Mutations Leading to Altered CheA Binding Cluster on a Face of CheY*

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CheY is the response regulator of Escherichia coli chemotaxis and is one of the best studied response regulators of the two-component signaling system. CheY can receive phosphate from the histidine kinase, CheA. Phospho-CheY interacts with the motor-switch complex to induce clockwise flagellar rotation, thus causing the cell to tumble. We used an enzyme-linked immunosorbent assay to study the direct interaction between the kinase, CheA, and the regulator, CheY. The products of random, suppressor, and site-specific cheY mutants were assayed for their ability to bind CheA. Nine mutants showed altered binding. We sequenced and mapped these point mutations on the crystal structure of CheY, and a high degree of spatial clustering was revealed, indicating that this region of CheY is involved in CheA binding. Interestingly, five of these altered binding mutants were previously defined as being involved in motor-switch binding interactions. This suggested a possible overlap between the motor-switch binding and CheA binding surfaces of CheY. Using CheY (Trp-58) fluorescence quenching, we determined the equilibrium dissociation constants of CheA (124–257) binding for these CheY mutants. The results from the fluorescence quenching are in close agreement with our initial enzyme-linked immunosorbent assay results. Therefore, we propose that the CheA and the motor binding surfaces on CheY partially overlap and that this overlap allows CheY to interact with either the CheA or the flagellar motor, depending on its signaling (phosphorylation) state.

Bacterial response to hostile environmental conditions is regulated by a complex network of interacting proteins, with the most predominant interactions being generated by members of two-component systems (1). In Escherichia coli chemotaxis, the interaction between the receptor-coupled histidine kinase, CheA, and the response regulator, CheY, controls the bacterial response to chemical environmental changes (1–4). In response to changes in the receptor’s occupancy and adaptation, CheA autophosphorylates (5, 6) and subsequently transfers its phosphate to either CheY or CheB (7, 8, 15). This phosphoryltransfer results from CheY’s intrinsic autophosphorylation activity and is not due to catalysis by CheA (9, 10). Studies indicate that phospho-CheY is the activated form that binds to a motor-switch complex, causing a clockwise flagellar rotation and a net change in the bacterial swimming direction (11–13).

Allosteric changes within a large complex containing a receptor dimer, a CheA dimer, and two copies of a small coupling protein, CheW, regulate CheA autophosphorylation and subsequent phosphotransfer to CheY (11, 14, 22, 23). The autophosphorylation site, His-48, lies on the N terminus of CheA, and the CheY binding determinants lie between residues 124 and 257, commonly known as the P2 domain (17). However, the CheA binding site on CheY is not defined. Structural studies indicate that CheY is a single domain protein that folds into a (ββα)5 topology, with five β-strands forming the hydrophobic core, surrounded by five α-helices (18, 19). Three aspartate residues, Asp-12, -13, and -57 form the molecule’s active site, with Asp-57 being the site where CheY receives the phosphate group from CheA His-48 (20, 21). Phosphorylated CheY loses its affinity for CheA (23) and shows high binding affinity for FliM, one of the motor-switch components (13).

It has been estimated that E. coli contains about 50 homologous two-component systems, which govern various cellular responses to stress (1, 3). Since the active site of response regulators is highly conserved (1), CheA must be able to differentiate CheY from other potential response regulators. Very recently, an NMR study on the interaction between an N-terminal fragment of CheA (1-233) and CheY has defined the CheA (1-233) binding site on CheY by measuring the chemical shift changes in CheY upon CheA (1-233) addition (26). Residues identified here lie distinct from the active site on α-4, β-4, α-5, and β-5 of CheY.

In the present study, we have employed a CheY-CheA binding assay, based on the ELISA, to characterize, in vitro, the interaction between full-length CheA and CheY and to screen CheY mutants for possible defects in CheA binding. The mutants that altered the CheA interaction had amino acid positions clustered on a face of the CheY protein, and this three-dimensional clustering suggested that this region of CheY is involved in CheA binding.

MATERIALS AND METHODS

Chemicals—Bacto-Tryptone, Bacto-Agar, and Bacto-yeast extract were from Difco. Molecular biology grade agarose was from International Biotechnologies, Inc. (New Haven, CT). Penicillin, ampicillin, hydroxylamine hydrochloride, 3-β-indoleacrylic acid, isopropanol-β-o-thiogalactoside, and anti-mouse IgG (Fc specific) were obtained from Sigma. Sequenase version II was obtained from U. S. Biochemical Corp.

Bacterial Strains and Media—Table I lists all bacterial strains and plasmids used in this study. Cultures were routinely grown in Luria broth (1% tryptone, 1% NaCl, and 0.5% yeast extract) containing penicillin (100 μg/ml). Screening of CheY mutants and swarm tests were performed on tryptone swarm agar plates (1% tryptone, 0.5% NaCl, and 0.3% agar, containing penicillin).

Overexpression and Purification of Proteins—Strains containing various vectors were grown in Luria broth at 37°C (30°C for CheY) to

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1 The abbreviation used is: ELISA, enzyme-linked immunosorbent assay.
mid-exponential phase, induced appropriately for 4–16 h, harvested, and sonicated to obtain homogenate. The homogenate was then centrifuged and the resulting supernatant was used in the binding assay.

The supernatant fraction was used as a source of CheY. The binding assay was performed by incubating microtiter plate wells with serially diluted rabbit anti-CheY antibody. The optical density was read at 405 nm in an ELISA reader.

The plasmid pRL22AZ was mutagenized using hydroxylamine mutagenesis (29). The mutagenized plasmid DNA was transformed into competent RP4079 cells, and transformants were plated by mixing with a tryptone swarm agar. After overnight incubation at 30°C, Che protein colonies appeared as miniswarms. Colonies exhibiting no swarm or miniswarm were picked as mutants, and their mutant phenotype was confirmed by transforming a fresh batch of RP4079 competent cells with DNA isolated from each of the colonies.

In Vitro Mutagenesis of the cheY Gene—The plasmid-carrying cheY gene (pRL22AZ) was mutagenized using hydroxylamine mutagenesis (29). The mutagenized plasmid DNA was transformed into competent RP4079 cells, and transformants were plated by mixing with a tryptone swarm agar. After overnight incubation at 30°C, Che bacterial colonies appeared as miniswarms. Colonies exhibiting no swarm or miniswarm were picked as mutants, and their mutant phenotype was confirmed by transforming a fresh batch of RP4079 competent cells with DNA isolated from each of the colonies.

DNA Sequencing—Double-stranded high copy number plasmid pRL22AZ, bearing putative cheY mutations, was DNA sequenced by a dideoxy chain termination method, using the protocol provided by the manufacturer of Sequenase, version 2.0 (U. S. Biochemical Corp.). Sequencing was facilitated by three appropriately situated, non-coding strand oligonucleotide primers that were specific for CheY.

Binding Assay—The binding assay was performed by coating microtiter plate wells with 100 μl of 2-fold, serially diluted rabbit anti-CheY antibody in 50 mM Tris, pH 7.9, and stored at –70°C until sonicated. Overexpressing lysates were obtained by thawing and centifuging them at 30,000 x g for 20 min. The supernatant fraction was used as a source of CheY. The binding assay was performed by incubating microtiter plate wells with serially diluted rabbit anti-CheY antibody. The optical density was read at 405 nm in an ELISA reader.

Detection of CheY-CheA Binding with ELISA—Based on ELISA, a sensitive and specific CheY-CheA binding assay was developed, which used a CheY-CheA complex as the antigen sandwiched between an anti-CheY antibody and an anti-CheA monoclonal antibody. This whole complex was detected by mouse-IgG-specific antibody conjugated with alkaline phosphatase (Fig. 1). Since the assay was done in microtiter plates,
it required a very small amount of the sample, and coating the microtiter wells with polyclonal rabbit anti-CheY antibody provided a simple means to affinity purify and concentrate the CheY from an overexpressed cell lysate (see "Materials and Methods").

The assay was performed using cell lysates overexpressing CheY and CheA/CheW. To measure background activity, lysates with either no CheY or a CheY deletion (Δ18–28) were used. As seen in Fig. 2, the wells containing wild-type CheY produced stronger signals than the negative controls, with the signal to noise ratio being at least 2 to 1. The signal obtained from the negative controls was due to a cross-reactivity of the anti-CheY antibody to CheA, which could not be completely eliminated. The mouse monoclonal antibody to CheA and the class-specific anti-mouse antibody did not cross-react with CheY (data not shown).

Isolation and Screening of CheY Mutants Resulting in Altered CheA Binding—65 random CheY mutants, generated by hydroxylamine mutagenesis, 6 CheY suppressors to motor-switch mutants, and 2 site-specific mutants, D13K and Y106W, were examined for CheA binding. 9 of these mutants exhibited altered binding properties, with 8 of the 9 showing decreased binding and 1 showing a slight increase in binding (Fig. 3). DNA sequencing revealed that all the random mutants, with altered CheA binding, carried a single-point mutation in the cheY gene (data not shown). The majority of the CheY mutants screened by ELISA demonstrated CheA binding similar to that of the wild-type CheY.

Mapping of Altered Binding CheY Mutants on the CheY Structure—Red atoms in Fig. 4 depict the positions on CheY where mutant CheY residues result in altered CheA binding. They cluster to a region, distinct from the active site on the CheY surface, and this high degree of clustering suggests that it is the CheA binding surface. The CheY mutants that do not alter CheA binding were not found on this proposed surface (Fig. 4, green atoms). This proposed CheA binding surface consists of solvent-accessible surfaces of the C terminus of β-4 (Thr-87), loop region between β-4 and α-4 (Ala-90, Glu-93), β-5 (Tyr-106, Val-108), the loop between β-5 and α-5 (Phe-111, Thr-112), and α-5 (Glu-117). Interestingly, several of these residues (Fig. 4, covered by stippling) were previously implicated (25) in motor-switch interactions. This identified a surface common to both interactions. All of these residues are surface located and solvent accessible. Their side chains, with the exceptions of Thr-87 and Phe-111, extend out to the surrounding solvent, toward a region where it can easily make contact with CheA. Asp-13 is not located on this proposed surface, and it seems possible that the altered CheA binding properties of the mutant D13K may be due to an altered conformation (see "Discussion").

The substitutions that affected binding also make sense chemically in the context of an altered protein-interaction surface. For example, the two glutamate-to-lysine substitutions (E93K, E117K) involve charge changes that could affect the electrostatic interactions between the interacting proteins.
The other substitutions involve side group volume changes (A90V, Y106W, V108M) as well as the hydrophobicity changes (T87I, T112I). Among these residues, only Thr-87 and Phe-111 have their side chains directed in toward CheY's hydrophobic core, and in these cases, only the backbone portion of these residues seems to contribute to the proposed CheA binding surface, so the influence of the side chain may be more indirect.

**Determination of Binding Affinities Using Trp-58 Fluorescence Quenching**—Tryptophan fluorescence quenching was used to quantitate the CheY mutant affinities for the CheY binding region of CheA. The P2 region of CheA has been shown to contain the major binding determinants for CheY interaction (16, 17). The P2 fragment is devoid of Trp and has been shown to quench the fluorescence of CheY, Trp-58, upon binding (26). Equilibrium dissociation constants ($K_D$) determined by fluorescence quenching of CheY (Table II) were found to be inversely correlated to the binding determined by ELISA (Fig. 3). For example, T87I showed slightly higher binding in the ELISA method and, as expected, 2-fold lower $K_D$. Whereas, E93K, Y106W, V108M, F111V, T112I, and E117K showed decreased binding in the ELISA method and severalfold increased $K_D$ values (Table II). D13K and A90V show no changes in their P2 binding affinity as determined by fluorescence quenching. Although it is known that D13K is defective in Mg$^{2+}$ binding (31) and that Mg$^{2+}$ binding quenches the Trp fluorescence (30), assaying P2 binding to D13K may not be comparable to the

**Table II**

| Protein | $K_D$ (μM) |
|---------|------------|
| Wild-Type | 1.2 ± 0.08 |
| D13K    | 1.3 ± 0.32 |
| T87I    | 0.7 ± 0.09 |
| A90V    | 1.4 ± 0.11 |
| E93K    | 4.8 ± 0.53 |
| Y106W   | 4.0 ± 0.60 |
| V108M   | 8.1 ± 0.71 |
| F111V   | 10.0 ± 0.76 |
| T112I   | 3.1 ± 0.30 |
| E117K   | 4.0 ± 0.21 |

Fig. 4. Stereographs showing the locations of some cheY mutations on the CheY (an α5β5 protein) structure (18). Highlighted residues are as follows: red atoms represent residues where mutation affects CheA binding, and green atoms represent residues where mutation does not affect CheA binding. The α-carbon backbone of CheY is in white. White stippling depicts solvent-accessible surfaces of the residues that were implicated in motor-switch binding (25). The top and bottom pictures are two different views, 90° apart from each other.
wild type or other mutants, it is also possible that the residues Ala-90 and Asp-13 may be critical for the intact CheA binding but not for the P2 binding.

**DISCUSSION**

The goal of this study was to define the CheA binding face of CheY. We screened cheY mutants for altered CheA interaction, using a modified version of the ELISA (32), which is a specific, economic, and sensitive assay. A technical problem with this method was a reduced signal to noise ratio caused by a high background. This was determined to be due to the cross-reactivity of the polyclonal anti-CheY antibody to CheA. Affinity purification of the CheY antibody on a CheY column reduced the activity of the polyclonal anti-CheY antibody to CheA. Affinity purification of the CheY antibody on a CheY column reduced the activity of the polyclonal anti-CheY antibody to CheA. Affinity purification of the CheY antibody on a CheY column reduced the activity of the polyclonal anti-CheY antibody to CheA. Affinity purification of the CheY antibody on a CheY column reduced the activity of the polyclonal anti-CheY antibody to CheA.

Use of this assay enabled us to identify nine point mutants, D13K, T87I, A90V, E93K, Y106W, V108M, F111V, T112I, and E117K, which showed altered CheA binding. The most striking result is their location on the CheY molecule and the fact that they clearly cluster on the face of CheY. This clustering becomes functionally more significant, since many of these residues were unique to each study. In our study, the mutagenesis may not have been saturated, despite the recurrence of T112I, and since we screened for total non-chemotaxis, it is possible that we might have missed some mutants that had reduced binding and partial chemotaxis function. In the NMR study, chemical shift changes in the backbone amide residues were measured, and only those chemical shift changes greater than 60 Hz were considered significant. It is possible that some residues, indicated in this study, may affect the binding through their solvent-accessible side chains but do not result in ΔHz greater than 60. The side chain of Phe-111 is buried inside CheY’s hydrophobic core (18) and may seem to be an exception, but NMR data show that Phe-111 shifts upon adding CheA(1-233), although the observed chemical shift change is less than 60 Hz. Since CheA has been shown to bind to CheY as a dimer (23), some of the residues we identified, which are not indicated by NMR studies, may be critical for CheA dimer binding.

The D13K mutant is a dominant tumbling mutation, also found to be defective in CheA binding. One possible explanation is that this mutant has acquired a conformation resembling phospho-CheY, and hence it is defective in CheA binding.

Welch et al. (13) found that it binds to FilM with higher affinity than the apo-CheY, and this is consistent with the possibility of a conformational similarity between this mutant and the phospho-CheY.

Our results identify mutations that can alter the CheY-CheA binding, and it is possible that some of our mutations may have introduced a structural change that sterically disrupted binding rather than remove an interaction contributing to the binding. Also, a mutation can alter CheA binding, either by specifically changing the interaction surface or by nonspecifically causing a change in folding. Since all of the mutants we characterized were overproduced in the stable form and can be phosphorylated by CheA (except D13K (data not shown)), the probability of any major folding defects occurring is reduced. Also, the crystal structures of T87I and Y106W do not show altered folding (33). The most striking evidence is derived from the clustering of these mutations on one face of CheY molecule. We also mapped the positions of five cheY mutants not displaying altered CheA binding, and these mutations are not found on our proposed CheA binding face (green atoms, Fig. 4).

It is clear that the CheA binding face of CheY that our study identified lies distinct from the active site of the molecule. Recognition by CheA, away from the highly conserved active site of CheY (3), may be a way of acquiring specificity for this interaction. On the other hand, evidence for an overlap between CheA binding face and motor-switch binding face makes the structural aspects of CheY more interesting. While more work will be needed for the absolute determination of the region of overlap between the two faces, it will be interesting to know the region of CheY involved in the binding of CheZ, the only protein besides CheA and motor-switch proteins, that is known to interact with CheY (34). This will provide a better understanding of the structural aspects of CheY’s activity and the regulation of chemotaxis.

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