The transmembrane, homodimeric aspartate receptor of *Escherichia coli* and *Salmonella typhimurium* controls the chemotactic response to aspartate, an attractant, by regulating the activity of a cytoplasmic histidine kinase. The cytoplasmic domain of the receptor plays a central role in both kinase regulation and sensory adaptation, although its structure and regulatory mechanisms are unknown. The present study utilizes cysteine and disulfide scanning to probe residues Leu-250 through Gln-309, a region that contains the first of two adaptive methylation segments within the cytoplasmic domain. Following the introduction of consecutive cysteine residues by scanning mutagenesis, the measurement of sulfhydryl chemical reactivities reveals an \( \alpha \)-helical pattern of exposed and buried positions spanning residues 270–309. This detected helix, termed the “first methylation helix,” is strongly amphiphilic; its exposed face is highly anionic and possesses three methylation sites, while its buried face is hydrophobic. In *vivo* and in *vitro* assays of receptor function indicate that inhibitory cysteine substitutions are most prevalent on the buried face of the first methylation helix, demonstrating that this face is involved in a critical packing interaction. The buried face is further analyzed by disulfide scanning, which reveals three “lock-on” disulfides that covalently trap the receptor in its kinase-activating state. Each of the lock-on disulfides cross-links the buried faces of the two symmetric first methylation helices of the dimer, placing these helices in direct contact at the subunit interface. Comparative sequence analysis of 56 related receptors suggests that the identified helix is a conserved feature of this large receptor family, wherein it is likely to play a general role in adaptation and kinase regulation. Interestingly, the rapid rates and promiscuous nature of disulfide formation reactions within the scanned region reveal that the cytoplasmic domain of the full-length, membrane-bound receptor has a highly dynamic structure. Overall, the results demonstrate that cysteine and disulfide scanning can identify secondary structure elements and functionally important packing interfaces, even in proteins that are inaccessible to other structural methods.

The aspartate receptor of *Escherichia coli* and *Salmonella typhimurium* is representative of a large family of cell-surface receptors that regulate two-component signaling pathways, which are widespread in prokaryotic and eukaryotic organisms (1–9). These receptors contain two putative transmembrane helices per subunit and, in all cases tested, form stable homodimers that signal via a transmembrane conformational change. Chimeric receptors containing the sensory domain of the aspartate receptor and the regulatory domain of another family member are functional, suggesting that members of this receptor family use a conserved mechanism of transmembrane signaling to regulate cytoplasmic histidine kinase activity (10–12). More generally, conformational transmembrane signals may provide an important component of kinase regulation in other, unrelated receptor families as well. For example, a chimera between the aspartate receptor and the human insulin receptor has been shown to possess aspartate-regulated tyrosine kinase activity, a result that has been interpreted to indicate that the cytoplasmic domain of the insulin receptor is regulated by an intramolecular conformational signal (13, 14).

The aspartate receptor, like the other transmembrane receptors of the bacterial chemotaxis pathway, associates with soluble components of the pathway to form a supermolecular signaling assembly. The core of this signaling assembly is a ternary complex involving the receptor and two cytoplasmic proteins: the histidine kinase CheA and the coupling protein CheW. Once formed, the ternary complex is kinetically stable for tens of minutes, and the stability is relatively independent of the receptor ligation state (15–17). The aporeceptor stimulates the autophosphorylation activity of the kinase, while attractant binding down-regulates the kinase over 102-fold (18, 19). Other cytoplasmic proteins associate transiently with the ternary complex, including the adaptation enzyme CheR, which binds to the C-terminal tail of the receptor (20) and serves to methylate specific receptor glutamate residues (21, 22). Another adaptation enzyme, CheB, hydrolytically demethylates these regulatory side chains, and the ternary complex stimulates this demethylation activity by phospho-activating the CheB protein (23–25). The resulting feedback adaptation loop enables the ternary complex to maintain its output at an optimal level, even in the presence of a constant background stimulus of chemoeffectant or repellent. Ultimately, the function of the ternary complex is to control the swimming state of the flagellar motor by phospho-activating the CheY protein (18, 26). Phospho-CheY subsequently dissociates from the ternary complex and diffuses to the flagellar motor, where it binds and alters the swimming behavior of the cell (for reviews of the chemotaxis pathway, see Refs. 1, 4–6, 9, 27, and 28).

The aspartate receptor is a 120-kDa homodimer both in the presence and absence of aspartate (29–31). The structures of the periplasmic and transmembrane domains have been extensively characterized. The 1.85-Å resolution crystal structure of the isolated periplasmic domain reveals a dimer of four-helix bundles (\( \alpha_1-\alpha_4 \) and \( \alpha_1'-\alpha_4' \), respectively) (32–34). Two symmetric attractant binding sites lie at the dimer interface, where they are coupled to yield negative cooperativity, such that occupancy of one site reduces or prevents occupancy of the...
The N- and C-terminal helices of each periplasmic subunit extend across the bilayer, yielding a membrane-spanning four-helix bundle. In both the periplasmic and transmembrane regions, the subunit interface is dominated by the packing interactions between the symmetric first transmembrane helices (α1/TM1 and α1/TM1') along the central axis of the dimer, while the second transmembrane helices (α4/TM2 and α4/TM2') pack along the periphery of the central pair (29, 31–33, 37–39). Previous studies have shown that the second transmembrane helix is the signaling helix that carries the ligand-induced signal across the bilayer (36, 38). Attractant binding to the receptor generates a piston (or swinging-piston) displacement of the signaling helix toward the cytoplasm (40). Parallel studies have shown that the related chemoreceptor for ribose and galactose shares the same helical structure and piston-type signaling mechanism, illustrating the generality of this transmembrane signaling motif at least within the bacterial chemoreceptor subfamily of two-transmembrane helix receptors (41–46).

In contrast to the periplasmic and transmembrane domains, the structure and mechanism of the cytoplasmic domain remains poorly understood. It has been demonstrated that the isolated cytoplasmic domain fragment, although functional, is highly dynamic and exhibits heterogeneous oligomeric states (47–50). These features have hindered attempts to determine the structure of the domain by crystallography and NMR. Thus, other structural methods are needed. Circular dichroism and hydrodynamic measurements have implied that the domain is an elongated bundle of α-helices (47, 48, 51), while comparative sequence analysis also suggests a predominantly helical structure. The latter analysis, which has yielded the predicted helical arrangement summarized in Fig. 1, is based on an alignment of 56 cytoplasmic domain sequences from chemo-, thermo-, and photo-receptors (52–57). The current study focuses on residues Leu-250 through Gln-309, which include the first methylation segment of the cytoplasmic domain. This segment contains three of the four methylation sites of the receptor (22) and lies between the signaling helix and the kinase interaction domain, suggesting that it is likely to play an important role in signal transduction. Genetic studies have underscored the importance of the first methylation segment to receptor signaling. Second site repressor mutations that counteract the deactivating mutant A19K, which lies in the first transmembrane helix, are found to cluster within this region (58), as do many signal-locking point mutations in the homologous receptor Tsr (59–61). Finally, biochemical studies analyzing the isolated cytoplasmic domain have implicated the first methylation segment in kinase regulation (49, 62). The periodicity of CheR recognition residues and comparative sequence analysis of homologous receptors suggest that the targeted region contains at least one α-helix (22, 53, 56, 57, 63), as shown in Fig. 1. These predictions remain to be tested, however.

The current study probes the structure of the first methylation segment in the full-length, membrane-bound receptor and tests specific mechanisms of kinase regulation involving this region. The experimental approach begins with cysteine-scanning mutagenesis, a systematic approach analogous to alanine-
scanning mutagenesis (64) used to identify secondary structure elements in proteins (43, 65–68). Analysis of the chemical reactivities and functional effects of the cysteines substituted at positions 250–309 reveals considerable α-helical structure in this region and also maps out a functionally critical buried face of an amphiphilic helix. Moreover, the disulfide-scanning method (29, 31, 37, 38, 41) confirms the previously observed dynamics of the cytoplasmic domain (50) and is used to discover intersubunit disulfide bonds that lock the kinase in its on-state. These lock-on disulfides identify a specific helix-helix contact that exists in the on-state of the receptor. Overall, the results provide the first experimental characterization of helical secondary structure and packing within cytoplasmic domain, and show that a helix-helix interface between receptor subunits plays an important role in the mechanism of kinase regulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—To prepare receptor-containing membranes, plasmid pSCF6 (see below) was expressed in *E. coli* RP3808 (ΔcheA-cheZDE2209Δ-tol-1u6B his-4 eda-50 rpsL316 (thi-1 Δgal-attI DE299 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78/∆mks)/Δmks)). To analyze receptor chemotaxis function in vivo, plasmid pSCF6 was expressed in *E. coli* RP6811 (Δ(tsr)DE7098,Δ(tsr::tap)DE2591,Δ(thi-1 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78/78)CP3624) of G. Hazelbauer via F. Dahlquist, pa/). Both strains were kindly provided by John S. Parkinson (University of Utah, Salt Lake City, UT; Ref. 69).

Plasmas and plasmids used for expression of the histidine kinase CheA (HB101/pM04) and the coupling protein CheW (HB101/pME5) were kindly provided by Jeff Stock (Princeton University, Princeton, NJ). The strain and plasmid used for expression of CheY (RRB455/pRRB40) were graciously provided by Bob Bouluer (University of North Carolina, Chapel Hill, NC). 5-Iodoacetamidofluorescein was purchased from Molecular Probes, Inc. [γ-32P](ATP (6000 Ci/mol)) was obtained from Amersham Corp. Mutagenic oligonucleotides were synthesized by Life Technologies, Inc.

**Cloning and Mutagenesis**—The gene encoding the *S. typhimurium* aspartate receptor under control of its natural promotor was cloned into the vector pBlueScript KS+ (Stratagene), yielding the plasmid pSCF6 as described previously (31). This plasmid was used for both mutagenesis and overexpression of wild-type and mutant receptors. Site-directed mutagenesis was carried out according to Kunkel et al. (70), with the modifications described by the Mutual Molecular mutagenesis kit (Bio-Rad). The resulting plasmids were transformed into *E. coli* LM1035 (a derivative of HB101), then isolated using QIAprep spin columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen). The sequences of the mutants were confirmed by plasmid DNA sequencing, performed by thermocycling with Sequith columns (Qiagen).

**Preparation of Membranes Containing the Aspartate Receptor**—Wild-type and mutant receptors were expressed in *E. coli* RP3808 bearing the appropriate version of pSCF6. Saturated liquid cultures were grown at 37 °C in the presence of 0.1% SDS. A typical 500-ml preparation yielded ~20 mg of total protein. To ascertain the fraction of this total protein comprised by the receptor, the membrane components were resolved on 10% SDS-PAGE gel with an acrylamide:bisacrylamide ratio of 40:0.2. The relative intensities of the Coomassie-stained protein bands were quantitated by laser densitometry (Ultroscan XL, Pharmacia Biotech Inc.). The receptor typically comprised 10–20% of the total protein concentration.

**Preparation of Soluble Chemotaxis Components**—CheY was purified as described previously (75). CheA (76) and CheW (77) were purified using an adaptation of the published protocols. Saturated liquid cultures of the appropriate strain/plasmid combination were grown in Tryptone Broth (10 g/liter tryptone, 4 g/liter NaCl, 0.6, approximately 3–5 mM H2O) overnight at 37 °C. Expression was induced by adding 3-β-indole acryl acid to 0.1 mg/ml. The cultures were then incubated for an additional 10–12 h before the cells were harvested by centrifugation (Beckman JA-10 rotor at 6000 rpm (6570 × g) for 10 min).

The isolated cells were resuspended in 20 ml of ice-cold TDEG (20 mM Tris, pH 7.5, with HCl, 1 mM EDTA, 2 mM DTT, 20% glycerol) containing 1 mM PMSF, and then the cells were lysed by French press and membranes and particulate matter were removed by ultracentrifugation (Beckman TLA100.3 rotor at 80,000 rpm (340,000 × g) for 20 min). To the supernatant, solid ammonium sulfate was added to 45% saturation. This solution was stirred on ice for 30–60 min, and then the precipitated protein was pelleted by centrifugation (Beckman JA-20 rotor at 13,000 rpm (20,400 × g) for 15 min). The pellet was resuspended in 4 ml of TDEG with 50 mM NaCl and 1 mM PMSF, dialyzed overnight against 500 ml of the same buffer, with one change of buffer. CheA or CheW was isolated from the dialyse by FPLC on a Q-Sepharose column (Pharmacia) with a gradient of 50 mM to 1 M NaCl in TDEG (50 mM Tris, pH 7.5, with HCl, 1 mM EDTA, 2 M DTT, 10% glycerol). The final column step was FPLC on Superdex 200 16/60 column (Pharmacia) for CheA or a Superdex 75 16/60 (Pharmacia) column for CheW, eluting with 20 mM Tris, pH 7.5, with HCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 750 mM NaCl. The pooled fractions from each column were concentrated by ultrafiltration (Amicon YM10 membrane) to a final volume of 1–2 ml yield, and then dialyzed overnight against 500 ml of TDEG with one change of buffer. Particular matter was removed from the dialyse by ultracentrifugation (Beckman TLA100.3 rotor at 100,000 rpm (540,000 × g) for 10 min). The sample was then aliquoted and snap-frozen in liquid nitrogen before storage at −70 °C.

The total protein yield was determined by a BCA assay as described above. To ascertain the purity of the protein, the sample was resolved on a 10% (CheA) or 15% (CheW) acrylamide SDS-PAGE gel, quantitating by laser densitometry. A typical 4-liter preparation yielded ~2 mg of CheA or CheW at ≥95% homogeneity.

**Analysis of Solvent Exposure**—Receptor-containing membrane samples were diluted to give 5 μM receptor monomer in 10 mM sodium phosphate, pH 6.5, with HCl, 50 mM NaCl, 50 mM KCl, 1 mM EDTA. Reactions were initiated by adding 5-iodoacetamidofluorescein (IAF) from a 10 mM stock in *N,N*-dimethylformamide to a final concentration of 500 μM. Each reaction was incubated at 25 °C for 10 min and then divided into two aliquots. One aliquot was quenched by addition of 12 M mercaptoethanol to 60 mM to destroy the remaining IAF, while the second aliquot was left unquenched. Subsequently, 12 M mercaptoethanol was added to both samples to denature the protein, followed by incubation at 95 °C for 3 min. Neither a longer incubation nor subsequent addition of IAF drove the unquenched aliquot to a higher extent of labeling, indicating that the labeling reaction in this aliquot had reached completion. Finally, 12 M-mercaptoethanol was added to 60 mM in the unquenched aliquot to destroy the unreacted IAF (and to improve
resolution by SDS-PAGE (for unknown reasons). Both samples were resolved on a 10% SDS-PAGE gel with an acrylamide:bisacrylamide ratio of 40:0.2. The unstained gel was photographed on a 302-nm transilluminator using a 470-nm long-pass filter and Polaroid 665 film (1-min exposure, F-stop 5.6) and then Coomassie-stained. The fluorescence was quantitated using a coupled phosphorylation assay, which monitors the reaction was inactivated by addition of sodium persulfate to 0.1 mM. The reaction was incubated at 25 °C for 60 min (1-min exposure, F-stop 5.6) and then Coomassie-stained gel. The normalized fluorescence of the sample from the 10-min, 25 °C incubation was then divided by the normalized fluorescence of the denatured reaction, yielding the ratio termed "chemical reactivity." As a negative control, the experiment was carried out with wild-type receptor, for which no significant fluorescent labeling was observed. Moreover, no competing reactions were observed under the conditions employed, since control reactions in which cysteine-containing receptors were denatured either immediately or after the standard 10-min incubation yielded the same extent of labeling. In particular, disulfide formation catalyzed by the presence of contaminating metals (sometimes termed "spontaneous" disulfide formation) was prevented by the presence of 1 mM EDTA.

**RESULTS**

**Construction of Cysteine-containing Receptors**—To carry out cysteine scanning in the targeted regions of the aspartate receptor, a series of single-cysteine substitutions were generated by oligonucleotide-directed mutagenesis of the receptor gene in the plasmid pSCF6. Each substitution yielded a unique cysteine side chain, since the native receptor contains no intrinsic cysteine residues. In the periplasmic domain, a set of nine positive control receptors were generated by scanning the cysteine through positions Thr-95 to His-103 in the known structure of helix 2. In the cytoplasmic domain, the single cysteine residue was scanned through 60 consecutive positions from Leu-250 to Gln-309, a region of unknown structure that includes the first methylation segment.

Each engineered receptor protein was overexpressed in *E. coli* and isolated using standard procedures. The expression strain chosen, RP3808, lacks chemoreceptors and the adaptation enzymes CheX and CheB (69). As a result, membranes isolated from this strain possessed no chemoreceptors besides the engineered receptor of interest, and the adaptation state of the isolated receptor population was well defined and homogeneous. In general, the mono-cysteine receptors were expressed at levels from 25 to 100% that observed for wild type. The only exceptions were the V265C and T266C receptors, for which no expressed protein was detectable. The adjacent nature of the latter positions suggests that they lie within a localized region that is essential for proper receptor folding or stability in vivo.

**Determination of Solvent Exposure: Experimental Strategy**—Under appropriate experimental conditions, the chemical reactivities of the cysteine-scanning residues provide direct information regarding solvent exposure and burial, as illustrated by previous studies of transmembrane receptors, transporters, and channels (65, 67, 83). In the present study, the chemical reactivity of each engineered sulphydryl was determined by quantitating its reaction with IAF, a large, aqueous, sulphydryl-specific alkylating agent. To measure this reactivity, isolated *E. coli* membranes containing the full-length receptor of interest (∼5 µM total monomer) were incubated with excess fluorescent probe (500 µM) for a fixed interval (10 min at 25 °C). Subsequently, one aliquot was removed and immediately quenched with β-mercaptoethanol to destroy the remaining IAF, while a second aliquot was denatured with SDS to allow the labeling reaction to proceed to completion in the unfolded receptor before quenching. The wild-type, cysteine-less receptor yielded no detectable labeling under these conditions. The cysteine-containing engineered receptors, by contrast, yielded substantial but variable levels of labeling depending on the cysteine location. Solvent-exposed cysteines were identified as those exhibiting similar extents of alkylation in the folded and unfolded receptor states, while buried cysteines were recognized as those that were protected from alkylation in the folded state. The latter buried residues yielded as much as 10-fold lower alkylation in the folded membrane-bound state than in the denatured state.

A chemical reactivity parameter was defined as the ratio of IAF labeling in the folded and unfolded receptor states. This parameter ranges from zero (for a cysteine fully protected from labeling in the folded state) to unity (for a fully solvent-exposed...
cysteine). The reaction conditions were optimized to provide a large dynamic range between the measured chemical reactivities while maintaining the average value below 0.5, thereby ensuring that most reactions did not approach completion in the unfolded receptor. The method can be adapted for other regions of the same or different proteins by adjusting the reaction conditions appropriately. In general, studies of buried structural elements will require stronger reaction conditions (increased label concentration, pH, temperature, or reaction time), while more exposed elements will entail milder conditions.

The ability of the chemical reactivity parameter to resolve solvent-exposed and buried positions was tested in a control surface helix of known structure, specifically the cysteine-scanning positions 95 through 103 in helix $\alpha_2$ of the periplasmic domain. Fig. 2 presents the relative chemical reactivity of each engineered cysteine, and also shows the calculated solvent accessibility of the $\beta$-carbon at the corresponding position in the periplasmic domain crystal structure (32, 82). The graph displays a striking correlation between the measured chemical reactivity and the calculated solvent exposure, indicating that the chemical reactivity is determined primarily by accessibility to the large, solvent-based probe. In principle, deviation from a perfect correlation could arise from (i) a minor deviation of the crystal structure from the structure of the native protein; (ii) local electrostatic or steric forces that, in addition to the solvent exposure, modulate the alkylation rate; or (iii) a structural perturbation triggered by a given cysteine substitution. Overall, the results indicate that such deviations are not typical and that the chemical reactivity can be used to accurately map out exposed and buried positions in a representative protein structure, even when the protein is embedded in a native membrane containing a mixture of protein components.

**Determination of Solvent Exposure for Targeted Cytoplasmic Positions**—Cysteines scanned through positions 250–309 of the cytoplasmic domain reveal an $\alpha$-helical pattern of chemical reactivities, as displayed in the cysteine-scanning plot of Fig. 3. Highly exposed positions are defined as those exhibiting a chemical reactivity above 0.6 (upper dashed line), while highly buried residues are those exhibiting a chemical reactivity below 0.3 (indicated by the upper and lower dashed lines in Fig. 3, respectively). Positions 270–306 display a clear oscillation between highly reactive and unreactive sulfhydryls, with a periodicity distinct from that expected for a $\beta$-strand but matching that exhibited by a surface-exposed $\alpha$-helix. In particular, local reactivity minima representing the most buried residues are located at residues 271, 278, 285, 292, 299, and 306, revealing a 7-fold periodicity characteristic of an $\alpha$-helix involved in a coiled-coil or four-helix bundle. The same 7-fold periodicity is observed for local reactivity maxima representing the most exposed residues at positions 274, 281, 288, 295, 302,
and 309, providing further evidence for α-helical secondary structure. Furthermore, when these highly exposed and buried positions are mapped onto a helical wheel, they fall on the opposite faces of an amphiphilic α-helix, respectively, such that the exposed positions lie on the charged face and the buried positions lie on the hydrophobic face (see model in Fig. 6A under “Discussion”). At the N-terminal end of the scanned region, residues 250–264 exhibit few highly buried positions, indicating that secondary structure elements that may be present in this region are largely solvent-exposed and thus not as easily recognized by oscillating chemical reactivities.

Throughout the scanned region, the observed chemical reactivities are unchanged, within error, by the addition of saturating aspartate (1 mM). Such insensitivity to ligand indicates that the pattern of solvent exposure and burial within the region is not detectably altered by the ligand-induced conformational change. Overall, the results reveal the presence of an amphiphilic α-helix with distinct buried and exposed faces, extending at least from positions 270 through 309. Hereafter, this helix is termed the “first methylation helix” to distinguish it from a second methylation segment also located in the cytoplasmic domain. The exposed face of the first methylation helix includes three regulatory methylation sites at positions 295, 302, and 309, while the opposite, hydrophobic face is buried in both the apo- and aspartate-occupied states of the receptor.

Identification of Side Chains Critical for Receptor Function in Vivo—The effect of each cytoplasmic cysteine substitution on receptor function in vivo was measured to identify critical side chain positions in the working, fully assembled receptor-kinase complex. The plasmid pSCF6 encoding a wild-type or engineered receptor was transformed into the E. coli strain RP8611, which lacks chemoreceptors but contains an otherwise complete chemotaxis system (69). Overexpression of a functional receptor restores the ability of cells to migrate or “swarm” up a self-generated concentration gradient of aspartate on semi-solid agar plates (31, 78). This relatively insensitive assay is designed to identify the most extreme receptor perturbations that largely destroy receptor function, while more subtle receptor perturbations are detected by a sensitive in vitro assay (see below). The present analysis focuses on the functional effects of the engineered cytoplasmic cysteines, which are presumed to exist in their free sulfhydryl state due to the reducing environment of the cytoplasm.

Fig. 4A summarizes the aspartate-specific swarm rates observed as the mutant cysteine is scanned through the targeted region of the cytoplasmic domain. Cysteine substitutions that suppress activity to less than 30% of the wild-type aspartate-specific swarm rate are classified as inhibitory (below the dashed line in Fig. 4A). Each of these inhibitory substitutions generates a substantial receptor perturbation that cannot be overcome by receptor adaptation or overexpression. Interestingly, all five of the cysteine substitutions from positions 262 through 266 virtually eliminate receptor function (I262C, D263C, and T264C) or expression (V265C and T266C). It follows that the side chains at these consecutive positions are critical to receptor assembly, function, or stability. Just C-terminal to these positions lies the first methylation helix identified by chemical reactivity measurements, spanning residues 270–309. Within this helix, nine inhibitory substitutions are observed to lie on the buried, hydrophobic face (see model in Fig. 6B under “Discussion”), while only two inhibitory cysteines lie on the solvent-exposed face. Thus, the in vivo functional assay reveals that the buried face of the first methylation helix is more easily perturbed by cysteine substitution.

Identification of Side Chains Critical for Receptor Function in Vitro—Further functional analysis of the engineered receptor utilized the reconstituted receptor-kinase signaling complex, which provides a sensitive in vitro assay for the effect of cysteine substitutions on receptor-mediated kinase regulation (18, 19, 31). The reconstituted complex consisted of the semi-purified receptor in isolated E. coli membranes to which the purified soluble components were added, including the coupling protein CheW, the histidine kinase CheA, and the aspartate kinase CheY. A sufficient molar excess of CheY was used to ensure that receptor-regulated histidine kinase activity was the rate-limiting step in the formation of phospho-CheY, which was quantified by its level of 32P incorporation. Under these conditions, the apo-wild-type receptor activates the bound histidine kinase, while aspartate binding to the periplasmic domain of the receptor inhibits the cytoplasmic kinase activity by a factor of 102- to 103-fold (18, 19, 31). Due to this large dynamic range, the in vitro phosphorylation assay is highly sensitive to subtle receptor perturbations.

Fig. 4B summarizes the effects of cysteine substitutions on kinase activation by the apo-receptor in its reduced state. Plotted in this figure are receptor-activated phosphorylation rates, where each rate has been converted to a specific activity by normalization to the rate observed for the same concentration of wild-type receptor. Interestingly, 10 of the 60 cysteine substitutions increase the specific phosphorylation activity at least 1.5-fold while, at the other extreme, 19 substitutions inhibit the phosphorylation activity over 10-fold. Within the
first methylation helix identified by chemical reactivities, 16 super-activating and inhibitory substitutions lie on the hydrophobic, buried face, while only 6 lie on the exposed face (see model in Fig. 6B under "Discussion"). Thus, the in vitro assay of receptor-mediated kinase activity indicates that the buried face of the first methylation helix is more easily perturbed by cysteine substitution than its exposed face, although the observation of several perturbing substitutions on the exposed face shows that this helix surface is also important to receptor structure or signaling.

Comparison of the in vivo and in vitro activity data (Fig. 4) reveals several cysteine substitutions that inhibit receptor function over 10-fold in both assays (substitution positions I262, R269, I275, I282, S290, R292, A297, and I301). Most of these perturbations are associated with non-conservative substitutions that could disrupt receptor structure or its interaction with other proteins, such as cysteine for isoleucine, arginine, or glutamate. Interestingly, all three cysteine for isoleucine substitutions completely block receptor function and yield intermediate solvent exposures in the chemical reactivity assay (Fig. 3). In the native receptor, these isoleucines may be partially solvent-exposed, or they may reside in highly buried packing interfaces that are partially disrupted by cysteine substitution. The latter type of structural perturbation, if present, is not sufficiently common to interfere with secondary structure identification. For example, many highly buried cysteines are observed on the hydrophobic faces of the control helix o2 and the first methylation helix (see Figs. 2–4 and the model in Fig. 6 under “Discussion”).

Identification of Functionally Important Helix-Helix Contacts—The cysteine-scanning results demonstrate the existence of the first methylation helix spanning at least positions 270–309, and illustrate the sensitivity of its buried surface to mutagenic perturbations. Cysteine scanning alone, however, could not identify the structure(s) against which the amphipathic helix is packed to generate its buried surface. To ascertain whether the packing interaction might lie at the interface between subunits, a disulfide mapping study was undertaken, making use of pairs of symmetric cysteines found within each molecule of the engineered, homodimeric receptor. Previous studies have successfully used disulfide formation rates or extents to map out helix-helix contacts or to detect thermal collisions between distal cysteines (29, 37, 41, 44, 46, 81, 83, 84). Surprisingly, mild oxidation of receptor-containing E. coli membranes, followed by detection of disulfide-linked dimers by a standard gel shift assay, yielded at least 90% disulfide formation in each of the 58 engineered receptors. Attempts to measure initial rates of disulfide formation formation were unsuccessful because all the rates were too fast to measure under standard reaction conditions (each rate constant was approximately 1 s⁻¹ molecule⁻¹ or faster). As a result, comparisons of disulfide formation rates could not be carried out to identify proximal and distal cysteine pairs. These findings indicate that the cytoplasmic domain of the membrane-bound receptor possesses an unusually dynamic structure in which even distal cysteine pairs can rapidly collide and form a disulfide bond. Such extensive backbone fluctuations are consistent with the dynamic structure previously observed for the isolated cytoplasmic domain in solution (50).

Although the unusually dynamic nature of the scanned region prevented the use of disulfide formation rates to probe structure, an intersubunit packing face was nevertheless detected by a function-based scanning approach. The successful strategy, termed “disulfide scanning,” measured the effect of each intersubunit disulfide bond on receptor function as previously illustrated by analogous studies of the periplasmic and transmembrane helices (31, 38, 42, 46, 83). Briefly, oxidized membranes containing disulfide-linked receptors were incubated with CheA, CheW, and CheY to reconstitute the receptor-kinase signaling complex, then the ability of each receptor to regulate kinase activity in vitro was measured as described earlier. In effect, this disulfide-scanning experiment systematically moved a unique intersubunit disulfide bond through consecutive positions of the targeted region and determined the functional impact of a covalent constraint at each position. Of the 58 disulfide bonds examined, three were found to lock the receptor in the kinase-activating state as illustrated under Fig. 5. These receptors contained a disulfide bond at position 278, 285, or 300 and retained 25–75% of the wild-type kinase activation, both in the apo state and in the presence of 1 mM aspartate. Separate binding measurements confirmed that 1 mM aspartate was sufficient to saturate each of these receptors with bound ligand.2 Thus, the three “lock-on” disulfide bonds appear to rigidly constrain or trap the cytoplasmic domain in its kinase-activating state, regardless of ligand occupancy. Furthermore, these three lock-on disulfides all cluster to the buried, hydrophobic face of the first methylation helix defined by chemical reactivity, thereby placing this face at an interface between two subunits in the kinase-activating state of the receptor.

More specifically, the lock-on disulfides covalently link the symmetric first methylation helices of the two subunits within the same dimer, as indicated by the following experiment. Three double-mutant receptors were generated, in which each subunit contained one cysteine at a lock-on disulfide position and a second cysteine at position 36 in the periplasmic domain (Cys-36/Cys-278; Cys-36/Cys-285; Cys-36/Cys-300). Previous studies of the isolated periplasmic domain have shown that upon oxidation, the interfacial Cys-36–Cys-36' disulfide bond forms rapidly between two subunits within the same dimer, thereby covalently stabilizing the native oligomeric structure (29, 31, 32). Thus, when a second intersubunit disulfide is introduced, the additional cross-link will either further stabilize the existing Cys-36–Cys-36' covalent dimer, or will form between existing dimers to yield covalent tetramers and higher order oligomers. In practice, the standard oxidation protocol is

FIG. 5. Effect of lock-on disulfides on receptor-mediated kinase regulation. In vitro activity was assayed as described in Fig. 4B. Shown are the relative rates of phospho-CheY production in the absence (open bar) and presence (shaded bar) of 1 mM aspartate. Assays utilized the indicated oxidized receptors in which intersubunit disulfide formation was driven to completion (see “Experimental Procedures”). No disulfide formation was observed for the wild-type receptor, which lacks cysteines.
found to convert each of the three double-mutants to a population of receptors containing over 90% covalent dimers, wherein a given dimer possesses two intradimer disulfide bonds. It follows that each lock-on disulfide forms between two subunits within the same dimer, thereby covalently trapping a native packing interaction between the symmetric pair of first methylation helices at the subunit interface.

DISCUSSION

The present study illustrates the use of cysteine and disulfide scanning to map out secondary structure and packing interactions within an unknown receptor structure, and to probe the roles of these structural features in receptor mechanism. The described cysteine-scanning and chemical reactivity measurements reveal an $\alpha$-helical pattern of solvent exposure for residues 270–309, wherein the most highly exposed and buried positions each exhibit the 7-fold repeating pattern of an $\alpha$-helix involved in a coiled-coil or four-helix bundle. The observed helix is strongly amphipathic, with the exposed and buried residues lying on opposite helix faces containing the majority of charged and hydrophobic side chains, respectively (see helix $\alpha_6$ in Fig. 6A). This helix, termed the first methylation helix, possesses three of the four receptor methylation sites on its exposed, highly anionic face. Its opposite buried face is more easily perturbed by cysteine substitutions than the exposed face (Fig. 6B), suggesting that the packing interactions of the first methylation helix exhibit critical tolerances that modulate receptor function. Disulfide scanning identifies three lock-on disulfide bonds that place the buried helix face at the subunit interface, where it packs against a symmetric helix face provided by the other subunit in the same dimer. The exclusive association of the lock-on disulfides with this interface indicates that the observed helix-helix packing interaction is central to kinase activation. Interestingly, the presumed regulation of this interface by the transmembrane signal may

$^3$ R. B. Bass and J. J. Falke, unpublished data.
The present results, however, provide strong experimental support for the existence of the first methylation helix in the periplasmic and transmembrane domains (40). Overall, the results demonstrate the existence of the first methylation helix and indicate its equilibrium stability at the subunit interface of the isolated, membrane-bound receptor.

The existence of the first methylation helix explains a number of previous observations, including the apparent helical periodicity of the methylation sites, as well as the periodicity of residues involved in recognition of the methyltransferase CheR (22, 63). The importance of this region to kinase regulation had been suggested by observation that neutralization of the three regulatory glutamates, either by methylation or amidation, stimulates kinase activity by a factor of as large as 10^2 to 10^3-fold (49, 62, 85). Moreover, most of the random, second-site substitutions in the first transmembrane helix (A19K) are located within this region (58), where they can be presumed to restore a critical structural or regulatory element. Finally, studies of the isolated cytoplasmic domain have previously implicated subunit-subunit interactions as important for kinase activation (49, 62). The present results, however, provide the first direct experimental evidence defining a specific α-helix in the cytoplasmic domain of a bacterial chemoreceptor. These results also identify a specific, functionally critical helix-helix packing interaction at the subunit interface of the dimeric receptor.

Despite the equilibrium stability of the first methylation helix, the scanned region of the cytoplasmic domain is remarkably dynamic. All of the 58 intersubunit cysteine pairs examined in this region are able to rapidly form disulfide bonds indicating that their sulphydryl-sulphydryl collision rates are considerably faster than observed in the periplasmic and transmembrane domains (51, 58). Moreover, even the most buried positions in the scanned region react at least 50-fold more rapidly with the aqueous alkylating agent than the most buried position detected in the periplasmic domain, which possesses a more static structure. Similarly, previous NMR results that have shown the isolated periplasmic domain to be better ordered than the isolated cytoplasmic domain, which exhibits characteristic features of a molten globule (36, 50). The new results demonstrate that the notable dynamics are not limited to the isolated domain, but are present in the full-length, membrane-bound receptor as well. It is not yet known whether the fully assembled receptor-CheW-CheA ternary complex retains these dynamics, or rather gains structural stability through quaternary contacts.

To determine whether the observed helix is conserved in a large class of related receptors, the aligned sequences of 56 homologous cytoplasmic domains can be compared (56, 57). Such an alignment reveals evidence of two α-helices, termed α5 and α6, in the region corresponding to residues 250–309 of the aspartate receptor (56, 57), as illustrated in Fig. 6A, where α6 contains the region identified herein as the first methylation helix. Both putative helices exhibit the heptad repeating pattern of hydrophobic and polar residues typical of helices involved in coiled-coils or four-helix bundles: a-b-c-d-e-f-g, where residues a and d are usually hydrophobic (53, 56, 86–89). The a and d positions are occasionally occupied by a polar residue, particularly Ser, Thr, Asn, or Gln, which can form a specific, interhelix hydrogen bond that stabilizes a specific register of the helix-helix packing interaction (90, 91). Putative helices α5 and α6 are further predicted to be separated by a short bend or linker between residues Leu-261 and Val-265, where a phase-shift is observed in the heptad repeating pattern of the eight enterobacterial chemotaxis receptors, including the aspartate receptor (56, 57). Such a phase-shift is incompatible with a continuous, canonical α-helix through this region. Moreover, an alignment of more distantly related prokaryotic receptors reveals 28 examples of insertions within the putative junction, suggesting that the junction can, in such cases, accommodate a larger loop (56, 57). Finally, flexible linker elements are often proteolytically susceptible, and it is notable that the principal trypsin cleavage site of the cytoplasmic domain, Arg-259, lies in the final, presumably dynamic turn of putative helix α5 just before the α5-α6 junction begins (47).

The present findings provide strong experimental support for the existence of the putative conserved helices α5 and α6 in the aspartate receptor, as well as the junction between them. As noted above, the chemical reactivity measurements directly demonstrate that the first methylation helix includes residues 270 through 309, which fall within the region identified as helix α6 by the sequence analysis (Fig. 6A, residues 270–309). The evidence for putative helix α5 is less convincing, since the tested region is smaller and exhibits a weaker pattern of oscillating solvent exposure. However, all six of the highly buried or exposed residues detected in this region map to the appropriate face of putative helix α5, consistent with the existence of α-helical secondary structure. The greater accessibility of buried positions on putative helix α5 suggests that this helix is more dynamic or more solvent-exposed than α6. It should be noted that, in general, chemical reactivity measurements do not ac-
curately define the ends of amphiphilic helices, since the helix termini can be relatively dynamic and therefore lack highly buried positions. Thus, helical regions defined by cysteine scanning may underestimate the lengths of individual helix elements.

Fig. 6B maps out the functional perturbations due to cysteine substitutions on the putative conserved helices α5 and α6. The buried face of the first methylation helix, corresponding to α6, is especially critical for kinase regulation, since 18 of the perturbing mutations associated with the methylation helix map to its buried face. The existence of seven perturbing mutations on its exposed face is consistent with the known importance of this face to receptor adaptation and recognition of the CheR adaptation enzyme (22, 24, 63). Interestingly, no single cysteine substitution at a methylation site destroys receptor function, an observation that lends further support to the previously proposed redundancy of the function, an observation that lends further support to the precedence of this face to receptor adaptation and recognition of the CheR adaptation enzyme (22, 24, 63). Interestingly, no single cysteine substitution at a methylation site destroys receptor function, an observation that lends further support to the

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