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Uncoupling of Ligand-binding Affinity of the Bacterial Serine Chemoreceptor from Methylation- and Temperature-modulated Signaling States*

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The *Escherichia coli* chemoreceptor Tsr mediates tactic responses to serine, repellents, and changes in temperature. We have previously shown that the serine-sensing ability of Tsr-T156C, which has a unique cysteine in place of threonine at residue 156, is specifically inactivated by thiol-modifying reagents and that L-serine protects the receptor from modification. In this study, we demonstrated the correlation between protective effects of various attractants and their potencies to elicit attractant responses. This indirect binding assay was used to monitor the affinity of the receptor for L-serine under various conditions. It has been demonstrated by *in vitro* assays that the ligand-binding affinities of Tsr and the related chemoreceptor Tar are unaffected by changes in the methylation state of the receptor. Using the serine protection assay, we re-examined this issue both *in vitro* and *in vivo*. The methylation levels of Tsr-T156C did not affect its ligand-binding affinity. We also showed both *in vitro* and *in vivo* that the ligand-binding affinity was unaffected by temperature. These results suggest that the structure of the periplasmic domain of the receptor is uncoupled from the signaling states of the cytoplasmic domain. This ligand-binding assay system should be applicable to other receptors.

In many sensory systems, desensitization or adaptation to a persisting stimulus plays a crucial role in highly sensitive detection of stimuli over a comprehensive range. Extracellular signals (*e.g.* binding of ligands) are received and transduced into intracellular signals (*e.g.* activation or inactivation of kinases) by cell surface receptors. Binding of a ligand to a receptor extracellular domain induces some changes in structure of a receptor intracellular domain and, hence, in its activity. By contrast, upon adaptation, the signaling activities of the intracellular domains are often down-regulated by intracellular feedback regulatory systems (typically via covalent modification of the receptors). The effects of receptor down-regulation may be limited to the intracellular domain or may induce a global structural change in the receptor.

Four closely related chemoreceptors of *Escherichia coli* serve as model systems for investigating both transmembrane signaling and down-regulation (for reviews, see Refs. 1–6), Tsr (for serine), Tar (for aspartate and maltose), Trg (for ribose and galactose), and Tap (for dipeptide). These chemoreceptors function as homodimers regardless of ligand occupancy state (7), and the homodimer forms a ternary complex with a homodimer of a cytoplasmic autokinase CheA and two molecules of an adaptor protein CheW (8). Each receptor monomer consists of an N-terminal periplasmic ligand-binding domain, a C-terminal cytoplasmic signaling domain, and two membrane-spanning segments. Binding of a ligand to the interface between the two periplasmic domains triggers some structural change within the receptor dimer. This structural change leads to activation or inactivation of autophosphorylation of CheA and phosphotransfer from CheA to CheY. Phosphorylated CheY binds to the flagellar motor and induce clockwise (CW)[1] rotation of the motor (causing tumbling of the cell), which otherwise rotates counterclockwise (CCW) (causing smooth swimming).

Adaptation is achieved via methylation and demethylation of the chemoreceptors (9). Each chemoreceptor contains four to six glutamic acid residues that are reversibly methylated. Methyltransferase CheR catalyzes transfer of a methyl group from *S*-adenosylmethionine to a glutamic acid side chain, and methylesterase CheB hydrolyzes the methyl ester bond of a methylated glutamate residue. The latter enzyme also serves as deamidase, converting specific glutamine residues of the nascent chemoreceptors to methylatable glutamic acid residues (10).

Recent *in vitro* studies (11–13) suggest that methylation of a receptor modulates its signaling state but has little effect on its ligand-binding affinity. This means that the structure of the periplasmic ligand-binding domain is uncoupled from the signaling state of the cytoplasmic domain under some conditions. However, contradictory results have been reported for receptor-containing membranes (14). To explain the discrepancy, Lin *et al.* (13) suggested that association of chemoreceptors with cytoplasmic proteins, such as CheA and CheW, might influence their ligand-binding behavior. Therefore, the effects of methylation and demethylation on ligand-binding affinity of receptors must be investigated under conditions closer to their native setting.

The four chemoreceptors are unique in that they function also as thermosensors (15, 16). Tsr, Tar, and Trg are warm

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[1] The abbreviations used are: CW, clockwise; CCW, counterclockwise; Em, a methylated glutamic acid residue; NEM, *N*-ethylmaleimide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TM1, the first transmembrane segment; TM2, the second transmembrane segment; Cm^r, resistance to chloramphenicol; Tc^r, resistance to tetracycline.

receptors that produce CCW signals upon a temperature increase and CW signals upon a temperature decrease, whereas Tap is a cold receptor with opposite signaling behavior (16). Temperature should affect the signaling states of the chemoreceptors, but it remains unclear how this can be achieved. Does a change in temperature cause a global change in receptor structure or just some local change within the cytoplasmic signaling domain?

In this study, we investigated the effects of methylation/amidation and temperature on the ligand-binding affinity of Tsr. We have previously shown that a Tsr-specific attractant L-serine protects Tsr-T156C, which has a unique cysteine residue at the ligand-binding site, from thiol-modifying reagents such as *N*-ethylmaleimide (NEM) in a dose-dependent manner (17). This protective effect of L-serine should reflect the affinity of Tsr-T156C for L-serine. If so, this assay can be used to monitor the ligand-binding affinity of the receptor under various conditions. In this report, we demonstrate a correlation between the stimulus strength of various amino acid attractants detected by Tsr-T156C and their abilities to protect Cys-156 from NEM modification. Using this assay system both *in vitro* and *in vivo*, we found that the ligand-binding affinity of Tsr is uncoupled from its signaling states modulated by covalent modification and temperature.

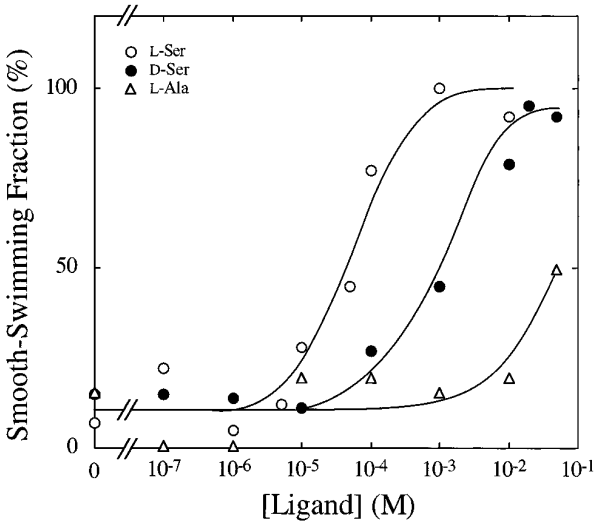

FIG. 1. **Sensing abilities of Tsr-T156C for various attractants.** HCB339 cells with Tsr-T156C were pretreated with 1 M glycerol and then stimulated with various concentrations of L-serine (*open circles*), D-serine (*closed circles*), or L-alanine (*open triangles*). Changes in smooth swimming cells after 30 s were measured. All manipulations were done at 25 °C. In this and the following figures, *lines* are drawn just by eye.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—All strains used in this work are derivatives of *E. coli* K-12. Strain HCB339 [Δ*tsr-7021* Δ(*tar-tap*)*5201 trg*::Tn*10 thr leu his met rpsL136*] (18) lacks all four chemoreceptors, and strain CP553 [*trg-100* Δ*tsr-7028* Δ(*tar-cheB*) *leu his rpsL lac xyl ara tonA tsx thi zab*::Tn*5*] (19) lacks CheB and CheR, as well as all chemoreceptors. Plasmid pGAN1 (17) carries the promoterless mutant *tsr* gene encoding the receptor protein with the single amino acid substitution, Thr-156 to Cys, placed downstream of the *tac* promoter. Plasmid pRAB1 (20) carries the methylesterase gene *cheB* and the tetracycline-resistant gene *tetA* (Tc^r). A *cheR*-overproducing plasmid pKB23 (11) and a *cheB*-overproducing plasmid pKB24 (11), both of which carry the chloramphenicol acetyltransferase gene *cat* (Cm^r), were provided by M. I. Simon of California Institute of Technology.

Chemicals—NEM, isopropyl-1-thio-β-D-galactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), and 1,10-phenanthroline were purchased from Wako Pure Chemical Industries (Osaka, Japan). Octyl-β-D-glucopyranoside was purchased from Dojindo Laboratories (Kumamoto, Japan). *N*-[ethyl-1-^{14}C]ethylmaleimide (40 mCi/mmol) and reagents for the bicinchoninic acid protein assay were products of Dupont NEN and from Pierce, respectively.

Assays of Chemo- and Thermoresponses—Cells were grown at 30 °C in tryptone-glycerol broth (1% Bacto-tryptone (Difco Laboratories, Detroit, MI), 0.5% NaCl, 0.5% glycerol) supplemented with 50 μg/ml ampicillin (and 25 μg/ml chloramphenicol, when necessary). At late exponential phase, cells were collected by centrifugation, washed twice with motility medium (10 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 10 mM sodium DL-lactate, 0.1 mM methionine), and resuspended in motility medium.

Temporal stimulation assays for chemoresponse were carried out as described previously (21). The cells suspended in motility medium were pretreated with a repellent, 1 M glycerol, and then stimulated with various concentrations of a Tsr-mediated attractant, L-serine, D-serine, or L-alanine. The change in the smooth swimming fraction after 30 s was measured. Addition of 1 M glycerol reduces the smooth swimming fraction of the cells to nearly zero. Therefore, an increase in the smooth swimming fraction upon addition of an attractant represents the magnitude of an attractant response of the cells.

Temporal stimulation assays for thermoresponse were carried out as described previously (20). Cells were cultured and washed as described above. A drop of cell suspension was placed on a glass slide mounted on a temperature control apparatus (22). When cells swam too smooth or too tumble, glycerol or L-serine was added to the suspension. The temperature was first increased from 20 to 30 °C and then decreased from 30 to 20 °C.

Preparation of Membranes and Solubilization of Membrane Proteins—Membranes were prepared by the method of Foster *et al.* (23). A fresh overnight culture of CP553 cells carrying pGAN1 was inoculated (1:100 dilution) into LB medium (1% Bacto-tryptone, 1% yeast extract, 0.5% NaCl) supplemented with 200 μg/ml ampicillin and grown at 30 °C. If the cells also carried pRAB1 (Tc^r) or pKB23 (Cm^r), 12.5 μg/ml tetracycline or 25 μg/ml chloramphenicol was added. After 3 h, 1 mM IPTG was added to the culture. After further incubation for 4 h, the cells were harvested by centrifugation, washed with motility medium, and were treated with 100 μM NEM in the presence of 10 mM serine for 60 min at 0 °C. The cells were then collected again, resuspended in lysis buffer (100 mM sodium phosphate, pH 7.2, 5 mM EDTA, 10% glycerol, 5 mM 1,10-phenanthroline, 2 mM PMSF) at 0 °C, and lysed by sonication (Heat Systems-Ultrasonics, Inc., model W-225). Unbroken cells and cell debris were removed by centrifugation for 20 min at 10,000 rpm in a Sakuma 7B rotor at 4 °C. The supernatant was centrifuged for 60 min at 40,000 rpm in an RP 50–2 rotor (Hitachi Koki Co., Ltd.) at 4 °C. The pellet was washed with wash buffer (50 mM sodium phosphate, pH 7.2, 2 M KCl, 10% glycerol, 5 mM EDTA, 5 mM 1,10-phenanthroline, 1 mM PMSF) and then resuspended in a buffer containing 50 mM Tris-HCl, pH 7.0, 10% glycerol, 5 mM 1,10-phenanthroline, and 1 mM PMSF at 0 °C. Protein concentration was determined by the bicinchoninic acid protein assay system using bovine serum albumin as a standard. The suspension was diluted with the same buffer to approximately 10 mg of protein/ml. Octylglucoside was added to 1.25%, and the mixture was incubated for 20 min at 0 °C and centrifuged for 60 min at 40,000 rpm (Hitachi RP65 rotor) at 4 °C. The supernatant was stored at 4 °C.

Modification with Radioactive NEM—For modification of Tsr-T156C in intact cells, *N*-[ethyl-1-^{14}C]maleimide (10 mCi/mmol; 50 μM final concentration) was added to the cells (A_{590} = 1.5), and the mixture was incubated at 25 °C for 30 min. For membranes or octylglucoside extracts, the mixture was incubated at 25 °C for 6 min. When necessary, various concentrations of L-serine, D-serine, or L-alanine were added prior to the addition of NEM. The reactions were terminated by the addition of 5% trichloroacetic acid, and the samples were collected by centrifugation. The precipitates were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21) and autoradiography. The labeled proteins was analyzed using Bio Imaging Analyzer (Fuji Photo Film Co., Ltd., BAS-1000).

RESULTS

Protection of Cys-156 from NEM Modification Reflects the Ligand-binding Affinity of Tsr-T156C—We first examined the sensing abilities of Tsr-T156C for various amino acid attractants. Plasmid pGAN1 carrying the *tsr-T156C* gene was introduced into strain HCB339, which is defective in all four chemoreceptor genes. As shown in Fig. 1, 40 μM L-serine or 1 mM D-serine was required for attractant responses in 50% of the cells. Only a small response was induced by 50 mM or higher

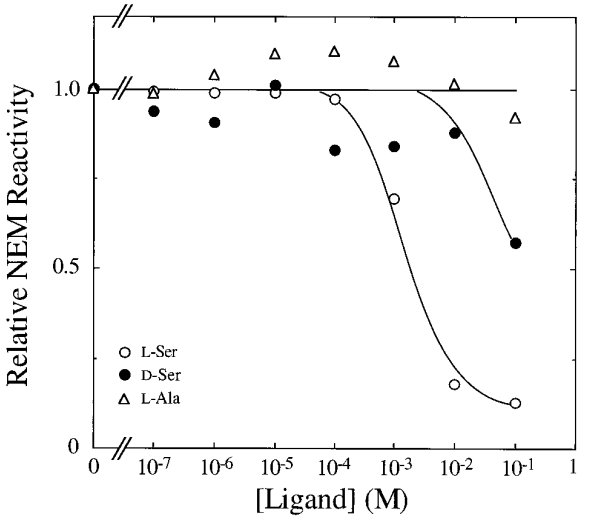

FIG. 2. **Protective effects of various attractants on modification of Tsr-T156C by radioactive NEM.** Membranes (containing approximately 4 μg of proteins) extracted from CheB$^-$ CheR$^-$ cells overproducing Tsr-T156C were solubilized with 1.25% octylglucoside. 50 μM [^{14}C]NEM was added to octylglucoside extracts in the presence of various concentrations of an attractant. The mixtures were incubated for 6 min at 25 °C and were subjected to SDS-PAGE followed by autoradiography with an imaging plate. The relative intensities of labeled Tsr-T156C were plotted. *Open circles*, L-serine; *closed circles*, D-serine; *open triangles*, L-alanine.

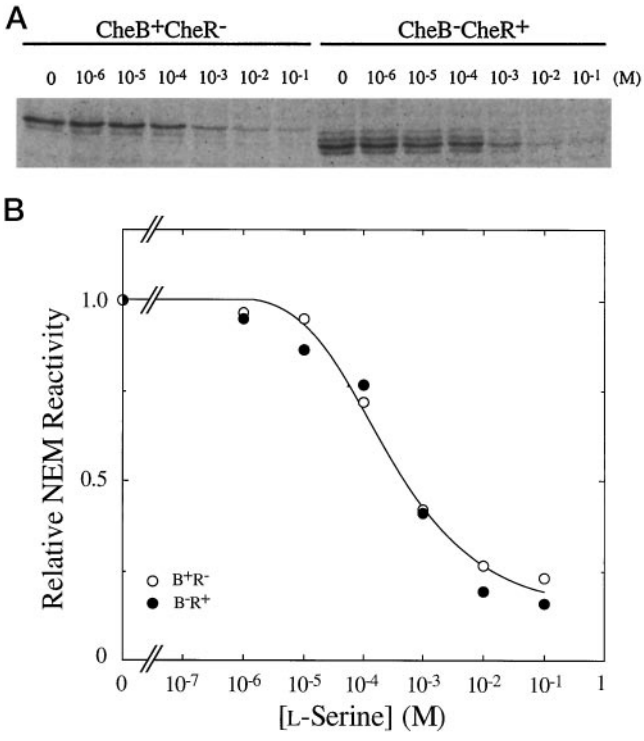

FIG. 3. **Effect of covalent modification on protection by L-serine.** Membranes (approximately 6 μg of proteins) from CheB$^+$ CheR$^-$ or CheB$^-$ CheR$^+$ cells were incubated with 50 μM [^{14}C]NEM for 6 min at 25 °C in the presence of various concentrations of L-serine. *A*, samples were subjected to SDS-PAGE and autoradiography. *B*, the relative intensities of labeled Tsr-T156C were plotted as described in the legend to Fig. 2. In the case of CheB$^-$ CheR$^+$ cells, the sum of all four bands was plotted. *Open circles*, membranes of CheB$^+$ CheR$^-$ cells; *closed circles*, membranes of CheB$^-$ CheR$^+$ cells.

concentrations of L-alanine. These results are essentially consistent with the published data for wild-type Tsr (24), that the affinity of Tsr for L-alanine is lower than that for L-serine by more than two orders of magnitude.

Next, we investigated the ability of various attractants to protect against NEM modification. As shown in Fig. 2, the addition of L-serine or D-serine to membrane proteins extracted with octylglucoside significantly prevented NEM from reacting with Tsr-T156C, but L-alanine appeared to have no protective effect. Similarly, glycine and α-aminoisobutyric acid, a nonmetabolizable attractant, had no effect (data not shown). The protective effect of D-serine was smaller than that of L-serine, consistent with the fact that the concentration of D-serine for a half-maximal response of cells expressing Tsr-T156C is about 10-fold higher than that of L-serine. Similarly, the inability of L-alanine to protect corresponds to the low apparent affinity of Tsr-T156C for L-alanine. Therefore, we conclude that the relative extent of protection by an attractant against NEM modification of Tsr-T156C reflects the affinity of Tsr for the attractant.

Effects of Covalent Modification on Serine Protection in Vitro—We next examined whether methylation of the chemoreceptor affects its affinity for attractants. Plasmid pGAN1 carrying the *tsr-T156C* gene was introduced into strain CP553, which lacks CheB and CheR as well as all four chemoreceptors. To modulate the methylation level of Tsr-T156C, the resulting strain was further transformed with plasmids carrying either the methylesterase/deamidase gene *cheB* or the methyltransferase gene *cheR*. Fig. 3*A* shows the effects of various concentrations of L-serine on NEM modification of Tsr-T156C in membrane preparations from CheB$^+$ CheR$^-$ or CheB$^-$ CheR$^-$ cells. In these strains, the five major methyl-accepting residues of Tsr (collectively designated as QEQEE) should be modified as follows. In CheB$^-$ CheR$^-$ cells, all five residues are unmodified (QEQEE); in CheB$^-$ CheR$^+$ cells, the Gln residues are not deamidated, and the Glu residues are methylated (QEmQEmEm); and in CheB$^+$ CheR$^-$ cells, the Gln residues are deamidated, and the Glu residues are not methylated (EEEEE). In fact, Tsr-T156C proteins in those cells showed distinct mobilities on SDS-PAGE due to methylation or deamidation (25). In each case, the extent of NEM labeling of Tsr-T156C decreased considerably in the presence of higher concentrations of L-serine. The L-serine concentration required for 50% protection is approximately 500 μM both for CheB$^-$ CheR$^+$ cells and for CheB$^+$ CheR$^-$ cells (Fig. 3*B*). Similar results were obtained using octylglucoside-solubilized Tsr-T156C proteins (data not shown). These results suggest that covalent modification of Tsr-T156C does not dramatically affect its affinity for L-serine in the absence of the cytoplasmic Che proteins, a conclusion consistent with recent studies on Tar (11, 12) and Tsr (13).

Effects of Covalent Modification on Serine Protection in Vivo—We then examined serine protection of Tsr-T156C from NEM modification in intact cells. As shown in Fig. 4*B*, the L-serine concentration required for 50% protection of Tsr-T156C was approximately 1 mM for CheB$^-$ CheR$^-$ (QEQEE), CheB$^-$ CheR$^+$ (QEmQEmEm), and CheB$^+$ CheR$^-$ (EEEEE) cells. This was not due to the ineffectiveness of the method since about 100 mM D-serine was required for 50% protection of Tsr-T156C expressed in CheB$^-$ CheR$^-$ cells (Fig. 4*B*). Four bands of Tsr-T156C were detected for samples from CheB$^-$ CheR$^+$ cells due to differential methylation levels (Fig. 4*A*). So, we further examined serine protection on each band. The slowest band on SDS-PAGE was considered to be the unmodified form (QEQEE) of Tsr (Fig. 4*C*). All bands were protected from NEM modification by L-serine to almost the same degree as the slowest band. Taken together, we conclude that covalent modification of Tsr-T156C does not dramatically change its affinity to serine even in the presence of the cytoplasmic Che proteins. However, since Tsr was overproduced, we cannot rule out the possibility that many of the receptor proteins might not form ternary complexes with CheW and CheA.

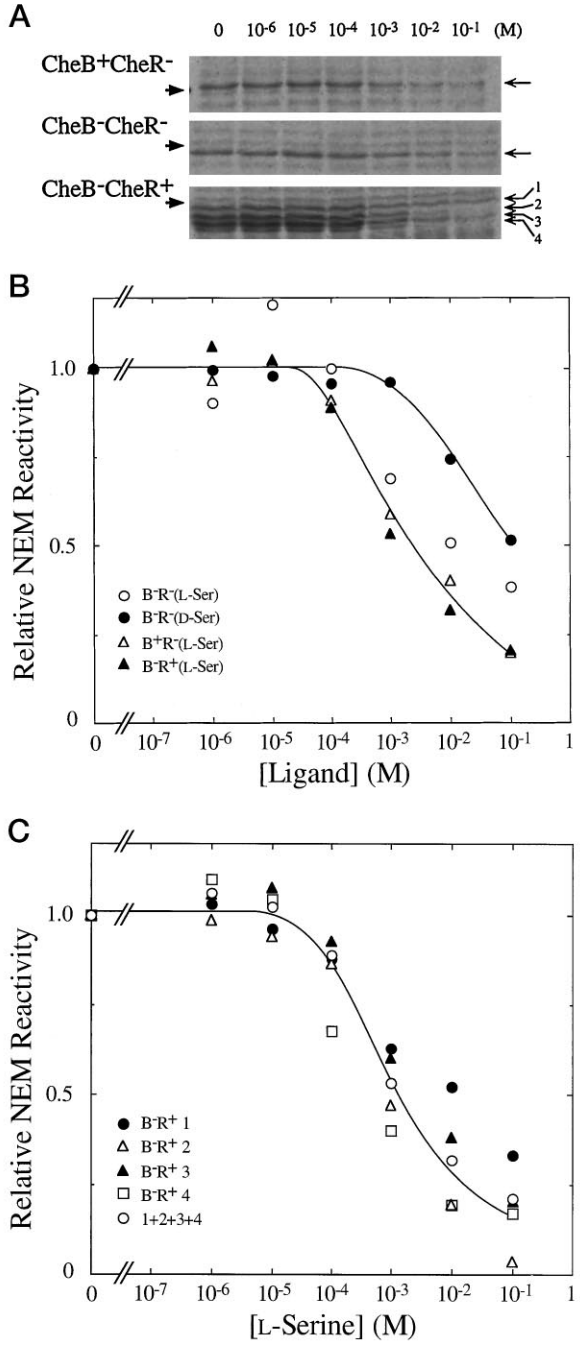

FIG. 4. **Effect of covalent modification on serine protection in intact cells.** $CheB^+$ $CheR^-$, $CheB^-$ $CheR^-$ or $CheB^-$ $CheR^+$ cells overproducing Tsr-T156C were incubated with 50 μM [^{14}C]NEM for 30 min at 25 °C in the presence of various concentrations of L-serine or D-serine. *A*, samples were subjected to SDS-PAGE and autoradiography. Each Tsr protein with differentially modified form is indicated by a *thin arrow* on the *right*. *Thick arrows* on the *left* represent the positions of bands, corresponding to the molecular weight of 66 kDa. *B*, the relative intensities of labeled Tsr-T156C were plotted as described in the legend to Fig. 2. *Open circles*, $CheB^-$ $CheR^-$ cells, L-serine; *closed circles*, $CheB^-$ $CheR^-$ cells, D-serine; *open triangles*, $CheB^+$ $CheR^-$ cells, L-serine; *closed triangles*, $CheB^-$ $CheR^+$ cells, L-serine. *C*, the relative protection by L-serine of different methylated forms in the $CheB^-$ $CheR^+$ cells. *Open circles*, total (bands 1–4); *closed circles*, band 1; *open triangles*, band 2; *closed triangles*, band 3; *open square*, band 4.

Effects of Temperature on Serine Protection—We next examined whether temperature influences the ligand-binding affinity of the chemoreceptor. First of all, we had to examine whether Tsr-T156C has thermosensing ability in the $CheB^-$ $CheR^-$ background since Tar does not have thermosensing ability in the same background (20). The results are summarized in Table I. CP553 cells (Tsr^- Tar^- Trg^- Tap^- $CheB^-$ $CheR^-$) were transformed with plasmid pJUN11, which carries the *tsr-T156C* gene. The resulting cells showed tumbling without any stimulus at 25 °C and did not change their swimming behavior upon temperature changes. When tumbling was reduced by the addition of 0.3 mM L-serine, the cells showed thermosensing abilities. The cells showed smooth swimming as the temperature increased (from 20 to 30 °C) and then tumbling as the temperature decreased (from 30 to 20 °C). Similar results were obtained for CP553 cells expressing wild-type Tsr in the presence of 120 μM L-serine. An analysis by Western blotting with anti-Tsr antiserum detected no change in mobility of either wild-type Tsr or Tsr-T156C during these behavioral responses, indicating that neither spontaneous deamidation nor degradation of the chemoreceptors had occurred (data not shown). These results suggest that, unlike Tar, the primary, unmodified translational product of Tsr can mediate a thermoresponse. That serine does not play an essential role in thermosensing by Tsr-T156C was verified as follows. To shift the signaling bias toward smooth swimming (CCW flagellar rotation) by deamidation, Tsr-T156C was expressed in CP553 cells carrying the CheB-overproducing plasmid pKB24 ($CheB^{++}$ $CheR^-$). The resulting cells swam very smoothly even when temperature was changed. However, in the presence of a repellent glycerol (10%), the deamidated form of Tsr-T156C mediated a thermoresponse. We conclude that both the unmodified and the deamidated forms of Tsr-T156C have thermosensing abilities.

Next, we subjected Tsr-T156C expressed in the $CheB^-$ $CheR^-$ background to NEM modification in the presence of various concentrations of L-serine. As shown in Fig. 5, both in a membrane preparation and in an octylglucoside extract, Tsr-T156C was protected by L-serine from NEM modification at 20, 25, and 30 °C to almost the same degree. By contrast, incubation at higher temperature (50 °C) almost completely abolished the protective effect of serine (data not shown), indicating (partial) denaturation of the protein. These results suggest that the ligand-binding affinity of the chemoreceptor is not significantly affected by a moderate change in temperature which causes thermotaxis.

TABLE I
Thermosensing abilities of Tsr and Tsr-T156C in the $CheR^-$ backgrounds

Thermoresponses of cells expressing Tsr or Tsr-T156C were analyzed. Strains CP553 ($CheB^-$ $CheR^-$: abbreviated as B^-R^-) and CP553 transformed with the CheB-overproducing plasmid pKB24 ($CheB^{++}$ $CheR^-$: abbreviated as $B^{++}R^-$) were used as hosts for plasmids carrying the *tsr* or *tsr-T156C* gene. Cells were incubated at 20 °C prior to the assays for themoresponses. Temperature was increased to 30 °C and then decreased to 20 °C as described under "Experimental Procedures." S, smooth swimming; R, random swimming; T, tumbling; NT, not tested; +, cells showed smooth swimming and tumbling upon an increase and a decrease in temperature, respectively; −, cells did not change their swimming behavior upon temperature changes.

Chemoreceptor/host	Swimming pattern at 25 °C	Thermoresponse		
		None	+ Serine	+ Glycerol
$Tsr/B^{++}R^-$	S/R	+	NT	NT
Tsr/B^-R^-	T	−	+[a]	NT
$Tsr\text{-}T156C/B^{++}R^-$	S/R	−	NT	+[c]
$Tsr\text{-}T156C/B^-R^-$	T	−	+[b]	NT

[a] Thermoresponses were measured in the presence of 120 μM L-serine.
[b] Thermoresponses were measured in the presence of 300 μM L-serine.
[c] Thermoresponses were measured in the presence of 10% glycerol.

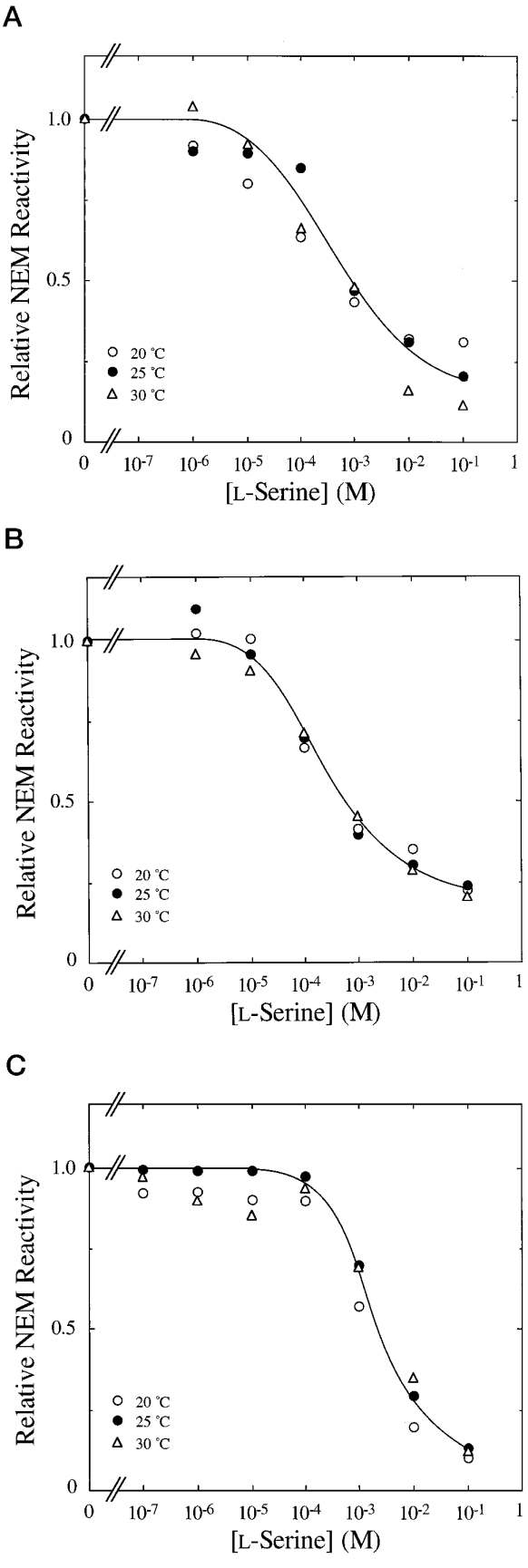

FIG. 5. **Effect of temperature on serine protection.** CheB$^-$ CheR$^-$ cells overproducing Tsr-T156C (*A*), and membranes (*B*) and solubilized membrane proteins (*C*) from the cells were incubated with 50 μM [^{14}C]NEM for 6 min at 20 °C (*open circles*), 25 °C (*closed circles*), or 30 °C (*open triangles*). The relative intensities of labeled Tsr-T156C were plotted as described in the legend to Fig. 2.

DISCUSSION

We used sulfhydryl modification to investigate the ligand-binding affinity of Tsr-T156C under various conditions. Whereas an attractant binds reversibly to Tsr, NEM covalently attaches to the cysteine residue. Therefore, the amount of NEM-labeled to Tsr-T156C increases even in the presence of the competitor during incubation. In this study, we could not determine absolute values of the dissociation constants. More detailed analyses would be required for obtaining such values as demonstrated for dissociation constants between an enzyme and its substrate or inhibitors (26). However, when Tsr-T156C was incubated with NEM for a fixed length of time in the presence of L-serine, D-serine, or L-alanine, the magnitudes of protection were consistent with the apparent affinities of Tsr-T156C for those attractants. Therefore, we could compare relative affinities of Tsr for L-serine under various conditions simply by monitoring the protective effect of L-serine on NEM modification during a fixed length of time.

Using this assay system, we found that the serine-binding affinity of Tsr is uncoupled from its methylation-modulated signaling states both *in vivo* and *in vitro*. This is consistent with recent *in vitro* studies on Tar (11, 12) and Tsr (13). These results suggest that "reverse transmembrane signaling" from the inside to the outside of the cell membrane does not happen in adaptation. However, a previous report (14) demonstrated that methylation of Tsr and Tar substantially decreased the ligand affinity of receptors in the membrane preparations. The discrepancy might be explained by the fact that the latter study did not monitor ligand binding to the receptors directly or by differences in the amount of the receptors in the membrane preparation. Alternatively, the cytoplasmic Che proteins, especially CheA and CheW, which form a ternary complex with the chemoreceptor, might contribute to reduction of the ligand-binding affinity upon methylation, which cannot be observed for the bare receptor, as has been discussed before (13). We cannot rule out this possibility since Tsr-T156C was overproduced in our experiments, and some would not be in ternary complexes with CheA and CheW.

More surprisingly, we found that the serine-binding affinity of Tsr is also uncoupled from its temperature-modulated signaling states *in vivo* and *in vitro*. Thus, a moderate change in temperature in the range between 20 and 30 °C may not cause a dramatic change in the receptor structure, such as monomer-dimer transition or a profound conformational change, but may cause some effects limited to the cytoplasmic domain. These local structural changes should be the nature of thermosensing by the chemoreceptor. The importance of the cytoplasmic domain in thermosensing by Tar has been suggested by a previous study (20), which showed that the primary translational product of *tar* does not have thermosensing ability and the unmethylated and the heavily methylated forms of Tar function as warm and cold receptors, respectively. In this regard, Tsr behaves differently. The unmodified form functions as a warm receptor (Table I), and it seems to lose thermosensing ability upon methylation (27, 28). These differences between Tsr and Tar might be related to differences in the number of methylation sites (25, 29). In any case, covalent modification in the cytoplasmic domain dramatically affects the thermosensing property of the receptor, a fact which is consistent with the notion that the structure of the cytoplasmic domain is altered by temperature changes.

Our results provide insight into the molecular architecture of the receptor. That the ligand-binding domain may be fairly stable in its overall organization is suggested by crystallographic analyses (30–32). There is only a small difference between the ligand-occupied and -free structures of a periplas-

mic fragment of Tar. Recent studies suggest that the first transmembrane helices (TM1 and TM1′) pair stably, and binding of the ligand causes some movement of the second transmembrane helix (TM2) relative to TM1-TM1′ (33–36). However, it remains unclear what happens to the cytoplasmic domain. Recent *in vitro* studies suggest that dimerization (or oligomerization) of the cytoplasmic domain of Tar plays a critical role (37, 38). On the other hand, the assays for genetic complementation between two mutant receptors suggest that only one intact cytoplasmic domain per receptor dimer is sufficient for signaling (39, 40). Therefore, the signaling mechanism may involve interactions between dimers, conformational changes within a single cytoplasmic domain, or both. In any case, binding of an attractant followed by a relatively small change in the periplasmic domain triggers a dramatic inactivation of CheA kinase. Therefore, it is reasonable to assume that the two different signaling states (CW and CCW; kinase on and off) of the receptor are caused by a relatively large structural change of the cytoplasmic domain. If so, covalent modification and temperature changes should cause similar structural changes in the cytoplasmic domain. However, under such conditions, the structure of the periplasmic domain remains unaffected, suggesting structural uncoupling between the two domains across the membrane.

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