Receptor-mediated protein kinase activation and the mechanism of transmembrane signaling in bacterial chemotaxis

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Chemotaxis responses of *Escherichia coli* and *Salmonella* are mediated by type I membrane receptors with N-terminal extracytoplasmic sensing domains connected by transmembrane helices to C-terminal signaling domains in the cytoplasm. Receptor signaling involves regulation of an associated protein kinase, CheA. Here we show that kinase activation by a soluble signaling domain construct involves the formation of a large complex, with ~14 receptor signaling domains per CheA dimer. Electron microscopic examination of these active complexes indicates a well defined bundle composed of numerous receptor filaments. Our findings suggest a mechanism for transmembrane signaling whereby stimulus-induced changes in lateral packing interactions within an array of receptor-sensing domains at the cell surface perturb an equilibrium between active and inactive receptor-kinase complexes within the cytoplasm.

Keywords: CheA/CheW/*Escherichia coli*/signal transduction/Tar

Introduction

Type I membrane receptors have a simple membrane topology, with an N-terminal sensing domain at the external surface of the cell linked by a membrane-spanning helix to a signaling domain in the cytoplasm (Ullrich and Schlessinger, 1990; Heldin, 1995). The signaling domains often have associated protein kinase activities. For instance, in vertebrate cells, insulin, growth hormone and cytokine receptors have associated tyrosine kinase activities (Schlessinger and Ullrich, 1992; Schindler and Darnell, 1995), whereas, in bacteria, type I receptor signaling domains generally have associated histidine kinase activities (Stock *et al.*, 1990; Bourret *et al.*, 1991; Parkinson, 1993). Although there is no evidence for any homology between histidine protein kinase- and tyrosine kinase-linked receptors, the two large families seem to use similar mechanisms for transmembrane signaling (Stock *et al.*, 1991). The best characterized bacterial type I receptor is Tar, the protein that mediates chemotaxis responses to aspartate in *Escherichia coli* and *Salmonella*

(Scott *et al.*, 1993; Chervitz and Falke, 1996; Le Moual and Koshland, 1996; Stock and Surette, 1996). As in the case of vertebrate tyrosine kinase-linked receptors, dimerization appears to be essential for Tar function (Milligan and Koshland, 1988; Kim, 1994; Cochran and Kim, 1996; Surette and Stock, 1996). Moreover, hybrid receptors with the extracellular aspartate-binding domain of Tar linked to the tyrosine kinase domain of the human insulin receptor exhibit changes in tyrosine kinase activity in response to aspartate (Moe *et al.*, 1989).

Tar is a 60 kDa membrane-spanning protein composed of an N-terminal uncleaved signal sequence that leads from the cytoplasm to an extracytoplasmic aspartate-binding domain of ~150 amino acids, which is in turn linked via a second transmembrane sequence to a C-terminal region of ~340 amino acids located within the cytoplasm. The aspartate-binding or sensing domain has been produced as an independently folded soluble protein and its structure has been determined by X-ray crystallographic methods in both the presence and absence of aspartate (Scott *et al.*, 1993). The domain is an up-down four helix bundle that forms a symmetric homodimer. There are two non-overlapping aspartate-binding sites at the dimer interface. Aspartate binding is negatively cooperative, with binding to one site within a homodimer causing an asymmetric conformational change in one subunit that interferes with binding to the symmetrically opposed site (Biemann and Koshland, 1994). Studies of subunit cross-linking by disulfide bond formation between engineered cysteines in the transmembrane or aspartate-binding domains of Tar indicate that the receptor in the membrane is predominantly dimeric (Milligan and Koshland, 1988), with aspartate binding causing a change in the relative positions of the transmembrane helices that link the extracytoplasmic aspartate-binding domains to the signaling domain at the other side of the membrane (Chervitz and Falke, 1996).

Tar generates chemotaxis responses by modulating the activity of the histidine protein kinase CheA (Borkovich *et al.*, 1989). CheA activation leads to an increase in the phosphorylation of the bacterial response regulator, CheY, which in turn interacts with the flagellar motor to regulate swimming behavior (for a recent review of chemotaxis signaling, see Stock and Surette, 1996). CheA, together with an adaptor protein, CheW, binds to the signaling domain of the receptor. The kinase activity of the isolated CheA protein is only ~1% that of CheA in Tar-CheW-CheA complexes formed by adding purified preparations of CheA and CheW to membranes enriched for Tar or the closely related serine chemoreceptor Tsr (Borkovich *et al.*, 1989; Ninfa *et al.*, 1991), or by incubating CheA and CheW with soluble genetically engineered receptor signaling domain constructs (Ames and Parkinson, 1994; Cochran and Kim, 1996; Surette and Stock, 1996). Tar

and CheA, which are predominantly dimeric, form 1:1 binary complexes with CheW, which is monomeric (Gegner and Dahlquist, 1991; Gegner *et al.*, 1992; Surette and Stock, 1996). It has therefore generally been assumed that active ternary complexes are composed of a 2:2:2 receptor–CheW–CheA assembly. This has led to the assumption that transmembrane signaling involves a rotational, scissors or piston motion of one receptor subunit within a 2:2:2 complex relative to the other subunit, thereby changing the relative positions of CheA subunits so as to modulate kinase activity (Pakula and Simon, 1992; Chervitz and Falke, 1996; Cochran and Kim, 1996). It has been difficult, however, to reconcile this view with data indicating that signaling can occur through receptor dimers that have been genetically engineered so that one Tar subunit lacks a signaling domain (Milligan and Koshland, 1991; Gardina and Mason, 1996; Stock, 1996; Tatsuno *et al.*, 1996). Moreover, immuno-electron microscopy (EM) studies indicate that the majority of the receptor–CheW–CheA complexes in cells are clustered into large arrays (Maddock and Shapiro, 1993). The formation of these receptor assemblies, which were localized predominantly to the cell poles, appears to depend on ternary complex assembly, since receptor clusters were not seen in mutant strains that lacked CheA or CheW.

We report a characterization of active soluble signaling complexes formed from the signaling domain of Tar, CheW and CheA. The results indicate a well defined particle which, from light-scattering data, has an M_r of ~1400 kDa and an effective hydrodynamic radius of ~20 nm. These values are consistent with the results of EM studies which show a structure with a diameter of ~12 nm and a length of ~47 nm. The complex has been purified, and kinase activity has been shown to be up to 100-fold higher than values obtained under comparable conditions with isolated CheA dimers. This is essentially the same degree of activation observed with complexes formed between CheA, CheW and intact receptors in the membrane. The complex contains 7 ± 1 signaling domain dimers and 3 ± 1 CheW monomers per CheA dimer. High concentrations of CheW and CheA inhibit the formation of active complexes and facilitate the disassembly of preformed complexes. These and other findings indicate a distinctly different mechanism for transmembrane signaling from what has hitherto been proposed. The putative 2:2:2 complex may not play a significant role in chemoreceptor signaling during chemotaxis. It seems more likely that active assemblies are composed of numerous receptor dimers, with higher order interactions functioning to control kinase activity. Environmental signals may initiate chemotaxis responses through their effects on packing interactions between receptor dimers that alter an equilibrium between distinctly different active and inactive signaling assemblies at the membrane–cytosol interface.

Results

Isolation and characterization of active ternary complexes between the receptor signaling domain, CheW and CheA

It has been shown previously that soluble active ternary complexes can be formed by incubating CheA and CheW

Fig. 1. Formation of active ternary complexes from mixtures of LZ-Tar_C, CheW and CheA. CheA, CheW and LZ-Tar_C were incubated for the indicated times at 30°C, and kinase activity was determined by measuring ATPase activity in a coupled assay in the presence of 50 μM CheY plus 1.0 mM ATP as described in Materials and methods. Activity is expressed as μM ATP hydrolyzed/s/μM CheA. Results are presented for mixtures that contained 5.0 μM CheA, 10 μM CheW and 45 μM all Q LZ-Tar_C (○), Q/E LZ-Tar_C (●) or all E LZ-Tar_C (▲); a mixture of 40 μM CheA, 15 μM CheW and 30 μM Q/E LZ-Tar_C (■); and a mixture of 5.0 μM CheA, 70 μM CheW and 30 μM Q/E LZ-Tar_C (□).

together with a variety of soluble receptor constructs that contain the signaling domain of Tar or Tsr (Ames and Parkinson, 1994; Cochran and Kim, 1996; Surette and Stock, 1996). The highest activities we obtained were with a construct, termed LZ-Tar_C, that has a leucine zipper dimerization domain fused to the N-terminus of the cytoplasmic portion of Tar. Since the signaling domain by itself tends to be monomeric (Long and Weis, 1992), we have assumed that the leucine zipper provides an essential dimerization function that is normally supplied by the sensing portion of the intact Tar protein. Tar contains two glutamates and two glutamines that are subject to methylation, demethylation and deamidation *in vivo*, and three variants of LZ-Tar_C were constructed to assess the effects of glutamyl modification: all E LZ-Tar_C in which all four residues are glutamates; Q/E LZ-Tar_C which corresponds to the sequence of the wild-type Tar protein; and all Q LZ-Tar_C in which all four residues are glutamines. As expected from results with intact receptors (Borkovich *et al.*, 1992), the all Q construct which corresponds to the fully methylated receptor was about twice as effective as the Q/E construct in its ability to stimulate kinase activity in the presence of CheW, and the all E construct was essentially inactive.

Ternary complexes between LZ-Tar_C, CheW and CheA were formed by incubating a mixture of the three purified soluble protein components at 30°C for 4 h (Figure 1). Complex formation was assayed routinely by following the associated increase in kinase activity. The complexes were purified from such mixtures by molecular sieve chromatography (Figure 2). Virtually all of the kinase activity eluted within a distinct peak that was well separated from LZ-Tar_C, CheW and CheA. The retention times of active ternary complexes during molecular sieve chromatography were essentially the same as that obtained with the 50S ribosomal subunit. Elution during molecular sieve chromatography depends on effective hydrodynamic

Fig. 2. Molecular sieve chromatography and light-scattering analysis of active complexes formed from all Q LZ-Tar_C, CheW and CheA. (A) A mixture containing 50 μ M all Q LZ-Tar_C, 10 μ M CheW and 10 μ M CheA was incubated at 30°C for 4 h and then subjected to molecular sieve chromatography on a TSK-Gel G5000PW_{XL} column (30 $\times$ 0.78 cm) as described in Materials and methods. Fractions were assayed for ATPase activity (bar graph), and analyzed for protein composition by Coomassie staining after 15% SDS-PAGE using purified components as standards. (B) Light-scattering profile and computed molecular weights (indicated by a scan of values obtained at different points in peak elution profiles) were obtained from data collected by a miniDAWN detector as described in Materials and methods. The molecular weight estimated from the region of the scattering profile of the peak of ternary complex where the ratio of UV absorption and scatter is approximately constant was 1 390 000 $\pm$ 10 000. The higher molecular weight values obtained at the leading edge of this peak is caused by overlaps with peaks that contain larger particles which, from EM analysis (see Figure 3A), appear to be aggregated complexes. The molecular weights of all Q LZ-Tar_C, CheA and CheW computed from the sequences of the corresponding genes are 36 900, 73 000 and 18 000, respectively. The ratio of components within ternary complex was estimated to be $\sim$ 7 all Q LZ-Tar_C and 1.5 CheW per CheA. Assuming four CheA subunits per complex, this gives an overall composition of $\sim$ 28 LZ-Tar_C:6 CheW:4 CheA, with a mol. wt of 1 430 000. Similar results were obtained with complexes formed from Q/E LZ-Tar_C.

radius rather than molecular weight. To measure the mass of the complex, an in-line light-scattering detector was connected to the chromatography column. This allowed assessment of both the mass of the particles in each elution peak and their effective radii of gyration. The results (Figure 2B) indicate that the complex has an M_r of $\sim$ 1400 kDa and a radius of gyration of $\sim$ 20 nm.

Analysis of the isolated complexes by SDS-PAGE indicated that they had a subunit composition of 7.0 ± 1.0 LZ-Tar_C and 1.5 ± 0.5 CheW per CheA monomer (Table I). Combining these data with the molecular mass estimates obtained by light scattering, the overall subunit composition appears to be 28 ± 4 LZ-Tar_C, 6 ± 2 CheW and 4 CheA. These values did not depend on the composition of the mixture from which active complexes were formed. As previously inferred from *in vitro* reconstitution experiments (Ninfa *et al.*, 1991; Gegner *et al.*, 1992; Ames and Parkinson, 1994) as well as from the effects of overproduction *in vivo* (Liu and Parkinson, 1991), high concentrations of CheW ($>10 \mu$ M) interfere with kinase activation (Figure 1). There was almost no ternary complex formation in mixtures that contained elevated levels of CheW, but the activity and composition of the few complexes that could be isolated under these conditions were indistinguishable from those obtained at lower concentrations of CheW. Moreover, complexes formed from either the all Q or the Q/E LZ-Tar_C constructs had approximately the same size and composition. The specific activity of CheA within the purified complexes formed from the all Q signaling domain was 20/s, and the activity of complexes formed from the Q/E signaling domain was slightly lower, 13/s. The specific activity of CheA dimers under these conditions is 0.20/s, so the activity of the isolated complexes corresponds to a 65- to 100-fold activation for the Q/E and all Q complexes, respectively. There was no evidence for CheA activation with the all E signaling domain (Figure 1), and we were unable to detect the formation of inactive complexes with the all E construct (data not shown).

Electron microscopic visualization of ternary complexes

Purified ternary complexes as well as mixtures of all Q or Q/E LZ-Tar_C, CheA and CheW that contained active ternary complexes were examined by EM. The results indicated an abundance of particles $\sim$ 47 nm long and 12 nm in diameter (Figure 3A and B). These particles were not observed if any one of the component proteins was omitted. LZ-Tar_C alone examined using the same

Table I. The subunit composition and activity of LZ-Tar_C-CheW-CheA complexes

Protein concentration in incubation mixture (μ M) Tar _C /CheW/CheA	Protein concentration in isolated complex (μ M) Tar _C /CheW/CheA	Molecular ratio Tar _C :CheW:CheA	Activity (/s)
50/10/10 ^a	3.03/0.586/0.399	7.6:1.5:1.0	20.1
50/25/10 ^a	0.844/0.127/0.105	8.0:1.2:1.0	24.6
50/10/5 ^a	2.47/0.475/0.371	6.7:1.3:1.0	18.1
50/10/25 ^a	2.80/0.617/0.393	7.1:1.6:1.0	20.8
50/10/10 ^b	1.32/0.246/0.192	6.9:1.3:1.0	13.1

The complexes were prepared, isolated and assayed for activity as described in Materials and methods.

^aAll Q LZ-Tar_C; ^bQEQE LZ-Tar_C.

Fig. 3. Electron micrographs of negatively stained LZ-Tar_C-CheW-CheA complexes. (A) A mixture of 45 μM LZ-Tar_C, 10 μM CheW and 5.0 μM CheA after incubation at 30°C for 4 h; (B) all Q LZ-Tar_C-CheW-CheA complexes purified from the mixture used in (A). (C) all Q LZ-Tar_C alone; (D) CheA alone; (E) CheW alone; (F) mixture containing 45 μM all Q LZ-Tar_C, 70 μM CheW and 5.0 μM CheA incubated at 30°C for 4 h. (G) Model for active ternary complexes wherein 7 ± 1 tetramers come together with CheA, and CheW bound to the CheW-binding regions at each end to form the structures seen in (A) and (B). The model of the single LZ-Tar_C tetrameric unit is based on a previously proposed model for monomeric Tar (Le Moual and Koshland, 1996). The tetramer is seen to be composed of two four-helix bundle dimers, with the two leucine zipper domains of each dimer forming end to end dimer-dimer contacts through another four-helix bundle. The proposed tetramer structure would be predicted to have the dimensions of the rods seen in (C).

procedure was composed of slightly shorter thin rods ($\sim 4 \text{ nm} \times \sim 35 \text{ nm}$, see Figure 3C). No rod-like particles could be detected with CheA or CheW in the absence of LZ-Tar_C (Figure 3D and E). HPLC coupled to in-line light-scattering measurements of LZ-Tar_C alone indicate that the protein is primarily tetrameric (data not shown).

The signaling domain appears to be predominantly α -helical (Le Moual and Koshland, 1996; Seeley *et al.*, 1996). It is composed of a central region that binds CheW (Liu and Parkinson, 1991; Surette and Stock, 1996), flanked by two sequences that are predicted to form coiled-coil structures (Lupas *et al.*, 1991; Stock *et al.*, 1991; Cochran and Kim, 1996; Surette and Stock, 1996). These sequences, termed methylated helices 1 and 2, MH1 and MH2, contain glutamate and glutamine residues that are subject to methylesterification, demethylation and

deamidation by a specific *S*-adenosylmethionine-dependent methyltransferase, CheR, and a specific esterase/amidase, CheB (Stock and Surette, 1996). The modified glutamyl groups are spaced in a heptad repeat so that they align along the solvent-exposed surface of the predicted coiled-coil. The uniform rod-shaped appearance of the LZ-Tar_C tetramers revealed by EM is consistent with a model wherein the tetramer is a coiled-coil structure with two dimers associated through interactions between LZ dimerization domains. Presumably, in the presence of CheA and CheW, 7 ± 1 tetramers coalesce to form a bundle with a CheA dimer and 3 ± 1 CheW monomers attached at each end (Figure 3G). We emphasize, however, that although this model fits the dimensions of the particles as well as structural information gleaned from previous analyses of signaling domain sequences, it should be

regarded only as a preliminary working hypothesis to aid the design of future experiments rather than as a low resolution structure based on solid biophysical data.

Glutamyl modification affects the stability of active ternary complexes

It has been observed previously that higher kinase activities are obtained when CheA and CheW are mixed with Tar that has higher levels of glutamate methylation or amidation (Ninfa *et al.*, 1991; Borkovich *et al.*, 1992; Cochran and Kim, 1996; Surette and Stock, 1996). We find that altered levels of glutamyl modification affect the stability of the preformed active complexes. Complexes formed from all Q LZ-Tar_C lost ~50% of their activity after a 24 h incubation at room temperature, and complexes formed from Q/E LZ-Tar_C lost ~80% of their activity over a similar period (Figure 4A). From EM examination and molecular sieve chromatography, it was apparent that the loss of kinase activity correlated with the dissociation of active complexes. No complexes could be detected with the all E LZ-Tar_C construct by either method (data not shown).

The CheB protein functions in the bacterial chemotaxis system to down-regulate receptor-associated kinase activity by catalyzing the deamidation/demethylation of glutamine and glutamyl methylester side chains. CheB is a monomeric enzyme composed of two domains, an N-terminal regulatory domain that is homologous to CheY, and a C-terminal methylesterase/amidase catalytic domain (Stock *et al.*, 1985; West *et al.*, 1995). CheA-dependent phosphorylation of the CheB regulatory domain activates the associated catalytic domain, thereby providing a negative feedback loop to down-regulate kinase activity (Lupas and Stock, 1989; Ninfa *et al.*, 1991). Cleavage of the regulatory domain mimics the effect of phosphorylation so that the isolated catalytic domain, CheB_C, is highly active. We used this constitutively active form of CheB to determine the effects of deamidation on the activity and stability of ternary complexes formed from the fully amidated receptor signaling domain construct, LZ-Tar_C (Figure 4B). The results clearly show that as CheB_C catalyzes the conversion of all Q LZ-Tar_C to various demethylated forms [seen as an increase in the apparent molecular weight during SDS-PAGE (Springer *et al.*, 1979)], there was a corresponding loss of kinase activity. Molecular sieve and EM analysis indicated that the loss of kinase activity correlates with ternary complex dissociation (data not shown). The Q/E LZ-Tar_C complexes generated by CheB_C-mediated deamidation appear to be considerably less stable than those produced from the genetically engineered Q/E LZ-Tar_C construct. The Q/E forms generated by CheB_C would be expected to differ from those in the Q/E construct since enzymatic deamidation is a stochastic process that involves deamidation at any of four essentially independent sites/monomer. The effects of glutamyl modification on complex stability are readily explicable in terms of our working hypothesis for the structure of the active ternary complex. Deamidation or demethylation would introduce anionic glutamate side chains within the MH1 and/or MH2 coiled-coils. This results in a large net negative charge in these regions that would be expected to destabilize the overall structure. One would expect amidation/methylation to have the opposite effect of stabilizing the active complexes.

Fig. 4. Effects of glutamyl modification and CheW on the kinase activity of purified ternary complexes. (A) Complexes formed from all Q LZ-Tar_C or Q/E LZ-Tar_C were purified by molecular sieve chromatography as described in the legend to Figure 2. Kinase activity was measured using the coupled ATPase assay in the presence of 50 μM CheY and 1 mM ATP after incubation at room temperature for the indicated times. All Q LZ-Tar_C (□), Q/E LZ-Tar_C (●), Q/E LZ-Tar_C plus 10 μM CheW (▲). Results are presented as percentage of activity of complexes measured 5 min after elution from the TSK-Gel G5000PW_{XL} column. (B) Effect of CheB_C on the activity of the all Q LZ-Tar_C-CheW-CheA complex. The all Q LZ-Tar_C-CheW-CheA complex was prepared and isolated as described in the legend to Figure 2. At time zero, 5.0 μM of the purified catalytic domain of CheB, CheB_C, was added to a fraction of the purified complex and, after the indicated times of incubation at room temperature, aliquots were removed and assayed for kinase ATPase activity (○) and for mobility of LZ-Tar_C during 10% SDS-PAGE. A control sample of complex was treated in the same way without addition of CheB_C (●).

Equilibria between active and inactive kinase signaling states

Previous results have indicated that ternary complex formation with either full-length receptors in membranes or soluble signaling domain constructs involves a reversible association of subunits so that the concentration of complex formed within mixtures reflects a steady-state between assembly and disassembly (Gegner *et al.*, 1992; Surette and Stock, 1996). Isolated ternary complexes formed from LZ-Tar_C, CheW and CheA were surprisingly stable,

however (Figure 4). It has been observed previously with Tar_C constructs (Surette and Stock, 1996) and with full-length Tar in membranes (Ninfa *et al.*, 1991; Gegner *et al.*, 1992) that high concentrations of CheW (>10 μ M) cause dramatic reductions in steady-state levels of ternary complex formation. The relative stability we have observed with isolated complexes as opposed to complexes in mixtures with CheW and CheA precursors raised the possibility that the inhibitory effect of high levels of CheW could be due, at least in part, to a stimulation of complex dissociation through an interaction of CheW with preformed complexes. An examination of the effects of CheW on the activity of purified complexes shows that CheW does in fact cause a dramatic increase in the rate of complex dissociation (Figure 4A). The level of CheW in cells has been estimated to be ~5.0 μ M (Gegner *et al.*, 1992; Wang and Matsumura, 1997), so CheW could function *in vivo* to help maintain a dynamic equilibrium between complex assembly and disassembly.

From previous studies of ternary complexes formed from intact Tar in membranes, it has been concluded that complexes can exist in two conformational states, one in which the kinase is activated ~100-fold and the other in which the kinase is essentially inactive (Borkovich and Simon, 1990). In the studies described above, we have only been able to isolate active ternary complexes, however. There was, for instance, no evidence for inactive complex formation in mixtures of all E LZ-Tar_C, CheW and CheA that were subjected to molecular sieve chromatography. Since isolated complexes formed from the Q/E LZ-Tar_C signaling domain construct, which have ~63% of the activity of ternary complexes formed from the all Q LZ-Tar_C, were significantly less stable than those formed from the all Q LZ-Tar_C construct, it seemed possible that our failure to detect inactive complexes was due to a relative instability which resulted in their dissociation during molecular sieve chromatography. We therefore developed an ultracentrifugation protocol that avoids sample dilution. Mixtures of Q/E LZ-Tar_C or all Q LZ-Tar_C, CheA and CheW that had been incubated to form active complexes were centrifuged, and the sedimentation of kinase activity was measured (Figure 5). The results indicated that, as expected from the molecular sieve chromatographic data, the active complexes were considerably larger than any of their component proteins. In control experiments where LZ-Tar_C, CheW or CheA was omitted, the sedimentation of the remaining pairs of components was negligible over the times and centrifugation speeds under which sedimentation of active complexes was observed.

To estimate the composition of undiluted complexes, mixtures were centrifuged for sufficient time to completely remove complexes from the meniscus, and the degree of depletion of LZ-Tar_C, CheA and CheW was measured. The results (Table II) indicated that over a range of different initial concentrations of LZ-Tar_C, CheW and CheA, although the amount of complex varied with the conditions of incubation, the subunit composition of the active ternary complexes that did form was roughly constant, with molar ratios of LZ-Tar_C:CheW:CheA similar to those obtained with complexes purified by molecular sieve chromatography. These experiments also provided an estimate of the fraction of total CheA incorporated into

Fig. 5. Sedimentation analysis of ternary complexes. Sedimentation profile obtained from a 80 μ l mixture of 60 μ M Q/E LZ-Tar_C, 15 μ M CheW and 5.0 μ M CheA incubated for 3 h at 30°C and then centrifuged in an Beckman Airfuge, A110 rotor at room temperature for 1.0 h at 22 p.s.i. Fractions of 10 μ l were removed from the top of the tube, assayed for ATPase activity using the coupled assay, and analyzed for protein composition by Coomassie staining of 15% SDS-PAGE using purified components as standards. ATPase activity expressed as μ M ATP hydrolyzed/s/ μ M CheA in the mixture (■), concentration of Q/E LZ-Tar_C (●), CheW (○), CheA (□).

complexes and, using this number, the specific activity of CheA in complexes could be determined (Table II). Again, the results with undiluted complexes were very similar to those obtained with purified preparations.

We next used the ultracentrifugation–meniscus depletion method to look for the formation of inactive complexes. We could obtain no evidence for the formation of large complexes in incubation mixtures that contained all E LZ-Tar_C (data not shown). Using the ultracentrifugation method, however, we were able to detect inactive higher molecular weight aggregates in mixtures of Q/E or all Q LZ-Tar_C plus CheA at concentrations of CheW that inhibit formation of active ternary complexes. Analysis of the compositions of this inactive material indicated that lower activity resulted from progressive increases in the stoichiometry of CheW binding (Figure 6A). Electron microscopic examination of these inactive mixtures indicated that the well-defined structures seen in active preparations were not, however, present (Figure 3F). Moreover, inactive complexes were not detected when these mixtures were subjected to molecular sieve chromatography.

Similar results were obtained in mixtures that contained high concentrations of CheA (Figures 1 and 6B). When the Q/E LZ-Tar_C construct was incubated at increasingly high CheA concentrations, kinase activation was lost as complexes formed that contained much higher amounts of CheA and CheW per receptor signaling domain subunit. The inhibitory effect of CheA was dependent on the ratio of CheW:Tar_C being significantly greater than 1.5:7 (data not shown). As at high concentrations of CheW, inactive complexes formed at high concentrations of CheA did not survive molecular sieve chromatography. The effects of elevated concentrations of CheA and CheW can be simply understood in terms of an equilibrium between active ternary complexes and inactive subassemblies with higher stoichiometries of CheA and CheW per Tar_C. The latter could include the putative 2 CheA:2 CheW:2 Tar complex as well as 2:1:2, 1:2:2 and 1:1:2 complexes. According to

Table II. Sedimentation studies of ternary complexes formed by Q/E LZ-Tar_C and all Q LZ-Tar_C

Protein concentration in original mixture (μM) Tar _C /CheW/CheA	Protein concentration in isolated complex (μM) Tar _C /CheW/CheA	Molecular ratio Tar _C :CheW:CheA	Activity (/s)
30/5/5 ^a	11.5/2.9/1.8	6.4:1.6:1	9.0
30/15/5 ^a	21.5/4.4/3.2	6.7:1.4:1	9.2
20/15/5 ^a	10.8/4.1/1.7	6.4:2.4:1	11.3
40/15/5 ^a	22.9/6.5/3.4	6.7:1.9:1	10.6
60/15/5 ^a	23.5/6.5/3.6	6.5:1.8:1	11.4
30/15/2 ^a	9.2/2.6/1.3	7.1:2.0:1	11.0
25/5/5 ^a	19.5/3.3/2.6	7.5:1.3:1	17.2
25/15/5 ^b	20.3/6.7/3.2	6.3:2.1:1	17.3
50/2.5/5 ^b	8.7/2.0/1.1	7.9:1.8:1	15.7
50/5/5 ^b	12.6/3.9/2.0	6.3:2.0:1	16.0
50/15/5 ^b	24.9/7.2/3.6	6.9:2.0:1	18.1

^aComplex formed by Q/E LZ-Tar_C, CheW and CheA.

^bComplex formed by all Q LZ-Tar_C, CheW and CheA.

Fig. 6. Effect of CheW and CheA concentration on ternary complex composition and activity. Kinase activities and subunit compositions of ternary complexes were determined by the meniscus depletion method (see Materials and methods). Kinase ATPase activity in μM ATP hydrolyzed/s/μM CheA in the complex (●); molar ratios of Tar:CheA (■) and CheW:CheA in complexes (▲). (A) Ternary complexes were formed by incubating mixtures containing either 25 (open symbols) or 50 μM (solid symbols) all Q LZ-Tar_C plus 5.0 μM CheA and the indicated concentrations of CheW at 30°C for 3 h. (B) Ternary complexes were formed by incubating mixtures containing either 30 μM Q/E LZ-Tar_C plus 15.0 μM CheW and the indicated concentrations of CheA at 30°C for 3 h.

this view, elevated CheA pushes the equilibrium toward the inactive state by pushing additional subunits of CheW into association with the receptor.

Discussion

We have characterized the structure and activity of ternary complexes formed between the kinase CheA, the adaptor protein CheW and the cytoplasmic signaling domain of the aspartate receptor Tar. The results indicate that complexes with high kinase activity are not formed by a simple 2:2:2 association of signaling domain and CheA dimers held together by two CheW subunits, as has previously been assumed, but instead involve a higher order structure with several receptor monomers per CheA. It previously has been shown that CheW binds both CheA and the signaling domain of Tar with 1:1 stoichiometry (Gegner and Dahlquist, 1991; Surette and Stock, 1996). Heterodimers formed between CheW and the Tar signaling domain do not appear to have a high affinity for CheA, however; and the association of CheW with CheA is weak (K_D of 20 μM) and does not affect CheA activity. These Tar–CheW and CheW–CheA binary interactions would therefore be expected to result in the formation of ternary complexes with 1:1:1 stoichiometry without activating CheA. Our results indicate that the formation of a completely different structure is required to activate CheA. The 100-fold increase in CheA activity within the isolated active complexes argues strongly for the idea that these soluble structures have direct relevance to the active complexes that have been produced from receptors in the membrane.

Structure of active signaling complexes

The active complexes that we have isolated are well defined structures that appear to contain two bundles of 12–16 receptor signaling domain monomers that intermesh to form structures that bind 2–4 CheWs and a single CheA dimer at each end (Figure 3). It seems likely that CheA and CheW nucleate bundle assembly at least in part by facilitating coiled-coil interactions between signaling domains that do not directly contact CheW or CheA. The leucine zipper dimerization domains probably act to help align the bundle and provide an additional surface for higher order polymer formation. The leucine zipper moiety

is not required for active complex formation, however. Ames and Parkinson (1994) showed that a construct of the Tsr signaling domain with the MH2 sequence deleted is also capable of forming active complexes and, furthermore, they observed that complex formation showed a higher order dependence on signaling domain concentration (Hill coefficient ~ 5). Moreover, immuno-EM and fluorescent labeling studies indicate that receptors in wild-type cells cluster into patches that contain hundreds of receptor monomers as well as CheA and CheW (Maddock and Shapiro, 1993). These clusters were not observed in mutant strains that lack CheA or CheW.

The signaling domain complexes we have isolated may represent essentially two symmetrically opposed unit cells of the membrane-associated receptor signaling network (Figure 3G). According to this model for the soluble complexes, the leucine zipper moiety may help organize the bundles as well as facilitate dimer formation. This function for the zipper region also fits with the observation that dimers produced from genetically engineered disulfide cross-links do not readily participate in active complex formation (M.G.Surette, unpublished results). It should also be noted that in electron micrographs of mixtures of LZ-Tar_C, CheW and CheA, we have generally observed larger aggregates that appear to be extended arrays formed through lateral interactions between numerous individual bundles (Figure 3A). These may be soluble correlates of the receptor clusters one sees in membranes.

Previous studies of CheA and CheW binding to receptors in membranes indicated that at saturation, a 1:1:1 stoichiometry was obtained (Gegner *et al.*, 1992). Although in this study it was reported that kinase activation was observed, no data were presented that correlated kinase activity with CheA binding. In membrane reconstitution experiments where kinase activation has been demonstrated, the concentration of CheA has invariably been at least an order of magnitude less than the concentration of receptors and CheW (Borkovich and Simon, 1991; Ninfa *et al.*, 1991). From our results with soluble receptor signaling domain constructs, it seems likely that at higher concentrations of CheA, inactive 1:1:1 associations predominate. On the other hand, it should be noted that the exact receptor stoichiometry per active CheA center in membranes may differ substantially from that seen in complexes formed from isolated signaling domain constructs. In the membrane there could be sharing of receptor subunits between adjacent unit cells in a larger array. The situation *in vivo* is complicated further by the continuous process of receptor methylation and demethylation that would be expected to keep the receptor signaling system poised in a dynamic equilibrium between active and inactive signaling states. The effects of methylation on kinase activity *in vivo* are readily explicable in terms of the differential stability of complexes formed from the all Q and Q/E signaling domains as well as the lack of formation of active ternary complexes from the all E construct. As proposed previously (Stock *et al.*, 1991), electrostatic repulsion between negatively charged glutamate residues probably interferes with the bundling of receptor coiled-coil structures that seems to be required for active complex assembly.

Aspartate binding to isolated sensory domain dimers or receptors in membranes exhibits a hyperbolic concentra-

tion dependence, with a K_D of ~ 1 μ M (Biemann and Koshland, 1994), and the inhibition of kinase activity by aspartate in reconstituted systems (Ninfa *et al.*, 1991; Borkovich *et al.*, 1992) as well as the swimming responses of intact cells (Clarke and Koshland, 1979) exhibit a first order dependence on aspartate binding. One might suppose that if there were a large number of receptor dimers per CheA active center there would be a higher order dependence of kinase activity on aspartate. This need not be the case, however; if aspartate binds with almost the same affinity to receptors in active and inactive complexes. For aspartate to induce a conformational change that inhibits kinase activity, it is necessary that it binds with higher affinity to inactive than to active complexes, but if the number of receptor dimers in active complexes is large, the difference in affinity need not be great. For instance, assuming seven receptor dimers per CheA dimer, a 2-fold increase in the K_D of aspartate bound to receptor dimers in active versus inactive complexes (i.e. a shift from 1–2 μ M) could produce a >100 -fold shift in the equilibrium between active and inactive complexes.

Implications for transmembrane signaling

Our findings are consistent with a model for kinase regulation that involves a transition between two essentially distinct membrane-associated structures. The relatively slow time scale for interconversions between active and inactive forms that we have detected with soluble receptor signaling domain complexes could occur much more rapidly under the constraints imposed by the two-dimensional environment of receptors in the membrane. Although the stoichiometry of components within active and inactive complexes appears to differ substantially, a transition between the two need not involve any significant binding or dissociation of CheW and CheA from receptors in the membrane. The data on CheW and CheA binding indicate that at the concentrations found in cells a large fraction of receptor dimers would have no associated subunits (Gegner *et al.*, 1992). One would therefore expect a range of different associations including free receptors, receptors with only CheW bound and receptors with both CheA and CheW bound. Our results indicate that CheA activation requires higher order interactions between receptor dimers. Presumably, dimers with no CheW and CheA bound interact with receptor dimers with CheW and CheA (inactive) bound to yield active complexes. If, as suggested from the large clusters of receptors observed in membranes, the active complexes that we have isolated correspond to unit cells of much more extensive arrays, it seems likely that active complexes in the membrane nucleate further active complex formation.

Tyrosine kinase receptors may use an analogous transmembrane signaling mechanism

Over the past several years, Tar has emerged as an important model system for understanding transmembrane signaling by type I receptors (Pakula and Simon, 1992; Kim, 1994; Stock, 1996). Research on tyrosine kinase receptors such as the epidermal growth factor receptor has indicated a simple mechanism that involves a hormone-induced transition from inactive monomers to active dimers (Heldin, 1995). Tar and insulin receptors locked in a dimeric state by disulfide cross-links can still regulate

their associated kinase activities, so although dimerization is a prerequisite for signaling it cannot be the whole story. Our results indicate that in the case of Tar, signal transduction involves higher order interactions between dimers. It seems likely that the slight perturbations in sensory domain conformation that have been observed in response to aspartate binding act by altering packing interactions between receptor subunits at the external surface of the membrane that perturb the equilibrium between different signaling complexes within the cell. This type of mechanism may be generally applicable to the analysis of type I receptor function. The important principle may be that transmembrane signaling need not involve the propagation of a specific conformational change through rigid connections between sensory elements on one side of the membrane and signaling activities on the other. Transmembrane sequences could function instead as flexible linkers that allow an equilibrium between conformational states at one surface to influence a distinctly different equilibrium between signaling states inside. According to this hypothesis, dimerization represents only the simplest manifestation of a very general mechanism whereby environmental signals perturb the dynamic organization of a sensory array on one side of a membrane to influence the dynamic organization of an associated signaling array on the opposing surface.

Materials and methods

Purification of proteins and determination of protein concentrations

The *Salmonella typhimurium* LZ-Tar_C, CheW, CheA and CheY proteins were purified as described previously (Surette and Stock, 1996). Protein concentrations in stock solutions were estimated using the BCA Protein Assay kit (Pierce) with bovine serum albumin as a standard and by UV absorption at 280 nm in 6 M guanidine chloride, 20 mM sodium phosphate, pH 6.5 (Gill and von Hippel, 1989). Extinction coefficients at 276 nm of 14.8 for CheA, 5.18 for CheW, 3.90 for CheA_H, 6.97 for CheY and 6.97/mM/cm for LZ-Tar_C were calculated from the protein sequences. The values obtained by BCA Protein Assay correlated well with those obtained by absorption methods. Protein concentrations of individual subunits in HPLC and ultracentrifugation fractions were estimated from Coomassie-stained 1.5 mm SDS-polyacrylamide gels where a range of known amounts (0.20–2.0 µg) of the corresponding pure proteins were used as standards. The gels were scanned with Color OneScanner (Apple) and the images were quantitated with NIH Image 1.60 software. Unless stated otherwise, all concentrations are expressed in terms of the indicated monomeric species.

Kinase activity assays

Unless indicated otherwise, rates of ATP hydrolysis were measured in 25 mM Tris-HCl, 50 mM potassium glutamate, 25 mM NaCl, 5.0 mM MgCl₂, 10% glycerol, 5.0% dimethyl sulfoxide (DMSO), pH 7.5 at 23°C in the presence of 2.0 mM ATP and 50 µM CheY using the spectroscopic pyruvate kinase/lactate dehydrogenase-coupled assay as described previously (Surette *et al.*, 1996). Activities are expressed as initial rates.

Isolation and characterization of LZ-Tar_C-CheW-CheA complexes

Typically, 50 µM LZ-Tar_C, 10 µM CheW and 10 µM CheA were mixed in a buffer containing 25 mM Tris-HCl, 50 mM potassium glutamate, 25 mM NaCl, 5.0 mM MgCl₂, 10% glycerol, 5.0% DMSO, 1.0 mM dithiothreitol (DTT), pH 7.5. The mixture was incubated for 4 h at 30°C, and 100 µl aliquots were subjected to chromatography on a TSK-Gel G5000PW_{XL} column (30×0.78 cm) with a TSK-Gel G4000PW guard column (TosoHaas) and eluted with 25 mM Tris-HCl, 50 mM potassium glutamate, 25 mM NaCl, 10% glycerol, 5.0% DMSO, pH 7.5, at a flow rate 0.5 ml/min. Absorbance at 280 nm was monitored with a Lambda-Max Model 481 LC Spectrophotometer (Waters).

Light-scattering measurements

The molecular mass and hydrodynamic radius of the LZ-Tar_C-CheW-CheA complex were determined using miniDAWN multiple angle laser light-scattering detector (Wyatt Technology) connected in-line with HPLC UV detector. The light-scattering and UV absorption data were processed with ASTRA software (Wyatt Technology) to obtain absolute estimates of molecular mass (for reviews, see Wyatt, 1993; Wen *et al.*, 1996). The concentration of eluted LZ-Tar_C-CheW-CheA complex required for the determination of molecular weight was estimated from UV absorption using the extinction coefficient of the ternary complex determined as following. The extinction coefficient of LZ-Tar_C-CheW-CheA complex was calculated from component proteins assuming 7:1:1 stoichiometry. The calculated extinction coefficient was corrected for the difference between the complex UV absorption in the elution buffer and in 6 M guanidine hydrochloride, 20 mM sodium phosphate, pH 6.5 (Gill and von Hippel, 1989). We used a value of 0.165 for the refractive index increment (dn/dc), and 0.133 for the solvent refractive index.

Electron microscopy

Specimens were prepared as described by the method of Steven *et al.* (1988). A thin carbon film was evaporated onto a freshly cleaved mica sheet. A 3 mm×3 mm square of mica was cut and pressed into a sheet of Parafilm, and 5 µl of sample was applied to the corner of the mica so that it penetrated under the carbon film. The sample was adsorbed to the carbon film and negatively stained in 1% uranyl acetate. The carbon film was placed on a 600-mesh copper grid and the specimen was observed in a Philip CM100 or a JEOL 100C electron microscope.

Sedimentation studies

LZ-Tar_C, CheW and CheA were mixed in the complex buffer and incubated for 3 h at 30°C. The complex mixture was spun briefly in a microfuge to remove precipitates. To estimate the stoichiometry, 80 µl of sample was centrifuged in a Beckman airfuge for 1 h at 22 p.s.i. The LZ-Tar_C-CheA mixture (without CheW) was spun under the same conditions as a control. The kinase activities and the concentrations of LZ-Tar_C, CheW and CheA in the meniscus before and after centrifugation were measured in the coupled assay and SDS-PAGE analysis respectively. The activity of ternary complex and concentrations of each component protein in the complex were calculated from the meniscus depletion. No depletion was observed after centrifugation of LZ-Tar_C-CheA mixtures.

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