Conserved Glycine Residues in the Cytoplasmic Domain of the Aspartate Receptor Play Essential Roles in Kinase Coupling and On−Off Switching[†]

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ABSTRACT: The aspartate receptor of the bacterial chemotaxis pathway serves as a scaffold for the formation of a multiprotein signaling complex containing the receptor and the cytoplasmic pathway components. Within this complex, the receptor regulates the autophosphorylation activity of histidine kinase CheA, thereby controlling the signals sent to the flagellar motor and the receptor adaptation system. The receptor cytoplasmic domain, which controls the on−off switching of CheA, possesses 14 glycine residues that are highly conserved in related receptors. In principle, these conserved glycines could be required for static turns, bends, or close packing in the cytoplasmic domain, or they could be required for conformational dynamics during receptor on−off switching. To determine which glycines are essential and to probe their functional roles, we have substituted each conserved glycine with both alanine and cysteine, and then measured the effects on receptor function in vivo and in vitro. The results reveal a subset of six glycines which are required for receptor function during cellular chemotaxis. Two of these essential glycines (G388 and G391) are located at a hairpin turn at the distal end of the folded cytoplasmic domain, where they are required for the tertiary fold of the signaling subdomain and for CheA kinase activation. Three other essential glycines (G338, G339, and G437) are located at the border between the adaptation and signaling subdomains, where they play key roles in CheA kinase activation and on−off switching. These three glycines form a ring around the four-helix bundle that comprises the receptor cytoplasmic domain, yielding a novel architectural feature termed a bundle hinge. The final essential glycine (G455) is located in the adaptation subdomain where it is required for on−off switching. Overall, the findings confirm that six of the 14 conserved cytoplasmic glycines are essential for receptor function because they enable helix turns and bends required for native receptor structure, and in some cases for switching between the on and off signaling states. An initial working model proposes that the novel bundle hinge enables the four-helix bundle to bend, perhaps during the assembly of the receptor trimer of dimers or during on−off switching. More generally, the findings predict that certain human disease states, including specific cancers, could be triggered by lock-on mutations at essential glycine positions that control the on−off switching of receptors and signaling proteins.

Glycine serves a unique structural function in proteins where it is often found in sites of sharp turns, bends, or close packing of adjacent secondary structure elements. In this well-characterized architectural role, glycine serves as a backbone structural element with unmatched angular range and a minimal side chain. Yet glycine can also play a dynamical role because of the enhanced conformational freedom it provides the protein backbone. In this dynamical role, glycine is likely to be essential for the switching of proteins between distinct functional states, such as the switching of a receptor or signaling protein between its on and off signaling states. These functional states could possess significantly different average conformations that require the flexibility of a glycine hinge for interconversion. Alternatively, the functional states could differ in their relative levels of thermal motion and entropy, with the more dynamic states requiring glycine for their backbone fluctuations. The potential importance of signaling-induced changes in conformational dynamics has been previously proposed for components of the chemotaxis pathway (*1*−*4*).

The transmembrane aspartate receptor of the bacterial chemotaxis pathway is an ideal protein in which to probe the essential functions of glycine in a typical signaling element that serves as an on−off switch. This receptor forms a stable signaling complex with the cytoplasmic proteins CheA, a histidine kinase, and CheW, a coupling protein (reviewed in refs *5*−*11*). Once the complex forms, it remains assembled for tens of minutes before dissociation, and its stability is independent of receptor ligand occupancy. In the absence of attractant, the apo receptor exists in its on state which stimulates the autophosphorylation of the associated CheA kinase. When aspartate binds to the periplasmic domain of the receptor, the receptor switches to its off state which inhibits autophosphorylation of the bound CheA. This signal is transmitted from the periplasmic domain through the bilayer by the transmembrane signaling helix, passes through the linker that couples this signaling helix to the

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Table 1: Conserved Glycine Positions in the Cytoplasmic Domains of Bacterial Chemoreceptors

	position (numbering for the *S. typhimurium* aspartate receptor)														
receptor[a]	271	278	285	338	339	344[b]	368	388	391	393	399	429	437	455	467
Tars	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Tare	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Tsre	G	G	G	G	G	N	G	G	G	G	G	G	G	G	G
Tcpe	N	G	G	G	G	N	G	G	G	G	G	G	G	G	G
Tape	G	G	G	G	G	T	G	G	G	G	G	G	A	G	G
Trge	G	G	G	G	G	G	G	G	G	G	S	G	G	Q	G
Aere	Q	G	G	G	G	T	S	G	G	G	G	G	G	A	G
TseEnt	G	G	G	G	G	N	V	G	G	A	G	G	G	G	G
TasEnt	G	G	G	R	−	D	G	G	G	G	G	G	G	G	G
McpArs	A	E	A	S	G	E	D	G	G	G	S	G	G	S	S
McpArc	T	E	A	G	E	Q	D	G	G	G	S	G	D	D	G
McpBrc	T	S	A	G	Q	R	D	G	V	G	S	G	G	A	G
McpAr1	A	G	S	S	G	S	E	G	G	G	Q	G	G	D	T
McpAc	S	G	A	S	G	Q	E	G	G	G	Q	G	G	T	G
McpEe	A	H	D	G	Q	H	G	G	G	G	D	S	A	A	V

[a] The first seven examples are *S. typhimurium* or *E. coli* chemoreceptors for which the designation ends in "s" or "e", respectively. The second eight examples are representative chemoreceptors from seven different eubacterial species (*15*). [b] Position 344 is a control glycine that is not highly conserved in chemoreceptors.

cytoplasmic domain, and is transmitted through the cytoplasmic domain to the CheA kinase docked at its distal end.

The receptor cytoplasmic domain, which is the focus of this study, is a four-helix bundle that plays a central role in signal transduction by transmitting both the attractant and adaptation signals to the associated CheA kinase (*8*, *12*−*14*). The domain possesses 14 positions at which glycine is from 67 to 100% conserved in the six chemotaxis receptors of *Escherichia coli* and *Salmonella typhimurium* (*15*), as summarized in Table 1. Figure 1 illustrates the locations of these 14 positions in the cytoplasmic domain. Table 1 further reveals that nine of these 14 positions exhibit at least 50% glycine conservation in a group of distantly related receptors from seven other eubacterial species (*15*). The roles of the conserved glycines in receptor structure and mechanism are not yet known, with the exception of three glycines found at the distal end of the cytoplasmic domain. These three glycines (Gly 388, Gly 391, and Gly 393) lie at or near the sharp turn that breaks the cytoplasmic helix and enables it to fold back on itself, thereby forming a helical hairpin (*12*, *13*). The two symmetric helical hairpins of the receptor homodimer, one provided by each subunit, pack together to form the extended four-helix bundle architecture of the cytoplasmic domain. Thus, these three glycines play a central architectural role by providing the backbone conformational freedom needed for the cytoplasmic hairpin turn.

The cytoplasmic domain is essential for the assembly of the receptor into higher-order oligomers. The receptor homodimer associates with other homodimers to form a trimer-of-dimers oligomeric structure stabilized by contacts at the distal end of the cytoplasmic domain near the hairpin turn (*13*, *16*−*18*). This trimer of dimers is believed to be the unit of receptor architecture that serves as the scaffold for the assembly of the signaling complex. The receptors and their signaling complexes cluster at the poles of the cell where cooperative interactions occur between individual signaling units (*16*, *19*−*26*), but it is not yet clear whether the receptor trimers of dimers in these clusters are loosely associated or form an extensive, ordered array.

To identify which of the 14 conserved glycine residues are essential for receptor function, and to begin to ascertain their functional roles, each has been replaced with both alanine and cysteine, and the resulting changes in receptor

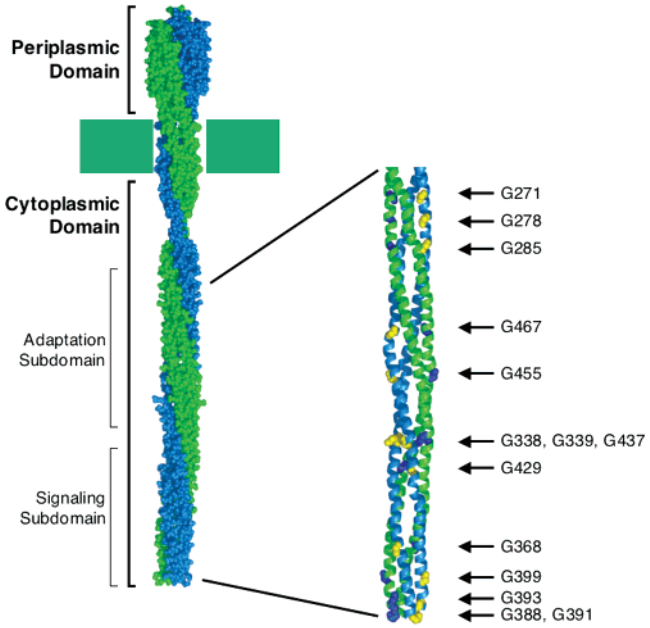

FIGURE 1: Model of chemoreceptor structure (*13*) illustrating the targeted glycine locations. Shown are the two identical subunits of the homodimer (green and blue) highlighting the positions of the 14 conserved cytoplasmic glycines (yellow and dark blue). Filled rectangles represent the plane of the membrane. The structures of the periplasmic domain (Tar residues 35−180) and the signaling subdomain (Tsr residues 340−439) are defined by high-resolution crystal structures (*13*, *39*), while the structures of the transmembrane region and the adaptation subdomain are modeled on the basis of structural information derived from cysteine and disulfide scanning (*8*, *12*, *14*, *29*, *31*−*33*, *40*, *41*). For simplicity, the linker between the transmembrane region and adaptation subdomain is modeled as a simple α-helix, but cysteine and disulfide scanning studies, as well as recent imaging studies, suggest that this linker has a more complex, compact structure (*33*, *42*). Not shown is the assembly of the receptor dimer into its higher-order trimer-of-dimers structure. [Indicated residue numbers are for the cytoplasmic domain of the *S. typhimurium* aspartate receptor (Tar) studied herein. The corresponding residue numbers for the highly homologous cytoplasmic domain of the *E. coli* serine receptor (Tsr) can be obtained by adding 2. The full-length receptor structural model uses the latter numbering system (*13*).]

function have been determined both in vivo and in vitro. Receptor function in vivo is measured using the chemotactic swarm assay which quantitates the ability of the mutant receptor to restore the ability of cells to chemotax in an aspartate gradient. An essential glycine residue is recognized by the loss of the ability of the receptor to direct cellular chemotaxis in this assay when the residue is substituted. The effect of the substitution on receptor-regulated kinase activity, measured in an in vitro assay, provides further information about the molecular mechanism of the receptor perturbation. In this assay, the receptor is isolated in native *E. coli* membranes and reconstituted with purified CheA and CheW. The activity of the resulting signaling complex can identify glycine substitutions that (i) block receptor-mediated kinase stimulation, (ii) cause the receptor to superactivate the kinase, or (iii) lock the receptor in the kinase-activating on state, thereby preventing switching to the off state when attractant binds. The results reveal that six of the 14 glycine residues are essential for receptor function, and show that mutations of these six glycines cause two basic types of receptor defects. One type of defect prevents receptor-mediated kinase activation, while the other type prevents on−off switching by locking the receptor in the kinase-activating on state. Three of the essential glycines form a novel architectural element termed a bundle hinge in the cytoplasmic four-helix bundle. Overall, the results provide strong evidence that the backbone conformational freedom provided by glycine is required for native receptor structure and function.

MATERIALS AND METHODS

Mutagenesis. Mutations of the pSCF6 plasmid were created using the previously described Kunkel method of site-directed mutagenesis (*27*). In all cases, the substitutions were glycine to alanine or cysteine, yielding a modified receptor gene possessing a single-point mutation. Automated DNA sequencing was used to confirm the presence of the desired mutations.

In Vivo Analysis of Receptor Function. Aspartate-specific chemotaxis of the mutant receptors was assessed using the previously described chemotaxis swarm assay (*28*). Briefly, *E. coli* strain RP8611, a strain lacking the aspartate receptor but containing all other soluble pathway components, was transformed with the mutant plasmids. Liquid cultures were grown for 6 h in Luria broth at 37 °C with shaking; 5 μL of culture was spotted onto agar minimal medium plates containing Vogel Bonner Citrate medium supplemented with 0.1% glycerol, 20 mM lactate, 40 μg/mL DL-histidine, 20 μg/mL L-leucine, 1 μg/mL thiamine, and 100 μg/mL ampicillin, with or without 0.1 mM aspartate. After overnight incubation at 30 °C, measurements of the swarm diameters were taken at 3 h intervals and normalized to that of the wild type to determine the relative rate of aspartate-specific cellular chemotaxis. For each mutant, from three to six independent rate measurements were carried out to determine the average and standard deviation.

Protein Expression and Isolation. Confirmed mutant plasmids were transformed into *E. coli* strains RP8611 and RP3808 and expressed as previously described (*29*). The overexpressed mutant receptors were isolated in native cell membranes using a published procedure (*29*). Following membrane isolation, receptor expression levels were examined by Laemmli SDS−PAGE[1] (10% acrylamide, 40:0.2 acrylamide:bisacrylamide ratio) and Coomassie staining. To quantitate expression levels, receptor bands were imaged with a digital camera and analyzed with integration software (Alpha Inotech). For each mutant−strain combination, three membrane preparations were analyzed to determine the average and standard deviation.

In Vitro Analysis of Receptor Function. Mutant receptors in isolated membranes from *E. coli* strain RP3808 were incubated with purified CheA, CheW, and CheY to yield the reconstituted signaling complex as previously described (*29*). Strain RP3808 lacks all major chemoreceptors and adaptation enzymes, ensuring that the receptor population was homogeneous. For cysteine-containing receptors, reconstituted signaling complexes were prepared containing both reduced and oxidized receptors using a published procedure (*30*). Subsequently, for each signaling complex the initial rate of receptor-regulated CheA autophosphorylation was measured in the presence and absence of the attractant aspartate, as previously described (*30*). The reaction conditions (excess CheY) ensured that the rate-determining step was CheA autophosphorylation, rather than phosphotransfer to CheY (*29*). For cysteine-containing receptors, the extent of disulfide bond formation present in the reduced and oxidized states was analyzed by Laemmli SDS−PAGE (10% acrylamide, 40:0.2 acrylamide:bisacrylamide ratio) and Coomassie staining. To quantitate the fractional receptor population in the disulfide-linked dimeric state, receptor bands were imaged with a digital camera and analyzed with integration software (Alpha Inotech). For each condition, from three to six independent rate measurements were carried out to determine the average and standard deviation.

RESULTS

Strategy for Probing the Role of Conserved Glycine Residues in the Receptor Cytoplasmic Domain. To test the hypothesis that the 14 conserved cytoplasmic glycine residues of the *S. typhimurium* aspartate receptor play an important role in receptor structure or function, each of these 14 positions (G271, G278, G285, G338, G339, G368, G388, G391, G393, G399, G429, G437, G455, and G467) was substituted with both alanine and cysteine. As a negative control, alanine and cysteine substitutions were also generated at one unconserved surface glycine position in the cytoplasmic domain (G344). Subsequently, the effect of each substitution on receptor function was analyzed in complementary in vivo and in vitro activity assays.

Design and Characterization of Mutant Receptors. Standard site-directed mutagenesis methods were employed to generate the alanine and cysteine substitutions at the 15 targeted locations in the *S. typhimurium* aspartate receptor gene (*27*), yielding 30 point mutants that were confirmed by DNA sequencing. [Seven of the mutants, G271C, G278C, G285C, G388C, G391C, G393C, and G399C, were previously constructed during the course of other published studies (*29*, *31*, *32*).] Subsequently, the pSCF6 expression plasmid containing a given mutant gene was transformed into *E. coli* strain RP3808, a chemoreceptor-deficient strain that lacks

[1] Abbreviations: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WT, wild type.

the aspartate receptor, the other major chemotaxis receptors, and the soluble pathway components, including the adaptation enzymes CheB and CheR. The lack of adaptation enzymes ensured the expression of a homogeneous population of aspartate receptors possessing identical adaptation sites that were not subjected to post-translational modification. The mutant receptor was overexpressed, and native cell membranes containing the receptor were isolated (*29*). SDS−PAGE analysis indicated that all 30 mutant receptors were expressed at substantial levels, indicating that each mutant receptor is a stable, membrane-imbedded protein.

Effects of Alanine and Cysteine Substitutions on Receptor Function in Vivo. The in vivo chemotactic function of the modified receptors was measured by the previously described chemotactic swarm plate assay (*28*). Each mutant receptor was expressed in *E. coli* strain RP8611, a strain that lacks the major chemoreceptors, including the aspartate receptor, but possesses all other essential components of the chemotaxis pathway. The chemotactic swarm assay measures the ability of a mutant aspartate receptor to restore cellular chemotaxis in response to a self-induced attractant gradient generated by metabolic depletion of aspartate in the medium. Because of the compensatory nature of the adaptation branch of the chemotaxis pathway which corrects many minor receptor defects, this *in vivo* swarm plate assay of cellular chemotaxis only reveals major receptor defects that significantly perturb receptor structure, stability, on−off switching, or adaptation.

Figure 2 shows that of the 30 mutant receptors that were tested, 12 exhibit the ability to restore cellular chemotaxis to within 50% of that of the wild type. Panels A and B of Figure 2 summarize the results for alanine and cysteine substitutions, respectively. At six positions, alanine substitution reduces chemotactic function by a factor substantially exceeding 2-fold (G338A, G339A, G388A, G391A, G437A, and G455A). Such >2-fold effects are highly significant, since the ability of the adaptation system to correct receptor defects effectively dampens the effects of many perturbations. Cysteine substitution reduces chemotactic function more than 2-fold at the same six positions (G338C, G339C, G388C, G391C, G437C, and G455C) and at an additional six positions (G271C, G285C, G368C, G393C, G429C, and G467C), indicating that the larger cysteine side chain is often more perturbing than alanine when introduced at conserved glycine positions. By contrast, alanine and cysteine substitutions at the control position (G344A and -C) have little or no inhibitory effect on receptor function in the cellular chemotaxis assay. At the opposite extreme, five substitutions increase the chemotactic swarm rate to a level at least 50% above normal (G271A, G278A, G285A, G344A, and G399C). These substitutions were also observed to reduce receptor expression levels approximately 2-fold relative to that of the wild-type receptor produced under identical overexpression conditions as summarized in Table 2. Previous studies have indicated that cells possessing an overexpressed aspartate receptor exhibit slower swarm rates than cells containing the native number of receptor molecules, and that reducing the level of receptor overexpression can increase the swarm rate (*29*, *31*−*36*). Thus, the enhanced swarm rates observed for five mutants likely stem from their lower levels of receptor overexpression.

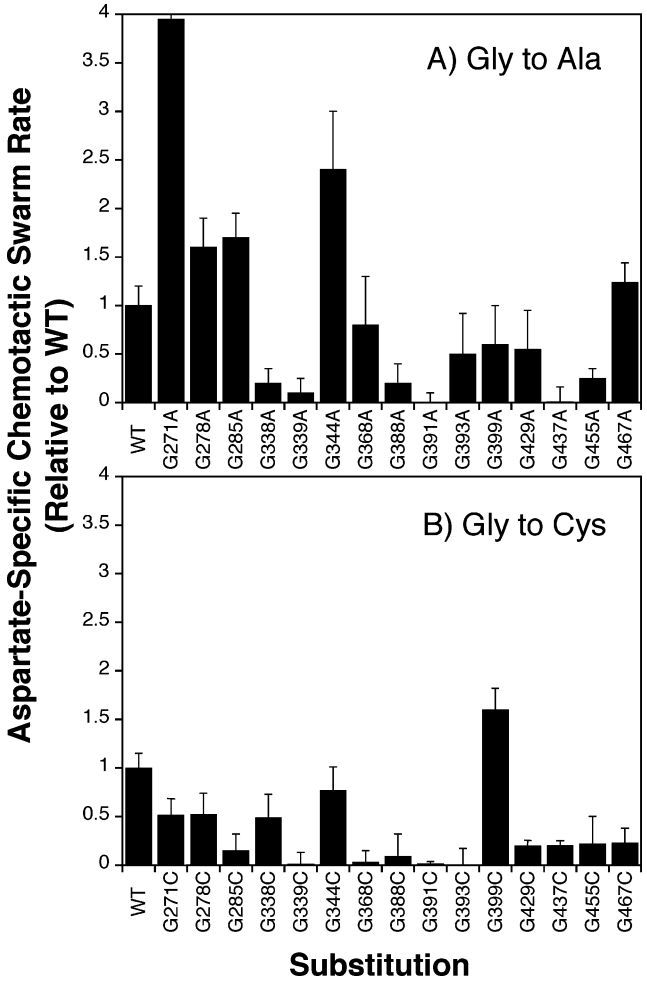

FIGURE 2: Effects of conserved glycine substitutions on cellular chemotaxis in vivo. Each conserved glycine was replaced with alanine and cysteine, yielding the indicated substitutions. As a control, an unconserved glycine at position 344 was also replaced with alanine and cysteine. Mutant receptors were expressed in an *E. coli* strain lacking the aspartate receptor, and their ability to restore in vivo chemotaxis in a self-induced gradient of aspartate was measured at 30 °C (see Materials and Methods). Each bar represents the aspartate-specific swarm rate for a given receptor normalized to the wild-type aspartate-specific rate. The two panels summarize the data for (A) alanine and (B) cysteine substitutions.

In short, the observation that alanine substitutions at six conserved glycine positions significantly inhibit receptor performance in vivo indicates that these six glycines are essential for normal receptor function. Substitution of the larger cysteine at these six positions also significantly inhibits receptor function in vivo. Each of the 12 receptors possessing analine or cysteine substitutions at these six positions was expressed at levels similar to that of the overexpressed wild-type receptor, indicating that the observed effects on receptor function were not due to variable expression levels. Instead, substitutions at these six glycine positions perturb receptor function in vivo by inhibiting receptor regulation of CheA kinase activity, or by inhibiting the receptor adaptation system. To rigorously determine the effect of the 17 substitutions on receptor-regulated kinase activity in the absence of adaptation effects, an in vitro approach was utilized.

Effects of Alanine and Cysteine Substitutions on Receptor-Regulated Kinase Activity in Vitro. The effects of alanine and cysteine substitutions on receptor regulation of kinase

Table 2: Effects of Glycine Substitutions on Receptor Expression Levels (relative to that of the wild type)

	strain RP8611		strain RP3808	
position	Ala substitution	Cys substitution	Ala substitution	Cys substitution
WT	1.0	1.0	1.0	1.0
G271	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
G278	0.4 ± 0.3	0.5 ± 0.3	0.5 ± 0.1	0.6 ± 0.1
G285	0.5 ± 0.2	0.5 ± 0.3	0.4 ± 0.1	0.4 ± 0.2
G338	1.0 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
G339	0.9 ± 0.3	0.5 ± 0.2	0.8 ± 0.2	0.6 ± 0.1
G344	0.6 ± 0.2	1.1 ± 0.3	0.6 ± 0.1	0.9 ± 0.2
G368	1.1 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.1
G388	0.8 ± 0.3	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
G391	1.0 ± 0.3	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
G393	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	0.9 ± 0.1
G399	0.9 ± 0.2	0.4 ± 0.2	1.0 ± 0.2	0.5 ± 0.1
G429	1.0 ± 0.2	0.8 ± 0.3	0.9 ± 0.2	1.0 ± 0.2
G437	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.1
G455	1.0 ± 0.3	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2
G467	1.1 ± 0.2	0.8 ± 0.3	0.9 ± 0.1	0.9 ± 0.2

activity were directly measured in the in vitro receptor-coupled kinase assay (*29*, *30*, *37*, *38*). This assay is well-suited for detecting even subtle effects of mutations on receptor modulation of kinase activity. Isolated *E. coli* membranes containing a given overexpressed receptor were reconstituted with purified CheA, CheW, and CheY to form the functional receptor−CheA−CheW signaling complex. Excess reducing agent (DTT) was included to ensure that all cysteine-containing receptors remained in their reduced states. Following addition of [γ-^{32}P]ATP, the initial rate of phospho-CheY formation was measured in the absence and presence of a saturating concentration of aspartate, thereby determining the abilities of the mutant receptors to activate and regulate the CheA kinase. The assay was carried out under conditions which ensured that CheA autophosphorylation was the rate-limiting step in phospho-CheY production (*29*); thus, the assay directly measured the ability of the receptor to modulate CheA activity. Since this in vitro system lacks adaptation enzymes CheR and CheB, the compensatory effects of the adaptation system will not mask subtle receptor perturbations. Moreover, the assay is carried out using a fixed receptor concentration, eliminating variations due to slightly different receptor expression levels (Table 2).

Panels A and B of Figure 3 summarize the effects of the alanine and cysteine substitutions on receptor-regulated CheA kinase activity in the reconstituted signaling complex, respectively. The findings place strong constraints on the molecular mechanisms by which the inhibitory substitutions perturb receptor function. These inhibitory mechanisms are of two types. The first type prevents normal receptor-mediated kinase activation, as illustrated in panels A and B of Figure 3 by the 11 substitutions which reduce kinase activity in the absence of ligand aspartate by at least 4-fold (G339A, G388A, G391A, G437A, G339C, G368C, G388C, G391C, G393C, G437C, and G455C). These 11 substitutions, termed inhibitory substitutions, were also observed to perturb in vivo receptor function in the chemotactic swarm assay as noted above (Figure 2A,B).

The second type of inhibitory mechanism locks the receptor in the kinase-activating state, thereby preventing the full inhibition of CheA kinase activity normally observed upon aspartate binding to the reconstituted signaling complex. Panels A and B of Figure 3 reveal that six substitutions, termed lock-on substitutions, retain substantial kinase activities even in the presence of a saturating concentration ofaspartate such that the remaining kinase activity exceeds 25% of the full activity observed for the apo wild-type signaling complex (G338A, G455A, G285C, G338C, G429C, and G467C). All six of these lock-on substitutions prevent normal on−off switching by trapping the receptor in the on state that stimulates kinase activity such that aspartate fails to trigger the normal kinase downregulation. Four of these lock-on substitutions also yield kinase activities in the absence of aspartate that are at least 50% higher than that of the apo wild-type signaling complex (G455A, G285C, G338C, and G467C), suggesting that these substitutions drive the receptor equilibrium toward the on state in both the apo and aspartate-occupied states. Finally, as one would expect for a receptor that serves as an on−off switch, five of the six lock-on substitutions are observed to inhibit in vivo receptor function in the chemotactic swarm assay as noted above (G338A, G455A, G285C, G338C, G429C, and G467C). The one exception is G338C which retains significant activity in vivo, most likely due to the ability of the cellular adaptation system to correct certain receptor defects.

Notably, the six essential glycine positions detected in the in vivo chemotactic swarm assay are all found to be essential for normal kinase regulation in the in vitro receptor-regulated kinase assay. Alanine or cysteine substitutions at positions G339, G388, G391, and G437 are observed to block kinase activation by the receptor (Figure 3A,B). By contrast, alanine or cysteine substitutions at position G338 are observed to lock the receptor in its kinase-activating on state, preventing full aspartate-triggered kinase inhibition. Finally, alanine substitution at position G437 yields a locked-on state, while cysteine substitution at this same position blocks kinase activation. Thus, the kinase activity of the reconstituted signaling complex provides molecular insights into the mechanistic roles played by the six essential glycine residues in receptor function.

Effects of Disulfide Bond Formation on Receptor-Regulated Kinase Activity in Vitro. In living cells, receptors containing engineered cysteines are maintained in their reduced state by the reducing environment of the cytoplasm; thus, the effect of disulfide bond formation on receptor function could not be determined in vivo. To examine the effects of disulfide bond formation on receptor-regulated kinase activity in vitro, isolated *E. coli* membranes containing a given overexpressed cysteine-substituted receptor were oxidized and reconstituted in the signaling complex. Since the native receptor contains no intrinsic cysteine residues, disulfide bonds were formed only between engineered cysteine pairs. Each engineered cysteine at a buried position within the receptor homodimer (G271C, G278C, G285C, G338C, G391C, G393C, G429C, and G467C) was expected to form an intradimer disulfide bond with the symmetric cysteine in the other monomer of the same dimer, while each cysteine at a solvent-exposed position (G339C, G344C, G368C, G388C, G399C, G437C, and G455C) was expected to form an interdimer disulfide bond with the homologous cysteine of a different dimer. Oxidation using Cu(II)-(1,10-phenanthroline)$_3$ as a redox catalyst (*30*) drove the disulfide formation reaction to at least 80% completion for each of the cysteine-containing receptors, as quantitated by standard

SDS−PAGE analysis of monomer and disulfide-linked dimer bands, and had no effect on the wild-type receptor.

Figure 3C summarizes the effects of disulfide bond formation on receptor-regulated CheA kinase activity in the reconstituted signaling complex. Only four of the 15 cysteine-containing receptors retain substantial kinase activation in the oxidized state (G271C, G278C, G285C, and G467C). All four of these disulfide bonds have been previously characterized in our published receptor-regulated kinase assays, and the results presented here confirm their previously observed activities (*12*, *29*, *31*, *32*). Moreover, all four positions have previously been shown to form intradimer disulfide bonds (*12*), as expected for their locations buried within the receptor homodimer. Two of the functional disulfide bonds retain normal receptor signaling (G271C and G467C), while the other two disulfides exhibit substantial lock-on character (G278C and G285C). At the 11 remaining cysteine positions, most of which are characterized here for the first time, disulfide bond formation largely eliminates any kinase activation observed in the reduced state.

DISCUSSION

The functional effects of alanine and cysteine substitutions at the 14 conserved glycine residues in the aspartate receptor cytoplasmic domain reveal six glycine positions that are essential for receptor activity in vivo. At each of these six positions, both alanine and glycine substitution is observed to substantially inhibit receptor-mediated cellular chemotaxis up an aspartate gradient. Receptor expression levels indicate that these substitutions do not significantly alter receptor copy number in the membrane, suggesting that receptor folding, membrane insertion, and stability are relatively unperturbed. Instead, each of the substitutions is observed to inhibit receptor-mediated kinase activation or on−off switching, indicating that the six glycines are essential for normal kinase regulation during cellular chemotaxis. The functional roles of these glycines are likely to be directly related to the conformational freedom they impart to the protein backbone, since in the crystal structure of the cytoplasmic domain all six are located at sites of bends or turns in the helical structure of the domain (*13*), as illustrated in Figure 4A. The magnitudes of these bends or turns range from ∼15° to ∼180°.

Two of the essential glycine residues, G388 and G391, lie at the hairpin turn that defines the helical hairpin architecture of the cytoplasmic domain (Figure 1) (*13*). Both alanine and cysteine substitutions at these two positions inhibit activation of CheA kinase in the receptor-coupled kinase assay, suggesting that they block the formation of the receptor kinase signaling complex or prevent normal complex activation in the absence of attractant. These two glycine residues are 100% conserved in the chemoreceptors of *E. coli* and *S. typhimurium*, and are 100 and 93% conserved in a larger representative group of 15 eubacterial chemoreceptors (Table 1) (*15*), providing further evidence for their proposed roles as essential architectural elements of the hairpin turn region. Besides its importance in the architecture of the cytoplasmic domain of the receptor homodimer, the hairpin turn is located in the highly conserved signaling subdomain that is known to associate with other dimers to form the receptor trimer of dimers, and

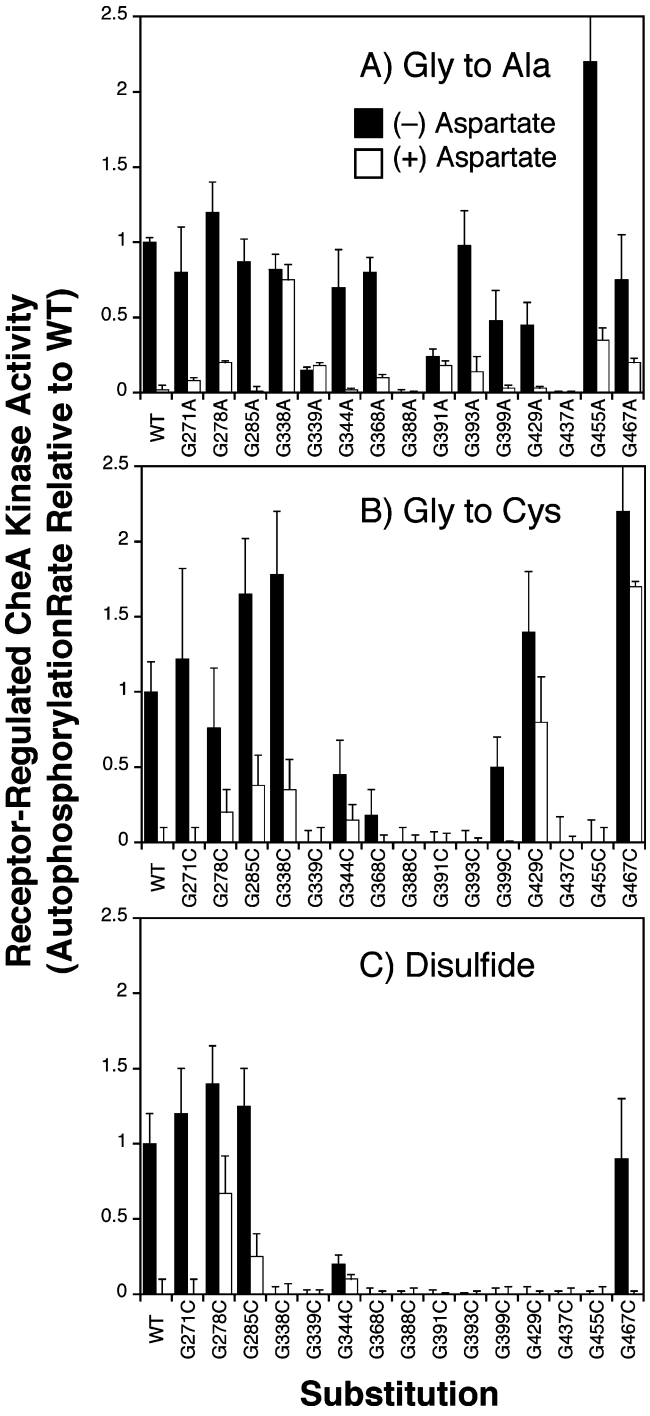

FIGURE 3: Effects of conserved glycine substitutions on receptor-coupled kinase activity in vitro. Mutant receptors in which glycine is replaced with (A) alanine or (B) cysteine were expressed in an *E. coli* strain lacking all soluble pathway components. Native cell membranes containing the overexpressed receptor were isolated and incubated with purified CheA, CheW, and CheY to reconstitute the signaling complex. The ability of each modified receptor to regulate the kinase in the reconstituted signaling complex was measured (see Materials and Methods). Each bar represents the observed receptor-coupled kinase activity in the presence (white bars) or absence (black bars) of aspartate, normalized to wild-type kinase activity in the absence of aspartate. Reaction mixtures at 22 °C contained 2 μM receptor monomer, 2 μM CheW monomer, 0.5 μM CheA monomer, and 10 μM CheY in buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, and 100 μM ATP. The three panels summarize the data for (A) alanine substitutions, (B) cysteine substitutions in the reduced state, and (C) cysteine substitutions oxidized to form intersubunit disulfide bonds.

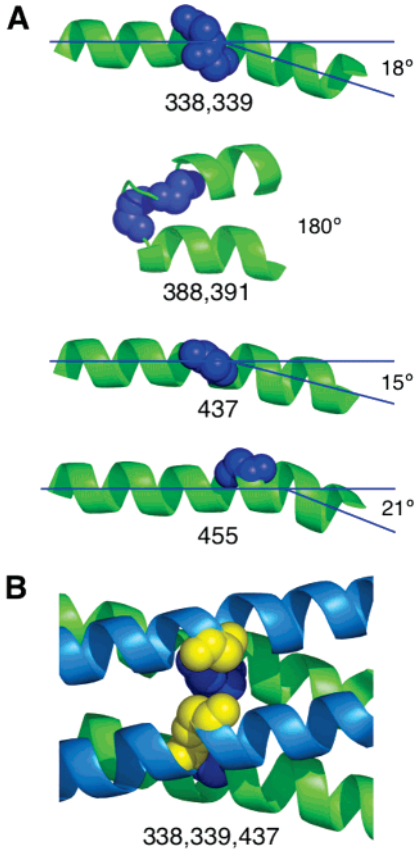

FIGURE 4: Local structures in the vicinities of six essential glycine residues. (A) Shown are the helical turns or bends associated with each of the six essential cytoplasmic glycines at positions 338, 339, 388, 391, 437, and 455 of a single receptor subunit. All of these residues lie within the region of the cytoplasmic domain homodimer defined by a high-resolution crystal structure (*13*). (B) Shown is the bundle hinge formed by the three essential glycines at positions 338, 339, and 437. These glycines form a ring around the four-helix bundle such that each helix possesses either one or two of the putative hinge glycines.

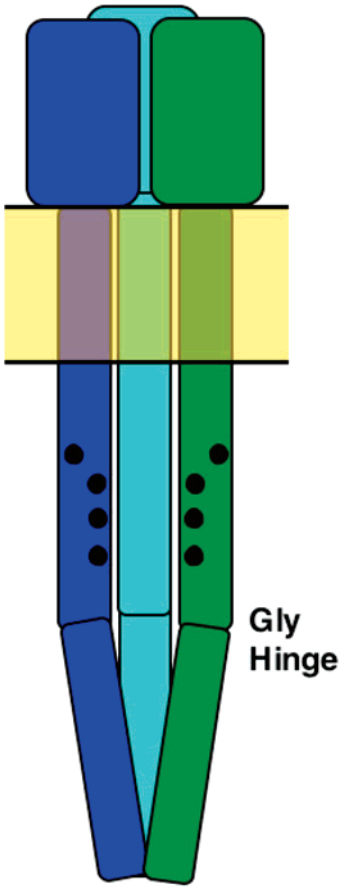

FIGURE 5: Proposed structural role of the bundle hinge in trimer-of-dimers assembly. Shown are the three receptor homodimers (different color cylinders) that assemble to form a trimer of dimers. The available evidence indicates that the trimer is stabilized by interdimer contacts at the distal end of the cytoplasmic domain as shown (*13*, *16*, *18*). Because of the larger diameter of the periplasmic domain, these contacts cannot form unless the cytoplasmic domain deviates from a linear axis normal to the membrane surface. The bundle hinge is hypothesized to enable the cytoplasmic four-helix bundle to bend so that the essential contacts are formed. Activity studies indicate that the bundle hinge could play a role in receptor on−off switching.

to bind CheA and CheW (*12*, *13*, *16*, *29*). The fact that CheA docks in the vicinity of the hairpin turn explains the importance of these two glycine positions to CheA coupling and activation.

Three other essential glycine residues, G338, G339, and G437, are located in a novel glycine hinge at the border between the adaptation and signaling subdomains of the cytoplasmic domain (Figure 1). In the receptor homodimer, these Gly residues form a ring encompassing all four helices of the four-helix bundle as illustrated in Figure 4B. To our knowledge, such a bundle hinge has not been previously described. Alanine and cysteine substitutions at two hinge positions (G339 and G437) block kinase activation, suggesting that they block the formation of the receptor kinase signaling complex or prevent normal complex activation in the absence of attractant. Alanine and cysteine substitutions at the third position (G338) lock the receptor in the kinase-activating on state, indicating that they block normal on−off switching. The two glycine residues at positions 338 and 339 are 100% conserved in the chemoreceptors of *E. coli* and *S. typhimurium*, and glycine is found at one or both of these positions in 93% of the larger representative group of 15 eubacterial chemoreceptors (Table 1) (*15*). Similarly, the glycine at position 437 is 86% conserved in the *E. coli* and *S. typhimurium* chemoreceptors, and is 80% conserved in the representative group of eubacterial receptors (*15*). Thus, the bundle hinge is a highly conserved architectural feature, although some receptors do not possess the full complement of three glycines.

Our current working model proposes that the bundle hinge enables the cytoplasmic domain of an individual dimer to bend during trimer-of-dimers assembly, in which one face of the signaling subdomain packs against the corresponding faces of two other dimers as shown in Figure 5. This figure presents each four-helix bundle of a given dimer as a bent cylinder. Examination of dimer geometry reveals that this packing would not be possible if the receptor was a purely linear molecule with its linear central axis oriented normal to the membrane. The location of the Gly hinge between the adaptation and signaling subdomains is ideal for inducing a bend that allows the needed packing interactions. Although the bend is not evident in the crystal structure of the isolated cytoplasmic domain (*13*), this is not surprising since the domain has been severed from the transmembrane helices, enabling it to adopt a relaxed, unbent conformation. Alternatively, the Gly hinge could play an essential mechanistic role in the conformational dynamics of on−off switching.

Suggestive evidence for this role is provided by alanine and cysteine substitutions at the G388 position which prevent receptor switching to the off state, thereby locking the receptor in the kinase-activating on state.

The remaining essential glycine residue, G455, lies in the adaptation subdomain containing the receptor adaptation sites that are covalently modified by adaptation enzymes CheR and CheB. Different side chain substitutions at this position yield different effects on kinase regulation: the alanine substitution yields partial lock-on character by driving the receptor equilibrium toward the kinase-activating on state in both the absence and presence of aspartate, while the cysteine substitution fully blocks kinase activation. This glycine residue is 71% conserved in the *E. coli* and *S. typhimurium* chemoreceptors, but is only 47% conserved in the representative group of eubacterial receptors (*15*). It follows that G455 is a specialized essential element of most *E. coli* and *S. typhimurium* chemoreceptors, but is not as widely important as the other five essential glycines, particularly in more distantly related members of the eubacterial chemoreceptor superfamily. It is possible that flexibility at this position plays a role in receptor on−off switching, since substitution of the small alanine side chain drives the receptor toward the on state and prevents full kinase inhibition by attractant binding. In this picture, the larger cysteine side chain would lock the switch in the off state or disrupt receptor structure in a more dramatic way that prevents coupling to CheA.

The findings also confirm that cysteine substitutions at four conserved glycine positions located at the subunit interface of the homodimer (G271, G278, G285, and G467) can be oxidized to form disulfide bonds that retain normal kinase regulation or lock the receptor in its kinase-activating on state as previously observed (*12*). Each of these disulfide bonds has been previously shown to form within the dimer, rather than between dimers (*12*). By contrast, the 11 other engineered disulfide bonds tested herein strongly inhibit receptor-mediated kinase activation, indicating that most intra- or interdimer disulfides engineered at conserved glycine positions prevent CheA coupling or stimulation, or trap the receptor in the off state. All four of the functional disulfides are located in the adaptation subdomain. The existence of signal-retaining and lock-on disulfide bonds in the adaptation subdomain is consistent with the current working model for a small rearrangement of helix packing within this subdomain during receptor on−off switching triggered by attractant binding or covalent adaptation (*8*, *12*). The small magnitude of the rearrangement allows its transmission through the inherent flexibility of certain disulfide bonds, while other disulfides lock the on or off state.

Overall, this study demonstrates that six of the 14 conserved glycine residues in the receptor cytoplasmic domain play especially critical roles in receptor function and can be considered essential. Even comparatively subtle alanine substitutions at these six positions have consistently dramatic effects on the ability of the receptor to dock to CheA kinase or to regulate kinase activity, thereby disrupting receptor function during cellular chemotaxis. Working models have been proposed for the molecular roles these six essential glycines play in receptor architecture and mechanism, but further studies are needed to test these models.

More generally, mutations at essential glycine positions could trigger pathway defects underlying certain disease states in higher animals. For example, these findings predict that specific human cancers could be triggered by mutations at glycine positions that trap an oncogenic receptor or signaling protein in its on signaling state, thereby constitutively activating a cellular growth pathway. Even in nononcogenic proteins, mutations at glycine positions could simply trap a specific conformational state, such as the inward- or outward-facing state of a transmembrane transporter (*43*), thereby destroying function. Thus, scanning mutagenesis studies of conserved glycines are likely to be generally useful in mechanistic studies of proteins that undergo backbone conformational changes during function.

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