The Bacillus subtilis chemoreceptor McpB is a dimer of identical subunits containing two transmembrane (TM) segments (TM1, residues 17–34: TM2, residues 280–302) in each monomer with a 2-fold axis of symmetry. To study the organization of the TM domains, the wild-type receptor was mutated systematically at the membrane bilayer/extracytoplasmic interface with 15 single cysteine (Cys) substitutions in each of the two TM domains. Each single Cys substitution was capable of complementing a null allele in vivo, suggesting that no significant perturbation of the native tertiary or quaternary structure of the chemoreceptor was introduced by the mutations. On the basis of patterns of disulfide crosslinking between subunits of the dimeric receptor, an α-helical interface was identified between TM1 and TM1 (containing residues 32, 36, 39, and 43) and between TM2 and TM2 (containing residues 276, 277, 280, 283 and 286). Pairs of cysteine substitutions (positions 34/280 and 38/273) in TM1 and TM2 were used to further elucidate specific contacts within a monomer subunit, enabling a model to be constructed defining the organization of the TM domain. Crosslinking of residues that were 150–180 Å removed from position 32 (positions 37, 41, and 44) suggested that the receptors may be organized as an array of trimers of dimers in vivo. All crosslinking was unaffected by deletion of cheB and cheR (loss of receptor demethyl-ation/methylation enzymes) or by deletion of cheW and cheV (loss of proteins that couple receptors with the autophosphorylating kinase). These findings indicate that the organization of the transmembrane region and the stability of the quaternary complex of receptors are independent of covalent modifications of the cytoplasmic domain and conformations in the cytoplasmic domain induced by the coupling proteins.

Keywords: chemotaxis; chemoreceptor; crosslinking; structure; helix-packing

*Corresponding author

Abbreviations used: TM, transmembrane; MCP, methyl-accepting chemotaxis protein; INDEL, insertion/deletion.

E-mail address of the corresponding author: ordal@uiuc.edu

Introduction

Transmembrane receptor proteins known as methyl-accepting chemotaxis proteins (MCPs) regulate bacterial chemotaxis. MCPs are stable homodimers regardless of the degree of ligand occupancy, and each homodimer exists as an extended helical bundle with a 2-fold axis of symmetry. MCPs bind extracellular ligand molecules and transduce this information to a cytoplasmic two-component signal transduction cascade. In the enteric organisms Escherichia coli and Salmonella typhimurium, ligand binding to the chemoreceptor is believed to induce a conformational change similar to a piston movement in helix 4 of the periplasmic domain in the dimeric chemoreceptor. The conformational change is believed to be translated to a vertical shift in the cytoplasmic domain of a monomer relative to the membrane plane. The resultant effect is down-regulation of the CheA autophosphorylation activity, which reduces the cytoplasmic concentration of CheY-P, leading to a smooth swimming event generated by counterclockwise rotation of the flagellar motor. Despite the sequence similarity among the
chemotaxis receptors, functional differences exist in the signaling mechanism between *E. coli* and *Bacillus subtilis* chemoreceptors. Although chemotaxis receptors in both *B. subtilis* and *E. coli* are subject to methylation by CheR methyltransferase and demethylation by CheB methylesterase to produce methanol during adaptation, only *B. subtilis* chemoreceptors give rise to enhanced methanol formation on both addition and removal of attractants or repellents. Enhanced methanol formation occurs from *E. coli* chemoreceptors only on removal of attractants and addition of repellents, and diminished methanol formation occurs on addition of attractants and removal of repellents. The basis of this difference is that chemoreceptor methylation is antagonistic in *B. subtilis* and synergistic in *E. coli*. To bring about adaptation, the *B. subtilis* chemoreceptor McpB undergoes methylation of Glu637 and demethylation of Glu630 on addition of attractant, and demethylation of Glu637 and methylation of Glu630 on removal. For adaptation in *E. coli*, attractants cause a net increase in chemoreceptor methylation and repellents cause a net decrease in receptor methylation.

This functional difference in operation of the receptors in these two organisms may be based, in part, on structural differences. The chemoreceptors are divided into three classes on the basis of the presence of defined insertion/deletion (INDEL) sequences, which add four turns of α-helix per INDEL. The *B. subtilis* receptors belong to the class III family of chemoreceptors, because they contain four INDELS. The *E. coli* receptors belong to the class I family and contain no INDELS, which are postulated to add overall length to the cytoplasmic domain without disrupting the predominantly anti-parallel α-helical coiled-coil nature of the chemotaxis receptor cytoplasmic methylation/signaling domains.

Due to the architecture of chemoreceptors, the transmembrane (TM) domain is an excellent region for analysis because it provides a structural link between the ligand-binding domain and the cytoplasmic signaling domain; therefore, conformational changes induced by ligand binding must be translated through this domain. The best-studied MCP in *B. subtilis* is the asparagine receptor McpB; this is a 72 kDa receptor with two well-defined TM domains bracketing the ligand-binding domain, followed by a conserved cytoplasmic domain containing the methylation and signaling domains (Figure 1). No structural study has been completed on this receptor molecule; however, on the basis of sequence and functional similarity, the TM domain is believed to exist as a loosely packed, four-helix bundle similar to *E. coli* receptors Tar and Trg, which have been demonstrated to transduce stimuli (see above).

---

**Figure 1.** Secondary structure model of McpB. McpB is shown as a full-length homodimer. Cylinders represent predicted stretches of α-helical regions in the protein on the basis of sequence alignment and known tertiary organization of homologous receptors. TM domains are numbered from the N terminus. Single cysteine substitutions and relative location in TM helix I and II are numbered and in bold-face.
Here, we investigated the structural organization of the TM domain of McpB using targeted disulfide crosslinking of engineered cysteine (Cys) substitutions with the oxidizing catalyst Cu^{2+}(phenanthroline)₃ (Cu^{2+}·Phe₃). We first constructed a map of the interacting helical faces of transmembrane helix 1 (TM1) and transmembrane helix 2 (TM2), and further elucidated the quaternary organization by creating selected double mutants in TM1 and TM2. Finally, we performed crosslinking studies on the TM domain in various genetic backgrounds to explore the effects of the methylation system and the coupling proteins had on TM1 and TM2 organization.

Results

In vivo complementation of McpB with single Cys replacement mutants

Previous experiments have shown that expression of wild-type McpB in a strain lacking all chemotaxis receptor homologs leads to functional complementation in vivo. Consistently, in vivo complementation was achieved for all 29 single Cys McpB chemoreceptors used in this study compared to OI3547 (Table 1). However, the S279C and T283 mutant chemoreceptors both failed to complement in one of the media used in the swarm assay. The apparent lack of complementation for both substitutions was not of importance because both complemented in the alternate medium used.

Crosslinking between TM1 and TM1

Despite the position of a single Cys substitution in McpB, non-crosslinked Cys-substituted McpB migrates at about 72 kDa, consistent with its calculated molecular mass of 71.8 kDa (Figure 2A or B, monomer). When cells containing McpB with a single Cys substitution at position 32 (helix 1) were oxidized with the catalyst Cu^{2+}·Phe₃, prior to electrophoresis, high-efficiency crosslinking was observed (Figure 2A), as evidenced by the appearance of a band migrating at the Mr of dimeric McpB (i.e., 144 kDa; lane 6). However, no crosslinking was detected for residues flanking position 32 (i.e. positions 30, 31, 33, and 34). In addition, residues that were positioned along the same face of a regular α helix as position 32 formed crosslinked species similarly when exposed to Cu^{2+}·Phe₃ (Table 1, positions 36, 39, and 43). Surprisingly, residues that were shifted 150–180 from the helical face of position 32 became increasingly susceptible to homologous crosslinking with increasing residue number (Table 1, residues 37, 41, and 44), leading us to believe that dimer–dimer interactions are responsible for this effect (see Discussion).

Crosslinking between TM2 and TM2

Receptors were constructed containing single Cys substitutions in TM2. Oxidation of McpB with single Cys residues at positions 276, 277, and 280 resulted in moderate to high levels of crosslinking of monomers to produce a dimer that migrated at

| Table 1. Crosslinking efficiencies and chemotaxis activity of McpB receptor with single cysteine substitutions |
|---------------------------------|---------------------------------|---------|---------|---------|---------|---------|
| Cys position (TM1)             | Crosslinking percentage (%)     | Swarm activity (%) | Cys position (TM2) | Crosslinking percentage (%) | Swarm activity (%) |
| (TM1)                          |                                 | mm⁴ TBr*           | (TM2)             |                                 | mm⁴ TBr*           |
| 30                             | 0                               | 109.7 91.0         | 272               | 0                               | 77.8 80.6          |
| 31                             | 0                               | 97.2 104.5         | 273               | 0                               | 95.8 92.5          |
| 32                             | 100                             | 106.9 86.6         | 274               | 0                               | 101.4 95.5         |
| 33                             | 0                               | 104.2 83.6         | 275               | 0                               | 105.6 89.6         |
| 34                             | 0                               | 84.7 95.5          | 276               | 51.7                            | 131.9 95.5         |
| 35                             | 0                               | 97.2 80.6          | 277               | 51.1                            | 92.6 87.6          |
| 36                             | 100                             | 91.7 88.1          | 278               | 0                               | 87.9 83.6          |
| 37                             | 52                              | 90.3 82.1          | 279               | 0                               | 97.2 62.7          |
| 38                             | 15                              | 93.1 92.5          | 280               | 95                              | 98.6 83.6          |
| 39                             | 74                              | 88.9 82.1          | 281               | 25.1                            | 84.7 83.6          |
| 40                             | nd                              | nd nd             | 282               | 0                               | 111.1 100.0        |
| 41                             | 100                             | 106.9 85.1         | 283               | 12.1                            | 68.1 95.5          |
| 42                             | 49.2                            | 94.4 97.0          | 284               | 0                               | 90.3 82.1          |
| 43                             | 42.3                            | 100.0 92.5         | 285               | 0                               | 98.6 95.5          |
| 44                             | 100                             | 88.9 100.0         | 286               | 18.2                            | 79.2 94.0          |

Swarm activity is measured as the rate of swarming and represented as percentage of OI3547 (see Materials and Methods). The OI3547 average swarm diameter was 24.0 mm after 15.5 hours for asparagine minimal swarm plates and 22.3 mm after ten hours for TBr swarm plates.

Bold type denotes helical face, which interacts within the dimer (Figure 6).

Percentage crosslinked receptor was calculated for duplicate experiments after five minutes of exposure to oxidizing reagent.

mm represents swarm assay in minimal medium to the attractant asparagine (see Materials and Methods).

TBr represents swarm assay in tryptone medium (see Materials and Methods).
slightly higher apparent molecular mass relative to Cys substitutions in TM1 (Figure 2B, M_r ~210 kDa). Similar to the crosslinking pattern found in TM1, residues near the same helical face as position 280 (Table 1, residues 281, 283, and 286) showed moderate levels of crosslinking.

Crosslinking between TM1 and TM2

The organization of TM1-TM1’ and TM2-TM2’ was implied from the relationship of the crosslinking efficiencies. However, determination of the organization of the dimeric receptor was incomplete without identification of the intramolecular contacts between TM1 and TM2. On the basis of previous reports for the aspartate and maltose chemoreceptors in E. coli, we anticipated a similar tertiary organization of McpB. Therefore, using the results we had obtained with the single mutants and the predicted tertiary organization of the receptor TM helices, we constructed double mutants in TM1 and TM2 that we predicted would demonstrate significant intramolecular crosslinking and minimal dimeric crosslinking.

Oxidation of McpB with paired Cys substitutions at position 38 (helix I) and position 273 (helix II) resulted in significant crosslinking, generating a faster migrating species (Figure 3), affording confirmation of the predicted tertiary contacts made within a monomer. Contacts were identified between position 34 and 280 (Figure 3). Surprisingly, double mutants constructed at position 35 in helix I with Cys in positions 277, 280, and 281 in helix II did not form crosslinked monomers as anticipated (data not shown). Paired substitutions were made for position 31 in helix I with all positions studied in helix II, and these did not produce intramolecular crosslinked monomers (data not shown). The failure of crosslinking may be due to the identity of the local amino acid residues around these positions in TM1 or TM2, which possibly do not afford the sites reactive to oxidation under the conditions tested. Another possibility is that TM1 is tilted away from TM1’ with increasing residue number and that TM2 coils around TM1 in the region of the transmembrane domain studied. This tertiary structure could explain why position 34 in TM1 can crosslink with position 280 in TM2 and position 38 in TM1 can crosslink with position 273 in TM2; yet positions 31 and 35 appear not to be in close enough proximity to react.

Neither the methylation system nor coupling proteins alters crosslinking contacts in the TM domain

The coupling proteins, CheW and CheV, and the methylation enzymes, CheR and CheB, have been shown to play a critical role in the excitation and adaptation processes of B. subtilis chemotaxis. However, the presence of cheRcheB or cheWcheV mutations did not affect crosslinking of L32C (Figure 4A). The same was true for the effect of these proteins on A276C crosslinking in TM2 (Figure 4B). The results were consistent for the remaining 13 residues in TM1 and 14 residues in TM2 (data not shown), demonstrating that the McpB TM domain organization is independent of methylation or coupling systems.

Discussion

In this study, site-directed thiol crosslinking was used to define the organization of the TM helical region of the chemotaxis receptor McpB from B. subtilis. To define residues that are close in three-dimensional space, Cu^{2+}-Phe-catalyzed disulfide formation was used for single Cys substitutions in TM1 and TM2 of the chemotaxis receptor McpB (Figure 2A and B). Disulfide bond formation can occur only if the thiol moiety is within the correct distance constraints. The β-carbon...
atom on the substituted Cys residue is consequently within ~3–5 Å if crosslinking occurs in a Cu^{2+}·Phe₃-dependent manner. As shown, Cu^{2+}·Phe₃ oxidation crosslinked Cys residues in a regular pattern along the helical face containing positions 32, 36, 39, and 43, indicating that Cys side-chains must be within ~3–5 Å in three-dimensional space (Figure 5). Consistently, residues that are not present on this helical face showed very little crosslinking, with the exception of three positions shifted approximately 150–180° from position 32 (see below) (Figure 6). A patch of residues in TM2 (positions 276, 277, 280, 281, 283, and 286) was identified that defined its dimeric interface. The structure of the TM domain was more fully elucidated by the construction of double mutants in TM1 and TM2 identifying the intramolecular contacts (Figure 3A and B).

In contrast to the regular pattern of crosslinking found along the L32C helical face, a second helical face of single Cys substitutions sensitive to oxidation was identified. Positions 37, 41, and 44 were oxidized significantly and were shifted ~150–180° from the L32C helical face, which was inconsistent with the observed pattern for a regular α helix. Additionally, the extent of crosslinking increased significantly over this stretch of amino acid residues, suggesting that the TM1 helix may be tilted away from its dimeric interface. Coincidentally, Pro28 in McpB is on the same helix face as position 32 and probably produces a kink in the helix. This prediction is consistent with the reduced crosslinking of residues with increasing residue number along the L32C helical face.

Previous findings in *E. coli* demonstrated that the serine chemotaxis receptor cytoplasmic domain crystallized as a trimer of dimers, and when residues predicted to be involved in forming the trimer of dimers were mutated, receptor function was impaired in vivo. Moreover, large supramolecular complexes of receptors C-terminal domain/CheA kinase/CheW coupling proteins can form in vitro that have enhanced CheA activity, indicating the importance of complexes. It is likely that dimers contact each other near the outside of the membrane, so that a large molecular array of chemoreceptors is generated. Here, we provide physical evidence of the dimer–dimer contacts near the outside of the membrane, supporting these predictions. (Presumably the cytoplasmic contacts that permit trimers of dimers to be formed exist also in *B. subtilis* receptors; otherwise, it is hard to imagine the dimer–dimer contacts we discovered would occur.) To illustrate this observation, we constructed a model of trimers of dimers using the dimeric transmembrane helical bundle as a rigid body and positioning residues

---

**Figure 3.** Crosslinking of McpB chemoreceptor containing paired Cys residues at positions 34 and 280, and positions 38 and 273. Protoplasts were prepared from *B. subtilis* strain OI3635 expressing McpB. Crosslinking was done as described in Materials and Methods. Samples were subjected to non-reducing SDS-7.5% PAGE followed by detection with anti-McpB antibody (1:25,000) (TM1-TM2 denotes an intramolecular disulfide within a monomer).

**Figure 4.** Crosslinking of Cys substitutions at position 32 in TM1 and position 276 in TM2 in various genetic backgrounds. Crosslinking was done as described in Materials and Methods. Samples were subjected to non-reducing SDS-12% PAGE followed by detection with anti-McpB antibody (1:25,000). A, Protoplasts were prepared from *B. subtilis* OI3545 (lanes 1 and 2), OI3635 (lanes 3 and 4), and OI3943 (lanes 5 and 6) expressing L32C from the *amyE* locus. B, Protoplasts were prepared from *B. subtilis* OI3545 (lanes 1 and 2), OI3635 (lanes 3 and 4), and OI3943 (lanes 5 and 6) expressing A276C from the *amyE* locus.
along positions 37, 41, and 44 in close proximity (Figure 7).31 Positioning of TM1 residues 37, 41, and 44 toward the interior of trimers of dimers was achieved, enabling close proximity of the Y37C helical face to make dimer–dimer contacts and maintain L32C helical contacts.

Recent studies show that the *E. coli* receptors, CheW coupling protein, and CheA kinase are all needed for a maximally active ternary complex to be formed.32 CheW has been demonstrated to be essential for the polar localization and dynamic clustering of receptor molecules.33 Additionally, the role methylation plays in receptor signaling along with the role of CheW and CheA in the formation of the ternary complex is crucial.

**Figure 5.** Packing of helices I and II, and helices I’ and II’ in the chemoreceptor McpB dimer viewed from the extracellular surface. The positions of local maxima identified in Table 1 are represented by light grey, filled circles. Dark grey, filled circles represent residues ~150–180° removed from position 32 (see the text).

**Figure 6.** Helical net diagram of TM1 and TM2, which are shown as two-dimensional representations of α helices with the position of each residue identified by a circle. The vertical axis indicates the residue number and the horizontal axis represents the rotational position of the residue. Positions of local maxima identified in Table 1 are represented by dark filled circles. Grey circles represent residues ~150–180° removed from position 32 (see the text).

**Figure 7.** Quaternary organization of a theoretical McpB trimer of dimers. A model of McpB trimer of dimers was constructed by positioning residues 37, 41, and 44 toward the center of the trimer of dimers, while keeping the dimeric molecules rigid.
Materials and Methods

Materials

Affinity-purified horseradish peroxidase conjugated goat anti-rabbit polyclonal IgG (H + L) antibody and SuperSignal® West Pico chemiluminescent substrate was purchased from Pierce Endogen (Rockford, IL). N-Ethylmaleimide (NEM), copper sulfate (CuSO₄), EDTA and phenanthroline were obtained from Sigma-Aldrich (St. Louis, MO). Anti-C-terminal antibody was prepared as described. Restriction endonucleases from CEL, BsrGI and DpnI were obtained from New England Biolabs (Beverly, MA). All other enzymes were purchased from Invitrogen Corporation (Carlsbad, CA). Pfu cloned polymerase was obtained from Stratagene (La Jolla, CA). Polymerase chain reaction (PCR) Qiaprep II-linearized pAIN750::mcpB plasmids selecting for spectinomycin resistance and amylose utilization deficiency to obtain the Cys substitutions in various genetic backgrounds.

Bacterial strains

Strain OI3545 has been reported. Strain OI3547 is strain OI3545 with a wild-type mcpB in the amyE locus under a wild-type promoter. Strain OI3653 is a methyltransferase and methylase knock-out of OI3545 derived from strains OI2680 and OI2836 respectively. OI3943 (this work) is an mcpA, mcpB, mcpC, tlpA, tlpB, cheW, and cheV knockout derived from strain OI3180 (M. M. Rosario & G.W.O., unpublished results), HB4007, and OI3270.

Construction of McpB receptor with single Cys residues

pAIN700 has been described. A single Cys replacement at positions 30–44 (TM1) and 272–286 (TM2) (Figure 1) was introduced into pAIN700 by a modified long PCR as described. Briefly, 100 ng of plasmid DNA was subjected to 25 cycles of amplification with Pfu polymerase using 250 ng of mutant phosphorylated primer pairs according to the manufacturer’s recommendations. Amplified DNA was column-purified using Qiaprep spin columns. Approximately 200 ng of purified mutated DNA was ligated overnight at 15 °C with phage T4 DNA ligase according to the manufacturer’s recommendations. Ligated products were heat-inactivated and desalted in buffer-exchanged MicroBio® spin columns and digested with DpnI. Ligated and DpnI-digested DNA was transformed into chemically competent TG-1. Mutant plasmids were isolated and verified by DNA sequencing at University of Illinois Biotechnology Center (Champaign, IL). Cysteine replacements were then subcloned into EcoRI/NotI-digested pAIN750 DNA. Double mutants were prepared by ligating a gel-purified BsrGI restriction fragment of TM2 DNA into a dephosphorylated TM1 gel-purified BsrGI fragment. Double mutants in the correct orientation were identified using a KpnI digest. B. subtilis strains OI3545, OI3653, and OI3943 were transformed with Aat II-linearized pAIN750:mcpB plasmids selecting for spectinomycin resistance and amylose utilization deficiency to obtain the Cys substitutions in various genetic backgrounds.

Expression of McpB single Cys replacement mutants

Cells were prepared for crosslinking by growing cultures (5 ml) in glucose minimal medium until late log phase. The cultures were then harvested and prepared as described.

Swarm assays

Strains were grown overnight on tryptose/blood/agar base plates. A single colony was transferred to a minimal asparagine swarm plate (0.1 x Spizizen’s salts, 0.7 mM sorbitol, 5 µg/ml of the required amino acids, 0.3 mM asparagine and 0.25% (w/v) agar) and incubated at 30 °C for 15 hours. Additionally, a single colony was transferred to a tryptone swarm plate (0.1 g/ml of Tryptone, 0.05 g/ml of NaCl, 0.27% agar) and incubated at 30 °C for ten hours. A two-tailed, two-sample, equal variance Student’s t test was used to determine the similarity of the individual swarm assays.

Crosslinking

Crosslinking was carried out at 30 °C for five minutes by adding Cu²⁺–Phe₃ (180 mM phenanthroline, 60 mM CuSO₄, 50 mM NaH₂PO₄ in water) to a final concentration of 300 µM to cells resuspended at A₅₆₀ of 1.0 in protoplast buffer. Reactions were terminated by transferring 1 ml of cell suspension into NEM to a final concentration of 1 mg/ml and EDTA to a final concentration of 5 µM. The samples were then placed in an ice/water bath and frozen in liquid nitrogen followed by storage at −20 °C. Cells were then thawed on ice, pelleted for ten minutes at 14,000g, and resuspended in 100 µl of 2 x non-reducing sample buffer (40 mM Tris–HCl (pH 7.8), 16 mM NaH₂PO₄, 2% (w/v) SDS, 50 µg/ml of bromphenol blue, 20 mM EDTA, 2 mg/ml of NEM, and 0.10 g/ml of sucrose). The samples were then subjected to SDS-PAGE at the indicated percentage of acrylamide. Protein was then transferred using a semi-dry blotting apparatus (Biorad). McpB expression was
probed using polyclonal antibody against the C terminus of the receptor and developed using Supersignal® reagent according to the manufacturer’s recommendations.

Acknowledgements

We thank the rest of the Ordal laboratory for intellectual contributions to this work. We thank Brian Beel for technical assistance with cross-linking experiments. The National Institutes for Health grant GM54365 supported this research.

References


Edited by I. B. Holland

(Received 9 April 2003; received in revised form 27 May 2003; accepted 29 May 2003)