Methyl esterification of glutamic acid residues of methyl-accepting chemotaxis proteins in *Bacillus subtilis*

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The amino acid residue modified in the reversible methylation of *Bacillus subtilis* methyl-accepting chemotaxis proteins was identified as glutamic acid; methylation results in the formation of glutamate 5-methyl ester. Identification was made by comparing the behaviour of a ^3H -labelled compound isolated from proteolytically hydrolysed methyl-accepting chemotaxis proteins labelled *in vivo* with that of authentic methylated amino acids by chromatographic and electrophoretic techniques. Also, the isolated compound on mild alkaline hydrolysis shows behaviour identical with that of authentic glutamate 5-methyl ester. ^3H Methanol released by mild alkaline hydrolysis was made to react with 3,5-dinitrobenzyl chloride to form ^3H methyl 3,5-dinitrobenzoate, which was identified by reverse-phase high-pressure liquid chromatography.

As chemotactic bacteria respond to attractant gradients in their environment, reversible methylation of specific integral membrane proteins called methyl-accepting chemotaxis proteins (MC proteins) occurs (Goy *et al.*, 1977; Wang & Koshland, 1980). The methyl group transferred has been demonstrated to have originated from methionine, which is intracellularly converted into the activated methyl donor *S*-adenosylmethionine (Kort *et al.*, 1975). In *Bacillus subtilis* this methylation event has been linked to the adaptational process, in that as bacteria adapt to certain amino acids, such as aspartate or histidine, methyl groups from specific MC proteins are removed to produce methanol (Goldman *et al.*, 1982). This event, the demethylation of MC proteins in response to stimulation by attractant, is uniquely different from that observed in the Gram-negative organism *Escherichia coli*, where stimulation by attractant increases the degree of methylation of MC proteins and the addition of repellents results in their demethylation (Goy *et al.*, 1977).

In *E. coli* and *Salmonella typhimurium*, the methylation of MC proteins involves the formation of a methyl ester at the C-5 position of glutamic acid residues (Kleene *et al.*, 1977; van der Werf &

Koshland, 1977). Evidence has been presented that one of at least three classes of MC proteins in *E. coli*, designated MC protein I, can be methylated at up to six residues, and that MC protein II can have up to four methyl ester groups (Kehry & Dahlquist, 1982). Previously reported work from this laboratory has demonstrated in *B. subtilis* that methylated MC proteins are demethylated, with the formation of methanol, in response to stimulation by attractant (Goldman *et al.*, 1982). Additional work *in vitro* has shown functional homology between *B. subtilis* methyltransferase II and the *cheR* protein of *E. coli*, in that both methyltransferases show methylation activity towards the MC protein substrates of either *E. coli* or *B. subtilis* (Burgess-Cassler & Ordal, 1982). This result suggests a strong conservation between the active site of methylation on the MC proteins of these two evolutionarily divergent organisms. Accordingly, we decided to investigate the chemical nature of methylation in *B. subtilis in vivo*. The present paper shows that the only detectable modified amino acid residue in *B. subtilis* MC proteins is the 5-methyl ester of a glutamic acid residue.

Materials and methods

Chemicals

L-[Me- ^3H]Methionine (78–93 Ci/mmol) was obtained from Amersham Corporation (Arlington

Abbreviation used: MC protein, methyl-accepting chemotaxis protein.

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Heights, IL, U.S.A.). Papain, leucine aminopeptidase and prolidase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification.

Media and buffers

Tryptone broth contained 1% tryptone and 0.5% NaCl; L broth contained 1% tryptone, 0.5% NaCl and 0.5% yeast extract. Chemotaxis buffer is 10 mM-potassium phosphate buffer, pH 7, containing 0.14 mM- CaCl_2 , 0.3 mM- $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM-EDTA, 5 mM-sodium lactate and 0.05% (v/v) glycerol. Protoplast buffer is 50 mM-potassium phosphate buffer, pH 7, containing 20 mM- MgCl_2 , 1 mM-EDTA, 30 mM-sodium lactate, 0.01% chloramphenicol and 20% (w/v) sucrose.

Growth of bacterial cultures

Bacillus subtilis OI 1085 was used for methylation experiments performed *in vivo* and has been previously described (Ullah & Ordal, 1981). Spores were subcultured overnight in tryptone broth and then grown in L broth to 150–175 Klett units (red filter). Cells were then washed twice in chemotaxis buffer and once in protoplast buffer, and resuspended in protoplast buffer supplemented with lysozyme (1 mg/ml) at an A_{600} of 0.75 for 30 min at 37°C. Methylation *in vivo* was initiated by adding 20 μl of L-[Me- ^3H]methionine/5 ml of cell suspension and incubating the mixture for 5 min at 37°C. The reaction was stopped by transferring the flask to an acetone/solid CO_2 bath. The mixture was thawed at 4°C and centrifuged to sediment the protoplasts.

Isolation of ^3H -labelled MC proteins

Sedimented protoplasts were solubilized in a minimal amount of Laemmli sample buffer (Laemmli, 1970) and then heated at 100°C for 3 min, and the sample was loaded into the wells of a 10%-acrylamide / 0.125% - NN' -methylenebisacrylamide sodium dodecyl sulphate/polyacrylamide gel. The gel was run at a constant 25 mA, and treated for fluorography by the method of Laskey & Mills (1975). The resulting fluorogram then served as a guide to remove from the dried gel the zone containing the MC proteins. This zone was rehydrated with Laemmli sample buffer and placed above the stacking gel of a second sodium dodecyl sulphate/polyacrylamide gel without wells, and electrophoresis was performed as described above. The zone containing the MC proteins was located on the basis of R_F value and removed from the wet gel.

The gel zone was mashed with a glass rod, and the protein was eluted into a 50 mM-sodium acetate buffer, pH 5.2, containing 0.2% (v/v) Triton X-100. This was incubated for 24 h at 4°C, after which the gel particles were removed by centrifugation. The supernatant was made 20% (w/v) in trichloroacetic

acid at 0°C, and the resulting precipitate washed twice in cold 5% trichloroacetic acid, once in 10 mM-HCl in acetone and twice in acetone at 0°C.

Enzymic hydrolysis of ^3H -labelled proteins

The protein precipitate from above was suspended in 50 mM-sodium acetate buffer, pH 5.2, containing 0.2 mM-EDTA and 1 mM-2-mercaptoethanol. Papain (0.5 mg/ml) was added, and the mixture was incubated at 37°C for 16–20 h. The solution was then acidified to pH 2 by titration with 1.0 M-HCl and freeze-dried. The residue was resuspended in 5 mM-Tris/HCl buffer, pH 8.5, containing 0.625 mM- MnCl_2 . Leucine aminopeptidase (100 $\mu\text{g}/\text{ml}$) and prolidase (200 $\mu\text{g}/\text{ml}$) were added, and this mixture was incubated at 37°C for 4 h. The sample was then acidified to pH 2 as described above and freeze-dried.

Chromatography and paper electrophoresis of protein digest

The protein digest was suspended in a minimal volume of water or 50% (v/v) methanol (to decrease the salt content; Kleene *et al.*, 1977). Cloudy samples were filtered through a 0.45 μm Millipore filter fitted to a syringe. Descending paper chromatography was done on Whatman 3MM paper (Whatman, Clifton, NJ, U.S.A.). Authentic standards (30–50 μg) were run and located by spraying the chromatogram with ninhydrin in acetone. A sample of the protein digest was run under identical conditions and the radioactivity profile determined by cutting the lane into 1 cm fractions, eluting the paper into 0.5 ml of water for 30 min, adding 4.5 ml of scintillation fluid [xylene/Triton X-114 (3:1, v/v) containing 0.4% 2,5-diphenyloxazole and 0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene] and counting for radioactivity in a liquid-scintillation spectrophotometer.

Electrophoresis was performed on Whatman 3MM paper with a Savant (Hicksville, NY, U.S.A.) flat-bed system with continuous cooling. Voltage was initially set at 100 V for 10 min, increased to 1000 V for 5 min and then increased to 2000 V for 200 min. Electrode buffer was 1.5 M-acetic acid/0.58 M-formic acid, pH 1.9 (Glazer *et al.*, 1975).

Test of methyl-linkage stability towards mild alkaline treatment

Alkaline hydrolysis of the protein digest was accomplished by adding a 30 μl sample of the digest to 2 ml of 10 mM-NaOH. A 60 μg portion of authentic glutamate 5-methyl ester was similarly treated. Control samples were added to 2 ml of 10 mM-NaCl. All samples were incubated for 1 h at room temperature, and then neutralized with 1.0 M-HCl and freeze-dried. The residue was dissolved in 75% (v/v) methanol and run on descending paper

chromatography in solvent 1 (see below). Standard was located with ninhydrin and radioactivity was measured as described above.

Formation of a derivative of the volatile product of alkaline hydrolysis

Fluorograms of sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of MC proteins labelled *in vivo* were used as guides to cut out the MC-protein-containing zone from dried gels. The zone was then placed in the outer well of a Conway diffusion cell and rehydrated with a minimal amount of 0.1 M-NaOH. The inner well contained 0.5 ml of water. Incubation proceeded for 3 h at room temperature, after which the water from the centre well was removed and replaced with another 0.5 ml of water, and the incubation was repeated as above; a third extraction was also performed. The three collections were pooled and treated with 3,5-dinitrobenzyl chloride in accordance with the method of Neish (1957). The product was recrystallized three times from a pyridine/water solution. The residue was then dissolved to 25 mg/ml in warm 50% (v/v) acetonitrile, and a 250 μ l sample was injected on to a Whatman Partisil PXS 10/25 ODS-3 reverse-phase high-pressure liquid-chromatography column developed at 1 ml/min in an isocratic 50%-acetonitrile gradient. The retention time of authentic methyl 3,5-dinitrobenzoate was determined spectrophotometrically at 254 nm with a Beckman (Palo Alto, CA, U.S.A.) model 25 spectrophotometer equipped with an 80 μ l flow cell. To determine the retention time of the derivative of the volatile component of alkaline hydrolysis, 30 s fractions were collected into scintillation vials, mixed with an additional 0.5 ml of 50% acetonitrile and 9 ml of scintillation fluid, and their radioactivities counted in a liquid-scintillation spectrophotometer.

Results

Total proteolytic hydrolysis of the isolated MC proteins that had been radiolabelled *in vivo* was accomplished by utilizing the method of Hill & Schmitt (1962) with some of the modifications described by Kleene *et al.* (1977), as well as some of our own. The amount of label recovered throughout the total digestion was typically 90% of the amount originally eluted from the gels. A portion of this digest was analysed by descending paper chromatography in two solvent systems and by high-voltage paper electrophoresis at pH 1.9.

Solvent 1 was butan-1-ol/acetic acid/water (12:3:5, by vol.). Authentic methylated amino acids were located by ninhydrin stain, and their mobilities relative to the migration of glutamate 5-methyl ester (R_{GluOMe}) are listed in Table 1. When a sample of the digest was analysed for radioactivity, only one peak

Table 1. Comparison of relative mobilities of methylated amino acid standards

For experimental details see the text.

	R_{GluOMe} on paper chromatography		R_{GluOMe} on high-voltage paper electrophoresis at pH 1.9 (origin at anode)
	Solvent 1	Solvent 2	
<i>N</i> ⁶ -Methylarginine	0.62	0.74	2.08
Aspartate 4-methyl ester	0.38	0.52	1.20
<i>N</i> ¹ -Methylhistidine	0.39	0.53	2.27
<i>N</i> ³ -Methylhistidine	0.49	0.65	2.27
<i>N</i> ^ε -Methyl-lysine	0.81	0.88	2.46

Fig. 1. Paper chromatography (a and b) and high-voltage paper electrophoresis (c) of the digest of *B. subtilis* MC proteins radiolabelled *in vivo*

Panel (a) is the profile in solvent 1 (solvent run off the end of the chromatogram to improve separation of slower-migrating standards; control runs showed no loss of radioactivity); panel (b) is the profile in solvent 2. Panel (c) is the result from paper electrophoresis for 4×10^5 V · min. Each fraction represents 1.0 cm. For experimental details see the text. In each case the solid bar represents the position of the ninhydrin-stained authentic glutamate 5-methyl ester standard.

was detected, which co-migrated with authentic glutamate 5-methyl ester (Fig. 1a). Solvent 1 clearly separates glutamate 5-methyl ester from aspartate 4-methyl ester, with the latter having a mobility relative to glutamate 5-methyl ester of 0.38.

Solvent 2 was butan-1-ol/pyridine/acetic acid/water (20:10:5:12, by vol.). The profile of radioactivity (Fig. 1b) again shows co-migration of the ^3H -containing compound from the digest of the MC protein with authentic glutamate 5-methyl ester standard. As with the result with solvent 1, no radioactivity is seen associated with any authentic methylated amino acid except glutamate 5-methyl ester.

Another method for the separation of amino acids is by high-voltage paper electrophoresis. The electrode buffer was pH 1.9, and standards were applied near the anode and electrophoresed for $4 \times 10^5 \text{ V} \cdot \text{min}$. Results obtained with this technique are shown in Fig. 1(c). In this system all of the authentic standards migrated faster than glutamate 5-methyl ester, unlike the situation with the paper-chromatographic systems. The radioactivity again co-migrates with authentic glutamate 5-methyl ester; thus the results obtained by using two different analytical techniques give the same conclusion that the ^3H -containing amino acid isolated from the MC proteins labelled *in vivo* is in the form of glutamate 5-methyl ester. This result is consistent with that found in *E. coli* and *S. typhimurium*. Kleene *et al.* (1977) tested for the presence of glutamate 1-methyl ester in digests of the *E. coli* MC proteins, but none was seen during amino acid analysis in which glutamate 1-methyl ester showed a difference in

retention time of almost 30 min on their amino acid analyser.

Alkaline treatment of the protein digest was carried out to examine the stability of the isolated radioactive compound. Methyl esters are unstable to alkaline treatment (Kleene *et al.*, 1977; van der Werf & Koshland, 1977), and so treatment of the protein digest should hydrolyse off the radioactivity, releasing it as methanol, if the linkage is in the form of an ester. As shown in Fig. 2, exposure of a sample of the digest to 10 mM NaOH for 1 h abolished the radioactive peak associated with authentic glutamate 5-methyl ester. Treatment of the standard resulted in a shift of ninhydrin-stainable material to a position

Fig. 2. Comparison of the result of alkaline treatment of a sample of the digest of *B. subtilis* MC proteins radio-labelled *in vivo* with untreated digest on paper chromatography

The profile was obtained with solvent 1 (solvent front not run off the end of the chromatogram). O, Untreated control; ●, result of 10 mM NaOH treatment. For experimental details see the text. Fractions are 1.0 cm each. The solid bar represents the position of authentic standard.

Fig. 3. High-pressure liquid chromatography of the derivative of the volatile product of alkaline hydrolysis of *B. subtilis* MC proteins labelled *in vivo* with authentic methyl 3,5-dinitrobenzoate

The ODS reverse-phase high-pressure liquid-chromatography column was developed in an isocratic 50% acetonitrile gradient. Panel (a) is the absorbance profile at 254 nm; panel (b) shows the results from collecting 30 s fractions and determining the radioactivity present by liquid scintillation. For experimental details see the text. The radioactivity profile shows a broader peak due to the quantity of material loaded on the column (6.25 mg); when the same quantity of authentic standard is run, the absorbance profile was identical with that for the derivative of the product (results not shown).

that co-migrated with authentic glutamic acid (results not shown). This demonstrates that these conditions do hydrolyse methyl esters, and that the loss of radioactivity is consistent with alkaline hydrolysis of glutamate 5-methyl ester to glutamic acid.

The nature of the ^3H -containing product of mild alkaline hydrolysis was also examined. Volatile product from the hydrolysis of MC proteins labelled *in vivo* was collected in the centre wells of Conway diffusion cells and made to react with 3,5-dinitrobenzyl chloride in accordance with the method of Neish (1957). The derivative was recrystallized three times by first dissolving it in warm pyridine then precipitating it with an excess of cold water. This material was chromatographed on a reverse-phase high-pressure liquid-chromatography column in an isocratic 50%-acetonitrile gradient. Results (Fig. 3) show that the radioactive derivative has a retention time identical with that of methyl 3,5-dinitrobenzoate, which is the derivative produced if the original form of the radioactive volatile product was methanol.

Discussion

All of the results presented here are consistent with the conclusion that methylation occurs during labelling of *B. subtilis* proteins *in vivo* in the form of glutamate 5-methyl ester formation. Work performed *in vitro* in this laboratory with purified methyltransferase II (Burgess-Cassler *et al.*, 1982) resulted in the formation of glutamate 5-methyl ester in MC proteins when proteolytically hydrolysed samples were examined by paper chromatography. Another result obtained in this laboratory showed that demethylation of MC proteins labelled *in vivo* resulted in the formation of methanol as analysed by g.l.c. (Goldman *et al.*, 1982).

Reversible modification of MC proteins has been shown to occur as a response to exposure to attractants in *B. subtilis* and to both attractants and repellents in *E. coli*. In *B. subtilis* the modification of MC proteins in response to attractants binding to their receptors has been correlated with a decrease in the degree of methylation of specific MC-protein

bands as examined on highly resolving sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Goldman *et al.*, 1982). How methylation changes actually mediate the signal through the sensory transduction system and the role of reversible modification of the MC proteins remains an unanswered question.

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