Isolation of Glutamic Acid Methyl Ester from an *Escherichia coli* Membrane Protein Involved in Chemotaxis*

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We have isolated glutamic acid 5-methyl ester from an *Escherichia coli* protein that is involved in chemotaxis. The bacteria were first incubated with [methyl-"Hl]methionine under conditions which are known to result in methylation of the protein. The protein, isolated by gel electrophoresis, was then digested by successive treatment with three proteolytic enzymes. One of the products was [methyl-"H]glutamic acid 5-methyl ester, identified by comparison with an authentic sample in the following studies: (a) chromatography on an automatic amino acid analyzer, (b) chromatography on paper in two solvent systems, (c) chromatography on paper of the N-acetyl derivatives, and (d) stability of the ester bond to various pH conditions. No aspartic acid 4-methyl ester was found in the enzymatic digest. Treatment of the methylated protein with alkali released the radioactivity as ["Hl]methanol, which was identified by gas chromatography and by preparation of the 3,5-dinitrobenzoate.

Recently this laboratory discovered that the methylation of a cytoplasmic membrane protein of *Escherichia coli* is involved in chemotaxis (1). The protein, methyl-accepting chemotaxis protein, acquires methyl groups derived from the methyl group of methionine. To further study this methylation, we wanted to identify the methylated residue of MCP.

We originally tried to isolate the methylated moiety by submitting MCP to a standard acid hydrolysis; however, this procedure resulted in a loss of the methyl groups of MCP as volatile material. We were aware that a mammalian enzyme, protein methylease II, catalyzes the formation of methylated proteins which, like MCP, are rapidly demethylated on acid or alkaline hydrolysis (2-4). Although the methylated amino acids have never been isolated from a protein methylease II product, there is strong evidence, mostly due to Kim and Paik, that the enzyme catalyzes the formation of methyl esters of aspartic and glutamic acid residues of proteins (2, 3, 5, 6).

To circumvent the problem of lability of the methylated residue of MCP to acid hydrolysis, we used an enzymatic procedure. By this means we have isolated [methyl-"H]glutamic acid 5-methyl ester from MCP which had been methylated in *vivo* by incubation with [methyl-"H]methionine. To our knowledge, this represents the first isolation of this amino acid from a natural source, and also the first report of pH-labile protein methylation in a prokaryote.

**MATERIALS AND METHODS**

Enzymes and Chemicals—*Papaya latex* papain was obtained from Worthington Biochemical Corp. as a crystalline suspension (10 units/mg). The enzyme aminopeptidase I (7) was the generous gift of Dr. K. Brew; 1 mg hydrolyzes 400 pmol of L-leucine p-nitroanilide/min under optimal conditions (7). An ammonium sulfate suspension of pig kidney prolidase (190 units/mg) was purchased from Sigma Chemical Co.

[methyl-"H]Methionine was obtained from Amersham/Searle Corp. [methyl-"H]Methanol and ['4C]methanol were purchased from New England Nuclear. Methyl amino acid standards were all commercial products, obtained from Calbiochem, Cyclo Chemical, and Sigma Chemical Co.

Preparation of [methyl-"HlMCP —[methyl-"HlMCP was prepared by the method of Kurt et al. (1) from *Escherichia coli* K-12, strain RP477 metF (1), a mutant that is unable to synthesize methionine. Methylation of the bacteria with [methyl-"H]methionine was allowed to proceed for 20 min under nongrowth conditions. Protein synthesis was blocked by omission of essential amino acids and by the presence of chloramphenicol. The envelope fraction of the bacteria was prepared and solubilized. From this, MCP was isolated by sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis on gels of 6.5% acrylamide (20 × 0.6 cm, diameter). More detailed procedures have been published previously (1).

Elution was accomplished by letting gel particles from the region of MCP (R, 0.56) stand for 24 h in 50 mM sodium acetate buffer (pH 5.2) and 0.25% Triton X-100, with occasional swirling by hand. The gel particles were then filtered out with suction on a Uni-Foam polycarbonate membrane (Bio-Rad Laboratories). Approximately 15% of the ["H]originally present in the gel particles was lost during this elution.

Enzymatic Digestion—A modification of the method of Hill and Schmidt (8) for complete proteolysis by enzymes was used. The
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filtered eluate (2.4 ml) containing [methyl-3H]MCP already included 120 μmol of sodium acetate buffer (pH 5.2). To this were added 7 μl of 0.1 M potassium ethylenediaminetetraacetic acid (adjusted to pH 7), 25 μl of 0.5 M β-mercaptoethanol, 200 μg (2 units) of papain, and distilled water to a volume of 3.5 ml. The pH was 5.7 due to the combination of acetate buffer with phosphate electrophoresis buffer eluted from the gel. The mixture was left at 40° for 18 h, adjusted to pH 2 with 1 M HCl (8), and lyophilized. The residue was dissolved in 1.5 ml of distilled water. To the resulting solution were added 45 μl each of 1 M CaCl₂ (cofactor for aminopeptidase I) and 1 M MnCl₂ (cofactor for prolidase). The pH was then adjusted to 7.9 to 8.1 with dilute NaOH. Finally, 50 μl of 0.5 M Tris hydrochloride buffer (pH 8.0), 100 μg of aminopeptidase I, 200 μg (38 units) of prolidase, and distilled water to a volume of 3.0 ml were added. (Aminopeptidase I was used because of its broad substrate specificity (7).) After a 4-h incubation at 40°, the mixture was centrifuged. The supernatant was adjusted to pH 2 with 1 M HCl and filtered through a Millipore filter, and the filtrate was lyophilized. The residue from lyophilization was the enzymatic digest of [methyl-3H]MCP used in all subsequent experiments.

There was no loss of 3H during the papain digestion stage. During the digestion with aminopeptidase I and prolidase, 30% of the radioactivity originally present in the eluate was converted to volatile material. The final recovery of 3H in nonvolatile form was 60% of that originally present in the gel particles.

Isolation of Volatile Alkaline Hydrolysis Product of [methyl-3H]MCP—Alkaline hydrolysis of the methyl linkages of [methyl-3H]MCP and recovery of the volatile radioactive product were both achieved with a standard Conway microdiffusion cell (9). The center well of the Conway cell contained 1.5 ml of distilled water. In the outer well were placed separately 1.0 to 1.5 ml of the eluate containing [methyl-3H]MCP and 2 ml of saturated aqueous K₂CO₃ (pH 12). The cell was left for 3 to 4 h at room temperature. We found that recovery of authentic [3H]methanol in the center well was 70% after 4 h.

Analytical Procedures—A Beckman 120C automatic amino acid analyzer with standard modifications for single column analysis (10) was used for amino acid analysis. Descending paper chromatography was done on strips (1 inch wide × 45 cm) of Whatman 1 paper. MCP digest was applied to the paper as a methanolic extract. This gave the same pattern of radioactivity as an aqueous extract, but less of the buffer salts were applied when methanol was used. Paper chromatograms were sprayed with ninhydrin (0.2% in acetone) to locate amino acids. For the experiments shown in Figs. 3 and 4, the resulting colored compounds were eluted into water and determined spectrophotometrically. 3H and 14C were measured by liquid scintillation spectrometry; samples were counted until at least 1000 disintegrations had been recorded.

Gas chromatography was done on a Varian Aerograph Autoprep model A-700 gas chromatograph equipped with a 6-foot stainless steel column (0.085 inch inner diameter). The column was packed with Carbowsieve B, 100 to 120 mesh (Supelco, Inc.). Helium was used as carrier gas at a flow rate of 90 ml/min, and the column temperature was 200°.

RESULTS

The protein MCP was eluted from a gel after electrophoresis and submitted to enzymatic proteolysis as described under "Materials and Methods."

Chromatography of Enzymatic Digest on Amino Acid Analyzer—Fig. 1 (top) shows the elution positions determined for 15 methyl amino acids on an automatic amino acid analyzer, as well as those of 16 common amino acids (arrows). Fig. 1 (bottom) shows the elution pattern of radioactivity from the enzymatic digest of [methyl-3H]MCP. Essentially all of the radioactivity in the enzymatic digest of MCP was recovered as two components, which we call Peak I and Peak II. Peak I eluted near glutamic acid 5-methyl ester, and further evidence in this paper confirms that Peak I is Glu(OMe). Peak II eluted near methionine, but evidence presented below indicates that little or no radioactive methionine was in fact present. When Peak II was collected from the amino acid analyzer and reincubated with aminopeptidase I and prolidase, the radioactivity gradually shifted from Peak II to Peak I. From this we conclude that Peak II is a peptide containing Glu(OMe). (When

Fig. 1. Chromatography of amino acids and enzymatic digest of MCP on an amino acid analyzer. Top, the elution positions of 11 methyl amino acids were determined with the analyzer ninhydrin system (A150). Abbreviations used are: Asp(OMe), aspartic acid 4-methyl ester; Glu(OMe), glutamic acid 5-methyl ester; His(πMe), N'-methylhistidine; Lys(πMe), N'-trimethyllysine; His(πOMe), N'-methylhistidine; Lys(πOMe), N'-dimethyllysine; Lys(πMe), N'-methyllysine; Met(Me), S-methylmethionine sulfonium; Arg(Me), N'-methylarginine; Arg(Me), N'-dimethylarginine; Arg(Me), N'-trimethylarginine; Arg(Me), N'-methylarginine. A 25-nmol sample of each methyl amino acid except Asp(OMe) was applied to the analyzer column. The latter gives a yellow product with ninhydrin which absorbs weakly at 570 nm, so a mixture of 14C amino acids and correlating the radioactivity maxima in the elution positions determined for 16 common amino acids (arrows). Fig. 1 (bottom) shows the elution pattern of radioactivity from the enzymatic digest of [methyl-3H]MCP. Essentially all of the radioactivity in the enzymatic digest of MCP was recovered as two components, which we call Peak I and Peak II. Peak I eluted near glutamic acid 5-methyl ester, and further evidence in this paper confirms that Peak I is Glu(OMe). Peak II eluted near methionine, but evidence presented below indicates that little or no radioactive methionine was in fact present. When Peak II was collected from the amino acid analyzer and reincubated with aminopeptidase I and prolidase, the radioactivity gradually shifted from Peak II to Peak I. From this we conclude that Peak II is a peptide containing Glu(OMe). (When
the MCP digestion with aminopeptidase I and prolidase was done for 48 h instead of the usual 4 h, the proportion of Peak II was greatly reduced. However, hydrolysis of the ester was also extensive by this time.) The enzymatic digest of MCP showed no radioactivity at the elution positions of aspartic acid 4-methyl ester or any of the known naturally occurring methyl amino acids (Fig. 1).

The radioactivity maximum of Peak I did not coincide exactly with the ninhydrin maximum of Glu(OMe) as shown in Fig. 2 (top). Fig. 2 (bottom) shows that the same shift of maxima occurred with authentic [methyl-"H]Glu(OMe). At the specific activity of the synthetic ester used, only 1 in 10<sup>3</sup> molecules contained any "H, so the ninhydrin reaction was due to nonradioactive molecules. Apparently, the shift is due to an isotope effect such as those described earlier (14). It can be said in conclusion that Peak I behaved the same as authentic [methyl-"H]Glu(OMe) on the amino acid analyzer; both eluted about 35 s earlier than nonradioactive Glu(OMe).

**Paper Chromatography of Enzymatic Digest—Authentic Glu(OMe) and enzymatic digest of [methyl-"H]MCP were chromatographed together on paper with n-butyl alcohol/pyridine/water (1:1:1) as the solvent (15). Again, two radioactive components were resolved (Fig. 3). The slower moving component (Peak II, R, 0.27) was found to coincide with Peak II when eluted from paper and chromatographed on the analyzer, and the faster moving component (Peak I, R, 0.47) coincided with Peak I on the analyzer. Peak I co-chromatographed with authentic Glu(OMe) (Fig. 3, A<sub>254</sub> maximum) in this paper system, as it did on the amino acid analyzer. Although Peak II eluted near methionine on the analyzer, the paper system confirms that Peak II (R, 0.27) and methionine (R, 0.57, not shown) are different compounds; evidence given above indicates that Peak II is a peptide containing Glu(OMe).

n-Butyl alcohol/acetic acid/water (12:3:5) was used as a second solvent system for paper chromatography (15). Peak I (isolated from a chromatogram such as that in Fig. 3) and Glu(OMe) (R, 0.49, first A<sub>254</sub> maximum of Fig. 4) were again found to co-chromatograph. The region isolated from the first chromatogram would also have included any radioactive methionine present, but Fig. 4 shows no radioactivity maximum which coincides with methionine (R, 0.58, second A<sub>254</sub> maximum). MCP digest in this system showed only one radioactivity maximum. Peak II had an R, of 0.66 and so was not well resolved from Peak I (R, 0.49).

**Paper Chromatography of N-Acetyl Derivatives of Peak I and of Glu(OMe)—** The N-acetyl derivatives of Peak I and Glu(OMe) were prepared and chromatographed together on paper (Fig. 5). It can be seen that no radioactivity remained at R, 0.49 (open bar), which indicates that Peak I had reacted with the acetic anhydride. A new peak of radioactivity was seen at R, 0.78, the same position occupied by the N-acetyl derivative of Glu(OMe) (solid bar).

**Comparison of pH Stabilities of Peak I, Glu(OMe), and MCP—** At extremes of pH, the methyl group of Peak I or Glu(OMe) (17) is lost as volatile material. Fig. 6 shows the stabilities of Peak I and authentic Glu(OMe) to be nearly equal.

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**Fig. 2.** Chromatography of Peak I and Glu(OMe) together on the amino acid analyzer. *Top,* enzymatic digest of [methyl-"H]MCP and 750 nmol of authentic Glu(OMe) were applied as a mixture to the analyzer. Fractions of 0.9 min (1.05 ml) each were collected in the region where Glu(OMe) elutes. The ninhydrin assay used here for quantitation of amino acids has been described previously (11, 12). Ninhydrin (A<sub>254</sub>, o--; "H (o--O)). *Bottom,* [methyl-"H]Glu(OMe) was synthesized by the method of Coleman (13) from glutamic acid and ["H]methanol (12.5 mCi/mmol). A 700-nmol sample of the synthetic radioactive Glu(OMe) was applied to the analyzer. Fractions of 0.2 min (0.23 ml) each were collected in the region where Glu(OMe) elutes. Ninhydrin (A<sub>254</sub>, o--; "H (o--O)). The method of Coleman (13) would be expected to produce glutamic acid 1-methyl ester as well as the b-methyl ester. An authentic sample of glutamic acid 1-methyl ester (Cycle Chemical) was found to have an elution time of 45 min (on the axes of this figure) and was thus well resolved from both maxima of this figure.

**Fig. 3.** Paper chromatography of enzymatic digest of [methyl-"H]MCP. A methanolic extract of the enzymatic digest of [methyl-"H]MCP was applied to the origin of a paper chromatogram. Also applied was a 0.2-μmol sample of authentic Glu(OMe). Development in n-butyl alcohol/pyridine/water (1:1:1) was allowed to proceed for 18 h, slightly longer than the time required for the solvent front to reach the end. Ninhydrin (A<sub>254</sub>, o--; "H (o--O)). Separate experiments (data not shown) identified the slower moving standard as Glu(OMe) and the faster moving one as methionine.

**Fig. 4.** Paper chromatography of Peak I in second solvent system. A region including Peak I (R, 0.47) and the methionine region (R, 0.57) was cut from a chromatogram like that in Fig. 3. A water eluate was prepared and applied to the origin of a paper chromatogram along with 0.2 μmol each of methionine and Glu(OMe). Development in n-butyl alcohol/acetic acid/water (12:3:5) proceeded for 40 h. (The solvent front had reached the end by 18 h.) We verified in a separate experiment that no compounds of interest ran off the end during this time.) Ninhydrin (A<sub>254</sub>, o--; "H (o--O)). Separate experiments (data not shown) identified the slower moving standard as Glu(OMe) and the faster moving one as methionine.
The pH stability of the methyl linkage of \( \text{[methyl-}^3\text{H}]\text{MCP} \) was also measured (Table I). The methyl linkage of MCP eluted into Triton X-100 is more stable than that of Peak I or was also studied (Table I). The methyl linkage of MCP eluted with the free ester (data not shown).

The pH stability of the methyl linkage of \( \text{[methyl-}^3\text{H}]\text{MCP} \) was also studied (Table I). The methyl linkage of MCP eluted into Triton X-100 is more stable than that of Peak I or Glu(OMe) at pH 9.6 and pH 13. It should be emphasized that this MCP had been denatured by boiling in sodium dodecyl sulfate prior to electrophoresis (1). We suspect that the anionic group of Glu(OMe) protected the MCP from attack by the hydroxide ions. Indeed, eluting the MCP into sodium dodecyl sulfate instead of Triton X-100 gave the methyl linkage greater stability toward alkali, while stability toward acid decreased (Table I).

Identification of Volatile Product of Alkaline Hydrolysis of MCP — The volatile product of alkaline hydrolysis of \( \text{[methyl-}^3\text{H}]\text{MCP} \) was collected in a Conway microdiffusion cell and identified as \( [\text{H}]\text{methanol} \) by two procedures. In the first procedure, gas chromatography (Fig. 7), the pattern of \( ^3\text{H} \) due to authentic \( ^3\text{H} \) was the only \( ^3\text{H} \) maximum seen.

In the second procedure, the contents of the Conway cell center well (1.5 ml) and 31 nmol of authentic, nonradioactive methanol were mixed and reacted with 3,5-dinitrobenzoic acid. The radioactivity maximum due to \( [\text{H}]\text{methanol} \) was the only \( ^3\text{H} \) maximum seen.

In methanol. Further evidence shows that all of the radioactivity in the volatile hydrolysis product of MCP was found in methanol. The original derivatization mixture contained 3880 cpm for each millimole of methanol added, and the specific activity of the final derivative was 3900 cpm/mmol.

**DISCUSSION**

In this report we describe the isolation and identification of glutamic acid 5-methyl ester from an *Escherichia coli* cytoplasmic membrane protein (methyl-accepting chemotaxis protein) that had been methylated in *vitro*. Proteolytic enzymes were used at moderate pH to accomplish cleavage of the peptide bonds with little hydrolysis of the ester linkage, which is labile to conventional acid or base proteolysis.

The ester was identified by comparing its behavior (detected by radioactivity) with that of authentic Glu(OMe) (detected by color reactions) in the following systems: I, chromatography
dodecyl sulfate with no added buffer and then incubated at the listed pH values (by addition of the same pH-controlled solutions described in the legend to Fig. 6). At various times, samples were dried under a stream of air to remove any volatile components, and the 3H remaining in nonvolatile form was determined. The decays were exponential, and the times given are for conversion of half the original label to volatile form. The half-times for Peak I (Glu(OMe)) from Fig. 6 are included for comparison.

<table>
<thead>
<tr>
<th>pH</th>
<th>SDS, sodium dodecyl sulfate.</th>
<th>MCP</th>
<th>MCP in Triton X-100</th>
<th>MCP in SDSa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>3 days</td>
<td>3 days</td>
<td>10 h</td>
<td></td>
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<tr>
<td>4.0</td>
<td>Stable</td>
<td>Stable</td>
<td>Stable</td>
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<tr>
<td>7.2</td>
<td>8 days</td>
<td>Stable</td>
<td>Stable</td>
<td></td>
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<tr>
<td>9.6</td>
<td>16 min</td>
<td>2.5 days</td>
<td>11 days</td>
<td></td>
</tr>
<tr>
<td>13.0</td>
<td>&lt;15 s</td>
<td>90 s</td>
<td>10 min</td>
<td></td>
</tr>
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* No detected hydrolysis by 3 days.

When E. coli bacteria are presented with an attractant, the methylation level of MCP increases (1). An enzymatic digest of MCP from cells stimulated with the attractant L-serine gave the same amino acid pattern as the digest shown in Fig. 1. Thus attractants appear to stimulate the methylation of more glutamic acid residues. We do not know whether attractant-stimulated methylation represents methylation of more molecules of MCP at the same site or a further methylation of the same MCP molecules at different sites. These bacteria also respond to some stimuli by demethylation of MCP (22). Since the methyl groups are attached by an ester linkage, they could be removed by a simple hydrolytic mechanism. The neutralization of negative charges on proteins by this type of methylation and the ease of removal of the methyl group are probably both of functional importance as suggested previously (5, 21, 24).

We feel that dicarboxylic acid esterification in proteins may be of general use in control mechanisms, where rapid and transient modifications are needed to respond to changes in cell environment. Dicarboxylic acid methylation may be a widespread biological phenomenon whose occurrence has not yet been fully recognized because of the lability of the methyl linkage. Enzymatic proteolysis as used here should be helpful in the detection and study of pH-labile protein methylations.

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