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Thermosensing Properties of Mutant Aspartate Chemoreceptors with Methyl-Accepting Sites Replaced Singly or Multiply by Alanine

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The aspartate chemoreceptor Tar has a thermosensing function that is modulated by covalent modification of its four methylation sites (Gln295, Glu302, Gln309, and Glu491). Without posttranslational deamidation, Tar has no thermosensing ability. When Gln295 and Gln309 are deamidated to Glu, the unmethylated and heavily methylated forms function as warm and cold sensors, respectively. In this study, we carried out alanine-scanning mutagenesis of the methylation sites. Although alanine substitutions influenced the signaling bias and the methylation level, all of the mutants retained aspartate-sensing function. Those with single substitutions had almost normal thermosensing properties, indicating that substitutions at any particular methylation site do not seriously impair thermosensing function. In the posttranslational modification-defective background, some of the alanine substitutions restored thermosensing ability. Warm sensors were found among mutants retaining two glutamate residues, and cold sensors were found among those with one or no glutamate residue. This result suggests that the negative charge at the methylation sites is one factor that determines thermosensor phenotypes, although the size and shape of the side chain may also be important. The warm, cold, and null thermosensor phenotypes were clearly differentiated, and no intermediate phenotypes were found. Thus, the different thermosensing phenotypes that result from covalent modification of the methylation sites may reflect distinct structural states. Broader implications for the thermosensing mechanism are also discussed.

Escherichia coli responds to small changes in temperature by altering its swimming behavior to migrate in spatial temperature gradients (22). This phenomenon, thermotaxis, is well suited to studies of the molecular mechanism of thermosensing. Four closely related transmembrane proteins have been identified as thermosensors (21, 27, 29). These "thermometer" proteins were originally identified as methyl-accepting chemotaxis proteins (MCPs) and as transducers responsible for chemotaxis (for reviews, see references 23, 32 and 40). In response to a repellent or an attractant, the receptor activates or inactivates the cytoplasmic histidine kinase CheA, which phosphorylates itself and serves as a phosphodonor for the cytoplasmic signaling protein CheY. Phospho-CheY interacts with the flagellar motor, which otherwise rotates counterclockwise (CCW), to cause clockwise (CW) rotation that results in loss of propulsive power and change of swimming direction (tumbling). A warm sensor mediates attractant and repellent responses upon increases and decreases in temperature, respectively, and a cold sensor mediates the opposite responses to the same stimuli (29).

Both in the presence and in the absence of its ligand, the receptors exist as homodimers (25) that form stable complexes with the CheA homodimer and two molecules of the coupling protein CheW (14, 34). The thermosensing mechanism is thought to involve temperature-dependent changes in the structure of the receptor-CheW-CheA ternary complex and/or in the interactions among multiple ternary complexes. Among the components of the ternary complex, the receptor itself is thought to be the primary thermosensor because (i) a receptorless strain never gives a thermotactic response under any condition tested (28) and (ii) each of the four receptors characteristically functions as either a warm sensor (Tsr, Tar, and Trg) or a cold sensor (Tar in the presence of attractants [see below] and Tap) (21, 27, 29).

The chemo/thermoreceptors are reversibly methylated at specific glutamate residues located in the C-terminal cytoplasmic domain (for reviews, see references 23, 32, and 40). This methylation is a key element of the adaptation process in chemo/thermotaxis. Receptor methylation and demethylation are catalyzed by the methyltransferase CheR and the methylesterase CheB. CheB also has a deamidase activity that converts glutamine residues at methylation sites to glutamate residues. Tar has four methylation sites, Gln295, Glu302, Gln309, and Glu491. Throughout this report, these individual residues will be referred to as sites 1, 2, 3, and 4, respectively, or collectively as QEQE. Modified sets of methylation sites will be described in a similar fashion. Sites 1, 2, and 3 are located on the same face of a putative α-helix (the first methylation helix [MH1]), and site 4 is on a second helix (MH2) (Fig. 1A).

Tar is a warm sensor in the absence of aspartate, but it is converted to a cold sensor after adapting to aspartate or maltose (27). We have recently reported that covalent modification of the methylation sites influences the thermosensing function (28) (Fig. 1B). The unmodified form of Tar (QEQE) mediates a chemoresponse but not a thermoresponse. It ac-

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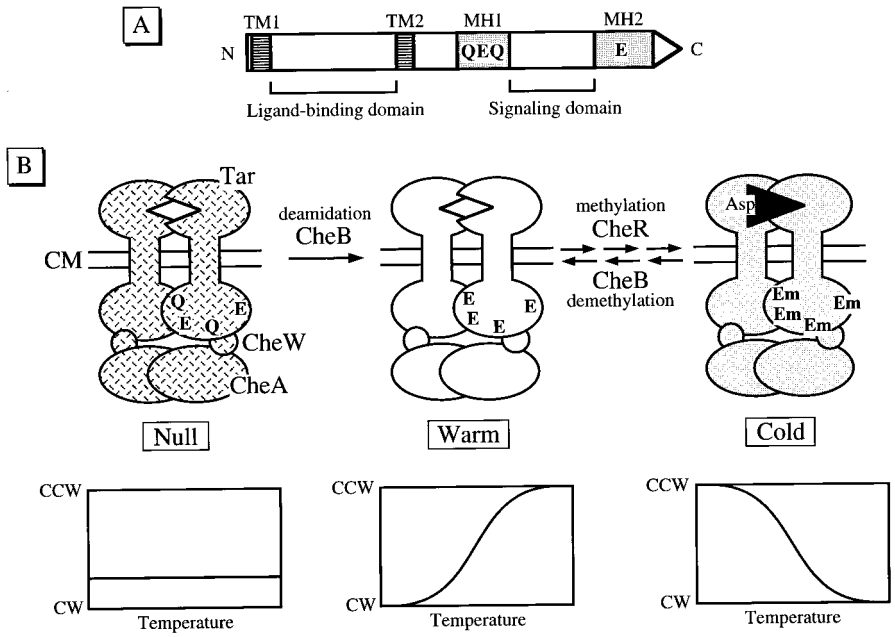

FIG. 1. Effects of covalent modification at methyl-accepting residues of Tar on its thermosensing function. (A) The Tar polypeptide. Two predicted α-helical regions containing methylation sites are indicated. MH1 and MH2, the first and second methylation helices; Q and E, methylation sites; N and C, the amino and carboxy termini of Tar; TM1 and TM2, the first and second transmembrane segments. (B) Schematic illustration of the three distinct thermosensor forms of Tar. Tar forms a homodimer that interacts stably with CheW and CheA. Equilibrium between the two signaling states of a null thermosensor should be unaffected by temperature. By contrast, increases in temperature should shift that of a warm sensor toward CheA inactivating (i.e., CCW rotation of flagella; smooth swimming) and that of a cold sensor toward CheA activating (i.e., CW rotation of flagella; tumbling). CM, cytoplasmic membrane; Q, glutamine residue; E, glutamate residue; Em, methylated glutamate residue; Null, null thermosensor form; Warm, warm-sensor form; Cold, cold-sensor form; Asp, aspartate.

quires thermosensing ability when it is deamidated by CheB: the unmethylated form (Tar-EEEE) functions as a warm sensor, and the heavily methylated form (represented by the fully methylated form Tar-EmEmEmEm, Em standing for a methylated glutamate residue) functions as a cold sensor. Therefore, Tar appears to exist in three distinct states, in which it functions as a warm, cold, or null thermosensor.

To investigate this modulation of thermosensing function further, we have constructed and characterized a series of mutant Tar proteins whose methylation sites are substituted with alanine singly or multiply in all possible combinations. In the CheB$^-$ CheR$^-$ background, warm sensors were found among mutants retaining two glutamate residues and cold sensors among those with one or no glutamate residue, suggesting that the negative charge at the methylation sites is one factor that determines thermosensor phenotypes, although the size and shape of the side chain may also be important.

MATERIALS AND METHODS

Bacterial strains. *E. coli* HCB339 [Δ*tsr-7021* Δ(*tar-tap*)*5201 trg*::Tn*10 thr leu his met rpsL136*] (45) and CP553 [Δ*trg-100 zab*::Tn*5* Δ(*tar-cheB*)*2234* Δ*tsr-7028*] (4), which lack four chemoreceptors (except Aer), were used as plasmid hosts.

Plasmids. Plasmid pAK101 (17) carries wild-type *tar*. Plasmids pRA111 to pRA126, constructed in this study from pAK101, contain mutant *tar* genes encoding receptors with altered methyl-accepting sites (Table 1). The following nucleotide substitutions were introduced by site-directed mutagenesis and verified by DNA sequencing: the codon for residue 295 (methylation site 1) of Tar was changed from CAG (Gln) to GCG (Ala); residue 302 (site 2) was changed from GAA (Glu) to GCA (Ala); residue 309 (site 3) was changed from CAG (Gln) to GCG (Ala); and residue 491 (site 4) was changed from GAA (Glu) to GCA (Ala).

Site-directed mutagenesis. Site-directed mutagenesis was performed by the method of Kunkel et al. (18). Oligonucleotides for mutagenesis and DNA sequencing were synthesized at the Center for Gene Research at Nagoya University.

Measurement of chemoresponse. Temporal stimulation assays were carried out essentially as described previously (42). Cells were grown at 30°C with vigorous shaking in tryptone-glycerol medium (1% Bacto Tryptone [Difco Laboratories, Detroit, Mich.], 0.5% NaCl, 0.5% glycerol) supplemented with ampicillin (50 μg/ml). After 4 h of cultivation, cells were harvested by centrifugation at room temperature and washed with motility medium consisting of 10 mM

TABLE 1. Thermoresponses mediated by the mutant receptors without modification

Plasmid[a]	Methyl-accepting site	Aspartate response (M)[b]	Thermoresponse[c]	
			No addition	+Aspartate[d]
pAK101	QEQE	10^{-6}	–	–
pRA111	AEQE	10^{-7}	Warm	–
pRA112	QAQE	10^{-6}	–	Cold
pRA113	QEAE	10^{-6}	–	–
pRA114	QEQA	10^{-5}	–	Cold
pRA121	AAQE	10^{-6}	–	–
pRA122	AEAE	10^{-7}	Warm	–
pRA123	AEQA	10^{-5}	–	–
pRA124	QAAE	10^{-6}	–	–
pRA125	QAQA	10^{-5}	–	Cold
pRA120	QEAA	10^{-6}	–	–
pRA119	AAAE	10^{-6}	–	–
pRA118	AAQA	10^{-5}	–	Cold
pRA117	AEAA	10^{-5}	–	–
pRA116	QAAA	10^{-6}	–	–
pRA126	AAAA	10^{-5}	–	Cold

[a] Each plasmid was introduced into strain CP553 (MCP$^-$ CheB$^-$ CheR$^-$). All cells harboring these plasmids showed a tumbly swimming pattern, as assayed at room temperature in motility medium without any chemoeffector.

[b] Concentration of aspartate giving a half-maximal chemoresponse.

[c] –, no thermoresponse; Warm, warm-sensor response; Cold, cold-sensor response.

[d] Aspartate was added at the concentrations given in the aspartate response column.

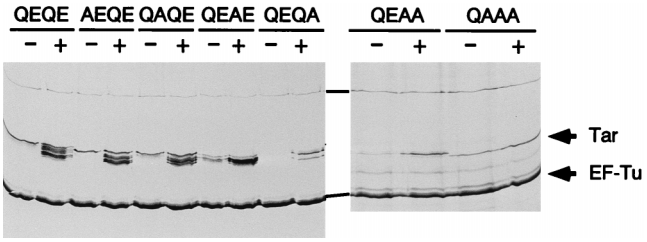

FIG. 2. In vivo methylation patterns of the wild-type and mutant Tar proteins in the presence or absence of aspartate. HCB339 ($CheB^+$ $CheR^+$) cells expressing wild-type or mutant Tar (indicated with their methylation sites before modification [QEQE, QEAE, etc.]) were incubated with 3.3 μM [*methyl*-^{3}H]methionine at 30°C for 30 min. H_2O (indicated with −) or aspartate (final concentration, 10 mM; indicated with +) was then added to the samples, which were incubated for a further 15 min. Note that the glutamine residues at sites 1 and 3 are converted into glutamate residues by CheB amidase activity in the host strain.

potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM sodium DL-lactate (pH 7.0), and 0.1 mM methionine. Cells were resuspended in motility medium and kept at room temperature. Immediately after addition of an attractant or a repellent to the cell suspension, the swimming pattern of the cells was observed with a dark-field microscope and recorded on videotape. In an image integrated for 1 s by using an Argus-10 image processor (Hamamatsu Photonics, K.K., Hamamatsu, Japan), smooth-swimming and tumbling cells gave line and blurred dot traces, respectively. The smooth-swimming fraction was defined as the percent fraction of smooth-swimming cells per total swimming cells that does not include cells attached to glass surface. The threshold concentrations of chemicals for tactic responses were estimated from their dose-response curves. Synthetic sodium L-aspartate, obtained from ICN Pharmaceuticals, Inc. (Plainview, N.Y.), was used for measurements of the chemoresponse.

Measurement of thermoresponse. Thermoresponses were assayed as described previously (28). A cell suspension was prepared as described above. If necessary, an appropriate concentration of the attractant aspartate or the repellent glycerol was added just before observation. A drop of the suspension was placed on a glass slide mounted on a temperature control apparatus (21). The temperature was changed from about 16°C to 27°C and returned to 16°C. Temperature changes were monitored by a Constantan-Chromel thermocouple inserted into the suspension. Changes in the swimming pattern of the cells were measured quantitatively as described above.

Analysis of methylation pattern. Methylation of Tar was measured essentially as described previously (42). In brief, cells in MLC medium (10 mM potassium phosphate buffer [pH 7.0], 0.1 mM EDTA, 10 mM DL-lactate, 0.2 mg of chloramphenicol per ml) were incubated at 30°C with vigorous shaking. After 30 min, 3.3 μM [*methyl*-^{3}H]methionine (12 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) was added to the cells, which were further shaken for 30 min. To enhance the methylation of Tar, aspartate was added to a final concentration of 10 mM, and the cells were shaken for 15 min. The reaction was terminated by adding trichloroacetic acid (final concentration, 5%). Methyl-labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography.

RESULTS

Methylation levels of alanine-substituted mutant Tar. Plasmids carrying the wild-type or mutant *tar* genes (Table 1) were introduced into *E. coli* host strains that are defective for the four chemoreceptor genes *tsr*, *tar*, *trg*, and *tap*. These strains themselves do not respond to temperature changes, although they have the recently identified *aer* gene, coding for the related receptor responsible for aerotaxis (2, 33). Thus, the only thermoreceptors present are encoded by the plasmid-borne *tar* genes. To make sure that all mutant proteins were expressed, we examined whole-cell extracts by Western blotting using antiserum raised against Tsr-T156C (16), which cross-reacts with Tar (data not shown). All mutant Tar receptors were present at levels similar to that of wild-type Tar.

Plasmid pAK101 (wild-type *tar*) and its mutant derivatives were introduced into a receptorless strain HCB339 ($CheB^+$ $CheR^+$). The resulting transformants were incubated with [*methyl*-^{3}H]methionine in the presence of 10 mM aspartate, and the whole-cell extracts were subjected to SDS-PAGE followed by fluorography (Fig. 2). It should be noted that the more a receptor is methylated, the faster it migrates in a gel (3, 5, 11, 12). As shown in Fig. 2, the mutant Tar proteins gave fewer bands, corresponding to the decrease in the number of glutamate residues available for methylation. All of the mutant proteins behaved similarly (data not shown). Since all of the mutant receptors except Tar-AAAA were methylated in response to aspartate, the substitutions with alanine apparently do not severely affect the chemotactic signaling functions of Tar, since methylation of the receptor is a consequence of signal production. The methylation patterns of the four mutant Tar proteins with single substitutions were different from each other (Fig. 2), indicating that the methyl-accepting abilities of the four residues are different, as had been demonstrated previously for Tar (37–39, 44) and for Trg (30).

Swarming ability of strains expressing mutant Tar proteins. To assess their functions as chemosensors, we examined the abilities of the mutant Tar proteins to support swarming of colonies in tryptone semisolid agar (Fig. 3). HCB339 cells expressing any mutant Tar spread slower than those expressing wild-type Tar (QEQE) but faster than those without any chemoreceptor. Colonies of cells expressing plasmid-encoded Tar-AEQE, -QAQE, -QEAE, -QEQA, -AEQA, -QAQA, -AAQA, or -AEAA formed clear chemotactic rings, but the ring diameters varied significantly among transformants carrying plasmids with these different *tar* alleles. The other mutant Tar

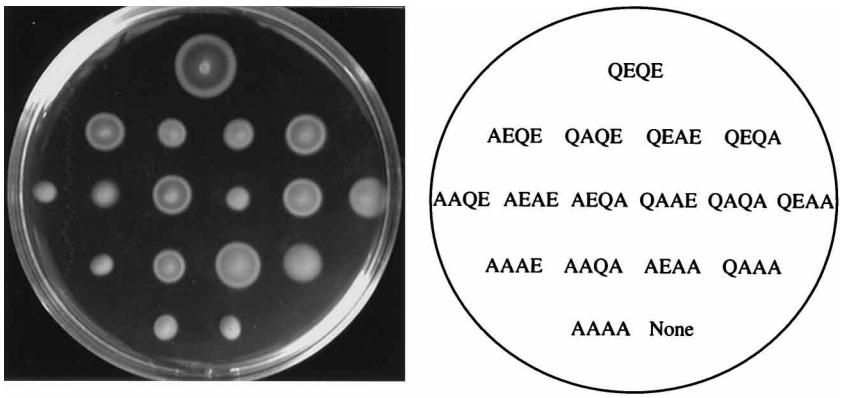

FIG. 3. Swarming behavior of cells expressing each mutant Tar receptor. An aliquot (2 μl) of fresh overnight culture of HCB339 ($CheB^+$ $CheR^+$) cells carrying pAK101, its derivative pRA plasmid, or the vector pBR322 was spotted on a tryptone semisolid agar plate supplemented with ampicillin (50 μg/ml). The plate was incubated at 30°C for 12 h. To the right of the photograph, the mutant receptors are indicated with their methylation site configurations before modification.

proteins seemed not to support good chemotaxis and had substitutions at site 2, site 3, or both: cells with Tar-QEAA or -QAAA spread well but formed only faint chemotactic rings, and cells expressing any other mutant proteins neither spread well nor formed clear chemotactic rings. In particular, all three mutant receptors with substitutions at sites 2 and 3 did not support chemotactic swarming, whereas cells expressing any mutant receptor that retains intact sites 2 and 3 were invariably chemotactic. This result is consistent with the notion that these sites play crucial roles in adaptation, since adaptation is essential for chemotaxis and requires methylation (see Discussion for details).

The swarm diameters did not correspond to receptor methylation levels. For example, Tar-QEQA was methylated much more poorly than Tar-QAQE and -QEAE (Fig. 2), but it supported better spreading. Cells expressing the nonmethylatable Tar-AAAA spread poorly but still significantly better than those expressing Tar-AAQE, -QAAE, or -AAAE. These results suggest that the signaling bias of the receptor is more important for spreading than its ability to be methylated. Such nonchemotactic swarming was found previously among strains deleted for the genes required for chemotaxis, and signaling bias seemed to be critical for the efficiency of such swarming (46). In this regard, site 4 seems different from the other three sites. Introduction of alanine at site 4 of Tar-AAQE, -AEAE, and -QAAE restored spreading, and even chemotaxis for Tar-AEAA and -AAQA, whereas alanine at the other sites had no effect or made chemotaxis worse.

Thermosensing properties of mutant receptors with single substitutions at the methylation sites. To assess the effects of alanine substitutions on thermosensing, we first examined the thermosensing abilities of Tar receptors with single substitutions. Tar-AEQE, -QAQE, -QEAE, and -QEQA were expressed in strain HCB339, and the thermotactic responses of the resulting strains were examined (Fig. 4). Although cells expressing Tar-QEQA showed extremely tumble-biased swimming and did not give a thermoresponse in the absence of aspartate, they produced a cold-sensor response in the presence of 0.1 mM aspartate. The other three strains showed warm- and cold-sensor responses in the absence and presence of aspartate, respectively. These results suggest that replacement of any single methylation site by alanine does not prevent Tar from sensing temperature.

Thermosensing properties of mutant receptors expressed in the modification-defective strain. We next examined thermosensing abilities of the mutant Tar proteins expressed in strain CP553 ($CheB^-$ $CheR^-$). In this host, a receptor protein is neither methylated nor deamidated. In temporal stimulation assays, Tar-QEAE, -AAQE, -AEQA, -QAAE, -QEAA, -AAAE, -AEAA, and -QAAA did not mediate a thermoresponse either in the absence or in the presence of aspartate, though all of them still mediated chemoresponses to aspartate (Table 1). By contrast, Tar-AEQE and -AEAE mediated warm-sensor responses in the absence of aspartate, whereas Tar-QAQE, -QEQA, -QAQA, -AAQA, and -AAAA mediated cold-sensor responses in the presence of aspartate (Table 1). Representative thermoresponses are shown in Fig. 5. Unlike the unmodified wild-type protein (Tar-QEQE), which does not mediate a thermoresponse (Table 1) (28), all three mutant Tar proteins that retain both glutamine residues (sites 1 and 3) functioned as cold sensors. These results demonstrate that suitable combinations of the methylation site modifications are required for thermosensing ability and that even subtle changes in the pattern of modification can prevent Tar from functioning as a thermosensor. It should be noted that we found no mutant Tar protein that could mediate a warm-sensor response under certain conditions and a cold-sensor response under others. Furthermore, we did not find any mutant form which mediated a degenerate thermoresponse. The mutant forms that mediated cold-sensor responses had one or no glutamate residue at the methylation sites (four of five lost glutamate at site 4), whereas those that mediated warm-sensor responses retained two glutamate residues.

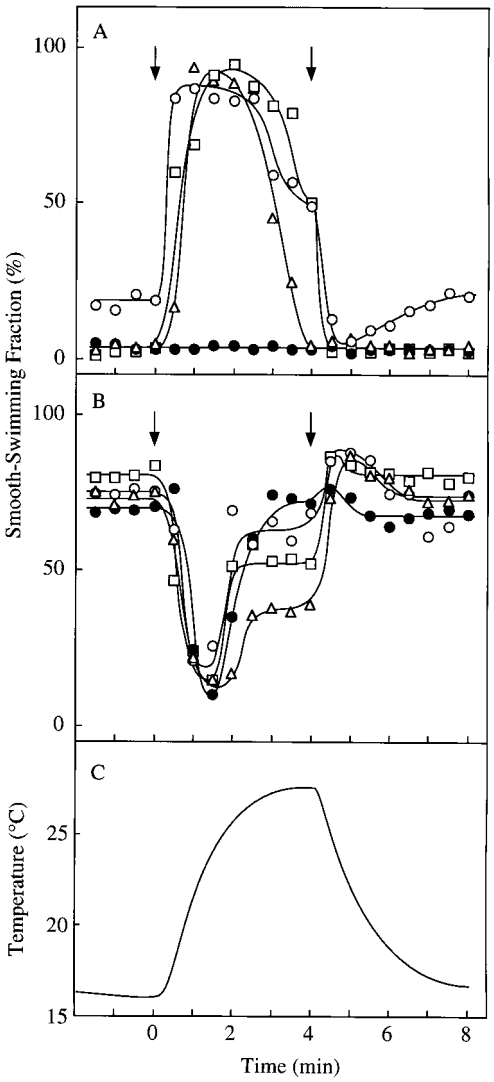

FIG. 4. Thermoresponses of HCB339 ($CheB^+$ $CheR^+$) cells expressing mutant Tar proteins with single alanine substitutions. The smooth-swimming fraction of the cells was measured in the absence (A) or presence (B) of aspartate. Aspartate was added at the concentrations giving about half-maximal chemoresponses (10 μM for Tar-AEQE, QAQE, and QEAE; 0.1 mM for QEQA). Arrows indicate the onset of temperature changes. Temperature (C) was monitored as described in Materials and Methods. Symbols: open circles, Tar-AEQE (pRA111); open squares, Tar-QAQE (pRA112); open triangles, Tar-QEAE (pRA113); closed circles, Tar-QEQA (pRA114). Note that the glutamine residues are converted to glutamate residues in the host strain.

Temperature dependences of signaling by mutant receptors expressed in the modification-defective strain. To characterize differential thermosensing properties further, we examined the steady-state swimming patterns of cells expressing mutant Tar proteins at various temperatures (Fig. 6). The smooth-swimming fraction of CP553 cells expressing Tar-AEQE or -AEAE was measured in the absence of any chemoeffector (Fig. 6A),

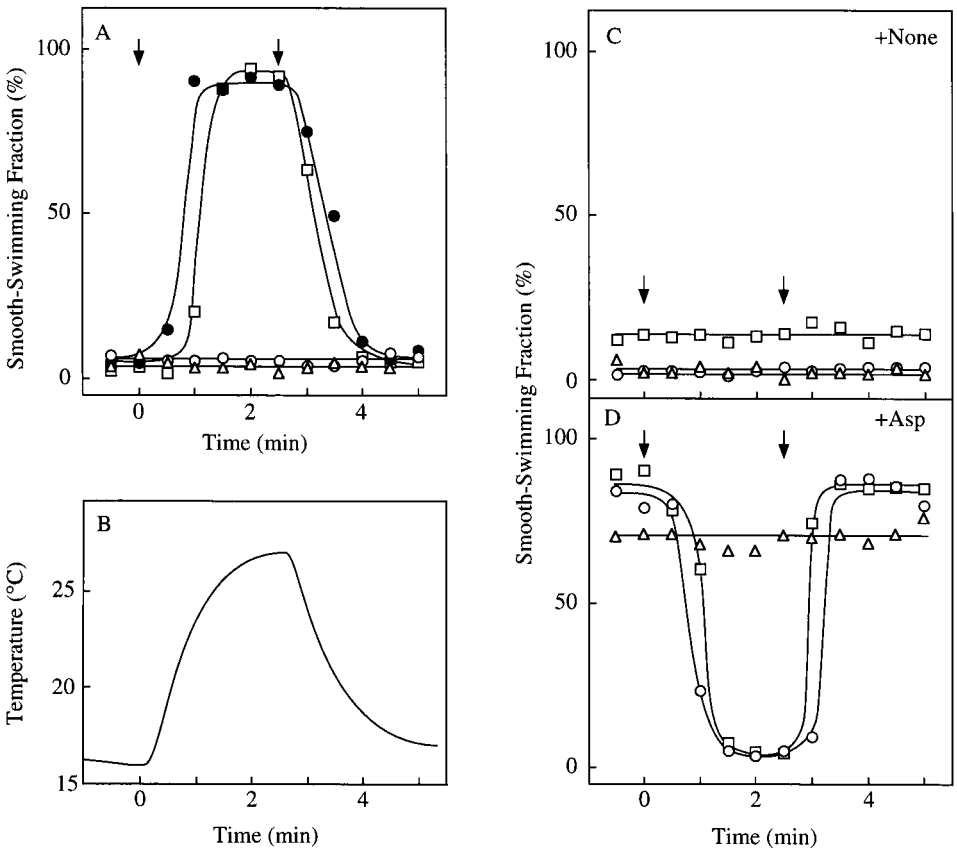

FIG. 5. Thermoresponses mediated by mutant Tar proteins expressed in the CheB$^-$ CheR$^-$ strain CP553. Thermoresponses were measured as described in the legend to Fig. 4. (A) Thermoresponses mediated by mutant proteins with two methyl-accepting glutamate residues in the absence of aspartate. Symbols: open circles, Tar-QEQE (pAK101); open squares, Tar-AEQE (pRA111); open triangles, Tar-QEAE (pRA113); closed circles, Tar-AEAE (pRA122). (B) Time course of the temperature change. (C and D) Thermoresponses mediated by mutant proteins with one or no glutamate residue in the absence (C) or presence (D) of 10 μM aspartate. Symbols: circles, Tar-QEQA (pRA114); squares, Tar-AAAA (pRA116); triangles, Tar-AAQE (pRA121).

and that of CP553 cells expressing Tar-QEQA or -AAAA was measured in the presence of 10 μM aspartate (Fig. 6B). As the incubation temperature increased, cells expressing Tar-AEQE or -AEAE showed a more smooth-biased swimming (Fig. 6A), and cells expressing Tar-QEQA or -AAAA showed a more tumble-biased swimming (Fig. 6B). These characteristics are consistent with the thermoresponses detected in temporal stimulation assays with these strains (Table 1).

DISCUSSION

In this study, we constructed and characterized a series of mutant Tar receptors whose four methyl-accepting sites are partly or fully substituted with alanine. All of the mutant Tar receptors mediated chemotactic responses to aspartate in the temporal assay, suggesting that substitutions with alanine do not per se disturb the function of Tar as a chemosensor. Still, each substitution can affect the methylation state and signaling bias during chemotaxis, and as a result, the abilities of the mutant receptors to support swarming in semisolid agar varied substantially. Since chemotaxis requires both excitation and adaptation, impaired methylation in a mutant receptor is expected to affect chemotaxis. Cells expressing any of the three mutant proteins possessing alanine substitutions at sites 2 and 3 were nonchemotactic, whereas those expressing any of the four mutant proteins retaining intact sites 2 and 3 were chemotactic, suggesting that these sites play crucial roles in adaptation. This conclusion is consistent with the following published results. Methylation and demethylation rates at sites 2 and 3 are much greater than those at the other two (38, 39, 44). Furthermore, an aspartate substitution at either site 2 or 3 decreases the overall methylation rate more than a double mutation at sites 1 and 4 does (37, 38). However, it should be noted that an alanine substitution at site 2 or 3 is not sufficient for the inhibition of chemotaxis. This might be because an alanine residue mimics a methylated glutamic acid residue (30). In contrast to these inhibitory effects of substitution at sites 2 and 3, the present study shows that an alanine substitution at site 4 can significantly increase colony spreading in agar plates, and in some cases, chemotactic swarming is enhanced as well. These effects of mutations at site 4 could be due to its location on MH2 rather than MH1.

As observed for chemotaxis, all of the mutant Tar proteins with a single alanine substitution retained nearly normal thermosensing properties in the CheB$^+$ CheR$^+$ background, demonstrating that these point substitutions also do not impair thermosensing function. When expressed in the CheB$^-$ CheR$^-$ background, roughly half of the mutant proteins did not function as thermosensors, but the others restored thermosensing function: some functioned as warm sensors, and the others functioned as cold sensors. These warm, cold, and null thermosensor phenotypes were discrete classes; i.e., we did not find any intermediate phenotypes. The null thermosensor forms did not mediate thermoresponses under any conditions

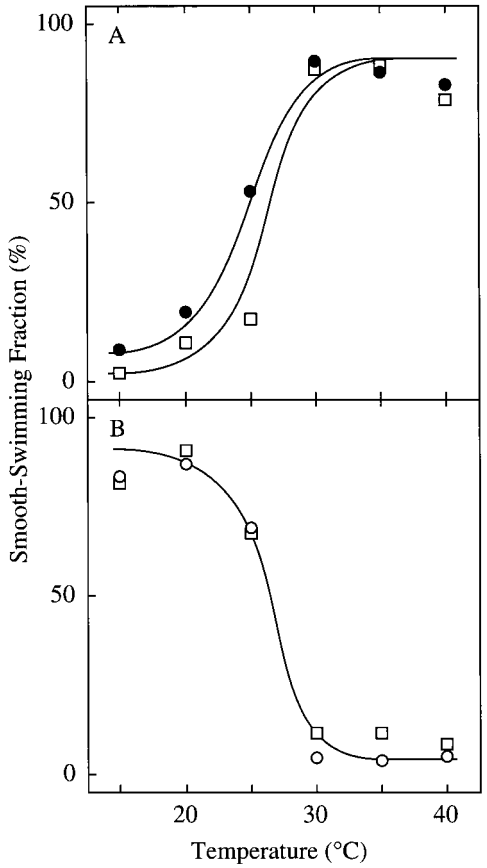

FIG. 6. Temperature dependences of the steady-state swimming patterns of CP553 (CheB$^-$ CheR$^-$) cells expressing mutant Tar proteins. The smooth-swimming fraction of the cells was measured at various temperatures in the absence (A) or presence (B) of 10 μM aspartate. (A) Symbols: open squares, Tar-AEQE (pRA111); closed circles, Tar-AEAE (pRA122). (B) Symbols: circles, Tar-QEQA (pRA114); squares, Tar-AAAA (pRA126).

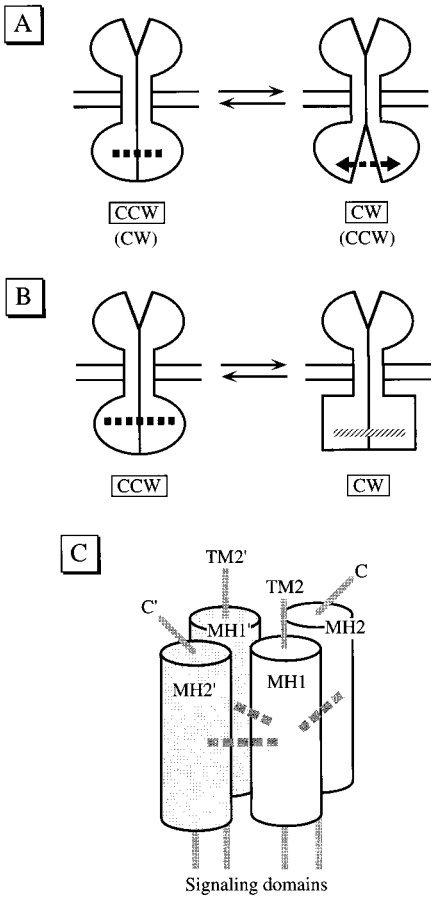

FIG. 7. Proposed models of thermotactic signaling. (A) Model assuming association and dissociation of the cytoplasmic domains to create the two signaling states. The thick dotted line and double-headed arrow indicate attractive and repulsive interactions between the cytoplasmic domains. (B) Model assuming two qualitatively different types of interaction (thick dotted and shaded lines) corresponding to the two signaling states. These interactions might occur within or between monomers. (C) Schematic illustration of the four methylation helices (MH1, MH1′, MH2, and MH2′) in the homodimer of Tar. Intra- and intersubunit interactions between these helices (gray dotted lines) are likely candidates for signaling-state-specific ones. Connections to the other parts of the protein (designated as in Fig. 1A) were indicated by gray solid lines.

tested. The two warm sensors (Tar-AEQE and -AEAE) had similar temperature dependences, as did the cold sensors (Tar-QEQA and -AAAA). Thus, subtle differences in the methylation sites seem to affect the thermosensing property of Tar in a quantized manner. The factors that determine thermosensor phenotypes may include the charge, size, and shape of the side chains of residues at the methylation sites. The charge could be the major determinant, since both mutant warm sensors possessed two glutamates, whereas the mutant cold sensors possessed one or no glutamate residue. In particular, Tar-AAAA (Fig. 5C and 6B) mediated a cold-sensor response similar to that of the fully methylated form. However, charge is not the whole story because the neutral side chains of glutamine and alanine produced slightly different effects on thermosensing, as they do on signaling (31) and on methylation (39).

Cold-sensor responses were observed only in the presence of aspartate. An attractant stimulus might be required only to shift the prestimulus behavior toward smooth swimming. Alternatively, the binding of aspartate to Tar might impose a structural constraint on the methylated forms that is required for the cold-sensor phenotype. In fact, Tar-AAAA functioned as a cold sensor in the presence of aspartate (Fig. 5C), but in the absence of aspartate (Fig. 5B), it produced a very weak and temperature-independent smooth signal. Thus aspartate, as well as neutralization of the methylation sites, may be required for the cold-sensor response.

What is the nature of the thermosensing mechanism? This and previous studies provide some insight. It has recently been shown that the ligand-binding affinity of Tsr was unaffected by temperature, suggesting that thermosensing does not involve a global change in receptor structure but rather involves local changes within the cytoplasmic signaling domain (16). Some models for the chemotactic signaling mechanism of the receptor propose that association and dissociation of the cytoplasmic domains correspond to kinase-inactivating (hence CCW rotation of flagella) and kinase-activating (hence CW rotation) signaling states (20, 35, 36, 47), or the other way around (9, 10, 41) (Fig. 7A). Models based solely on association-dissociation can explain thermosensing as a balance between attractive and repulsive forces that have different temperature dependences. For example, under certain conditions, heating could increase the interaction between domains via the hydrophobic effect or could weaken the interaction by melting their structures. Models based on this premise would propose that the methylation

state (and possibly the presence or absence of aspartate) determines which type of temperature dependence is dominant.

Other models argue for conformational changes within a cytoplasmic domain. Tar heterodimers with a single intact cytoplasmic domain can signal in vitro (26) and in vivo (13, 43), suggesting that the chemotactic signaling mechanism may involve interactions between dimers, conformational changes within a single cytoplasmic domain, or both. It remains to be seen whether thermosensing can occur in a receptor possessing only one cytoplasmic domain per dimer. If it is assumed that CCW- and CW-signaling forms exhibit different types of intra- or intersubunit interaction (Fig. 7B), thermosensing would require that these interactions have different temperature dependences. If the CW-specific interaction is more sensitive to temperature than the CCW-specific interaction, the CW form would become less stable beyond a certain temperature, and hence the receptor would function as a warm sensor. The opposite relation between the CW- and CCW-specific interactions would result in a cold-sensor phenotype.

A likely candidate for intra- or intersubunit interactions that are signaling-state specific would be the interactions between the methylation helices themselves (Fig. 7C), although these helices are not required for signal production by cytoplasmic fragments of Tsr (1). Dimerization of Tar cytoplasmic fragments through leucine zipper motifs that are fused upstream of MH1 still allows modulation of signaling by covalent modification of methylation sites (9, 41). In these constructs, modifications at the methylation sites could cause subtle rearrangements of the MH1-MH1′, MH1-MH2, or MH1-MH2′ helix pairing. Similarly, these helix-helix interactions could be modulated by displacement of MH1 during transmembrane signaling in chemotaxis (e.g., in response to aspartate), since MH1 is coupled to the membrane-spanning helix TM2 (Fig. 7C), which is believed to be the mobile transmembrane signaling element in chemotaxis (6–8, 15, 19, 24). How temperature influences the stability of these helix-helix interactions remains to be seen. In any case, further studies of thermosensing should shed new light on the general mechanisms of receptor signaling and adaptation.

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