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Genetics

Analysis of mutations in the transmembrane region of the aspartate chemoreceptor in *Escherichia coli*

(chemotaxis/signal transduction/site-specific mutagenesis/membrane assembly)

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ABSTRACT **Site-specific mutagenesis was used to replace an alanine with a lysine residue and to create a deletion of seven amino acids into the first transmembrane region (TMI region) of the aspartate chemoreceptor in *Escherichia coli*. The mutations resulted in the loss of aspartate chemotaxis on tryptone motility plates. However, both mutant proteins were able to associate with the membrane and to bind aspartate. They were both refractory to methylation or to modification of the C-terminal region of the protein by the *cheB* gene product. These results suggested that the integrity of the TMI domain of the protein was required to maintain the function of the cytoplasmic portion of the receptor. The Lys-19 mutant retained the ability to generate a repellent response. Analysis of suppressor mutations of the Lys-19 mutation suggested that formation of an ion pair or specific changes in a 40 amino acid stretch in the cytoplasmic region of the protein (from amino acid 264 to amino acid 303) could suppress the effects of the Lys-19 mutation. The TMI region of the protein may be involved in transmembrane transmission of signals from the periplasmic portion of the cell to the cytoplasmic portion of the Tar protein.**

The signal transducers for bacterial chemotaxis are transmembrane proteins. The genes encoding five different bacterial receptor-transducer proteins were cloned, the amino acid sequences of the gene products were deduced from the nucleic acid sequences (1–4), and one of the transmembrane proteins was isolated in homogeneous form (5). On the basis of these data, it has been suggested that the chemotaxis signal transducing proteins are composed of a series of differentiated functional and structural domains. These include a periplasmic component involved in ligand binding, a transmembrane segment, and a cytoplasmic domain required for signal transduction. Fig. 1 shows the distribution of these domains along the linear amino acid sequence of the *tar* gene product.

The Tar protein is the chemoreceptor responsible for binding aspartate and regulating the cells' response to environmental changes in concentrations of aspartate, a number of related amino acids, and maltose (6). The Tar protein also controls responses to repellents, such as nickel ion and glycerol (6, 14). Its sequence includes two stretches of hydrophobic amino acids that have been called the first and second transmembrane regions (TMI and TMII) (Fig. 1). The hydrophobic stretch including amino acids 7–37 constitutes a "signal sequence" that apparently is not processed and remains a part of the mature protein (3). The second sequence, TMII, is thought to traverse the membrane and function in the assembly of the rest of the protein into the cytoplasmic space. The regions labeled K1 and R1 (Fig. 1) indicate portions of the protein that include specific sites that are modified by reversible methylation (7–9). The periplasmic portion of the protein is thought to include the ligand binding site. Information is transmitted across the membrane in response to changes in aspartate binding, resulting in methylation or demethylation at the K1 and R1 sites. A second process is also initiated, which results in the generation of a signal that informs the flagellar motor of changes in ligand concentration as a function of time at the surface of the cell. To more clearly delineate the functions of the structural domains of the Tar transmembrane protein, we have initiated a series of studies involving site-specific mutagenesis. In this paper, we describe and characterize two mutations in the TMI region. The first involves a deletion of seven amino acids. The second involves the introduction of a potentially charged amino acid residue in the middle of the hydrophobic region. Our results suggest that the TMI region may play a role in the assembly and maintenance of the structure of the protein and mediate its transmembrane signaling function.

MATERIALS AND METHODS

Strains and Vectors. *Escherichia coli* strains used in this work were RP4372 *recA* [*tsr-1* Δ(*tar-tap*) *5201*] (8), HB294 *recA* [*tsr*::*Tn5* Δ (*tar-cheB*)], and JM103 (10). All plasmids were derivatives of pAK101 (8). M13 mp10 for site-specific mutagenesis and M13 mp18 and M13 mp19 for DNA sequencing were purchased from Pharmacia. The DNA encoding Tar was inserted into the *Eco*RI site of λgt11 (11); the phage was used for transduction of *tar* and for the construction of strains carrying *tar* in single copy.

Media and Chemicals. Tryptone broth contained 1% Bactotryptone (Difco) and 0.5% NaCl. Tryptone motility plates contained 0.25% Bactoagar (Difco) in tryptone broth. Restriction enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, or New England Biolabs. L-[*methyl*-^{3}H]Methionine (15.0 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN and L-[U-^{14}C]aspartic acid (224 mCi/mmol) was from Amersham.

Methods. The oligonucleotides for site-specific mutagenesis shown in Fig. 2 were chemically synthesized on an Applied Biosystems oligonucleotide synthesizer by Suzanna Horvath of the California Institute of Technology. Mutagenesis was performed according to Newman *et al.* (12). After the mutations were verified by sequencing, the *Eco*RI–*Xba* I fragment carrying the mutation was inserted into the *tar* gene on pAK101. To select pseudorevertants, RP4372 *recA* carrying pAK101 with either the Lys-19 mutation or the deletion was inoculated into tryptone motility plates. After 24 hr, "flares" emerged from the initial inoculum; one or two of these were picked from each plate. The DNA was isolated and retransformed at low concentration into RP4372 *recA*. "Swarmers" were picked and DNA was isolated from these strains. The strategy for mapping

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Abbreviation: TMI and TMII, first and second transmembrane regions.

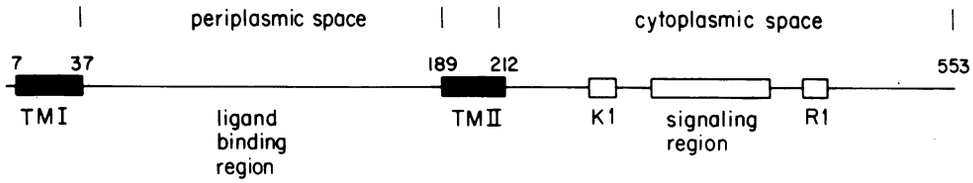

FIG. 1. Structure of the Tar protein. The Tar protein has 553 amino acid residues. TMI refers to the region between amino acid residues 7 and 37 and TMII includes amino acids 189–212. The K1 and R1 regions include the sites that are methylated and demethylated by methyltransferase (CheR) and methylesterase (CheB).

mutations, for DNA sequencing, and for ^{3}H-*methyl* labeling of Tar protein was basically the same as described (13).

The procedure for the separation of cytoplasm and membrane was as follows. Cells were grown at 30°C in T broth to OD_{590} = 0.6–0.8, washed with motility buffer (10 mM potassium phosphate buffer, pH 7.0/0.1 mM EDTA/10 mM sodium lactate) twice, and sonicated for 3 min on ice. Whole cells were removed by centrifugation at 5000 × *g* for 5 min at 4°C. The membrane fraction was separated from cytoplasm by centrifugation at 100,000 × *g* for 30 min at 4°C.

The procedure for measuring the dissociation constant (K_d) of L-aspartate from Tar was described by Clarke and Koshland (15). The tethered cell assay was performed as described (8).

RESULTS

Site-Specific Mutagenesis. Fig. 2 describes the oligonucleotides that were prepared to introduce mutations into the TMI region. The first mutation involved the substitution of a lysine at position 19 for an existing alanine residue. The change was affected by introducing a thymine and two adenine residues. This resulted in a change of Ala-19 to a lysine and in the formation of a *Dra* I restriction endonuclease digestion site. The deletion was prepared by using an oligonucleotide with 15 base pairs of homology on either side of the region to be deleted (Fig. 2). The deletion resulted in the removal of a stretch of amino acids from Val-7 to Met-13. The cloned DNA that carried the mutations was sequenced after introduction into the plasmid pAK101 and the plasmid was subsequently introduced into *E. coli* RP4372 *recA*. This strain is deleted for two chemoreceptors, *tar* and *tap*, and has a small deletion in the *tsr* receptor (16). The only intact receptor protein in this strain is the product of the *trg* gene, and this does not affect the behavior of the cells on tryptone motility plates. In subsequent experiments, a further mutation eliminating *trg* was introduced without perceptibly changing the results. Fig. 3 shows that the deletion and the introduction of a lysine residue result in the absence of chemotaxis behavior on tryptone motility plates.

Membrane Assembly of Mutant Proteins. To test for the association of the mutated gene products with the membrane, cells (RP4372 *recA* with or without pAK101, pAK101 Lys-19, or pAK101Δ7-13) were grown and fractionated into membrane and cytoplasm, and the components of each were separated by electrophoresis and tested for the ability to react with Tar-specific antibodies (13). Fig. 4*A* shows that the Tar protein is associated, almost entirely, with the membrane fraction. The same general distribution is found for the deletion mutant and for the Lys-19 mutant. The mobility of the Lys-19 gene product is slightly greater than that of the wild type or the deletion.

Although these data indicate that the mutant and the wild-type proteins associate with the membrane fraction, it is possible that they may have been incorrectly assembled into the membrane. To test for the integrity of the N-terminal portion of the Tar protein, the ability to bind aspartate was measured. The K_d determined for the wild-type aspartate receptor was similar to that described (15)—i.e., 11 μM—whereas that for the Lys-19 mutant was 25 μM and that for the 7 amino acid deletion was 14 μM. These results suggest that the mutant proteins were able to assemble and form the appropriate ligand binding site. Furthermore, behavioral studies were undertaken to test for residual responses to

A. ala-19 → lys-19

			15					22	
Wild type	amino acid sequence		leu	gly	val	phe	ala	leu	leu gln
	nucleotide sequence		CTG	GGG	GTA	TTC	GCA	CTG	TTA CAG
Oligonucleotide sequence		3'	GAC	CCC	CAT	AAA	TTT	GAC	AAT GTC 5'
Mutant	nucleotide sequence		CTG	GGG	GTA	TTT	AAA	CTG	TTA CAG
	amino acid sequence		leu	gly	val	phe	lys	leu	leu gln

B. deletion val-7 to met-13

			2															18
Wild type	amino acid sequence		ile	asn	arg	ile	arg	val	val	thr	leu	leu	val	met	val	leu	gly	val phe
	nucleotide sequence		ATT	AAC	CGT	ATC	CGC	GTA	GTC	ACG	CTG	TTG	GTA	ATG	GTG	CTG	GGG	GTA TTC
Oligonucleotide sequence		3'	TAA	TTG	GCA	TAG	GCG								CAC	GAC	CCC	CAT AAG 5'
Mutant	nucleotide sequence		ATT	AAC	CGT	ATC	CGC								GTG	CTG	GGG	GTA TTG
	amino acid sequence		ile	asn	arg	ile	arg								val	leu	gly	val phe

FIG. 2. Oligonucleotides used for site-specific mutagenesis. (*A*) The oligonucleotide used to introduce lysine to replace Ala-19 was made of 24 nucleotides and also introduced a new restriction site for *Dra* I as well as the lysine codon at position 19. (*B*) The oligonucleotide used in making the deletion from Val-7 to Met-12 was 30 nucleotides long.

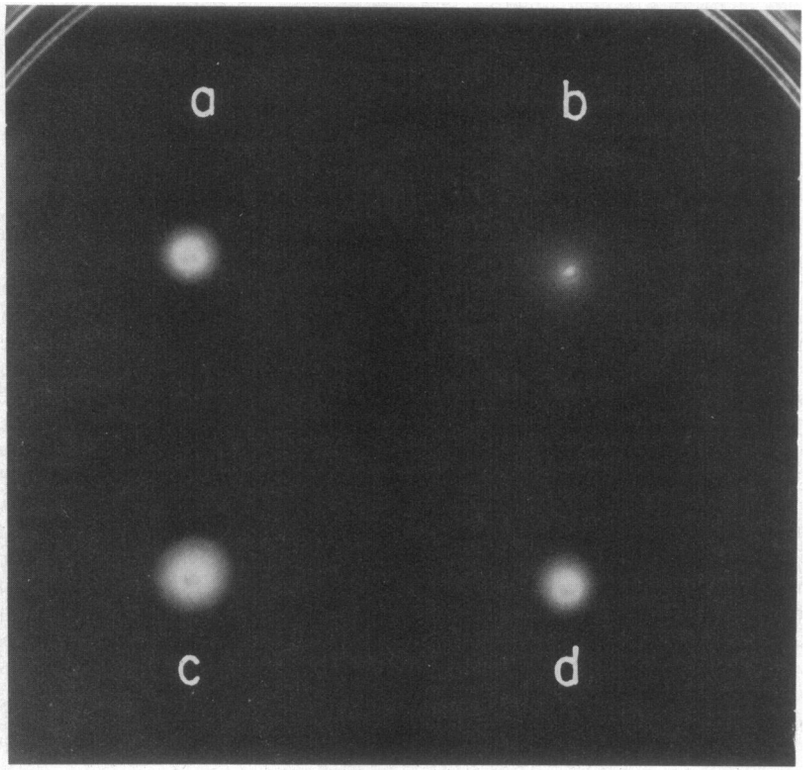

FIG. 3. Chemotaxis of strains carrying mutant genes. The cells were inoculated into tryptone motility agar. (*a*) RP4372 *recA* without plasmid. (*b*) RP4372 *recA* with pAK101. (*c*) RP4372 *recA* with pAK101 carrying the mutation Lys-19. (*d*) RP4372 *recA* with pAK101 carrying a deletion from Val-7 to Met-13.

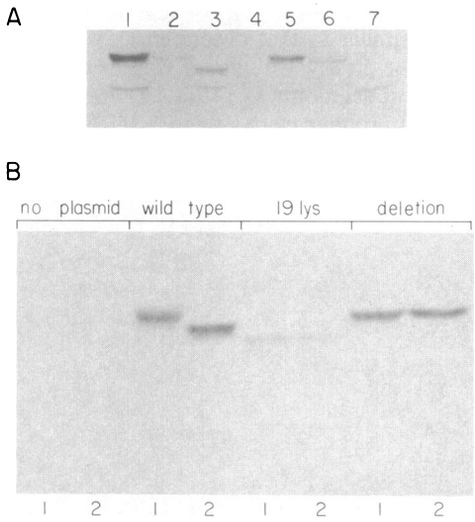

FIG. 4. Detection of the Tar protein by immunoblotting. (*A*) Localization of mutant Tar protein. The membrane fraction was separated from the cytoplasmic fraction after sonication by centrifugation. The Tar protein was detected by immunoblotting with anti-Tar antiserum. The lower band in the lanes that include membrane-derived protein is the result of the crossreaction of our antibody with a non-Tar derived protein. Lanes 1 and 2, RP4372 *recA* with pAK101; lanes 3 and 4, RP4372 *recA* with pAK101 carrying the Lys-19 mutation; lanes 5 and 6, RP4372 *recA* with pAK101 carrying the deletion from Val-7 to Met-13; lane 7, RP4372 *recA* without plasmid; lanes 1, 3, 5, and 7 represent the membrane fraction; lanes 2, 4, and 6 represent the cytoplasmic fraction. (*B*) Effects of modification of the Tar protein by the CheR and CheB proteins. Lanes 1, the host cell was RP4372 *recA* (*cheR*$^+$, *cheB*$^+$); lanes 2, the host cell was HB294 *recA* (*cheR*$^-$, *cheB*$^-$).

aspartate. The deletion showed no attractant response upon the addition of aspartate to tethered cells. The attractant response of the Lys-19 mutant was difficult to test since it showed only counterclockwise rotation when tethered. However, when aspartate was washed away, the Lys-19 mutant showed an immediate transient repellent response. Taken together, these data suggest that the ligand binding region of the mutant gene products is able to function in a manner similar to the wild-type protein. Furthermore, the Lys-19 mutant is able to initiate a repellent response.

To further test the properties of the mutant gene products, we looked for functions that are controlled by the cytoplasmic portion of the molecule. Neither the Lys-19 mutant nor the deletion mutant product were found to be methylated under ordinary growth conditions. It is known (17, 22) that the Tar protein undergoes modification catalyzed by the product of the *cheB* gene. This results in the specific deamination of glutamine residues in the K1 and R1 domains of the protein. To determine if the cytoplasmic portions of the mutant proteins were still subject to modification, they were compared in background strains that were proficient for *cheB* modification and *cheR*-mediated methylation and in other strains that lacked the *cheR* and *cheB* gene functions. Fig. 4*B* shows that the wild-type polypeptide is modified in its mobility in the *cheR*, *cheB* background strains, whereas the mobility of the Lys-19 and of the deletion gene product is exactly the same in both backgrounds. This suggests that the mutant polypeptides are not good substrates for modification by the *cheB* gene product. Thus, the structure of the C-terminal portion of the molecule may be distorted in the mutants so that it no longer can be recognized by the modification enzymes.

Isolation of Pseudorevertants. Pseudorevertants were selected on tryptone motility plates. After incubation for 24 hr, swarms emerged from an initial inoculum of the mutant strain. To determine the nature of the pseudorevertants, DNA was extracted and used to transform RP4372 *recA*; a

cloned transformant was subsequently retested for chemotaxis. To map the suppressor mutations, a number of techniques were used. Since the Lys-19 mutation had an extra *Dra* I site, nucleotide changes at that position resulted in changes in the *Dra* I restriction pattern. Second, the gene was subdivided into three segments by using the restriction enzymes *Xba* I, *Kpn* I, and *Nsi* I. Each of these fragments was tested for the presence of suppressor activity by reinsertion into a mutant plasmid. Once the suppressor was localized, the segment carrying the suppressor was sequenced and the exact nature of the mutation was determined.

We found no suppressors that mapped within the *tar* gene that changed the phenotype of the deletion mutation. On the other hand, a large number of pseudorevertants were found for the Lys-19 mutation. Thirty-five of these were sequenced. The results are tabulated in Fig. 5 and illustrated graphically in Fig. 6. The suppressors were found to be localized in three regions of the molecule. Seven of them were found in the TMI region. These included six same-site revertants where the Lys-19 residue was found to be changed to a relatively hydrophobic amino acid, glutamine, isoleucine, or threonine. No changes to charged amino acids were found at this site. A pseudorevertant in the TMI region was found to be the result of mutation of Val-17 to Glu-17. This suggests that opposite charged residues might form ion pairs and thus suppress the effects of the Lys-19 mutation. The second cluster of suppressors was found in TMII. They included five suppressor mutations—three of them resulting in changes of a hydrophobic amino acid to a glutamate residue, again suggesting that one mechanism of suppression may be the formation of an ion pair in the membrane. Most of the suppressor mutations were found in the region of the molecule between amino acids 264 and 303. Many of these were found multiple times. Thus, for example, Gly-271 was found to be changed to alanine, cysteine, serine, or valine. Furthermore, Thr-279 was found in five separate instances to be changed to Ile-279.

A.

Wild type		Mutant		Revertant		No.
GCA	ala-19	AAA	lys-19	CAA	gln-19	2
				ATA	ile-19	1
				ACA	thr-19	3

B.

Wild type		Suppressor		No.
GTA	val-17	GAA	glu-17	1
TGG	trp-192	CGG	arg-192	1
GCG	ala-198	GAG	glu-198	1
GTC	val-201	GAG	glu-201	2
GTA	val-202	TTA	leu-202	1
ACC	thr-264	ATC	ile-264	3
CAT	his-267	GAT	asp-267	1
GGT	gly-271	GCT	ala-271	1
GGT	gly-271	TGT	cys-271	3
GGT	gly-271	AGT	ser-271	1
GGT	gly-271	GTT	val-271	1
GGT	gly-278	GCT	ala-278	1
ACC	thr-279	ATC	ile-279	5
GAA	glu-281	AAA	lys-281	2
GAT	asp-288	GCT	ala-288	1
GAT	asp-288	GTT	val-288	1
ACT	thr-293	ATT	ile-293	2
ACT	thr-303	ATT	ile-303	1

FIG. 5. Revertants and suppressors of the Lys-19 mutation.

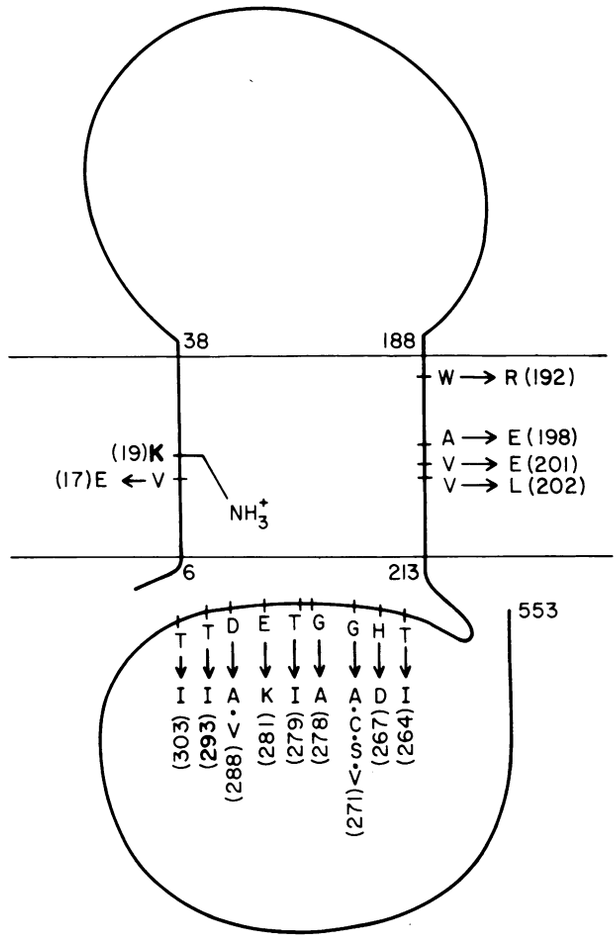

FIG. 6. Distribution of suppressor mutations of Lys-19 mutation. The cartoon represents the distribution of the Tar polypeptide chain in the periplasmic, membrane, and cytoplasmic regions of the cell. One-letter codes for amino acid residues are A for alanine, C for cysteine, D for aspartic acid, E for glutamic acid, G for glycine, H for histidine, I for isoleucine, K for lysine, L for leucine, R for arginine, S for serine, T for threonine, V for valine, and W for tryptophan. The numbers in parentheses represent amino acid residues numbered from the N-terminal end.

To test for effects of gene dosage on suppression, some of the suppressor mutations were moved onto bacteriophage λ and introduced into the cells as single copy genes. They were found to permit complete chemotaxis function. The suppressors were also tested for their ability to be modified and methylated. All of them showed methylation and CheR-, CheB-mediated modification (data not shown). Finally, a number of the suppressors, particularly those in the transmembrane region and the C-terminal portion of the molecule, were separated by *in vitro* recombination from the original Lys-19 mutation and tested for their ability to mediate chemotaxis. Most of the suppressor mutations were themselves found to be functional (data not shown).

DISCUSSION

The results of introducing a potentially charged amino acid or a relatively large deletion in the TMI region suggest that it plays multiple roles in the function of the receptor. A portion of TMI may be important for the insertion of the protein into the membrane (N. Mutoh and M.S., unpublished data). In addition, TMI appears to play a role in maintaining the function and structure of the C-terminal portion of the receptor molecule. The deletion of seven amino acids from the start of the TMI region or the introduction of a lysine

residue into the middle of TMI does not effectively change the association of the protein with the membrane. The mutations, however, result in the loss of chemotaxis function, which is accompanied by a distortion in the structure of the cytoplasmic part of the receptor so that it no longer functions as a substrate for modification or methylation. Some form of transmembrane signaling is still possible with the Lys-19 mutant. It responds to some repellents and to the removal of aspartate; however, it does not show any response to attractants. Studies by Bogonez and Koshland (18) of the solubilized Tar receptor protein showed that in the absence of high concentrations of glycerol and appropriate lipids, the purified receptor was not able to act as a substrate for methylation; thus, the appropriate stabilization of the transmembrane region may be required for the integrity of the C-terminal portion of the protein.

The introduction of a lysine residue into the transmembrane region could require alterations in TMI structure to accommodate the presence of a charge in the membrane. These changes, for example, could result from the association of the γ-amino group of Lys-19 with head groups of the phospholipids in the membrane, distorting the structure of the TMI region and thus affecting the C-terminal region of the molecule or the ability of the molecule to assemble into the correct oligomeric form. If this notion is correct, then we would predict that the Lys-19 mutation could be suppressed in a number of ways. One type of suppression would result from the formation of an ion pair that would be more stable than a single charge in the hydrophobic context of the membrane. The suppressors that map in the TMII region suggest that the two transmembrane regions are closely associated so that nearby amino acid residues that form glutamate moieties could pair with Lys-19 (see Fig. 6). The finding that most of the suppressors cluster in the cytoplasmic portion of the protein—i.e., between amino acids 264 and 303—can be rationalized if this portion of the molecule were intimately associated with part of the TMI sequence or the membrane adjacent to the TMI sequence. Changes in structure in the region between amino acids 264 and 303 could compensate for conformational changes introduced by the presence of the Lys-19 mutation. A portion of the C terminus of the Tar molecule may interact with TMI and thus act as a functional link between the transmembrane region and the signaling region. TMI may play a specific role in the transmembrane transmission of signals and in generating a cytoplasmic response to ligand binding events that occur in the periplasmic space.

It is clear that changes in the structure of the transmembrane region—i.e., deletions or the introduction of potentially charged residues—can have marked effects on the signaling function of the chemotaxis transmembrane protein. There have been a number of other examples of mutations resulting in charged residues that are stabilized in the transmembrane portion of specific proteins (19, 20). Most recently, a variant of a transmembrane receptor with a glutamate residue in a portion of the molecule that is thought to traverse the membrane was found to have oncogenic properties (21). One interpretation of these results is that the charged residue distorted the structure of the transmembrane protein and fixed the protein in a signaling mode. In much the same way, the Lys-19 mutation results in a protein that is defective in signaling. It is capable of signaling the presence of repellent stimuli but is insensitive to attractants. The bacterial chemotaxis receptors provide an interesting model system whereby the extensive tools of molecular genetics can be applied to questions of the mechanisms of signal transduction.

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