THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 30, Issue of July 26, pp. 17932–17936, 1996

Printed in U.S.A.

Modulation of the Thermosensing Profile of the *Escherichia coli* Aspartate Receptor Tar by Covalent Modification of Its Methyl-accepting Sites*

(Received for publication, May 31, 1995, and in revised form, April 30, 1996)

Toshifumi Nara‡, Ikuro Kawagishi§, So-ichiro Nishiyama, Michio Homma, and Yasuo Imae†

From the Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

The *Escherichia coli* aspartate receptor Tar is involved in the thermotactic response. We have studied how its thermosensing function is affected by the modification of the four methyl-accepting residues (Gln^{295}, Glu^{302}, Gln^{309}, and Glu^{491}), which play essential roles in adaptation. We found that the primary translational product of *tar* mediates a chemoresponse, but not a thermoresponse, and that Tar comes to function as a thermoreceptor, once Gln^{295} or Gln^{309} is deamidated. This is the first identification of a thermosensing-specific mutant form, suggesting that the methylation sites of Tar constitute at least a part of the region required for thermoreception, signaling, or both. We have also investigated the inverted thermoresponse mediated by Tar in the presence of aspartate. We found that, whereas the deamidated-and-unmethylated form functions as a warm receptor, eliciting a smooth-swimming signal upon increase of temperature, the heavily methylated form functions as a cold receptor, eliciting a smooth-swimming signal upon decrease of temperature. Thus, it is suggested that Tar exists in at least three distinct states, each of which allows it to function as a warm, cold, or null thermoreceptor, depending on the modification patterns of its methylation sites.

Temperature is one of the crucial environmental parameters that restrict growth and other biological activities in any organism. Therefore, virtually all organisms show some kind of response to an increase or decrease in temperature. It is, however, generally very difficult to investigate how an organism senses temperature shifts, since most higher organisms have no specialized thermosensing organ, and temperature affects a wide variety of cell functions. Thermotaxis in *Escherichia coli*, however, has been well characterized in terms of physiology, genetics, and biochemistry (1). Thermal stimuli are sensed by the four closely related transmembrane receptors (2–6).

These receptors, also known as methyl-accepting chemotaxis proteins (MCPs),[1] were originally identified as chemosensory receptor/transducers (7–13). They are reversibly methylated at four or five glutamate residues within their C-terminal cytoplasmic domains (14–16). Methylation and demethylation are the basis of adaptation in chemo/thermotaxis and are catalyzed by CheR and CheB, respectively (17, 18). An attractant stimulus, whether chemical or thermal, causes elevated methylation of the receptors, which shuts off the attractant signal output. In contrast, a repellent stimulus brings about lowered levels of methylation to counteract the repellent signal (15). Increases and decreases in methylation levels correspond to the magnitudes of attractant and repellent stimuli, respectively. It should be noted that some of the methyl-accepting glutamate residues are derived from glutamine residues in the primary translational products (19, 20). CheB is also responsible for the specific and irreversible deamidation of these groups.

Extensive genetic and biochemical studies on bacterial chemotaxis have identified all of the protein components involved in the chemotactic signal transduction pathway from the detection of attractants and repellents to the control of swimming behavior (for review, see Refs. 10–13). In brief, when a chemoreceptor binds to a repellent, it activates the cytoplasmic autophosphorylating protein kinase CheA that can transfer phosphate group to the cytoplasmic signaling protein CheY and the receptor methylesterase CheB. Phospho-CheY interacts with switch component(s) of the flagellar motor to cause its clockwise rotation; phospho-CheB demethylates chemoreceptor molecules to shut off the tumbling signal. On the other hand, binding of an attractant to a receptor causes inhibition of CheA kinase, which results in a decrease in CheY and CheB phosphorylation. The same signaling pathway (from the receptor to the flagellar motor) is utilized in thermotaxis, but the mechanism underlying thermosensing has not been elucidated.

Each receptor forms a ternary complex with CheA and the coupling protein CheW (21). Since the complex is stable *in vitro* (21, 22) and *in vivo* (23), receptor-mediated control of CheA activity is thought to occur through structural changes within this receptor-CheW-CheA complex. Therefore, it is reasonable to assume that the thermosensing mechanism involves similar structural changes in the ternary complex to those caused by increases and decreases in temperature. It should be stressed that temperature dependence of autophosphorylation, phosphotransfer, or dephosphorylation cannot simply explain thermotaxis. Rather, the receptors seem to be primary thermosensors (2–6): they fall into two classes, namely warm receptors (Tsr, Tar, and Trg) and cold receptors (Tar in the presence of attractants (see below) and Tap). A warm receptor produces attractant and repellent signals upon increases and

* This work was supported in part by grants-in-aid for scientific research (to I. K. and S. N.) from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Professor Yasuo Imae, who was a pioneering researcher in the fields such as bacterial thermotaxis and sodium-driven flagellar motor, died unexpectedly on July 2, 1993. This article is dedicated to him by the rest of the authors with deep sorrow, respect, and affection.

‡ Present address: Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060, Japan.

§ To whom all correspondence should be addressed: Dept. of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan. Tel.: 81-52-789-2993; Fax: 81-52-789-3001; E-mail: i45406a@nucc.cc.nagoya-u.ac.jp.

[1] The abbreviation used is: MCP, methyl-accepting chemotaxis protein.

decreases in temperature, respectively, whereas a cold receptor mediates the opposite responses to the same stimuli (6).

Interestingly, two lines of evidence suggest that covalent modification of the methyl-accepting sites might influence the thermosensing properties of the receptors. First, *cheB*-defective strains, which can respond to but cannot adapt to chemotactic stimuli, show no thermoresponse, whereas *cheR*-defective strains, which cannot adapt either, retain thermosensing abilities (2, 24). The absence of a thermoresponse in *cheB* mutants might be due to the absence of deamidation and/or demethylation of the receptors. Alternatively, CheB might be involved in the thermosensing/signaling process: *e.g.* it might constitute a thermosensor complex with the receptor, CheW and CheA. Second, Tar produces inverted thermotactic signals in the presence of any of its specific attractants, such as aspartate and maltose (3, 5). Whereas it usually functions as a warm receptor, Tar appears to be converted into a cold receptor after adapting to an attractant. Enhanced methylation of Tar has been proposed to cause this attractant-dependent conversion of thermoreceptor function.

In this study, we have studied the roles of the methyl-accepting residues (Gln^{295}, Glu^{302}, Gln^{309}, and Glu^{491}: the set of the four residues will be referred to as [QEQE] throughout this paper) in the thermosensing function of Tar, by examining thermosensing profiles in the various genetic background.

EXPERIMENTAL PROCEDURES

Bacterial Strains—All of the bacterial strains used in this study are derivatives of *E. coli* K-12. Strains HCB339 (Δ*tsr-7021* Δ*(tar-tap)5201 trg*::Tn*10 thr leu his met rpsL136*) (25), CP553 (Δ*trg-100 zab*::Tn*5* Δ(*tar-cheB)2234* Δ*tsr-7028*) (26), and RP1245 (*cheR*)[2] were provided by H. C. Berg of Harvard University, C. Park of Korea Advanced Institute of Science and Technology, and J. S. Parkinson of University of Utah, respectively.

Plasmids—A pBR322-based plasmid pAK101 (27), which carries *tar* and *cheW*, was provided by M. I. Simon of California Institute of Technology. Plasmids pRA130, pRA131, and pRA132, coding for the mutant Tar proteins whose Gln^{295} and Gln^{309}, Gln^{295} alone, and Gln^{309} alone, respectively, are replaced by Glu, were constructed from pAK101 in this study as follows. The codons for residues 295 and 309 were changed from CAG (Gln) to GAG (Glu) using site-directed mutagenesis. The mutations were verified by nucleotide sequencing. Plasmid pNI130, which was constructed from pRA101 and pLAN931 (28), is the pBR322-based plasmid that carries the same mutant *tar* gene as pRA130 but lacks most of the *cheW* sequence.

A *cheR*-carrying plasmid pRAR1 was constructed as follows. The 2.3 kb *Nru*I-*Pvu*II fragment of plasmid pDV2,[3] provided by P. Matsumura of University of Illinois, Chicago, was blunt-ended and ligated with *Eco*RV-digested pACYC184. The ligation mixture was introduced into the *cheR* mutant RP1245, and chloramphenicol-resistant $CheR^+$ transformants were selected. Plasmid DNA was extracted from the clones and examined by restriction enzyme digestion.

DNA Manipulations—Routine DNA manipulations were carried out according to standard procedures (29). Restriction endonucleases and other enzymes for DNA manipulations were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Site-directed mutagenesis was performed by the method of Kunkel *et al.* (30). 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 as described previously (5). Cells were grown at 30 °C with vigorous shaking in tryptone-glycerol broth (1% Bacto-tryptone (Difco Laboratories, Detroit, MI), 0.5% NaCl, and 0.5% glycerol) supplemented with ampicillin (50 μg/ml) and, if necessary, chloramphenicol (25 μg/ml). After 5 h of cultivation, cells were harvested by centrifugation at room temperature and washed with motility medium (10 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM sodium DL-lactate (pH 7.0), and 0.1 mM L-methionine). Cells were resuspended in motility medium and kept at room temperature. Immediately after adding 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. The smooth-swimming fraction was measured photographically as described previously (31). 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, NY) was used throughout the measurement of chemoresponse.

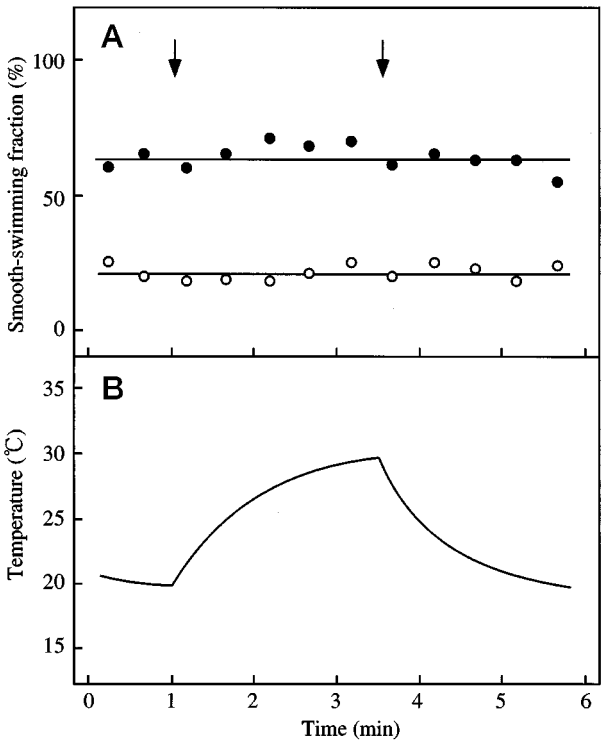

FIG. 1. **Absence of thermotactic response of the CP553 ($CheB^-$ $CheR^-$) cells carrying pAK101 which contains the wild-type *tar* gene.** *A*, swimming behavior of the cells with (*closed circles*) or without (*open circles*) 10 μM aspartate. *B*, the time course of the temperature.

Measurement of Thermoresponse—A cell suspension was prepared as described in the previous section. If necessary, an appropriate concentration of aspartate or glycerol was added to cells suspended in motility medium. A drop of the suspension was placed on a glass slide mounted on a temperature control apparatus as described previously (2). The temperature was changed from 20 to 30 °C and returned to 20 °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 in the previous section.

RESULTS

Inhibition of the Thermosensing Ability of Tar by the Remaining Glutamine Residues—To address the role of CheB in thermosensing, we first examined whether Tar with [QEQE] functions as a thermoreceptor, rather than [QEmQEm] (Em standing for a methylated glutamate residue), which is expressed in *cheB*-defective strains and does not mediate thermoresponse. The plasmid pAK101 carrying wild-type *tar* was introduced into strain CP553, which is defective in *cheB* and *cheR* as well as all four receptor genes (in all experiments in this study, the *E. coli* host strains are defective in all four receptor genes and the only receptors derive from the plasmid-borne *tar* genes). In this strain, the methylation/amidation state of Tar is fixed at [QEQE]. The resultant strain pAK101/CP553 showed extremely tumbly motility and showed no thermoresponse upon temperature shift (Fig. 1*A*, *open circles*). Even when the smooth-swimming fraction of the cells was increased by adding aspartate, no thermoresponse was seen (Fig. 1*A*, *closed circles*). Since neither the methylated [QEmQEm] nor the unmethylated [QEQE] form of Tar has thermosensing

[2] J. S. Parkinson, personal communication.
[3] P. Matsumura, personal communication.

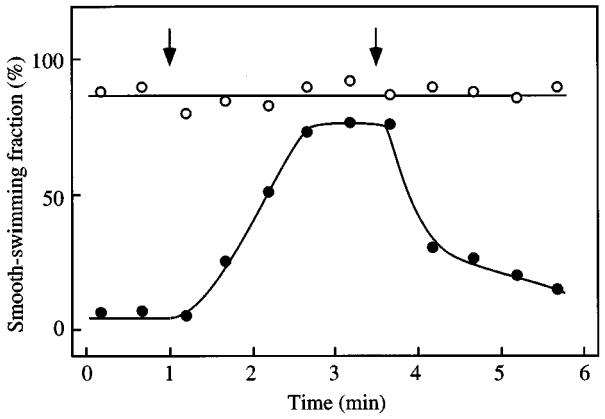

FIG. 2. **Thermoresponses mediated by the deamidated form ([EEEE]) of Tar.** Smooth-swimming fraction of pRA130/CP553 ($CheB^-$ $CheR^-$) cells was measured in the presence (*closed circles*) or absence (*open circles*) of glycerol. *Arrows* indicate the beginning of temperature change.

ability, failure of demethylation of the receptors in *cheB* mutants *cannot* explain their inability to respond to temperature shift.

We then examined whether the absence of deamidating activity in *cheB* mutants is responsible for this phenomenon. We constructed a *tar* gene coding with two glutamine codons in the methylation sites replaced by glutamate and expressed the resulting "genetically deamidated" receptor ([EEEE]) in the *cheB cheR* background (CP553). The resulting cells (pRA130/CP553) swam extremely smoothly regardless of temperature change (Fig. 2), but showed a normal thermoresponse when the smooth-swimming fraction was decreased by adding glycerol, a repellent recognized by Tar (31, 32) (Fig. 2). It should be noted that CP553 cells which do not have any receptor swam always smoothly and did not respond to temperature change even in the presence of glycerol (data not shown). Moreover, $CheB^+$ $CheR^+$ cells expressing wild-type Tsr, Tar, Trg, or Tap show thermoresponses in the absence of glycerol (2–6). Therefore, temperature-dependent changes in the intracellular concentration of acetyl phosphate, which can donate a phosphate group to CheY (33), is not likely to be the cause of thermotaxis. The fact that even a *cheB*-defective strain can respond to temperature changes indicates that CheB is *not* a thermosensor nor a part of a thermosensor complex. Rather, Gln^{295}, Gln^{309}, or both seems to prevent thermosensing function of Tar.

To examine which glutamine residue has the inhibitory effect on thermosensing ability, Gln^{295} and Gln^{309} of Tar were replaced by Glu singly: *i.e.* [EEQE] and [QEEE]. In the *cheB cheR* background, both of them mediated normal thermoresponses when smooth-swimming fraction of the cells was decreased by adding glycerol (Table I). These results suggest that the presence of both Gln^{295} and Gln^{309} in the methyl-accepting sites prevents Tar from thermosensing in the *cheB*-defective strains. We therefore concluded that in the wild-type strain, the primary translational product of Tar ([QEQE]) cannot function as a thermoreceptor, although it has the chemosensing ability, and that Tar acquires thermoreceptor function, once either of its glutamine residues is deamidated by CheB.

Methylation of Tar in the Presence of Aspartate Results in Its Conversion from a Warm to a Cold Receptor—We now turn to the question how Tar is converted to a cold receptor in the presence of a specific attractant such as aspartate. We first examined whether the genetically deamidated mutant Tar receptor ([EEEE]) has the same thermosensing characteristics as the wild-type Tar ([QEQE]) in the $CheR^+$ $CheB^+$ background. Plasmid pNI130 coding for Tar with [EEEE] was introduced into *E. coli* strain HCB339, which lacks all four chemoreceptor. As shown in Fig. 3, the resultant cells showed an essentially similar aspartate dependence of thermoresponses as the wild-type Tar. In the absence of aspartate, the cells showed attractant and repellent responses to increases and decreases in temperature, respectively. Even after adapting to 1 μM aspartate (for 30 min), the cells showed similar responses to thermal stimuli. However, when aspartate was added at a concentration of 10 μM, the cells showed a repellent response to increased temperature (Fig. 3). They did not seem to respond to decreased temperature, since they swam too smoothly even after prolonged incubation with aspartate. However, when glycerol was added further to adjust their swimming bias toward tumbling, they showed a clear inverted thermoresponse (data not shown).

TABLE I
Effect of the glutamine residues in the methylation sites on thermosensing ability of Tar

Plasmid[a]	Methyl-accepting sites of Tar	Swimming pattern[b]	Thermoresponse[c]
pAK101	[QEQE]	Tumble	−
pRA131	[EEQE]	Random	+
pRA132	[QEEE]	Random	+

[a] Each plasmid was introduced into the host strain CP553 ($CheB^-$ $CheR^-$).
[b] Swimming patterns of the cells were assayed in the motility medium without any chemoeffector.
[c] Thermoresponses were measured as described under "Experimental Procedures."

The concentration of aspartate required for the inverted thermoresponse of pNI130/HCB339 is 1 order of magnitude lower than that of the *tsr*-defective mutants (3). However, this should not be due to the mutations of *tar* (Gln^{295} to Glu and Gln^{309} to Glu) on pNI130, since the HCB339 cells carrying the wild-type *tar*-containing plasmid pAK101 also require 10 μM aspartate for the inverted thermoresponse (5). Thus, Tar with [EEEE] can be used to assess the role of methylation in the inversion of thermoresponses.

It has been considered that the attractant-induced methylation of Tar might be responsible for the inverted thermoresponse (1, 5). Therefore, we next compared thermosensing abilities of the methylated and unmethylated forms of Tar as follows. As described previously, CP553 (*cheB cheR*) cells carrying pBR322-based plasmid pRA130 encoding Tar with [EEEE] showed extremely smooth-biased basal swimming pattern, but normal thermoresponse in the presence of glycerol (Fig. 2). The cells never showed an inverted thermoresponse under the conditions tested. We now constructed the pACYC184-based plasmid pRAR1 carrying the methyltransferase gene (*cheR*). The pRA130/CP553 cells were further transformed with pRAR1. In the transformant (pRA130/pRAR1/CP553) cells, Tar is expected to be heavily methylated, since the cells retain methyltransferase but lack methylesterase. In fact, Borkovich *et al.* (34) have demonstrated that Tar is fully methylated in the *cheB*-defective but *cheR*-overexpressing cells. Since we did not examine experimentally whether Tar was fully methylated in the transformant cells, we can only state that Tar must be heavily methylated (this can be predicted from the cells' tumbly biased swimming behavior). For simplicity, we will hereafter describe Tar as fully methylated ([EmEmEmEm]) in these cells. The cells showed inverted thermoresponses in the presence of 10 μM aspartate (Fig. 4). These results indicate that receptor methylation causes the inverted thermoresponse mediated by Tar in the presence of aspartate.

Temperature Dependencies of the Steady-state Signaling Biases Produced by the Heavily Methylated and the Unmethylated Forms of Tar—To further characterize the different thermosensing profiles induced by the unmethylated and the heavily

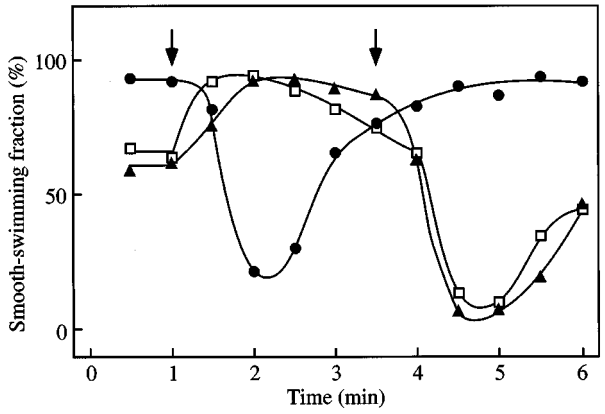

FIG. 3. **Aspartate-dependent inversion of thermoresponse mediated by the genetically deamidated mutant Tar ([EEEE]).** Thermoresponse of the pNI130/HCB339 ($CheB^+$ $CheR^+$) cells was measured in the absence of any chemoeffector (*open squares*) or 30 min after addition of 1 μM (*closed triangles*) or 10 μM (*closed circles*) aspartate. *Arrows* indicate the beginning of temperature change.

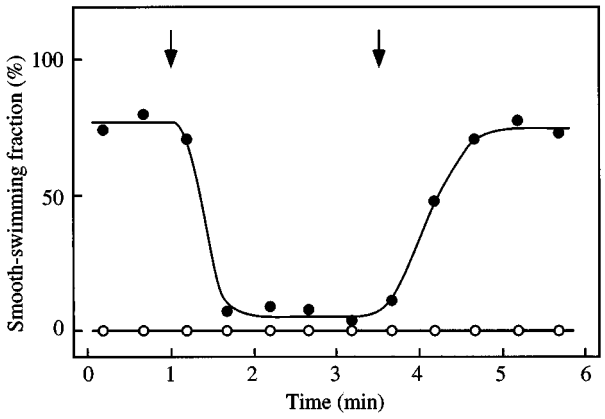

FIG. 4. **Thermoresponses mediated by the heavily methylated form ([EmEmEmEm]) of Tar.** Smooth-swimming fraction of the pRA130/pRAR1/CP553 ($CheB^-$ $CheR^+$) cells was measured in the presence (*closed circles*) or absence (*open circles*) of 10 μM aspartate. *Arrows* indicate the beginning of temperature change.

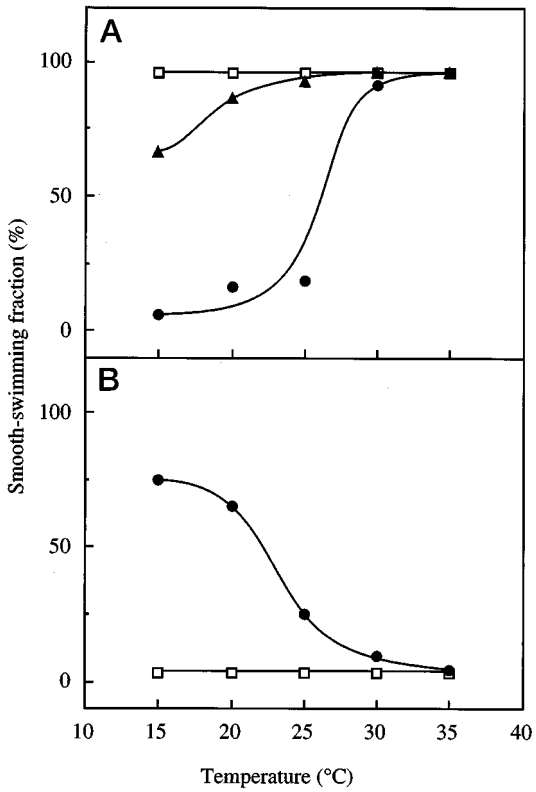

FIG. 5. **Steady-state swimming patterns of cells having the unmethylated ([EEEE]) or the heavily methylated ([EmEmEmEm]) form of Tar at various temperatures.** Smooth-swimming fraction of the pNI130/CP553 ($CheB^-$ $CheR^-$; *A*) or pNI130/pRAR1/CP553 ($CheB^-$ $CheR^+$; *B*) cells was measured without addition of any chemoeffector (*open squares*) or 5 min after addition of 5% (*closed triangles*) or 10% (*closed circles*) glycerol (*A*) or 0.1 mM aspartate (*closed circles* in *B*).

methylated forms of Tar, we then examined the steady-state swimming patterns of cells with either of these two forms of Tar at various temperatures (Fig. 5).

The cells expressing Tar with [EEEE] (pNI130/CP553: $CheB^-$ $CheR^-$) showed exclusively smooth swimming at all temperatures tested (from 15 to 35 °C). In the presence of 10% glycerol, however, they showed a temperature-dependent swimming pattern: almost all cells were tumbling at 15 °C, and as the temperature increased, the smooth-swimming fraction of the cells gradually increased and finally became almost 100% at about 30 °C or above. This profile of the unmethylated form of Tar is consistent with its warm receptor function. Moreover, the cells swam extremely smoothly in the presence of 1 or 10 μM aspartate at all temperatures tested (data not shown). This indicates that in the absence of methylation, aspartate cannot convert Tar from a warm to a cold receptor.

The cells expressing Tar with [EmEmEmEm] (pNI130/pRAR1/CP553: $CheB^-$ $CheR^+$) showed exclusively tumbling behavior at all temperatures tested (from 15 to 35 °C). In the presence of 0.1 mM aspartate, however, they showed a temperature-dependent swimming pattern: almost all cells tumbled at 30 °C or above and as the temperature decreased, the smooth-swimming fraction of the cells increased and became about 75% at 15 °C. This profile is consistent with a cold receptor function. Thus, we conclude that the methyl esterification of the specific glutamate residues is required for the conversion of Tar from a warm to a cold receptor in the presence of aspartate.

DISCUSSION

In this study, we have demonstrated that the primary translational product of *tar* (Tar with [QEQE]) does not have thermosensing ability (Fig. 1), but acquires it by the deamidation of either Gln^{295} or Gln^{309} (Table I). The multifunctional nature of bacterial chemoreceptors has been exploited to isolate mutants defective only in the response for a specific chemoeffector, leading to the identification of the amino acid residues responsible for its recognition (for Tar and Tsr, see Refs. 5 and 35–41). This is the first identification of a thermosensing-specific mutant form of the receptor, since it has been reported for Tar (35, 42) and Tsr (43) that covalent modifications of a receptor do not severely affect its ligand binding affinity.

One interpretation of the results presented here is that the methylation sites of Tar constitute at least a part of the region required for the recognition of temperature or thermotactic signal production. In this regard, it would be interesting to test whether a cytoplasmic fragment of Tar can mediate thermoresponse at all, since some cytoplasmic fragments of Tar and Tsr have been shown to retain abilities to produce chemotactic signals (44, 45).

We have also shown that the unmethylated ([EEEE]) and the heavily methylated ([EmEmEmEm]) forms of Tar function as a warm receptor and a cold receptor, respectively. The unmethylated form of Tar mediated a normal thermoresponse in the presence of glycerol (Fig. 2). However, under no conditions

tested, did it show any characteristics of a cold receptor in the *cheB cheR* background. In contrast, the heavily methylated form (*i.e.* the genetically deamidated Tar expressed in the *cheB* background) mediated an inverted thermoresponse in the presence of aspartate (Fig. 4). Under no conditions tested did it show any characteristics of a warm receptor. Thus, the specific methylation, and possibly the presence of aspartate or any of the other Tar-mediated attractants also, are required for the conversion of Tar from a warm to a cold receptor.

In summary, covalent modification of the four methyl-accepting residues of Tar modulates thermosensing properties of the receptor. These findings reinforce the notion that thermotaxis is resulted from a temperature-dependent shift in an equilibrium between the two signaling states (corresponding to smooth swimming and tumbling) of the receptor itself. Further investigation of the effects of covalent modification would provide an important clue to elucidate what kind of structural change of the receptor plays an essential role in thermosensing.

Acknowledgments—We thank Drs. H. C. Berg, P. Matsumura, C. Park, J. S. Parkinson, and M. I. Simon for providing us with bacterial strains and plasmids and N. Nishioka for drawing. We especially thank Dr. R. M. Macnab of Yale University for critically reading the manuscript.

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