Diagnostic cross-linking of paired cysteine pairs demonstrates homologous structures for two chemoreceptor domains with low sequence identity

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Abstract

Hundreds of bacterial chemoreceptors from many species have periplasmic, ligand-recognition domains of approximately the same size, but little or no sequence identity. The only structure determined is for the periplasmic domain of chemoreceptor Tar from *Salmonella* and *Escherichia coli*. Do sequence-divergent but similarly sized chemoreceptor periplasmic domains have related structures? We addressed this issue for the periplasmic domain of chemoreceptor Trg_E from *E. coli*, which has a low level of sequence similarity to Tar, by combining homology modeling and diagnostic cross-linking between pairs of introduced cysteines. A homology model of the Trg_E domain was created using the homodimeric, four-helix bundle structure of the Tar_S domain from *Salmonella*. In this model, we chose four pairs of positions at which introduced cysteines would be sufficiently close to form disulfides across each of four different helical interfaces. For each pair we chose a second pair, in which one cysteine of the original pair was shifted by one position around the helix and thus would be less favorably placed for disulfide formation. We created genes coding for proteins containing four such pairs of cysteine pairs and investigated disulfide formation in vivo as well as functional consequences of the substitutions and disulfides between neighboring helices. Results of the experimental tests provided strong support for the accuracy of the model, indicating that the Trg_E periplasmic domain is very similar to the Tar_S domain. Diagnostic cross-linking of paired pairs of introduced cysteines could be applied generally as a stringent test of homology models.

Keywords: bacterial chemotaxis; transmembrane receptors; homology modeling; ligand-binding domains; cysteine cross-linking

Bacterial chemoreceptors contain two modules: a carboxyl-terminal signaling-and-adaptation domain and an amino-terminal, ligand-recognition domain (Zhulin

2001). More than 2000 chemoreceptor genes have been identified in the sequences of bacterial genomes, and ~70% of these are deduced to code for transmembrane proteins in which the signaling-and-adaptation domain is in the cytoplasm, the ligand-recognition domain is in the periplasm, and there are two transmembrane segments: one near the amino-terminus, and the other, the direct connection between the periplasmic and cytoplasmic domains (Zhulin 2001; R. Alexander and I. Zhulin, pers. comm.). Signaling-and-adaptation domains from

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all these receptors have related sequences, centered on a highly conserved segment that is the signature of a bacterial taxis receptor. Common primary sequences imply a common three-dimensional structure. Thus, the X-ray structure of the signaling-and-adaptation domain of one receptor (Kim et al. 1999) is considered representative of the general structure of chemoreceptor signaling-and-adaptation modules.

The situation is less clear for periplasmic, ligand-recognition domains since their sequences vary greatly and most exhibit only modest or no significant relatedness (Zhulin 2001; R. Alexander and I. Zhulin, pers. comm.). However, for the >1200 chemoreceptors deduced by sequence analysis to have periplasmic domains, over half of these domains are 160 ± 20 residues (Zhulin 2001; R. Alexander and I. Zhulin, pers. comm.). In this group, the structures of the same ligand-recognition domain from two species have been determined by X-ray crystallography: Tar_S from *Salmonella enterica* Serovar typhimurium (Milburn et al. 1991) and Tar_E from *Escherichia coli* (Bowie et al. 1995). The two structures are very similar: a homodimer for which each monomer is a four-helix bundle in which helix 1 is the extension of transmembrane segment 1 and helix 4 continues into the bilayer, forming transmembrane segment 2. Do other chemoreceptor periplasmic domains, particularly those of approximately the same size, have similar three-dimensional structures? In the current study we addressed this issue for chemoreceptor Trg_E from *E. coli* by combining homology modeling

and an experimental strategy that provides stringent tests for a homology model.

Results

Modeling the periplasmic domain of Trg_E

The first step in creating a homology model for the periplasmic domain of Trg from *E. coli* was alignment of the sequence of the Trg_E domain with the sequence of a Tar periplasmic domain of known structure. However, there are few residue matches between the Trg_E domain and either Tar domain. To achieve the best alignment we used an iterative PSI-BLAST search to find sequences related to the periplasmic domain of Trg_E and found a family limited to eight at the time we did the search (Peach et al. 2002). This family would be much larger if a search were performed with current databases, but this is not crucial for our purposes, since the original family included both periplasmic domains of known structure: Tar_S (Milburn et al. 1991) and Tar_E (Bowie et al. 1995). We refined alignment of the eight sequences by assigning a strong penalty for gaps in regions corresponding to helices in the known structures. This confined gaps to loops between the four helices, where they are most likely to occur (Barton and Sternberg 1987). In the resulting multiple-sequence alignment (Fig. 1), the sequence of the Trg_E periplasmic domain had the most residue identities with the Tar_S domain: 22 identical residues and three gaps (four, one, and three residues) among the 159 residues of the Trg_E domain. The 14%

Figure 1. Alignment of a Trg-related family of chemoreceptor periplasmic domain sequences. See text for explanations of alignment and identification of the family. Sequences are periplasmic domains of chemoreceptors Trg_E, Tar_E, Tap, and Tsr_E of *E. coli*; Tar_S and Tsp of *Salmonella*; and Tas and Tse of *Enterobacter aerogenes*. We have defined the beginning and the end of periplasmic domains using membrane boundaries determined experimentally for Trg_E (Boldog and Hazelbauer 2004). The cartoon above the sequences indicates positions of helices and loops in the three-dimensional structure of the domains of Tar_S and Tar_E (Milburn et al. 1991; Bowie et al. 1995). Boxes enclose residues identical between the Trg_E and Tar_S sequences.

Figure 2. Model of Trg_E periplasmic domain with positions of diagnostic paired pairs of introduced cysteines. (A) The polypeptide backbone of the modeled homodimer, viewed parallel to the cytoplasmic membrane (Membrane) depicted using Swiss-PDBViewer (Guex and Peitsch 1997). (B) Cartoon of helical positioning for a monomer of the homodimer, viewed normal to the membrane from the periplasm. In both panels, different helices are color-coded, and positions of introduced cysteines are marked by residue number. In A, these cysteines are also marked by balls: blue for the closer residue of each paired pair, and brown for the more distant residue. Cysteine pairs are connected by lines: solid for pairs predicted to be sufficiently close for effective disulfide formation, and dotted for the paired pair more distant in the model. For clarity, in A specific cysteine pairs are shown in one, not both, monomers.

match compared to an 11% match, and the same gaps for alignment with the Tar_E sequence. Thus, we used a structure of the Tar_S periplasmic domain to create a homology model of the periplasmic domain of Trg_E. Among several structures determined for this domain, we used 1LIH (Milburn et al. 1991) because it was most likely to reflect the domain structure in the intact chemoreceptor (Peach et al. 2002). We placed Trg_E side chains onto a backbone template of the four-helix bundle of structure 1LIH with loops deleted, rebuilt the loops with Trg_E residues, and refined the structure (Peach et al. 2002). The result was a homology model of the periplasmic domain of Trg_E based on the Tar_S structure: a homodimer of two four-helix bundles of helices α -1 through α -4, in which α -1 emerges from the membrane and α -4 extends back into it (Fig. 2A).

Testing a low-match homology model

Although the technique of homology modeling for building a three-dimensional model for a protein domain of unknown structure is well-established, in a situation

where the target shares <25% sequence identity with the template structure, homology modeling may still be less than straightforward. Aside from the degree of structural similarity between template and target, the quality of a homology model depends most significantly on the correctness of the alignment of the target sequence to the template (Tramontano and Morea 2003). We addressed this issue for our model of the Trg_E periplasmic domain by diagnostic cross-linking between pairs of paired, introduced cysteines. The strategy was to choose pairs of positions for which the structural model predicted that introduced cysteines would be on adjacent helices, sufficiently close to allow ready formation of a disulfide bond, and then chose a control pair, a closely related variant of the initial pair in which one of the cysteines was displaced by a single position. Thus, if the Trg_E sequence were correctly aligned to the helices in the Tar_S structure, the first pair of cysteines in each set should cross-link readily, whereas in the control pair the cysteine displaced by a single position in the sequence should be located on the opposite side of the helix, and cross-linking should not occur (Fig. 2). We identified four such sets of paired positions for cysteine introduction, each of which was predicted by the model to be positioned across the interface between a different pair of helices in the four-helix bundle (Fig. 2). Table 1 lists the four paired pairs and their model-predicted separations.

We created plasmid-borne genes under the control of a modified *lac* promoter that coded for the eight cysteine-substituted forms of Trg_E listed in Table 1 and introduced each plasmid into a strain lacking other receptor genes as well as the enzymes of receptor modification. The relative cellular content of these altered receptors was examined by immunoblotting SDS-polyacrylamide gels of reduced samples taken directly from cultures growing in conditions that result in cellular Trg_E at a level comparable to the wild-type complement of chemoreceptors (Fig. 3A). The cellular contents varied to some extent, but still allowed visualization using the same immunoblot conditions. Trg178C/130C and Trg178C/131C exhibited slightly altered electrophoretic mobility.

Table 1. Predicted distances between cysteine pairs

Interface	Cysteine substitutions	Modeled C _{α} -C _{α}	
		Class	Distance [Å]
$\alpha 1$ - $\alpha 2$	66-108	farther	8.1
	66-109	close	5.5
$\alpha 1$ - $\alpha 3$	77-146	farther	10.5
	77-145	close	7.0
$\alpha 2$ - $\alpha 3$	96-150	farther	11.1
	96-149	close	7.2
$\alpha 4$ - $\alpha 3$	178-131	farther	8.7
	178-130	close	5.1

Figure 3. Cellular content and disulfide formation in vivo for Trg_E containing pairs of introduced cysteines. The panels show segments corresponding to ~60,000 *M_r* of immunoblots of SDS-polyacrylamide gels displaying samples from the same log-phase cultures processed in sample buffer containing (A) or lacking (B) the reducing agent β-mercaptoethanol. Samples are grouped as the related pairs of cysteine pairs with the pair predicted to be closer following the more distantly separated pair. The position of the Trg_E monomer devoid of disulfides is marked by an arrow. Introduction of cysteine at position 130 slightly retards electrophoretic mobility of Trg_E. Positions of introduced cysteines and model-postulated distances between α-carbons are shown below B. All samples in A represent the same number of log-phase cells, as determined by optical density. Samples for B were adjusted to compensate for cellular content and interchain cross-links (see text). Relative to the samples for A, these were 66–108, 2; 66–109, 1; 77–146, 2.5; 77–145, 1; 96–150, 2; 96–149, 1; 178–131, 2.5; 178–130, 2.5. Trg_E with an intrachain disulfide between cysteines migrates more rapidly than the unrestrained polypeptide. This effect can vary, and for cysteines at 178–130 or 178–131, it is less than for the other cysteine pairs.

We investigated disulfide formation between the introduced cysteine pairs by analysis of the same samples as for Figure 3A, but without treatment with reducing agent (Fig. 3B). In such blots, Trg_E polypeptides with an internal disulfide migrate below unmodified polypeptide (Lee et al. 1994). For all four sets of cysteine pairs, the pair predicted to be closer formed a disulfide readily, even in the absence of added oxidant or catalyst, and did so to a greater extent than the companion pair, predicted to be farther apart. Disulfide formation was over 95% for three cysteine pairs—66–109, 77–145, and 96–149—predicted to be sufficiently close to form a disulfide readily across the α1–α2, α1–α3, or α2–α3 interface, respectively, and was minimal for the three related pairs in which one of the cysteines was displaced by a single residue position, the cysteine pairs 66–108, 77–146, and 96–150. For the fourth set of cysteine pairs, cross-linking across the α4–α3 interface occurred between position 178 and both positions on helix α3 but was greater for position 130, predicted to be closer. Extensive cross-linking of cysteine pairs predicted to be sufficiently close to form disulfides across four different helical interfaces at different segments of the structure provided strong evidence the general organization predicted by the structural model was correct, particularly since the reactions occurred without added oxidant or catalyst. Moreover, the greatly reduced cross-linking between three of the

four related pairs, predicted to be more distant, validated details of helical positioning and packing of those helices. Significant cross-linking for the more distant pair across the α4–α3 interface implied less tight packing at that interface, consistent with the well-documented helical sliding of α4 in transmembrane signaling (Falke and Hazelbauer 2001; Peach et al. 2002). Cross-linking between the diagnostic cysteine pairs at the α4–α3 interface might have been influenced by two nearby arginines, but it is unlikely that local electrostatics was the primary origin of differences between cross-linking patterns across this and other interfaces, since diagnostic pairs spanning other interfaces also had neighboring charged groups.

Cysteine-containing forms of Trg_E for which intrachain disulfides were not predominant formed interchain disulfides, creating electrophoretic species at apparent molecular weights ≥120 kDa (Lee et al. 1994). Approximately 40% of the cellular content of Trg77C/146C participated in interchain cross-links, as did ~20% of Trg178C/130C and ~10% of Trg_E with cysteines at 66–108, 96–150, or 178–131.

Functional activity of cysteine-substituted and disulfide cross-linked forms of Trg_E

It was important to assess the functional activity of cysteine-substituted receptors. Thus, we assayed their ability to signal across the membrane and to mediate chemotaxis. To test chemotaxis, we examined formation of chemotactic rings on semisolid agar plates containing ribose or galactose, the two Trg-linked attractants, in cells containing all other components of the taxis system, including other receptors. All singly substituted and six of eight doubly substituted forms mediated chemotactic ring formation. For four doubly substituted forms, the two with 96C and the two with 178C, effective ring formation required an increased level of the inducer IPTG, 50 μM rather than 20 μM (for the 96C receptors, this was the case only for galactose rings). Trg_E with cysteines at positions 66 and 109 or at 77 and 146 did not mediate tactic response to either ribose or galactose (Fig. 4). Essentially 100% of the cellular content of Trg66C/109C has an intrachain disulfide (Fig. 3), and most of Trg77C/146C was cross-linked in interchain disulfides (see above). Cleaving those disulfides by adding dithiothreitol to the soft agar plates allowed the two double-cysteine forms to mediate formation of chemotactic rings (Fig. 5). This reducing agent also allowed ring formation mediated by double-cysteine Trg_E with cysteines at 96 or 178 even when induced with only 20 μM IPTG (data not shown).

We characterized transmembrane signaling in vivo by assessing the ability of ligand occupancy in the

Figure 4. Chemotaxis mediated by Trg_E containing introduced cysteines. Panels show images of chemotactic rings 7 h after inoculation of semisolid agar plates containing minimal salts, required amino acids, 20 or 50 (*) μ M IPTG and either 50 μ M galactose (Gal) or 100 μ M ribose (Rib), and incubation at 35°C. Plates were inoculated with derivatives of CP177 (deleted for chromosomal *trg*) harboring a plasmid coding for a form of Trg_E containing zero, one, or two cysteines as indicated *below* each panel.

periplasmic domain to increase methylation of the cytoplasmic domain and thus shift in the pattern of the multiple electrophoretic forms of the receptor to more methylated, more rapidly migrating species (Yaghmai and Hazelbauer 1992). All but Trg66C/109C exhibited the shift indicative of transmembrane signaling, although the extent varied (Fig. 6A). Addition of dithiothreitol to Trg66C/109C cells allowed signaling by this receptor (Fig. 6B).

Discussion

Experimental validation of a homology model

Patterns of oxidative cross-linking *in vivo* between the sets of cysteine pairs (Fig. 3B) provided strong support for the validity of a homology model of the Trg_E periplasmic domain. We devised this experimental strategy as a demanding test of homology models, and our Trg_E model passed. The cysteine substitutions themselves allowed receptor function, although in some cases only after adjusting test conditions to correct for cellular content or restrictions imposed by disulfides, implying that the extent of disulfide formation reflected the native position of the residue in the three-dimensional structure. The model was derived from the crystal structure of an isolated Tar_S periplasmic fragment, but the experimental tests, performed on native receptor in unperturbed, actively growing cells, demonstrated its validity for the intact receptor.

All four cysteine pairs that the model predicted should form disulfides across four different helical interfaces were cross-linked at high efficiency, even in the absence of added oxidant or catalyst. For three pairs, disulfide

formation was $\sim 100\%$; for the fourth it was $\sim 50\%$. Strikingly, the three associated control cysteine pairs for the positions exhibiting $\sim 100\%$ disulfide formation were hardly cross-linked. Thus, not only did the Trg_E model place specific cysteines in the predicted proximity, but the drastic difference between cross-linking to adjacent cysteines provided strong support for specific packing faces and the precise helical positions postulated by the model. For the $\alpha 4$ – $\alpha 3$ interface, the pair predicted to be sufficiently close to form a disulfide cross-linked to $\sim 50\%$ rather than $\sim 100\%$. The companion pair, predicted to be more distant, exhibited less but significant cross-linking. These observations are consistent with well-documented movements of $\alpha 4$ relative to the rest of the subunit, both the axial sliding that mediates transmembrane signaling (Falke and Hazelbauer 2001) and sliding in the absence of ligand (Peach et al. 2002). Thus, the $\alpha 4$ – $\alpha 3$ interface would be expected to be less tightly packed than the other helical interfaces, providing sufficient mobility to reduce disulfide formation for the most favorably positioned cysteine pair and increase it for the less favorably positioned pair.

Considering all the data, we conclude that the periplasmic domain of Trg_E has essentially the same four-helix bundle structure as the known structures of the Tar_S domain, even though the extent of residue identity is minimal.

The reduced level of disulfide formation across the $\alpha 4$ – $\alpha 3$ interface in comparison to other interfaces is an example of the influence of a factor in addition to distance between the introduced sulfhydryls. An appropriately short distance is required for disulfide formation, but other factors—such as motion, local geometry, and local electrostatics—can influence disulfide formation. Predicting the extent or even the direction of such influences is not straightforward, but the degree to which they result in incorrect interpretations can be kept to a minimum by the use of pairs of cysteine pairs and, as necessary, by increasing the number of diagnostic paired pairs and making conclusions based on overall patterns rather than one or a few cysteine cross-links.

Figure 5. Restoration of chemotaxis by the reducing agent dithiothreitol. Experimental procedure, images and labels as for Figure 4 with the addition of 1 mM DTT (+) to one set of plates.

Figure 6. Transmembrane signaling by Trg_E. The images are immunoblots of samples from actively growing cultures (as for Fig. 3A) of strains lacking chromosomal genes for the methyl-accepting chemotaxis proteins but harboring a plasmid coding for Trg_E containing zero, one, or two cysteines. Cells were grown in the presence (+) or the absence (–) of Trg-mediated attractant ribose (see Materials and Methods for details). Exposure to attractant results in a shift to more rapidly migrating electrophoretic forms of Trg_E, corresponding to the increased methylation that mediates sensory adaptation. The growth medium contained 20 μ M IPTG with two exceptions (*) for which 50 μ M was required to provide sufficient cellular content of Trg_E. (A) Transmembrane signaling by Trg_E containing the eight pairs of cysteine pairs or their constituent individual cysteines. (B) Effect of reducing agent dithiothreitol (DTT) on signaling of Trg_E with cysteines at positions 66 and 109.

In the following discussion, we consider implications of our observations for the structure of other periplasmic domains and for testing homology models.

Structures of chemoreceptor periplasmic domains

Over half of the > 1200 chemoreceptors currently identified by sequence analysis appear to have periplasmic domains of 160 ± 20 residues (Zhulin 2001; R. Alexander and I. Zhulin, pers. comm.). Do these domains, with few or no significant sequence matches, share a common three-dimensional structure with the 159-residue Tar periplasmic domains of known structure (Milburn et al. 1991; Bowie et al. 1995)? We have presented strong evidence that this is the case for a periplasmic domain with only 14% sequence identity with Tar_S. We identified a family of eight periplasmic domains related by sequence (Fig. 1). Some years ago, it was suggested that periplasmic domains of *E. coli* and *Salmonella* chemoreceptors had related structures (Mowbray and Sandgren 1998) and a model for one of those domains was constructed once the Tar_S structure was available (Jeffery and Koshland 1993), but neither the general suggestion nor the specific model were tested experimentally. Within the family of sequence-related periplasmic domains, the Trg_E domain is one of the least related by sequence matches to the domains of known structure. Yet our data indicate that the Trg_E domain has a structure very similar to the Tar_S domain. Thus, we conclude that the

periplasmic domains of Tcp, Tsr_E, Tas, Tse, and Tap likely also have closely related structures.

What about the hundreds of chemoreceptor periplasmic domains that are not part of the immediate family related to Trg_E and Tar? The > 600 which have ~160 residues are very good candidates for having the same basic structure as the Tar domains. Diagnostic cross-linking at paired cysteine pairs provides a powerful experimental test for homology models that could be performed for such chemoreceptor domains.

Unassisted disulfide formation as an indication of close and stable positioning

In many studies of oxidative cross-linking between introduced cysteines, an oxidant or oxidative catalyst is added, because without such treatments the extent of disulfide formation is often minimal. For example, this was the case for the initial study of disulfide formation in vitro between introduced cysteines in a chemoreceptor, which focused on the periplasmic domain (Falke and Koshland 1987) as well as for many subsequent studies of chemoreceptor transmembrane domains in vivo (Lee et al. 1994, 1995; Hughson and Hazelbauer 1996) and in vitro (Lynch and Koshland 1991; Pakula and Simon 1992), and of receptor cytoplasmic domains in vitro (Danielson et al. 1997; Bass and Falke 1998, 1999; Butler and Falke 1998). Thus, it is notable that no added

oxidant or oxidative catalyst was necessary to generate essentially complete cross-linking *in vivo* for three of the four cysteine pairs in the periplasmic domain of Trg_E predicted to be well-positioned for disulfide formation. Such high propensity for oxidative cross-linking must reflect not only positioning of the side chains sufficiently close to form disulfides but also a narrow distribution around an average distance as the result of stable positioning of structural units containing the cysteines. As discussed above, the fourth cysteine pair predicted to be well-positioned bridged a helical interface that undergoes specific movements (Falke and Hazelbauer 2001; Peach et al. 2002), consistent with < 100% cross-linking efficiency. However, even in that case, the extent of unassisted disulfide formation was substantially greater than observed in all but one previous study of chemoreceptors (Maruyama et al. 1995).

The notably higher extent of unassisted oxidative cross-linking between introduced cysteines in the periplasmic domain versus the transmembrane or cytoplasmic domains suggests significant differences in dynamics of helical packing in the different domains. The high efficiency and striking differences we observed in disulfide formation for related pairs of cysteine pairs implies that three of the four helices in the periplasmic domain are tightly packed in a stable structure with minimal dynamic movement between helices. This is consistent with the relative protease resistance of these domains (Mowbray et al. 1985; Feng et al. 1997). In contrast, chemoreceptor transmembrane (Barnakov et al. 2002) and cytoplasmic (Wu et al. 1995) domains are more loosely packed and dynamic, consistent with low levels of unassisted formation *in vitro* of disulfides between introduced cysteines in those domains.

Testing homology models

Besides direct structural determination, how can homology models be tested experimentally? Among the few approaches are mutational analysis of postulated binding or active site residues and assessment of oxidative cross-linking between introduced cysteines postulated to be sufficiently close to form disulfides. The former approach is limited to one or a few regions of the protein. The latter approach is not, but to be convincing, formation of a postulated disulfide requires experimental assurance that the cross-link reflects proximity in the average structure, not trapping of low probability excursions produced by dynamic movement, a phenomenon that has been observed for chemoreceptors (Falke and Koshland 1987; Danielson et al. 1997). In the current work, we apply an experimental approach that addresses this concern. Specifically, we designed controls for each introduced cysteine pair by creating a related pair in

which one cysteine of the original pair was shifted by one position around the helix and thus would be less favorably placed for disulfide formation. When introduced at crucial points throughout a postulated structure, such sets of introduced cysteine pairs can provide stringent tests of a homology model. Our success in validating a model of the periplasmic domain of Trg_E indicates that this strategy could be widely applied.

Materials and methods

Sequence alignment and model construction

Details of our sequence alignment and homology modeling of Trg_E using the 1LIH coordinates for the periplasmic domain of Tar_S have been described (Peach et al. 2002).

Strains and plasmids

E. coli K-12 strains CP177, CP362 (Park and Hazelbauer 1986), and CP553 (Burrows et al. 1989) are deleted, respectively, of the chromosomal genes for *trg*, for all four methyl-accepting chemotaxis proteins, and for those chemoreceptor genes plus *cheR* and *cheB*, coding for the two enzymes of receptor covalent modification. pAL1 contains *trg_E* under the control of a modified *lac* promoter/operator as well as *lacI^f* (Feng et al. 1999). Cells harboring pAL1 and grown in the presence of 20 μ M isopropyl- β -D-thiogalactoside (IPTG) contain Trg_E at a level that approximates the amount produced from the chromosomal gene (Feng et al. 1999). Derivatives of pAL1 were constructed by PCR-based, oligonucleotide-directed mutagenesis to code for forms of Trg_E with serine in place of its native cys23 and one or two cysteines introduced elsewhere.

In vivo cross-linking

We analyzed cells at mid-logarithmic phase ($\sim 2.5 \times 10^8$ cells/mL) at 35°C in H1 minimal salts medium (Hazelbauer and Harayama 1979) containing required amino acids at 0.5 mM, 26 mM ribose, 50 μ g/mL ampicillin, and 20 μ M IPTG (or as indicated 50 μ M). Samples of CP553 harboring a plasmid coding for a two-cysteine form of Trg_E were taken from a culture actively growing in 4 mL of medium contained in a 20-mL, 16-mm (OD) test tube tipped far on its side in a reciprocating shaker to ensure efficient aeration; centrifuged in a refrigerated microcentrifuge; suspended in 20 mM Tris/8 mM NaH₂PO₄ (pH 7.8), 10 mM EDTA, 10 mM *N*-ethylmaleimide, 1% SDS, 5% sucrose, and 2.5 μ g/mL bromophenol blue; boiled for 4 min; and analyzed by polyacrylamide (10%) gel electrophoresis and immunoblotting with anti-Trg_E.

Functional assays

Formation of chemotactic rings (Feng et al. 1997) and transmembrane signaling (Beel and Hazelbauer 2001) were assayed as described, using CP177 or CP362, respectively, harboring an appropriate plasmid coding for a form of Trg_E. Where indicated, dithiothreitol (DTT) was present in the semisolid agar plates at 1 mM. For the signaling assay, growth was as

described for in vivo cross-linking with either ribose or succinate as the sole source of carbon and energy. Samples were taken from actively growing cultures in mid-logarithmic phase, mixed with ice-cold TCA at a final concentration of ~7.5%, and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. Effects of DTT were assessed 2 h after addition of 2.5 mM reducing agent to an actively growing culture.

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