Identification of the Reactive Sulfhydryl Groups of S-Adenosylmethionine Synthetase*

(Received for publication, December 28, 1987)

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S-Adenosylmethionine synthetase from *Escherichia coli* is rapidly inactivated by N-ethylmaleimide. In the presence of excess N-ethylmaleimide inactivation follows pseudo first-order kinetics, and loss of enzyme activity correlates with the incorporation of 2 eq of N-[ethyl-2-3H]maleimide/subunit. Preincubation of the enzyme with methionine and the ATP analog adenylylimidodiphosphate reduced the rate of N-ethylmaleimide incorporation more than 30-fold. Two N-[ethyl-2-3H]maleimide-labeled tryptic peptides were purified from the modified enzyme by reverse phase high performance liquid chromatography. The modified residues were identified as cysteine 90 and cysteine 240 by comparison of the amino acid compositions of these peptides with the protein sequence. These are the first residues to be implicated in the activity and/or structure of the enzyme.

N-Ethylmaleimide-modified S-adenosylmethionine synthetase exists mainly as a dimer in conditions where the native enzyme is a tetramer. Accumulation of the dimer parallels the loss of the enzyme activity. When an enzyme sample was partially inactivated, separation of tetrameric and dimeric enzyme forms by gel filtration revealed that the residual enzyme activity was solely present in the tetramer and N-[ethyl-2-3H]maleimide was present predominantly in the dimer. Gel filtration studies of the tetramer-dimer equilibrium for the native enzyme indicated that the dissociation constant between the tetramer and dimers is \( < 6 \times 10^{-11} \) M. Similar studies for the N-ethylmaleimide-modified protein indicated that the dissociation constant of the tetramer is \( \sim 4 \times 10^{-4} \) M. Upon modification the strength of dimer-dimer interactions is diminished by at least 9 kcal/mol.

*S-Adenosylmethionine (AdoMet)* synthetase (ATP:L-methionine S-adenosyltransferase) catalyzes the only known reaction of AdoMet, the primary alkylating agent in biological systems (1). We have now been engaged in structural and mechanistic studies of the enzyme from *Escherichia coli* which is a tetramer of identical 383-residue polypeptide chains (2, 3). The three-dimensional structure of the protein is not yet known, and roles for any particular amino acid in enzyme function or stability have not been identified. It was previously reported that incubation of AdoMet synthetase with N-methylmaleimide rapidly inactivated both the AdoMet synthetase and tripyrophosphatase activities of the enzyme; however, detailed studies of the modification were not undertaken at that time (2). We now report identification of the amino acid residues modified by N-ethylmaleimide (NEM). We have found that reaction with NEM results both in inactivation of the enzyme and in displacement of the tetramer-dimer equilibrium of the protein toward the dimer by at least 7 orders of magnitude.

**MATERIALS AND METHODS**

AdoMet synthetase was purified and assayed as described previously (2, 4). Protein concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 1.3 (mg/ml)\(^{-1}\) cm\(^{-1}\) (2). N-[ethyl-2-3H]Maleimide and [3H]iodoacetic acid were purchased from Du Pont-New England Nuclear. Unlabeled NEM and TPCK-trypsin were purchased from Sigma. NEM concentrations were determined from the absorbance at 305 nm (using an extinction coefficient of 620 M\(^{-1}\) cm\(^{-1}\)) (5). [3H]NEM and [3H]iodoacetic acid were used at a specific activity of 2.4 mCi/mmol. Other reagents were obtained from commercial sources. Experiments were performed at 22 °C unless noted.

**Stoichiometry of NEM Modification—Incorporation of [3H]NEM into the protein was monitored by incubation of enzyme with [3H]NEM for periods between 10 s and 15 min in 50 mM Hepes/KOH, 50 mM KCl, 10 mM MgCl\(_2\). Aliquots of the reaction mixture were diluted 25-fold into 10 mM dithiothreitol in 50 mM Hepes/KOH, 50 mM KCl. From the diluted samples, aliquots were removed for assay of enzyme activity. From a second portion of the diluted enzyme the protein was precipitated by addition of an equal volume of 10% trichloroacetic acid. After centrifugation, the supernatant was removed, and the pellet was washed with 10% trichloroacetic acid. Aquasol scintillation fluid (Du Pont-New England Nuclear) was added to the tube containing the pellet, and radioactivity was determined by scintillation counting.

In experiments measuring protection by substrates and products against NEM modification, reaction with NEM was followed by the decrease in absorbance at 305 nm accompanying sulphydryl reaction with NEM (5).

**Preparation of Samples for Peptide Purification and Analysis—**1.0 ml of 5.9 mg/ml AdoMet synthetase in 50 mM Hepes/KOH, 50 mM KCl, 10 mM MgCl\(_2\) was treated with 0.8 mM [3H]NEM for 45 min. At the end of the incubation less than 5% of the original enzyme activity remained. Excess NEM was removed by gel filtration on a 1 x 20-cm column of Sephadex G-25 equilibrated and eluted with 50 mM NH\(_4\)HCO\(_3\), pH 8.0. The incorporation of radioactivity into the protein peak indicated that 2.1 ± 0.1 eq of NEM adduct were formed per subunit.

The solution of modified AdoMet synthetase was adjusted to 8 mM urea and 10 mM iodoacetic acid by addition of solid reagents. After 90 min, iodoacetic acid and urea were removed by three cycles of 20-fold concentration, and dilution into 50 mM NH\(_4\)HCO\(_3\), pH 8.0. The incorporation of radioactivity into the protein peak indicated that 2.1 ± 0.1 eq of NEM adduct were formed per subunit.

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onto a Rainin C8 reverse phase HPLC column attached to a Beckman model 320 HPLC system. The column was eluted at 1.5 ml/min with a gradient of 0-40% CH₃CN in 50 mM NH₄HCO₃ over 90 min. Elution was monitored at 229 nm, and 0.5-ml samples were collected. Aliquots (50 μl) were analyzed by scintillation counting. Two radioactive peaks eluting at 19 and 23% acetonitrile were obtained. Together these peaks contributed about 99% of the applied radioactivity. The two peptide fractions (each 1 ml) were denoted NEM-1 and NEM-2. The peptides were separately taken to dryness under vacuum and dissolved in 2 ml of 0.1% trifluoroacetic acid. The peptides were separately chromatographed again on the C8 column which was equilibrated in 0.1% trifluoroacetic acid and eluted with a gradient of 0-30% acetonitrile over 60 min. Elution was monitored at 229 nm, and radioactivity in each fraction was determined as described above. The radioactive component of peptide NEM-1 eluted at 34% acetonitrile, and the radioactive component of peptide NEM-2 eluted at 29% acetonitrile; in both cases the radioactive component chromatographed with the major UV-absorbing peak. The peptides were taken to dryness under vacuum. The amino acid compositions of aliquots of the peptides (between 1 and 0.25 nmol) were determined using the Picotag method and instrumentation of Waters Chromatography Division (Millipore Corp., Milford, MA) with minor modifications. The peptides were hydrolyzed at 110 °C for 17 h in the vials containing unknowns, amino acid standards, and protein standards were processed together in the same reaction chamber through the steps of hydrolysis and derivatization with phenylisothiocyanate. The amino acid contents of the unknowns were calculated based on the co-hydrolyzed and co-derivatized standards and are, therefore, corrected for hydrolytic losses. Data for protein standards show that the standard errors for most amino acids are <3% for amounts greater than 100 pmol; for serine, threonine, cysteine, valine, and isoleucine the standard errors are between 5 and 10%.

Gel Filtration Studies—Molecular weight determinations were performed by gel filtration using a Pharmacia LKB Biotechnology Inc. fast protein liquid chromatography system equipped with a Superose 12 column (1 x 30 cm) eluted at 0.5 ml/min with 50 mM KCl, 10 mM MgCl₂, 50 mM Hepes/KOH, pH 8.0. Sample volumes of 25 or 200 μl were injected, and elution was monitored at 280 nm. To investigate the dissociation of AdoMet synthetase into subunits at protein concentrations below 0.5 μM subunits, 50 mM Tris/Cl was substituted for Hepes/KOH. The lower absorption of Tris allowed elution to be monitored at 214 nm and permitted concentrations as low as 20 nm subunits to be used. In a few studies in which rapid separation of protein species was desired, a Beckman model 320 HPLC system equipped with a 0.75 x 30-cm TSK-250 gel filtration column (Bio-Rad, Richmond, CA) was used. The column was eluted at 0.5 ml/min with 50 mM potassium phosphate, 0.1 mM sodium sulfate, pH 7.0. 50-μl samples were injected, and 280-nm detection was used. A complete chromatogram was obtained in 16 min by HPLC whereas 40 min were required for the fast protein liquid chromatography separation.

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RESULTS

Rate and Stoichiometry of Modification with N-Ethylmaleimide—Fig. 1A shows the time course for inactivation of 0.09 mM AdoMet synthetase (subunit concentration) incubated with 0.7 mM NEM. Loss of activity followed pseudo first-order kinetics. Pseudo first-order kinetics of NEM modification were also observed when the reaction was followed by monitoring the decrease in the absorbance of NEM at 305 nm. The rate of inactivation was proportional to the concentration of NEM. The second order rate constant for inactivation is 0.31 M⁻¹·min⁻¹, determined from the rate of inactivation at 0.25, 0.5, 1.0, and 2.0 mM N-Ethylmaleimide. When the number of NEM groups incorporated as a function of inactivation was determined using [3H]NEM (see “Materials and Methods”), the data in Fig. 1B were obtained. Inactivation correlates with incorporation of 2 eq of NEM/enzyme subunit; the magnitude of the decrease in NEM absorbance at 305 nm upon reaction with the enzyme also indicates that 2 eq of NEM react per subunit.

Protection by saturating concentrations of substrates and products of the reaction was explored, and the results are presented in Table I. Substantial protection was obtained with PP₁ and the ATP analog AMP-PNP (ATP was not used due to the ATPase activity of the enzyme (2)), but not with methionine or AdoMet. In the presence of PP₁ or AMP-PNP, the rate of NEM incorporation followed pseudo first-order kinetics, and the magnitude of the change in absorbance at 305 nm indicated that 2 eq of NEM were incorporated per subunit. Greater protection was obtained after preincubation of the enzyme with AMP-PNP and methionine when an enzyme-AdoMet-O₂PO₄NHPO₄ complex that dissociates at a rate of 6 x 10⁻⁴ s⁻¹ (2) is formed. This complex reacts with NEM at about 3% of the rate of unliganded enzyme, as monitored both by the disappearance of NEM absorbance at...
Identification of the Modified Amino Acid Residues—In order to identify which groups were modified by NEM, a tryptic digest of AdoMet synthetase modified with [3H]NEM was prepared. Peptides were purified in two steps by reverse phase HPLC chromatography (see "Materials and Methods"). The amino acid compositions of the peptides were determined and are given in Table II. Comparison of the peptide compositions with compositions of all tryptic peptides predicted from the amino acid sequence (3) uniquely identified peptide NEM-1 as containing residues 75–98 and peptide NEM-2 as containing residues 231–245. In both cases the cysteine predicted from the sequence was missing from the modified peptide, as anticipated. The modified groups are, therefore, cysteine 90 and cysteine 240, 2 of the 5 cysteine residues present in each subunit.

Modification Induces Dimer Accumulation—The observation that two NEM molecules react with each enzyme subunit at the same apparent rate suggested that modification of one cysteine by NEM might lead to a conformational change in the protein that exposed a previously inaccessible cysteine which then rapidly reacted with NEM. To investigate the possibility of change in aggregation state, the enzyme was analyzed by gel filtration as a function of modification. Fig. 2A shows that unmodified protein chromatographed as a single peak which had the expected retention time for a tetramer. Fig. 2B shows that protein which is ~35% inactivated chromatographs as two peaks; the first peak contains the enzyme activity and the second contains 3H from NEM. The retention time of the second peak corresponds to that expected for a dimer; monomer was not observed. Fig. 2C shows that at ~90% modification, the two protein peaks remain visible in the chromatogram; a small amount of 3H from NEM (~2–5% of the total 3H incorporated) is present in the tetramer peak which also contains unmodified protein since there is enzyme activity. The dimer peak contains <95% of the 3H from NEM and has no detectable enzymatic activity.

From the amount of 3H chromatographing in the dimer region in Fig. 2C, a stoichiometry of 1.8 ± 0.3 eq of NEM incorporated per subunit is estimated, whereas in the tetramer region the stoichiometry is estimated to be 0.5 ± 0.2 eq of NEM/subunit. These stoichiometries indicate that essentially all of the protein chromatographing as a dimer has two NEM modifications/subunit. From the distribution of 3H between the dimer and tetramer peaks, the dissociation constant for a modified tetramer to yield two modified dimers is estimated to be ~4 × 10^{-4} M. To further investigate the correlation of dimer appearance with loss of enzyme activity, enzyme inactivated to various extents was analyzed by gel filtration to determine the amount of enzyme remaining as tetramer. Fig. 3 shows a linear correlation between modification by NEM and accumulation of dimeric protein. Thus, dimer accumulation as well as inactivation correlates with modification by NEM. Unfortunately solutions of the modified protein become turbid on standing for long periods (>1 h), and aggregates of ~6 × 10^6 daltons are observed by gel filtration, preventing detailed structural studies of the modified protein.

Investigation of Other Sulphhydrol Reagents—Other sulphhydrol reagents were tested for inactivation or induction of subunit dissociation. After a 1-h incubation of 0.08 mM enzyme with 5 mM [3H]iodoacetic acid, <0.1 eq of iodoacetate/subunit were incorporated, and no dissociation of the enzyme was detected by gel filtration. These observations agree with the previously reported lack of inactivation by iodoacetic acid and iodoacetamide (2). In an attempt to determine whether the two NEM-reactive sulphhydrols reacted sequentially with only the first being selective toward NEM, 4 mM [3H]iodoacetic acid was included in a 2-h incubation of 0.06 mM NEM with 0.06 mM enzyme subunits. After gel filtration, no incorporation of [3H]iodoacetate (<0.2 eq/subunit) was detected above that observed in a control sample lacking NEM. Incubation of 0.1 mM enzyme with the small reversible sulphhydrol reagent methyl methanethiosulfonate (6) (8 mM) for 1 h did not result in inactivation or dimer accumulation and did not protect against subsequent modification by NEM.

Dimer-Tetramer Dissociation Constants for Native and Modified AdoMet Synthetase—In view of the dissociation of the enzyme to a dimer upon NEM modification, the dimer-tetramer equilibrium constant for the unmodified enzyme was investigated. Enzyme was incubated for 1 h at various concentrations and was then analyzed by gel filtration chromatography under the same conditions used for studies of the NEM-modified enzyme. At subunit concentrations from 200 μM to 22 nM the peak position and peak width of the tetramer were unchanged, and no other aggregates were observed. Assuming that at the lowest protein concentration 10% of the protein was present as a dimer but had escaped detection, an upper limit on the dissociation constant for a tetramer to two dimers is 6 × 10^{-13} M.

DISCUSSION

The present study shows that reaction of AdoMet synthetase with NEM results in concurrent inactivation and modification of cysteine 90 and cysteine 240; cysteines 41, 134, and 295 were not detectably modified. These results provide the first insight into residues which may play roles in the structure and/or activity of the enzyme. Since only 50% of the enzyme is inactivated when on the average one NEM/subunit has been incorporated, either 1) one group reacts with NEM at the observed rate and the second group on the same subunit subsequently reacts faster (inactivation could result from modification of either group) or 2) two groups react with NEM independently and fortuitously at the same rate, but modification of only one of the groups affects the enzyme activity. If the two groups react independently, inactivation results from modification of only one of the residues since at 50% modification (an average of one adduct/subunit) the

| Peptide | Predicted Peptide NE-M-1 | Predicted Peptide NE-M-2 |
|---------|---------------------------|--------------------------|
| Lys     | 1.0                       | Arg 1                    |
| Asp + Asn| 2.6                       | Asp 1                    |
| Glu     | 1.1                       | Gly 5                    |
| Ser     | 3.1                       | Thr 1                    |
| Gly     | 3.2                       | Pro 1                    |
| His     | 1.1                       | Val 1                    |
| Ala     | 3.2                       | Met 1                    |
| Tyr     | 1.1                       | Ile 1                    |
| Val     | 2.3                       | Leu 1                    |
| Met     | 1.0                       | Phe 1                    |
| Ile     | 2.1                       | Cys 1                    |
| Leu     | 1.1                       | All others' 0            |
| Phe     | 1.1                       | <0.1                     |
| Cys     | 1.0                       | <0.1                     |
| All others' 0 | <0.1                     |<0.1                     |

* Predictions are based on the amino acid sequence of the enzyme (3). No other peptides have similar compositions.
* Compositions are the average of two determinations and are normalized to the content of lysine or arginine as appropriate.
* Tryptophan was not determined.
FIG. 2. Dissociation of modified AdoMet synthetase from a tetramer to a dimer. Native AdoMet synthetase and modified forms were separated by gel filtration on Superose 12 (as described under "Materials and Methods"). Elution was monitored at 280 nm. Fractions of 0.4 ml were collected. The enzyme activity and [3H]NEM content of each fraction which contained protein were determined using 5- and 200-μl aliquots, respectively. The panels shown correspond to chromatograms obtained after incubations of 0.05 mM enzyme subunits with: A, no NEM addition; B, 0.5 mM NEM for 3 min (~35% inactivation); C, 0.5 mM NEM for 10 min (~90% inactivation). The smooth line is the A280 profile; ○ denotes AdoMet synthetase activity; ●, [3H] from NEM.

FIG. 3. Correlation of fraction of dimeric AdoMet synthetase and inactivation. AdoMet synthetase (0.08 mM subunits) was incubated with 1.0 mM NEM for various lengths of time in 50 mM Hepes/KOH, 50 mM KCl, 10 mM MgCl2, pH 8.0. The incorporation of NEM was estimated from the decrease in NEM absorbance at 305 nm. Reactions were stopped by a 2-fold dilution into 50 mM Hepes/KOH, 50 mM KCl, 10 mM dithiothreitol, pH 8.0. The samples were chromatographed on a gel filtration HPLC column (TSK-250) to separate different aggregation states of the enzyme. The fraction of enzyme present as the tetramer was estimated from the height of the peak eluting at the position of the unmodified enzyme. The line is the best linear regression fit to the data and has slope 0.994.

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S. H. K. and M. C. C. are present as equal fractions of unmodified enzyme, enzyme modified at group 1, enzyme modified at group 2, and enzyme modified at both groups 1 and 2. If modification at either group alone inactivated the enzyme, 75% of the enzyme activity would be lost, in contrast to the observed 50% inactivation (Case I of Ray and Koshland (7)). Substrates which protect against NEM modification equally protect both sulfhydryl groups; therefore, it appears likely that modification of the two groups occurs sequentially with the first modification rendering the second group reactive; the alternative that both groups are independently and fortuitously modified at the same rate under a variety of conditions seems less probable.

Interpretation of the cause of inactivation is complicated by both the formation of two NEM adducts/subunit and the destabilization of the tetrameric native enzyme relative to a dimer. Since there is no evidence as to whether an unmodified dimeric enzyme is catalytically active, it is not possible to attribute enzyme inactivation to modification of a catalytically important group or even a steric effect due to the modification reagent. The enzyme is composed of identical subunits and does not display cooperativity in kinetics or substrate binding (2), and it was surprising that the modified protein dissociated to dimers rather than monomers. However, it is noteworthy that in the absence of substrates the protein crystallizes with two subunits/asymmetric unit suggesting that the interactions among the four subunits of the tetramer are not identical (8).

Since the NEM-modified enzyme exists primarily as a dimer, it appeared possible that a small amount of the native enzyme exists as a dimer, and this was the species which reacted with NEM. Gel filtration studies of the native enzyme indicate that the tetramer-dimer equilibrium lies far toward the tetramer (Kd < 6 × 10−11 M) under the conditions of rapid NEM activation. Since the rate of enzyme inactivation is proportional to the concentration of NEM, if the dimer were the species which reacts with NEM, the rate of tetramer dissociation cannot be rate-determining in inactivation and must be significantly greater than the fastest observed pseudo first-order rate of NEM inactivation, 0.1 s−1. However, if the dimer were the NEM-reactive species, the rate of NEM reaction would be proportional to the dimer concentration and, therefore, proportional to (Kd [tetramer])21 where Kd is the dissociation constant for the tetramer-dimer equilibrium. The observation of a first-order dependence of the rate of inactivation on protein concentration, rather than a square root dependence, indicates that NEM does not react solely with the dimeric enzyme.

It, therefore, appears probable that the tetramer is the species with which NEM reacts. The first modification by NEM may render the second sulfhydryl reactive, possibly as a result of tetramer dissociation. When partial enzyme activity is lost from a sample, all of the residual activity appears in the tetramer, and the ≥95% of the radioactivity from NEM appear in the dimer. Therefore, if the tetramer is the NEM-reactive species the unmodified dimers formed upon dissociation are able to reaggregate to form an enzymatically active tetramer. Our inability to modify the sulfhydryls with other reagents including NEM-derived nitroxide spin labels5 has thwarted attempts to determine which sulfhydryl reacts first or the spatial proximity of the cysteines.

An indication of the possible importance of the cysteines comes from comparison of the sequence of the E. coli enzyme

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with the highly similar sequences (52% identical residues) of the two tetrameric AdoMet synthetases coded for by the SAM1 and SAM2 genes of *Saccharomyces cerevisiae* (9, 10). Cysteine 90 is conserved in all three sequences while cysteine 240 is replaced by threonine (11) or alanine (1) in the SAM1 and SAM2 gene products, respectively. In each sequence cysteine 90 closely precedes the sequence Gly-Ala-Gly-Asp-Gln-Gly, which conforms to the Gly-X-Gly-X-Gly sequence found in many nucleotide binding sites (12). The outcome of site-directed mutagenesis studies investigating the roles of these residues in AdoMet synthetase may yield further insight into interactions involved in protein oligomerization and activity.

Acknowledgments—We would like to thank Dr. Y. Surdin-Kerjan for communication of the sequences of the two AdoMet synthetase genes of *S. cerevisiae* prior to publication. We also thank Dr. T. S. Leyh for many helpful discussions, and A. Pomenti and Dr. A. Zweidler for the amino acid analyses.

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