Detection of a Conserved $\alpha$-Helix in the Kinase-docking Region of the Aspartate Receptor by Cysteine and Disulfide Scanning*

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The transmembrane aspartate receptor of Escherichia coli and Salmonella typhimurium propagates extracellular signals to the cytoplasm, where its cytoplasmic domain regulates the histidine kinase, CheA. Different signaling states of the cytoplasmic domain modulate the kinase autophosphorylation rate over at least a 100-fold range. Biochemical and genetic studies have implicated a specific region of the cytoplasmic domain, termed the signaling subdomain, as the region that transmits regulation from the receptor to the kinase. Here cysteine and disulfide scanning are applied to the N-terminal half of the signaling subdomain to probe its secondary structure, solvent exposure, and protein-protein interactions. The chemical reactivities of the scanned cysteines exhibit the characteristic periodicity of an $\alpha$-helix with distinct solvent-exposed and buried faces. This helix, termed $\alpha_7$, ranges approximately from residue 355 through 386. Activity measurements probing the effects of cysteine substitutions in vivo and in vitro reveal that both faces of helix $\alpha_7$ are critical for kinase activation, while the buried face is especially critical for kinase down-regulation. Disulfide scanning of the region suggests that helix $\alpha_7$ is not in direct contact with its symmetric partner ($\alpha_7'$) from the other subunit; presently, the structural element that packs against the buried face of the helix remains unidentified. Finally, a novel approach termed "protein interactions by cysteine modification" indicates that the exposed C-terminal face of helix $\alpha_7$ provides an essential docking site for the kinase CheA or for the coupling protein CheW.

A fundamental question in signaling biology concerns the mechanism by which cell surface receptors regulate cytoplasmic kinases. Certain receptors activate their associated kinases by dimerization, but in other cases kinase activation is triggered by a transmembrane conformational change. One class of receptors that generates such conformational regulation is the large group of cell surface receptors that modulate histidine kinases in prokaryotic and eukaryotic two-component signaling pathways (reviewed in Refs. 1–9). The aspartate receptor of bacterial chemotaxis is representative of this diverse class. The aspartate receptor is one of the ligand specific di-meric receptors utilized by the related chemotaxis pathways of Escherichia coli and Salmonella typhimurium to recognize periplasmic attractant and repellent molecules. This receptor binds aspartate, an attractant, in the periplasm and propagates a signal across the bilayer to an associated histidine kinase in the cytoplasm. Receptor-kinase coupling is provided by the formation of a kinetically stable ternary complex between the cytoplasmic domain of the receptor, the coupling protein CheW, and the histidine kinase CheA (10, 11). The cytoplasmic phosphorylation cascade is initiated by receptor-stimulated CheA autophosphorylation; subsequently, phospho-CheA becomes the substrate for a phospho-transfer reaction that phosphorylates one of two response regulator proteins, CheY or CheB (12, 13). Phospho-CheY controls the swimming activity of the cell by docking to the flagellar motor, while phospho-CheB regulates the adaptation branch of the chemotaxis pathway by hydrolyzing the methyl esters of specific glutamates that serve as receptor adaptation sites (14–17). The level of receptor-mediated CheA kinase activation or down-regulation is determined by the sum of the signals generated by ligand binding to the receptor in the periplasm, and the level of receptor methylation in the cytoplasm (12, 13). Since aspartate binding down-regulates the kinase activity while receptor methylation stimulates the kinase, the two signals oppose each other to provide a negative feedback loop that optimizes the signal output of the ternary complex (2).

The bacterial chemoreceptors, including the aspartate receptor, belong to the taxis receptor subfamily of the two-component receptor superfamily. The taxis receptor subfamily spans at least 60 known homologues proposed to initiate the thermo-, photo-, osmo-, redox-, and chemotaxis pathways of a wide variety of prokaryotic organisms (18–25). The primary structures of subfamily members diverge greatly in their periplasmic domains, which are specific for different stimuli; however, their cytoplasmic domains are highly conserved (18, 19). These receptors also possess a conserved mechanism of transmembrane signaling and kinase regulation, as demonstrated by the formation of active chimeric receptors via the fusion of receptor domains from distantly related pathways (26–29). Moreover, an active chimera has been generated by fusing the transmembrane signaling domain of the aspartate receptor and the tyrosine kinase domain of the human insulin receptor, indicating that even these unrelated receptors may use similar mechanisms of conformational transmembrane kinase regulation (30, 31).

The periplasmic and transmembrane regions of the aspartate receptor are structurally well characterized. The receptor is a homodimer of two stably associated 60-kDa subunits (32). A high resolution crystal structure of the isolated periplasmic domain has revealed a dimer of two four-helix bundles ($\alpha_1$-$\alpha_4$ and $\alpha_1'$-$\alpha_4'$), where the symmetric helices $\alpha_1$ and $\alpha_1'$ form a tightly associated coiled coil at the dimer interface (1). The structure of the transmembrane region has been characterized by disulfide mapping and consists of a bundle of the four membrane-spanning helices $\alpha_1$/$TM1$, $\alpha_1'/TM1'$, $\alpha_4/TM2$, and $\alpha_4'/TM2'$ (33–42). The mechanism of transmembrane signaling is a subtle piston-type displacement of the signaling helix.

* This work was supported by National Institutes of Health Grant GM40731 (to J. J. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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α4/TM2 toward the cytoplasm, as observed in the superimposed crystal structures of the apo- and aspartate-occupied periplasmic domain (43). Further evidence for the piston displacement is provided by engineered disulfide bonds that lock the membrane-bound receptor in the on and off states, by ligand-induced changes in disulfide formation rates detected in a closely related receptor for ribose and galactose, and, most recently, by spin-labeled ESR measurements (34, 39, 43–45).

Until recently, comparatively little structural information has been available for the cytoplasmic domain of the receptor, because its unusually dynamic conformation has prevented analysis by high resolution methods (46). Earlier hydrodynamic studies indicated that the cytoplasmic domain is highly elongated, while circular dichroism studies revealed a high degree of α-helicity (47, 48). The cytoplasmic domain is functionally critical, because it contains the adaptive methylation sites and the docking sites for the kinase CheA and coupling protein CheW in the active ternary complex. Once formed, the ternary complex containing the receptor, CheW, and CheA, is stable for tens of minutes, regardless of the receptor ligand occupancy and methylation state (10, 11). The adaptive methylation sites are glutamate side chains located at positions 295, 302, 309, and 491 within the cytoplasmic domain (49). These residues are methyl-esterified by the methyltransferase CheR, which binds to a specific site at the receptor C terminus, and are demethylated by phospho-CheB (50–52). The signaling subdomain, extending approximately from residue 348 through 427, lies between the third and fourth methylation sites (18, 19). This subdomain appears to constitute an independent folding unit that binds CheW and CheA and modulates the rate of CheA autophosphorylation (53–56).

Recent cysteine and disulfide scanning studies have revealed helices α4, α5, and α6 as well as structurally linked latches between these helices within the cytoplasmic domain (57–59). Helices α5 and α6 were determined by disulfide mapping to be at the dimer interface. Helix α6 contains three of the four sites of adaptive methylation at positions 295, 302, and 309. However, despite its functional significance, the structure of the signaling subdomain remains unknown. Genetic studies have shown that this subdomain contains the site(s) of interaction with the coupling protein CheW, and biochemical studies have implicated it as a major locus of CheA interactions as well (53–55, 60). Finally, the signaling subdomain of a related receptor has been shown to be unusually susceptible to mutations that lock the swimming behavior of cells (61). Given these observations, the signaling subdomain is a prime target for structural studies designed to characterize the mechanism by which a receptor-mediated transmembrane signal is translated by the cytoplasmic domain into kinase regulation.

Cysteine and disulfide scanning has proven to be an invaluable tool for the determination of protein structure and has the advantage of not being limited by the size of the macromolecule to be studied (33–35, 41, 42, 44, 62, 63). This technique has been successfully employed in previous structural and mechanistic studies of the bacterial chemoreceptors that revealed the aforementioned helices α4–α6 of the cytoplasmic domain (57, 58). The present study extends the cysteine and disulfide scanning approach to the N-terminal half of the signaling domain, where the goal is to elucidate the secondary structure, packing, and function of the region. The results reveal an α-helix with distinct exposed and buried faces. Both of these faces are critical for kinase activation, while the buried face is particularly critical for kinase down-regulation. The exposed face near the C terminus of the helix is found to provide a docking site for CheW, CheA, or both, as revealed by the protein interactions by cysteine modification (PICM)1 method.

EXPERIMENTAL PROCEDURES

Materials—The E. coli strains utilized were kindly provided by Dr. John S. Parkinson (University of Utah, Salt Lake City, UT). Strains used for receptor expression and characterization were DR3808 (∆cheA-cheZ)/DE2209 trs-1 leuB6 his-4 eda-50 rpsL136 [thl-1 Δ(gal-attl)/DE99 ara-14 lacY1 mtl-1 xyl-5 tonA31 tpx-78]/(mks) and RP8611(Δ/CP362 of G. Hazelbauer via F. Dahlquist, pa/)

(64). The receptor expression plasmid pSCF6 has been previously described (33). Expression strains and plasmids used to produce CheA (HB101/pMO4), CheW (HB101/ pME5), and CheR (JM109/pME43) were generously provided by Dr. Jeff Stock (Princeton University, Princeton, NJ). The strain and plasmid used to generate CheY, (RBB455/pRB440), were kindly provided by Dr. Bob Bourret (University of North Carolina, Chapel Hill, NC). Chemical reactivity probes, 5-IAF, 5-FM, and IANBD were obtained from Molecular Probes, Inc. (Eugene, OR). S-Adenosyl-l-[methyl-3H]methionine and [γ-32P]ATP were purchased from Amersham Pharmacia Biotech. Deoxynucleotide triphosphates were synthesized by Life Technologies, Inc. Kunkel mutagenesis reagents (T7 DNA polymerase, T4 DNA ligase, and deoxynucleotide triphosphates) were purchased from Bio-Rad. Unless specifically noted, all other reagents were obtained from Sigma and were reagent grade.

Creation of Cysteine-containing Receptors—Individual cysteine-containing receptors were created by oligonucleotide-directed mutagenesis of the plasmid pSCF6 by the method of Kunkel et al. as modified by the Bio-Rad phagemid mutagenesis kit (33, 65). Mutagenesis reactions were transformed into RP8611, and plasmids were isolated using Qiagen spin columns. Receptor genes encoding the appropriate cysteine substitution were verified by PCR plasmid sequencing using a modification of the Sanger method employing a thermocycler and reagents from Epicentre Technologies (66).

Purification of Engineered Receptors—Plasmids encoding individual cysteine substitutions were transformed into the strain RP3808 (64). Receptors were purified as described previously (57) with the following modifications. 18 h after inoculation with saturated 2-ml Luria broth cultures, 500-ml Vogel-Bonner citrate minimal media cultures (67) were harvested by centrifugation in a GS-3 rotor (Sorvall) at 6000 rpm (6080 × g) for 10 min. Cells were resuspended in 6 ml of ice-cold low salt buffer containing 20 mM sodium phosphate, pH 7.0, with NaOH, 10% (v/v) glycerol, 10 mM EDTA, 50 mM DTT, 2.5 mM 1,10-phenanthroline, and 0.5 mM phenylmethylsulfonyl fluoride in thin walled ultracentrifuge tubes (Beckman) in an ice/salt water bath and lysed by sonication (3 × 20-s bursts with 20-s cooling intervals using a Mysonics model W-385 sonicator equipped with a macrotip). Cell debris was pelleted in a TLA 100.3 rotor (Beckman) at 15,000 rpm (12,000 × g) for 20 min. Supernatants were transferred to fresh tubes, and membranes were pelleted in a TLA100.3 rotor at 100,000 rpm (540,000 × g) for 15 min. Pellets were resuspended in 0.5 ml of high salt buffer, 20 mM sodium phosphate, pH 7.0, with NaOH, 2 mM KCl, 10% (v/v) glycerol, 10 mM EDTA, 5 mM DTT, 2.5 mM 1,10-phenanthroline, and 0.5 mM phenylmethylsulfonyl fluoride. Re-suspension was achieved by placing tubes in an ice/salt water bath and sonicating with the aforementioned sonicator equipped with a microtip (2 × 20-s bursts and 20-s cooling intervals using a Mysonics model W-385 sonicator equipped with a macrotip).

1 The abbreviations used are: PICM, protein interactions by cysteine modification; DTT, dithiothreitol; 5-IAF, 5-iodoacetamidofluorescein; 5-FM fluorescein-5-maleimide; IANBD, N’-(2-iodoacetoxy)ethyl-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole; DMF, N,N-dimethylformamide.
intervals). Membranes were diluted to 3 ml in high salt buffer, pelletted again, and resuspended as above, except DTG and phenanthroline were omitted. Finally, receptor-containing membranes were resuspended in 0.5 ml of final buffer containing 20 mM sodium phosphate, pH 7.0, with NaOH, 10% (v/v) glycerol, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride before diluting to 3 ml in final buffer, pelletting as above, and resuspending in 300 μl of final buffer. The resulting membranes were snap frozen in liquid nitrogen and stored at −80 °C.

Protein samples were assayed for total protein using the BCA assay (Pierce) calibrated against bovine serum albumin standards (Pierce). After development, absorbance measurements were made using a microplate reader (Molecular Devices, Inc.). Protein purity was determined by quantitating the receptor and nonreceptor bands on a Coomassie-stained 10% Laemmli SDS-polyacrylamide gel (acrylamide:bisacrylamide ratio of 40:0.2) using a digital camera (Alpha Innotec) (68).

Preparation of Cytoplasmic Chemotaxis Components—CheA, CheW, CheY, and CheR were produced and isolated as described previously (33, 57).

Chemical Reactivity Assays—Reactivity assays were performed as described previously (58) with the following modifications. All quantitation was performed using a digital camera (Alpha Innotec). Alkylation of each engineered receptor was performed with 5-IAF except in the case of the periplasmic control positions Thr39 through His103, where both 5-IAF and IANBD were used as labels. Reaction conditions were essentially as described previously (58). Briefly, the assay utilized a 40-μl reaction containing 5 μM receptor monomer in reaction buffer (10 mM sodium phosphate, pH 6.5, with NaOH, 50 mM NaCl, 50 mM KCl, and 10 mM EDTA), to which was added 300 μM 5-IAF in DMF. The reaction was allowed to proceed at 25 °C for 5 min. At that point, the reaction mixture was split, and half of the sample was quenched with 1.25 μl of β-mercaptoethanol to react with any remaining probe. The remainder of the sample was denatured with 0.6% (w/v) SDS at 95 °C, allowed to react for an additional 3 min, and then quenched as before. To each reaction was added 5 μl of 4× nonreducing Laemmli sample buffer, and then each was heated to 95 °C for 2 min prior to resolving on a 10% Laemmli SDS-polyacrylamide gel (acrylamide:bisacrylamide ratio of 40:0.2). Fluorescent receptor bands were visualized on a UV light box (Alpha Innotec) and quantitated with a digital camera (Alpha Innotec). Gels were subsequently stained with Coomassie, and the resulting receptor bands were quantitated with a digital camera to provide normalization of the fluorescent intensities for variations in the amount of receptor. Chemical reactivity was defined as the ratio of receptor alkylation in the folded versus the unfolded states.

In Vivo Activity Assays—Chemotaxis swarm assays were performed in vivo as described previously (57, 69). Controls using vector alone (pBluescript) and vector carrying the wild type receptor (pSCF6) were performed to determine the swarm rates of receptorless cells and cells possessing the native receptor, respectively. All swarm rates were determined on minimal media agar plates lacking or containing 100 μM aspartate. The aspartate-specific swarm rates were determined by subtracting the minus aspartate swarm rate from the plus aspartate swarm rate to correct for pseudotaxis (58), and the resulting rate was then normalized to the wild type rate for comparison.

In Vitro Activity Assays—The in vitro receptor-coupled kinase assay was performed essentially as described with the following modifications (12, 13, 57). Standard membranes containing the reduced receptor were utilized except in experiments testing the effects of engineered disulfide bonds (see below). The receptor-coupled kinase reaction was initiated by the addition of [γ-32P]ATP to the reaction mixture containing receptor, CheW, CheA, and excess CheY. After 10 s, aliquots were removed and quenched with 2× Laemmli sample buffer supplemented with 25 mM EDTA. The concentration of proteins used in the standard assay ensures that receptor-regulated CheA autophosphorylation is the rate-determining step, such that the rate of phosphotransfer to CheY is linearly proportional to the CheA autophosphorylation rate. 32P-Phosphorylated CheY was resolved on a 15% Laemmli SDS-polyacrylamide gel (acrylamide:bisacrylamide ratio of 40:1.25). Gels were dried and quantitated by PhosphorImager analysis (Molecular Dynamics, Inc.). In a 10-μl wild type reaction mixture containing 60 pmol of receptor monomer, 20 pmol of CheY, 100 pmol of CheA, 2.6 pmol of CheA, and 1000 pmol of ATP and reaction buffer (50 mM Tris, pH 7.5, with HCl, 50 mM KCl, and 5 mM MgCl2), phospho-CheY was produced at a rate of 0.02 pmol s−1.

In some assays the engineered cysteines were oxidized to form an intersubunit disulfide bond in order to test the effect of this cross-link on receptor-mediated kinase regulation (33, 34). Briefly, membrane samples containing 12 μM receptor monomer were incubated with 0.2 mM Cu(II)(1,10-phenanthroline)3 and ambient O2 (approximately 200 μM) for 20 min at 37 °C. This oxidation was inactivated by the addition of 0.1 mM sodium persulfate, and the resulting membranes were used in the receptor-coupled kinase assay as above. The extent of disulfide formation was determined by analyzing an aliquot of the oxidized membranes on a 10% nonreducing Laemmli SDS-polyacrylamide gel (acrylamide:bisacrylamide ratio of 40:0.2). Gels were subsequently Coomassie-stained, monomer and dimer bands were quantitated with a digital camera, and the percentage of dimer formation was calculated.

Disulfide Formation Rates—Each full-length, membrane-bound cysteine-containing receptor was diluted to a final concentration of approximately 2 μM in 20 mM sodium phosphate, pH 7.0, with NaOH, 10% (v/v) glycerol, 5 mM EDTA. The oxidation reaction was initiated by the addition of 1 mM Cu(II)(1,10-phenanthroline)3 and ambient O2 (approximately 200 μM) for 20 min at 37 °C. The oxidation reaction was stopped by adding an equal volume of 10 mM N-ethylmaleimide (33, 34, 59, 68). Samples were resolved on a 10% Laemmli SDS-polyacrylamide gel as in the chemical reactivity assay and Coomassie-stained, and the monomer and dimer bands were quantitated with a digital camera.

PICM—For each engineered receptor, a 15-μl reaction mixture containing 12 μM membrane-bound receptor monomer was mixed with either 150 μM 5-FM in DMF (final concentration) or, in the case of a control, 0.5 μM of DMF. The modification reaction was allowed to proceed at 25 °C for 1 min and then quenched with 35 mM DTT. These reaction tubes were held on ice until used just as in the in vitro receptor-coupled kinase assay as described above. As before, the membrane samples were incubated with CheA, CheW, and CheY in reaction buffer, followed by the addition of radiolabeled ATP, and the phosphotransfer reactions were allowed to proceed for 10 s before quenching. The resulting phospho-CheY bands were visualized and quantitated by PhosphorImager analysis.

As a control to determine whether the 5-FM labeling proceeded to completion, control reactions were performed as was in the chemical reactivity assay. A 40-μl reaction mixture prepared containing 5 μM receptor monomer was mixed with 125 μM 5-FM in DMF (final concentration). The reaction was allowed to proceed, as before, for 1 min at 25 °C; half of the sample was quenched with 50 mM DTT; and the remainder of the sample was denatured in 0.6% SDS at 95 °C and allowed to
react for an additional 3 min before quenching. The latter sample served as a control for maximum label incorporation. The extent of labeling was determined by quantitating the fluorescence in the receptor band using a digital camera.

In Vitro Methylation Assays—Receptor-containing membranes were first diluted to make an 11-μl stock of 10 μM receptor monomer. After labeling with 125 μM 5-FM in DMF and quenching (see above), the methylation reaction was carried out largely as described (70). Briefly, to the above sample was added 1 μl of CheR-containing cytosolic extract (15–20 mg/ml total protein) and potassium phosphate (pH 7.0 with NaOH) to 40 mM. The reactions were then allowed to equilibrate for at least 30 min at 25 °C to allow the receptor-CheR complex to form. The methylation reaction was initiated by the addition of a 1:1 ratio of 2 mM S-adenosyl-L-methionine to S-adenosyl-L-[methyl-3H]methionine (55–85 Ci/mmol, 1 μCi/μl), to achieve a final S-adenosyl-L-methionine concentration of 0.1 mM. Aliquots of the reaction were removed at 50, 60, and 70 s, spotted on 1 3 1-cm Whatman filter papers, and immediately placed in a rapidly stirring 10% (w/v) trichloroacetic acid bath for 10 min at 25 °C. The trichloroacetic acid wash was repeated twice, followed by washing in methanol for 2 min to remove residual trichloroacetic acid. The methanol wash was repeated, and filter papers were allowed to air dry for 5 min. Individual filter papers were placed in 0.65-ml Eppendorf tubes and then floated in 4 ml of Ecoscint H (National Diagnostics) in a scintillation vial. To the filter paper was added 100 μl of 1 M NaOH to hydrolyze the methyl esters, and the scintillation vials were capped. Liberated methanol partitioned into the scintillation mixture during an 18-h incubation at 37 °C, at which point the vials were scintillation-counted.

Standard Deviation—The error ranges given represent the S.D. for n ≥ 3.

FIG. 1. A schematic model of the full-length membrane-bound aspartate receptor, illustrating the various domains and subdomains. Cylinders represent helices determined by previous studies employing crystallography (1) and cysteine and disulfide scanning (53–56, 41, 42, 57, 58). The two 60-kDa subunits of the homodimer are depicted in white and gray, respectively. Filled circles represent the sites of adaptive methylation on each subunit (49). The open box denotes the region of the signaling subdomain probed by cysteine and disulfide scanning in the current study (residues Thr348 through Ala387).

RESULTS

Creation of Cysteine Library—To probe the signaling region of the cytoplasmic domain, a library of mutant receptors was created by cysteine scanning. Using oligonucleotide-directed mutagenesis to create a unique cysteine substitution in each receptor, the position of substitution was scanned from Thr348 through Ala387, representing approximately the N-terminal half of the signaling subdomain. Fig. 1 presents a schematic diagram of the receptor structure, illustrating the location of the scanned positions. Each engineered receptor was generated via plasmid overexpression in an E. coli strain that lacks receptors and the methylation and demethylation enzymes CheR and CheB, respectively (RP3808) (64). As a result, the expressed receptor population is homogenous with respect to the modification states of the four cytoplasmic methylation sites (Gln295, Glu302, Gln309, and Glu491). Altogether, 40 single cysteine-containing receptors were generated, 37 of which were expressed at or near wild type levels. The remaining three mutant receptors, A353C, N374C, and E383C did not accumulate at detectable levels in the membrane; thus, the native side chains at these positions are critical for receptor expression, folding, or stability.

The Chemical Reactivity Assay—Previous studies have demonstrated that the solvent-exposure pattern displayed by a library of scanned cysteines can map out surface-exposed secondary structure elements (57, 58). In the present study, the solvent exposure of each engineered cysteine was determined by assaying its chemical reactivity with 5-IAF, a large anionic sulfhydryl-specific probe. The charge and size of this aqueous probe virtually exclude it from the cores of proteins so that it reacts slowly with buried cysteines and rapidly with solvent-exposed cysteines.
To carry out the chemical reactivity assay, isolated *E. coli* membranes containing a given receptor were incubated with the 5-IAF probe at 25 °C for 5 min. Following this incubation, which was designed to yield less than 50% full labeling at the majority of cysteine positions, one half of the sample was quenched. The remaining half was denatured with SDS at 95 °C and allowed to react for an additional 3 min to ensure full labeling. Finally, both samples were run on a SDS-polyacrylamide gel, and the receptor fluorescence was quantitated with a digital camera. The ratio of fluorescence in the native to the denatured states yielded the chemical reactivity, which ranged from a value of 1 for a highly solvent-exposed sulfhydryl to a value of 0 for a fully buried sulfhydryl.

The ability of the chemical reactivity assay to detect the periodic fluctuation of solvent exposure due to local secondary structure elements was tested by a control study of helix a2, which lies within the known structure of the periplasmic domain. Using a library of receptors in which cysteine was scanned from positions Thr95 through His103 in helix a2, the measured chemical reaction ratio was found to be highly correlated with the solvent exposure calculated from the crystal structure coordinates of the periplasmic domain as illustrated in Fig. 2 (1, 71). Both the anionic probe 5-IAF and the neutral probe IANBD yielded similar results (Fig. 2), indicating that the chemical reactivity ratio is determined mainly by solvent exposure, rather than by interactions of the probe with local electrostatic fields.

**Chemical Reactivities of Scanned Cysteines in the Cytoplasmic Domain**—The measured chemical reactivity ratios for the scanned cysteines T348C through A387C within the signaling subdomain are summarized in Fig. 3. This figure also illustrates the reactivity levels used to operationally define highly buried, intermediate, and highly exposed cysteines. Eight cysteines were determined to be highly buried, yielding a native: denatured reactivity ratio of below 15% (S355C, S356C, I362C, I363C, T373C, N379C, A380C, and N386C). Nine positions were highly exposed, displaying reactivities greater than 35% (M349C, K357C, V365C, D367C, G368C, F371C, I375C, A381C, and A385C). When these positions were mapped onto different secondary structure models, it was found that the best segregation of highly buried and exposed residues occurs on an α-helix (see Fig. 7A). Thus, the results indicate the presence of an α-helix with distinct buried and exposed faces spanning approximately positions 355–386 in the signaling subdomain. No significant ligand-induced change in the reactivity was found for any position in this region (data not shown), indicating that no radical rearrangement of this region is triggered by ligand binding to the full-length, membrane-bound receptor under these assay conditions.

**Effects of Cysteine Substitutions on in Vivo Receptor Function**—Positions critical for function in *vivo* were found by assaying the ability of the engineered receptors to mediate chemotaxis. Each wild type or engineered receptor was generated by plasmid overexpression in an *E. coli* strain that lacks functional aspartate and serine chemoreceptors (RP6111) (64). The ability of an engineered receptor to restore chemotactic swimming up an aspartate gradient in semisolid agar directly measured the functional effect of its engineered cysteine (69). This assay is designed to detect only the most deleterious cysteine substitutions, since subtle receptor defects are corrected by receptor overexpression or by receptor adaptation via methylation (33, 34). Inhibitory substitutions were defined as those that reduced the aspartate-specific swarm rate at least 2-fold relative to the wild type receptor. Fig. 4 summarizes the observed relative, aspartate-specific swarm rates as well as the operational definition of inhibitory and noninhibitory substitutions. The 24 inhibitory substitutions were found to lie on both the buried and exposed faces of the helix identified by chemical reactivity measurements (see Fig. 7B), indicating that both faces of the helix are critical for function. One cysteine substitution, A360C, was found to mediate a swarm rate 3-fold faster than wild type, revealing that the native receptor is not fully optimized for aspartate-specific chemotaxis in the standard chemotaxis assay. Overall, the data illustrate an extremely high density of critical contacts in the signaling subdomain relative to other regions of the cytoplasmic domain examined in previous studies (see “Discussion”).

**Effects of Cysteine Substitutions on in Vitro Receptor Function**—Additional activity measurements were carried out using an *in vitro* assay designed to detect more subtle perturbations...
Engineered receptors were expressed in an *E. coli* strain lacking the aspartate receptor, and the ability of each receptor to restore chemotaxis up an aspartate gradient was measured by the swarm assay (33, 69). The aspartate-specific swarm rate represents the difference between the chemotactic swarm rates measured on minimal media plates containing and lacking aspartate, respectively. In addition, the indicated rate differences have been normalized to the corresponding difference observed for cells expressing the wild type receptor. Receptors yielding rates below the dashed line (50% of wild type) are classified as inhibitory.

of receptor-mediated kinase regulation (12, 13). Reconstitution of the active ternary signaling complex was achieved by adding highly purified coupling protein CheW and the histidine kinase CheA to isolated *E. coli* membranes containing the receptor of interest. Activity of the reconstituted complex was assayed by observing the rate of phosphotransfer to a saturating concentration of the response regulator CheY. Under these conditions, the autophosphorylation of CheA is the rate-limiting step. The maximal autophosphorylation rate is stimulated by the apo receptor, while aspartate binding to the complex slows autophosphorylation over 100-fold.

Fig. 5 illustrates that in the absence of attractant, 21 of the 37 engineered receptors retained the ability to stimulate CheA autophosphorylation to at least 20% of the wild type level. In seven of these receptors, however, the cysteine substitution prevented the normal ligand-induced down-regulation of CheA autophosphorylation (E351C, S356C, K358C, I363C, I369C, A377C, and A387C). Each of these substitutions yielded retention of at least 15% of the maximal native autophosphorylation rate even in the presence of 1 mM aspartate. This partial rate seen in the presence of 1 mM aspartate. This partial rate is diminished to undetectable levels.

16 inhibitory cysteines were found on both the exposed and buried faces of the newly identified helix (see Fig. 7B) It follows that both faces of the helix are essential to kinase activation.

**Disulfide Formation and Extents**—Since each engineered receptor possesses a pair of cysteines located at symmetric positions in the two subunits of the homodimer, cysteines at the subunit interface can be identified by their rapid rate of disulfide formation and in certain cases by their minimal effect on receptor activity (2, 33, 34). Mild oxidation of cysteine pairs to disulfides was initiated by the addition of the catalyst Cu(II)(1,10-phenanthroline)$_3$ to purified receptor-containing *E. coli* membranes in the presence of a Cu(II) buffer, EDTA (59).

For cysteine pairs located within the signaling subdomain, the maximal extent of disulfide formation was 30% under mild oxidation conditions. Significantly, no cysteine pairs approached the level of 60% disulfide formation achieved under identical conditions for interfacial cysteines in the previously identified a5 and a6 helices (57, 58). These results suggest that the newly identified helix does not lie at the subunit interface.

Strong oxidation conditions were able to drive all cysteines in the signaling domain to over 60% disulfide formation, enabling analysis of the affect of each disulfide on kinase regulation in the *in vitro* assay. In general, the intersubunit disulfide bonds formed between symmetric cysteines in the signaling domain were found to block kinase activation, yielding at least 100-fold inhibition of autophosphorylation relative to the oxidized wild type receptor. Only one disulfide generated in the scanned region, between Cys$^{387}$ and Cys$^{387}$, was found to retain both kinase activation and aspartate regulation. Table 1 shows that when the Cys$^{387}$-Cys$^{387}$ disulfide bond was driven to over 90% completion, the disulfide-containing receptor maintained over 40% of the wild type kinase activation and yielded native aspartate-induced down-regulation. The signal-retaining...
The Signaling Subdomain of the Aspartate Receptor

Cys\textsuperscript{387}-Cys\textsuperscript{387} disulfide was formed between the two subunits in the same dimer, rather than between colliding dimers, since a double cysteine mutant, N36C/A387C, yielded disulfide-linked dimers rather than oligomers upon oxidation (data not shown). In the latter experiment, the dimer containing both the Cys\textsuperscript{394}, Cys\textsuperscript{396} and Cys\textsuperscript{397}, Cys\textsuperscript{387} disulfide bonds was identified by its characteristic mobility on SDS-polyacrylamide gel electrophoresis, in between the two control dimers that contain the Cys\textsuperscript{395}, Cys\textsuperscript{396}, or Cys\textsuperscript{397}-Cys\textsuperscript{387} cross-link alone. Overall, the failure of most intersubunit disulfides to retain kinase activation provides additional evidence that the bulk of the scanned region does not lie at the subunit interface. The C-terminal end of the scanned region, however, may lie nearby the subunit interface, thereby enabling the signal-retaining behavior of the Cys\textsuperscript{387}, Cys\textsuperscript{387} disulfide.

**PICM**—In order to identify positions that contact CheA or the coupling protein CheW, seven mutant receptors that possessed a surface-exposed cysteine within the signaling subdomain and that retained function \textit{in vitro} were selected (H350C, D354C, K357C, A360C, S364C, A381C, and A385C). These seven receptors were labeled with 5-FM, a sulfhydryl-specific probe, and then tested for the effect of probe attachment on receptor function in the \textit{in vitro} kinase regulation assay. Covalent modification with a large probe at a CheW or CheA docking position was predicted to block activation of CheA autophosphorylation. This approach is termed PICM.

The results demonstrate the usefulness of PICM as a tool to map out a functionally significant docking surface. The stoichiometry of labeling was quantitated as in the solvent exposure assay and was found to range from 0.8 to 1.0 probe molecule/receptor monomer, indicating that each mutant cysteine was essentially fully labeled. Fig. 6 shows that four of the labeled receptors, H350C, D354C, K357C, and A360C, maintained at least 50% of the unlabeled kinase activation and full aspartate-down-regulation. Labeling at the remaining two positions, A381C and A385C, blocked kinase activation at least 90%, suggesting that these positions lie within the CheA or CheW docking surface.

An alternative explanation, namely that these inhibitory labels simply disrupt the overall conformation or stability of the cytoplasmic domain, was disfavored by the observation that the 5-FM labeled S364C, A381C, and A385C receptors all retained wild type or better methylation rates in the \textit{in vitro} methylation assay (data not shown). Since this assay is highly sensitive to perturbations of the cytoplasmic domain conformation, the simplest explanation for the observed kinase inhibition is that the covalent label causes a local steric or electrostatic perturbation of the CheA or CheW docking surface (33, 72).

**DISCUSSION**

The present study has employed cysteine scanning to probe the secondary structure, packing, and function of residues Thr\textsuperscript{345} through Ala\textsuperscript{387} in the cytoplasmic domain of the aspartate receptor, corresponding to the N-terminal region of the signaling subdomain. The measured chemical reactivities of the scanned positions reveal a pattern of highly buried and exposed residues beginning at approximately position Ser\textsuperscript{355} and continuing without detectable breaks through Arg\textsuperscript{386}. The periodicity is consistent with that of an a-helix, termed a7, as shown in Fig. 7A. The best segregation of highly buried and exposed residues is observed for a helical model with 3.5 rather than 3.6 residues per turn, suggesting that the observed helix has the heptad repeating pattern of a coiled coil (Fig. 7A). An alignment of homologous receptors reveals that the same heptad repeat is a conserved feature of the scanned region, providing strong evidence that the observed coiled-coil helix is a conserved structural element in this large receptor class (18, 19). Helix a7 is partially amphiphilic, since its charged residues fall predominantly on its exposed face. Other charges, such as Lys\textsuperscript{355} and Arg\textsuperscript{386}, lie at the edge of the observed packing surface while Glu\textsuperscript{383} lies less than one turn from the predicted helix C terminus. The long side chains of the residues could enable their charges to reach the solvent or to form charge pairs (for example between Glu\textsuperscript{383} and Arg\textsuperscript{386}). Significantly, the a and d positions of the conserved heptad repeat possess no charges and exhibit the highest frequency of hydrophobic residues (Fig. 7A), consistent with the conclusion that these positions lie on a conserved packing face.

The docking partner that packs against the buried face of helix a7 has not yet been identified. Helix a7 does not appear to intimately pack against its symmetric partner to form an a7-a7' interaction at the dimer interface, since a7-a7' intersubunit disulfides are formed inefficiently and block receptor-mediated kinase activation. In contrast, the previously characterized helices a5 and a6 exhibit efficient intersubunit

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**TABLE I**

Effects of intersubunit disulfide formation on the A387C receptor and control receptors

| Receptor       | Aspartate  | Relative kinase activitya | Reducedb | Oxidizedc |
|----------------|------------|--------------------------|----------|-----------|
| Wild type      | 1.0 ± 0.05 | 1.0 ± 0.08               | >0.01    | >0.01     |
| V365C         | 1.2 ± 0.2  | >0.01                    | >0.01    | >0.01     |
| A387C         | 4.0 ± 0.3  | 0.44 ± 0.04              | >0.01    | >0.01     |
|                | 0.21 ± 0.02| 0.21 ± 0.02              |          |           |

a Relative rate of CheA autophosphorylation in the \textit{in vitro} receptor-coupled kinase assay, normalized to the rate observed for the apo wild type receptor (see “Experimental Procedures” for details).

b Rate for receptor-containing membranes prepared under standard reducing conditions (see “Experimental Procedures”).

c Rate for receptor-containing membranes exposed to oxidizing conditions to drive intersubunit disulfide formation to completion (see “Experimental Procedures”).

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FIG. 6. The effects of covalent modification at specific surface positions on kinase activation \textit{in vitro}. The indicated receptors in isolated \textit{E. coli} membranes were modified with the cysteine-specific probe 5-FM or unmodified, respectively. The \textit{in vitro} activities of the resulting receptors were measured in the receptor-coupled kinase assay (see legend to Fig. 5). The resulting rates of phospho-CheY formation observed in the absence of aspartate are indicated for each unmodified (filled bars) and modified (open bars) receptor.
disulfide formation, and, in some cases, these disulfides retain kinase activation (57, 58). The C terminus of helix α7 may lie near the subunit interface, thereby enabling the Cys387-Cys387 intersubunit disulfide to form and retain a significant level of kinase activation and regulation. No evidence for an aspartate-induced displacement of the helix α7 packing face has been observed, since no aspartate-induced change in cysteine labeling rates or α7-α7′ disulfide formation rates has been observed. This insensitivity to aspartate suggests that the signal-induced rearrangement of the signaling subdomain is small (consistent with the subtle conformational shift observed in the periplasmic domain (1, 43)); alternatively, a larger rearrangement of the subdomain could occur in the full ternary complex.

Activity measurements for the library of cysteine-containing receptors, carried out both in vitro and in vivo, underscore the functional importance of helix α7. Of the 37 cysteine-containing receptors that accumulate to wild type levels in the membrane, 65% are unable to mediate chemotaxis in vivo, while 43% were unable to activate CheA autophosphorylation in vitro. This is the highest density of inhibitory cysteine substitutions yet observed in the scanned regions of the cytoplasmic domain, where previous studies have yielded an average inhibition frequency of 34% in vivo and 30% in vitro (57, 58). Another unusual feature of helix α7 is the sensitivity of its solvent-exposed face to perturbations by cysteines; 29% of the inhibitory cysteines observed in vivo and 40% observed in vitro fall on the exposed face of the helix. In contrast, previous in vivo and in vitro studies of other regions of the cytoplasmic domain revealed that only 6 and 11%, respectively, of the inhibitory cysteines were located on exposed helical faces (57, 58). Together, these results suggest that unlike other regions of the cytoplasmic domain, both the exposed and buried faces of the helix α7 are extensively involved in critical contacts necessary for receptor-mediated kinase activation. In particular, the in vitro receptor-coupled kinase assay results demonstrate that cysteines on both helix faces can block receptor stimulation of CheA autophosphorylation. Interestingly, the lock-on cysteine substitutions that prevent full aspartate-induced down-regulation of kinase activity in vitro were observed only on the buried face of helix α7, suggesting that this face plays the central role in attractant-induced kinase inhibition.

It is not surprising that the buried face of helix α7 is critical for both kinase activation and down-regulation, since cysteine substitutions that perturb helix packing may inhibit the critical function of the signaling subdomain. However, one may ask why the exposed face of helix α7 is so essential for kinase activation. PICM analysis of the exposed cysteines, in which a large probe (5-FM) is covalently attached to a series of surface-exposed cysteines, reveals that modification of three positions near the C-terminal end of the helix inhibits kinase activation in vitro. The inhibition is not due to simple perturbation of cytoplasmic structure, since the inhibitory chemical modifications do not significantly reduce the rate of receptor methylation in the in vitro adaptation assay. Instead, the PICM results begin to map out a docking surface on the exposed face of helix α7. The simplest interpretation is that CheA, CheW, or both must dock to this exposed face to form the functional ternary
Further support for the identification of helix $\alpha 7$ as a CheA or CheW docking element is provided by sequence analysis and genetic data. First, exposed positions in helix $\alpha 7$ are 100% identical between the aspartate receptors of $S. typhimurium$ and $E. coli$, whose chemotaxis pathways share functionally interchangeable CheA and CheW (2). In contrast, much lower sequence conservation is observed for the exposed faces of the previously characterized helices $\alpha 4$, $\alpha 5$, and $\alpha 6$ where exactly half of the exposed residues are identical (18, 19, 57, 58). The highly conserved nature of the exposed surface of helix $\alpha 7$ is consistent with its proposed role in CheA or CheW docking. Second, exactly half of the residues on the exposed face of helix $\alpha 7$ are hydrophobic (Fig. 7A), a much higher frequency than observed on the exposed faces of helix $\alpha 5$ and $\alpha 6$ (0 and 16%, respectively). Such exposed hydrophobic residues, which are among the most highly conserved residues in the scanned subdomain, could facilitate intermolecular docking via the hydrophobic effect. Third, genetic studies have previously implicated involvement of the signaling subdomain in CheW docking. Within the scanned region, the inhibitory effect of mutations at three receptor positions could be overcome by compensatory mutations in CheW (60). Strikingly, all three of these receptor positions lie on the newly identified exposed face of helix $\alpha 7$ (Fig. 8), suggesting that this face directly contacts the CheW molecule.

Together, these findings underscore the importance of helix $\alpha 7$ in kinase docking activation and regulation. The results also provide further illustrations of the usefulness of cysteine and disulfide scanning in studies defining secondary structure and molecular packing faces in proteins inaccessible to high resolution structural methods, including transmembrane receptors. The current work also has expanded this battery of applications to include the mapping of intermolecular protein-protein interaction surfaces via PICM analysis. Currently, it appears that this cysteine and disulfide engineering approaches may yield the first chemically defined, low resolution structural model of an independently folded protein domain.

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