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Receptor structure in the bacterial sensing system

(chemotaxis/membranes/serine receptor/aspartate receptor/methyl-accepting chemotaxis proteins)

ELIZABETH A. WANG AND DANIEL E. KOSHLAND, JR.

Department of Biochemistry, University of California, Berkeley, California 94720

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ABSTRACT **The primary receptors for aspartate and serine in bacterial chemotaxis have been shown to be the 60,000-dalton proteins encoded by the *tar* and *tsr* genes. The evidence is: (*i*) overproduction of the *tar* gene product at various levels by recombinant DNA techniques produces proportionate increases in aspartate binding; (*ii*) aspartate binding copurifies with [^{3}H]methyl-labeled *tar* gene product; (*iii*) antibody to tar and tsr protein fragments precipitates a single species of protein (60,000 daltons) which retains binding capacity and [^{3}H]carboxymethyl label. Partially purified *tar* gene product can be reconstituted into artificial vesicles and retains aspartate binding and aspartate-sensitive methylation and demethylation. These results show that the aspartate and serine receptors are transmembrane proteins of a single polypeptide chain with the receptor recognition site on the outside of the membrane and the covalent methylation site on the inside.**

Receptors are the molecules that receive signals from the exterior of a cell and transmit information to its interior. When the cell is on the outside of an organism, the receptors record environmental conditions relevant to the survival of the species. When the cell is in the interior of the organism, the receptors record signals from other cells as part of the communication and regulatory system of a differentiated organism.

The structure of receptors in mammalian systems is beginning to become apparent. Some, such as the cyclic nucleotide-dependent hormone receptors, are composed of multiple peptide chains, usually a hormone-binding peptide, a GTP-binding peptide, and adenyl cyclase (1). The acetylcholine receptors appear to contain four peptides (2). Others may contain only a single peptide, as in the case of rhodopsin (3) or antibody receptor (4). In bacterial systems, the receptors are also part of complex networks usually involved in transport or sensing (5, 6). In each case, the manner in which the peptides interact to achieve the recognition and transmission of the signal is crucially dependent on receptor structure.

The bacterial sensing system offers a special advantage for the study of receptor structure and function. The repertoire of receptors is extensive because the single cell also represents the whole organism. Approximately 30 different receptor recognition types have been identified in *Escherichia coli* (5) and a similar number appear to be involved in *Salmonella typhimurium* (6). Some are periplasmic proteins (7–9); others appear to be membrane bound (10).

Two 60,000-dalton proteins in the bacterial membrane have been shown (11, 12) to be part of the network in signal transmission and products of the *tar* and *tsr* genes. On the basis of binding and genetic studies, Clarke and Koshland (10) suggested that in the case of the *tar* and *tsr* gene products the peptides recognizing the chemoeffector and producing the transmembrane signal were the same. This type of genetic evidence, however, cannot be conclusive *per se* in determining the primary receptor because it can be argued that the transmembrane proteins are essential to maintain the conformation of a second recognition component. Definitive evidence in regard to the role of the 60,000-dalton *tar* and *tsr* gene products in binding was needed, and it was obtained as described below by a combination of recombinant DNA techniques and protein purification.

MATERIALS AND METHODS

Materials. All amino acids and analogues were obtained from Sigma, Calbiochem, or Vega Fox (Tucson, AZ). L-[^{14}C]-Aspartic acid (225.0 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels), L-[^{14}C]serine (162.0 mCi/mmol), *S*-[*methyl*-^{3}H]adenosyl-L-methionine (AdoMet; 11.8 Ci/mmol), and L-[^{35}S]methionine (1149.9 Ci/mmol) were obtained from New England Nuclear. IgGSORB (*Staphylococcus aureus*) was obtained from New England Enzyme Center. Soybean phosphatidylcholine was purchased from Sigma. Chromatography media were purchased from Pharmacia.

Bacteria. The wild-type chemotaxis parent *E. coli* RP437 and the *tar tsr* double mutant RP4372 were obtained from J. S. Parkinson (University of Utah). The *S. typhimurium* strain ST422 pDK1 has been described (13). The vectors λ*che*$_S$1 and pDK1, containing the *Salmonella cheR, cheB, cheY, cheZ,* and *tar* genes have been described (14). The plasmid pGK3 containing the *cheR* and *tar* genes was derived by genetic techniques, described in ref. 14, by N. Gutterson-Cahill.

Membrane Preparation. Cells were grown in L broth (0.5% Bacto yeast extract/0.5% NaCl/1.0% Bacto tryptone) at 30°C and harvested in late exponential phase. Membranes for aspartate and serine binding and for methylation were prepared as described (10).

Assays. Membrane methylatable tar and tsr were measured by incubating 0.1–0.5 mg of prepared membranes (or 0.01–0.05 mg of reconstituted membranes), 5–10 units (1 unit = 1 pmol of [^{3}H]methyl groups per min per mg) of methyltransferase from a crude *Salmonella* cytoplasmic fraction, 10 nmol of [^{3}H]AdoMet (typically, diluted to 200 cpm/pmol), and 10 μmol of $NaPO_4$ at pH 7.0 in 0.1 ml at 30°C. The reaction was stopped at various intervals. The level of methylation was determined by the method of Stock *et al.* (J. B. Stock, personal communication). An asymptotic value of ^{3}H incorporation was usually reached by 60 min.

Transferase assays were performed similarly except that 1–2 mg of unmethylated membranes [ST1038 (15)] and 0.5–2.0

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; MCP, methyl-accepting chemotaxis protein.

units of transferase from cytoplasmic extracts were used. Methylesterase assays were executed as above.

Amino acid binding to membranes was measured as described (10).

Protein was determined by a modified Lowry assay (16).

tar and tsr Purification. Membranes were methylated by [^{3}H]AdoMet as described (10). Washed membranes were solubilized in 0.5% Triton X-100. The solubilized membrane extract was assayed by measuring ^{3}H incorporation into protein carboxymethyl esters and analyzed by $NaDodSO_4$ gel electrophoresis (17) and fluorography (18). Polyacrylamide gels were quantitated by slicing, solubilizing, and assaying the gel as described (19). The cholate dialysis procedure (20) was used for reconstitution.

Antibody Preparation and Techniques. Rabbit antibody to 300-fold-purified tar and tsr 27,000- and 21,000-dalton fragments was prepared by standard methods. Antibody purified by ammonium sulfate precipitation was used in crossed immunoelectrophoresis (21), Ochterlony immunodiffusion (22), *S. aureus* precipitation (23), and amino acid binding.

RESULTS

Overproduction of the *tar* Gene Product. As a first step in determining whether the *tar* gene product alone is the aspartate receptor, the protein was overproduced at several levels and the level of aspartate binding was determined. Where a phage containing *tar*, *cheR*, *cheB*, *cheY*, and *cheZ* (14) was introduced into RP4372, a *tar tsr* mutant that shows low levels of aspartate binding, the level of aspartate binding increased in proportion to the amount of tar protein (Table 1). The level of production was determined by (*a*) the level of ^{3}H-labeled methyl groups incorporated into the *tar* gene product, and (*b*) assay for the chemotaxis-specific methyltransferase which is expressed with the tar protein. The aspartate binding was performed in crude membranes which show no transport or metabolism of aspartate and serine under the assay conditions (10). The increase in aspartate binding in proportion to tar protein overproduction indicates that the tar protein is an essential part of the receptor.

If the tar protein were part of an α,β pair requiring both components for aspartate binding and if the non-tar subunit were in excess, the proportionality described above would not be conclusive. Therefore, the tar protein was further overproduced by using two plasmids, pDK1 [containing *tar*, *cheR*, *cheB*, *cheY*, and *cheZ*, 9.6×10^6 daltons (14)] and pGK3 [containing *tar* and *cheR* only, 5.6×10^6 daltons (N. Gutterson-Cahill, personal communication)] which is expected to have a higher copy number. Strains containing pDK1 and pGK3 showed a further increase in aspartate binding over the λche_S1 lysogen (Table 1). The 22-fold and 149-fold increases in aspartate binding in pDK1 and pGK3 were proportional to the increase in the tar protein produced. Serine binding remained the same in all these strains, showing lack of a general effect of these vectors on membrane binding. The hypothesis theorizing a second subunit in the aspartate receptor therefore would require the other subunit to be in 150-fold excess in the *tar tsr* mutant.

Purification of the tar Protein Fragments and Antibody. To eliminate the remaining possibility of a second subunit containing the recognition sites, the *tar* gene product was purified by a somewhat novel procedure. Initial attempts to purify the protein revealed proteolytic digestion. Because attempts to prevent the proteolysis were initially unsuccessful, we decided to take advantage of adversity and purify the digestion products. Antibodies prepared against purified digestion products reacted with the intact tar protein, aiding both assay and purification. Membranes from ST422 pDK1, which overproduce tar about 4-fold over ST422 (14), were used as the source of methyl-accepting chemotaxis protein (MCP), and MCPs were assayed by means of their radioactive methyl groups. The *in vitro* methylation is specific for MCPs (15) but does not discriminate between the *tar* and *tsr* gene products which may copurify (see Table 2). Final purification yielded 300-fold-purified 27,000- and 21,000-dalton fragments which retained [^{3}H]methyl groups.

Antibody prepared against these fragments gave a single arc (radioactive) in crossed immunoelectrophoresis against crude solubilized membrane in which tar and tsr protein had been specifically labeled *in vitro*. The same antibody was then used against a cell preparation in which all proteins were labeled with [^{35}S]methionine. When the immunoprecipitate from this mixture was run on a $NaDodSO_4$ gel, only one major band, at 60,000 daltons, was obtained. Moreover, this band was multiple as found for authentic tar protein (14). No single protein was present in an amount greater than 5% of that of the 60,000-dalton protein, assuming the same number of methionines.

The antigen–antibody complex retained aspartate binding activity. Solubilized membrane protein was precipitated by antibody and *S. aureus* (23) and assayed for aspartate binding. The level of aspartate binding (Table 3) was similar to that obtained with crude solubilized protein reconstituted into artificial phosphatidylcholine vesicles (see below). Thus, it appears that the antibody carries down a single 60,000-dalton protein which retains aspartate binding activity.

Evidence for the Serine Receptor. To determine the crossreactivity of the antibody, serine binding was also assayed. The antibody precipitated serine-binding activity as well as aspartate-binding activity in both *Salmonella* and wild-type *E. coli* membrane extracts (Table 3). Thus, the antibodies to the *Salmonella tar tsr* fragments crossreact with the analogous *E. coli* proteins. The fact that serine-binding activity is precipitated shows that the tsr protein is the serine receptor, in analogy to the tar protein being the aspartate receptor.

Table 1. Overproduction of *tar* gene product correlates with aspartate binding

Strain	Max. ^{3}H incorporation,* pmol/mg	Methyltransferase activity, pmol/min/mg	Aspartate Max. binding, pmol/mg	Aspartate K_d, μM	Serine Max. binding, pmol/mg	Serine K_d, μM
RP 437 (wild type)	39	0.5	34	7	81	7
RP 4372 (tar^-tsr^-)	2	2.4	8	4	54	4
RP 4372 λche_S1	69	26.7	98	5	114	3
RP 4372 pDK1	252	56.1	176	9	47	3
RP 4372 pGK3	1739	111.1	1194	10	37	2

Methylation, transferase assays, and amino acid binding were performed as described. The K_d and the maximum number of binding sites were calculated from a Lineweaver–Burk graphical analysis of binding performed at five or six concentrations of amino acid (1.0–8.3 μM).

* Incorporation into methyl-accepting chemotaxis protein.

Table 2. Copurification of aspartate-binding protein with [^{3}H]MCP

Fraction	Max. L-Asp bound, pmol/mg (corrected)	Corr.*	Purification of Asp binding, -fold	Purification of [^{3}H]MCP, -fold
Crude membranes	555	1.00	1.00	1.00
Membranes, washed	538	1.00	0.97	1.06
Solubilized supernatant†	2,524	1.06	4.54	3.09
Ethanol supernatant†	1,901	1.90	3.42	3.34
DEAE I pool†	11,690	8.47	21.04	18.62
DEAE II, peak fraction†	62,540	33.3	112.6	115.3

ST 422 pDK1 membranes were methylated (10) and then washed in 2 M KCl/20 mM KPO_4/1 mM EDTA, pH 7.0. The membranes were solubilized at 0°C for 2 hr in 50 mM $NaPO_4$/0.5% Triton X-100. The soluble membrane supernatant was brought to 20% saturation with cold ethanol, allowed to equilibrate for 30 min, and then centrifuged. This supernatant (ethanol supernatant) was dialyzed overnight in 20 mM Tris, pH 7.0/0.1% Triton X-100; 315 mg was applied to a 3.0 × 30 cm DEAE-Sepharose column equilibrated with the same buffer. ^{3}H-Labeled protein was eluted with a 1200-ml gradient of 0.05–0.50 M NaCl. The pool of labeled protein, dialyzed against the next column buffer, was applied to a 1.5 × 30 cm DEAE-Sepharose column in 20 mM $NaPO_4$, pH 7.15/0.1% Triton X-100 and eluted with a 300-ml gradient of 0.0–0.45 M NaCl.

* Factor correcting for fraction of [^{3}H]MCP >27,000 daltons.

† Soluble protein was reconstituted into phosphatidylcholine vesicles, and aspartate binding was measured.

Correlation of Binding with Purification of Tar. Purification of the *tar* gene product from ST422 pDK1 was pursued to correlate binding with purification. The detergent-solubilized ^{3}H-labeled membrane fraction containing intact tar and some methylated fragments was reconstituted by the cholate dialysis procedure. The amount of tar present was determined in two ways: (*a*) by the amount of aspartate binding remaining in ^{3}H-labeled tar protein which was present in fragments larger than 35,000 daltons (the 27,000- and 21,000-dalton purified fragments retained no binding activity) and (*b*) the number of methylated carboxyl groups. The relationship between these two measurements is shown in Table 2. The correspondence was excellent up to 115-fold purification. The K_d values for aspartate binding were also checked at each stage of purification and always remained at about 5 μM, a value in agreement with the K_d of the intact native protein (10).

Table 3. Aspartate- and serine-binding mediated by antibody

Membrane origin	Aspartate Added, μM	Aspartate Bound, pmol	Serine Added, μM	Serine Bound, pmol
RP 4372 (*tar*$^-$*tsr*$^-$)	7.3	13	—	—
ST 422 pDK1 (wild-type + plasmid)	3.0	122	3.0	88
RP 437 (wild-type *E. coli*)	7.3	73	7.3	79

Antiserum was incubated with an excess of 10% IgGSORB. The pelleted cells were incubated with crude solubilized membrane fraction for 30 min at 0°C and then washed with 100 mM $NaPO_4$/1 mM EDTA, pH 7.0. Amino acid binding at the concentration indicated was measured as described except that variation in the volumes was calculated by weighing the tubes before and after removal of the supernatant. A control sample containing preimmune serum did not bind aspartate or serine. The *E. coli* extracts were added in 2- to 3-fold excess; the *Salmonella* extract was added in 1.2-fold excess. Normalized to this value, the binding of the *Salmonella* extract was 332 pmol/mg of protein added for aspartate and 226 for serine.

Further evidence that the right protein was being purified was obtained by determining the specificity. The competitive inhibition values for L-glutamate, D-aspartate, and α-methyl-D,L-aspartate remained constant with purification.

Reconstitution of Aspartate-Coupled Reaction. The cholate reconstitution procedure was able to reconstitute binding activity of the tar protein at various levels of purification in phosphatidylcholine vesicles. In addition, the proteins could be re-formed in conformations that were substrates for the highly chemotaxis-specific methyltransferase enzyme. Unreconstituted protein was not methylated. As final evidence that we had retained the aspartate receptor, we tested whether the methylation of reconstituted protein showed coupling to the chemoeffector aspartate, as had been observed *in vitro* (24, 25).

Addition of aspartate increased methylation of the reconstituted protein by a small but definite amount (Fig. 1). Aspartate also inhibited demethylation by 400-fold purified esterase (26, 27) by almost 50%, and it is possible that decreased demethylation in the crude transferase extract (i.e., decreased turnover) accounts for the aspartate coupling seen in methylation. This is exactly the behavior one would require for the pure protein because the whole cells show increased methylase activity and decreased methylesterase activity in the presence of attractant (28). The degree of esterase coupling is quite large—in fact, better than in previous observations on *in vitro* coupling (24, 25). Electrophoresis of the methylated reconstituted protein showed ^{3}H incorporation in one band centering at 60,000 daltons. Moreover, the addition of other chemoef-

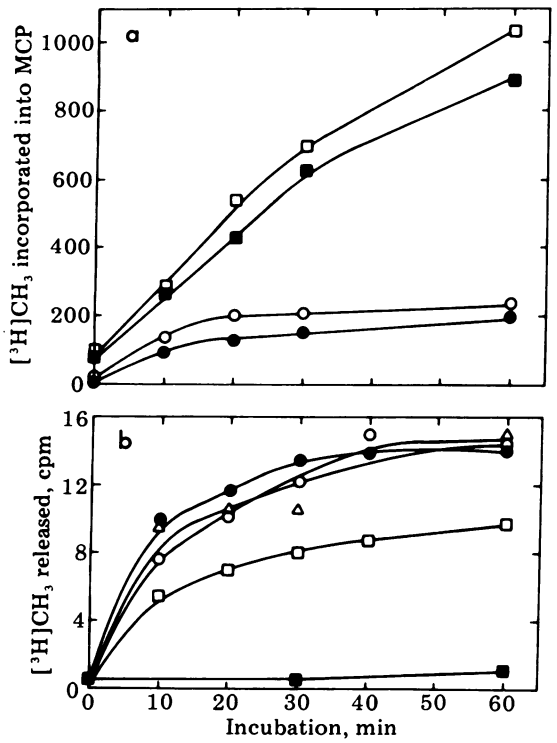

FIG. 1. Effect of chemoeffectors on tar methylation and demethylation. (*Upper*) Methylation of crude membranes and reconstituted partially purified tar by a crude *Salmonella* cytoplasmic extract containing transferase. Ordinate is expressed in pmol of methyl groups per mg of membrane protein. ●, Crude membrane; ○, crude membranes + 1 mM aspartate; □, reconstituted membranes; ■, reconstituted membranes + 1 mM aspartate. (*Lower*) Demethylation of reconstituted partially purified tar by 400-fold purified *Salmonella* extract. Ordinate is expressed in cpm per μg of esterase. ■, No esterase; □, esterase + 1 mM aspartate; △, esterase + 1 mM $NiSO_4$; ○, esterase + 1 mM serine; ●, esterase alone.

fectors that do not affect tar, such as serine and certain repellents, had no effect on the rate of methylation or demethylation of the 60,000-dalton protein.

DISCUSSION

Evidence that the *tar* gene product, an integral membrane protein of 60,000 daltons, is the primary receptor for aspartate in bacterial sensing appears to be complete. The protein has been purified, and the binding capacity for aspartate correlates with the methyl-labeled protein over a 100-fold purification range. The protein can be precipitated by a specific antibody, and the antibody–tar complex continues to bind aspartate. The antibody binds only 60,000-dalton protein. Overproduction of the *tar* gene product by factors of 22- and 149-fold relative to the *tar tsr* mutant, by using recombinant DNA, increased aspartate binding capacity by the same relative amounts. Mutants lacking the tar protein lack aspartate binding. The K_d of the aspartate binding correlates with the K_d of the behavioral profile of the bacteria (10). Thus, it seems certain that the *tar* gene product is the primary receptor for aspartate in bacterial chemotaxis. Because the antibody prepared against the tar tsr fragments also precipitates serine-binding activity, it was also possible to confirm that the *tsr* product is the primary receptor for serine.

Fig. 2 shows a schematic version of the receptor based on our current knowledge. The "floating receptor" mechanism appears to be well established for the interaction of the ribose-binding protein and the galactose-binding protein with the *trg* gene product (29, 30). Competition studies show that the galactose–galactose-binding-protein complex and the ribose–ribose-binding-protein complex compete for a limited number of *trg* sites in the membrane (29). Mutations affecting the galactose-binding protein eliminate galactose-taxis (9); mutations affecting the ribose-binding protein eliminate ribose-taxis (7), and mutations affecting the trg protein eliminate both (31). Similarly, mutations affecting the tar protein eliminate both aspartate- and maltose-taxis (11) and mutations affecting the maltose-binding protein eliminate maltose-taxis (8). Thus, the three sugars act on the transmembrane proteins through protein–chemoeffector complexes whereas aspartate acts directly on the protein.

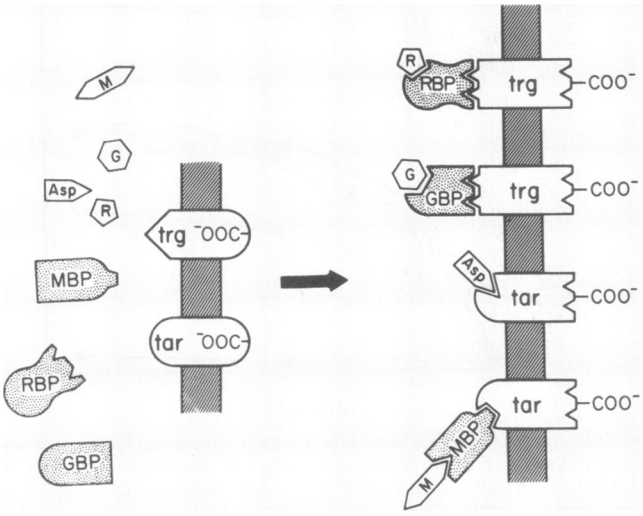

FIG. 2. Binding induces conformational change. The MCPs, encoded by the *tar* and *trg* genes, undergo a conformational change upon binding ligand. This change is direct in the case of aspartate or indirect as mediated by primary receptor proteins, which expose the carboxyl groups to methyltransferase. Asp, aspartate; G, galactose; R, ribose; M, maltose; GBP, RBP, MBP, the periplasmic proteins that bind these carbohydrates.

The finding that the *tar* and *tsr* gene products are the primary receptors allows us to reexamine previous genetic studies with additional perspective. Clarke and Koshland (10) described a serine-taxis mutant lacking serine binding. Hedblom and Adler (32) isolated a serine-taxis mutant that retained group I repellent taxis and lacked serine binding. In other work in this laboratory (unpublished data), two mutants defective in serine-taxis and binding and another mutant that shows no aspartate-taxis but does bind aspartate normally have been isolated. Our results, combined with these data, suggest that the *tar* and *tsr* gene products have separate, but not independent, domains for amino acid binding and taxis functions: a point mutation in the taxis domain need not necessarily affect amino acid binding but a mutation that eliminates binding will necessarily destroy specific taxis. Preliminary evidence with partially purified tar protein reconstituted into membrane vesicles supports this notion: vesicles reconstituted with 27,000-dalton and 21,000-dalton fragments, which retain methyl groups, do not bind aspartate but vesicles reconstituted with partially purified methylated tar containing 60,000- or 35,000-dalton fragment do bind aspartate. However, reconstitution of methyl-accepting ability does require intact 60,000-dalton tar protein.

This two-domain hypothesis suggests that the tar protein is a transmembrane receptor. The periplasmic maltose-binding protein interacts with the maltose-binding protein receptor site on the outside of the inner membrane. Methylation must occur on the inner side of the inner membrane because the chemotaxis-specific methyltransferase is a cytoplasmic enzyme (15). Aspartate binding probably also occurs on the outside face of the inner membrane because aspartate chemoreception is independent of aspartate transport and because levels of aspartate in metabolism are expected to fluctuate. Thus, it appears that both aspartate and the maltose-receptor-chemoeffector complex bind to the tar protein where it protrudes into the periplasmic space, as shown in Fig. 2.

The induced conformational change travels an appreciable distance. The membrane is 50–90 Å wide and the aspartyl-induced conformation change must therefore travel 50–90 Å. In the case of maltose, the chemoeffector-induced conformational change must alter the maltose-binding protein and then the tar protein (33). The galactose conformational change can travel 30–40 Å (34), and similar changes might be expected in the closely related maltose-binding protein. Conformational changes induced by immunoglobulin (35) may conceivably be carried over 100 Å, based on the distance between binding site and complement-fixing site (36). Like rhodopsin, therefore, the serine and aspartate receptors are transmembrane proteins which transmit a signal by an extensive conformational change and become covalently modified in the sensing and adaptation process.

Such a long-distance conformational change must be the result of a protein programmed to change from one thermodynamic minimum in the absence of ligand to a second stable conformation in its presence. The ligand must trigger the change, which cannot be a single distortion in the sense of strain energy. The latter would be too rapidly dissipated in such a long pathway. Rather a domino effect in which amino acid residues or domains slide into new conformations preprogrammed to be stabilized by the ligand seems to be required.

A reasonable hypothesis on the evolution of such a system is that the tar protein initially began as a protein that transmitted information in regard to aspartate in the environment. Because there were large amounts of maltose-binding protein present in the periplasm, a mutational event that allowed the maltose-binding protein to interact with the aspartate receptor re-

sulted in transmission of a chemotactic signal to the interior of the cell. This bacterium, having an advantage in being able to respond to maltose, a good carbon source, then incorporated this new function into its machinery and thus improved its survival at the expense of less-efficient organisms. The same would be true of the tsr protein which would initially have been only a serine receptor and would later have become susceptible to protons, indole, and other molecules inducing similar conformational changes. We do not yet know which of these latter stimuli operate through their own specific binding proteins and which have binding sites on the tsr protein itself.

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