Methyl-Accepting Chemotaxis Proteins of *Escherichia coli*: Methylated at Three Sites in a Single Tryptic Fragment†

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ABSTRACT: The location of the sites of methylation of the methyl-accepting chemotaxis proteins (MCP) of *Escherichia coli* has been investigated by trypsin digestion and mapping of the peptides. The two principal MCPs, MCP I and MCP II, were found to have very similar methyl-labeled peptide patterns. Each produced three major species of methylated peptide. Both MCPs migrate on high-resolution sodium dodecyl sulfate–polyacrylamide slab gels as a cluster of discrete protein bands. Each band produced one of the three major methylated peptides as the unique or dominant species. Progressive demethylation of the least acidic peptide produced, sequentially, the intermediate and most acidic peptides before complete loss of the methyl label. This is consistent with a single peptide backbone carrying from one to three methyl groups. The possible significance of three methyl-accepting sites in a single tryptic peptide is discussed.

Reversible protein methylation reactions have been identified in a wide variety of organisms and cell types (Kort et al., 1975; Dilberto & Axelrod, 1976; Pike et al., 1978). These modifications are characterized by the catalyzed transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to form the methyl ester of a carboxylic acid, typically on a glutamyl residue. Release of the methyl group, either by spontaneous or catalyzed hydrolysis, results in production of methanol (Kim & Paik, 1971; Liss et al., 1969). The best characterized example of such methylations plays a central role in bacterial chemotaxis and is the focus of our work. Many other examples have been bound, and the apparent universality of reversible methylations has sparked a great deal of recent interest in the field. Because of the inherent instability of the methyl ester, however, methanol production is often the assay of these reactions in many instances. Production of methanol has been found in most mammalian tissue, especially in the pituitary gland (Dilberto & Axelrod, 1976; Kim & Li, 1979a). In support of this observation, the pituitary hormone, corticotropin has been methylated by Kim & Li (1979b) in vitro using an extract containing methyltransferase II activity. While the studies in eucaryotes are just being developed, much progress has been made in *Escherichia coli* and *Salmonella typhimurium*.

Bacteria can achieve directional movement by regulated switching of their flagellar rotation from counterclockwise to clockwise. Counterclockwise rotation produces smooth swimming while clockwise rotation causes tumbling (Silverman & Simon, 1974; Larsen et al., 1974). In this way, they can migrate toward a variety of chemical attractants and away from a number of repellents. At least 20 receptors detect these stimuli (Adler, 1975) and, in turn, interact with one of three methyl-accepting chemotaxis proteins (MCP) (Koiwai & Hayashi, 1979). An increase in attractant concentration induces a slow increase in methylation of the respective MCP. This lasts up to several minutes and plateaus at a value related to the concentration of stimulus. A decrease in attractant or increase in repellent concentration causes a rapid demethylation (Kort et al., 1975; Silverman & Simon, 1977; Kondoh et al., 1979). The methylation site thus serves as an indicator of previous receptor occupancy and acts as a simple form of memory. The MCP may then compare previous to existing receptor occupancy and communicate this difference to the flagellar tumble regulator. In the absence of methylation the cells can respond behaviorally to a change in stimulus concentration but cannot adapt to its presence, having no way to make a comparison. Thus methylation is required for bacterial cells to adapt to their environment (Adler & Dahl, 1967; Aswad & Koshland, 1974).

The behavior of bacteria, which respond to stimuli by regulating the frequency of tumbles, has been compared to the response of neurons which alter their frequency of action potentials following chemical excitation (Goy & Springer, 1978). High levels of methylating activity in the brain therefore stimulated our interest in this regulatory modification beyond the already intrinsically exciting phenomenon of chemotaxis. Study of the methylation event in bacteria should provide clues as to its function and mechanism of action in higher organisms. It has already been shown that the product of *cheX* in *E. coli* is responsible for transfer of the methyl moiety of SAM to glutamyl residues on the MCPs (Springer & Koshland, 1977; DeFranco et al., 1979; Van Der Werf & Koshland, 1977). A methylesterase has also been found which demethylates the ester to produce methanol and recover the free carboxyl of the glutamic acid (Stock & Koshland, 1978; Toews & Adler, 1979). We have recently shown that more than one methylation takes place on each MCP molecule (Chelsky & Dahlquist, 1980). In this communication, we present evidence which demonstrates the existence of three distinct sites of methylation in a single tryptic fragment for both MCP I and MCP II. The possible implications of such a structural element in the MCPs is discussed.

Methods

Strains. *E. coli* MS5234(λ)*leu his thr tsr* and MS5234(λ)*leu his thr tar* were used for methyl incorporation into the MCPs. *E. coli* 159(λind⁻) was used as a host for hybrid phage infection. MS5228(λ)*leu his thr tar tsr* could be used for expression of hybrid phage genes under the conditions described below. *E. coli* λ hybrid phage, λ*fla91* and λ*fla3Δ14*, carry the genes *tsr* and *tar*, respectively (Silverman et al., 1977). All bacteria and hybrid phages were the gift of M. Silverman and M. Simon.

Labeling Procedures. Hybrid phage directed synthesis of chemotaxis proteins was carried out by a modification of the method of Jaskunas et al. (1975). *E. coli* 159(λ) was grown

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on AB minimal medium (Clark & Maaloe, 1967) with 0.4% maltose and 1 μg/mL thiamin from an overnight culture to 4×10^8/mL. The cells were centrifuged and resuspended in the same medium at 1×10^9/mL and 5 mL was placed in a 10-cm glass petri plate and exposed to one 40-W germicidal lamp (Sylvania) from 40 cm at 37 °C for 10 min. $MgSO_4$ was added (0.02 M final concentration) followed by addition of the hybrid phage at a ratio of 10:1 phage to bacteria. This was incubated for 15 min at 34 °C followed by shaking for an additional 15 min. L-[^{35}S]Methionine (10 μCi; 1000 Ci/mmol, Amersham) was added to 1 mL of cells and the incubation continued for 15 min after which the cells were pelleted and taken up in sodium dodecyl sulfate ($NaDodSO_4$) dissociation buffer or isoelectric focusing (IEF) lysis buffer.

The MCPs were methyl labeled by a modification of the method of Kort et al. (1975). MS5234*tsr*$^-$ or MS5235*tar*$^-$ (Silverman & Simon, 1977) were grown in tryptone (Difco) and 1% glycerol from an overnight culture to 4×10^8/mL and washed twice in 10 mM Tris-HCl, pH 7.1, and 0.1 mM Na_2EDTA. The cells were then resuspended in the same buffer at 1×10^9/mL with 0.2 mg/mL chloramphenicol and 25 mM succinate and incubated with shaking for 10 min at 34 °C. L-[*methyl*-^{3}H]Methionine (80 Ci/mmol, New England Nuclear) was then added (15 μCi) to 1 mL of cells and the incubation continued for 50 min at 34 °C. Attractant was added at 30 min and formalin was added at 50 min (2.5% final concentration). The cells were then pelleted and taken up in $NaDodSO_4$ dissociation buffer.

For experiments involving both ^{35}S and [^{3}H]methyl labeling of MCP II, *E. coli* MS5228 *tar*$^-$ *tsr*$^-$ was grown on Tryptone broth and 1% glycerol from a 1:50 dilution of an overnight culture to 4×10^8/mL at 34 °C. The cells were washed 3 times in AB minimal medium, 0.4% maltose, and 1 μg/mL thiamin followed by shaking at 34 °C for 45 min in order to deplete required amino acids. MS5228 is a λ ind$^+$ lysogen, and this treatment prevented ultraviolet light induced lysis during the next step.

A 5-mL suspension of starved cells at 1×10^9/mL was ultraviolet irradiated as described above. This was followed by the addition of $MgSO_4$ (0.02 M, final concentration) and λfla3Δ14 (1×10^{10} phage) to 1×10^9 cells in 1 mL. After 15 min at 34 °C, 0.3 mL of AB minimal medium supplemented with leucine, histidine, and threonine (0.1 mg/mL each) was added and the incubation continued for an additional 15 min with shaking. L-[^{35}S]Methionine (12 μCi) was added and 20 min later the cells were washed 3 times by centrifugation with 10 mM Tris-HCl, pH 7.1, and 0.1 mg/mL EDTA and resuspended in the same buffer with the addition of 25 mM succinate and 200 μg/mL chloramphenicol. After 10 min at 34 °C, 20 μCi of L-[*methyl*-^{3}H]methionine was added followed by 0.5 mM aspartate 30 min later. After a 5-min incubation, the cells were pelleted and resuspended in aspartate-free buffer for 2 min. This was intended to demethylate the MCP, thereby removing any unlabeled methyl groups. Aspartate (10 mM) and L-[*methyl*-^{3}H]methionine (10 μCi) were then added again to ensure maximal labeling of the cells. After 20 min, formalin (2.5% final concentration) was added and the cells were pelleted and dissolved in $NaDodSO_4$ dissociation buffer. The proteins were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis [7.5% acrylamide, 0.05% *N,N'*-methylenebis(acrylamide)]. The slab gel was fixed in 7% acetic acid, dried, and autoradiographed. The MCP II bands were cut out and eluted into 0.5 M urea, 50 mM $NaHCO_3$, and 0.1% $NaDodSO_4$ for 8 h followed by addition of scintillation fluor for double channel counting.

Two-dimensional electrophoresis was carried out as described by O'Farrell (1975). The IEF tube gel measured 3 mm i.d. × 10 cm. The $NaDodSO_4$ slab gel (Laemmli, 1970) consisted of a running gel which measured 13 × 20 cm and a stacking gel, 13 × 2 cm. The gap between the IEF tube gel on top and the stacking gel was less than 5 mm and was filled with 1% agarose (type I: low EEO, Sigma) in stacking gel buffer. When single dimension slab gels were used, a comb was inserted in the stacking gel before polymerization. An acrylamide: *N,N'*-methylenebis(acrylamide) ratio of 150:1 was used at a total concentration of 7.5%. Glass-distilled water and electrophoresis grade chemicals were used throughout.

Isolation of Methylated Tryptic Peptides. Samples for trypsin treatment were eluted directly from dried slab gels which had been fixed in 7% acetic acid for 30 min. ^{35}S-labeled MCPs were used as a standard for locating the [^{3}H]methyl-labeled samples. Pieces of gel were added to 0.1 M ammonium bicarbonate (200 μL/0.1 mm^2 slice), pH 7.8, after the paper backing was removed, and eluted in the presence of 2 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (Worthington, 260 units/mg) for 8 h at 22 °C. The slices were then removed, 2 μg of trypsin was added, and the incubation was continued for another 5 h. After this treatment at least 95% of the radioactivity equilibrated with the buffer.

Trypsin-treated samples were lyophilized, taken up in 10 μL of water, and spotted on chromatography paper (Whatman, 3 MM). Electrophoresis was carried out in pyridine–H_2O–acetic acid (10:90:0.4, pH 6.5) at 2300 V for 2 h. Slices (6 mm) were cut out of each lane and eluted in 0.3 mL of the urea, sodium bicarbonate, and $NaDodSO_4$ elution buffer discribed above for scintillation counting.

Peptide Demethylation. Methylated tryptic peptides were separated by paper electrophoresis. For spontaneous demethylation studies, the least acidic peptide was eluted from the paper in 5 mM ammonium bicarbonate, pH 7.8, at 20 °C for 2 h. The paper was then removed and the sample lyophilized. This was then taken up to 0.1 M triethylamine titrated to pH 8.4 with CO_2, divided into four aliquots, and incubated at 100 °C. Samples were frozen at the indicated times and lyophilized for a second separation by paper electrophoresis.

Results and Discussion

MCP I and MCP II each migrate on $NaDodSO_4$–polyacrylamide slab gels as a set of closely spaced bands. The exact number of these bands is dependent on the resolution of the gel system. We have previously reported (Chelsky & Dahlquist, 1980) that MCP II resolves as five bands using a Laemmli (1970) gel with a ratio of acrylamide to *N,N'*-methylenebis(acrylamide) of 33:1. In this communication we have used a higher ratio of acrylamide to bisacrylamide, 150:1, which resolves MCP II into at least ten bands and MCP I into at least seven (A. Boyd, personal communication), as shown in Figure 1.

A comparison was made of methyl-labeled and phage-directed protein under neutral and attractant conditions. Phage-directed protein is [^{35}S]methionine labeled and represents both methylated and unmethylated forms while the appropriate use of tritiated methylmethionine results in labeling of only the methylated forms. Figure 1 shows an autofluorograph of the MCP region of a $NaDodSO_4$–polyacrylamide slab gel. MCP II bands appear in lanes 1 and 2 after treating a *tsr*$^-$ strain with [*methyl*-^{3}H]methionine. The corresponding phage-directed protein bands are in lanes 3 and 4. MCP I bands from a methyl-labeled *tar*$^-$ strain are in lanes 7 and 8 while phage directed MCP I is in lanes 5 and 6. Lanes

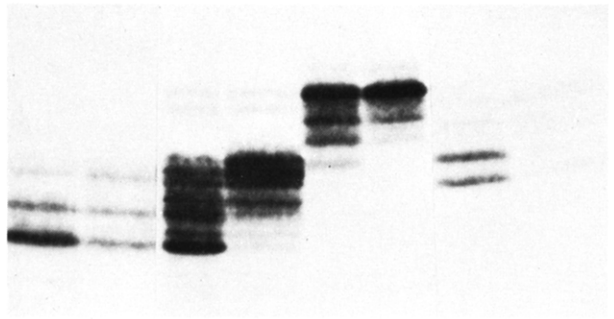

FIGURE 1: Comparison of methyl-labeled and phage-directed banding patterns of MCP I and MCP II before and after stimulation of the cells. Shown is an autofluorograph of the MCP region of a NaDodSO$_4$–polyacrylamide slab gel (7.5% acrylamide). Lanes 1 and 2: [*methyl*-^{3}H]methionine-labeled MS5234*tsr*$^-$; 3, 4: λfla3Δ14 directed synthesis of MCP II in *E. coli* 159(*i*) in the presence of [^{35}S]methionine; 5, 6: λfla91 directed synthesis of MCP I; 7, 8: methyl-labeled MS5235*tar*$^-$. Cells were stimulated with 5 mM aspartate for lanes 1 and 3 and with 5 mM serine for lanes 5 and 7.

1, 3, 5, and 7 contain attractant-stimulated cells. It has been shown earlier that, in addition to an increase in methylation level after attractant stimulation, there is a reversible shift in the distribution of protein into the faster migrating bands as a result of this methylation (Chelsky & Dahlquist, 1980). The exact distribution is dependent on the strain and its environment prior to solubilizing in NaDodSO$_4$. Since three different strains were used to produce Figure 1, the relative amount of protein in the phage-labeled bands may not always correspond to the methyl-labeled bands. This is most notably the case for the fastest migrating band of MCP I.

Examination of phage-directed MCP II indicates ten resolved bands. These are numbered 1 through 10, with the fastest migrating band designated as band 10. All but the three slowest migrating bands (bands 1–3) are methylated. Of these, the two slowest are very minor products and do not change their intensity with attractant or repellent stimulation. Thus, it is likely that only one unmethylated band is present which is responsive to the presence of attractant. This is also the case for MCP I. Of the methylated bands, only a few appear to be dominant. These are well resolved and allow for an accurate analysis of their composition.

The number of methyl groups per protein in each band from NaDodSO$_4$–polyacrylamide gel electrophoresis was estimated by labeling phage-directed MCP II with [^{35}S]methionine followed by methylation of that protein with [*methyl*-^{3}H]-methionine. The strain MS5228 was used since its genes for both major MCPs are defective. The phage-directed protein was therefore the only methyl-labeled protein in this region of the gel.

The data in Table I indicate that the three slowest migrating bands are not methylated. As for the methylated bands, the ratio ^{3}H:^{35}S appears to occur in relative increments of 1, 2, and 3. The trend is toward increasing amounts of methyl groups with faster migrating bands. Only band 9 deviates from this trend, with a relative ratio of 0.8. For two reasons, to be discussed later, we fell that this number is misleading. As for the major methylated species, bands 5, 8, and 10, we have assigned one, two, and three methyl groups per molecule, respectively. Further evidence in support of this claim is given below.

Methylation of the MCPs involves the conversion of the γ-carboxyl of a glutamyl residue to a methyl ester (Van Der Werf & Koshland, 1977; Kleene et al., 1977). Thus, there

Table I: Methyl Esters per Molecule[a]

band	^{3}H (cpm)	^{35}S (cpm)	^{3}H/^{35}S	relative ratio (band 5 = 1.0)
1	0	593	0	0
2	0	628	0	0
3	15	1267	0.01	0
4	588	3961	0.15	0.8
5	877	4994	0.18	1.0
6	322	1732	0.19	1.1
7	328	2350	0.14	0.8
8	1650	5374	0.31	1.7
9	393	2695	0.15	0.8
10	3433	6291	0.55	3.1

[a] Phage-directed MCP II was labeled with [^{35}S]methionine and subsequently methyl labeled with [*methyl*-^{3}H]methionine. The band numbers refer to migration on NaDodSO$_4$–polyacrylamide slab gels with 1 referring to the slowest migrating band. Band 5 was used to determine the relative ratios since it was the most labeled of the slower migrating bands. The gross counts are the summation of two separate experiments.

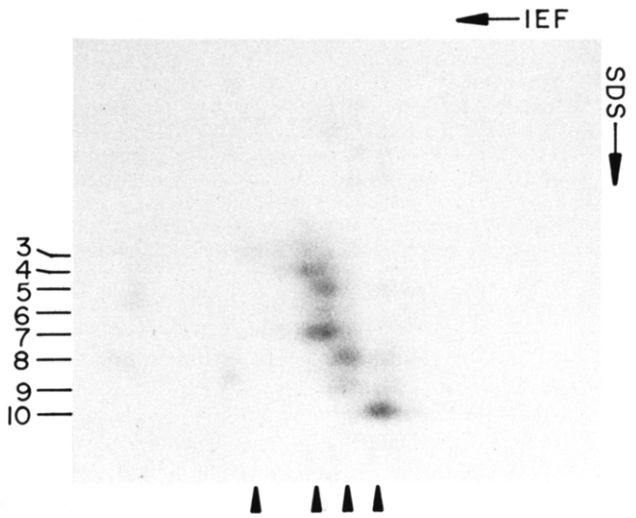

FIGURE 2: Two-dimensional separation of MCP II. λfla3Δ14 directed, [^{35}S]methionine-labeled, MCP II was first separated by charge on an isoelectric focusing gel followed by NaDodSO$_4$–polyacrylamide gel electrophoresis as described by O'Farrell (1975). An autoradiograph of the resulting pattern is shown with the arrows indicating charge groups and numbers assigned to the spots in order of increasing migration on NaDodSO$_4$–polyacrylamide gel electrophoresis. Spots 1 and 2 are too faint to be seen.

is net change in charge of +1. If there are three possible methylation sites, there should be four possible charged states of the protein. Furthermore, if each band on NaDodSO$_4$–polyacrylamide slab gels corresponds to a particular level of methylation, each species should have a uniform charge. This theory was tested by a two-dimensional separation using the technique of O'Farrell (1975). Methionine-labeled MCP II was first separated according to charge by isoelectric focusing. The focusing gel was then equilibrated with NaDodSO$_4$ and the proteins were further separated by NaDodSO$_4$–polyacrylamide gel electrophoresis in the second dimension. The two-dimensional pattern that is produced is shown in Figure 2. As predicted, the protein bands migrate in one of four charged states in the isoelectric focusing dimension. These are indicated by the arrows below the gel. The unmethylated band, 3, runs as the most acidic species while band 10 migrates as the least acidic species. According to our theory, bands 4, 5, 6, and 7 are singly methylated while bands 8 and 9 are doubly methylated and band 10 has three methyl groups/molecule. This is consistent with the double label results with

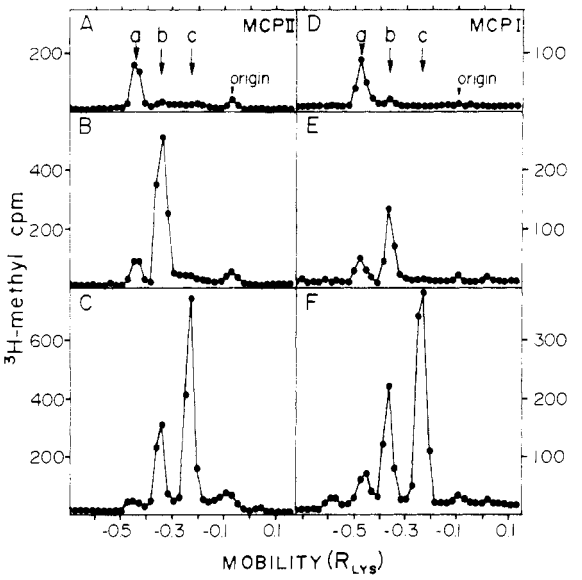

FIGURE 3: Tryptic maps of individual, methyl labeled, $NaDodSO_4$–polyacrylamide gel electrophoresis bands of MCP I and MCP II. The major methylated species of MCP II as seen in Figure 1 were cut out of the $NaDodSO_4$ gel and trypsin treated. All peptide samples were then separated at the same time by paper electrophoresis. Samples A and D are derived from the slowest migrating MCP bands while samples C and F were from the fastest. The major methylated peptides are designated *a*, *b*, and *c* for purposes of reference and are used to describe the corresponding species in both MCP I and MCP II because of their apparently identical behavior. Zero mobility refers to the location of the neutral standard, Dnp-lysine, following the run.

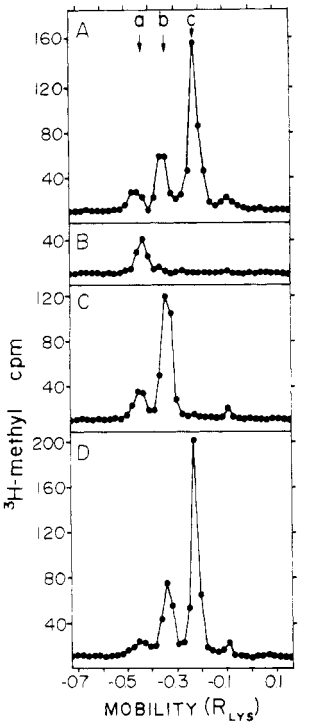

FIGURE 4: Paper electrophoresis of isolated methyl-labeled tryptic peptides. (A) [^{3}H]methyl-labeled MCP II was trypsin digested and separated by paper electrophoresis. The peak fractions of *a*, *b*, and *c* were eluted and subjected to a second separation (B, C, and D, respectively).

the exception of band 9. Further inspection of the gel, however, reveals additional labeled spots running coincidently with band 9, in the $NaDodSO_4$ dimension. The additional spots are not shown in the figure. Since there is very little methylation of band 9, this additional protein, which may not be related to MCP II, could significantly alter the ^3H:^{35}S ratio, resulting in a low estimate for the actual number of methyl groups per molecule in this region.

We have also occasionally observed additional charge groups for MCP II in the IEF dimension. This may be an artifact of the gel or may represent additional charge related events in the MCPs. While our yields are good for recovery of the methyl label, the presence of a minor methylated site, in addition to the three proposed here, cannot be completely ruled out.

Tryptic digestion of methyl-labeled MCP I and MCP II with subsequent separation by paper electrophoresis has revealed the presence of three distinct methyl-labeled species in each protein (Chelsky & Dahlquist, 1980). By use of the separation possible with the $NaDodSO_4$–polyacrylamide slab gels, each individual band has been digested and subjected to paper electrophoresis. Three patterns are produced which are shown in Figure 3. Both MCP I and MCP II produce very similar patterns. Bands 4, 6, and 7 of MCP II produce the same pattern as band 5 (Figure 3A), which agress with the double label results suggesting that these bands are similarly methylated. Band 9 has a peptide pattern identical with that of band 8 (Figure 3B), which again suggests that band 9 has two, rather than one, methyl group per molecule. Band 10 has a unique pattern (Figure 3C), being the only band to produce the *c* peptide.

The three peptides produced are all acidic, with bands 4–7 producing the most acidic peptide, *a*. Bands 8 and 9 produce a peptide of intermediate rate of migration, *b*, with a small amount of *a*. Band 10 produces the least acidic peptide as the dominant species. While band 10 produces some of the more acidic peptides as well, they are only minor products. However, they are very reproducible, and we fell that they do not represent contamination of one band by another. Trailing on the $NaDodSO_4$–slab gel would cause band 5 to be contaiminated with the peptides of bands 8 and 10 while band 8 should contain some of the peptide from band 10. This is not found to be the case, and it seems more likely that the more acidic peptides can be derived from their less acidic neighbors. This would be consistent with the possibility that each methylated species represents the same peptide backbone with a different number of methyl groups incorporated. The most methylated would be the least acidic since it would have the fewest free carboxyls as described earlier. The presence of small amounts of the lesser methylated peptides would then be the result of demethylation during the tryptic digest. According to this prediction, if we were to run a tryptic map of the entire MCP II, elute the fractions, and rerun the peaks, we would produce pure peptide *a* but never be able to get peptides *b* and *c* in pure form since even neutral pH and 20 °C are sufficient to spontaneously demethylated the peptides. This experiment was performed and the results, as shown in Figure 4, were as predicted. The total methyl-labeled trypsin digest of MCP II was separated as shown in Figure 4A. The peak fractions of *a*, *b*, and *c* were then eluted and rerun as shown in parts B, C, and D, respectively, of Figure 4.

Further evidence in support of our theory comes from in vitro demethylation of the least acidic peptide. This peptide was isolated from a tryptic map and heated in a slightly basic, triethylamine–carbonic acid buffer for up to 90 min, enough time for almost complete loss of the methyl label. At several time intervals, a sample was withdrawn and frozen. A second electrophoretic separation of these samples is shown in Figure 5. The data are represented as relative number of molecules rather than gross counts since the least methylated species contains only one-third the counts per molecule as the most methylated. This method of representation does not alter the

qualitative nature of the profiles. It can be seen that as peptide *c* disappears, peptides *b* and *a* grow in size. Later, peptide *b* subsides with a further increase in *a*. Finally peptide *a* begins to disappear as all of the counts are liberated as methanol. Peptide *b*, when demethylated in this way, produces peptide *a* while peptide *a* loses its label to methanol without conversion to the other peptides.

While the three peptides are clearly derived from a common peptide backbone, examination of the loss of methyl label as a function of alteration in migration is necessary to establish that these phenomena are directly related. The appearance of the di-, mono-, and unmethylated species following the spontaneous demethylation of the trimethylated form can be predicted by simple mathematical formulas. For a given time, *t*, the amount of trimethylated (*C*), dimethylated (*B*), monomethylated (*A*), and unmethylated species (*D*) relative to the original amount of the trimethylated form can be derived as follows:

$$C = e^{-3t}$$

$$B = 3(e^{-2t} - A)$$

$$A = 3(A - 2e^{-2t} + e^{-t})$$

$$D = 1 - A - B - C$$

These equations are the solutions to the kinetic scheme:

$$C \xrightarrow{3k} B \xrightarrow{2k} A \xrightarrow{k} D$$

Here, the conversion of C into B proceeds three times as fast as the demethylation of A since C has three methyl groups as compared to one for A. These predicted values have been compared (solid lines) to the data (symbols) as shown in Figure 6. The close fit of the actual and theoretical values further supports our interpretation of three methylation sites in this peptide.

Conclusion

We have previously reported that the methyl-accepting chemotaxis proteins, MCP I and MCP II, have more than one site of methylation. In this report we demonstrate that there are three methyl-accepting sites per molecule, located on a single tryptic peptide. In both proteins, the mono-, di-, and trimethylated peptides can be resolved, and all are acidic. The most methylated peptides are the least acidic, which is consistent with the progressive methyl esterification of glutamyl residues. Since the trimethylated form is still acidic, it appears that at least one additional acidic residue is present in this peptide.

MCP I and MCP II both migrate on $NaDodSO_4$–polyacrylamide gels as sets of closely spaced protein bands. Investigation of MCP II has shown that these multiple forms are related, at least in part, to the methylated state of the molecule. While only one trimethylated protein band is apparent, two dimethylated and four monomethylated bands are resolved. In explanation of these bands, it is possible that the high-reolution polyacrylamide gels can distinguish between methylation at different sites on the molecule as well as different numbers of methyl groups per molecule. If this were the case, one would expect single bands of the un- and trimethylated species while the mono- dimethylated species would each be expected to exist as three positional isomers. This would explain the existence of all but the fourth monomethylated band. If such an explanation were correct, the fact that we see only one dominant band at each methylation level would suggest that an ordered sequence of methylation may exist for these sites.

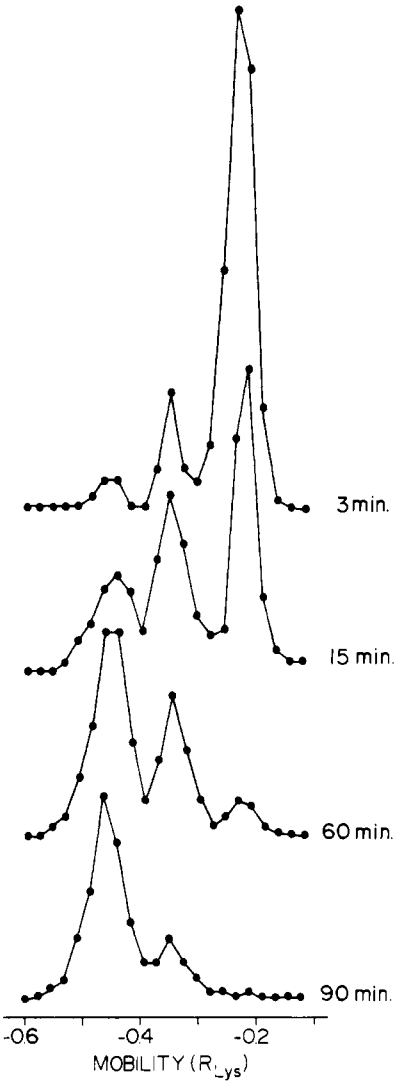

FIGURE 5: Time course of peptide demethylation. Methyl-labeled peptide *c* from MCP II was isolated by paper electrophoresis and dissolved in triethylamine titrated to pH 8.4 with CO_2. This was divided into four aliquots and heated to 100 °C for the times indicated. Samples were frozen, lyophilized, and again separated by paper electrophoresis. Results are shown in relative number of molecules rather than absolute ^3H counts taking into account the reduced number of methyl groups per molecule in peptides *a* and *b*. This does not alter the qualitative changes in peak size.

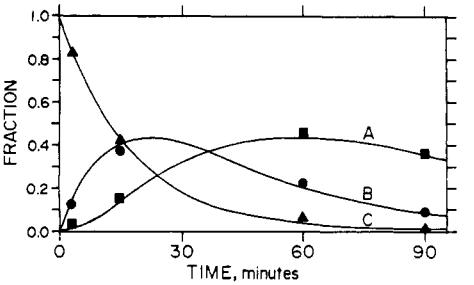

FIGURE 6: Time course of peptide demethylation: a comparison of experimental and theoretical values. The amount of mono- (A), di- (B), and trimethylated (C) peptide relative to the original amount of the trimethylated form is plotted (solid lines) vs. time based on a simple exponential decay as described in the text. Experimental values derived from Figure 5 are plotted with symbols.

It is intriguing to consider the role placed by the three sites of methylation in a single tryptic fragment. The change from a charged to a neutral moiety in the molecule could effect large changes in local structure which could, in turn, affect inter-

molecular interactions. This would be possible with only one methylation event, however, and we believe that the triple methyl-accepting site has a particular significance. Multiple carboxyl groups in close proximity serve as calcium binding sites in a number of proteins. Bovine prothrombin, for example, has two adjacent glutamic acids. Conversion to the calcium binding state involves the carboxylation of these residues to form four adjacent γ-carboxyls (Stenflo & Suttie, 1977). Thermolysin has four calcium binding sites, all of which chelate the ion with a combination of carbonyls and glutamyl and aspartyl carboxyls (Matthews & Weaver, 1974). Two calciums are bond by the carp muscle binding protein (parvalbumin). Both sites contain "four carboxylate groups with no lysine or arginine residues near enough to make formal electrical neutrality" (Kretsinger & Nockolds, 1973). Finally, in Staphylococcal nuclease, the calcium ion is coordinated by a square array of carboxylate groups (Cotton et al., 1971). The three carboxyls on a single peptide in the MCPs fit well with these structures, with the added ability to displace the bound ion by reversible methylation of the chelating groups. Equivalent electrostatic interactions could take place as well.

There is some indication for a role of calcium in the chemotaxis event. Swimming behavior in *Bacillus subtilis* has been reported to be modulated by altering the external calcium concentration in the presence of a calcium ionophore (Ordal, 1977). A low concentration produces smooth swimming while a high concentration results in tumbling. An intermediate level of calcium allows the cells to maintain a random swimming pattern. Thus, the methyl-accepting region could function to reduce the internal free calcium concentration (Ordal & Fields, 1977). Alternatively, it could function as a gate, blocking ion diffusion through an MCP pore.

We are currently investigating the structural and physiological role of the multiple methylations. With reversible methylation emerging as a widespread phenomenon, this problem is becoming increasingly important to solve.

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