid Residue Modified by Phenylglyoxal—Phenylglyoxal reacts with arginine residues in proteins in a highly selective manner, and the reaction can be followed as a loss of arginine in amino acid analysis after acid hydrolysis (7, 8). S-Adenosylhomocysteine contains as many as 14 arginine residues per subunit (1). Therefore, to quantitate a small change in the amino acid content, analysis was carried out in triplicate on three different samples. The analyses on the enzyme that had been inactivated to less than 2% of the original activity revealed a loss of 2.0 mol of arginine/mol of enzyme subunit on the average. No significant change was noted in the contents of other amino acids (Table I).

Takahashi reported that the product of reaction of phenylglyoxal with free arginine is di(phenylglyoxal)arginine and this 2:1 stoichiometry is normally obtained for arginine residues in proteins (7). When S-adenosylhomocysteine was incubated with [7,14C]phenylglyoxal, incorporation of radioactivity into the protein was observed concomitant with loss of enzyme activity. Incorporation of 4.2 mol of phenylglyoxal/mol of enzyme subunit was obtained for complete inactivation.

Treatment of S-adenosylhomocysteine with phenylglyoxal resulted in a marked change in its ultraviolet absorbance spectrum (Fig. 3). The difference spectrum between the modified enzyme and the native enzyme (Fig. 3, Curve 3) showed a maximum at 250 nm and was identical in shape with the spectrum of di(phenylglyoxal)arginine. Using a molar absorptivity of 11,000 for di(phenylglyoxal)arginine (8), the completely inactivated enzyme was calculated to contain 2.3 modified arginine residues/subunit. Although there are some examples in which phenylglyoxal reacts with an arginine residue in a 1:1 stoichiometry (9-14), the results described above, when taken together, indicate that each of the modifiable arginine residues of S-adenosylhomocysteine reacts with 2 eq of phenylglyoxal, and the modification reaction occurs only at arginine residues.

Relationship between Inactivation and Number of Residues Modified—The relationship between loss of activity and the number of arginine residues modified was examined by incorporation of the radiolabel from [7,14C]phenylglyoxal, using a stoichiometry of 2 mol of phenylglyoxal/mol of arginine. Whereas the modification of 2 arginine residues/subunit is
Essential Arginine Residue of S-Adenosylhomocysteinate

In an attempt to obtain the number of essential residues, S-adenosylhomocysteinate was incubated with 5 mM [7-\textsuperscript{14}C]-phenylglyoxal in the presence (100 \mu M) and absence of adenosine. At 25 min, when the enzyme incubated in the absence of adenosine was almost completely inactivated, the reaction mixtures were subjected to gel filtration and the radioactivity fixed to the protein was measured as described under "Experimental Procedures." Under these conditions, the enzyme incubated in the presence of adenosine lost 20% of enzyme activity with the incorporation of 1.6 mol of phenylglyoxal/mol of subunit. The enzyme incubated without adenosine showed the incorporation of 3.95 mol of phenylglyoxal, as expected. If both of two modifiable residues/subunit are essential for activity (in the sense that the modification of either one totally abolishes the catalytic activity), the amount of phenylglyoxal incorporated cannot exceed 0.8 mol/mol of subunit for the 20% inactivated enzyme. (Two molecules of phenylglyoxal react with a single arginine residue.) Thus, the incorporation of phenylglyoxal above this limit indicates that only one arginine residue is essential. The result also shows that the reaction of phenylglyoxal at nonessential residue is retarded when the enzyme is complexed with the ligand.

Effect of Adenosine on Inactivation—The rate of inactivation of S-adenosylhomocysteinate by phenylglyoxal was decreased in the presence of the substrate adenosine (Fig. 5). In the presence of 100 \mu M adenosine, the apparent first order rate constant for inactivation was 0.01 min\(^{-1}\), whereas a value of 0.15 min\(^{-1}\) was obtained in its absence. At 100 \mu M adenosine, and the enzyme concentration used in the experiment, more than 98% of the enzyme may be considered to exist as the enzyme-ligand complex\(^2\) as calculated with a \(K_d\) of 0.8 \mu M (3). The inability of adenosine to fully protect the enzyme against inactivation is not due to the lowering of adenosine concentration by the reaction with phenylglyoxal, since exactly the same inactivation kinetics was observed when the enzyme was added to the mixture in which adenosine and phenylglyoxal were preincubated for 30 min. Therefore, to establish that the slow inactivation is a property of the enzyme-ligand complex and that the partial protection is caused by the specific interaction of adenosine at the active site, we studied the inactivation kinetics at lower adenosine concentrations. As shown in the figure, at subsaturating concentrations of adenosine, the experimental points fell on the theoretical curve calculated on the assumption that the enzyme-ligand complex (the concentration being calculated with \(K_d = 0.8 \mu M\)) reacts with the reagent at a rate constant of 0.01 min\(^{-1}\) and the free enzyme at 0.15 min\(^{-1}\). Thus, the binding of ligand at the active site greatly slows but does not prevent the reaction.

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\(^{1}\) Adenosine when bound to S-adenosylhomocysteinate undergoes a series of reactions ultimately to form 4',5'-dehydroadenosine (2, 3). The intermediates in this transformation are tightly bound by the enzyme (2). The term ligand is used here to indicate these intermediates.

\(^{2}\) These values include both free and amidated residues.

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**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Native enzyme</th>
<th>Phenylglyoxal-treated enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol/mol of enzyme monomer</td>
<td>mol/mol of enzyme monomer</td>
</tr>
<tr>
<td>Aspartic acid(^*)</td>
<td>46.3</td>
<td>47.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>21.9</td>
<td>22.1</td>
</tr>
<tr>
<td>Serine</td>
<td>11.0</td>
<td>11.5</td>
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<tr>
<td>Glutamic acid(^*)</td>
<td>40.4</td>
<td>41.6</td>
</tr>
<tr>
<td>Proline</td>
<td>26.4</td>
<td>25.8</td>
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<tr>
<td>Glycine</td>
<td>37.3</td>
<td>36.5</td>
</tr>
<tr>
<td>Alanine</td>
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</tr>
<tr>
<td>Valine</td>
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<tr>
<td>Methionine</td>
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<td>15.1</td>
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</tr>
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</tr>
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<td>Histidine</td>
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</tr>
<tr>
<td>Lysine</td>
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<td>29.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>14.1</td>
<td>12.1</td>
</tr>
</tbody>
</table>

\(^*\) These values include both free and amidated residues.
zyme (1). The coenzyme, which is involved in the oxidation of 3'-hydroxyl of adenosine and S-adenosylhomocysteine (3, 15), is held very tightly by the enzyme and cannot be removed by gel filtration or dialysis at neutral pH. Since arginine residue is implicated in a number of enzymes to function as a cationic group in the binding of a negatively charged coenzyme or substrate, the possibility may be considered that the modification of arginine results in the dissociation of NAD\(^+\) from S-adenosylhomocysteinase. However, the measurement of NAD\(^+\) content of the modified enzyme as described under "Experimental Procedures" showed that the coenzyme was not appreciably released. The enzyme that had been inactivated to less than 5% of the original activity contained 0.78 mol of NAD\(^+\)/mol of subunit, compared with 0.85 mol/mol of subunit of the untreated enzyme. The reversibility of inactivation also indicates that phenylglyoxal treatment does not result in the modification or release of the coenzyme.

The quaternary structure of the enzyme was unchanged by the arginine modification as evidenced by Sephadex G-200 gel filtration chromatography of the inactivated enzyme. Although the activity was partially restored during chromatography at 4 °C, the protein was eluted from the column as a single symmetrical peak at the position expected for the native enzyme (data not shown).

**Fluorescence Property of the Modified Enzyme**—As shown in Fig. 6, S-adenosylhomocysteinase exhibits fluorescence with a maximum at 337 nm. A considerable portion (~63%) of this fluorescence has been shown to be quenched when adenosine is added (1). The phenylglyoxal treatment caused diminution of the fluorescence with a shift of the maximum to shorter wavelength (327 nm) (Fig. 6). When compared at their peak wavelengths, the fluorescence intensity of the modified enzyme was about 17% that of the native enzyme. The fluorescence was not changed by the addition of adenosine.

**Lack of Interaction of Adenosine with the Modified Enzyme**—The binding of adenosine to the inactivated enzyme was examined by the equilibrium dialysis method as described under "Experimental Procedures." The experiment was performed at 0 °C in order to minimize the reversal of modification. During the 15-h period of experiment, the inactivated enzyme recovered about 7% of the activity. When the contribution by the regenerated enzyme was taken into account, the modified enzyme showed no binding of adenosine. The native enzyme under the same conditions bound adenosine with an apparent \(K_d\) of 0.31 \(\mu\)M.

### DISCUSSION

An arginine-modification reagent phenylglyoxal inactivates S-adenosylhomocysteinase in an all-or-none fashion. Two other commonly used reagents, cyclohexanedione and 2,3-butanedione, do not inactivate the enzyme appreciably when tested at a concentration of 10 mM in 0.1 M borate buffer, pH 8.0. The inactivation by phenylglyoxal does not involve the modification or release of the bound coenzyme nor the change in quaternary structure of the enzyme. Thus, it may be concluded that the inactivation is due to the modification of amino acid residue(s) essential for catalytic activity.

Although phenylglyoxal is quite specific for arginine, its reaction with NH\(_2\)-terminal amino group or \(\epsilon\)-amino group of lysine residue is reported (7, 8). Reaction of phenylglyoxal with these groups probably involves Schiff base formation and transamination, the latter transformation being irreversible (7). Since the inactivation of S-adenosylhomocysteinase is reversible, the occurrence of transamination reaction is unlikely. S-Adenosylhomocysteinase contains 2 modifiable arginine residues per subunit (Table I). The spectrum of the modified enzyme shows that each of these residues reacts with phenylglyoxal in a 1:2 ratio. This fact and the finding that 4 mol of phenylglyoxal are incorporated per subunit indicate that the reaction of phenylglyoxal is restricted to arginine residues and rule out the possibility that the inactivation is due to the formation of a labile Schiff base.

The reaction leading to the formation of di(phenylglyoxal)arginine conceivably proceeds in two steps; a molecule of phenylglyoxal first condenses with the guanido group to form a cis-diol adduct which rapidly adds a second equivalent to give the final product. The second order kinetics of inactivation with respect to phenylglyoxal could arise in two situations. First, when a first molecule of the reagent forms a freely reversible adduct with the arginine residue followed by a rate-limiting addition of a second molecule, a second order inactivation kinetics would be obtained provided that the dissociation constant of phenylglyoxal from the reversible

![Fig. 6. Fluorescence emission spectra of the native (Curve 1) and phenylglyoxal-treated (Curve 2) enzymes. The phenylglyoxal-treated enzyme was prepared as described in Fig. 3. Both spectra were taken in 50 mM potassium phosphate buffer, pH 8.0, at the protein concentration of 0.23 mg/ml. Excitation wavelength, 285 nm.](http://www.jbc.org/lookup/doi/10.1074/jbc.7377)
complex is substantially greater than the reagent concentration. The reversible adduct may or may not be enzymically active. The second alternative is when the first adduct formed in a rate-limiting reaction retains enzyme activity which is lost by subsequent reaction with second molecule. It is difficult or impossible to distinguish between these alternatives, but the latter seems unlikely in view of a number of examples in which reaction of one molecule of phenylglyoxal is sufficient for inactivation (9–14).

The pseudo-first order loss of enzyme activity indicates that there is no cooperativity in the reaction of phenylglyoxal with arginine residues and eliminates the possibility that modification of more than one residue is required for complete inactivation (17, 18). Since, under these circumstances, the pseudo-first order rate constant for inactivation is the sum, not the product, of individual rate constant for each essential residue (17), the reaction order of two with respect to phenylglyoxal is not an indication of the existence of two essential residues. The relationship between loss of enzyme activity and the number of residues modified (Fig. 4) shows that, of the two modifiable arginine residues per subunit, one residue (at least this residue is essential) reacts with the reagent much faster than the other. When there are two modifiable residues and one is essential, the number of residues modified at a given stage of modification (m) may be expressed as

$$m = 2 - (A/A_0) - (A/A_0)^\alpha. \tag{1}$$

When two residues are essential,

$$m = 2 - (A/A_0)^{1+\alpha} - (A/A_0)^{1+\alpha} \tag{2}$$

where $\alpha$ is the ratio of rate constant for the slow-reacting residue to that for the fast-reacting residue. If $\alpha$ is very small ($\alpha \ll 1$) as in the present case, no distinction can be made between two possibilities. The number of phenylglyoxal incorporated into the enzyme in the presence of adenosine which is in excess over that expected when the two residues are essential, however, shows that S-adenosylhomocysteine contains only one essential arginine residue/subunit.

In a number of cases, arginine residue is thought to serve as a cationic site in the binding of a negatively charged group of substrate or coenzyme. Several examples are also known in which arginine is apparently involved in catalysis (19–22). Despite the absence of negative charge, the equilibrium binding study shows that adenosine does not bind to the arginine-modified S-adenosylhomocysteine. In accordance with this observation, the modified enzyme fails to catalyze partial reactions with adenosine (the reduction of bound NAD$^+$ and the $4'$-proton exchange reaction) (2–4) (data not shown). As shown in Fig. 6, the intrinsic tryptophan fluorescence of the enzyme is largely lost upon modification. Since the modification of tryptophan apparently does not occur, this may be considered due to a conformational change or to a direct interaction of the introduced group with the fluorescing tryptophan. The previous finding that a considerable portion of the fluorescence is quenched with adenosine (1) suggests that the tryptophan(s) occur(s) at or near the adenosine-binding site. From these considerations, it may be suggested that the phenylglyoxal modification results in the loss of adenosine binding ability through a conformational change or by a steric hindrance at the nucleoside-binding site.

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