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Enzymatic deamidation of methyl-accepting chemotaxis proteins in *Escherichia coli* catalyzed by the *cheB* gene product

(methylation/CheB-dependent modification/sensory adaptation)

MARILYN R. KEHRY*, MARTHA W. BOND†‡, MICHAEL W. HUNKAPILLER†, AND FREDERICK W. DAHLQUIST*

*Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403; and †Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125

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ABSTRACT **The methyl-accepting chemotaxis proteins (MCPs) of *Escherichia coli* undergo reversible methylation that has been correlated with adaptation of cells to environmental stimuli. MCPI, the product of the *tsr* gene, accepts methyl groups at multiple sites that are located on two tryptic peptides, denoted K1 and R1. A second modification of the MCPs, which is not methylation, has been designated the CheB-dependent modification. A CheB-dependent modification occurs on methyl-accepting peptide K1 and allows additional methyl groups to be incorporated into this peptide. We have performed partial amino acid sequence analyses on radiolabeled peptides K1 and R1 derived from MCPI and have identified several methyl-accepting sites. We found that, in the absence of CheB-dependent modification, a site in peptide K1 is unable to accept methyl groups. Correlation of this protein sequence data with the nucleotide sequence of the *tsr* gene [Boyd, A., Kendall, K. & Simon, M. I. (1983) *Nature (London)* 301, 623–626] suggests that CheB-dependent modification of MCPI is the enzymatic deamidation of glutamine to methyl-accepting glutamic acid. Possible roles for this deamidation in bacterial chemotaxis are discussed.**

Escherichia coli swim in a random walk resulting from alternating clockwise (tumbling behavior) and counterclockwise (straight swimming behavior) flagellar rotation. The bacteria respond to changes in their environment by modulating the probabilities of clockwise and counterclockwise rotations. Cells suppress their tumbling in response to attractants and increase their tumbling in the presence of repellents. Subsequently, the cells undergo a process of adaptation, which entails a return to their prestimulus swimming pattern (1–3).

These behavioral responses of *E. coli* to certain stimuli are mediated by a family of transmembrane proteins, the methyl-accepting chemotaxis proteins (MCPs) (4). At least three different MCPs are essential in the sensory transduction process. MCPI, MCPII, and MCPIII, encoded by the *tsr*, *tar*, and *trg* genes, respectively, mediate cellular responses to different subsets of attractants and repellents (5–8). A fourth gene, *tap*, seems to encode an additional MCP whose functional role in chemotaxis has not yet been defined (9–11). The behavioral response is initiated by interaction of the effector molecule or an effector–receptor complex in the periplasm with the appropriate MCP (12–16).

MCPs undergo reversible methylations in the cell cytoplasm, and changes in the level of methylation correlate with the adaptation of cells to environmental stimuli (6, 17–19). Methylation is mediated by a protein methyltransferase encoded by the *cheR* gene and results in the formation of the γ-methyl ester of glutamic acid (20–23). A protein methylesterase encoded by the *cheB* gene balances the methyltransferase activity by removing methyl groups (24, 25). Each MCP has multiple sites at which methylation and demethylation may occur (26–29). For example, MCPI may accept a maximum of six methyl groups, and MCPII accepts at least four methyl groups (30, 31).

A second covalent modification of the MCPs also occurs at multiple sites and is dependent on the presence of the *cheB* gene (32, 33). The chemical nature of the modification, denoted the CheB-dependent modification, had not been identified previously, and its specific role in chemotaxis is unclear. However, several properties suggest that CheB-dependent modification may be the enzymatic conversion of glutamine to glutamic acid residues (32). Each CheB modification of MCPs results in the addition of one net negative charge to the protein and allows additional methyl groups to be accepted by the MCP (31, 32).

We have previously shown (30) that two tryptic peptides accept methyl groups in MCPI and MCPII. One of these, designated peptide K1, contains lysine and methionine; the other, designated peptide R1, contains arginine. In addition, peptide K1 derived from MCPI contains at least one CheB-modifiable site. To identify the CheB-modifiable amino acid(s) and elucidate the chemical nature of the CheB-dependent modification, we have performed protein sequence determinations on the radiolabeled K1 and R1 peptides derived from MCPI. One of the methylated positions observed in peptide K1 that is methylated in protein obtained from a wild-type background remains completely unmethylated in protein synthesized in a *cheB* mutant. In conjunction with the amino acid sequence of MCPI deduced from the nucleotide sequence of the *tsr* gene (34), these data provide compelling evidence that CheB modification is the enzymatic deamidation of glutamine to glutamic acid. Possible functions for this deamidation in chemotaxis are discussed.

MATERIALS AND METHODS

Strains. *E. coli* strains and λ–*E. coli* hybrid phage were as described (30, 31). All strains and phages were the generous gifts of J. S. Parkinson and M. Simon.

Radiolabeling of MCPs, Electrophoresis, Enzyme Digestions, HPLC Analysis, and Isolation of Peptides. These procedures were exactly as described (30, 31). Neutralized HPLC fractions containing radiolabeled peptides K1 and R1 were pooled and lyophilized directly without removal of sodium phosphate.

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Abbreviation: MCP, methyl-accepting chemotaxis protein.

‡ Present address: DNAX Research Institute of Molecular and Cellular Biology, Inc., 1450 Page Mill Rd., Palo Alto, CA 94304.

Radiolabeled Sequence Analysis. The sequences shown in Fig. 1 were determined on a spinning cup sequenator with a single cleavage program as described (35, 36). To minimize hydrolysis of the methyl esters during sequence determinations on [^{3}H]methyl-labeled peptides, the sequenator was modified to bypass the conversion flask; the anilinothiazolinone derivatives were extracted from the cup with 1-chlorobutane/0.1% dithiothreitol and collected. For each sequenator cycle, the fraction that contained the derivatized amino acid was dried under argon and dissolved in 0.2 ml of acetonitrile. Radioactivity in the entire fraction was determined by liquid scintillation counting. All samples were loaded in 0.15% $NaDodSO_4$ (Bio-Rad, twice recrystallized), extracted with 4 ml of 1-chlorobutane/0.1% dithiothreitol, and double coupled with an extended first cleavage time. Sequence analysis of [^{35}S]methionine-labeled peptide K1 was performed in duplicate and yielded similar results both times.

The sequences shown in Fig. 2 were determined on a gas-phase sequenator as described (37). Samples were loaded with 0.3 mg of sperm whale myoglobin in 1% $NaDodSO_4$. To minimize hydrolysis of the methyl esters, delivery of the 1 M HCl/methanol was turned off, and after delivery of 1-chlorobutane the extracted anilinothiazolinone derivatives (≈0.3 ml) were collected. No wash was performed, and recovery was estimated to be 80%. For each sequenator cycle, the sample was transferred to a scintillation vial and 4 ml of Liquifluor (New England Nuclear) was added for determination of radioactivity by scintillation counting (50 min per vial). Sequenator lag, which is observed as a significant yield (>10%) of the amino acid from one cycle in the following cycle, resulted from the large quantity of sodium phosphate (pH 2.2) in the samples, which changed the pH of the coupling buffer.

Several [^{3}H]methyl-labeled peptides were incubated for various times under the conditions used for coupling in the sequenator (pH 9.0 at 54°C). These base-catalyzed hydrolyses indicated that the half-life of the methyl esters averaged 7 hr. The coupling time for each sequenator cycle was a total of 30 min in the spinning cup sequenator and 15 min in the gas-phase sequenator. Thus, it would be possible to carry out at least 12 sequenator cycles on the [^{3}H]methyl-labeled peptide samples before the radioactivity was lost due to nonspecific hydrolysis. As can be seen in the data of Figs. 1 and 2, assignment of methylated residues beyond cycle 16 is tentative.

Amino Acid Analyses. Pronase digestion of radiolabeled peptides K1 and R1 was performed in 0.2 M *N*-ethylmorpholine acetate (pH 7.0) at 34°C. Two micrograms of Pronase (10 mg/ml in 0.2 M *N*-ethylmorpholine acetate, pH 7.5) was added four times over the course of 8 hr. Samples were lyophilized, and the amino acids were dissolved in 20–30 μl of 0.4 M sodium borate (pH 9.5), derivatized with *o*-phthalaldehyde, and separated by reverse-phase HPLC as described (38, 39). Fractions (0.5 min) were collected into scintillation vials from the time of injection, and radioactivity was determined. Elution positions of 19 amino acids (except proline) in addition to glutamic acid γ-methyl ester and aspartic acid β-methyl ester were determined with known amino acid standards and were reproducible (39). Elution times of derivatized tyrosine, methyl aspartate, methyl glutamate, and methionine were 39, 39.5, 43, and 48 min, respectively.

Preliminary Pronase treatment of [^{35}S]methionine-labeled tryptic peptides derived from MCPIII showed that >85% of the radiolabel recovered was free methionine. The remainder eluted as several peaks that did not correspond to any of the standard amino acids and probably represented incompletely cleaved small peptides.

RESULTS

Positions of Methyl-Accepting Sites in Tryptic Peptides from MCPI. Methyl-accepting sites on the two methyl-accepting tryptic peptides from MCPI, K1 and R1, have been extensively characterized with respect to their identity and number (30). The methylated forms of peptides K1 and R1 have been designated T1–T6 in order of elution from a reverse-phase HPLC column. Methylated peptide T4 corresponds to the dimethylated form of peptide R1. Peptides T2, T3, T5, and T6 represent the mono-, di-, tri-, and tetramethylated forms of CheB-modified peptide K1, respectively, and are not observed in MCPI produced in a *cheB* mutant background (30, 31). Peptide T1 is the monomethylated form of peptide K1 that does not contain a CheB modification; peptide T1 is only observed as a major methylated form in MCPI produced in *cheB* cells and does not accept more than one methyl group (31).

To localize the positions of the methyl-accepting sites in the amino acid sequences of the peptides, radiolabeled peptides were isolated from MCPI synthesized in wild-type cells and subjected to protein sequence determinations. Sequence analysis was performed on [^{3}H]methyl-labeled peptide K1 that was CheB-modified (peptides T3 and T5 combined) and established methylated residues at positions 3, 10, and perhaps 17 (Figs. 1 and 2). A methylated residue also was found at position 11 in the second methyl-accepting peptide, R1, from MCPI. Deter-

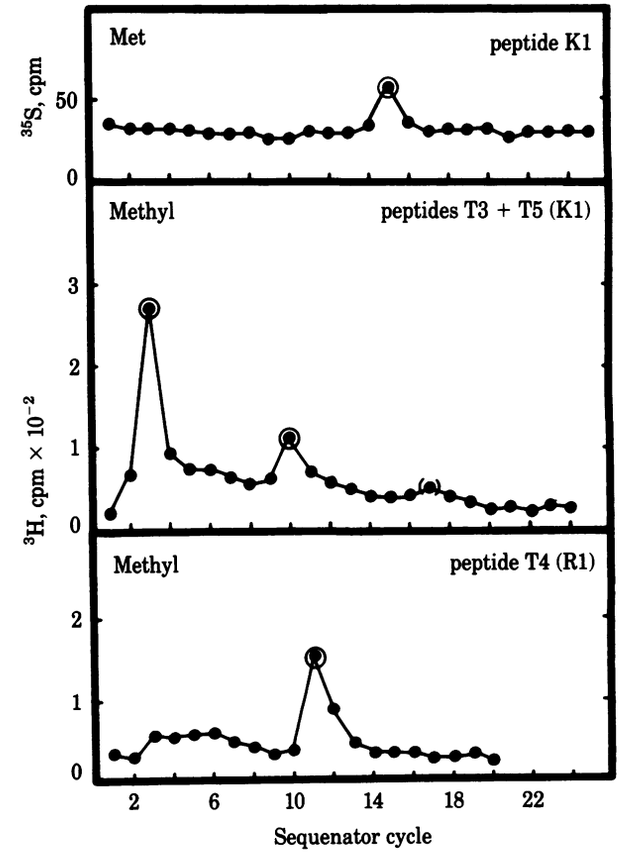

FIG. 1. Radiolabeled protein sequence analysis of methyl-accepting tryptic peptides from MCPI. (*Top*) Sequenator run on [^{35}S]methionine-labeled peptide K1 isolated from MCPI synthesized in *cheRB*-deleted cells (RP2859 λ*ind*$^-$ infected with λ*fla91*). (*Middle*) Sequenator run on [^{3}H]methyl-labeled peptides T3 and T5 (K1) isolated from MCPI produced in *tar* cells (MS5235). Equal amounts of radioactivity were present in the T3 and T5 peptides (4,000 ^{3}H cpm each) used in the analysis. (*Bottom*) Sequence determination on [^{3}H]methyl-labeled peptide T4 (R1) isolated as for peptides T3 and T5 (≈8,000 ^{3}H cpm).

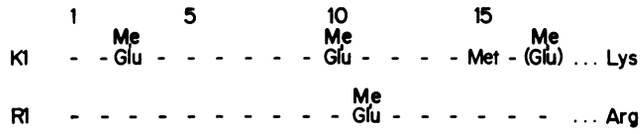

FIG. 2. Sequence results on peptides K1 and R1 summarized. The carboxyl-terminal lysine and arginine residues were determined by incorporation of these ^{3}H-labeled amino acids into the peptides (30).

mination of more than 16 or 17 residues of sequence on the [^{3}H]methyl-labeled peptides was not possible due to the lability of the methyl esters under the conditions used in the sequenator coupling step. Similar analyses of [^{35}S]methionine-labeled peptide K1 indicated that residue 15 contained methionine.

The identities of the methylated amino acids in peptides K1 and R1 were confirmed as methylglutamyl residues by amino acid analysis. A mixture of [^{3}H]methyl-labeled peptides T3 and T4 (in a 1:1 ratio as judged by ^{3}H incorporation) was digested with Pronase. Quantitation of free amino acids after derivatization with *o*-phthalaldehyde (38, 39) showed that 25% of the recovered radiolabel comigrated with glutamic acid γ-methyl ester. Approximately 60% of the radiolabel was found in the flow-through peak of the analysis and most likely represented [^{3}H]methanol released during the *o*-phthalaldehyde derivatization at pH 9.5. No radioactivity was associated with methyl aspartate in this amino acid digest of [^{3}H]methyl-labeled K1 and R1 peptides (data not shown). The remainder of the radiolabel eluted in four peaks that did not correspond to any of the standard amino acids and may be incompletely cleaved small peptides (data not shown). We conclude that MCPI synthesized in a wild-type background accepts methyl groups on glutamate residues at the positions in peptides K1 and R1 indicated in Fig. 2.

Unmodified Peptide K1 Lacks a Methyl-Accepting Site. We compared the methylated positions in CheB-modified and unmodified peptide K1 directly by subjecting [^{3}H]methyl-labeled peptides T1 and T2 to protein sequence analyses. Each peptide contained one methyl ester, but peptide T1 did not contain a CheB modification. Sequence determination on CheB-modified peptide T2 showed the presence of methyl esters at position 3 (Fig. 3). Position 10 may also be methylated, suggesting that, as isolated by HPLC from a population of MCPI molecules, peptide T2 may consist of a mixture of monomethylated isomers, each of which is methylated at one of the possible methyl-accepting glutamate residues.

In contrast, sequence analysis of the unmodified [^{3}H]methyl-labeled peptide T1 showed the absence of methyl esters at position 3 (Fig. 3). The data suggest that this peptide contained a methylated residue at position 10, although the peak is broad. This is most likely due to "sequenator lag" which results from incomplete coupling at any or all of the preceding sequenator cycles. In the analyses shown in Fig. 3, the peptide T1 sample contained twice as much radioactivity as the peptide T2 sample. We therefore believe that the absence of a methyl ester at position 3 in peptide T1 reflects the inability of this residue to accept methyl groups in the absence of CheB-dependent modification. Due to hydrolysis of the methyl esters during sequence analysis, the presence or absence of methyl esters cannot be confirmed at position 17 in the two sequences (Fig. 3).

DISCUSSION

MCPI is multiply methylated in wild-type *E. coli* cells, and the methyl esters are found on two tryptic peptides, K1 and R1 (30). We have established positions of methylated glutamic acid residues and a methionine residue in methyl-accepting peptides derived from MCPI produced in wild-type cells and have cor-

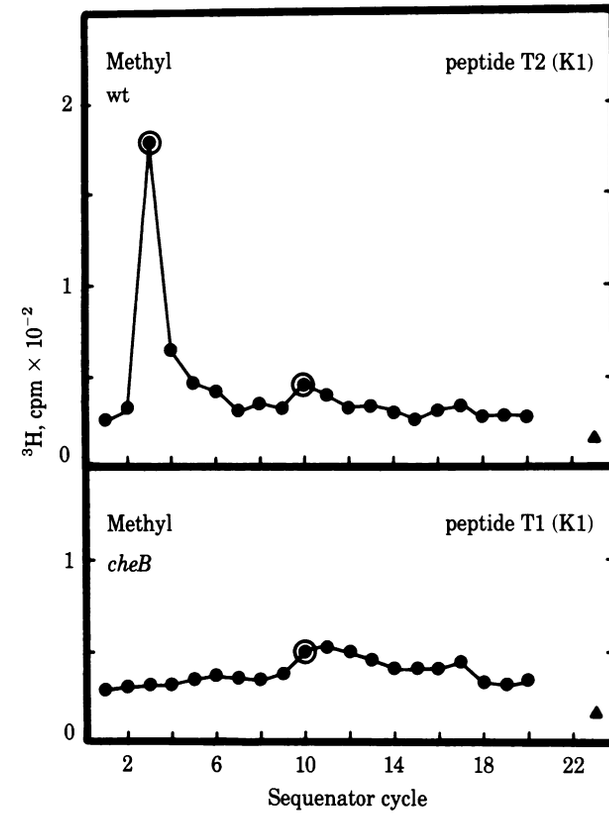

FIG. 3. Methyl-accepting sites in CheB-modified and unmodified peptide K1 from MCPI. (*Upper*) Radioactivity in cycles of a sequenator run on [^{3}H]methyl-labeled peptide T2 isolated from MCPI produced in *tar* cells (MS5235). This peptide was CheB-modified and contained ≈3,000 ^{3}H cpm. wt, Wild type. (*Lower*) Sequence determination on [^{3}H]methyl-labeled peptide T1 (≈6,000 ^{3}H cpm) isolated from MCPI produced in a *cheB* background (RP4209). ▲, Background radioactivity in a blank cycle prior to sample loading.

related this information with the nucleotide sequence of the *tsr* gene recently determined by Boyd *et al.* (34). Only one sequence in the single open reading frame of the *tsr* gene contained a methionine residue 15 amino acids from a tryptic cleavage site. This amino acid sequence derived from the nucleotide sequence of *tsr* is shown in Fig. 4 and has several features that unambiguously identify it as methyl-accepting peptide K1. The peptide is 23 amino acids long and ends in lysine, in agreement with its chromatographic properties on HPLC and the incorporation of [^{3}H]lysine (30).

The predicted sequence contains glutamic acid at position 10 and glutamine at positions 3 and 17 (Fig. 4; see below). These positions are observed as methylated sites in peptide K1. The

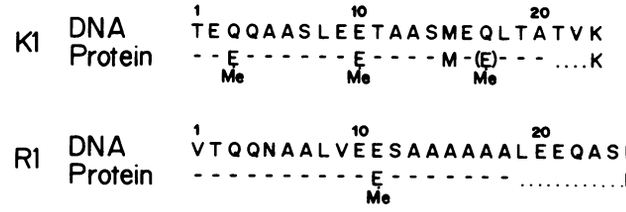

FIG. 4. Amino acid sequences of methyl-accepting peptides deduced from the nucleotide sequence of *tsr* (34). Amino acid sequences corresponding to the radiolabeled protein sequence determinations in Fig. 1 are shown. Dashes indicate the absence of a radiolabeled residue at that position (see Fig. 2). The single-letter code for amino acids is given in Dayhoff (40); Q and E denote glutamine and glutamic acid, respectively.

glutamine positions are compatible with our suggestion that the CheB modification may be the conversion of glutamine to glutamic acid. This does not exclude other sites (residues 4, 9, and 16) as possible methyl-accepting sites. The extensive homology in this region of the *tsr* and *tar* genes (41) supports the idea that this sequence contains sites for methyltransferase activity.

The nucleotide sequence of *tsr* predicts that position 3 is a glutamine (Fig. 4). The identification of positions of methylated residues shows that residue 3 in peptide K1 derived from MCPI synthesized in wild-type cells is a methyl-accepting glutamic acid (Fig. 2). However, the same residue in the unmodified peptide from MCPI produced in *cheB* mutants is not methylated. We conclude that the CheB modification of peptide K1 may be the enzymatic conversion of the glutamine at position 3 to methyl-accepting glutamic acid. The CheB-modified and unmodified forms of peptide K1 both accept methyl groups at position 10, which is encoded as glutamic acid in the *tsr* gene. At this position the methyl-accepting glutamic acid is adjacent to another glutamic acid at position 9 and may account for methylation of the unmodified form. This is consistent with the idea that pairs of glutamic acid residues create a methyl-accepting site recognized by the methyltransferase (34). At position 3, the CheB modification of glutamine to glutamic acid thus results in the formation of a pair of glutamic acid residues (positions 2 and 3), of which position 3 is a methyl-accepting site.

All other properties of the CheB-dependent modification are consistent with its being a deamidation process. Each CheB modification results in the addition of one net negative charge to MCPs (31, 32). pH-dependent changes in the chromatographic elution position of the modifiable peptide suggest that the CheB-modified group has a pKa similar to that of glutamic acid (42). CheB modification is irreversible and is stable to base-catalyzed demethylation conditions (31, 32). The modification allows additional methyl groups to be incorporated into methyl-accepting peptide K1 (31). Because the data are so extensive, we suggest the use of "deamidation" or "CheB-dependent deamidation" rather than the term "modification" for this process.

The sequences shown in Fig. 4 also suggest that the deamidation might occur at a second site in peptide K1 (residue 17) to produce a third methyl-accepting glutamic acid. We previously determined that peptide K1 is subject to only one deamidation yet it may accept two or three additional methyl groups subsequent to this single deamidation (31). Because the data regarding residue 17 in peptides T1 and T2 are ambiguous (Fig. 3) we can only propose explanations for the apparent discrepancy. First, either residue 3 or residue 17 may be CheB modifiable but, when one site has been modified, the second site is no longer reactive. This would be consistent with the possible methyl-accepting residue at position 17 (Fig. 2). Second, MCPI could acquire two CheB-dependent deamidations on peptide K1 (residues 3 and 17) only in the presence of CheR, the methyltransferase. We quantitate the number of deamidations on MCPs produced in cells that are *cheR* (31–33); therefore, this possibility cannot be excluded. Interestingly, the analogous peptide from MCPIII synthesized in *cheR* cells is subject to two deamidations, suggesting that this limitation or control may no longer hold in minicells (42). Finally, it is possible that methyl groups (up to three or four total) may be placed with a lower frequency on other glutamate residues in peptide K1 in addition to those at positions 3, 10, and 17. Due to the high background that results from hydrolysis of the methyl esters during sequence analysis, these lower-preference methyl-accepting sites would not have been detected in our determinations.

Sequence comparisons within the deduced amino acid sequence of MCPI identified a second sequence approximately 200 amino acids carboxyl-terminal to peptide K1 that is homologous to peptide K1 (34). The homologous amino acid sequence (shown in Fig. 4) seems to correspond to peptide R1. The peptide is 25 amino acids long, ends in arginine, contains no methionine, and contains potential methyl-accepting glutamic acid residues at positions 11 and 21 (Fig. 2). The predicted sequence of peptide R1 also is conserved in the *tsr* and *tar* gene sequences (41). In addition, the amino acid sequences of peptide R1 deduced from the *tsr* and *tar* nucleotide sequences (41) are in accordance with the number of methyl-accepting sites in peptides R1 derived from MCPI and MCPII. Peptide R1 from MCPI accepts two methyl groups (30), whereas peptide R1 derived from MCPII only accepts one methyl group (31). Comparison of the deduced amino acid sequences of peptide R1 from MCPI and from MCPII reveals the substitution of a glutamine in MCPII for the glutamic acid at residue 10 in MCPI (39). Because the only other amino acid difference is a serine for the alanine at residue 7, it is likely that the single glutamic acid at position 11 in MCPII is unable to serve as a methyl-accepting site. Two pairs of glutamic acid residues that could serve as such recognition sites for methyltransferase are present in the predicted sequence of peptide R1 from MCPI but only one site is found in the predicted sequence of peptide R1 from MCPII (41).

There are several roles for the CheB-dependent deamidation in chemotaxis. First, it is possible that the deamidation may have a structural function. All of the MCPs acquire multiple deamidations (10, 32, 33, 42, 43). MCPs synthesized in wild-type cells are maximally deamidated (31), which implies that CheB-dependent deamidation occurs shortly after synthesis of MCPs. Perhaps the neutral glutamine residues are required for proper membrane insertion or assembly of newly synthesized MCPs. Additionally, a more neutral environment in this region of the MCP molecule may enhance CheB–MCP associations required for proper formation of an active transducer complex. In this regard, an *in vitro* methylation system constructed from cytoplasm and membranes has not been formed in a functional state when *cheR* or *cheB* membranes are combined with wild-type cytoplasmic extracts (44). This suggests that the components (MCP, CheR, CheB) may be assembled during synthesis, and CheB-dependent deamidation may facilitate the association process.

Second, the deamidation activity of the *cheB* gene product may originally have functioned as a primitive adaptation mechanism prior to the existence of methylation. Recent work by Parkinson *et al.* (10) and Stock *et al.* (45) on methylation-deficient chemotactic pseudorevertants of *cheR* mutants supports the idea that a methylation-independent, CheB-dependent adaptation process is sufficient to allow rudimentary MCP-mediated chemotaxis. In the absence of methylation the irreversible nature of the deamidation process would require that MCPs turn over and be continually resynthesized. A role for the deamidation in adaptation thus is indicated by the idea that the useful life-time of the MCP was maximized by providing multiple sites for CheB-dependent deamidation on each MCP.

That the CheB-dependent deamidation still plays a primary role in bacterial responses is suggested by the drastic effect of *cheB* mutations on the methylation-independent chemotaxis to oxygen and certain sugars (46, 47). We envision the existence of additional, as yet unidentified, sensory transducer proteins that mediate other types of taxis and that are deamidated by CheB.

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