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Constitutively Signaling Fragments of Tsr, the *E. coli* Serine Chemoreceptor

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ABSTRACT

Tsr, the serine chemoreceptor of *E. coli*, has two signaling modes. One augments clockwise (CW) flagellar rotation, the other augments counter-clockwise (CCW) rotation. To identify the portion of the Tsr molecule responsible for these activities, we isolated soluble fragments of the Tsr cytoplasmic domain that could alter the flagellar rotation patterns of unstimulated wild-type cells. Residues 290-470 from wild-type Tsr generated a CW signal, whereas the same fragment with a single amino acid replacement (alanine 413 to valine) produced a CCW signal. The soluble components of the chemotaxis phosphorelay system needed for expression of these Tsr fragment signals were identified by epistasis analysis. Like full-length receptors, the fragments appeared to generate signals through interactions with the CheA autokinase and the CheW coupling factor. CheA was required for both signaling activities, whereas CheW was needed only for CW signaling. Purified Tsr fragments were also examined for effects on CheA autophosphorylation activity *in vitro*. Consistent with the *in vivo* findings, the CW fragment stimulated CheA, whereas the CCW fragment inhibited CheA. CheW was required for stimulation, but not for inhibition. These findings demonstrate that a 180 residue segment of the Tsr cytoplasmic domain can produce two *active* signals. The CCW signal involves a direct contact between the receptor and the CheA kinase, whereas the CW signal requires participation of CheW as well. The correlation between the *in vitro* effects of Tsr signaling fragments on CheA activity and their *in vivo* behavioral effects lends convincing support to the phosphorelay model of chemotactic signaling.

INTRODUCTION

Methyl-accepting chemotaxis proteins (MCPs) mediate chemotactic responses in a variety of motile bacteria, ranging from Archaebacteria to Gram-positive and Gram-negative Eubacteria (reviewed in (11)). These sensory transducers offer tractable models for molecular studies of transmembrane and intracellular signaling. *E. coli* has four different MCPs that detect the attractants serine (Tsr), aspartate (Tar), dipeptides (Tap), and ribose and galactose (Trg). MCP molecules are about 550 amino acids in length and span the cytoplasmic membrane. They have a periplasmic ligand-binding domain that monitors attractant levels in the environment and a cytoplasmic signaling domain that controls rotation of the flagellar motors (14, 21). Occupancy changes in the sensing domain modulate the output activity of the signaling domain to elicit chemotactic movements. For example, attractant increases promote smooth, forward swimming by reducing the probability of the episodes of clockwise (CW) flagellar rotation that cause turns or tumbling movements. Ensuing changes in MCP methylation state cancel excitatory stimulus signals to achieve sensory adaptation, thereby enabling the organism to detect further changes in its chemical environment (31).

MCPs in *E. coli* appear to control swimming behavior by modulating the autophosphorylation activity of CheA, a cytoplasmic autokinase that in turn activates the response regulator proteins CheY and CheB by donating its phosphate groups to them (12). Phospho-CheY interacts with switching components of the flagellar motors to augment CW rotation (4, 34); phospho-CheB demethylates MCP molecules to regulate the sensory adaptation process (19). In *in vitro* coupling assays, ligand-free receptors stimulate CheA autophosphorylation, whereas attractant-occupied receptors inhibit CheA activity (5, 25). Single amino acid replacements at sites throughout the receptor molecule can lock it into one or the other of these signaling states. Mutant receptors locked in the inhibitory mode cause exclusively counter-clockwise (CCW) flagellar rotation and smooth swimming, whereas receptors locked in the stimulatory mode cause predominantly CW rotation and incessant tumbling (3, 24). CheW, a small cytoplasmic protein, is essential for stimulatory receptor coupling and promotes formation of complexes between MCP and CheA molecules (5, 10, 17). Once formed, MCP/CheW/CheA complexes are quite stable both *in vitro* (10) and *in vivo* (20), suggesting that CheA activity is primarily regulated by conformational changes within the ternary complexes, rather than through their assembly and disassembly.

Many important mechanistic questions about chemoreceptor signaling have yet to be answered. Is CheW needed for inhibitory coupling? Where are the sites of contact between MCP, CheW, and CheA molecules? Do intermolecular contacts serve only to control CheA conformation, or do they also contribute groups that participate directly in the CheA autophosphorylation reaction in some manner? To facilitate experimental approaches to these issues, we generated and characterized soluble fragments of the Tsr signaling domain that retain CW- or CCW-signaling activity *in vivo* and a corresponding ability to stimulate or inhibit CheA autophosphorylation rates *in vitro*. These Tsr fragments evidently contain the contact sites needed to modulate the CheA autokinase activity, and should prove to be informative subjects for structural and functional studies of chemoreceptor signaling.

MATERIALS AND METHODS

Bacterial Strains. All of the strains used in this work were derived from RP437, an *E. coli* K-12 strain that is wild type for chemotaxis (28). The *in vivo* effects of Tsr fragments were studied in RP5700, which carries a complete deletion of the *tsr* locus [*Δ(tsr)7028*], but is otherwise chemotactic (7). RP3098 [*Δ(flhD-flhA)4*] (30) was used to synthesize proteins from expression plasmids for subsequent purification. Strains with multiple chemotaxis defects for epistasis studies are listed in the legend of Figure 4.

Construction of Tsr Fragment Plasmids. DNA fragments of the wild-type *tsr* gene were obtained from plasmid pPA420 (3). Fragments from a pPA420 derivative carrying the *tsr-1006* allele were the source of the Tsr-AV413 mutant (alanine to valine replacement at residue 413) (3). Plasmid DNAs were digested with different combinations of restriction enzymes and the resulting fragments were inserted into the expression site of plasmid pTM30. Details of the various constructions are given in the legend of Figure 1.

Behavioral Assays. All physiological experiments were conducted at 35°C. Chemotactic ability was assessed on tryptone semisolid agar (swarm) plates (27) containing 50 μg/ml ampicillin and, when necessary to induce expression of Tsr fragments, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Flagellar rotation patterns were determined by cell tethering, as described (27).

Measurement of Tsr Fragment Expression Levels. Cells of strain RP5700 carrying pPA56 or pPA58 were grown in tryptone broth containing 100 μg/ml ampicillin and various concentrations of IPTG. At mid-log phase, 1 ml samples of the cultures were pelleted by centrifugation and washed once with ice-cold 10 mM potassium phosphate buffer, pH7.0, 1 mM EDTA. The cells were pelleted again and resuspended in 100 μl of SDS sample loading buffer (15) and lysed by boiling for five minutes. Lysate samples were electrophoresed in 15% SDS-containing polyacrylamide gels (SDS-PAGE), blotted to polyvinylidine difluouride (PVDF) membranes, and subjected to western analysis. Tsr-related bands were detected with rabbit antibody prepared against the purified `Tsr´-CW protein. The plasmid-encoded β-lactamase, which served as an internal standard, was detected with antiserum kindly provided by G. Georgiou (University of Texas-Austin). Antibody-reactive bands were visualized with ^{35}S-Protein A (Amersham) and quantified with a Molecular Dynamics PhosphorImager™.

Purification of Tsr Fragments. The Tsr fragments made by plasmids pPA56 and pPA58 were purified by a combination of ammonium sulfate precipitation, DEAE-anion exchange chromatography, and gel exclusion chromatography. Cultures of RP3098 harboring either pPA56 or pPA58 were grown overnight at 37°C in H1 salts minimal medium (27) containing 0.4% glycerol, 1 mM each of the required amino acids threonine, leucine, histidine, and methionine, and 100 μg/ml ampicillin. 50 ml of this saturated culture were added to two liters of the same medium supplemented with 1% casamino acids (Difco). The cultures were incubated with vigorous shaking at 37°C until cell density was about 10^8/ml, at which time IPTG was added to 1mM and shaking was continued for a further 3.5-4 hr. The cells were chilled on

ice, harvested by centrifugation, washed twice with ice-cold 25 mM Tris, pH8, 1 mM EDTA, and then resuspended in 1/50th of their original volume in cold 25 mM Tris, pH8, 5 mM EDTA. The cells were lysed by two passages through a French press, the large debris was removed by centrifugation at 5,000 g for 10 min in an SS-34 rotor at 4°C, and the lysates were clarified further by centrifugation at 100,000 g for 1 hr. Solid ammonium sulfate was added to the supernatants to 25% saturation, and the mixture incubated on ice for 2 hr. The precipitated material was pelleted by centrifugation at 10,000 g for 20 min, and the pellets redissolved in 2-4 ml of cold 25 mM Tris, pH8, 1 mM EDTA and dialyzed overnight against 2000 volumes of the same buffer at 4°C. The dialyzed protein was applied to a DEAE-Sepharose (Pharmacia) column, equilibrated with the dialysis buffer, and the column washed with 10 volumes of the same buffer. A 0-500 mM linear gradient of NaCl in 25 mM Tris, pH8, 1 mM EDTA was applied in 8 column volumes. Eluted fractions were assayed for total protein and for the Tsr fragments by SDS-15% polyacrylamide gel electrophoresis. Both Tsr fragments eluted over a broad (100-400 mM) NaCl concentration range. Those fractions which contained the Tsr fragments were pooled, and concentrated by ultrafiltration using Centriprep-10 devices (Amicon). The concentrated pool was applied to a Sephacryl S-300 (Pharmacia) column equilibrated with 25 mM Tris, pH8, 1 mM EDTA, and the column developed with the same buffer. Fractions were assayed for total protein, and those containing the Tsr fragments, identified by SDS-PAGE, were pooled and concentrated by ultrafiltration as above. Typical yields from a two-liter preparation were 5-10 mg of Tsr fragment of greater than 95% purity as determined by SDS-PAGE analysis and Coomassie staining.

Purification of CheA, CheW, and CheY Proteins. CheA was purified from cultures of RP3098 carrying plasmid pDV4 essentially as described (13). CheW was purified from cultures of RP3098 carrying plasmid pJL63 (18) essentially as described (32). CheY was purified from cultures of RP3098 carrying plasmid pRL22 essentially as described (22). All proteins were greater than 95% pure by SDS-PAGE analysis and Coomassie staining.

***In Vitro* Coupling Assays of Tsr Fragment Function.** Purified reaction components in TKMD buffer (50 mM Tris, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol) were mixed in various combinations and molar ratios in a total volume of 20 µl and preincubated at room temperature for 60 minutes. Phosphorylation reactions were initiated by addition of 1 mM [γ-^{32}P]ATP (~1000 cpm/pmol) and stopped after 10 seconds by addition of SDS-PAGE loading buffer. Reactants were separated by electrophoresis on SDS-15% polyacrylamide gels, and the distribution of label was quantified with a Molecular Dynamics PhosphorImager™. The concentrations and ratios of components used in different experiments are given in the legends of Figures 5, 6, and 7.

Preparation of Phospho-CheA for Dephosphorylation and Phosphotransfer Assays. Radiolabeled phospho-CheA was prepared by combining 700 µg of purified CheA in TKMD buffer with 100 µM [γ-^{32}P] ATP (~5×10^5 cpm/pmol). The reaction was incubated for two hours at room temperature and terminated by addition of solid ammonium sulfate to 55% saturation. This mixture was kept on ice for 2 hr before recovering the precipitate by centrifugation at 14,000 g in a microcentrifuge at 4°C. The labeled protein was washed three times with cold 60% saturated ammonium sulfate, then resuspended in 1 ml of TKMD buffer and

dialyzed overnight at 4°C against 1000 volumes of the same buffer. Phospho-CheA was then concentrated by ultrafiltration and used as a reagent to test Tsr fragments for effects on phospho-CheA stability and on the phosphotransfer rate between CheA and CheY, using simple variants of the coupling assays described above.

Methylation of Tsr Fragments. The ability of Tsr fragments to accept methyl groups was assessed by labeling with ^{3}H-methyl methionine, essentially as described for full-length MCPs (8).

RESULTS

Isolation of Tsr Signaling Fragments. The membrane topology and domain organization of Tsr, the serine chemoreceptor, are representative of the MCP family of transducers (Fig. 1A). The periplasmic ligand-binding domain, flanked by membrane-spanning segments, comprises about 200 residues at the N-terminus of the molecule. The remaining 350 or so C-terminal residues are located in the cytoplasm, where they interact with CheA and CheW to generate the signals that control flagellar rotation, and with CheR and CheB, the enzymes that methylate or demethylate the receptor to promote sensory adaptation. The methylation sites are located in two segments of the receptor (K1 and R1) that flank a highly conserved region, which probably contains CheA and CheW contact sites, based on intergenic suppression studies (16, 18). Many mutations that lock the Tsr molecule into a CW or CCW signaling mode also fall within this region, implying that it is involved in modulating or generating flagellar signals (3).

To determine whether the putative signaling domain of Tsr was sufficient for generating signals, we subcloned segments of the *tsr* gene and examined their polypeptide products for signaling activities. Coding fragments corresponding to various portions of the Tsr cytoplasmic domain (Fig. 1A) were inserted into pTM30 (23), a multicopy plasmid that provided an IPTG-inducible promoter (P_{tac}) and efficient translation initiation signals (Fig. 1B). The fragments were inserted in-frame with the translation start site in pTM30, yielding Tsr polypeptides with three vector-encoded residues at their N-terminus (Fig. 1B). Polypeptides made from coding segments that lacked the normal *tsr* stop codon also contained nine vector-encoded residues at their C-terminus (Fig. 1b). To assess the signaling properties of these Tsr fragments, recombinant plasmids were introduced into chemotactic recipients and tested for ability to inhibit chemotaxis upon induction with IPTG. We reasoned that Tsr fragments capable of interacting with signaling components such as CheA or CheW should interfere with chemotaxis by titrating their activities. Moreover, any such fragments that are able to produce a CCW or CW flagellar signal might also perturb the cell's unstimulated flagellar rotation pattern.

All of the *tsr* subclones depicted in Figure 1 severely inhibited the swarming of chemotactic strains (RP437 or RP5700) on soft agar plates containing 1 mM IPTG (data not shown). At this induction level, Tsr fragments were expressed in roughly 100-fold excess of the full-length receptors in the cell, but growth rates were unchanged (data not shown), indicating that the reduction in swarm speed was due to impaired chemotaxis. The effects of these Tsr fragments on the flagellar rotation pattern of RP5700 are summarized in Figure 2. Plasmid-

containing cells grown in medium without IPTG exhibited wild-type rotational behavior, characterized by frequent reversals in at least 80% of the individuals. However, upon growth in medium containing 1 mM IPTG, the same strains had substantially lower reversal rates and pronounced rotational biases.

Four fragments derived from the wild-type *tsr* gene imparted a CW rotational bias. The largest of them, $Tsr_{176-551}$, contains the entire cytoplasmic domain; the smallest, $Tsr_{290-470}$, contains only the putative signaling domain and the adjacent K1 methylation segment (Fig. 2). We draw two tentative conclusions from these results. First, the periplasmic sensing domain of Tsr inhibits CW signal output in the intact molecule because its removal leads to unmodulated CW output. Second, neither the R1 methylation segment nor the linker are essential for CW signaling, consistent with the premise that the signaling domain alone is responsible for output activity. One or more determinants critical for CW signaling are evidently located near the N-terminus of $Tsr_{290-470}$ because a somewhat shorter fragment, $Tsr_{354-470}$, caused a CCW rotational bias (Fig. 2). The 290-354 segment of the signaling domain might be directly involved in generating CW output, or it might play a less direct role, for example, in controlling transitions between the CW and CCW signaling modes.

To study the CW signaling process in more detail, we focused on the $Tsr_{290-470}$ fragment made by plasmid pPA56. We found that the CW signaling activity of this Tsr fragment could be converted to CCW output by single amino acid changes that lock the intact receptor into a CCW signaling mode (Fig. 2). An alanine to valine replacement at residue 413 (AV413), for example, conferred CCW signaling ability on $Tsr_{290-470}$ and $Tsr_{290-551}$ that was indistinguishable from that of $Tsr_{354-470}$ (Fig. 2). All three fragments had a dominant effect on rotational behavior, demonstrating that their CCW signaling is an active process, not simply the absence of CW signaling. Although the mechanistic basis of the AV413 signaling defect is unknown, it affects the full-length Tsr molecule and its signaling domain fragments in the same way. Accordingly, we used the AV413 derivative of $Tsr_{290-470}$, made by plasmid pPA58, for subsequent studies of the CCW signaling process. For simplicity, we will refer to the CW and CCW versions of the $Tsr_{290-470}$ fragment as `Tsr´-CW and `Tsr´-CCW.

Components Required *In Vivo* for Tsr Fragment Signaling. To evaluate the relative potency of `Tsr´-CW and `Tsr´-CCW signals, strain RP5700 carrying pPA56 or pPA58 was grown in medium with different IPTG concentrations to manipulate fragment levels. At mid-log phase the cells were tethered to coverslips by their flagella to examine their rotation patterns, and protein extracts were made from parallel samples to quantify Tsr fragment levels by immunoblotting. The flagellar rotation effects of the two fragments at different expression levels are summarized in Fig. 3. The uninduced expression levels of the fragments are comparable to those of the chromosomally-encoded native receptors, whereas at full induction the fragments are roughly in 100-fold excess (data not shown). The `Tsr´-CCW plasmid (pPA58) produced substantial rotational bias at four-fold lower IPTG concentrations than did the `Tsr´-CW plasmid (pPA56) (Fig. 3A). However, the two plasmids made comparable amounts of fragment at each induction level (Fig. 3B). The higher specific activity of the `Tsr´-CCW fragment suggests that the mechanisms of CCW and CW signaling may differ in important respects.

According to the phosphorelay model of chemotactic signaling, chemoreceptors of the MCP family control flagellar rotation by modulating the phosphorylation state of CheY. The default rotational state of the flagellar motors is CCW. Interaction of phospho-CheY with the FliG/FliM switch proteins causes CW rotation. CheY acquires its phosphates from CheA, whereas the CheZ protein opposes CheA activity by accelerating the rate of CheY dephosphorylation (12). The autokinase activity of CheA is in turn regulated through direct interactions with MCPs and CheW (6). Thus, CW signaling reflects stimulation of CheA activity, CCW signaling reflects inhibition of CheA. Do the Tsr fragments signal by the same mechanism? To explore this question *in vivo*, we tested the ability of `Tsr´-CW and `Tsr´-CCW to influence flagellar rotation in cells lacking various components of the MCP-dependent signaling pathway (Fig. 4).

Strains deleted for all MCP receptors are extremely CCW-biased due to their inability to stimulate CheA autokinase activity and to the CheY dephosphorylating action of CheZ. In such a strain, pPA58 (`Tsr´-CCW) had no effect on rotation pattern, even when fully induced (Fig. 4, line 1). However, induction of pPA56 (`Tsr´-CW) caused a considerable CW shift (Fig. 4, line 1). This demonstrates that the `Tsr´-CW fragment does not require intact MCP molecules to generate signals, and that it can act directly on the soluble signaling components. A similar result was obtained in an MCP-less strain that was also deleted for CheR and CheB, the MCP methyltransferase and methylesterase involved in sensory adaptation (data not shown). If the signal output of `Tsr´-CW were subject to attenuation by the adaptation enzymes, we might have seen a more pronounced CW bias in their absence. The K1 methylation region is present in these fragments, but neither was significantly methylated in wild-type cells, indicating that they are poor substrates for the methylating enzyme or good substrates for the demethylating enzyme (data not shown).

Strains lacking both MCPs and CheZ exhibit intermediate levels of CW rotation that reflect the unstimulated, unopposed autophosphorylation activity of CheA. Upon induction in such a strain pPA56 imposed a more CW bias, whereas pPA58 imposed a more CCW bias (Fig. 4, line 2). This demonstrates that both `Tsr´-CW and `Tsr´-CCW can generate signals in the absence of MCPs and CheZ. However, their behavior was very different in a strain deleted for CheW in addition to the MCPs and CheZ (Fig. 4, line 3). Upon induction, both plasmids shifted the rotation pattern toward the CCW extreme, demonstrating that the CW-signaling Tsr fragment requires CheW for its activity, while the CCW-signaling fragment does not. Without CheW, `Tsr´-CW seemed to have moderate CCW signaling activity.

Strains lacking CheA or CheY are completely CCW-biased, and did not respond to expression of either receptor fragment (data not shown). These findings support the idea that `Tsr´-CW signaling is mediated through CheA-dependent phosphorylation of CheY. To determine if `Tsr´-CCW signaling might use the same pathway, we asked whether pPA58 could impose a CCW rotation pattern on a flagellar switch mutant ($FliM^{CW}$) that was capable of CheY-independent CW rotation. In the absence of CheY, neither pPA58 nor pPA56 had any effect on $FliM^{CW}$ rotational behavior (Fig. 4, line 4), implying that both signaling pathways operate through CheY, and presumably through CheA as well.

In summary, these *in vivo* studies suggest that Tsr fragments use the same signaling pathway and mechanisms as native chemoreceptors. If so, then `Tsr´-CW should show CheW-dependent stimulation of CheA autophosphorylation activity, and `Tsr´-CCW should show CheW-independent inhibition of CheA autophosphorylation. We tested these predictions with *in vitro* assays.

Signaling-Related Activities of Tsr Fragments *In Vitro*. The purified `Tsr´-CW fragment was tested for its effects on CheA autophosphorylation activity with an assay similar to one used to study intact receptors in membrane vesicles (6). Because receptors can accelerate CheA autophosphorylation by 100-fold or more, it is expedient to measure stimulated rates by following the overall transfer of CheA phosphate groups to CheY. When excess CheY is present, autophosphorylation of CheA is the rate-limiting step in CheY phosphorylation. Thus, the initial appearance of phosphate in CheY, before dephosphorylation becomes appreciable, provides an accurate measure of the rate of CheA autophosphorylation.

Under these assay conditions, `Tsr´-CW stimulated CheY-phosphate production approximately 100-fold (Fig. 5). Stimulation was negligible at low fragment concentrations, but then rose rapidly, attaining a maximum at 160 μM. The sigmoidal response to increasing fragment concentration indicates cooperative stimulation of CheA by `Tsr´-CW, implying that more than one fragment molecule participates in the activation process. Perhaps two or more fragment molecules are needed to form the active complex because CheA functions as a dimer (9, 33, 35). It seems unlikely that many more than two fragments are required because native receptors also function as dimers. Instead, the high stoichiometries needed for maximal stimulation (16-fold molar excess of fragment to CheA and CheW) may reflect relatively weak binding between `Tsr´-CW and other components of the activated complex. Consistent with this view, we found that even at optimal stoichiometries preincubation of the reaction mixture for up to 90 minutes greatly enhanced the stimulatory effect, implying that assembly of active complexes is relatively slow (data not shown).

Stimulation of CheA by `Tsr´-CW proved to be CheW-dependent, as was CW signaling by the fragment *in vivo* (Fig. 6). At optimal fragment levels, an equimolar ratio of CheW to CheA produced maximal stimulation. With limiting or excess CheW, stimulation dropped off sharply, and was negligible at 1:10 and 10:1 stoichiometries (Fig. 6). A similar decline in CheA stimulation at high CheW levels also occurs in reactions using intact receptors, and has been ascribed to saturation of CheW sites on both CheA and receptor molecules (10). We infer from these results that the `Tsr´-CW fragment contains a CheW binding site needed for stimulatory coupling to CheA.

The purified `Tsr´-CCW fragment evinced signaling activities in several assays. In the complete reaction system (CheW/CheA/CheY), `Tsr´-CCW was a potent inhibitor of CheA stimulation by `Tsr´-CW (Fig. 7A). Substantial inhibition occurred even when the CW fragment was in molar excess, consistent with the stronger signaling activity of the CCW fragment observed *in vivo*. These results indicate that `Tsr´-CCW may have a higher affinity than `Tsr´-CW for CheA or CheW. In addition, inhibition might require a lower fragment

stoichiometry. For example, binding of one `Tsr´-CCW molecule could be sufficient to inactivate a stimulated CheA complex.

The `Tsr´-CCW fragment was also tested for direct, CheW-independent inhibition of CheA, as suggested by its *in vivo* behavior. After mixing `Tsr´-CCW and CheA, the autophosphorylation rate was measured by monitoring the initial transfer of phosphate label from ATP to CheA. The unstimulated rate is slow enough to measure accurately in this manner. `Tsr´-CCW inhibited the initial autophosphorylation rate of CheA (Fig. 7B). At a 16-fold molar excess of fragment, the CheA autophosphorylation rate was reduced to about 20% of normal, but even at stoichiometries of 2:1, the rate was only 50% of normal. The strength of this inhibitory effect suggests that the `Tsr´-CCW fragment might bind directly to CheA with fairly high affinity.

We also tested `Tsr´-CCW for two other activities that could conceivably contribute to its CCW signaling properties *in vivo*. The fragment had no effect on the stability of phosphorylated CheA, nor did it influence the rate of phosphotransfer from phospho-CheA to CheY (data not shown). Thus, the principal effect of this fragment is to inhibit the autophosphorylation of CheA.

DISCUSSION

A Two-State Model of MCP Signaling. The Tsr fragments characterized in this study promise to provide new insight into the workings of bacterial chemoreceptors. In summarizing their signaling properties, we will interpret their activities in terms of a two-state model of MCP signaling, previously proposed to account for Tsr mutants with locked or biased signaling behavior (1, 3). According to the model, MCP molecules can exist in either of two signaling states, which respectively augment CCW and CW flagellar rotation (Fig. 8). The proportion of molecules in each output state determines the overall swimming behavior of the cell. The CCW/CW distribution is modulated by changes in chemoeffector occupancy (excitation) and by changes in MCP methylation (adaptation). For example, binding of an attractant ligand shifts MCPs to the CCW signaling mode to initiate a smooth swimming response. A subsequent increase in MCP methylation shifts a fraction of the receptors to the CW signaling mode to restore pre-stimulus behavior. MCPs appear to control flagellar rotation by regulating the CheA-dependent transfer of phosphate groups to CheY. Receptor molecules in the CW signaling mode stimulate CheA autophosphorylation, whereas those in the CCW state inhibit CheA.

The Signal-Generating Region of Tsr. Tsr fragments containing residues 290-470 of the cytoplasmic domain were capable of generating both types of flagellar signals. The 290-470 fragment of wild-type Tsr produced a CW signal. The same fragment with a single amino acid replacement (AV413) produced a CCW signal. These fragments encompass a highly conserved core sequence (residues 375-425) that probably specifies the sites of interaction with CheA and CheW. The methylation sites that border both ends of the core region in intact receptors are evidently not essential for either signaling activity. $Tsr_{290\text{-}470}$ fragments lack the C-terminal R1 methylation segment, and although they contain the N-terminal K1 segment, the fragments do not appear to be methylated at those sites. Moreover, their signaling efficiency is unaltered in hosts deleted for the CheR and CheB enzymes. Most importantly, Tsr fragments lacking both

methylation segments still exhibit signaling activity. $Tsr_{354-470}$ generates a CCW signal (this work), and similar fragments with just a few additional residues at the N-terminus can generate a CW signal (36). We conclude that all determinants essential for CCW and CW signaling are located within or near the core region of the Tsr signaling domain.

CW Signaling. At normal intracellular concentrations, CheA and MCP molecules form ternary complexes with CheW. Such complexes are relatively long-lived *in vitro* (10), and probably *in vivo* as well (20), suggesting that receptor-mediated control of CheA activity involves conformational changes in pre-existing ternary complexes rather than formation or disruption of such complexes (10). In the CW signaling mode, the receptor and CheW might serve to position CheA in a highly active conformation. Alternatively, one or both of these components might contribute directly to catalysis of CheA phosphorylation. Although the mechanistic details are still unclear, intact receptors and the CW-signaling Tsr fragment seem to stimulate CheA in the same manner.

$Tsr_{290-470}$ imparted a pronounced CW rotational bias in cells with a functional phosphorelay system. `Tsr´-CW signaling was not dependent on other MCPs or on CheZ, suggesting that its *in vivo* target is CheA or CheY, both of which are essential for CW flagellar rotation. The receptor coupling factor, CheW, was also required, implying that this Tsr fragment generates a CW signal by forming ternary complexes with CheW and CheA. Indeed, in *in vitro* assays `Tsr´-CW stimulated the autophosphorylation rate of CheA in a CheW-dependent fashion. High levels of CheW reduced the stimulatory effect, indicating that `Tsr´-CW, like full-length MCPs, contains a saturable CheW-binding site involved in the control of CheA. Because CheA also binds directly to CheW (9), an excess of CheW molecules could uncouple CheA from receptor control by blocking ternary complex formation. The slow timecourse of CheA activation by `Tsr´-CW and the high fragment concentrations needed for maximal stimulation indicate that the binding of CheW and CheA to `Tsr´-CW is considerably weaker than to native receptors. Nevertheless, this Tsr fragment stimulates CheA as well as a full-length Tsr molecule, and in both cases the CheW coupling factor plays an essential role in that control. Thus, the `Tsr´-CW fragment could provide a useful model for molecular investigations of the CW signaling process. In particular, its solubility and relatively small size should facilitate structural studies of the stimulatory complex.

CCW Signaling. Upon binding of an attractant ligand to ternary CheA/CheW/receptor complexes, CheA assumes a “sequestered” state in which autophosphorylation is blocked (6). Mutant receptors locked in the CCW signaling mode produce similar, but ligand-independent, effects on CheA activity (2, 6). Thus, CCW signaling involves a conformational change in the MCP/CheW/CheA complex that prevents CheA autophosphorylation (Fig. 8). The receptor or CheW might simply hold CheA in an inactive conformation, or might interfere more directly with the autophosphorylation reaction, for example, by concealing CheA’s substrate site or catalytic center. Given the stability of terrnary complexes and the inability to detect direct binding interactions between CCW-signaling receptors and CheA, Gegner et al. (10) concluded that CheW was essential for inhibitory coupling control. The signaling properties of the `Tsr´-CCW fragment demonstrate that this is not the case.

`Tsr´-CCW ($Tsr_{290-470}$ with the AV413 replacement) blocked CW flagellar rotation *in vivo* and inhibited CheA autophosphorylation activity *in vitro*. Both signaling effects occurred equally well in the presence or absence of CheW, demonstrating that `Tsr´-CCW interacts directly with CheA to block its enzymatic activity. Inhibition presumably occurs through low-affinity binding, because high levels of fragment were required for maximal effect. The inability of CheW to enhance this inhibitory effect indicates either that `Tsr´-CCW cannot form ternary complexes, or that ternary complex formation neither stabilizes the inhibited configuration nor provides additional inhibitory contacts to CheA. Assuming that native MCPs and the `Tsr´-CCW fragment utilize the same mechanism of CheA control, we conclude that CheW plays no direct role in the inhibitory coupling process.

Regulation of Tsr Signal Output. Our current view of MCP signaling (Fig. 8) holds that all receptor-mediated control of CheA activity occurs through conformational changes in MCP/CheW/CheA complexes. In the CCW output mode the receptor signaling domain makes direct contact with CheA, inhibiting its autophosphorylation. When the receptor shifts to the CW conformation, the inhibitory contact is broken. The receptor signaling domain, and possibly CheW, then interact with CheA to stimulate its autophosphorylation activity. Thus, the receptor signaling domain contains three distinct sets of output determinants: binding sites for inhibiting CheA; allosteric or catalytic sites for stimulating CheA; and CheW contact sites needed for ternary complex formation. All of these determinants are evidently specified by residues 290-470 of Tsr, because this segment of the molecule is capable of generating both CCW and CW output signals. In addition, the signaling domain may contain structural features, a conformational hinge, for example, that play important mechanical roles in the transitions between signaling states. Amino acid changes, such as the AV413 mutation used in the present study, could conceivably bias or lock the receptor's signal output by altering any of these signaling domain elements.

Other regions of the receptor, particularly the periplasmic sensing domain and the cytoplasmic methylation sites, must control the output activity of the signaling domain by regulating transitions between its CCW and CW signaling conformations. When the wild-type signaling domain of Tsr is freed of these controls, it assumes the CW signaling mode, which could represent a conformationally relaxed state. Removal of the Tsr sensing domain yields maximal CW activity from the liberated cytoplasmic domain, indicating that the ligand-binding portion of the receptor is instrumental in promoting transitions to the CCW conformation. The situation may be somewhat different in Tar, the aspartate receptor, because its liberated cytoplasmic domain did not exhibit CW signaling activity (26). However, the Tar fragment was evidently studied at lower expression levels than our Tsr fragments, which could account for the behavioral difference. We suggest that other MCPs most likely use output control mechanisms like those in Tsr. If so, structural studies of CW- and CCW-signaling Tsr fragments should serve to elucidate the conformational differences between MCP signaling states and the principles of output control in bacterial chemoreceptors.

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Fig. 1. Strategy for constructing Tsr fragment plasmids. (A) Domain organization of the Tsr protein. Numbers indicate amino acid positions of restriction site landmarks in the *tsr* coding sequence. The indicated *tsr* coding segments were subcloned from plasmid pPA420 into the pTM30 expression vector (B) as follows: codons 176-551 in a 1.9 Kb *Pvu*I /S1 (i.e., cut with *Pvu*I then with S1 nuclease to produce a blunt end) - *Hin*dIII fragment ligated to pTM30 cut with *Bam*H1/S1 and *Hin*dIII; codons 259-551 in a 1.6 Kb *Nde*I/S1 - *Hin*dIII fragment ligated to pTM30 cut with *Pst*I /S1 and *Hin*dIII; codons 290-551 in a 1.5 Kb fragment produced by limited *Sau*3a digestion of the 1.6 Kb *Nde*I/S1 - *Hin*dIII fragment and ligated into pTM30 cut with *Bam*HI plus *Hin*dIII; codons 290-470 in a 543 bp *Sau*3a fragment ligated into the *Bam*HI site of pTM30; codons 354-470 in a 350 bp fragment produced by cutting the 543 bp *Sau*3a fragment with *Eco*RV, then ligating it to pTM30 cut with *Bam*HI, treating the linear product with S1 to polish the ends, and then ligating the blunt ends.

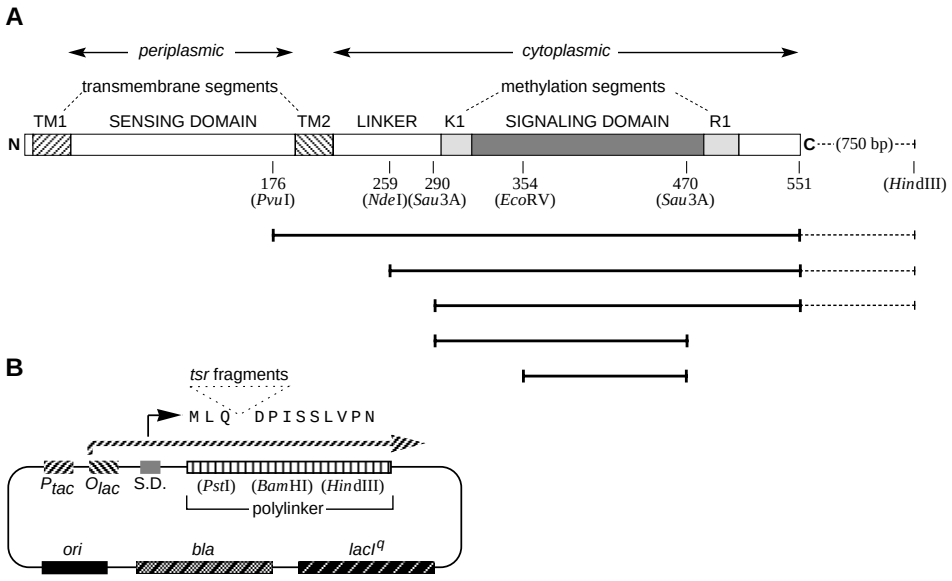
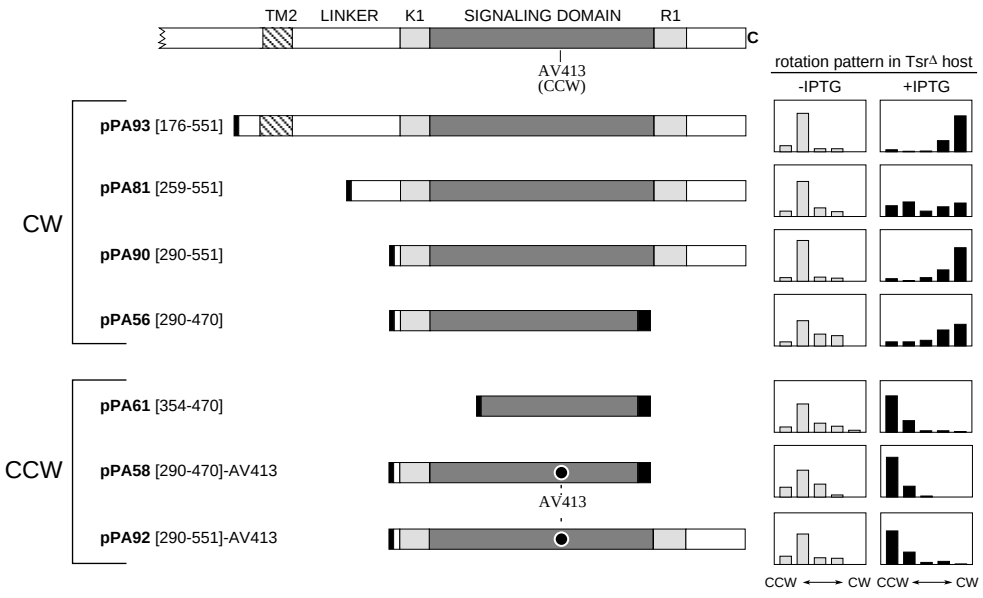


Fig. 2. Inducible signaling activities of Tsr fragment plasmids. Plasmids carrying the indicated *tsr* coding segments were transformed into RP5700 to evaluate their effects on flagellar rotation pattern. Cells were tethered to microscope cover slips with flagellar antibody and classified into one of five rotational categories. The histograms show the relative proportion of rotating cells in each category, listed from left to right: exclusively CCW; predominantly CCW, with occasional reversals; reversing frequently with no evident directional bias; predominantly CW, with occasional reversals; and exclusively CW. When grown with 1 mM IPTG to induce expression of their Tsr fragment, four plasmids (pPA93, pPA81, pPA90, pPA56) shifted rotation in the CW direction, three others (pPA61, pPA58, pPA92) shifted rotation in the CCW direction.



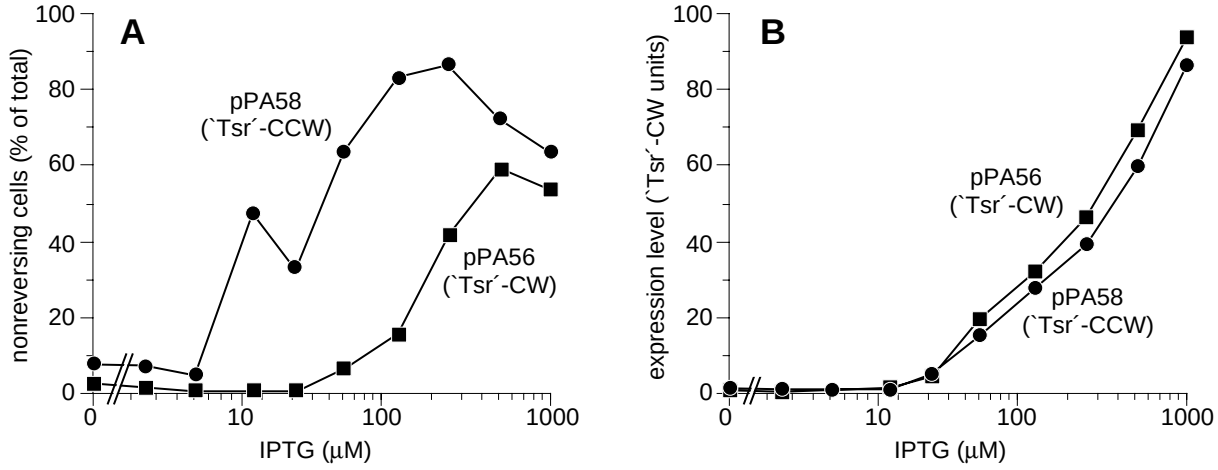
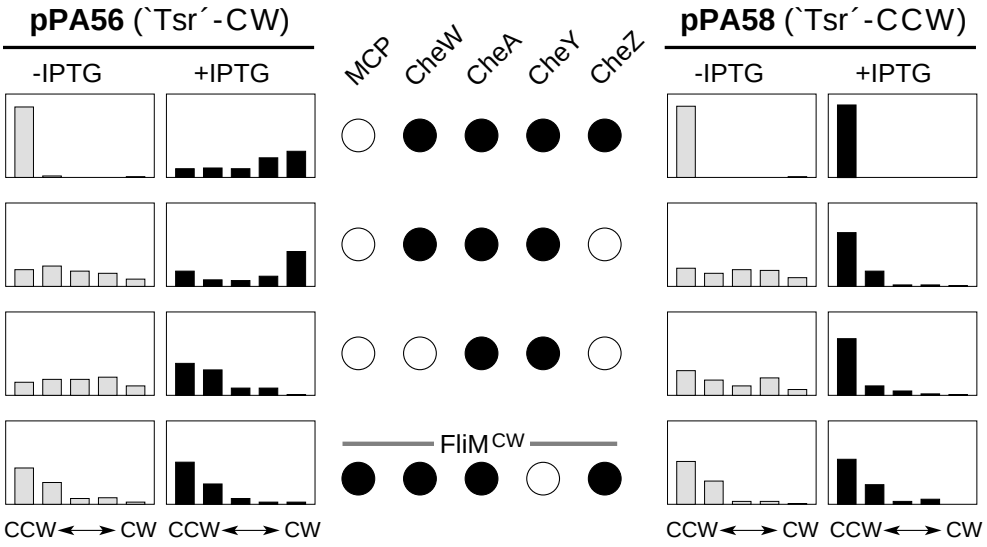


Fig. 3. Induction curves of pPA56 (`Tsr´-CW) and pPA58 (`Tsr´-CCW). Strain RP5700 carrying pPA56 or pPA58 was grown in tryptone broth containing various concentrations of IPTG and examined for flagellar rotation pattern (A) and for intracellular level of Tsr fragment (B). Flagellar rotation was evaluated by antibody tethering. The curves show the percent of rotating cells in the population that were exclusively CCW (pPA58) or CW (pPA56). Fragment expression was measured by immunoblotting, using the plasmid-encoded β-lactamase protein as an internal standard to compensate for any changes in plasmid copy number under different induction conditions. Fragment expression values are defined relative to the uninduced level of Tsr-CW fragment (set as one unit). Values are the mean of two independent determinations.



. **Fig. 4.** Epistatic analysis of components needed for Tsr fragment signaling. pPA56 and pPA58 were transformed into various host strains and examined for their effects on flagellar rotation pattern with and without IPTG induction. Each histogram presents the rotation profile of a cell population, defined as in Fig. 2. The signaling components present in the host strains are indicated by filled circles, those missing are shown with unfilled circles. The four host strains were (from top to bottom): RP8611 [*Δ(tsr)7028 Δ(tar-tap)5201 Δ(trg)100*] (17); RP9352 [*Δ(tsr)7028 Δ(tar-tap)5201 Δ(trg)100 Δ(cheZ)6725*] (17); RP9411 [*Δ(tsr)7028 Δ(cheW-tap)2217 Δ(trg)100 Δ(cheZ)6725*] (17); RP3199 [*Δ(cheY)6021 scyB10*] (29)

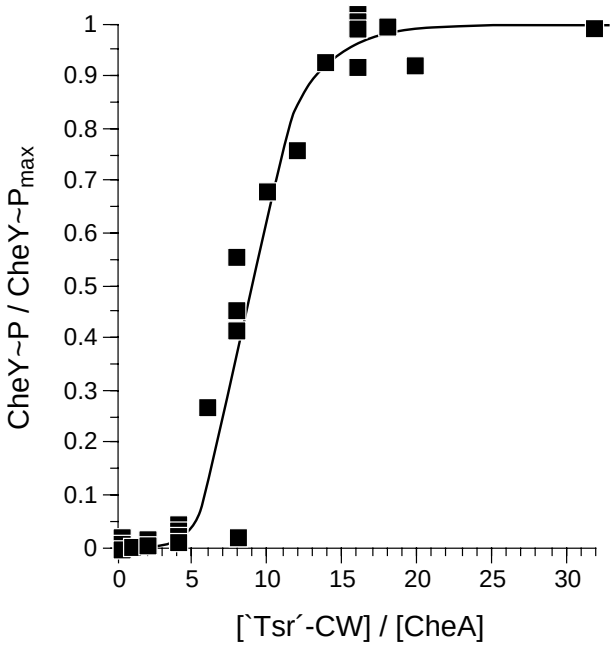


Fig. 5. Stimulation of CheA autophosphorylation by `Tsr´-CW. Reactions contained CheA (10 μM), CheW (10 μM), CheY (250 μM), and different amounts of the `Tsr´-CW fragment. At these stoichiometries, the initial rate of CheY phosphorylation reflects the CheA autophosphorylation rate (see text). Maximal rates of CheY phosphorylation were attained with a 16-fold molar excess of `Tsr´-CW to CheA. Values at other stoichiometries are expressed relative to this maximum. The data points represent values from four different experiments. The line drawn through the points is defined by the Hill equation $y = kx^n / (1 + kx^n)$, with $k = 10^{-5}$ and $n = 5.3$. These parameter values give an R^2 fit to the data points of 0.98

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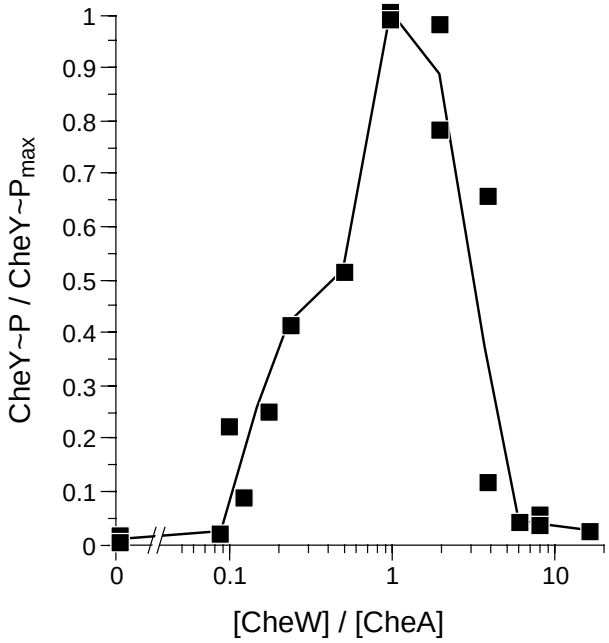

Fig. 6. CheW requirement for stimulation of CheA by `Tsr´-CW. Reactions contained ATP (1 mM), CheA (10 μM), CheY (250 μM), `Tsr´-CW (160 μM) and different amounts of CheW. The maximal rate of CheY phosphorylation was attained at equimolar ratios of CheW to CheA. Values at other stoichiometries are expressed relative to this maximum. The data points represent values from two different experiments. The line connecting the points was drawn manually.

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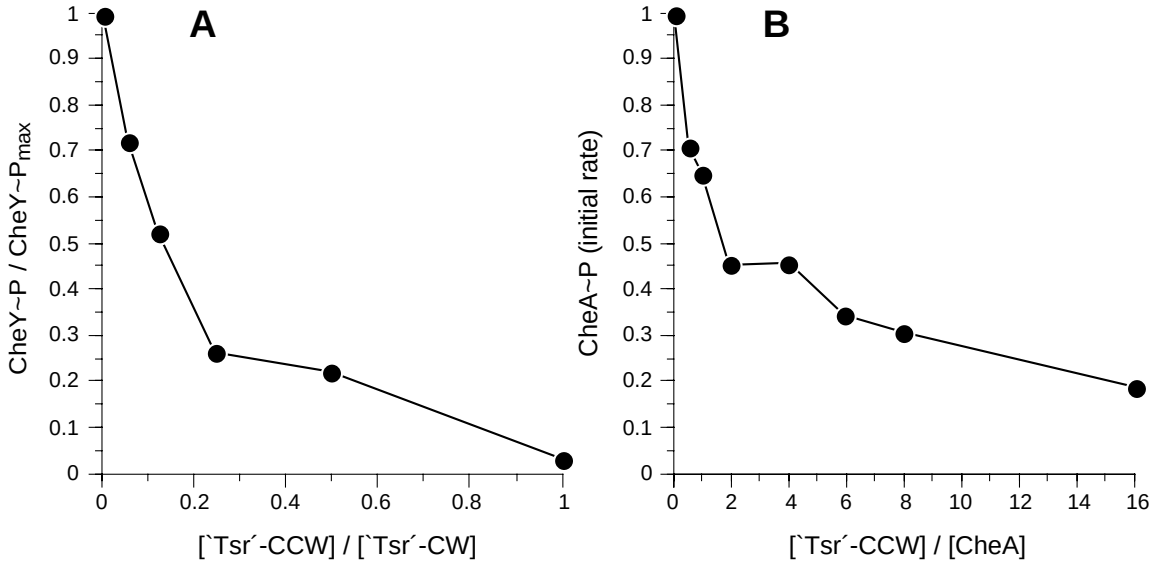

Fig. 7. Inhibitory *in vitro* effects of `Tsr´-CCW. (A) Interference with the stimulatory action of `Tsr´-CW. Reactions contained ATP (1 mM), CheA (10 μM), CheW (10 μM), CheY (250 μM), `Tsr´-CW (160 μM) and different amounts of `Tsr´-CCW. CheA autophosphorylation rate was followed as the initial appearance of phospho-CheY (see text). (B) Inhibition of CheA autophosphorylation. Reactions contained ATP (1 mM), CheA (10 μM) and different amounts of `Tsr´-CCW. CheA autophosphorylation rate was estimated from the first three time points (10, 20, 30 seconds) after addition of [γ-^{32}P]ATP.

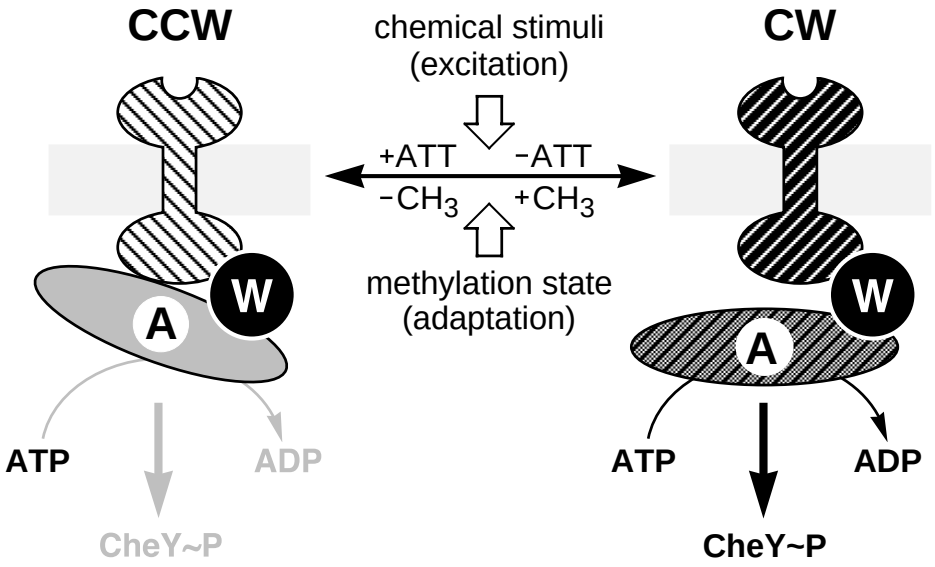

Fig. 8. A two-state model of MCP signaling. The CCW signaling mode inhibits CheA autophosphorylation, the CW mode stimulates CheA activity. Shifts in the distribution of MCP molecules in the CCW and CW states, elicited by changes in ligand occupancy or methylation state, determine the overall rotational behavior of the flagellar motors. See text for additional details.