CheA Kinase and Chemoreceptor Interaction Surfaces on CheW*

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CheW has four known activities in vitro: (i) binding to CheA; (ii) binding to MCPs, such as Tar; (iii) promoting formation of CheA-CheW-MCP ternary complexes; and (iv) enabling MCPs to stimulate and/or inhibit CheA autokinase activity (9, 10, 12, 18–22, 26, 27). Based on these observations, current models depict CheW as an “adapter protein” that serves to tether CheA to the MCPs (18, 20, 28). While such a simple adapter role would account for the known in vitro activities of CheW, it is important to emphasize that the relationships among these activities and their roles in vivo have not been clearly established. In particular, the functional importance of CheW’s binding activities has not been clearly established by, for example, determining whether disrupting the CheW ↔ CheA binding interaction affects formation of the CheA-CheW-MCP ternary complex and/or activation of CheA within this complex.

In previous work (29), we followed a traditional mutational approach in an attempt to investigate the functional significance of CheW binding interactions with CheA and MCPs: we identified mutations that disrupted CheW’s ability to support chemotaxis, and then we examined the effect of these mutations on the ability of the protein to bind to CheA and MCPs. Each of the mutants we isolated following this approach was defective in formation of an activated ternary complex and exhibited defects in both binding interactions. While those results sug-

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The abbreviations used are: MCP, methyl-accepting chemotaxis protein; DITA, differential interaction trap assay; 5-FOA, 5-fluoroorotic acid; LZ-cTar, a construct that has a leucine zipper fused to the cytoplasmic (soluble) portion of Tar; F*-CheW, fluorescein-labeled CheW; BSA, bovine serum albumin.

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gested that CheW’s binding activities are indeed important for its role in chemotaxis signal transduction, they did not allow us to examine the effects of eliminating just one binding interaction at a time; nor did they allow us to identify which segments of CheW might be involved specifically in interactions with CheA and which with MCPs. To examine more rigorously the role(s) of CheW’s binding activities and to begin defining potential interaction interfaces with CheA and Tar, we turned to the DITA approach of Inouye et al. (30) with the goal of identifying CheW mutants lacking just one of the two binding activities. Our results, in conjunction with the recently solved three-dimensional structure of CheW (31), indicate clustering of mutation sites that cause disruption of CheW ↔ CheA interactions and therefore suggest the location of a possible CheA binding interface on CheW. In addition, our results help to further elucidate the CheW surface that appears to mediate contacts with MCPs, first defined by the genetic suppressor studies of Liu and Parkinson (26).

**EXPERIMENTAL PROCEDURES**

**Materials and Assays—**5-Fluoroornic acid (5-FOA) was purchased from Tokyo Chemicals, Inc. Fluorescein 5-isothiocyanate was purchased Molecular Probes, Inc. (Eugene, OR). All other reagents were purchased from standard sources and were of reagent grade.

The following procedures were carried out using previously published procedures: purification of CheA, CheW, and CheY (13, 18, 29, 32); isolation of Tar-containing inner membrane vesicles (18, 33); fluorescence-monitored assays of CheW binding to CheA and Tar (29); coupled assays of CheA activity (11, 21, 34); and assay and screening for nucleotide misincorporation by dNTPs and a high concentration of Mn2+ (29).

**Strains and Plasmids—**Saccharomyces cerevisiae strain YCJ4 and plasmids pLex202PL, pAS-Snfl, and pLex-bicid were kindly provided by Jeremy Thorner (University of California, Berkeley). Strains RP5098 (ΔfibA-αR;Δ) and D392 (ΔcheA W339) are Escherichia coli K-12 derivatives kindly provided by J. S. Parkinson (University of Utah) and F. W. Dahlquist (University of Oregon), respectively. Strain D392 was used for swarm assays, whereas strain RP5098, lacking all chemotaxis genes, was used for purification of most of the proteins used in this study.

Plasmid pGAD:CheW encoded a CheW-Gal4BD fusion and was created using vector pGAD424 (CLONTech). Wild type cheW was PCR-amplified from pCW (27) using primers that generated EcoRI and Sall sites upstream and downstream, respectively, of cheW. The resulting PCR fragment was digested with EcoRI and Sall and then ligated into corresponding sites of pGAD424. Plasmid pLexTar encoding cTar-LexA fusion was created by ligating a fragment of Tar encoding amino acids 257–553 into EcoRI and BamHI sites of plasmid pLex202PL. Plasmid pGBT9:CheA encoding CheA-Gal4AD fusion was created by inserting full-length cheA into vector pGBl9 (CLONTech, Inc.) between Sall and Sall sites.

The plasmid pCE (29) was used for overexpression and purification of CheW mutant proteins carrying a Met–His–Cys extension at their N-terminus. Mutant cheW alleles were excised from corresponding pGAD:CheW vectors using AgeI and SallI and ligated into corresponding sites of pCn029 (29). All plasmids created in the course of this work were verified by dyeoxy sequencing (performed at the University of Maryland Center for Agricultural Biotechnology).

**Mutagenesis and Screening—**Random cheW mutants were generated using an error-prone PCR procedure in which an unequal ratio of dNTPs and a high concentration of Mn2+ created conditions favorable for nucleotide misincorporation by Taq polymerase (40). The PCR-generated, mutagenized cheW DNA fragment was gel-purified and then transformed along with EcoRI and SallI-digested pGAD424 into YCJ4 strain carrying pGBT9:CheA and pLex-Tar (30). pGAD:CheW plasmid was restored in vivo in the resulting YCJ4 transformants by a gap repair mechanism (41). Half of the transformation mixture was plated on Yc medium lacking histidine, tryptophan, and leucine (Yc-his, trp-leu (selectable) plasmids pGBT9:CheA, pGAD:CheW, and pGBT9:CheA, respectively) and including 5-FOA. Colonies capable of growth in the presence of 5-FOA were transferred to Yc-his, trp-leu plates lacking uracil, and the results of growth on these plates were evaluated after 3 days at 30 °C. The colonies that displayed a URA+ phenotype were then restreaked on Yc-his, trp-leu plates; after a 2-day incubation at 30 °C, these colonies were tested for β-galactosidase activity using a colony lift assay (42).

The second half of the transformation mixture was plated on Yc-his, trp-leu medium in which the amount of uracil was reduced from the normal level of 0.01 to 0.0008%; this selected for URA+ cells (attempts to use medium completely lacking uracil were unsuccessful).

Among the mutants growing on this type of medium, we then identified strains with GalA+ colonies as described above, pGAD:CheW from selected colonies was then isolated and retransformed into YCJ4 strain expressing cTar and CheA fusions. The resulting transformants were assayed for URA3 and lacZ expression to confirm the phenotype of the strains observed in the differential interaction trap assay. Nucleotide changes in these alleles were determined by dyeoxy sequencing at the University of Maryland.

**Measuring CheW Affinities for CheA and Tar—**These assays utilized wild-type and mutant CheW proteins that had been engineered to include an N-terminal His6-Cys tag, as described previously (29). The purified His6-Cys-CheW proteins were modified by covalently attaching fluorescein maleimide (Molecular Probes) to the single cysteine side chain. Fluorescently labeled His6-Cys-CheW (hereafter referred to as F*-CheW) was then used for binding assays. Binding of wild-type or mutant F*-CheW to CheA was monitored using fluorescence anisotropy as described previously (29). In short, these assays involved measuring the increase in fluorescence anisotropy (λ2 = 492 nm, λm = 517 nm) of the F*-CheW sample as increasing concentrations of CheA were added. These experiments were performed in TEND buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 100 mM NaCl, 0.5 mM diethiothreitol) containing calf thymus DNA (0.2 mg/ml). The magnitude of the observed anisotropy increase as a function of the CheA concentration was used to generate a binding isotherm that was fitted to a simple binding equation to generate an estimate of Kd, the dissociation constant for the F*-CheW-CheA complex. Details of this essay and analysis procedure have been published previously (29).

Assays of the binding of wild-type or mutant CheW proteins to Tar were performed using the “pull-down competition” approach detailed previously (29). These assays made use of inner membrane vesicles carrying overproduced levels of Tar (18). Such vesicles are easily pelleted by centrifugation and carry with them, into the pellet, F*-CheW in the expected amounts based on CheW binding to Tar. To assess binding of mutant CheW proteins binding to Tar, competition experiments were performed in which wild-type or mutant CheW (final concentration 2 μM) was premissed with a particular CheW mutant protein (0–50 μM). Then the inner membrane vesicles were added to this mixture, generating a final Tar concentration of 11 μM in TEND buffer containing 1 mg ml−1 BSA. The resulting protein mixtures were allowed to equilibrate (in the dark, with gentle mixing) for 10 min at 25 °C, and then the vesicles (and associated F*-CheW) were sedimented by centrifugation. The fluorescein anisotropy of the supernatants of the supernatant samples were then measured (λ2 = 492 nm, λm = 517 nm). The data generated in these experiments showed the expected progressive increase in signal intensity (reflecting increasing levels of free F*-CheW) at increasing concentrations of competitor (unlabeled, mutant CheW). Analyses of these titration curves allowed us to define the Kd of the complex formed by Tar with the mutant CheW (29).

**RESULTS**

**Efficacy of the Differential Interaction Trap Assay—**We used the DITA of Inouye et al. (30) to quickly and easily observe CheW interactions with CheA and Tar. This approach is a three-hybrid modification of the yeast two-hybrid assay (43), and it involved simultaneous expression of three chimeric proteins, carried by three distinct plasmids, in S. cerevisiae strain YCJ4. The three chimeric fusions were as follows: CheW fused to the transcription activation domain of Gal4 (CheW-Gal4AD); CheA fused to the DNA-binding domain of Gal4 (CheA-Gal4BD); and the cytoplasmic domain of Tar (amino acids 257–553) fused to the DNA-binding protein LexA (cTar-LexA). As depicted in Fig. 1, interaction of CheW-Gal4AD with CheA-Gal4BD is expected to result in expression of the URA3 reporter gene in YCJ4, and interaction of CheW-Gal4AD with cTar-LexA is expected to drive expression of the lacZ reporter gene in this yeast strain. Our goal was to use this system to identify two distinct classes of cheW mutations: (i) those that diminished CheW binding to CheA, but not to Tar and (ii) those that decreased the affinity of CheW for Tar but not for CheA. Before proceeding with such a mutant search, we first exam-


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![Schematic diagram of the differential interaction trap assay](Image)

**FIG. 1. Schematic diagram of the differential interaction trap assay (30).** Fusions of CheW to the Gal4 activation domain (Gal4AD), CheA to the Gal4 DNA-binding domain (Gal4BD), and cTar (amino acids 257–553) to LexA (a DNA-binding protein) were expressed in the yeast strain YCJ4. This strain has two chromosomally integrated reporter genes: lacZ, located downstream of the lexA operator, and URA3, located downstream of the GAL4 UAS (30). Pairwise CheW ↔ Tar and CheW ↔ CheA interactions were assessed by expression of lacZ and URA3, respectively.

To assess the sensitivity of the DITA in our system, we evaluated the effects of six cheW mutations that we had previously shown to cause moderate to severe decreases in the affinity of CheW for CheA and Tar (29). Our results (Table I) indicate that expression of the URA3 reporter gene is sensitive to moderate (and large) changes of somewhat greater sensitivity for altered expression of the Tar; a 3-fold increase in reporter gene in response to changes in the affinity of CheW for CheA and Tar (29). Our results (Table I) previously shown to cause moderate to severe decreases in the cheW variants. To support these results and quantify the


tar interaction: mutations in CheW that affected in vitro binding to full-length Tar (in membrane vesicles) had the expected effect on the two-hybrid interaction.

**Isolation of cheW Point Mutants Using DITA**—We subjected the entire cheW gene to random mutagenesis using error-prone PCR (40); PCR products were introduced into appropriately cleaved plasmid pGAD424 (CLONTECH) and expressed as CheW-Gal4AD fusions in YCJ4 cells that already carried plasmids encoding CheA-Gal4BD and cTar-LexA. To isolate variants of CheW that exhibited weakened affinity for CheA, but not for Tar, we plated transformation mixtures onto medium containing 5-FOA, a nucleotide analog that inhibits growth of yeast cells expressing URA3 (50). Colonies that grew on these plates were then tested for β-galactosidase activity (42). +, production of blue color when permeabilized cells were exposed to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal); −, the absence of color change; −/+ , a faint blue color was observed in half of the assays performed with this mutant (the total number of trials was 4).

| CheW variant | K<sub>D, CheA</sub> | K<sub>D, Tar</sub> | URA3 expression<sup>a</sup> | lacZ expression<sup>a</sup> |
|--------------|-----------------|-----------------|----------------------|----------------------|
| Wild type    | 6.0 ± 0.2       | 11.0 ± 0.5      | +                    | +                    |
| G57D         | >130            | 33 ± 6          | −                    | −                    |
| V36M         | 47 ± 2          | >230            | −                    | −                    |
| G13E         | 75 ± 5          | 45 ± 6          | +                    | −                    |
| 154ocr       | 43 ± 4          | 3.6 ± 0.2       | +                    | −                    |
| R62H         | 14.7 ± 0.5      | 23 ± 4          | +                    | −/+                  |
| G41D         | 20 ± 1          | 37 ± 3          | +                    | −                    |

<sup>a</sup>URA3 expression was assessed as growth on selective medium lacking uracil. +, growth after 3 days of incubation at 30°C; −, absence of growth under such conditions.

<sup>b</sup>lacZ expression was assessed by a colony lift assay of β-galactosidase activity (42): +, production of blue color when permeabilized cells were exposed to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal); −, the absence of color change; −/+ , a faint blue color was observed in half of the assays performed with this mutant (the total number of trials was 4).

To identify variants of CheW that exhibited diminished affinity for Tar, but not for CheA, we first plated YCJ4 transformation mixtures onto medium depleted of uracil and then screened −2,000 of these to identify four that were β-Gal<sup>−</sup>. These Ura<sup>−</sup> β-Gal<sup>−</sup> mutants (hereafter referred to as Class I) were subjected to DNA sequence analysis and retained for further analysis.

To identify variants of CheW that exhibited diminished affinity for Tar, but not for CheA, we first plated YCJ4 transformation mixtures onto medium depleted of uracil and then screened −2,000 of these to identify four that were β-Gal<sup>−</sup>. These Ura<sup>−</sup> β-Gal<sup>−</sup> mutants (hereafter referred to as Class II) were subjected to DNA sequence analysis and retained for further analysis.

The nucleotide sequences of three of the Class II mutants exhibited single point mutations, whereas the remaining Class II mutant and all nine Class I mutants had two or more nucleotide substitutions. For each of these multiple mutants, a single mutation was responsible for the observed DITA phenotype. This was determined by separating the mutations using convenient restriction sites and, in some instances, by recreating the mutations using oligonucleotide-directed mutagenesis. The amino acid changes associated with the Class I and Class II mutations are summarized in Table II.

**Biochemical Analysis of cheW Mutant Proteins**—The DITA results suggested that specific mutations selectively inhibited CheW binding to CheA or Tar. This approach, while extremely convenient for initial identification of mutants, provided only an indirect and qualitative assessment of binding defects for the CheW variants. To support these results and quantify the
Amino acid changes in CheW mutant proteins isolated using the differential interaction trap assay and affinities of mutants for CheA and Tar

Affinities were determined using fluorescence anisotropy measurements (to define $K_d$) and competition pull-down assays (to define $K_d$) as described under “Experimental Procedures” and as detailed by Boukhvalova et al. (29). Each value represents the average of at least two independent titration experiments ± S.E.

| DITA selection CheW variant | $K_d$ (µM) CheA | $K_d$ (µM) Tar |
|-----------------------------|-----------------|----------------|
| Class I URA+ lacZ+          |                 |                |
| Wild type                   | 6.0 ± 0.2       | 11.0 ± 0.5     |
| V45L                        | 52 ± 2          | 14 ± 2         |
| T46S                        | 34 ± 1          | 15 ± 2         |
| T46A                        | 34 ± 1          | 15 ± 2         |
| T51A                        | 54 ± 2          | 18 ± 2         |
| T51P                        | 74 ± 10         | 12 ± 3         |
| I65N                        | 48 ± 3          | 7.6 ± 0.6      |
| M156V                       | 20 ± 10         | 150 ± 30       |
| L158Q                       | 17 ± 1          | 40 ± 10        |

Class II (URA+ lacZ+)  

| Wild type                   | 6.0 ± 0.2       | 11.0 ± 0.5     |
| T46A                        | 34 ± 1          | 15 ± 2         |
| T51A                        | 54 ± 2          | 18 ± 2         |
| T51P                        | 74 ± 10         | 12 ± 3         |
| I65N                        | 48 ± 3          | 7.6 ± 0.6      |
| M156V                       | 20 ± 10         | 150 ± 30       |
| L158Q                       | 17 ± 1          | 40 ± 10        |

Effects of the mutations on CheW binding affinities, we performed in vitro binding titrations that utilized purified, fluoresein-labeled versions of the wild-type and mutant variants of CheW (29). To evaluate CheW binding affinity for CheA, we monitored the increase in fluorescence anisotropy exhibited by labeled CheW when it binds CheA (Fig. 2) (29). To assess CheW binding affinity for Tar, we used pull-down experiments in which membrane vesicles carrying high levels of Tar were used to sediment fluoresein-labeled CheW out of solution via centrifugation (Fig. 3) (18, 29). These experiments defined binding isotherms that we analyzed to estimate $K_d$ (the dissociation constant for the CheW-CheA complex) and $K_d$ (the dissociation constant for the CheW-Tar complex). The observed $K_d$ values (Table II) indicated that the DITA selection CheW variant had successfully identified mutations that specifically diminished CheW affinity for CheA (without affecting its affinity for Tar); Class I mutants exhibited $K_d$ values ranging from 5- to 10-fold higher than the wild-type $K_d$ value, whereas $K_d$ values for these mutants remained within 30% of the wild-type value. Class II mutants, as expected, exhibited significant increases in $K_d$ values (values ranged from 4 to 14 times the wild-type $K_d$ value); however, this was accompanied by a moderate (~3-fold) increase in $K_d$. Several factors could have contributed to this lack of specificity with the Class II mutants, such as an inadequately sensitive reporter system for CheW ↔ CheA interactions and/or the nature of the Tar binding interface, a possibility that is considered in greater detail under “Discussion.”

Effect of CheW Mutations on Tar-CheW-CheA Ternary Complexes—In the presence of CheW and MCPs, CheA exhibits a dramatically enhanced autokinase activity (9, 11, 12, 21, 22, 51, 52). This activation of CheA appears to result from formation of an MCP-CheW-CheA ternary complex in which CheW serves as a “coupling factor.” Little is known about how CheW accomplishes this receptor-kinase coupling or how it promotes formation of the ternary complex. As a first step toward improving our understanding of the functional role of CheW, we used our set of binding-defective CheW proteins to determine whether both of CheW’s binding interactions are necessary for it to accomplish receptor-kinase coupling. We examined the ability of our mutant CheW variants to accomplish CheA activation by assaying CheA autokinase activity in mixtures containing purified CheW, CheA, the cytoplasmic domain of Tar (LZ-cTar), and CheY in the presence of an ATPase coupling system (34, 51, 52). These results (Fig. 4) indicate that each of our mutant CheW variants was less effective than the wild-type CheW in mediating MCP-CheA coupling in the concentration range tested. Although assays at higher CheW concentrations would have been informative for some of the mutant proteins, we were unable to pursue such assays because of concentration limitations for CheW, CheA, and CheY; adding higher CheW concentrations would have required corresponding decreases in the volumes of CheA, CheY, and/or ATPase coupling reagents, but we were unable to increase the stock concentrations of these proteins enough to accommodate the necessary changes.

For the Class I mutants (Fig. 4A), we observed a consistent qualitative correlation between the affinity of CheW for CheA and the observed coupling effectiveness; mutant K561 had the highest $K_d$ CheA value and performed the worst in the coupling assays; mutant T46A had the least affected $K_d$ CheA value and was the most effective Class I mutant in coupling assays. Other Class I mutants, with intermediate $K_d$ CheA values, exhibited intermediate coupling abilities. These results suggest that the...
Altered Binding Affinities

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Class II mutants also exhibited diminished coupling activities (Fig. 4B). However, attributing this decrease to a reduced affinity of CheW for Tar is complicated by the fact that these mutants also have $K_d$ values that are $\sim$3-fold higher than the wild-type value. For assessing the effects of an increase in $K_d$, we found it helpful to use the titration profile of mutant T46A (a Class I mutant) as a standard for comparison; T46A exhibits a $K_d$ value that is $\sim$6-fold higher than the wild-type value and a $K_d^{\text{tar}}$ value that differs from the wild-type value by only $\sim$30%. Both of the Class II mutants that we studied in detail exhibited coupling activities that were considerably worse than that of T46A. If the affinity of CheW for Tar had no effect on coupling ability, then we would have expected Class II mutants to perform at least as well as T46A and perhaps considerably better because they bound CheA somewhat better than did T46A. This was not the case: Class II mutants E38D and V87A both performed considerably worse than T46A. This was not the case: Class II mutants to perform at least as well as T46A and perhaps considerably better because they bound CheA somewhat better than did T46A. This was not the case: Class II mutants E38D and V87A both performed considerably worse than T46A. This was not the case: Class II mutants E38D and V87A both performed considerably worse than T46A. If the affinity of CheW for Tar was assessed the chemotactic abilities of the resulting transformants by examining their migration rates in semisolid "swarm" agar (Fig. 5). These results indicated that the mutant variants were surprisingly effective, supporting swarm rates that were close to that observed with wild-type CheW. These results indicate that in vivo activity of CheW is surprisingly insensitive to moderate changes in the affinity of CheW for CheA and MCPs.

Under conditions of CheW overproduction, some differences were observed with several of the mutants. Elevated levels of wild-type CheW resulted in severe inhibition of chemotactic activity (Fig. 5), a phenomenon that has been reported previously (14, 53). Class II mutants lacked this inhibitory ability, whereas one of the Class I mutants (L158Q) appeared to have enhanced inhibitory ability (Fig. 5B and data not shown). L158Q exhibited an affinity for Tar that is slightly higher (30%) than that of wild-type CheW. In previous work, we reported a similar enhancement of inhibition with cheW154coc, a mutant that exhibited a 3-fold increased affinity for Tar compared with...
the wild-type CheW (29). These results indicate a correlation between the affinity of CheW for MCPs and its effectiveness as an inhibitor of chemotaxis at elevated levels of expression; mutants with significantly diminished affinity for Tar are ineffective as inhibitors, whereas mutants with even moderately enhanced affinity for Tar are more effective inhibitors.

**DISCUSSION**

**Rationale, Advantages, and Limitations of the DITA Approach for Analysis of CheW Binding**—To identify CheW mutants lacking just one of the two binding activities, we applied the DITA approach of Inouye et al. (30). One advantage of this selection/screen is that it allowed us to focus specifically on the protein-protein interactions of interest in a heterologous reporter system without possible interference from other components of the chemotaxis system or from unknown in vivo activities of CheW. In addition, this approach selected against several types of mutations that were uninteresting for our purposes, including those that caused large scale perturbations of the three-dimensional structure (e.g. mutants with disrupted folding patterns), nonsense mutations, and mutations that caused significant changes in CheW stability, turnover, or expression level.

This approach led to identification of several mutations (Class I) that diminished CheW affinity for CheA without simultaneously affecting its interaction with Tar. The existence of such mutants and their location on the three-dimensional structure of CheW (see below) suggests that the binding interfaces for CheA and Tar involve distinct faces of the CheW surface. The DITA approach was less successful in identifying mutations that specifically influenced CheW ↔ MCP binding interactions; each of our Class II mutants exhibited a moderately decreased ability to bind CheA in addition to the anticipated decrease in affinity for Tar. Nonetheless, we were able to isolate one mutant (E38D) that exhibited a large defect in Tar binding and a considerably smaller defect in CheA binding. Our two classes of mutants allowed us to address several basic questions about the functional role(s) of CheW’s binding interactions. These questions are considered below.

**Mutations That Affect CheW Pairwise Binding Interactions Have Corresponding Effects on Its Receptor-Kinase Coupling Activity**—Titration of CheA with increasing concentrations of CheW gives rise to a simple hyperbolic binding isotherm; there are no indications of multiple affinities of binding sites or cooperativity. Similar results are observed for titrations that monitor binding of wild-type CheW to MCPs (18, 29, 52). We isolated mutants that exhibit diminished binding affinities in these simple pairwise interactions, and these mutants allowed us to address a simple question: Do these mutations also affect the ability of CheW to promote formation of the CheA-CheW-MCP ternary complex that is thought to mediate key events in chemotaxis signal transduction? At first glance, this question might seem trivial and its answer obvious. However, it is important to emphasize the potential complexities of CheW binding interactions in CheA-CheW-MCP complexes. In contrast to the apparent simplicity of CheW pairwise binding interactions, when CheW is placed in mixtures of CheA and MCPs, there appear to be multiple competing binding equilibria that result in formation of several distinct ternary complexes. These different versions of the ternary complex have different stoichiometries, different kinase activities, and different stabilities (21). This complexity suggests that the component proteins can interact with one another in a variety of different ways, some of which lead to a ternary complex in which CheA autokinase activity is very high (the “activated ternary complex”) and some of which lead to a ternary complex in which CheA is essentially inactive (the “inactive ternary complex”). In view of this complexity, we deemed it important to investigate the relationship between the pairwise interactions and those that mediate formation of the activated ternary complex.

Our results clearly indicate that mutations that diminish the affinity of the pairwise interactions have a corresponding effect on the ability of CheW to promote formation of the activated ternary complex. In theory, the differences observed between the wild-type and mutant titration profiles (Fig. 4) could reflect either diminished levels of activated ternary complex or diminished activity of CheA within this complex. We did not pursue experiments to distinguish between these two possibilities; however, it is notable that the shapes of the profiles and the observed magnitudes of the maximal activities match fairly closely with those predicted by computer simulations generated by assuming diminished complex formation.

These results suggest that CheW coupling activity involves both of its binding interactions (i.e. CheW must interact with both CheA and Tar to enable formation of the active complex). Moreover, these findings are consistent with the idea that the binding contacts that mediate pairwise CheW ↔ CheA and CheW ↔ Tar interactions are closely related to (perhaps even identical to) the contacts that are required for formation of the activated ternary complex.

Two of our mutant CheW proteins (V87A and E38D) generated a sigmoidal relationship between the concentration of CheW and the levels of activated complex, suggesting that activated complex formation is a cooperative process, at least with these mutants (see Fig. 4B). We noted a similar effect with the mutant CheW154ocr in previous work (29). CheW154ocr and the Class II mutants analyzed here had altered affinity for receptors. However, pairwise titration experiments using these mutants and Tar gave rise to simple hyperbolic binding curves; only in the presence of CheA (in the kinase activation assays) did we observe the apparent cooperativity. Cooperativity in regulation of CheA activity by receptors has been reported previously (12, 21, 22, 54) and may be linked to the phenomenon of receptor clustering (19, 45).

Our results suggest that CheW might contribute to this phenomenon through its binding interactions with receptors. Binding of CheW to an MCP dimer might, for example, generate or uncover additional contact sites that promote formation of a complex involving CheW, CheA, and a cluster of MCPs. These “revealed sites” might include MCP positions that become available for interaction with CheA, similar sites on CheA that become available for interaction with MCPs, or possibly some additional sites on CheW itself that allow it to act as a nucleator of higher-order macromolecular aggregates. It is puzzling that we observed apparent cooperativity with several CheW mutant variants but not with other mutants and not with wild-type CheW. Further detailed analysis of these mutants may provide insight into the molecular mechanism by which CheW promotes formation of activated ternary complexes.

In Vivo Chemotactic Signaling Tolerates Significant Decreases in CheW Binding Affinities—We examined the ability of our binding-defective CheW variants to support chemotaxis in vivo. When examining the results of these complementation experiments, it is useful to consider two mutants as representative test cases: K56E for examining the effect of diminishing the affinity of CheW for CheA and E38D for examining the effect of diminishing the affinity of CheW for Tar. The K56E mutant exhibits an affinity for CheA that is weaker than that of wild-type CheW by a factor of ~12, but its affinity for Tar is essentially the same as wild type. The E38D mutant has a diminished affinity for Tar by a factor of ~14, whereas its ability

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2 R. C. Stewart and M. Boukhvalova, unpublished observations.
to bind CheA is influenced to a lesser extent (−3-fold). Both of these mutants exhibited diminished abilities to promote formation of the activated ternary complex in vitro, as discussed above. Therefore, we were surprised to find that these and other such mutants performed quite well when tested by in vivo complementation (swarm) assays; the mutant proteins supported swarming rates that were comparable with that supported by wild-type CheW. One conceivable, albeit extreme, interpretation of these findings would be that in vitro binding, ternary complex formation, and CheW coupling activity are in vitro artifacts that have little to do with CheW activity in vivo. However, in previous work we demonstrated that several cheW null alleles (isolated by virtue of their Che− phenotype in swarm assays) encode variants of the CheW protein that have severely disrupted binding activities and that completely lack coupling activity in vitro. This observation and the dramatic increase in CheA activity that results from ternary complex formation (9, 21) argue against the extreme interpretation suggested above. A second possibility is that the chemotaxis signaling pathway is able to make due with a severely diminished level of activated ternary complex. Several recent publications have demonstrated that the signaling circuitry of the chemotaxis system is a “robust” network that can maintain responsiveness and sensitivity despite large fluctuations in the activities and/or concentrations of key signaling components (55–57). In previous work (58), we observed that CheA active site mutants having autokinase activities as low as 6% of the wild-type activity can support chemotaxis (as measured by swarm assays). Thus, the 10- or 20-fold reduction in the amount of activated ternary complex expected for our E38D and K56I mutants might be accommodated by this robust network. Such a situation would explain why most cheW null alleles (isolated by screening for a Che− phenotype (29)) have very large effects on both $K_{d}^{\text{CheA}}$ and $K_{d}^{\text{Tar}}$; less drastic effects on CheW binding would have no phenotype in swarm assays and would never be identified for further analysis. Yet a third possible explanation of the surprising efficacy of our weakly binding CheW mutants is that in vivo conditions compensate in some other way for the poor binding abilities of the mutant CheW proteins, perhaps as a result of macromolecular crowding in the cytoplasm (59). Receptor clustering (19, 60) or adjustments of MCP methylation levels (55, 61, 62) might also contribute to the ability of the in vivo chemotaxis system to overcome defects in CheW binding affinity. However, our qualitative examination of MCP methylation patterns in cells expressing these cheW alleles did not reveal any clear differences from methylation patterns observed in cells expressing wild-type cheW, leading us to doubt the contribution of methylation as a compensating factor (results not shown).

**Mutation Sites Define Possible Interaction Interfaces for CheW Binding to CheA and MCPs**—One of the goals of this study was to define the segments of CheW that mediate its binding to CheA and to MCPs. The results discussed above suggest that the binding surfaces used in pairwise CheW-CheA and CheW-Tar complexes are also utilized in the activated ternary complex, so identifying sites that are required for pairwise binding interactions is a viable approach for defining sites that play important roles in the protein-protein interactions of the activated ternary complex. The three-dimensional structure of CheW from *Thermotoga maritima* has recently been determined by NMR methods in the Dahlquist laboratory (31). This structure indicates that CheW consists of two five-stranded β-barrels surrounding a hydrophobic core (Fig. 6). The *T. maritima* protein has an amino acid sequence that is quite similar to that of the *E. coli* protein (63); therefore, it seems likely that they have similar structures. Such similarity has been observed in the structures of another component of the chemotaxis system (CheY) from *E. coli* and *T. maritima* (64). Mapping our mutation sites onto the structure of *T. maritima* CheW allowed us to visualize the relative orientation, in three-dimensional space, of the amino acid positions altered by the mutations. One interesting observation is the clustering of the Class I mutation sites on one face of CheW (Fig. 6A). We propose that this surface of CheW serves as a binding interface that mediates interactions between CheW and CheA in pairwise combinations as well as in the activated ternary complex. The same surface of CheW was proposed to mediate its interaction with CheA based on NMR chemical shift changes observed in *T. maritima* CheW in the presence of CheA (31). Possible CheA ↔ CheW interaction sites have also been
proposed as a result of computer models generated by assuming
that CheW adopts a three-dimensional structure resembling
that of the C-terminal regulatory domain of CheA (28, 65). Our results support the prediction of Bilwes et al. (65) that the $\beta_2$-$\beta_4$ region of CheW (corresponding to $\beta_2$ and $\beta_1$ of CheA) mediates CheW $\leftrightarrow$ CheA binding interactions.

We were successful in identifying only four Class II mutants. With such a small sample size, “clustering” of these on the three-dimensional structure of CheW (Fig. 6B) is not necessarily a surprising or insightful finding. However, there are numerous cheW mutations that were identified by Liu and Parkinson (26) as suppressors of MCP mutations. Presumably, these mutations affect positions that participate in CheW-MCP contacts. Mapping these mutation sites and our Class II sites onto the structure of T. maritima CheW defines an extended surface adjacent to the putative CheA binding site of CheW (Fig. 6). We suggest that this extended surface mediates CheW $\leftrightarrow$ MCP binding in both the pairwise CheW $\leftrightarrow$ MCP complexes as well as in the activated ternary complex.

Our approach identified mutations that altered CheW $\leftrightarrow$ CheA or CheW $\leftrightarrow$ Tar binding. Several different types of changes in protein structure might underlie the observed binding defects: removal of binding contacts, steric disruption of binding contacts, nonspecific changes in global structure, and/or protein folding. In this regard, it is important to note that the Class I mutants exhibited normal affinities for Tar, so it seems unlikely that the binding phenotype of these mutants (diminished affinity for CheA) arose from nonspecific alterations of CheW structure. The results generated with the Class II mutants are less clear cut; these mutations had the expected effects on Tar binding affinity but also caused small decreases in the affinity of CheW for CheA. The Class II mutations affect $\beta$-strands located on a common surface of CheW and might cause subtle structural changes in the orientations of these strands, changes that could be propagated to the CheA-binding interface of CheW. In this regard, it is interesting to note that the C-terminal $\alpha$-helix of CheW and $\beta$-strand 10 (connected to the N-terminal end of this helix) together form a structure that runs along the entire length of the CheW molecule and contacts most of the other secondary structural elements. This might provide a physical link that conveys structural perturbations (such as those caused by mutations) from the MCP-binding interface of CheW to its CheA-binding interface. Deleting most of this helix (in CheW1546cr) has the interesting effect of enhancing the affinity of CheW for Tar by a factor of 3 (compared with wild-type CheW) (29). Perhaps, in wild-type CheW, this helix mediates communication between the two binding interfaces of the protein.

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