Transmembrane Signal Transduction in Bacterial Chemotaxis Involves Ligand-Dependent Activation of Phosphate Group Transfer

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Transmembrane signal transduction in bacterial chemotaxis involves ligand-dependent activation of phosphate group transfer

(excitation/reconstitution/receptor/chemotaxis proteins/aspartate)

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ABSTRACT **Signal transduction in *Escherichia coli* involves the interaction of transmembrane receptor proteins such as the aspartate receptor, Tar, and the products of four chemotaxis genes, *cheA*, *cheY*, *cheW*, and *cheZ*. It was previously shown that the *cheA* gene product is an autophosphorylating protein kinase that transfers phosphate to CheY, whereas the *cheZ* gene product acts as a specific CheY phosphatase. Here we report that the system can be reconstituted *in vitro* and receptor function can be coupled to CheY phosphorylation. Coupling requires the presence of the CheW protein, the appropriate form of the receptor, and the CheA and CheY proteins. Under these conditions the accumulation of CheY-phosphate is enhanced ≈300-fold. This rate enhancement is seen in reactions using wild-type and "tumble" mutant receptors but not "smooth" mutant receptors. The increased accumulation of phosphoprotein was inhibited by micromolar concentrations of aspartate, using wild-type, but not tumble, receptors. These results provide evidence that the signal transduction pathway in bacterial chemotaxis involves receptor-mediated alteration of the levels of phosphorylated proteins. They suggest that CheW acts as the coupling factor between receptor and phosphorylation. The results also support the suggestion that CheY-phosphate is the tumble signal.**

Bacteria such as *Escherichia coli* or *Salmonella typhimurium* sense their environment through a series of transmembrane receptor proteins. Each of these binds a specific subset of ligands that may act as attractant or repellent. Changes in ligand concentration initiate two responses (for reviews of bacterial chemotaxis, see refs. 1 and 2). First, a rapid excitation response occurs that modulates the frequency of changes in bacterial flagellar rotation, and second, an adaptation response is initiated that presumably modifies the sensitivity of the receptor. The excitation response can be manifested in two ways. Increase in concentration of an attractant ligand may decrease flagellar-rotation-reversal frequency, leading to "smooth" swimming of the cell. Alternatively, an increase in repellent concentration can result in a transient increase in flagellar reversal, leading to "tumbly" swimming behavior. In addition to the receptor, the excitation response requires the presence of the products of four chemotaxis genes, *cheA*, *cheY*, *cheZ*, and *cheW* (3–6). We have shown that the CheA protein is an autophosphorylating protein kinase that in the presence of ATP phosphorylates histidine residue 48 (7–10). Once CheA is phosphorylated, it is able to very rapidly transfer phosphate to the *cheY* gene product. CheY-phosphate or a derivative of CheY is thought to interact with proteins at the base of the flagellar motor to increase the frequency of reversal of rotation (11–13). The *cheZ* gene product specifically dephosphorylates CheY (7). Thus, these results suggest a plausible scheme for how the *che* gene products might generate a "tumble" regulator. However, little is known about how the receptor interacts with the phosphorylating system or how CheW functions.

We have suggested that the transmembrane receptors when activated by repellents or when fixed by mutation in a mode that generates tumbles would accelerate the rate of formation of CheY-phosphate (8, 10). On the other hand, the receptor when it has attractant bound to it should decrease the rate of formation of CheY-phosphate. Physiological experiments suggest that the coupling of the transmembrane receptor to the excitation response also requires the *cheW* gene product (3, 5). In this paper we present the results of experiments designed to reconstitute the coupling of the transmembrane receptor to phosphorylation. We describe a system where the nature of the receptor and its ligand regulate the rate of CheY-phosphate formation and the presence of CheW is required for efficient coupling of the receptor to the phosphate transfer system.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *tar* gene was excised as an *Eco*RI–*Ava* I fragment from plasmid pNM13, and both ends were filled in using the Klenow fragment of DNA polymerase. This fragment was inserted in the *Sma* I site of pUC9 to give plasmid pNT2. pNT2 was cut with *Eco*RI, and the end was filled in using the Klenow fragment and then cut with *Bam*HI. A derivative of pBR322 containing a tac promoter was cut with *Hin*dIII, filled in using the Klenow fragment, and cut with *Bam*HI, and the above fragment from pNT2 was inserted. The resulting plasmid, pNT201, contained the wild-type *tar* gene under control of a tac promoter and was used to overexpress the Tar protein. Corresponding smooth (allele 325L) and tumble (allele 346M) (14) mutant Tar protein-encoding vectors were constructed by replacement of a restriction fragment of the wild-type *tar* gene with the appropriate fragment carrying the dominant mutation. The plasmids were maintained in *E. coli* derivative KO607 (Δtar-tap-5201, Δtsr-7021, Δtrg-100) (15).

Growth of Cells, Preparation of Plasma Membranes, and Protein Purification. Cells were cultivated in L broth (1% tryptone/0.5% yeast extract/1% NaCl) containing ampicillin at 100 μg/ml at 37°C. Tar proteins were induced during late logarithmic phase using 1 mM isopropyl β-D-thiogalactoside. Membranes were prepared by a modification of the procedure described by Bogonez and Koshland (16). Cells were harvested by centrifugation and washed in a buffer containing 100 mM Tris·HCl, 5 mM EDTA, 5 mM *o*-phenanthroline, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol (pH 7.5). Lysates were prepared by sonicating the cells in the same solution, followed by centrifugation twice at 12,000 × *g* for 10 min to remove debris. The lysate was fractionated into soluble and membrane fractions by centrif-

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ugation at 100,000 × *g* for 1 hr. The pellet was washed by resuspension in a solution containing 50 mM Tris·HCl, 2 M KCl, 5 mM EDTA, 3 mM *o*-phenanthroline, 1 mM PMSF, and 10% glycerol (pH 7.5) and pelleting at 12,000 × *g* for 15 min. This step was repeated once. The membranes were washed once more in a buffer containing 50 mM Tris·HCl, 1 mM *o*-phenanthroline, 1 mM PMSF, and 10% glycerol (pH 7.5). The pellet was resuspended in the same solution and used as the source of membranes for experiments without addition of detergent.

The levels of the different membranes used are difficult to compare with each other since the amount of receptor was different in each preparation. Therefore, for the later experiments described in this paper, 3 μl of wild-type receptor preparation (diluted with 3 μl of membranes derived from KO607 with no plasmid, referred to as "control" membranes) or 6 μl of each of the tumble, smooth, or control receptor preparations shown in Fig. 1 was used in all the assays unless otherwise indicated.

CheA, CheY, and CheZ were purified as described (8, 17). CheW was purified as described (18), except for the inclusion of a blue dye ligand chromatography step (8). Protein concentrations were determined by using the Bio-Rad protein reagent concentrate, with bovine serum albumin as the standard.

Electrophoretic Transfer (Western) Blotting. Samples of the final washed plasma membranes from the various strains were subjected to SDS/PAGE (12.5% acrylamide gel), and the gels were stained using Coomassie blue or electroblotted onto nitrocellulose. The blotted gel was allowed to react with a Tar-specific antiserum (14); this was followed by reaction with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Promega). The Tar proteins were visualized after a final reaction of the blot using the ProtoBlot system color development reagent (Promega).

Protein Labeling. Phosphorylation reaction mixtures contained 50 mM Tris·HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (≈1000 cpm/pmol), purified proteins, plasma membranes, and other components as indicated in a total volume of 20 μl. Early experiments contained 0.1 mM ADP; it was later determined that this had no effect on the amount of phosphorylation seen. Reactions were terminated by the addition of Laemmli SDS sample buffer (19). The samples were subjected to SDS/PAGE (12.5% acrylamide gel), after which the gels were Coomassie-stained, destained, dried under vacuum, and autoradiographed. Phosphate incorporation into a given protein was quantitated by cutting the stained band out of the dried gel and assaying for radioactivity in scintillation fluid. For cpm <1000, there was an error of ≈10–15%.

RESULTS

Expression and Isolation of Receptor-Containing Membrane Fragments. Bogonez and Koshland (16) were able to use solubilized membrane preparations containing the aspartate receptor to demonstrate coupling between the addition of ligand and receptor methylation that occurs during the adaptation response. To test for reconstitution of the excitation response we prepared membranes from cells that were deleted for all four chemotaxis receptors, cells that overproduced only the Tar receptor, and cells that overproduced each of the two (*tar346* and *tar325*) dominant aspartate receptor mutants. Mutant receptor 346M induces continuous tumbling behavior in cells even in the presence of wild-type Tar or of the other chemotaxis receptors (14). Thus, it behaves as if it were fixed in a form that constantly generates tumble signals. The mutant receptor 325L behaves as if it were fixed in a state that can only generate a smooth-swimming signal (14). Plasma membrane preparations of the four strains were checked for expression and for the size of the Tar protein by Coomassie staining and immunoblot analysis after separation of proteins on polyacrylamide gels (Fig. 1). The proteins are of the same mobility range as noted previously for Tar (14). The smooth mutant (325L) protein migrates slightly faster (lane 3) and the tumble mutant (346M) migrates more slowly (lane 4) than the wild-type protein (lane 2). The control strain minus plasmid possesses no protein that reacts with the antibody (lane 1). The three Tar proteins are expressed to a high level and can be visualized by Coomassie staining (lanes 6–8). The wild-type protein (lane 6) is present at 3- to 5-fold higher levels in membranes than the two mutants, which are each at about the same abundance in the membrane (lanes 7 and 8).

Stimulation of Phosphorylation in the Presence of Receptor and CheW. To examine the effects of the different chemotaxis proteins on phosphorylation, each of the purified proteins was added to salt-washed membranes containing receptors in the presence of [γ-^{32}P]ATP. After reaction the mixture was separated on SDS/PAGE and the regions containing the CheA and CheY proteins were excised from the gel and assayed for radioactivity. The addition of membranes did not influence the phosphorylation of CheA in the presence or absence of CheW during a 2.5-min assay (Table 1). The same result was seen in 10-sec assays under these conditions (data not shown). Additionally, the K_m of CheA for ATP was 300–400 μM in the presence or absence of any of the plasma membranes and CheW (data not shown). Thus, under these conditions the autophosphorylation rate of CheA is independent of the presence of transducer-containing membranes. When CheY was added to reaction mixtures containing CheA and membranes, a relatively small difference in the amount of CheY-phosphate that accumulated could be seen. In assays with the wild-type or tumble receptor there was a 2- to 3-fold increase in the amount of CheY-phosphate (Table 1). However, if CheW was supplied to the coupled system there was an increase in CheY-phosphate of approximately 5- to 7-fold for the wild-type and tumble receptors. Moreover, in these assays CheA-phosphate also accumulated at appreciable levels. If CheZ was added to phosphorylation reaction mixtures containing CheA, CheY, and membranes, there was a great reduction in the level of CheY-phosphate, which is consistent with results noted previously (7, 8).

The enhancement of CheY phosphorylation appears to be tightly coupled—i.e., to get a striking receptor-mediated response, all four components, CheA, CheY, CheW, and wild-type or tumbly receptor, are necessary. Taken together, the above observations are consistent with the hypothesis that (*i*) receptor and CheW regulate the rate of phosphorylation in the reconstituted system, (*ii*) wild-type or tumble receptor stimulates the level of phosphorylation, whereas smooth receptor gives little, if any, enhancement, and (*iii*)

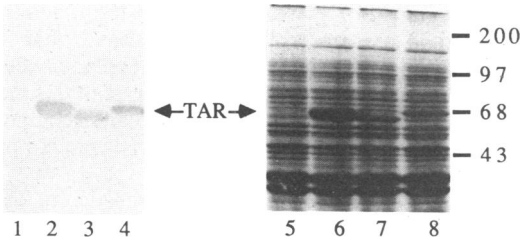

FIG. 1. Expression of wild-type and mutant Tar proteins in membranes. Salt-washed membranes of the four strains were prepared and subjected to SDS/PAGE. Lanes 1–4 show an immunoblot analysis of the gel using Tar-specific antiserum, whereas lanes 5–8 are of a duplicate gel that has been Coomassie-stained. Lanes 1 and 5, KO607; lanes 2 and 6, KO607 with pNT201 (wild type); lanes 3 and 7, KO607 with pNT201-325L (smooth); and lanes 4 and 8, KO607 with pNT201-346M (tumble). Sizes are shown in kDa.

Table 1. Phosphorylation of CheA and/or CheY in the presence of the other components

Purified proteins	Membrane source	cpm incorporated	
		CheA	CheY
CheA	Minus receptor	2061	0
CheA	Wild type	1803	0
CheA	Tumble	1388	0
CheA	Smooth	1596	0
CheA + CheW	Minus receptor	2399	0
CheA + CheW	Wild type	2041	0
CheA + CheW	Tumble	2285	0
CheA + CheW	Smooth	1888	0
CheA + CheY	Minus receptor	0	982
CheA + CheY	Wild type	0	2,380
CheA + CheY	Tumble	24	3,192
CheA + CheY	Smooth	0	727
CheA + CheW + CheY	Minus receptor	11	1,571
CheA + CheW + CheY	Wild type	726	10,885
CheA + CheW + CheY	Tumble	331	7,923
CheA + CheW + CheY	Smooth	37	1,902
CheA + CheY + CheZ	Minus receptor	4	28
CheA + CheY + CheZ	Wild type	63	72
CheA + CheY + CheZ	Tumble	32	161
CheA + CheY + CheZ	Smooth	20	31
CheA + CheW + CheY + CheZ	Minus receptor	0	13
CheA + CheW + CheY + CheZ	Wild type	0	760
CheA + CheW + CheY + CheZ	Tumble	0	394
CheA + CheW + CheY + CheZ	Smooth	0	136

Reactions were performed under the standard conditions with 100 μM ADP, 4 μl of membranes, 40 pmol of CheA, 40 pmol of CheW, 50 pmol of CheY, and 10 pmol of CheZ where indicated. Reaction time was 2.5 min.

CheW is required to efficiently couple the "state" of the receptor to the transfer of phosphate through CheA to CheY.

Dependence of Phosphorylation on the Components of the Reconstituted System. To understand the receptor-mediated regulation of phosphorylation it is necessary to define the role of each of the components in the overall reaction. Therefore, a number of titration experiments were undertaken. In reaction mixtures containing 100 pmol of CheY and 40 pmol of CheW, the production of CheY-phosphate was linear with increasing concentrations of CheA up to ≈5 pmol of CheA added (Fig. 2*A*). At this point, CheA-phosphate became detectable. The level of CheA-phosphate continued to increase with increasing concentrations of added CheA. We have shown that CheA-phosphate is an obligatory intermediate in the phosphorylation of CheY (8). The data in Fig. 2*A* indicate that in the coupled system—i.e., in the presence of Tar-containing membranes and CheW—the formation of CheY-phosphate requires CheA. The large difference between the amount of CheY-phosphate in the reaction with receptor and in the control reactions shows that Tar is specifically required to increase CheY-phosphate accumulation. CheY appears to behave as the substrate in this reaction and under the conditions shown in Fig. 2*C*, the accumulation of CheY-phosphate was linear with increased added protein up to 200 pmol.

The phosphorylation of CheY in the presence of Tar was dependent on the concentration of CheW (Fig. 2*B*). At low levels of CheW (5 pmol), where it is stoichiometric with CheA, there was very little increased phosphorylation of CheY. The amount of CheY-phosphate obtained increased with increasing concentrations of CheW and was not completely saturated at 80 pmol in the assay. It is difficult to interpret this result in an unequivocal manner because of the relative complexity of the reconstituted system. Increasing concentrations of CheW could drive the formation of a transient complex between CheW, CheA, and CheY. CheW could also interact with the receptor or CheY to facilitate the activation of CheA and the transfer of phosphate to CheY.

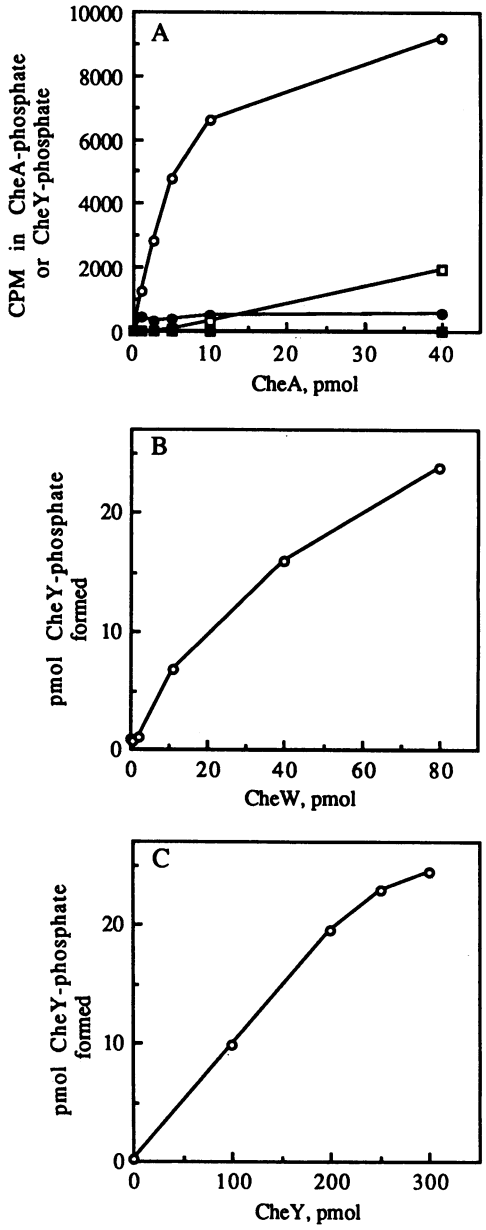

FIG. 2. Dependence of the phosphorylation reaction on the concentrations of CheA, CheW, and CheY. (*A*) Dependence on CheA level. Ten-second phosphorylation assays were performed under the standard conditions using 3 μl of wild-type (open symbols) or control (closed symbols) membranes, 100 pmol of CheY, 40 pmol of CheW, and various amounts of CheA in a total volume of 20 μl. The amount of CheY-phosphate (circles) and CheA-phosphate (squares) produced was determined as described in the text. (*B*) Dependence on CheW level. The standard phosphorylation reaction was performed using 3 μl of wild-type membranes diluted with 3 μl of control membranes, 5 pmol of CheA, 200 pmol of CheY, and the indicated levels of CheW in a volume of 20 μl. The amount of CheY-phosphate produced in 10 sec was quantitated as described in *A*. (*C*) Dependence on CheY level. The phosphorylation reaction was performed as described for *B*, except that CheW was held constant at 40 pmol and CheY was varied. The amount of CheY-phosphate was determined as described in *A*.

Kinetics of the Phosphorylation Reaction. Under reaction conditions where the extent of phosphorylation of CheY is dependent on the rate of CheA phosphorylation—i.e., where there is no accumulated CheA phosphate—we can approxi-

mately measure the rate enhancement that results from the absence or presence of various receptors (Fig. 3). By 10 sec, the amount of CheY-phosphate is at a maximum in the reaction containing wild-type membranes. The rate of CheY phosphorylation is also high using tumble mutant receptor membranes; the amounts of receptor protein in the two preparations are different, which may account for the differences in stimulation. Even though the conditions used for this reaction may be suboptimal, if we compare CheY-phosphate production after 10 sec of reaction in the presence of wild-type versus control membranes, we find a 300-fold increase in phosphorylated CheY. After this burst, the amount of CheY-phosphate decreases steadily. This decrease may be due to a drop in the concentration of ATP resulting from endogenous ATPase activity or to the autodephosphorylation of CheY or both.

Dependence of the Phosphorylation Reaction upon $MgCl_2$ and Aspartate. By using the assay conditions described above and 10-sec reactions, the dependence of the phosphorylation reaction was further studied. The reaction was dependent on the presence of $MgCl_2$ (Table 2). Additionally, the maximum production of CheY-phosphate required the presence of ATP, CheA, CheW, CheY, and receptor, as noted previously (Table 1).

Since aspartate acts *in vivo* as an attractant at micromolar concentrations via the Tar transducer (20), according to our model, addition of 1 mM aspartate to the wild-type reconstituted system should result in decreased phosphorylation. Fulfilling this prediction, reactions containing wild-type receptor were inhibited 95% in the presence of 1 mM aspartate, whereas the tumble mutant reactions were unaffected (Table 2). There was no significant difference in phosphorylation observed with the two membrane preparations derived from cells with no receptors and cells with the smooth mutant receptor. Thus, the inhibition by aspartate of phosphorylation *in vitro* correlates with its ability to stimulate smooth-swimming chemotactic behavior *in vivo*. It was difficult to test for repellent responses due to the presence of glycerol in the membrane preparations; glycerol has been shown to be a potent repellent in chemotaxis assays.

The dependence of CheY-phosphate production upon aspartate was determined over four orders of magnitude of aspartate concentration (Fig. 4). At 10^{-3} M, the reaction was almost completely inhibited, consistent with the results in Table 2. A marked response to added ligand occurred between 10^{-6} and 10^{-5} M aspartate, the same concentration range observed to strongly influence chemotaxis *in vivo* (21). A double-reciprocal plot of the data gave a K_d for aspartate inhibition of 3 μM. This value is identical to the binding constant of solubilized Tar for [^{3}H]aspartate (14) and similar to the same binding constant for native receptor (21).

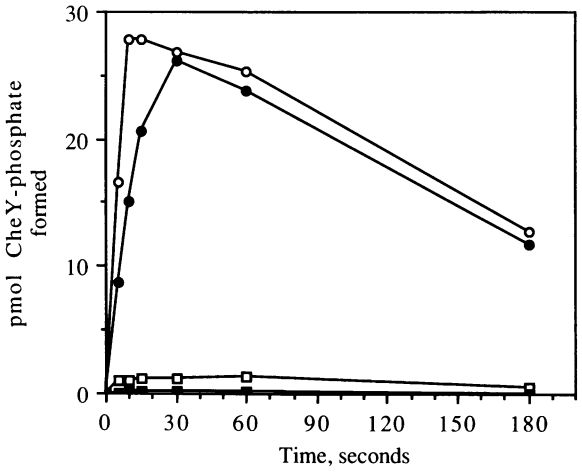

FIG. 3. Kinetics of the phosphorylation reaction using the four membrane preparations. The standard phosphorylation assay was performed using 5 pmol of CheA, 200 pmol of CheY, 40 pmol of CheW, and 3 μl of wild-type membranes diluted with 3 μl of control membranes (open circles), 6 μl of tumble mutant membranes (closed circles), 6 μl of smooth mutant membranes (open squares), or 6 μl of control membranes (closed squares). The amount of CheY-phosphate produced was quantitated as described in the legend to Fig. 2.

Table 2. Dependence of CheY-phosphate production upon $MgCl_2$, CheA, CheW, receptor, and aspartate

Purified proteins	Membrane source	% maximum CheY-phosphate production
CheA + CheW + CheY	Wild type	100
CheA + CheW + CheY − $MgCl_2$ + 25 mM EDTA	Wild type	0
None	Wild type	1
CheY	Wild type	3
CheY + CheW	Wild type	5
CheA + CheY	Wild type	3
CheA + CheW + CheY	Minus receptor	1
CheA + CheW + CheY + 1 mM aspartate	Minus receptor	0
CheA + CheW + CheY	Smooth	5
CheA + CheW + CheY + 1 mM aspartate	Smooth	1
CheA + CheW + CheY	Tumble	48
CheA + CheW + CheY + 1 mM aspartate	Tumble	43
CheA + CheW + CheY	Wild type	100
CheA + CheW + CheY + 1 mM aspartate	Wild type	6

Reactions were performed under the conditions described for Fig. 3, using 10-sec assays. In these experiments, the maximum CheY-phosphate produced was 16 pmol.

DISCUSSION

One of the major difficulties in understanding the mechanism involved in transmembrane transfer of information is the question of how the state of the cell surface receptor generates a rapid intracellular signal. In the case of the bacterial chemotaxis system current evidence suggests that the intracellular signal may involve the accumulation of

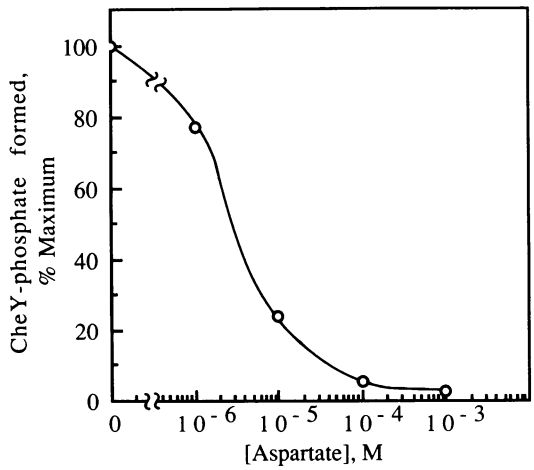

FIG. 4. Inhibition of phosphorylation by aspartate. Standard phosphorylation reaction mixtures contained the amounts of proteins and wild-type membranes described in the legend to Fig. 3 and the indicated concentrations of L-aspartate. Incubation was for 10 sec, and CheY-phosphate was quantitated as described in the legend to Fig. 2.

phosphorylated CheY. In this report we have shown that we can reconstitute *in vitro* a system in which the rate of CheA autophosphorylation and the accumulation of CheY-phosphate are dependent on the nature of the ligand bound to the Tar receptor or the mutated form of the receptor. The *cheW* gene product plays a critical role as coupling factor. In the presence of the appropriate form of the receptor, CheW, CheA, and CheY, enhancements in the amounts of phosphorylated CheY of 300-fold were observed even under suboptimal reaction conditions. However, we have not been able to demonstrate receptor-mediated enhancement of the rate of CheA autophosphorylation when the *cheY* gene product is absent (Table 1). Thus, the receptor and CheW may act on a rate-limiting step in the reaction that involves a complex of CheA and CheY.

How could CheW and receptor function? One notion is that the activated receptor assembles a catalytic complex including CheA and CheW, with CheY molecules acting as substrate that can cycle through the complex. Another model, in analogy with the function of guanine nucleotide-binding proteins as signal transducers in eukaryotic systems (22), suggests that the activated receptor catalyzes ATP binding to the CheW protein, which has been shown to have a highly conserved amino acid sequence that could act as a nucleotide-binding site (18). In this model CheW with nucleotide bound increases the rate of CheY phosphorylation by mediating the exchange of ATP for ADP bound in the transient CheA-CheY complex or by facilitating the release of CheY-phosphate. We have found that ADP can act as an effective competitive inhibitor of CheA autophosphorylation (K.A.B. and M.I.S., unpublished); thus exchange of ADP for ATP might be rate limiting. Finally, it is possible that activated CheW or receptor could generate a small molecule (e.g., a lipid metabolite) that would act as an activator of CheA. Thus, although we do not yet understand how the state of the receptor is communicated through CheW to the CheA-CheY complex, we do have in hand a reconstituted system that makes this process accessible to experimentation.

A number of lines of evidence suggest that this system accurately reflects the excitation process. First, the dependence of the stimulation of phosphorylation on the level of aspartate added to receptor is similar to the known concentration dependence of aspartate binding to receptor. Second, the receptor carrying the dominant tumble mutation behaved in the same fashion as the aspartate receptor in the absence of ligand, but the activity of the dominant mutant was relatively insensitive to added ligand. Finally, the magnitude of the rate enhancement and the rapidity of the reaction all suggest that it could account for the generation of "tumble regulator" seen in the excitation response. Although we have not demonstrated any effect of the smooth mutant receptor there could be a second signal generated that blocks or inhibits the formation of CheY-phosphate. For example, we might imagine that sequestration or modification of the CheW protein or activation of the CheZ phosphatase could act to "suppress tumbles." We have shown previously that there are structural differences between the proteins that correspond to the signaling forms of the tumbly and the smooth aspartate receptor mutants (23). These altered forms could be involved in interaction with CheW.

In addition to phosphorylating CheY, CheA can transfer phosphate to CheB, increasing receptor demethylation and adaptation.

Eventually, it should be possible to reconstitute *in vitro* the excitation and the adaptation responses in order to study the coordinated response of the receptor to changes in ligand concentration and thus get a better picture of the mechanisms involved in signal transduction.

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