Dual Recognition of the Bacterial Chemoreceptor by Chemotaxis-specific Domains of the CheR Methyltransferase*

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Adaptation to persisting stimulation is required for highly sensitive detection of temporal changes of stimuli, and often involves covalent modification of receptors. Therefore, it is of vital importance to understand how a receptor and its cognate modifying enzyme(s) modulate each other through specific protein-protein interactions. In the chemotaxis of Escherichia coli, adaptation requires methylation of chemoreceptors (e.g. Tar) catalyzed by the CheR methyltransferase. CheR binds to the C-terminal NWETF sequence of a chemoreceptor that is distinct from the methylation sites. However, little is known about how CheR recognizes its methylation sites or how it is distributed in a cell. In this study, we used comparative genomics to demonstrate that the CheR chemotaxis methyltransferase contains three structurally and functionally distinct motifs: (i) the catalytic domain common to a methyltransferase superfamily; (ii) the N-terminal domain; and (iii) the subdomain of the catalytic domain, both of which are found exclusively in chemotaxis methyltransferases. The only evolutionary conserved motif specific to CheR is the positively charged face of helix α2 in the N-terminal domain. The three-dimensional structure of CheR of Salmonella typhimurium revealed that the monomeric protein consists of two domains (the N-terminal domain with no assigned function and the MTase domain) and one subdomain (the β-subdomain) (13). The co-crystal of CheR and the pentapeptide revealed that the β-subdomain binds to the NWETF sequence (11). Mutagenesis of the NWETF sequence of Tar demonstrated that it binds to CheR mainly through hydrophobic interaction (12). However, it is not clear how the CheR molecule is oriented when it binds to CheR. The NWETF sequence might not be essential for its catalytic activity itself.

In many sensory systems, transmembrane receptors recognize extracellular stimuli and transduce them into cytoplasmic signals to trigger defined physiological responses. These initial responses often diminish during persisting stimulation. The latter process, termed adaptation or desensitization, is essential for the detection of temporal changes of stimuli and/or the highly sensitive detection of stimuli over a comprehensive range. Covalent modifications of a receptor are often required for adaptation. In such cases, it is of vital importance to understand a regulated interplay between a receptor and modifying enzyme(s), including their mutual recognition and their subcellular localization, that assures spatially and temporally organized information processing.

Molecular mechanisms of adaptation have been well characterized in the chemotaxis of Escherichia coli and Salmonella typhimurium (1–6). The transmembrane chemoreceptors, also known as the methyl-accepting chemotaxis proteins (MCPs),1 are methylated by the S-adenosylmethionine (AdoMet)-dependent methyltransferase (MTase) CheR and demethylated by the methyltransferase CheB. In the resting state, an MCP is in equilibrium between methylation and demethylation. An attractant shifts the equilibrium toward methylation and a repellent toward demethylation. Each MCP has 4–5 glutamate residues located in two separate α helices (the first and second methylation helices (MH1 and MH2)) in the cytoplasmic domain (7, 8). CheR has to recognize these residues (i.e. the substrate sites), but this interaction between CheR and an MCP has not been detected to date probably because it is weak and/or transient.

In contrast, binding of CheR to the C-terminal pentapeptide sequence (NWETF) of the two high abundance receptors, the serine chemoreceptor Tsr and the aspartate chemoreceptor Tar, has been well characterized (9–12). However, the low abundance chemoreceptors (the ribose-galactose transducer Trg and the dipeptide transducer Tap) do not have the sequence, indicating that the binding of CheR to the NWETF sequence might not be essential for its catalytic activity itself.

The three-dimensional structure of CheR of S. typhimurium revealed that the monomeric protein consists of two domains (the N-terminal domain with no assigned function and the MTase domain) and one subdomain (the β-subdomain) (13). The co-crystal of CheR and the pentapeptide revealed that the β-subdomain binds to the NWETF sequence (11). Mutagenesis of the NWETF sequence of Tar demonstrated that it binds to CheR mainly through hydrophobic interaction (12). However, it is not clear how the CheR molecule is oriented when it binds to a chemoreceptor, nor how the NWETF sequence is oriented relative to the other part of the chemoreceptor molecule.

Recently, subcellular localization of some proteins involved in chemotactic signal transduction (MCPs and Che proteins) has been studied using immunoelectron and immunofluorescence microscopy and YFP fusion proteins (14–18). These studies demonstrated that MCPs cluster with the histidine kinase CheA and the adaptor protein CheW at cell poles. The localization and the clustering depend, at least to some extent, on CheA and CheW, but not on CheR or CheB. The localization and clustering of the chemotactic machinery at cell poles are proposed to be essential for amplification of input signals and...
for efficient methylation. The latter hypothesis assumes a high local concentration of CheR around the receptor/kinase cluster to provide a molecular basis of efficient methylation of both high abundance and low abundance receptors. Previous studies (10, 12, 19–21) suggest that the NWETF peptide may serve to concentrate CheR around MCPs, but no direct evidence has been obtained.

Moreover, the information about the structure-function relation of CheR was limited although the three-dimensional structure of S. typhimurium CheR has been determined in the absence and presence of the NWETF peptide and the mutagenesis of the cysteine residues of S. typhimurium CheR was carried out. In this study, we took advantage of comparative genomic analysis to identify evolutionary conserved and therefore structurally and functionally important residues in the CheR protein family. Mutagenesis of some conserved residues in E. coli CheR demonstrated that some of them are functionally important. Characterization of GFP-CheR revealed that CheR localizes to cell poles through the interaction between its β-subdomain and the NWETF sequence of the chemoreceptor. Dissulfide cross-linking assay was employed to examine the interaction between CheR and Tar and demonstrated that the positively charged residues in helix α2 of CheR are involved in the recognition of MH1. Thus, CheR interacts with the chemoreceptor through two distinct chemotaxis-specific modules to achieve efficient adaptation.

EXPERIMENTAL PROCEDURES

Data Base Searches and Protein Sequence Analysis—BLAST searches (23) of nonredundant and individual microbial genome data bases at the National Center for Biotechnology Information (Bethesda, MD) were performed with default parameters. Position-specific iterative BLAST searches (23) were performed with defined parameters (BLOSUM62 matrix, an inclusion threshold of E = 0.01 and composition based statistics). Searches were iterated to convergence and repeated with all newly found homologues as queries. CheR and related sequences from unfinished microbial genomes were identified in tBLASTn searches at www.ncbi.nlm.nih.gov/Entrez/genom_table.cgi followed by gene finding and translation using the FramePlot program at www.nih.gov/∼jnci/cgi-bin/frameplot.pl. Profile hidden-Markov-model searches against SMART (24) and Pfam (25) databases were performed with default parameters. Multiple alignments were constructed using the ClustalX program (26). Visualization of the three-dimensional structure of CheR was achieved using a Swiss-PDB viewer constructed using the ClustalW program (27). The dimensional structure of CheR was limited although the three-dimensional structure of S. typhimurium CheR was achieved using a Swiss-PDB viewer constructed using the ClustalX program (26). Visualization of the three-dimensional structure was performed with default parameters. Multiple alignments were performed with all newly found homologues as queries. CheR and related proteins with a core domain corresponding to that of CheR (Fig 1). Searches with the catalytic domain (residues 553–593) was introduced into the vector pMAL-c2. The pC4742-95 strain identified not only homologous CheR proteins from a variety of species, but also several types of multidomain proteins with a core domain corresponding to that of CheR (Fig 1).

Bacterial Strains and Plasmids—All strains used in this work are derivatives of strain MG1655. Strain RP4944 (ΔcheR his::pyrC46 thyA araD139 lacU169 naIa rpsL thi) lacks CheR, and RP58691 (Δtar -7021 sec::Tn10 cheR leuB6 his-4 metF159Am eda-50 rpsL163 thi-1 ara-14 lacYI mit-1 xyl-5 tonA31 tre-76) lacks CheR and Tar (29). Strain HCB436 (Δtar -7021 tar- cheB2234 stry-100 zbd tar thi leu his met rpsL136) lacks CheR and CheR as well as MCPs, and strain HCB1262 (ΔcheA-cheZ::zeor Tar thi leu his met rpsL136) lacks all of the Che proteins involved in general chemotaxis as well as MCPs. Plasmids used in this study are listed in Table I. Plasmid pHrChB1 (Invitrogen) carries the trc promoter, the lacY and bla genes. Plasmid pBAD24 (31), which was provided by J. Beckwith, carries the araBAD promoter, the araC gene encoding the positive and negative regulator, and the bla gene. Plasmid pMAL-c2 (New England Biolabs) carries the mutant malE gene, which encodes the mature maltose-binding protein (MBP) without its leader sequence, under the control of the tac promoter, the lac promoter, and the bla gene. Plasmid pACYC184 and its derivatives were used to clone the gene. The pSU21-based plasmid pBAD24 was used as the wild-type cheR plasmid. The pACYC184-based plasmid pACYC184 was used as the wild-type tar gene under control of the nahG promoter. Plasmid pEGFP (CLONTECH) encodes the enhanced green fluorescent protein.

Site-directed mutagenesis of the cheR and tar genes was performed according to the two-step PCR method (34) using primers synthesized by Life Tech Oriental (Tokyo) and Pyrobest DNA polymerase (Takara Shuzo, Kyoto). For the tight regulation of CheR proteins (i.e., CheR, HisA-CheR, and GFP-CheR proteins), the coding regions were placed downstream of the araBAD promoter on vector pBAD24. For the expression of MBP-Tar, the 3′ region of tar (encoding the cytoplasmic domain, i.e., residues 215–553) was introduced into the vector pMAL-c2.

Swarm Assay of Chemotaxis—Swarm assays were performed essentially as described previously (10) using tryptone semisolid agar supplemented with appropriate antibiotics and, when necessary, with various concentrations of arabinose and fucose.

Analysis of Receptor Methylation by Immunoblotting—Receptor methylation was monitored by immunoblotting as described previously (10) with anti-Tsr serum (38), which cross-reacts with Tar, and alkaline phosphatase-conjugated anti-rabbit IgG (Vector Laboratories) as the first and the second antibodies (at the dilution of 1:3000).

Observation of Subcellular Localization of GFP-CheR—HCB436 cells expressing any of the HisA-tagged CheR proteins were suspended in 2 ml of lysate buffer (50 mM NaHPO4 (pH 8.0), 300 mM NaCl, and 10 mM imidazole) containing 10 mM dithiothreitol, and sonicated in short pulses (10 s each) on ice. After low speed (17,000 × g at 4 °C for 10 min) and high speed (100,000 × g at 4 °C for 30 min) centrifugation, the resulting supernatant was applied to a nickel-nitrotriacetic acid–agarose column (Qiagen). Each HisA-tagged CheR protein was eluted with 250 mM imidazole. HCB1262 cells expressing MBP-Tar-E308C were suspended in 2 ml of column buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA) containing 10 mM dithiothreitol, and sonicated in short pulses on ice. After low speed and high speed centrifugation, the supernatant was applied to the amylase column (New England BioLabs). MBP-Tar-E308C was eluted with 10 mM maltose. Each HisA-tagged CheR protein and MBP-Tar-E308C were mixed and incubated at 37 °C for 20 min in the presence or absence of 0.1 mM IPTG. The mixture was divided into two aliquots, which were boiled at 100 °C for 3 min with or without 2-mercaptoethanol and were subjected to SDS-PAGE followed by immunoblotting with anti-CheR serum (12), at a dilution of 1:2000, or anti-MBP serum (New England BioLabs) at a dilution of 1:10000. The first antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG (New England BioLabs) at the dilution of 1:30000 and with an ECL detection kit (Amersham Biosciences).

RESULTS

Comparative Protein Sequence Analysis of the CheR Protein—Initial analysis of the domain architecture of the CheR protein identified not only homologous CheR proteins from a variety of species, but also several types of multidomain proteins with a core domain corresponding to that of CheR. (Fig 1). Position-specific iterative BLAST searches with the N-terminal domain (residues 1–90 of S. typhimurium CheR (13) retrieved only corresponding domains from homologous CheR proteins from bacterial and archaeal species, indicating that this domain is present exclusively in chemotaxis-related MTases (Figs. 1 and 2A). Searches with the catalytic domain (residues 91–286) retrieved sequences of nonchemotactic AdoMet-dependent MTases, including DNA MTases and RNA dimethyltransferases, from both prokaryotes and eukaryotes (Figs. 1 and 2B); hundreds of functionally divergent MTases were identified in the third and fourth iterations with a high degree of statistical significance (expectation value E ranged from 10−4 to 10−7).

Domain organization of all types of MTases retrieved during the data base searches is shown in Fig. 1. FzrF of Myxococcus xanthus, a known functional CheR homolog (39), contains sev-
eral tetratrico peptide repeats in its C terminus. Tetratrico peptide repeats is thought to facilitate protein-protein interactions in various signal transduction proteins (40, 41). An unusual fusion protein containing catalytic domains of methylesterase and MTase as well as several PAS domains that may also facilitate protein-protein interactions (42) have been identified in several proteobacterial species, e.g. in Sinorhizobium meliloti (Fig. 1). Finally, a CheR-like protein from spirochetes lacks the N-terminal domain of a classical CheR, but instead has a CheW domain in its N terminus. The CheW protein is known to directly interact with MCPs (43).

Multiple sequence alignments of the N-terminal CheR-specific domains (Fig. 2A) revealed a conservation pattern centered on hydrophobic and turn-like residues comprising /H9251-helices. Residue Gly-39, which determines a crucial turn following helix /H92511, is strictly conserved in all CheR homologs. The only two other positions where strict conservation occurs are positively charged residues Lys/Arg-46 and Arg-53 of helix /H92512. Such strict conservation of a positive charge across long evolutionary distances clearly indicates functional significance, especially for Arg-53.

Catalytic domains from most AdoMet-dependent MTases, including CheR, share a signature sequence (G/A)X(G/A/S)XG (Fig. 2B) involved in the binding of AdoMet (44). Interestingly, the β-subdomain is present in all CheR homologs and CheR-related proteins, such as PilK, FrzF, CheW3, and Smb20515, but is missing from all nonchemotactic single-domain MTases, such as COQ3 (Figs. 1 and 2B). This finding is consistent with the functional role of the β-subdomain, i.e. interaction with terase and MTase as well as several PAS domains that may also facilitate protein-protein interactions (42) have been identified in several proteobacterial species, e.g. in Sinorhizobium meliloti (Fig. 1). Finally, a CheR-like protein from spirochetes lacks the N-terminal domain of a classical CheR, but instead has a CheW domain in its N terminus. The CheW protein is known to directly interact with MCPs (43).
FIG. 2. Multiple sequence alignments of the CheR-specific N-terminal domain (A) and a portion of the MTase (catalytic) domain including the β-subdomain (B). Each sequence is identified by the NCBI gene identification number or by the sequencing center preliminary identification number (shown in the last column). The secondary structure (β for β-sheets and H for α-helices) shown above the alignments is based on the known three-dimensional structure of Salmonella typhimurium CheR. The signature sequence involved in binding to AdoMet (44) is indicated by three stars, and a critical histidine residue (His-192) in the β-subdomain is highlighted in black (panel B). Conserved amino acid residues are
MCPs (11). CheR proteins from all chemotactic species retain the β-subdomain, although in many species all MCPs lack the NWETF-type sequence (data not shown). On the other hand, residues His-192 and Arg-197 of S. typhimurium CheR, which interact with the Trp residue of the NWETF pentapeptide (11), and residue Arg-187, which forms a salt bridge with the Glu residue of the pentapeptide (11), are absent from many CheR homologs (Fig. 2B).

**Mutagenesis of Some Conserved Residues in E. coli CheR**—We replaced some conserved residues of E. coli CheR by Ala or Glu. The resulting mutant proteins were expressed in strain RP4944 (∆CheR) at levels similar to that of wild-type CheR (data not shown). Swarming abilities of these mutants were examined (Fig. 3A). RP4944 cells expressing either MTase domain mutants (R98A or D154A) failed to swarm, as expected. Among the β-subdomain mutations, H192A, R197A, and H192A/R197A significantly slowed the swarming rate. The defects in swarming were suppressed by the overproduction of these three mutant CheR proteins (data not shown). Thus, these three mutations may decrease the affinity of CheR for an MCP, consistent with the crystallographic study (11), which suggested that His-192 and Arg-197 interact with the Trp residue of the pentapeptide sequence. In contrast, R187A and R187E did not affect the ability of CheR to support swarming. Residue Arg-187 is suggested to form a salt bridge with the Glu residue of the NWETF sequence (11), but the E551A mutation of E. coli Tar had little effect on its methylation (12). Taken together, the salt bridge, if it is formed, may not play a critical role in the CheR function in vivo. Among the N-terminal domain mutations, V50A and L54A had no effect, but R53A and Δ1–89, in which residues 1–89 were deleted, impaired swarming, fully supporting predictions that resulted from our in silico analysis.

Next, we examined methylation levels of Tar in RP8691 (ΔTar ∆CheR) cells expressing the mutant CheR proteins by immunoblotting with anti-Tar, which cross-reacts with Tar (Fig. 3B). In RP8691 cells carrying the vector, methylation of Tar was hardly detected in the absence of aspartate but was slightly enhanced by the addition of aspartate, suggesting that the host strain has a residual activity of CheR. In the absence of aspartate, the methylation level of Tar in RP8691 cells expressing wild-type CheR was not much different from that of cells carrying the vector, but was greatly increased by the addition of aspartate, as expected. The MTase domain mutant (CheR-D154A) did not show any methylating activity, nor did the N-terminal domain mutant (CheR-R53A). Among the β-subdomain mutants, CheR-R197A and CheR-H192A/R197A were slightly impaired in the methylation activity. These results are consistent with those of the swarming assay (Fig. 3A).

**Subcellular Localization of GFP-CheR**—Previous studies demonstrated that MCPs together with CheA and CheW form clusters and localize to cell poles (14, 16, 17), and that CheY and CheZ also localize to cell poles in the presence of MCPs (18). CheR may also target to the receptor/kinase cluster. To visualize subcellular localization of CheR, we constructed a plasmid encoding GFP-CheR. Swarming ability of RP4944 cells expressing GFP-CheR was similar to those of cells expressing CheR (data not shown). We then examined subcellular localization of GFP-CheR in the presence of 1 mM arabinose (Fig. 4).

With the lower concentrations of arabinose, the fluorescence bleached quickly, but arabinose concentration did not seem to affect localization of GFP-CheR (data not shown). In HCB436 (ΔMCPs ΔCheR ΔCheB) cells, GFP-CheR localized to cell poles in the presence of wild-type Tar, but not in the presence of the mutant Tar protein lacking the C-terminal NWETF sequence (Fig. 4, left panels). These results demonstrate that CheR is targeted to the MCP cluster through its binding to the NWETF sequence.

We next examined whether mutations in the CheR part of GFP-CheR affect its localization. The three severe mutations characterized above (R53A, D154A, and H192A/R197A), each representing one of the three domains, were tested. GFP-CheR with the D154A mutation (in the catalytic domain) localized to cell poles (Fig. 4, right middle panel) although the D154A mutation impairs the MTase activity (see Fig. 3, A and B).
Receptor Recognition by Methyltransferase

GFP-CheR with the H192A/R197A mutation (in the β-subdomain) did not localize to cell poles (Fig. 4, right lower panel), supporting the previous conclusion that the mutation may decrease the affinity of CheR for the NWETF sequence. Interestingly, GFP-CheR with the R53A mutation (in the N-terminal domain) localized to cell poles (Fig. 4, right upper panel), although the mutation severely impaired the CheR activity (Fig. 3, A and B). These results suggest that the targeting of CheR to cell poles does not depend on the MTase activity and the function of the N-terminal domain of CheR, but depends primarily on the binding of the β-subdomain of CheR to the C-terminal NWETF sequence of high abundance MCPs.

Mutagenesis of Positively Charged Residues in Helix α2 of CheR—The R53A mutation severely impaired the CheR function without affecting its subcellular localization. However, cells expressing CheR-R53A produced larger swarm with increasing levels of expression (Table II), raising a possibility that residue Arg-53 is not directly involved in the catalytic activity. Helix α2 of CheR that contains Arg-53 has a face with many positively charged residues (Fig. 5, left) that constitutes a part of the “receptor interaction opening” together with the active center and the β-subdomain (13). On the other hand, the first methylation helix (MH1) of Tar that contains three of the four methylation sites has many residues with negatively charged or polar side chains (Fig. 5, right). Therefore, it is reasonable to speculate that the positively charged face of helix α2 may interact with the negatively charged face of MH1. To examine this possibility, we substituted Ala or Glu for some of the positively charged residues in helix α2 of CheR (K46A/E, R53A/E, and R57A/E).

Swarming abilities of RP4944 cells (ΔCheR) expressing the mutant CheR proteins were examined (Table II). Expression of wild-type CheR with 0.1 mM arabinose and 0.1 mM fucose resulted in the largest swarm ring. Overproduction of CheR impaired swarming presumably because overmethylation of MCPs caused tumbling-biased swimming and/or the titration of AdoMet by excess CheR impaired cell growth. Cells expressing CheR-K46A or R53A required higher concentrations of arabinose for swarming than cells expressing wild-type CheR. As the concentration of arabinose increased, the former cells swarmed faster. However, cells expressing CheR-K46E or R53E failed to swarm even in the presence of higher concentrations of arabinose. Thus, a Glu substitution seems to be more severe than an Ala substitution. Overproduction of CheR-R57A or R57E impaired swarming presumably because these proteins have weak activities or are unstable. These results suggest that the positive charges of helix α2 are important for the CheR function. Especially, Arg-53 seems to be critical, which is consistent with the results described above (Fig. 3).

Disulfide Cross-linking between CheR and MH1 of Tar—To detect directly the interaction between CheR and MH1 of Tar...
that had been eluded from conventional biochemical assays, we employed a disulfide cross-linking assay. Wild-type CheR has two Cys residues (Cys-7 and Cys-229), but consistent with previous reports (22, 45), the substitution of Ser (C7S/C229S) had little effect on CheR function (data not shown). Cys-scanning mutagenesis was carried out for residues (Lys-46 and Arg-53 through Ser-60) in helix of His6-tagged Cys-less CheR (named His6-CheR-CS). All of the resulting proteins were functional, but the L54C and R57C versions were not used in the following assay because of their low yields (data not shown).

Residue Glu-308 (underlined) of E. coli Tar lies in the consensus sequence around methylation sites (bold) ((E/Q)(E/Q)XXA/S/TX) (46). This position was used as a target for cross-linking with CheR. A cytoplasmic fragment (residues 215-553) of the deamidated (EEEE) or the amidated (QQQQ) derivative of Tar-E308C was fused to the cytoplasmic version of the maltose-binding protein (named MBP-Tar-EEEE or QQQQ) (46). Gln residues are known to mimic methylated Glu residues. RP437 (wild type for chemotaxis) cells expressing each MBP-Tar protein failed to swarm (data not shown), a dominant negative effect that suggests that Tar fragments are correctly folded to interact with some Che proteins.

We first identified the Tar-CheR cross-linked product. MBP-Tar-EEEE-E308C (about 79 kDa) was mixed with His6-CheR-CS-R53C (about 34 kDa) in the presence of 0.1 mM I$_2$ (an oxidant) and detected by immunoblotting with anti-CheR and anti-MBP sera (Fig. 6A). The R53C protein, but not its Cys-less parental protein, gave a Tar-CheR cross-linked product with an apparent molecular mass a little higher than expected (about 175 kDa).  

**Fig. 6.** Disulfide cross-linking between CheR and Tar. A, disulfide cross-linking between His$_6$-CheR-CS-R53C (lanes labeled with R53C) and MBP-Tar-EEEE-E308C with (lanes labeled with +) or without (lanes labeled with −) an oxidant (0.1 mM I$_2$). The Cys-less parental protein His$_6$-CheR-CS (lanes labeled with CS) was also tested as a control. B, disulfide cross-linking between the Cys-replaced derivatives of His$_6$-CheR-CS-CheR and MBP-Tar-EEEE-E308C. Lane 1, His$_6$-CheR-CS; lane 2, -K46C; lane 3, -R53C; lane 4, -V55C; lane 5, -R56C; lane 6, -L58C; lane 7, -R59C; lane 8, -S60C; lane 9, -K46C/R53A. C, effect of amidation of Tar on its interaction with CheR. The K46C, R53C, or S60C derivatives of His$_6$-CheR-CS were mixed with the EEEE (lanes labeled with E) or QQQQ (lanes labeled with Q) version of MBP-Tar-E308C. The samples were subjected to SDS-PAGE followed by immunoblotting with anti-CheR or anti-MBP serum as indicated.
CheR (Ref. 11 and this study). The positively charged face in helix 2 and an MH is predicted to be weakly cross-linked to Tar (lane 2). The cross-linking of the L58C protein to Tar was only barely detectable and the V55C protein was much less effectively cross-linked to Tar than the K46C, R56C, R59C, and S60C proteins (lanes 2 and 4–8). The R53C protein was not effectively cross-linked to Tar (lane 3) presumably because Arg-53 is particularly important to recognize MH1. Indeed, the introduction of the R53A mutation into the K46C protein reduced the efficiency of cross-linking (compare lanes 2 and 9). These results suggest that the Cys residues in the positively charged face of α2 are more efficient for cross-linking to Tar than those in the opposite face and that the cross-linking reflects the ability of CheR to recognize MH1.

We also examined the effect of amidation of Tar on cross-linking with CheR. The His8-CheR-CS proteins with K46C, R53C, or S60C were mixed with the EEEE or QQQQ version of MBP-Tar-E308C (Fig. 6C). The K46C protein was effectively cross-linked to MBP-Tar-E308C regardless of its amidation state. However, the R53C and S60C proteins were less effectively cross-linked to the amidated (QQQQ) fragment than to the deamidated (EEEE) one. This result reinforces the electrostatic nature of the interaction between MHs of CheR and helix α2 of CheR.

**DISCUSSION**

In this study, we examined how the MTase CheR interacts with its substrate, i.e., the chemoreceptor (MCP). More specifically, we asked how CheR is targeted to the receptor/kinase clusters at cell poles and how CheR recognizes the methylation sites of the chemoreceptor. We found that these two processes result from the distinct functions of CheR that reside in its two distinct domains.

Comparative protein sequence analysis of CheR homologs and related proteins revealed that all of the CheR proteins have two domains and one subdomain: the CheR-specific N-terminal domain, the catalytic domain, and the β-subdomain. This organization is consistent with the crystallography of *S. typhimurium* CheR, suggesting that all of the CheR proteins share a common three-dimensional structure. It is also possible that they share common basic mechanisms for catalysis and substrate recognition. However, MCPs of many bacteria lack the C-terminal NWETF-type sequence that serves as a primary binding site of CheR in *E. coli* and *S. typhimurium* although all of the CheR proteins have the β-subdomain, which binds to the NWETF sequence in the case of *S. typhimurium* and *E. coli* CheR (Ref. 11 and this study). The β-subdomain can be divided into two groups (11) (Fig. 2B): longer β-loops (e.g. *E. coli*, *S. typhimurium*, and *S. meliloti*) and shorter β-loops (e.g. *Vibrio cholerae* and *Bacillus subtilis*). The CheR proteins of the bacteria whose MCPs contain the C-terminal NWETF-like motif belong to the former group. This difference in the length of the β-loop might reflect the differences in the mode of receptor recognition by CheR.

In contrast to the recognition of the C-terminal tail of MCPs, little was known about the recognition of the methylation sites of MCPs by CheR. X-ray crystallography raised a possibility that the positively charged face of helix α2 of CheR might be involved in the interaction with MCPs (13). However, such an interaction had not been detected biochemically. Here, the mutagenesis and the disulfide cross-linking analyses indicated that the positively charged face in helix α2 of CheR is involved in the recognition of MH1 of Tar. This is the first direct demonstration of the interaction between CheR and a methylation helix of any MCP. Among the residues tested, Arg-53 seems to be the most important residue for the recognition of MH1. This residue is strictly conserved among all of the CheR proteins except for the *Campylobacter jejuni* homolog, in which the corresponding residue is Lys.

Thus, effective methylation requires two types of interaction between CheR and MCPs: one between the β-subdomain and the NWETF sequence (for the targeting of CheR to cell poles) and the other between the positively charged face in helix α2 and the negatively charged face of an MH (for the recognition of substrate sites) (Fig. 7). An *E. coli* cell expresses some 5,000 monomers of MCPs and only several hundred molecules of CheR (47). Therefore, the targeting of CheR to the C-terminal tail of MCPs may be required to concentrate CheR molecules around receptor/kinase clusters at cell poles. Increased probability of CheR to collide with MCP molecules may then allow it to interact with the negatively charged face of an MH. This interaction between helix α2 and an MH is predicted to be weak and/or transient. CheR might slide on the negatively charged face of MH1, which contains three methylation sites with intervals of two turns of the helix, to monitor the methylation sites.

It is still unknown how CheR is oriented when it binds to an MCP. The binding of the NWETF sequence to the β-subdomain was visualized by x-ray crystallography (11). However, it is unclear how the rest of the MCP molecule is oriented and whether CheR can catalyze methylation of an MCP molecule while it is anchored to the C-terminal tail of the same molecule or the partner subunit of the same dimer. It was also suggested that CheR can catalyze methylation of a neighboring MCP molecule within an MCP cluster (19, 20). This interdimer methylation explains why a low abundance MCP can be methylated efficiently in the presence of a high abundance MCP (48). Again, it is not clear whether this can be achieved without dissociation of CheR from the NWETF sequence. In any case, the
simultaneous anchoring and catalysis would require large flexibility of the MCP molecule. Because the affinity of CheR for the pentapeptide is not very high, it is also possible that CheR is dissociated from the NWETF sequence during catalysis.

We also examined subcellular localization of CheR. GFP-CheR localized to cell poles only in the presence of an MCP with the NWETF sequence. The mutagenesis of the CheR part suggested that the targeting of a CheR to cell poles depends primarily on the interaction between CheR and the NWETF sequence. These results are consistent with the hypothesis that the NWETF sequence serves to concentrate CheR around MCPs at cell poles. It should be noted that the abilities of the wild-type and mutant versions of GFP-CheR to localize to cell poles (Fig. 4, right panels) appeared to vary: wild-type > R53A > D154A. In the experimental conditions applied, the GFP-CheR proteins were mildly overproduced relative to chromosome-encoded CheR. Therefore, the methylation level of Tar would be different from one strain to another. This may suggest two possibilities: the methylation levels of MCPs might affect the subcellular localization of (i) CheR and/or (ii) MCPs themselves. The high abundance MCPs (Tar and Ter) localize to cell poles and form clusters with CheA and CheW, whereas the low abundance MCPs (Tap and Tryg) also localize to cell poles but do not form a cluster (16). The latter receptors are poor substrates for CheR because they lack the C-terminal NWETF sequence. Taken together, it is possible that the methylation levels of MCPs would be critical for their clustering at cell poles. However, the polar localization of the high abundance MCPs does not seem to require CheR and CheB (15). Further analyses are required to clarify this issue.

The Cys-substituted CheR proteins were more effectively cross-linked to Tar-EEEE than to Tar-QQQQ. This may result from the electrostatic nature of the interaction between MHs of MCPs and helix a2 of CheR. It is also possible that receptor amidation (and hence methylation) alters the conformation of the MHs to reduce its affinity to helix a2 of CheR. In any case, this finding is consistent with the notion that upon methylation, an MCP becomes a poorer substrate of CheR.

CheB also binds to the C-terminal NWETF sequence of MCPs (49, 50) and has to recognize MHs of MCPs. However, CheB may be different from CheR in these respects. The affinity of CheB for the pentapeptide is much lower than that of CheR and the cellular concentration of CheB is much higher than that of CheR. Therefore, it is hard to imagine that the NWETF sequence serves to recruit CheB around MCPs. As for the substrate recognition, CheR recognizes methylated Glu residues to hydrolyze them, whereas CheB recognizes unmethylated Glu residues. Consistent with this consideration, E. coli CheB does not have a positively charged cluster in the primary sequence. Thus, it is intriguing to compare the mechanisms of receptor recognition of CheB with those of CheR.

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