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Mutational Analysis of a Transmembrane Segment in a Bacterial Chemoreceptor

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Trg is a member of a family of receptors that mediates chemotaxis by *Escherichia coli*. Its transmembrane domain is a loose four-helix bundle consisting of two helices from each of the two identical subunits. This domain mediates transmembrane signaling through a conformational change in which the second transmembrane segment (TM2) is thought to move relative to TM1, but mutational analysis of TM2 by cysteine scanning had identified only a few positions at which substitutions perturbed function or induced signaling. Thus, we performed mutational analysis by random mutagenesis and screening. Among 42 single-residue substitutions in TM2 that detectably altered function, 16 had drastic effects on receptor activity. These substitutions defined a helical face of TM2. This functionally important surface was directed into the protein interior of the transmembrane domain, where TM2 faces the helices of the other subunit. The functionally perturbing substitutions did not appear to cause general disruption of receptor structure but rather had more specific effects, altering aspects of transmembrane signaling. An in vivo assay of signaling identified some substitutions that reduced and others that induced signaling. These two classes were distributed along adjacent helical faces in a pattern that strongly supports the notion that conformational signaling involves movement between TM2 and TM1 and that signaling is optimal when stable interactions are maintained across the interface between the homologous helices in the transmembrane domain. Our mutational analysis also revealed a striking tolerance of the chemoreceptor for substitutions, including charged residues, usually considered to be disruptive of transmembrane segments.

A family of transmembrane receptor proteins mediates the chemotactic response in *Escherichia coli* and *Salmonella typhimurium* (see references 3, 10, and 32 for more detailed discussions and for the primary references for the general information provided in this and the following paragraph). Chemoreceptors recognize specific attractants and repellents and generate intracellular signals that alter swimming behavior. The ability to sense temporal gradients and the related phenomenon of sensory adaptation are mediated by covalent modification of the receptors, specifically the formation of carboxylmethyl esters on particular glutamyl side chains. These receptors control the activity of a histidine kinase, CheA, and correspondingly control the extent of phosphorylation of a response regulator, CheY. CheY-phosphate interacts with the flagellum to induce clockwise rotation of an otherwise counterclockwise-rotating motor. Counterclockwise rotation corresponds to runs, i.e., coordinated forward swimming, and clockwise rotation corresponds to tumbles, i.e., uncoordinated thrashings in place that reorient the cell to new, randomly chosen directions. An appropriate balance of runs and tumbles results in a biased random walk toward a favorable chemical environment.

Chemoreceptors are homodimers (22, 23, 35). Each 60-kDa monomer has two transmembrane segments that connect a periplasmic, ligand-binding domain to a cytoplasmic, signaling domain. Three-dimensional structural information is available for Tar, which mediates chemotaxis toward aspartate by direct binding of that small molecule, and for Trg, which mediates chemotaxis towards galactose and ribose by recognition of the respective sugar-occupied binding proteins. Structures of the isolated periplasmic domains of the aspartate receptors from *S. typhimurium* (Tar_S) and *E. coli* (Tar_E) have been determined by X-ray crystallography (4, 22, 35), and the structures of the transmembrane domains of the intact chemoreceptors Tar_E (29) and Trg_E (16) have been deduced from patterns of oxidative cross-linking in receptor proteins containing cysteine substitutions. These data provide a consistent picture of chemoreceptor structure. The periplasmic domain is an elongated, helical structure extending more than 70 Å (7 nm) normal to the membrane, with its ligand-binding site at the membrane-distal end. Each monomer of the homodimeric structure is a four-helix bundle consisting of two longer helices (α1 and α4) and two shorter helices (α2 and α3). The longer helices form a quasi-four-helix bundle along the subunit interface that passes through the membrane, with α1 extending from transmembrane helix 1 (TM1) and α4 becoming TM2. Cross-linking studies indicate that there are extensive interactions between TM1 and TM2 within a subunit and between TM1 and TM1′ across the subunit interface (16, 18, 29), in a pattern that suggests a splayed bundle of the four transmembrane helices.

Transmembrane signaling by chemoreceptors is thought to involve conformational change within a stable homodimer (23). What is the nature of that conformational change? The effect of constraining movement between pairs of helices in the periplasmic or transmembrane domains by disulfide cross-links indicated that signaling required significant movement between the long helical pair within a subunit but not between helices in different subunits (5, 7, 19, 20), a conclusion consistent with earlier assays of signaling by heterologous receptor dimers with one complete and one truncated subunit (24) and supported by additional analysis of the periplasmic fragment of a chemoreceptor (6, 8). Can mutational analysis provide information relevant to the nature of transmembrane signaling? Individual substitutions in a transmembrane segment have been found to eliminate receptor activity (14, 28) or even

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induce signaling (1), but those individual observations were not sufficient to deduce specific features of conformational signaling. However, analysis by cysteine scanning of the entire sequence of the two transmembrane segments of Trg revealed coherent patterns (17). Cysteine substitutions that reduced receptor efficiency (none inactivated the protein) were distributed on a single face of each helix, and these faces corresponded to the interior of the four-helix structure of the transmembrane domain deduced from patterns of oxidative cross-linking (16), suggesting that functionally disruptive cysteines perturbed interactions between the transmembrane helices. In fact, each functionally disruptive substitution altered receptor signaling, either reducing signaling efficiency or inducing signaling in the absence of ligand occupancy. Cysteine substitutions in TM1 that reduced signaling clustered along the apparent packing face of TM1 on its homologous partner in the other subunit, TM1′, and those that induced signaling clustered along the adjacent face of TM1-TM2 interaction. We reasoned that altering a side chain in an interaction face between helices would usually weaken that interaction rather than strengthen it, so we should base our interpretation of reduction and induction of signaling by cysteine substitutions on the principle that in both cases helix-helix interaction was being weakened. By this reasoning we concluded that optimal signaling required stable helical packing across the subunit interface (TM1-TM1′ interaction) and that signaling involved a relative movement within a subunit (TM2 relative to TM1). Cysteine scanning of TM2 was less informative, since few cysteine substitutions disrupted function. However, several cysteine substitutions in TM2 did reduce signaling and, like for TM1, clustered along the intersubunit interface where TM2 faces its homologous partner, TM2′. Only two cysteine substitutions in TM2 induced signaling, which is not sufficient to define a pattern. In summary, mutational analysis (17) and assays of signaling activity by constrained or truncated chemoreceptors (5, 7, 17, 20, 24) all indicated that a crucial aspect of the conformational change of signaling was relative movement with a subunit between TM2 and TM1. Whether this involved a movement of TM2 relative to a static TM1, of TM1 relative to a static TM2, or of both helices relative to other parts of the receptor could not be determined unequivocally from those data. However, models based on mutational and structural analyses as well as on the positions of interhelical cross-links that induced signaling (6, 20) have postulated that TM2 is the active agent of the intrasubunit conformational change, although the two models suggest different forms of movement. No matter what the detailed movement is, there is a strong case for the intimate involvement of TM2 in conformational signaling. If that is true, then why did so few cysteine substitutions in TM2 of Trg have a discernible effect on receptor function, and why did so few induce signaling? Perhaps the characteristics of the cysteine side chain, which is intermediate in size and polarity among those likely to be found in a transmembrane sequence, reduced the likelihood of packing face disruption. This reasoning suggested that an analysis of random substitutions in TM2 might be informative. Thus, in this study, we have created random mutations in the segment of *trg* that codes for TM2 and analyzed the effects of the resulting substitutions.

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MATERIALS AND METHODS

Strains and plasmids. CP177 and CP362 are derivatives of *E. coli* OW1 (27) containing, respectively, Δ(*trg-100*) or that deletion plus Δ(*tsr-7026*) and Δ(*tar-tap*)*5201* (30). pMG2 is a pUC13 derivative carrying *trg* under the control of its native promoter (26).

Mutagenesis and screening. A mixture of mutagenic 96-mers corresponding to *trg* codons 195 to 227 was synthesized by adding to each reagent used for oligonucleotide synthesis a total of 1.025% (1/96) of the other three nucleotide reagents (9, 25). The resulting, gel-purified oligonucleotides were used as primers for in vitro DNA synthesis directed by an M13mp11-*trg* hybrid produced from the *dut ung* strain CJ236 (15). In an experiment designed to determine the frequency of mutagenesis, a sample of the reaction mixture was used to transfect the Dut^+ Ung^+ strain JM101 (21), single-stranded DNA was isolated from 30 transfectants, and the sequence of the relevant *trg* segment was determined for each. One-sixth of the transfectants contained nucleotide substitutions in the mutagenized region, a frequency sufficiently high to allow identification of mutations that altered receptor function by screening instead of selection. Individual mutagenesis experiments were then performed in which the synthesis mixture was treated with *Rsr*II and *Afl*II, which cleave *trg* at codons 60 and 259, respectively, and the 0.6-kb fragment carrying the mutagenized region was isolated by agarose gel electrophoresis and ligated to pMG2 cleaved with the same enzymes. The resulting pool of plasmids was used to transform CP362. Isolated transformants were screened for Trg-mediated chemotaxis by using semisolid agar plates containing tryptone.

Assays. Semisolid agar swarm plates containing tryptone broth plus 100 μg of ampicillin per ml or containing minimal salts, required amino acids, 50 μg of ampicillin per ml, and either 0.05 mM galactose, 0.1 mM ribose, or 0.1 mM maltose were inoculated with highly motile, logarithmic-phase cultures in tryptone broth at 2.5×10^8 to 5×10^8 cells per ml and placed in a humid incubator at 35°C (12). Plates inoculated with CP362 containing plasmid-borne *trg* were examined after 17 to 24 h. For CP177 containing plasmid-borne *trg*, ring diameters were measured each hour for at least 6 h, beginning soon after a ring was first evident (3 to 4 h after inoculation). Swimming behavior at room temperature was assessed by viewing, with a phase-contrast microscope, cells grown to mid-logarithmic phase in tryptone broth containing 100 μg of ampicillin per ml, pelleted by gentle centrifugation, suspended in 10 mM potassium phosphate (pH 7)–0.1 mM EDTA–1 μM methionine, centrifuged, and suspended again in the same buffer. The immunoblot assay (33) was performed with CP362/pMG2 derivatives grown in tryptone broth at 35°C in the absence or presence of 0.2% (13 mM) ribose. At a density of $\sim 2.5 \times 10^8$ cells per ml (in logarithmic growth), a sample of 2.5×10^7 cells was added to trichloroacetic acid (10% final concentration). Precipitated material was separated by polyacrylamide (11%, with 0.073% bisacrylamide) gel electrophoresis, electroblotted onto nitrocellulose, treated with anti-Trg serum and a peroxidase-coupled secondary antibody (Bio-Rad, Richmond, Calif.), and incubated with hydrogen peroxide and 4-chloro-1-naphthol.

RESULTS

Isolation of mutants. We synthesized a mixture of oligonucleotides corresponding to the segment of *trg* coding for TM2 but containing random substitutions, and we used the mixture as the primer for in vitro synthesis of *trg*. The products of this synthesis, carried in a derivative of pUC13, were introduced into a strain with the chromosomal copies of the chemoreceptor genes deleted but otherwise wild type for chemotaxis and motility. A total of 1,650 transformed strains, representing derivatives from 12 independent mutagenesis experiments, were tested for the ability to form Trg-mediated chemotactic rings on semisolid agar plates, and 90 appeared to be defective. The nucleotide sequence of the segment of *trg* corresponding to TM2 was determined for the 90 genes (Table 1). Seventy-three of the genes coded for a single amino acid substitution in Trg, 13 altered two residues, and 4 created three substitutions. Among independently isolated, single-residue substitutions, 16 were found more than once in independent experiments, resulting in the identification of 42 different substitutions. This collection included substitutions at all but 2 of the 26 positions from R-198 through R-223, the two charged residues that bracket TM2.

Characterization of receptor function. A detailed phenotypic analysis was performed for the 42 singly substituted Trg proteins. Receptor function was assessed by examining the formation of chemotactic rings on semisolid plates containing galactose, ribose, or tryptone. Trg mediates an attractant response to the two sugars as well as formation of a chemotactic ring in response to an unidentified compound present when

TABLE 1. Mutations creating single substitutions in TM2

Residue	Mutational change		
	Codon	Multiple isolates	Amino acid
198	CGC→CTC		R→L
199	None isolated		
200	GGT→TGT		G→C
	GGT→GTT		G→V
201	GGG→TGG	2	G→W
	GGG→GAG		G→E
	GGG→GCG		G→A
202	ATG→ACG		M→T
	ATG→AGG		M→R
203	TTC→ATC		F→I
	TTC→CTC		F→L
	TTC→TTA	3	F→L
	TTC→TAC	2	F→Y
204	ATG→ATA	6	M→I
205	ATT→AAT		I→N
	ATT→AGT	3	I→S
206	GGC→TGC	2	G→C
207	GCG→ACG	2	A→T
208	TTT→TCT		F→S
	TTT→TTG		F→L
209	GTG→GAG		V→E
210	None isolated		
211	GCC→ACC		A→T
	GCC→CCC		A→P
	GCC→GGC	2	A→G
212	CTG→CAG		L→Q
213	GTC→GGC		V→G
214	ATG→ATA		M→I
	ATG→ATT		M→I
	ATG→GTG		M→V
215	ACG→AAG		T→K
	ACG→AGG		T→R
	ACG→ATG	2	T→M
216	CTG→CGG	3	L→R
217	ATA→CTA		I→L
	ATA→TTA		I→L
	ATA→AGA		I→R
	ATA→ATG		I→M
218	ACA→ATA		T→I
219	TTT→TGT	2	F→C
	TTT→TTA	3	F→L
	TTT→TTC		F→L
	TTT→TTG		F→L
220	ATG→AGG	3	M→R
	ATG→ATA		M→I
221	GTG→TTG	2	V→L
222	CTA→CAA	2	L→Q
	CTA→CCA		L→P
223	CGT→CTT	3	R→P

cells grow on tryptone. This latter response is evident on semisolid agar plates containing tryptone for strains containing Trg as the sole chemoreceptor and particularly for strains with an amplified amount of Trg. The test cells had the chromosomal chemoreceptor genes deleted but carried the altered *trg* on a derivative of pUC13. For such strains, in which Trg is the only receptor species, the tumble frequency is low and thus taxis is relatively inefficient. This allowed the detection of relatively subtle defects but made it difficult to obtain useful measurements of rates of ring movement. Thus, chemotactic rings were rated qualitatively (Table 2). Examples of chemotactic rings formed on the three test plates by cells containing wild-type *trg* or representative mutants forms are shown in Fig. 1. Of the 42 substitutions identified by the original screens, all but two, L-212→Q (L212Q) and I217M, were found upon detailed examination to cause a discernible reduction in at least some aspect of Trg function. With only one exception (F219C), all substitutions had the same relative effect on the responses to galactose and to ribose. This is consistent with the deduction from mutational analysis (30, 33, 34) that responses to the two attractants are mediated by interaction of the respective sugar-occupied binding proteins at closely related, overlapping binding sites at the membrane-distal end of the extended periplasmic domain, substantially distant from the region of the mutational substitutions in TM2. In contrast, approximately half of the substitutions had an effect on the Trg-mediated ring observed on a tryptone plate that was different from the effect on the responses to the two attractant sugars. Many of these differences were modest, but they may reflect a different recognition site or mode of signaling for whatever compound elicits the response observed on tryptone. The active compound may be a repellent, perhaps a product of cellular metabolism.

The 16 substitutions that had a drastic effect on all three responses, eliminating chemotactic rings or allowing only a marginal ring to form, were not randomly distributed along the TM2 sequence but rather occurred at positions related to the periodicity of an alpha helix. This is illustrated in Fig. 2, in which the positions are shown on a helical net diagram of TM2. The helical face defined by the perturbing substitutions faces the protein interior of the four-helix organization of the transmembrane domain (16), where TM2 apposes TM1′ and TM2′ (Fig. 3).

Characterization of receptor defects. What was altered by substitutions that drastically reduced receptor function? Defective taxis could reflect a disruption of protein stability that in turn would reduce the cellular content of an altered receptor. To address this possibility, receptor content was investigated by immunoblot analysis. Thirty-six of the 42 proteins were present at approximately the same level as the wild type in host cells lacking other chemoreceptors but harboring a Trg-producing derivative of pUC13. Six were present at 20 to 50% of the wild-type level, but this difference did not appear to be the origin of defective receptor function since one (Trg-I217M) was indistinguishable from the wild type in our functional tests and the others (Trg-A207T, Trg-F208S, Trg-T218I, Trg-M220I, and Trg-V221L) exhibited improved functional ability when produced from the identical plasmid but in a host containing active chromosomal genes for the other chemoreceptors (see below), a situation that did not result in an increased dosage of plasmid-produced Trg. Thus, none of the substitutions in TM2 appeared to cause a phenotypic defect solely as the result of a lowered cellular content of the altered protein.

Alternatively, it was possible that substitutions could disrupt structure, and thus function, by perturbing the transmembrane helix in which they occurred. Candidates for such general perturbations are noted in Table 2. They include two substitutions that eliminated one of the two charged side chains that bracket (and may anchor) the hydrophobic sequence of TM2, eight substitutions that introduced a normally charged residue into the hydrophobic transmembrane sequence, and three substitutions that introduced a potentially helix-disrupting proline. Among these 12 potentially perturbing substitutions (R223P occurs in two categories), only 4 resulted in drastic defects in chemotaxis. These were the two substitutions, one a proline, for the bracketing charged residues (R198L and R223P), an introduced proline (L222P), and an introduced charge (T215K). For the latter two, the functional disruption may not be simply the result of the presence of a proline or a potential

TABLE 2. Phenotypic effects of substitutions in TM2

Mutation	Possible disruption by:	Receptor-minus host[a]					Receptor-plus host[a]			
		Ring quality[b,c] with:			Altered swimming[d]	Altered signaling[e]	Ring rate[b,f] with:			Altered swimming[d]
		Gal	Rib	Tryp			Gal	Rib	Mal	
R198L	Charge loss	−	−	−		Induced	−	−	−	
G200C		−	−	−		Induced	−	−	−	
G200V		−	−	−		Induced	−	−	−	
G201A	Charge gain	(+)	(+)	(+)			+++	+++	+++	
G201E		+	+	(+)	T	Reduced	++++	++++	++++	t
G201W		(−)	(−)	−		Induced	++	++	++++	
M202R	Charge gain	(+)	(+)	+			+++	+++	++++	
M202T		(+)	(+)	(+)		Induced	++	++	++++	
F203I		(+)	(+)	+		(Reduced)	++++	++++	++++	
F203L		(+)	(+)	(+)		(Reduced)	++++	++++	++++	
F203Y		(+)	(+)	(+)		(Reduced)	+++	+++	++++	
M204I		+	+	(+)		(Reduced)	+++	+++	++++	
I205N		(−)	(−)	(−)		Induced	−	−	−	
I205S		(−)	(−)	(−)			++	++	+++	
G206C		(+)	(+)	(+)			++++	+++	++++	
A207T		(+)	(+)	(+)			++++	++++	++++	
F208L		(+)	(+)	+			++	++	++++	
F208S		−	−	(−)	T	Reduced	++	+++	+++	T
V209E	Charge gain	(+)	(+)	+			+++	+++	++++	
A211G		(+)	(+)	(+)	(T)	(Reduced)	+++	+++	++++	t
A211P	Proline	(+)	(+)	+			++++	+++	++++	
A211T		−	−	−	T	Reduced	−	++	−	T
L212Q		+	+	+						
V213G		(+)	(+)	+			+++	++	+++	
M214I		(+)	(+)	+			++++	+++	+++	
M214V		(+)	(+)	(+)	(T)	(Reduced)	++++	++++	++++	t
T215K	Charge gain	−	−	−	(T)	Reduced	++++	++++	++++	t
T215M		−	−	−	T	(Reduced)	−	−	++++	t
T215R	Charge gain	+	+	(+)	T	Reduced	++++	++++	++++	t
L216R	Charge gain	(+)	(+)	(+)		(Induced)	++++	+++	++++	
I217L		(+)	(+)	+			++++	++++	++++	
I217M		+	+	+						
I217R	Charge gain	(+)	(+)	+			++++	++++	++++	
T218I		−	−	(−)	T	Reduced	++++	++++	++++	T
F219C		(−)	−	(−)		Induced	++	++	++++	
F219L		(−)	(−)	−		Induced	−	−	−	
M220I		(+)	(+)	(−)	T	(Reduced)	++++	++++	++++	T
M220R	Charge gain	(+)	(+)	+			++	++	++++	
V221L		+	+	(+)		Reduced	++++	++++	++++	
L222P	Proline	−	−	−	t	(Induced)	++++	++++	++++	
L222Q		(−)	(−)	−	(T)	Induced	++++	++++	++++	t
R223P	Charge loss or proline	−	−	−		Induced	+++	++	++++	

[a] The host was CP362 (receptor minus) or CP177 (receptor plus) harboring derivatives of pUC13.
[b] In semisolid agar plates containing minimal medium plus galactose (Gal), ribose (Rib), or maltose (Mal) or in plates containing tryptone (Tryp).
[c] +, (+), (−), and −, the chemotactic ring was essentially wild type, present but distinctly defective, marginal, or absent, respectively. The ratings reflect consistent results of multiple trials.
[d] t, (T), and T, in comparison with a control strain containing wild-type Trg, the tumbling frequency was detectably greater, frequent, or constant, respectively.
[e] Induced, (Induced), Reduced, and (Reduced), the substitution created a signaling phenotype of induced, partially induced, reduced, and partially reduced, respectively.
[f] Rates are expressed as a percentage of the rate mediated by wild-type Trg: ++++, 76 to 100%; +++, 51 to 75%; ++, 26 to 50%; +, ≤25%; −, no ring formed.

charge, since substitution of a different side chain at the same position also resulted in a drastic defect. In Fig. 2, the dashed boxes indicate the two positions (198 and 222) at which a drastic functional defect could be due simply to a general structural perturbation. The remaining 12 substitutions that caused drastic functional defects appeared to act by relatively subtle disruptions, most likely perturbations of side chain interactions between units of essentially unperturbed secondary structure.

Single amino acid changes in a chemoreceptor can cause a persistently altered signaling state, perturbing the normal swim-tumble balance of the host cell and thus affecting the ability to migrate in spatial gradients (1, 2, 31). We checked for substantial perturbations in the swim-tumble balance by the substitutions in TM2. Most strains containing a substituted Trg as the sole chemoreceptor exhibited the low frequency of tumbles characteristic of comparable cells containing the wild-type protein, but 12 exhibited detectably greater, frequent, or constant tumbling (Table 2). Among these 12 strains, 7 were drastically defective in the chemotactic response and 3 more were changed at positions that were sites of other mutational alterations that resulted in drastic defects. Thus, there was substantial, albeit incomplete, correspondence between an increased tumble frequency and a drastic defect in chemotaxis; the dis-

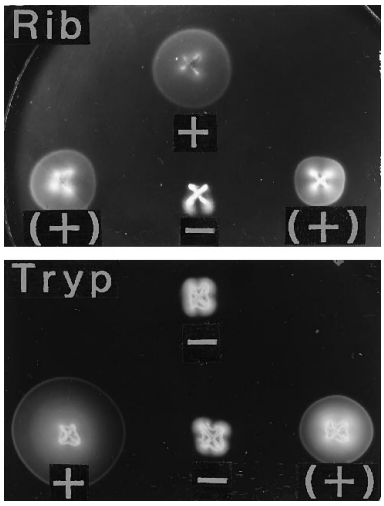


FIG. 1. Chemotactic responses on semisolid agar plates. Examples of responses that were wild type, present but distinctly defective, or absent [+, (+), or −, respectively] on plates containing tryptone broth (Tryp) or minimal salts medium plus ribose (Rib) are shown. Plates were inoculated with derivatives of CP362 harboring a pUC19 derivative containing a wild-type or mutated form of *trg* by stabbing the agar twice, at right angles, with a loop containing a cell suspension (hence the tubrid "x" at the position of each inoculation). The ring on the tryptone plate mediated by wild-type Trg moved at approximately half the rate of a "serine ring" mediated by Tsr produced from a comparable plasmid in the same strain.

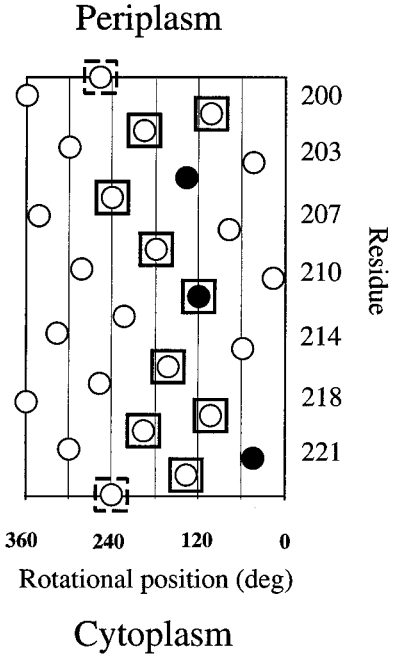
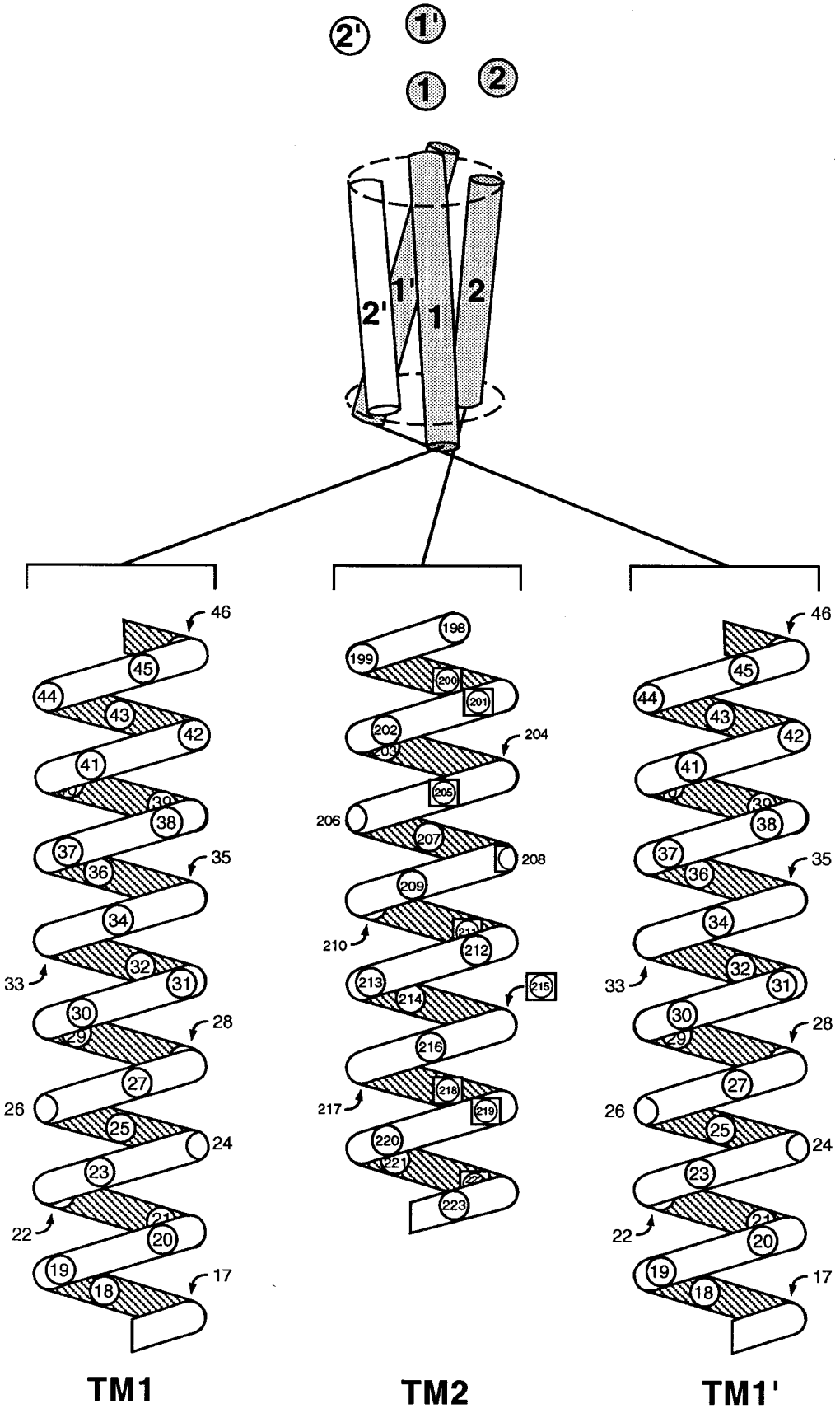


FIG. 2. Positions of substitutions with drastic effects on Trg function displayed on a helical net diagram of TM2. The sequence of TM2 from R-198 through R-223 is oriented with the cytoplasmic end down and the periplasmic end up. Positions at which a substitution had a drastic effect on receptor function (Table 2) are boxed. Dashed lines indicate substitutions that might reduce function as the result of perturbing a general feature of a transmembrane helix. For positions at which different substitutions created different phenotypic effects, the most extreme effect is indicated. Filled circles mark positions of local maxima for oxidative cross-linking between homologous cysteines in the two subunits of the receptor dimer (16); these identify the face along which TM2 and TM2′ are most closely apposed. deg, degree.

tribution of the two effects defined almost the same helical face on TM2 (Fig. 4). Only position 220, at which a substitution created a tumbly phenotype, was located outside this face.

The phenotypic severity of a *trg* mutation can be affected by the cellular context in which the altered protein is produced (12, 31). Particularly important are the predominant chemoreceptors Tsr and Tar, the presence of which provides a ratio of runs to tumbles characteristic of a wild-type cell. Cells containing Trg as the sole chemoreceptor exhibit a low tumble frequency (11). The collection of Trg proteins listed in Table 2, with the exception of the two that were essentially indistinguishable from the wild type, were tested for activity in cells carrying chromosomal copies of the other chemoreceptors genes and thus exhibiting a wild-type run-tumble balance. With these strains we could also examine the possibility that the mutational substitutions in Trg significantly perturbed the balance of the sensory system and thus would affect taxis mediated by an independent chemoreceptor. The 40 relevant plasmids were transferred individually into a host strain with the chromosomal copy of *trg* deleted but otherwise wild type for chemotaxis. For each strain, the rates of movement of chemotactic rings in response to galactose, ribose, and maltose were determined and expressed relative to the rate mediated by the wild-type protein (Table 2). The response to maltose is mediated by Tar, a chemoreceptor distinct from Trg. For simplicity, the relative rates were classified into quartiles (0 to 25%, 26 to 50%, 51 to 75%, and 76-100%) of the wild-type rate. Of the 16 substitutions in TM2 that had a drastic effect on Trg function in an otherwise receptorless cell, 6 also eliminated detectable Trg-mediated taxis in a cell containing the normal complement of the other chemoreceptors, and 1 allowed a reduced response to ribose but no response to galactose. Of the remaining nine substitutions, five resulted in reduced but detectable taxis and four allowed taxis approximately as good as that mediated by the wild-type protein. It was particularly striking that all but one of the substitutions that eliminated or drastically reduced Trg-mediated taxis by cells with a normal swim-tumble balance also eliminated the response to maltose, indicating that the overall sensory system was perturbed by those specific alterations in Trg. In contrast, none of the other altered Trg proteins had a significant effect on maltose taxis.

Analysis of receptor signaling. Transmembrane signaling by Trg can be assessed in vivo by monitoring the extent of receptor methylation in the absence and presence of an attractant (33). An increase in ligand occupancy of the periplasmic domain has two effects on the cytoplasmic domain and the noncovalently associated kinase, a transient inhibition of kinase autophosphorylation and a persistent increase in receptor methylation. The change in kinase activity is transient because the increase in methylation counteracts the effect of ligand occupancy to restore the null state of activity. Thus, a comparison of the extent of receptor methylation in unstimulated cells with the extent of methylation after stimulation and adaptation provides a measure of transmembrane signaling. Since added methyl groups result in increased electrophoretic mobility of the Trg polypeptide (27), the comparison can be accomplished by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with anti-Trg serum (33). In such assays the wild-type receptor appears as a series of electrophoretic species reflecting a low average level of methylation when assessed in unstimulated cells and as primarily the fastest-migrating, most methylated electrophoretic species of the unstimulated pattern when assessed in cells stimulated with a saturating amount of ribose and allowed to adapt. An examination of the 42 altered Trg proteins by this procedure re-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TM1'

FIG. 3. Three-dimensional organization of the transmembrane domain of Trg. The upper part of the figure is a schematic diagram showing a periplasmic view and a side view of the four helical segments of the domain. The circles and cylinders have diameters proportional to the dimensions of a helical backbone; side chains would extend out from these circumferences and would come in contact directly or as the result of dynamic fluctuations of the structure. The lower part shows a flattened view of the detailed orientation of TM2 relative to TM1 and TM1′ seen from the lipid-facing side of TM2. Positions at which a substitution had a drastic effect on receptor function (Table 2) are boxed. They lie along the surface where TM2 faces TM1′ (right side of TM2 in the lower diagram) or where TM2 faces TM2′ (back side of TM2 in the lower diagram). The helical orientations are based on data in reference 16.

vealed three general classes, examples of which are shown in Fig. 5. Some resembled the wild-type protein and thus appeared normal in signaling (Fig. 5, top row), some exhibited a reduced shift to more-methylated forms upon stimulation and thus were inefficient in signaling (bottom row), and some were in the highly methylated form even in the absence of stimulation and thus appeared to signal even in the absence of ligand (middle row). Table 2 indicates those receptors with reduced or induced signaling as well as those with a signaling phenotype intermediate between those classes and the wild-type class. Figure 6 shows in helical net diagrams the locations of substitutions that caused induced or reduced signaling, with the TM2-TM2′ interface shown for reference.

DISCUSSION

Mutational phenotypes and helical faces. Using random, region-specific mutagenesis, we explored the impact of a wide array of substitutions in TM2 on the chemoreceptor function of Trg. Substitutions in a transmembrane helix might be disruptive in two different ways, by being unfavorable for some general feature of the helix or its transmembrane character or by disturbing specific side chain packing involved in helix-helix interaction. The first class could include substitutions that disrupted helical secondary structure, eliminated a charged side chain at a putative membrane border, or introduced a charged side chain into the hydrophobic interior. Disruption of Trg action by such changes in TM2 would not provide insight into specific features of the chemoreceptor organization. In contrast, drastic effects by substitutions of the latter class, in which a hydrophobic residue was changed to another hydrophobic or neutral side chain, would be more likely to reflect specific features of interactions between transmembrane helices in the domain. Fortunately, 12 of the 16 substitutions that caused drastic defects in Trg function were in the latter, more informative class. In any case, whether all disrupting substitutions or only those likely to disrupt specifically are considered, the positions define the same distinct helical face (Fig. 2). This face is thus implicated as being important for helix-helix interactions within the transmembrane domain. In the cysteine-scanning analysis of TM2 in Trg (17), only 4 of 24 cysteine substitutions caused a statistically significant (in most cases moderate) reduction in receptor efficiency. Those four are contained within the helical face defined by the current, more widely based analysis and correspond to four of the positions identified in Fig. 2. In addition, the structural model of the transmembrane domain of Trg, deduced from patterns of disulfide cross-links between pairs of cysteines introduced into the native dimer (16), places the TM2 face identified by the current mutational analysis within the central region of potential helix-helix interaction in the membrane-spanning, four-helix bundle (Fig. 3).

Benign functional consequences of potentially disruptive substitutions. Eight of the 12 substitutions that would be expected to disrupt general features of a helix or its transmembrane character had moderate or minimal effects on receptor function (Table 2). One of the eight introduced a proline in the

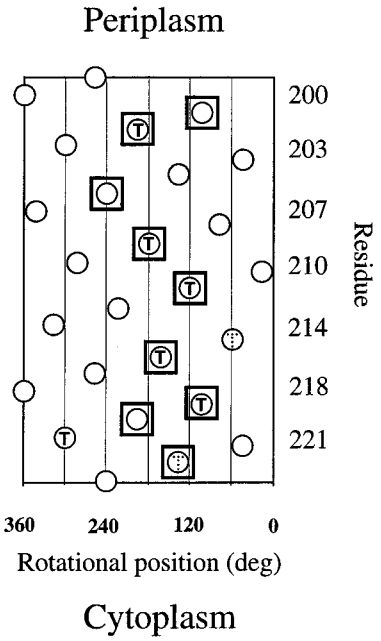

FIG. 4. Positions of substitutions that induced persistent tumbling in relation to those with a drastic effect on function. The helical net diagram of TM2 is as described for Fig. 2. Positions at which substitutions had drastic effects on receptor function are boxed, and those at which a substitution caused frequent or constant tumbles are marked by a dashed or solid T, respectively. For positions at which different substitutions created different phenotypic effects, the most extreme effect is indicated. deg, degree.

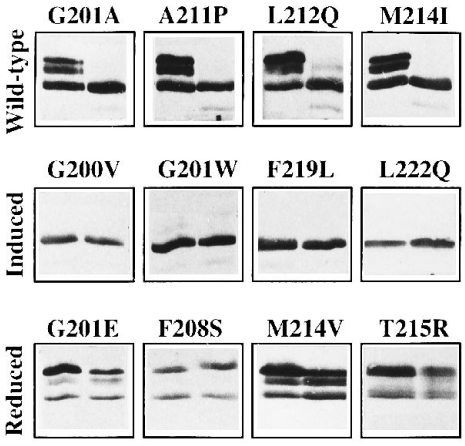

FIG. 5. Signaling classes of substituted Trg proteins. Immunoblots of cells producing a particular Trg as the only chemoreceptor are shown in the region including the various electrophoretic forms of Trg. For each strain, two conditions are shown: no Trg-mediated attractant (left) and saturating levels of ribose (right). Examples are shown for the three classes of signaling phenotypes, i.e., wild type, induced, and reduced. The single band visible for the induced class is at the same position as the lowest band in the patterns from unstimulated cells of the other two classes and corresponds to an apparent M_r of approximately 60,000.

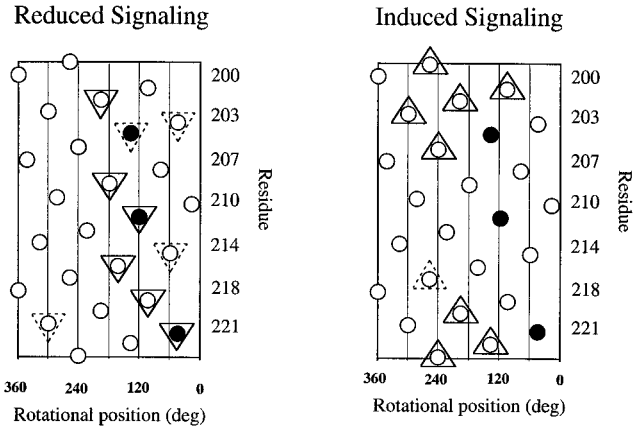

FIG. 6. Altered signaling related to structural faces of TM2. The helical net diagrams of TM2 are as described for Fig. 2. Positions at which a substitution caused signaling to be induced or reduced (Table 2 and Fig. 5) are enclosed in upward- or downward-pointing triangles, respectively. Dashed triangles indicate moderately abnormal phenotypes. For positions at which different substitutions created different phenotypic effects, the most extreme effect is indicated. Filled circles identify the face along which TM2 and TM2′ are most closely apposed. deg, degree.

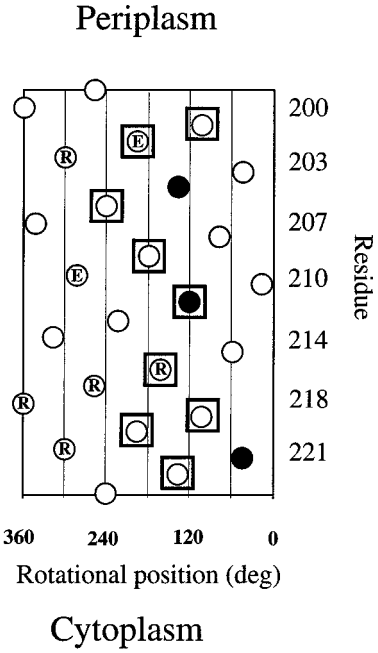

FIG. 7. Positions at which introduction of a normally charged side chain had a minimal effect on receptor function. The helical net diagram of TM2, boxes, and filled circles are as described for Fig. 2. Positions at which an argininyl (R) or glutamyl (E) reside was introduced with little detectable effect on function (Table 2) are indicated. deg, degree.

middle of TM2, but since prolines do not necessarily break, but can simply kink, transmembrane helices (13), the absence of a substantial effect by this substitution may reflect such a moderate perturbation. The other seven substitutions introduced side chains, charged at physiological pH, into a hydrophobic membrane-spanning helix. It is striking that the chemoreceptor accommodates, with little apparent effect on function, the negative thermodynamic contributions of introducing such side chains into the hydrophobic environment of the membrane, even at positions near the membrane center. Such accommodation may be limited to specific side chains. All of the benign substitutions introduced arginine or glutamate; none introduced lysine or aspartate. Glutamate might be favored over aspartate because of its longer hydrocarbon chain, and arginine might be favored over lysine because of the delocalized polarity. Note that at position 215 arginine was tolerated with little effect on receptor function, but lysine caused substantial disruption. The positions of the benign charge substitutions are shown on the helical net diagram in Fig. 7. They lie along a face of TM2 that is in large part distinct from the face defined by functionally disruptive substitutions. In our model of the transmembrane domain (16), this face is in contact with lipid, not with other protein segments, and the side chains, presumably in their unchanged states, could extend into the hydrocarbon environment of the lipid bilayer and not interfere with helix-helix interactions. Thus, the distribution of benign change substitutions provides additional support for our model of the transmembrane domain.

Functional disruption and altered signaling. What is altered by the TM2 substitutions that disrupt Trg function? It may not be possible to explain all of the mutational effects observed, but many can be understood in the context of transmembrane signaling. The transmembrane domain contains no known sites for binding or catalysis but instead functions by conveying conformational changes from the periplasmic to the cytoplasmic domain. Thus, a substitution in a transmembrane segment that drastically reduces function is likely to do so as the result of a perturbation in transmembrane signaling. Several lines of evidence indicate that this is the case for almost all of the functionally disrupting substitutions in TM2. Our in vivo assay for transmembrane signaling showed that all but 1 (I205S) of the 16 substitutions that essentially eliminated Trg-mediated taxis in a strain lacking other chemoreceptors either induced (10 cases) or reduced (5 cases) signaling (Table 2). All five of this latter group (F208S, A211T, T215K, T215M, and T218I) affected steady-state swimming behavior by increasing the tumble frequency, implying that the substitutions induced a repellent signaling state that thus reduced the effect of an attractant-induced signal. Mutational effects on signaling are also implied by the taxis phenotypes of strains containing altered Trg proteins and the normal complement of the other chemoreceptors. Nine of the 16 altered receptors that were unable to mediate a significant response to a spatial gradient of galactose or ribose by a host devoid of the other receptors (G201W, I205S, F208S, T215K, T218I, F219C, L222P, L222Q, and R225P) mediated a substantial, and in some cases apparently normal, response to both sugars by cells containing the other receptors. Thus, the functional disruptions created by these substitutions could be partially or completely corrected by the presence of the other receptors, implying that the altered Trg proteins generated persistent signals that caused crucial perturbations of the sensory balance in cells devoid of other chemoreceptors but that these perturbations could be compensated for by the buffering effects of other receptor proteins. Seven substitutions (R198L, G200C, G200V, I205N, A211T, T215M, and F219L) had drastic effects on Trg-mediated taxis even in cells containing the other chemoreceptors. Six of these Trg substitutions also blocked Tar-mediated taxis towards maltose, indicating that the altered Trg proteins, present in amplified amounts, perturbed cellular behavior, presumably by persistent signaling to an extent sufficient to prohibit a response mediated by an independent receptor. For one substitution (A211T), the induction of continual tumbling indicated that the persistent signaling was kinase activation leading to

clockwise (tumble mode) flagellar rotation. For the other five, the persistent signaling was likely to be kinase inhibition, leading to counterclockwise (run mode) rotation, as indicated by the induced-signaling phenotype noted in the in vivo assay. Of all the mutational substitutions characterized in the study, only T215M appeared to eliminate Trg function in a way that neither was corrected by the influence of other chemoreceptors nor perturbed the action of those receptors.

Reduction and induction of transmembrane signaling. Analysis by cysteine scanning revealed that the introduction of a cysteine at seven positions in TM2 reduced the efficiency of transmembrane signaling by Trg (17). The positions defined a helical face centered along the monomer-monomer interface where TM2 and TM2′ were apposed, as identified by cross-linking studies (16). Similarly, in TM1, cysteines that reduced signaling clustered along the intersubunit TM1-TM1′ interface. We concluded that perturbation of subunit packing in the transmembrane domain reduced signaling efficiency. However, we did not know to what extent this focused distribution was specific for cysteines. The results of the current study, in which any side chain could be introduced, provide strong evidence for the general significance of the focused distribution. Of the 15 random substitutions in TM2 that reduced signaling efficiency as measured in our in vivo assay, all but one (M220I) clustered along the same TM2-TM2′ face identified by cysteine scanning (Fig. 6), in most cases at exactly the same positions (both analyses identified positions 203, 204, 208, 211, 214, and 215; cysteine scanning also identified position 222; and random substitutions also identified positions 201, 218, and 221).

In the cysteine-scanning study, signaling was induced by introduction of a cysteine at six positions in TM1 but only two in TM2. The positions in TM1 were distributed toward the two ends of the helix and along the TM1-TM2 interface. The positions in TM2 were too few to define a pattern. The current study identified a sufficient number of random substitutions that induced signaling for a pattern to be discerned. Twelve substitutions at nine different TM2 positions induced signaling (Fig. 5). Like the analogous TM1 substitutions, the TM2 substitutions clustered near the two membrane surfaces and were distributed broadly across a face apposing another helix, in this case TM1′. These observations strengthen and extend the conclusions from the cysteine-scanning study (17), in which we deduced that interactions of TM1 with TM1′ and of TM2 with TM2′ should be stable for optimal signaling whereas interactions of TM1 with TM2 are altered in signaling (see the introduction). The distribution of signal-inducing substitutions in TM2 thus suggests that TM2-TM1′ interactions are also altered in signaling, a movement predicted by a pattern of stable interactions across the TM1-TM1′ and TM2-TM2′ interfaces and relative movement of TM1 and TM2. As noted for TM1 (17), the clustering of substitutions that induced signaling toward the membrane surfaces implies that movement between transmembrane segments is most extensive or most easily induced at the peripheries of the helices. At present we do not understand the significance of signal-inducing substitutions clustering along the TM1-TM2 interaction face of TM1 and along the TM2-TM1′ apposition face of TM2.

Mutational analysis of transmembrane segments. Our analysis of the transmembrane domain of Trg by cysteine scanning (17) provided important insights into the roles of helical interfaces in transmembrane signaling, but since few cysteine substitutions in TM2 disrupted function or induced signaling, this particular genetic analysis left an important gap in the correspondence of mutational and biochemical analyses of transmembrane signaling by chemoreceptors (see the introduction). The application of random mutagenesis to the analysis of TM2 of Trg has now filled that gap. For both TM2 and TM1, and for random as well as cysteine substitutions, transmembrane signaling is disrupted by perturbing interactions between homologous helices in different subunits and can be induced by perturbations of interactions of heterologous helices in the same or different subunits. This is wholely consistent with the conclusions from biochemical and physiological experiments indicating that transmembrane signaling involves little movement between transmembrane helices in different subunits but significant movement between subunits (5–8, 17, 20, 24). Models of the nature of the movement between heterologous helices have been proposed (6, 20), but choosing between these or other models will require additional data from both genetic and biochemical experiments.

It is noteworthy that mutational analysis of the transmembrane segments of a chemoreceptor, whether by cysteine scanning or random mutagenesis, provided useful and coherent information. Many substitutions caused only subtle perturbations, and many substitutions that had the character of drastic structural alterations (for example, introduction of a charged side chain) had little if any effect on receptor function. However, identification and characterization of altered receptors under sufficiently sensitive conditions, combined with consideration of the patterns of effects of many substitutions, provided important information about a protein domain that functions neither by catalysis nor by binding but rather by a conformational change.

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