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Characterization of alkylamine-sensitive site in α_2-macroglobulin

(blood protein/protease inhibitor/active-site peptide/protein modification/γ-glutamylmethylamide)

RICHARD P. SWENSON* AND JAMES BRYANT HOWARD†

Department of Biochemistry, University of Minnesota, 227 Millard Hall, 435 Delaware Street, Minneapolis, Minnesota 55455

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ABSTRACT **Methylamine reacts with the plasma protease inhibitor, α_2-macroglobulin, to form an irreversible, covalent modification. Quantitation of the reaction indicates 3.9 ± (SD) 0.4 reactive sites per native tetrameric protein (M_r = 725,000) or one site per subunit. The reaction is selective and specific in that only 1 or 2 labeled peptides are observed on radioautography of peptide maps derived from [^{14}C]methylamine-treated α_2-macroglobulin. A single chymotryptic peptide was isolated in 56% overall yield from the labeled protein. The peptide sequence by Edman degradation was found to be Gly-Cys-Gly-Glu-X-Asn-Met-(Val, Leu), in which X was the only radiolabeled phenylthiohydantoin derivative. Amino acid analysis and mass spectral analysis of the derivative suggests that X is γ-glutamylmethylamide. Because glutamic acid and glutamine residues do not normally react with alkylamines, this work presents presumptive evidence for an alternative activated center in selected proteins.**

The plasma protease inhibitor, α_2-macroglobulin (α_2M), and the complement proteins, C-3, C-4, and C-5, have been shown to be inactivated by ammonium ion, hydrazine, and alkylamines (1–4). The unique sensitivity of these few plasma proteins to amines has been long established and is used as a selective means of their inactivation—e.g., hydrazine is used to inactivate C-3 and C-4 in plasma for the assay of these complement components (4). However, at the molecular level, there is no recognized mechanism for the inactivation of these proteins by alkylamines. It has been considered to be either reversible (3) or irreversible (refs. 1, 2, and 4) and to be either a covalent reaction—e.g., a reaction with a hypothetical carbonyl function (2)—or a noncovalent, specific charge effect (5). Because the amine reaction is limited to a few plasma proteins, alkylamines would appear to be a useful probe for the study of the mechanism of these proteins.

We have undertaken a systematic study of the methylamine reaction with α_2M to elucidate the nature of the alkylamine reaction. In this communication, we establish that the reaction is selective, covalent, irreversible, and stoichiometric. We present evidence that the methylamine-sensitive site does not involve a carbonyl function, but is at an activated glutamyl residue. Thus, α_2M must contain a functional modification not previously described in proteins. A preliminary account of a portion of this work has been presented (6).

MATERIALS AND METHODS

α_2M was purified from outdated human plasma (University of Minnesota Hospital Blood Bank) and assayed as described (7). Amino acid sequences were determined by automated Edman degradation and by using a modified dilute Quadrol program (8). High performance liquid chromatography was used to identify and to isolate the phenylthiohydantoin (PTH) derivatives (8). All other methods are included in legends. [^{14}C]Methylamine was obtained from Amersham.

RESULTS AND DISCUSSION

To test whether the alkylamine reaction with α_2M resulted in a covalent modification of the protein, we incubated α_2M (2 mg/ml) with 25 mM [^{14}C]methylamine (27,000 cpm/nmol) for 6 hr at 25°C and pH 8.0. During the reaction, α_2M was >95% inactivated with respect to trypsin binding capacity. The reaction mixture was divided into two portions, one of which was reduced with 10 mM $NaBH_4$ for 18 hr at 8.0 and 4°C. The protein was separated from unreacted methylamine by gel filtration, as shown in Fig. 1*A*. Both the reduced and nonreduced methylamine-treated α_2M had radioactivity associated with the protein, which suggested that either the methylamine was covalently incorporated or that α_2M had a specific, noncovalent binding site for methylamine. If the latter were true or if the covalent binding were through a Schiff's base with a carbonyl function, then the [^{14}C]methylamine should exchange with unlabeled methylamine. When [^{14}C]methylamine-modified α_2M was incubated in 150 mM unlabeled methylamine, no significant loss of radioactivity from the protein was detected, even after 96 hr at 25°C (see Table 1). Likewise, there was no apparent difference in methylamine exchange whether the methylamine–α_2M adduct had or had not been previously reduced with $NaBH_4$.

To further demonstrate that the methylamine incorporation was covalent, [^{14}C]methylamine-labeled α_2M was denatured and reduced in 6 M guanidine·HCl/10 mM dithiothreitol for 3 hr at pH 8.0 and 40°C (7). After carboxymethylation (7), the protein was rechromatographed by gel filtration, as shown in Fig. 1*B*. Greater than 80% of the radioactivity remained with the protein. Based upon the amount of [^{14}C]methylamine retained in the carboxymethylated α_2M, there are 3.9 ± (SD) 0.4 (six determinations) sites of reaction per native protein (M_r = 725,000) (9). Recently, we (7) showed that α_2M is a tetramer of identical subunits (M_r = 185,000), which can bind two trypsin molecules per tetramer. The stoichiometry of methylamine incorporation indicates that each of the four subunits may contain a reactive site.

Carboxymethylated, methylamine-treated α_2M was digested with various proteases and the resultant peptides were mapped by electrophoresis and partition chromatography on paper (see Fig. 2). The radiolabeled peptides were identified by radioautography (8). Both tryptic and chymotryptic peptide maps of α_2M indicated a limited number (1 or 2) of unique radiolabeled peptides. The tryptic map was consistent with a single site of methylamine reaction (a small amount of radiodensity at the origin was probably due to partially digested protein). The chymotryptic map excluded more than two unique sites of methylamine reaction. Complete enzymatic digestion gave essentially a single radiodense spot. The combination of the

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Abbreviations: α_2M, α_2-macroglobulin; PTH, phenylthiohydantoin.

* Present address, Department of Biochemistry, University of Michigan, Ann Arbor, MI 48104.
† To whom reprint requests should be addressed.

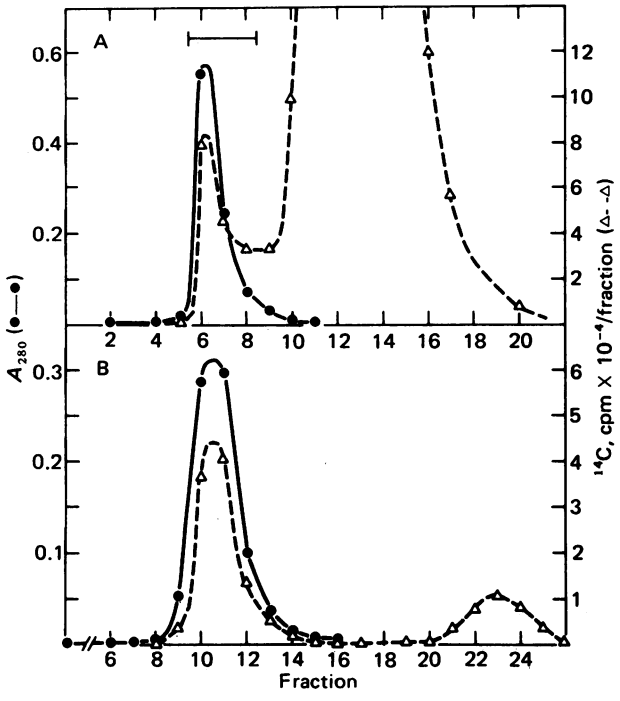

FIG. 1. (*A*) Chromatography of methylamine-reacted α_2M (1 mg) on a 1 × 16 cm column of Sephadex G-25 equilibrated with 0.1 M Tris·HCl, pH 8.0. (*B*) Chromatography of carboxymethylated, methylamine-reacted α_2M on a 1 × 25 cm column of Sephadex G-25 equilibrated with 0.05 M NH_4AcO, pH 8.0. Fraction size for both columns was 1.0 ml.

three maps, each having its own unique radiolabeled peptides, was consistent with a single covalent site of reaction and excluded a random reaction or a large number of sites.

To identify the site of covalent reaction and to characterize the specific amino acid residue that was modified by methyl-

Table 1. Exchange of unlabeled methylamine with [^{14}C] methylamine-treated α_2M

Exchange reaction time, hr	Radioactivity retained in α_2M,* %	
	Nonreduced	$NaBH_4$-reduced
0	100	100
1	94	92
7	90	85
24	105	88
40	109	88
72	102	80
96	95	83

* [^{14}C]Methylamine-treated α_2M (0.4 mg, 5500 cpm) from Fig. 1*A* was incubated at 25°C in 1.0 ml of 150 mM unlabeled methylamine (pH 8.0). At the specified times of incubation, 100-μl samples were removed and rapidly filtered through 0.50 ml of DEAE-Sephadex A-25 equilibrated with 50 mM Tris·HCl, pH 8.0. Under these conditions, methylamine passes through the resin whereas α_2M is retained. After washing with the same buffer, the resin was transferred to a scintillation vial and the protein was solubilized by 1.0 ml of 0.50 M NaCl. The radioactivity was determined after the addition of 5 ml of Aquasol-2 (New England Nuclear) counting fluid. To demonstrate that free methylamine was not retained by the DEAE-Sephadex resin, a large excess of [^{14}C]methylamine was added to methylamine-treated α_2M and filtered. The radioactivity retained on the resin was linear with ^{14}C-labeled α_2M and independent of methylamine concentration. [^{14}C]Methylamine-treated α_2M that was reduced with $NaBH_4$ prior to gel filtration (Fig. 1*A*) was treated in an identical manner as the nonreduced sample.

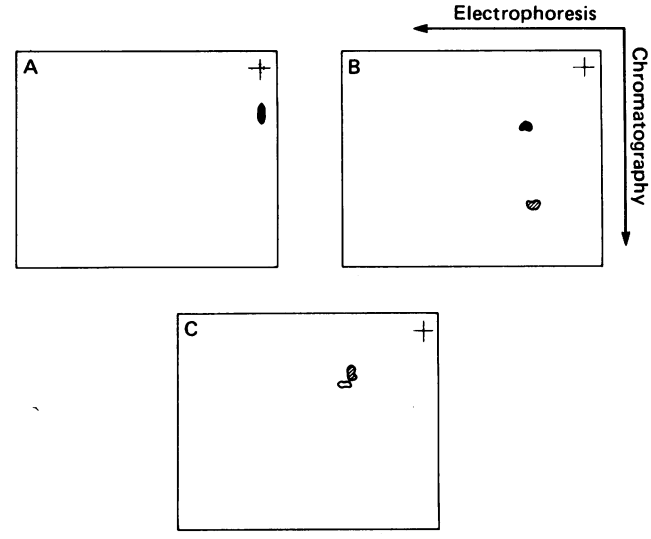

FIG. 2. Radioautography of peptide maps of carboxymethylated, [^{14}C]methylamine-treated α_2M. Electrophoresis at pH 1.9 was followed by descending chromatography in pyridine/1-butanol/acetic acid/water (10:15:3:12, vol/vol). Each digest contained 225 μg of α_2M and 21,000 cpm. (*A*) Trypsin (3% by weight) digestion was at pH 8.0 for 6 hr at 37°C. (*B*) Chymotrypsin (8% by weight) digestion was at pH 8.0 for 18 hr at 37°C. (*C*) Complete digestion of α_2M by trypsin (6% by weight) and chymotrypsin (4% by weight) at pH 8.0 for 18 hr followed by carboxypeptidase Y (0.8 units) for 6 hr at pH 6.0 and leucine aminopeptidase (20 units) at pH 8.0 for 18 hr.

amine, we isolated the radiolabeled chymotryptic peptide. Thirty-five milligrams (190 nmol) of carboxymethylated, [^{14}C]methylamine-inactivated α_2M (138 cpm/nmol) was digested with 4% (wt/wt) chymotrypsin for 15 hr at 25°C. The digest was fractionated on Sephadex G-50 as shown in Fig. 3. A single peak was found which accounted for 85% of the original radioactivity. The pooled peak was chromatographed on DEAE-Sephadex A-25 as shown in Fig. 4. Two radioactive peaks were found. The major peak, which accounted for 78% of the total radioactivity, was desalted and separated from several small peptides on Sephadex G-25 (85% recovery). Including the material used to count the fractions from the chromatography steps, the overall yield of the peptide was 56%. The amino acid composition of the peptide is shown in Table 2. It should be noted that methylamine was identified in the analysis and no unidentified residues were observed. This suggested that the methylamine had been incorporated as an

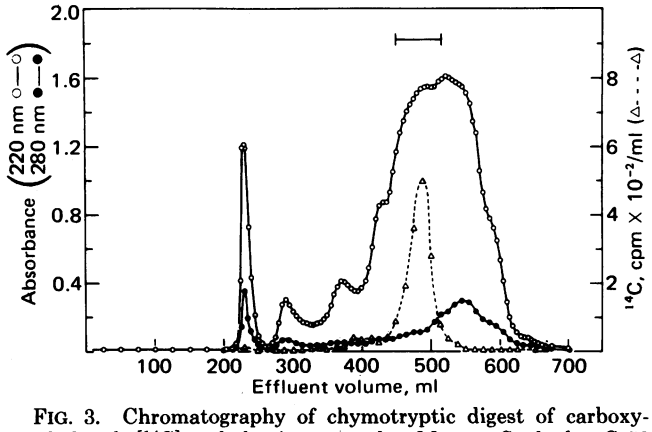

FIG. 3. Chromatography of chymotryptic digest of carboxymethylated, [^{14}C]methylamine-treated α_2M on a Sephadex G-50 column (2.5 × 100 cm) at pH 8.5 in 0.05 M ammonium acetate. The radioactive peptide was pooled as indicated by the bar.

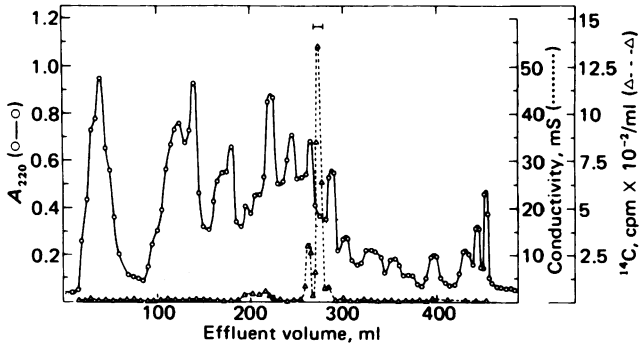

FIG. 4. DEAE-Sephadex A-25 ion exchange chromatography (1 × 54 cm column) of the radioactive material from Fig. 3. The column was equilibrated in 50 mM Tris·HCl, pH 8.0, and a gradient from equilibrating buffer to 0.35 M NaCl (200 ml each) was run. The main radioactive peak was pooled as indicated by the bar.

acid labile component of a normal amino acid. The specific radioactivity of the peptide (140 cpm/nmol) was the same as the starting protein, which indicated that the peptide was representive of the total labeling of the protein.

The sequence of the radiolabeled peptide was determined by automated Edman degradation. The PTH derivatives were separated by high performance liquid chromatography and the radioactivity for each cycle of degradation was determined. The results are shown in Fig. 5. A radioactive PTH derivative for cycle 5 (and as "out-of-step" residues in cycles 6 and 7) was observed that coeluted with PTH-glycine (7.1 min on our chromatographic system) (8). The radioactive PTH derivative was separated from PTH-glycine by rechromatography by using gradient 7 rather than gradient 3 of the standard separation (8). The derivative had a specific activity of 136 cpm/nmol when the extinction coefficient for PTH-glycine was used. Acid hydrolysis of the PTH derivative [6 M HCl containing 0.1% $SnCl_2$ for 4 hr at 150°C (10)] gave 85% conversion to glutamic acid and methylamine in equal amounts. No other amino acids were observed. This is consistent with one extra glutamic acid in the peptide as determined by amino acid analysis when compared to the sequence determination.

The isolated PTH derivative was subjected to low resolution mass spectrometry and the results are shown in Fig. 6. PTH derivatives of amino acids are good derivatives for mass spectral analysis because they have prominent molecular ions and characteristic fragments at mass to charge (m/e) ratios = 135 and 192 (11). For the PTH derivative of the methylamine-containing amino acid, the molecular ion of m/e = 277 is 63% of the m/e = 135 ion. A mass of 277 is 14 larger than expected for PTH-glutamine and 13 larger than PTH-glutamic acid—namely, one methyl group larger than PTH-glutamine or exactly that predicted for PTH-γ-glutamylmethylamide. A characteristic ion of glutamyl and glutaminyl derivatives in the mass spectrometer is the ketene ion, which is obtained by elimination at the γ-carboxyl group (12). For the PTH derivative, m/e = 246 is the expected ketene ion and is a major fragment observed in the spectra of the sample (Fig. 6). This confirms that m/e = 277 is the molecular ion and that the site of substitution is the γ-carboxyl group. Ions m/e = 135 and 192 are consistent with the derivative's being a PTH amino acid.

Thus, the preponderance of evidence suggests that methylamine is incorporated into a unique glutamic acid residue to give γ-glutamylmethylamide. This modification fully inactivates α_2M as a protease inhibitor. However, it can be assumed that the site of methylamine incorporation is not a simple glutamine or glutamic acid in the unreacted protein. The unusual nature of the methylamine incorporation requires that the glutamic acid residue be activated. Several possibilities can be suggested; the glutamic acid could be modified by a postribosomal modification—e.g., as an activated ester—or the

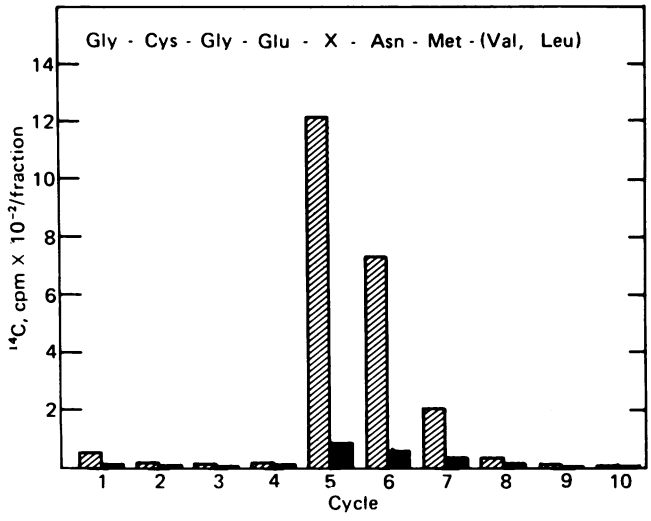

FIG. 5. Summary of automated Edman degradation of the radiolabeled chymotryptic peptide from α_2M. The initial coupling for 35 nmol of peptide was 70% and the repetitive yield was 90% without correction for "out-of-stepness." The yields of the PTH amino acids for the individual cycles were: cycle 1, 20 nmol; cycle 2, 17 nmol; cycle 3, 16 nmol; cycle 4, 17 nmol; cycle 5, 14 nmol; cycle 6, 8 nmol; cycle 7, 3 nmol. Each residue was identified by high performance liquid chromatography (8) and the radioactivity was determined by scintillation counting. Valine and leucine could not be identified, probably because of washout of the carboxyl-terminal hydrophobic dipeptide. Cross-hatched bars indicate the radioactivity in the ethylacetate extract from the 1.0 M HCl conversion (8) and the solid bars are the radioactivity remaining in the aqueous acid.

Table 2. Amino acid composition of the radiolabeled chymotryptic peptide

Amino acid	Residues
Carboxymethylcysteine	0.8
Aspartic acid	1.0
Glutamic acid	2.2
Glycine	1.8
Valine	1.0
Methionine	0.8
Leucine	1.0
Methylamine	0.7

Hydrolysis was for 24 hr in 6 M HCl at 110°C. Composition was calculated for a single leucine residue. Methylamine eluted 10 min after ammonia and has a color constant of 0.29 × leucine.

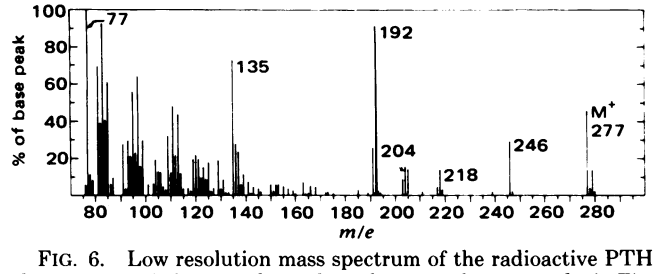

FIG. 6. Low resolution mass spectrum of the radioactive PTH derivative pooled from Edman degradation cycles 5, 6, and 7 in Fig. 5. The spectrum was determined with 5 nmol by using an AEI MS 30 spectrometer in the University of Minnesota, Department of Chemistry, Mass Spectroscopy Laboratory. Ionization potential was 70 eV.

glutamic acid could be part of an activated center involving other side chains—e.g., a pseudo enzyme active site. An alternative hypothesis that the methylamine incorporation might be catalyzed by factor XIII (transglutaminase) has been eliminated. The α_2M reaction, unlike factor XIII-catalyzed transamidation (13), is pseudo first order with respect to methylamine, is not inhibited by EDTA, and is not activated by calcium (unpublished results). In addition, we are using a highly purified α_2M preparation.

The role of this functional center can only be contemplated. One would not expect that the methylamine reaction would result in α_2M inhibition solely by steric hinderance. A more attractive hypothesis is that this site may serve for the covalent attachment of the protease to the inhibitor. Although several groups have presented evidence that suggests that α_2M may covalently bind proteases, there is no direct chemical characterization of the linkage (14, 15). Alternatively, the methylamine reaction may induce a major conformational change in α_2M similar to that observed upon binding of proteases (16). The similarity of the α_2M site to the methylamine site in complement proteins has yet to be established. However, the formation of the irreversible complex of C-3 and C-4 with cell membranes may also utilize a covalent bond (17). Such an interaction might be predicted to involve a similar methylamine-sensitive site.

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