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Transmembrane Signaling by Bacterial Chemoreceptors: E. coli Transducers with Locked Signal Output

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Summary

Methyl-accepting chemotaxis proteins (MCPs) function as transmembrane signalers in bacteria. We isolated and characterized mutants of the E. coli Tsr protein that produce output signals in the absence of overt stimuli and that are refractory to sensory adaptation. The properties of these "locked" transducers indicate that MCP molecules are capable of generating signals that actively augment clockwise and counterclockwise rotation of the flagellar motors. Transitions between MCP signaling states can be influenced by amino acid replacements in many parts of the molecule, including the methylation sites, at least one of the two membrane-spanning segments, and a linker region connecting the receptor and signaling domains. These findings suggest that transmembrane signaling may involve direct propagation of conformational changes between the periplasmic and cytoplasmic portions of the MCP molecule.

Introduction

Transmembrane signaling systems mediate a variety of cellular responses to chemical stimuli, ranging from hormonal growth control to chemotactic behavior. The methyl-accepting chemotaxis proteins (MCPs) of E. coli offer useful models for investigating such signaling events at the molecular level (Springer et al., 1979). These sensory transducers contain an extracellular receptor domain that monitors the levels of attractant and repellent chemicals in the environment and an intracellular signaling domain that controls the rotational behavior of the flagellar motors. Changes in receptor occupancy trigger chemotactic responses by modulating signal output from the cytoplasmic domain. Subsequent changes in transducer methylation state, catalyzed by cytoplasmic enzymes, restore signal output to pre-stimulus levels and bring about sensory adaptation.

Although MCP molecules have a simple transmembrane structural organization (Figure 1), it is not yet clear how sensory stimuli and covalent modification control their signaling activities. Sequence analyses of MCP structural genes (Boyd et al., 1983; Krikos et al., 1983; Russo and Koshland, 1983; Bollinger et al., 1984), the properties of chimeric transducers (Krikos et al., 1985; Slocum et al., 1987), and studies of MCP membrane topology (Manoil and Beckwith, 1986) indicate that the N-terminal half of the transducer molecule, flanked by membrane-spanning segments (TM1 and TM2), comprises a periplasmic chemoreceptor domain, whereas the C-terminal half of the molecule, bordered by segments (K1 and R1) containing the methylation sites, comprises a cytoplasmic signaling domain. The cytoplasmic portion of the molecule between TM2 and K1, which joins the receptor and signaling domains, has received little attention to date but could conceivably play an important part in the transmembrane signaling process (Oosawa and Simon, 1986). (We will refer to this segment as the "linker" to avoid undue functional connotations.)

To investigate the roles of these various structural features in regulating MCP signaling activity, we isolated and characterized mutations that were capable of "locking" the transducer in an active signaling state, enabling it to generate flagellar signals in the absence of overt stimuli and to resist the signal-damping action of the sensory adaptation machinery. In contrast to null mutants, many of which are likely to be defective in signal generation, transducers with locked outputs should be defective in signal control and were expected to reveal regions of the molecule that are important in modulating its signaling behavior. We determined the sequence changes and associated signaling properties of 58 locked output mutants of the E. coli Tsr transducer, which mediates responses to serine, related attractants, and several repellents (Reader et al., 1979). The nature and location of the inferred structural alterations in the mutant proteins provide novel insight into the functional architecture of transducer molecules and how their output might be controlled.

These locked output defects are closely analogous to those in oncogenic variants of eukaryotic sensory transducers, which also have unregulated signaling behavior. Thus, it should be possible to obtain similar mutants in other transducer systems as a means of investigating the structural and functional bases of their signaling properties.

Results

Isolation of Locked Transducer Mutants

There are four MCP species in E. coli (Tar, Tap, Trg, and Tsr), each of which mediates responses to a different set of chemoeffector compounds. Mutations that inactivate one of these transducers eliminate only a subset of chemotactic responses (Harayama et al., 1979; Parkinson, 1980; Slocum and Parkinson, 1985). In contrast, mutations that lock one of the transducers in a constant signaling mode should cause a general defect in chemotactic ability if the mutant signal is strong enough to drown out sensory input from wild-type transducers. Since the aberrant functional properties of the mutant transducers must prevail over both their wild-type counterparts (dominance) and over heterologous transducers (epistasis), we looked for MCP alterations that were capable of blocking chemotactic behavior in a cell containing several species of wild-type transducers. For simplicity, we will refer to such mutants as having dominant transducer defects. The Tsr transducer was chosen for this work because previous

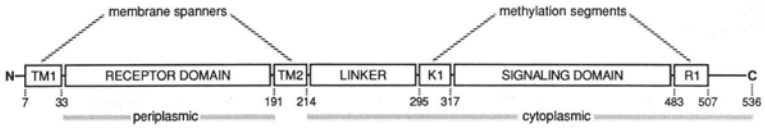

Figure 1. Structural Features of MCP Molecules

The Tsr protein is shown, but all four MCP species in E. coli exhibit essentially the same organization. The figure is drawn to scale; the numbers indicate the residue positions of key structural landmarks.

studies had indicated that it could give rise to mutants of this sort (Parkinson, 1980; Callahan and Parkinson, 1985).

A multicopy plasmid carrying the *tsr* locus was randomly mutagenized in vitro with hydroxylamine and transferred into a tester strain in which the chromosomal *tsr* locus was deleted. The chemotactic behavior of individual transformants was then assessed on semi-solid agar swarm plates to identify ones with defects in the plasmid-encoded Tsr protein that blocked chemotactic responses to compounds normally handled by the other transducers in the cell. These screens yielded a total of 41 independent *tsr* mutants with dominant defects that might be due to locked signal production. These, and 17 comparable mutants obtained previously with other mutagens (Parkinson, 1980; Callahan and Parkinson, 1985), were characterized with respect to DNA sequence changes within the *tsr* gene, flagellar rotation patterns, and steady-state and stimulated MCP methylation levels (Table 1).

DNA Sequence Changes of Locked Transducer Mutants

The approximate physical position of each mutation was established in mapping crosses against a series of deletions with defined endpoints in the *tsr* coding sequence. On the basis of those results, appropriate oligonucleotide primers were chosen for determination of the DNA sequence in the vicinity of the mutation site. Sequence changes were identified in all 58 mutants, yielding 40 different mutations in 36 different codons (Table 1). Every mutant exhibited a single base pair substitution within the sequenced portion of the *tsr* gene, and the mutational changes were consistent with the mutagenic treatments used in the mutant isolations, indicating that we had identified the coding changes responsible for the aberrant transducer behaviors. Three of the mutants have nonsense mutations and presumably produce truncated Tsr proteins. The remainder arose through missense mutations and should have single amino acid replacements in the Tsr protein. To facilitate subsequent discussion, the missense mutations will be designated by their inferred amino acid changes. For example, "FS18" refers to a mutation that results in a phenylalanine to serine replacement at residue number 18.

Flagellar Rotation Patterns of Locked Transducer Mutants

Flagellar motors can rotate in the counterclockwise (CCW) direction, which produces smooth swimming, and in the clockwise (CW) direction, which causes abrupt turning movements or tumbles. Wild-type cells exhibit frequent reversals of motor rotation, which are transiently suppressed by transducer signals during chemotactic responses. Attractant increases and repellent decreases favor CCW rotation; the opposite stimuli favor CW rotation. Thus, transducers locked in an active signaling state should cause a permanent CCW or CW rotational bias, with few reversals. The flagellar rotation patterns of the dominant *tsr* mutants were examined by cell tethering and proved consistent with this expectation (Table 1). Whereas wild-type cells reverse their direction of rotation about once per second, reversal episodes were much less common in the transducer mutants: 30 out of 40 strains had a pronounced CCW bias, with roughly 30%–100% of the cells rotating exclusively in the CCW direction during a 15 sec observation period; 7 out of 40 were CW-biased, with about 30%–50% of the cells rotating exclusively in the CW direction; and 3 out of 40 (dubbed "slow switchers") exhibited both CW-and CCW-biased individuals. The slow switcher mutants evidently are capable of rotating their flagella in either direction but seem to be defective in switching from one mode of rotation to the other, whereas the CW- and CCW-biased mutants seem to be more or less stuck in one rotational state. Since complete inactivation of the Tsr transducer has no effect on rotational behavior when the cell contains other normal transducers (Parkinson, 1980), these rotation patterns must be caused by an aberrant activity of the mutant Tsr molecules.

Steady-State MCP Methylation Levels of Locked Transducer Mutants

The flagellar signals generated by locked transducers cannot, by definition, be shut off by the sensory adaptation system, implying that locked transducers are refractory to the effects of methylation changes. On the one hand, they might be unsuitable substrates for the methylation or demethylation enzymes. On the other hand, changes in methylation state may no longer regulate their signal output. To distinguish these alternatives, we examined the ability of locked transducers to accept methyl groups. In order to focus on the mutant transducers, plasmids bearing the *tsr* mutations were transferred into a strain lacking all but one of the MCP structural genes. The lone remaining transducer, Trg, is not expressed at high enough levels to be seen under the assay conditions used. We found that the three mutants with nonsense mutations gave no detectable methylation, possibly because of instability of their truncated Tsr molecules. All of the other mutant transducers were capable of accepting methyl groups; however, their steady-state methylation levels varied greatly, ranging from much lower to much

Table 1. Mutational Changes and Behavioral Consequences of Locked Transducer Mutants

Mutant[a]	Base Change	*tsr* Alleles[b]	Flagellar Rotation[c]			Methylation[d]	
			CCW	CCW-CW	CW	– SER	+ SER
FS18	T → C	*303*	96	3	1	>	0
AV233	C → T	*1093*	4	79	17	<	0
GD235	G → A	*1081*	100	0	0	<	0
EK248	G → A	*1008, 1091*	27	73	0	<	0
MI259	G → A	*1020, 1045*	43	56	1	<	0
RC271	C → T	*1066*	98	2	0	>	+
AT276	G → A	*1011*	84	15	1	>	+
GD280	G → A	*331, 1063*	57	42	1	>	+
EK296	G → A	*1002*	44	55	1	>	±
MI309	G → A	*1065, 1098*	28	71	1	=	+
AT323	G → A	*364*	96	4	0	>	+
AT337	G → A	*193, 1059*	100	0	0	>	+
AV337	C → T	*191*	66	34	0	>	+
GD341	G → A	*1099*	26	74	0	>	+
SN357	G → A	*1004, 1035*	96	1	3	<	0
IT371	T → C	*221, 224, 229, 231*	4	67	29	<	0
IT377	T → C	*385*	82	17	1	ND	ND
AV382	C → T	*1007*	84	12	4	<	+
AT387	G → A	*1012, 1090*	45	21	34	<	0
RC388	C → T	*1092*	50	49	1	>	0
AV389	C → T	*1088*	30	42	28	<	+
Qoc392	C → T	*1080*	60	40	0	0	0
RC394	C → T	*313*	2	57	41	=	+
RH394	G → A	*1031*	53	47	0	>	+
AT397	G → A	*1037*	65	35	0	>	+
VM398	G → A	*1084*	77	23	0	>	+
AT407	G → A	*1058*	70	26	4	<	0
AV407	C → T	*1005*	3	45	52	<	0
AV411	C → T	*1100*	80	20	0	>	+
AT413	G → A	*192, 394, 1026, 1071*	87	13	0	>	+
AV413	C → T	*1006, 1023*	100	0	0	>	±
AV414	C → T	*1056*	80	18	2	=	+
LQ420	T → A	*518*	72	28	0	>	+
GS431	G → A	*1003, 1028*	2	58	40	<	0
AT438	G → A	*359*	3	48	49	<	0
VM449	G → A	*1095*	23	45	32	<	0
SF463	C → T	*34, 352, 1038*	86	14	0	>	+
Qam466	C → T	*1044*	12	61	27	0	0
Qoc485	C → T	*1024, 1060*	64	33	3	0	0
VM491	G → A	*1033*	92	7	1	>	±

[a] Mutants are designated by the coding changes inferred from their DNA sequence. The notation used gives the original amino acid, the mutant amino acid or nonsense codon (am = amber; oc = ochre), and the position of the altered residue in the Tsr protein.

[b] Origin of mutant alleles: *34, 518* (nitrosoguanidine) (Parkinson, 1980); *101–199* (ethyl methane sulfonate) (Parkinson, 1980); *201–399* (*mutD*) (Callahan and Parkinson, 1985); and *1001–1100* (hydroxylamine) (this study).

[c] Rotational patterns were examined by antibody tethering of RP5700 (Δ*tsr-7028*) strain carrying *tsr* mutations on plasmid pPA418. Each rotating cell was observed for 15 sec and classified as either not reversing (i.e., exclusively CCW or CW) or with one or more reversal episodes during the observation period (CCW-CW). The percentages of individuals in each category are indicated. There is considerable variation in the reversing class because one reversal episode was sufficient to assign a cell to this category, whereas the overall reversal rate was still much lower than in wild-type controls.

[d] MCP methylation was measured in RP5869 strains (Δ(*tar-tap*)*5201* Δ*tsr-7021*) carrying *tsr* mutations on plasmid pPA418. Methylation levels in the absence of a serine stimulus (– SER) were classified as follows: no detectable methylation (0), less than wild-type (<) (e.g., lanes a–d, Figure 2), wild-type (=) (lane e, Figure 2), greater than wild-type (>) (e.g., lanes f–h, Figure 2). Methylation increases in response to a serine stimulus (+ SER) were classified as follows: no detectable increase (0) (e.g., lanes a and c, Figure 2), marginal increase (±) (e.g., lanes b, g, and h, Figure 2), substantial increase (+) (e.g., lanes d–f, Figure 2). ND = not determined.

higher than wild-type (Table 1). Examples of the mutant methylation patterns are shown in Figure 2.

In comparison with the wild-type methylation pattern (Figure 2, lane e), most of the transducer mutants with CCW rotational biases (19 out of 29) exhibited high methylation levels (similar to the examples in lanes f–h in Figure 2), whereas the majority of the CW-biased mutants (6 out of 7) had low methylation levels (similar to the examples in lanes a–d in Figure 2). These aberrant methylation patterns are consistent with the notion that the mutant transducers are locked in an active signaling mode, because in wild-type cells stimuli that produce CCW rotational responses evoke methylation increases but CW stimuli elicit methylation decreases. Thus, it appears that the adaptation machinery in the mutants may be engaged in a futile effort to cancel the locked transducer signals.

The main exceptions to this pattern were CCW-biased mutants (8 out of 29) with below normal methylation levels

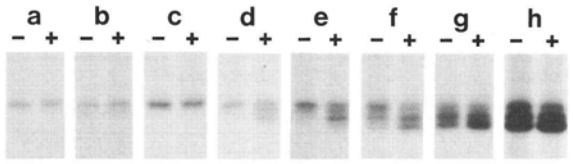

Figure 2. MCP Methylation Patterns in Locked Transducer Mutants

Each lane shows the SDS–PAGE banding pattern of ^{3}H-methyl-labeled Tsr molecules from a different transducer mutant, either before (–) or after (+) stimulation of the cells with the attractant serine. The *tsr* mutations were: SN357 (lane a); GS431 (lane b); AV233 (lane c); AV389 (lane d); wild-type control (lane e); RH394 (lane f); AV413 (lane g); and VM491 (lane h).

(see Table 1), but these could well represent extreme cases of the same underlying mechanism for the following reason. MCP methylation levels are determined by the relative activities of two cytoplasmic enzymes: CheR, the methyltransferase (Springer and Koshland, 1977), and CheB, the methylesterase (Stock and Koshland, 1978). CheB activity can be regulated by sensory signals through a cytoplasmic feedback circuit, producing net changes in methylation state. Transducers that generate exceptionally strong CCW responses would be expected to inhibit CheB activity to a great extent, resulting in slow turnover of MCP methyl groups and apparent inability to incorporate label into them.

Stimulus-Induced Methylation Changes in Locked Transducer Mutants

Many of the transducer mutants, particularly those with CCW-biased output, exhibited detectable methylation increases when subjected to a serine stimulus (see Table 1 and examples in Figure 2, lanes b and d–h), indicating that they retain some sensitivity to stimuli. These responses could reflect changes in the CheR and CheB substrate properties of the transducer molecules themselves or feedback control of methylesterase activity through the intracellular signaling circuitry. In either case, the "locked" transducers in these strains are evidently still capable of undergoing stimulus-induced conformational changes and should not be viewed as completely inflexible with no vestiges of normal functionality.

Discussion

Methyl-accepting proteins probably serve as sensory transducers in a variety of bacterial species in addition to E. coli and Salmonella typhimurium, including Bacillus subtilis (Goldman et al., 1982), Caulobacter crescentus (Shaw et al., 1983), Rhodospirillum rubrum (Sockett et al., 1987), and Spirochaeta aurantia (Kathariou and Greenberg, 1983). The primary structures of MCP molecules in E. coli and S. typhimurium have been deduced from DNA sequence data and seem to be organized into discrete structural and functional domains, as shown in Figure 1. Although little is yet known about the higher order structures of these proteins, it might be possible to deduce some basic principles of their operation through studies of transducer mutants. With few biochemical approaches available, the limiting factor in such analyses is the ability to ascertain the functional defects of the mutants. The mutants described in this report should be useful because they have rather specific functional alterations. Two properties indicate that their transducers are "locked" in active signaling states that interfere with other sensory input pathways. First, they cause predominantly CW or CCW rotation of the flagellar motors, consistent with a constant production of reversal-suppressing signals like those transiently generated during chemotactic responses. Second, the mutant transducers have abnormal methylation states, consistent with permanent activation of the sensory adaptation machinery through the feedback control circuitry.

The properties of locked transducer mutants warrant three general conclusions. First, MCP molecules generate two sorts of flagellar signals. The two signaling modes probably correspond to alternative conformational states. Second, MCP signals are generated by determinants in the cytoplasmic signaling domain. Third, the signal output of MCP molecules is probably directly modulated by the K1 and R1 methylation segments. Control by the receptor domain is effected through the membrane spanners and the linker region. After developing these points in the following sections, we discuss the possible molecular mechanisms underlying the generation and control of MCP signals and present a working model of transmembrane signaling by bacterial transducers.

MCP Molecules Generate Two Kinds of Output Signals

Locked transducers clamp the flagellar motors in either a CW or CCW rotational mode, indicating that MCP molecules can generate signals that augment rotation in either direction. The dominant phenotypes of these mutants imply that both behaviors are due to aberrant signaling activities rather than a loss of function. Transducers must play an active role in eliciting CW rotation because strains devoid of MCP molecules are extremely CCW-biased (Hazelbauer and Engström, 1980). However, transducer mutants locked in a CCW rotational mode could conceivably have dominant null defects in which the nonfunctional transducer subunits aggregate with and inactivate heterologous transducer subunits, resulting in loss of CW signals and a consequent CCW rotational bias. This possibility cannot be rigorously excluded, but three lines of evidence argue against it. First, the dominant properties of locked transducer mutants are relatively insensitive to changes in gene dosage (unpublished data), implying that titration of other transducer subunits is probably not involved. Second, although MCP molecules appear to function as dimers, there is no evidence that they form mixed complexes (Milligan and Koshland, 1988). Third, at least some of the CCW-biased transducers are capable of undergoing stimulus-induced changes in methylation state, indicating that they retain many of the functions of normal transducers. Thus, we propose that MCP molecules normally alternate between two active conformations that correspond to CCW and CW signaling states.

MCP transducers probably mediate their behavioral ef-

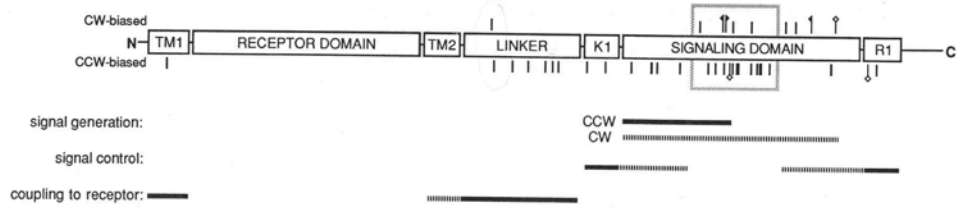

Figure 3. Distribution and Probable Functional Defects of Locked Transducer Mutants

The figure is drawn to scale, with the position of each mutation indicated by a vertical line. Unfilled diamonds show the locations of three nonsense mutations; filled triangular flags show the locations of three slow-switcher mutations. The boxed region in the central portion of the signaling domain contains a variety of rotational phenotypes and may comprise a conformational hinge (see text). The deduced functions of other transducer regions are summarized at the bottom of the figure. Solid bars indicate regions that are clearly implicated in a particular activity, broken bars indicate regions that might be involved.

fects through a phosphorylation cascade involving the cytoplasmic CheA, CheB, and CheY proteins (Hess et al., 1988). The signal relay begins with CheA, which is capable of autophosphorylation (Hess et al., 1987). CheA phosphoryl groups are subsequently transferred to CheB and CheY and probably serve to regulate their functional activities (Hess et al., 1988). Phosphorylated CheB may be the catalytically active form of the MCP methylesterase, accounting for feedback control of the adaptation system. Phosphorylated CheY may produce CW rotation of the flagellar motors, providing the mechanism for eliciting changes in rotational behavior. Since the phosphoryl groups on CheB and CheY are labile, the steady-state levels of the phosphorylated forms should reflect the rate at which phosphate can be cycled through CheA. The CheA autophosphorylation reaction is, therefore, the most likely point of transducer control (Oosawa et al., 1988a). In the CW signaling mode, MCP molecules might activate CheA for autophosphorylation; in the CCW signaling mode they might inactivate CheA.

Transducer Regions Required for Signal Generation

Three of our locked transducer mutants had nonsense mutations, implying that the C terminus of MCP molecules is involved in output control but is not essential for signaling activity. The positions of those mutations (indicated by diamonds in Figure 3) delimit the transducer regions involved in signal production and control. A nonsense mutation at codon 485, at the beginning of the R1 methylation segment, resulted in CCW-biased rotation, indicating that the segment from residue 485 to the C terminus, most likely the R1 region itself, plays a role in output control. Truncation of the Tar transducer at a similar position also results in aberrant signaling behavior (Koshland et al., 1983; Russo and Koshland, 1983). A nonsense mutation at codon 466, near the C-terminal end of the signaling domain, caused CW-biased rotation, demonstrating that the segment between residues 466 and 485 also contributes to output control. Conversely, all determinants necessary for generating CW output must be located to the N-terminal side of residue 466. Truncation of the Tar transducer at a similar position results in identical signaling defects (Krikos et al., 1985). A nonsense mutation at codon 392, in the center of the signaling domain, resulted in CCW-biased flagellar rotation, demonstrating that all functions needed for CCW signaling must be encoded on the N-terminal side of residue 392. By inference, the region between residues 392 and 466 must contain one or more determinants involved in the generation or control of CW signals.

Oosawa et al. (1988b) recently showed that soluble fragments of the Tar transducer, from the linker through the C terminus, are capable of generating flagellar signals. Since this portion of MCP molecules is highly conserved in sequence, this finding most likely applies to Tsr as well. However, the behavioral properties of truncated Tar and Tsr molecules demonstrate that the region from the R1 segment through the C terminus is not needed for signal production. Since the R1 and K1 segments have similar structures and functions, it seems likely that the K1 region is also not involved in generating flagellar signals. Thus, we conclude that the signaling domain alone probably contains all the information needed to generate CW and CCW flagellar signals and that the flanking methylation regions are primarily involved in controlling signal output (Figure 3).

Functional Defects in Locked Transducer Mutants

If MCP molecules have two signaling modes, net signal output from a population of transducers should reflect the time-averaged proportion of molecules in each signaling state. Any transducer alteration that shifts this equilibrium should lead to a locked output condition. In principle, this could happen through changes in the signal control mechanism used in the excitatory or adaptive responses, through structural alterations that prevent conformational transitions, and possibly through changes in the signal-generating sites themselves. The transducer regions susceptible to locked output mutations indicate that most of these defects may be represented among our mutants. The overall distribution of mutations and the conclusions we draw from it are summarized in Figure 3.

The most striking feature of this pattern is the clustering of mutations in the middle of the signaling domain (boxed area in Figure 3). Alterations in this portion of the transducer can lead to both CW- and CCW-biased mutants and

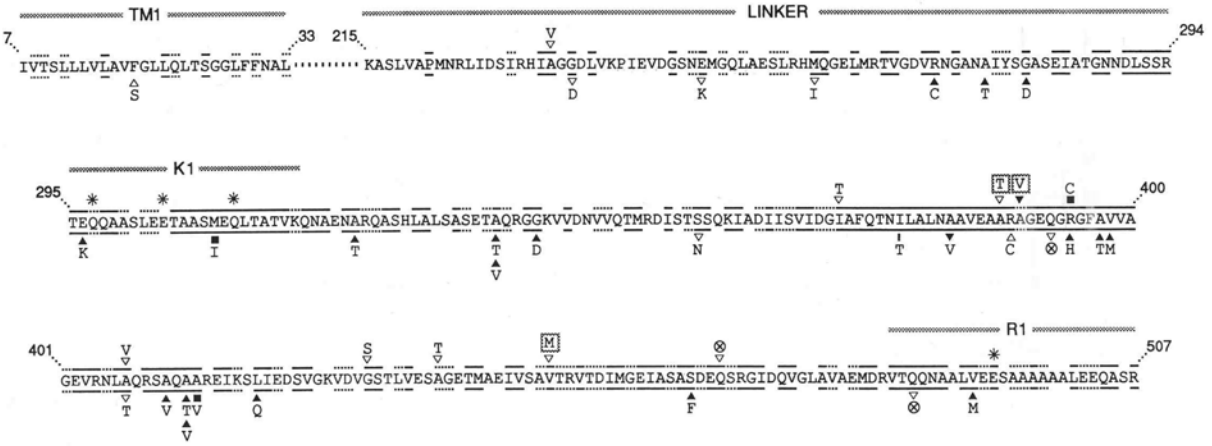

Figure 4. Primary Structure Changes and Associated Phenotypes of Locked Transducer Mutations

The deduced amino acid replacements in the locked transducer mutants are indicated above or below the corresponding wild-type residue in the Tsr sequence. CW-biased mutants and slow-switchers (boxed) are listed above the line; CCW-biased mutants are listed below the line. The ⊗ symbol indicates the positions of stop codons in nonsense mutants; asterisks indicate the positions of the methylation sites. The unstimulated MCP methylation level in each mutant is indicated as follows: less than wild-type (▽,▼); approximately wild-type (□,■); greater than wild-type (△,▲). An unfilled symbol indicates that MCP methylation in that mutant did not increase detectably after application of a serine stimulus, whereas a filled symbol indicates a discernable stimulus-induced increase in methylation level. Residues with solid horizontal lines above and below are conserved in all four MCP species; those with dotted lines are conserved in three out of four MCP species.

to slow-switchers (indicated by triangular flags in Figure 3). The variety of signaling defects and the interspersion of the CW and CCW types suggest that this segment plays an important role in mediating transitions between the CW and CCW conformations. Since the slow-switchers seem to be specifically defective in carrying out these transitions, this region may serve as a "conformational hinge" that enables the molecule to switch signaling modes. This "switch" region is flanked by segments that yielded relatively few locked mutants (Figure 3), suggesting that those portions of the transducer may be essential for generating CCW and CW output signals. Thus, the majority of mutational lesions in these output segments would be expected to reduce or eliminate signaling activity, whereas much more specific alterations would be required to shift the equilibrium proportions of the CCW and CW signaling modes sufficiently to cause a locked output condition.

The K1 and R1 methylation segments modulate signal output as part of the sensory adaptation mechanism. The few locked signal mutations found in these regions might represent alterations that mimic or interfere with the control exerted by changes in methylation state. The remainder of the locked output mutations, which lie in the N-terminal half of the transducer, probably act through the communication mechanism that modulates signal output in response to receptor input. We surmise that both the linker region and the first membrane spanner play an important role in coupling the receptor domain to the signaling domain. The second membrane spanner adjacent to the linker could be involved as well.

Structural Alterations in Locked Transducers

All but three of the locked transducers arose through missense mutations, most of which affect residues that are identical in all four MCP species of E. coli (Figure 4). Nearly half of these mutations (17 out of 37) are located in the switch region, between amino acids 371 and 420 of the signaling domain (Figure 4). Some of these switch mutations have been observed in other transducers. Three of them (AT387, RC394, and AV411) are identical to ones obtained in the Tar transducer, although it is not known if the Tar mutants have locked output signals (Mutoh et al., 1986). Another (AT413) is identical to a mutation in the Trg transducer that does cause aberrant signaling behavior (Park and Hazelbauer, 1986). The switch is the most highly conserved portion of MCP molecules, with 43 out of 50 identities. The flanking regions of the signaling domain, although substantially longer, are less conserved (53 out of 115 identical residues) and yield fewer mutations (10 out of 37). The proportion of conserved residues is somewhat higher in the methylation regions (16 out of 23 in K1 and 14 out of 25 in R1) but much lower in the linker segment (25 out of 80). Nevertheless, even the linker mutations occur at conserved positions (6 out of 7, see Figure 4).

These conserved residues are obviously of critical importance to transducer function. On the one hand, any sort of amino acid replacement at these positions may cause a profound change in transducer structure, severely disrupting function. On the other hand, the seemingly innocous nature of many of the amino acid replacements in the mutants suggests that perhaps even relatively minor structural perturbations have drastic effects on the signaling properties of MCP molecules. These observations imply that the CW-CCW transition could be delicately poised and may involve fairly subtle conformational shifts. Several mutations in the switch region provide compelling support for this notion: the RC394 and AV407 mutants were CW-biased, whereas the RH394 and AT407 mutants

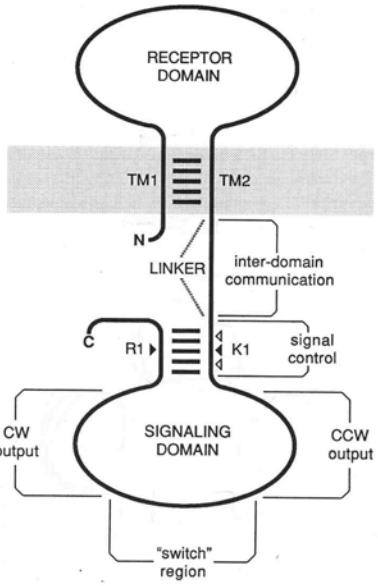

Figure 5. Possible Structure-Function Relationships in MCP Molecules

The shaded area represents the cytoplasmic membrane. The four methylation sites in Tsr are indicated by triangles, with the two open symbols representing the sites that must undergo deamidation before they can accept methyl groups (Kehry et al., 1983). Horizontal lines between TM1 and TM2 and between the methylation segments indicate possible interactions of those regions that may be involved in controlling signal output.

were CCW-biased (Figure 4). Thus, different amino acid changes at the same position can lock the signaling domain in different output modes.

Much of the signaling domain, as well as the K1 and R1 methylation segments, is predicted, by several standard algorithms (Chou and Fasman, 1978; Garnier et al., 1978), to have an α-helical secondary structure. The amino acid changes within these regions that lead to locked signal output are predicted to reduce helix-forming potential and may act by destabilizing or disrupting one of these helical stretches. The switch region, due to glycine residues at positions 390, 393, 395, and 401, is predicted to have a less rigid configuration, consistent with its putative role as a conformational hinge. The mutational changes in the linker region entail no obvious effects on predicted secondary structure; however, four of the seven amino acid replacements involve charge changes (Figure 4), which suggests that electrostatic forces might play a role in linker function.

The TM1 and TM2 regions have a high content of nonpolar and hydrophobic amino acids and appear to be membrane-spanning segments (Manoil and Beckwith, 1986). We obtained one locked output mutant (FS18) with a phenylalanine to serine replacement in the middle of TM1, which should cause a decrease in overall hydrophobicity of the segment. However, its signaling defect is probably not caused by a failure in membrane insertion because Oosawa and Simon (1986) constructed a mutant (AK19) of the Tar transducer with an even more drastic change in TM1 and showed that the altered protein was still inserted in the membrane. The aberrant signaling behavior of the Tar mutant could be alleviated by suppressor mutations in TM2, suggesting that the two membrane spanners might interact with one another (Oosawa and Simon, 1986). Thus, the signaling properties of TM1 mutants may reflect improper interactions with TM2 that ultimately lead to a conformational change in the signaling domain.

Molecular Mechanisms of Signal Generation and Output Control

A model summarizing our analysis of locked transducer mutants is presented in Figure 5. We propose that the cytoplasmic signaling domain contains regions essential for generating CW and CCW output. Both segments may specify binding sites for CheA or one of the other soluble proteins (e.g., CheW or CheZ) involved in the signaling pathway. Interactions with the CW region would enhance the autophosphorylation rate of CheA, whereas interactions with the CCW region would lead to inhibition of CheA autophosphorylation. Locked output defects in these segments are relatively rare and might arise through changes in the relative affinities of the various binding sites. In contrast, the switch region separating the CW and CCW domains is much more susceptible to locked output mutations. It may function mainly as a "conformational hinge" to permit transitions between the CW and CCW signaling states but might also include sites that contribute to each of these signaling activities. In any case, the transition from one signaling conformation to another must entail either a direct structural transformation of the binding sites or rearrangements that expose or mask appropriate components of the sites.

We propose that transducer output is controlled by modulating the equilibrium proportions of the CW and CCW forms and that the methylation segments flanking the signaling domain are primarily responsible for this control. Since the methylation sites are not utilized in strict sequential order (Kehry and Dahlquist, 1982) and their effects on signaling behavior are roughly additive (Springer et al., 1979), signal output may be regulated by interactions between the methylation regions. For example, the K1 and R1 regions might associate through electrostatic or hydrophobic interactions along complementary faces of α-helixes. Changes in methylation state would alter the mutual affinity or relative alignment of these segments, leading to a shift in the CW-CCW equilibrium of the signaling domain. The locked output mutations in the K1 and R1 segments may affect these interactions by mimicking a permanent change in methylation state. Accordingly, structural changes in other parts of the transducer molecule that perturb the interaction of the methylation segments should also give rise to locked transducer mutants. This could account for locked signal defects in the linker and TM1 segments and suggests a simple mechanism for transmembrane signaling.

Transmembrane Signaling by MCP Transducers

We propose that communication between the receptor and signaling domains occurs by direct propagation of

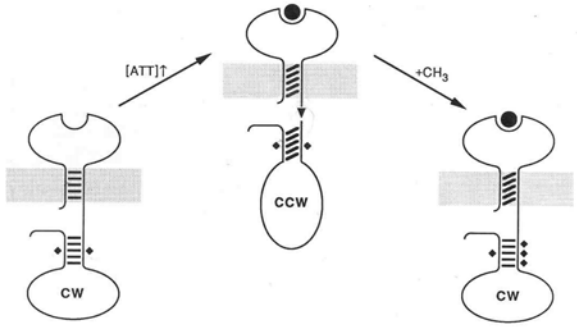

Figure 6. A Model of Transmembrane Signaling by MCPs

Each transducer molecule can assume either a CW or CCW signaling conformation, with overall signal output reflecting the proportion of molecules in each state. In the example shown, a molecule in the CW state is converted to the CCW conformation by ligand binding, and subsequently returned to the CW conformation by an increase in methylation state. (Occupied methylation sites are indicated by solid diamonds.) Transitions between signaling states are triggered by conformational strain between interacting segments of the molecule (indicated by a series of slanted lines) and transmitted between the receptor and signaling domains through the linker region (▼).

conformational changes through the linker segment (Figure 6). The conformation of the receptor domain may be maintained by interactions between the membrane-spanning segments (Oosawa and Simon, 1986), just as interactions between the methylation regions may regulate the conformation of the signaling domain. Conformational changes in the receptor domain, triggered by changes in ligand occupancy (Falke and Koshland, 1987), could affect the alignment of TM1 and TM2, resulting in movement of the linker segment. A shift in linker orientation could in turn alter the interactions between the methylation segments, leading to a change in signal output. Subsequent changes in transducer methylation state during the adaptation phase probably induce compensating changes in the methylation segments to cancel the excitatory signals.

The locked output mutations described in this report provide a useful, but still very incomplete, view of structure-function organization in MCP transducers. However, it is now feasible to test specific predictions of these models with more refined genetic approaches, such as site-directed mutagenesis and second-site suppression analyses. Those studies, combined with additional information about the tertiary structures of MCP molecules, could soon lead to a molecular understanding of transmembrane signaling by bacterial transducers.

Experimental Procedures

Strains and Plasmids

Bacterial mutants used in this work were from our laboratory collection and were derived from RP437, a chemotactically wild-type strain of E. coli K-12 (Parkinson and Houts, 1982). Plasmids were derived either from pUC118 (Vieira and Messing, 1987) or pACYC184 (Chang and Cohen, 1978).

Growth Media

Tryptone broth, plates, and swarm agar (Parkinson, 1980) were used throughout. When needed, ampicillin and chloramphenicol were added to tryptone media at 100 μg/ml and 34 μg/ml, respectively. Stocks of λtsr70 phages (Callahan et al., 1987) were prepared by growth on strain RP5838 (Δ*tsr-7021* (Δ*tar-tap*)*5201*) in NZYM medium as previously described (Parkinson and Houts, 1982).

Enzymes and Reagents

Restriction enzymes were purchased from Bethesda Research Laboratories and from New England Biolabs. T4 DNA ligase, DNA polymerase (Klenow fragment), and deoxy-and dideoxynucleotides were from Boehringer Mannheim. Exonuclease III, S1 nuclease, and acrylamide were purchased from Bethesda Research Laboratories. Hydroxylamine hydrochloride (grade 1) was from Sigma Chemical Company, ^{3}H-methionine was from Amersham, and [^{32}P]dATP was from Dupont/New England Nuclear. All enzymes were used according to the supplier's recommendations.

Isolation of Dominant *tsr* Mutations

New *tsr* mutations were isolated directly on plasmid pPA418 to facilitate subsequent characterization and sequence analysis. This plasmid, which carries the entire *tsr* locus, was constructed by introducing a 5.1 kb HindIII–EcoRI restriction fragment from λtsr70 (Callahan et al., 1987) into pUC118. Mutations were induced in pPA418 DNA by in vitro treatment with hydroxylamine hydrochloride as previously described (Wolff and Parkinson, 1988). The treated DNA was used to transform strain RP5700 (Δ*tsr-7028*), and individual ampicillin-resistant colonies were scored for chemotaxis ability on tryptone swarm plates. Generally nonchemotactic mutants with dominant *tsr* defects occurred at a frequency of about 0.5%.

Dominant *tsr* mutations that had been isolated and characterized in previous work (Parkinson, 1980; Callahan and Parkinson, 1985) were transferred to plasmids to facilitate comparison with the pPA418 mutants described above. A 5.1 kb HindIII–EcoRI restriction fragment containing each of these *tsr* alleles was obtained from the corresponding λtsr70 derivative (Callahan et al., 1987) and inserted into pUC118. The presence of the mutant alleles in the resultant plasmids was confirmed by fine-structure mapping against chromosomal *tsr* mutations (Callahan and Parkinson, 1985).

Flagellar Rotation Assays

RP5700 strains (Δ*tsr-7028*) carrying pPA418 or pUC118 derivatives with *tsr* mutations were grown in tryptone broth containing ampicillin and tethered with flagellar antibodies, as previously described (Parkinson, 1976). At least 100 rotating cells were observed for a period of 15 sec each and classified as exclusively CW or CCW or reversing, as described (Slocum and Parkinson, 1985).

MCP Methylation Assays

RP5869 strains ((Δ*tar-tap*)*5201* Δ*tsr-7021*) carrying *tsr* mutations on plasmid pPA418 were grown and labeled with ^{3}H-methyl methionine, as previously described (Engström and Hazelbauer, 1980). Protein samples were analyzed by electrophoresis in sodium dodecyl sulfate–containing polyacrylamide gels (SDS–PAGE) (Laemmli, 1970).

Deletion Mapping of *tsr* Mutations

Mutations carried on pPA418 were mapped against *tsr* deletions carried on derivatives of a compatible plasmid, pACYC184. Two approaches were used to construct deletions of *tsr* in the pACYC184 vector. First, partial HindIII–Sau3A restriction fragments from pPA418 were inserted into pACYC184 DNA cut with HindIII and BamHI. The *tsr* gene in pPA418 contains nine Sau3A sites, with the HindIII site at its 3′ end, so these fragments should represent nested segments from the promoter-distal end of the *tsr* coding region. Second, DNA from plasmid pPA144, a pACYC184 derivative carrying the entire *tsr* gene, was opened at its unique SalI site, treated for various times with exonuclease III, and recircularized by S1 polishing and blunt end ligation. Since the SalI site is located at the 5′ end of the *tsr* insert, these deletions remove nested pieces of the promoter-proximal end of the *tsr* coding region. Plasmids from both of these procedures were transformed into RP5869 (Δ*tsr-7021* (Δ*tar-tap*)*5201* (λ)) and tested for *tsr* content by high-resolution crosses to λtsr70 phages carrying previously mapped *tsr* point mutations (Callahan et al., 1987). The physical positions of the deletion endpoints were then determined by restriction mapping and DNA sequence analysis.

The pPA418 *tsr* mutations were mapped against the pACYC184 *tsr* deletions in RP5869, which carries a deletion of the entire *tsr* locus, and cannot contribute to the production of tsr^+ recombinants. Strains containing both plasmids were prepared by transformation and streaked across the surface of swarm plates containing ampicillin and chloramphenicol. Chemotactic recombinants were scored after incubation for 24–30 hr at 30°C.

DNA Sequence Determinations

Nucleotide sequences were determined by the dideoxy method of Sanger et al. (1977), using either single- or double-stranded plasmid DNA templates. Oligonucleotide primers were 20 bases in length and synthesized at the University of Utah.

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