In Vivo Sulfhydryl Modification of the Ligand-Binding Site of Tsr, the *Escherichia coli* Serine Chemoreceptor†

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The *Escherichia coli* chemoreceptor Tsr mediates an attractant response to serine. We substituted Cys for Thr-156, one of the residues involved in serine sensing. The mutant receptor Tsr-T156C retained serine- and repellent-sensing abilities. However, it lost serine-sensing ability when it was treated in vivo with sulfhydryl-modifying reagents such as *N*-ethylmaleimide (NEM). Serine protected Tsr-T156C from these reagents. We showed that [³H]NEM bound to Tsr-T156C and that binding decreased in the presence of serine. By pretreating cells with serine and cold NEM, Tsr-T156C was selectively labeled with radioactive NEM. These results are consistent with the location of Thr-156 in the serine-binding site. Chemical modification of the Tsr ligand-binding site provides a basis for simple purification and should assist further in vivo and in vitro investigations of this chemoreceptor protein.

Escherichia coli has four closely related chemoreceptors (for reviews, see references 3, 10, 22, 25, 31, 35, and 36); each consists of an N-terminal periplasmic ligand recognition domain, a C-terminal cytoplasmic signaling domain, and two transmembrane segments. Each one detects various chemical attractants in a highly sensitive and specific manner (12). For instance, mutants that lack the serine receptor (Tsr) but retain the aspartate receptor (Tar) do not respond to serine.

The initial steps toward elucidating the mechanism that underlies this precise molecular recognition were to isolate and characterize mutant receptors which were defective in attractant detection but retained other sensing abilities, such as repellent- (14, 30, 34, 38, 39) and temperature-sensing (13, 23, 29) abilities. In Tar, Arg-64, Arg-69, Thr-154, and several other residues have been implicated in aspartate sensing (7–9, 18, 19, 28, 41). The crystal structure of the ligand-binding domain of *Salmonella typhimurium* Tar has been determined in the presence and absence of aspartate (26, 33, 42). In the cocrystal, aspartate is intercalated between the two subunits of the Tar homodimer, and only one of the two nonoverlapping aspartate-binding sites is filled.

In contrast, Tsr has not been extensively studied in terms of ligand binding since earlier characterizations of mutants (11, 20). Recently, isothermal titration calorimetry (21) and computer simulation (15) have been used to analyze ligand binding by Tsr. Site-directed sulfhydryl modification analyses should provide valuable information complementary to these studies, as they were successfully used to investigate the aspartate-binding site in Tar (8, 9). Therefore, we substituted Cys for Thr-156, which has been implicated in serine sensing. If ligand binding can be specifically inactivated in vivo, Tsr function can be analyzed in its native setting.

Effects of the Thr-156-to-Cys replacement on receptor functions. This site-directed replacement (Thr-156→Cys) was carried out as described previously (19) with the *tsr*-carrying plasmid pJFG5 (24). The mutant gene and its product are referred to hereafter as *tsr-T156C* and Tsr-T156C, respectively. The mutant gene *tsr-T156S* was described previously (19). Plasmids that carried *tsr*, *tsr-T156S*, and *tsr-T156C* were transferred into strain HCB339 (40), which lacks all four receptors. Wild-type Tsr, Tsr-T156S, and Tsr-T156C had comparable glycerol-sensing abilities (Fig. 1). The serine concentration required for one-half of the maximal attractant response was 2 μM for cells that expressed wild-type Tsr or Tsr-T156S and 40 μM for cells that expressed Tsr-T156C (Fig. 1). These results indicate that the replacement of Thr-156 (or Ser-156) with Cys, i.e., the change from OH to SH, does not drastically affect Tsr receptor functions, including serine-sensing ability.

SH modification and serine protection of Cys-156. SH modification and chemotaxis assays were carried out as described

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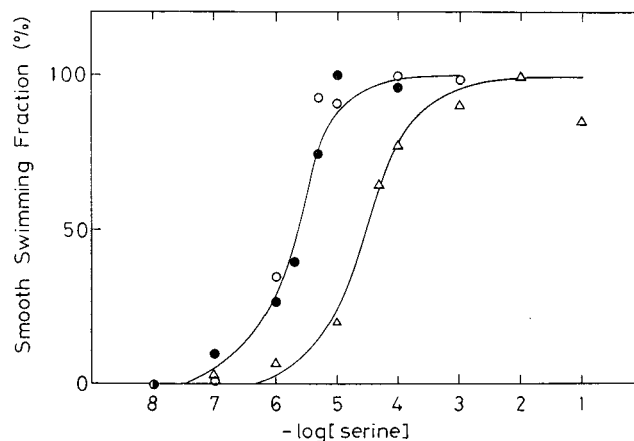
FIG. 1. Serine-sensing abilities of mutant Tsr receptors. HCB339 cells that expressed wild-type Tsr (open circles), Tsr-T156S (closed circles), or Tsr-T156C (open triangles) were pretreated with 1 M glycerol and then stimulated with various concentrations of serine. The changes in smoothly swimming cells after 30 s were measured. All manipulations were done at 25°C.
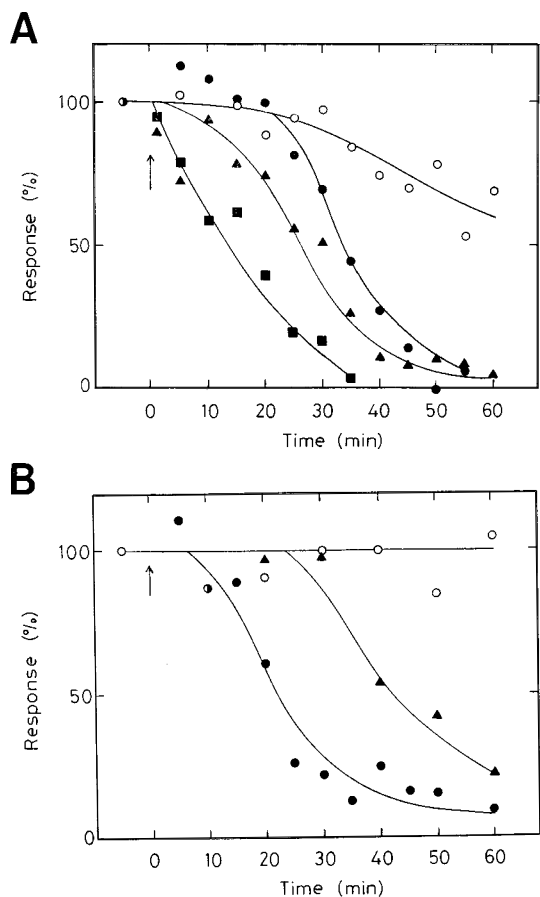
A

B

FIG. 2. Inactivation by sulfhydryl-modifying reagents of the serine-sensing ability of Tsr-T156C. Various concentrations of NEM (A) or DTNB (B) were added at the time indicated by the arrow in each panel to cells that expressed Tsr-T156C (closed symbols). Cells that expressed Tsr-T156S (open circles) were treated with 50 μM NEM (A) or 200 μM DTNB (B). At intervals, aliquots were taken and serine responses were measured by adding 10 mM serine as described in the legend to Fig. 1. (A) Closed circles, 30 μM; closed triangles, 40 μM; closed squares, 50 μM. (B) Closed triangles, 100 μM; closed circles, 200 μM.

FIG. 3. Protective effect of serine on NEM modification of Tsr-T156C. At the time indicated by the arrow, 40 μM NEM was added to HCB339 cells that expressed Tsr-T156C in the absence (open circles) or presence of 1 mM serine (closed triangles) or aspartate (closed circles). After serine or aspartate was washed out, serine responses were measured as described in the legend to Fig. 2.

previously (8). In the presence of 50 μM N-ethylmaleimide (NEM), the serine-sensing ability of Tsr-T156C decreased drastically, whereas that of Tsr-T156S decreased only slightly (Fig. 2A). Glycerol-sensing abilities were not affected by NEM. 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) produced similar effects (Fig. 2B). The inhibitory effect of DTNB was reversed by the reductant dithiothreitol (1 mM; data not shown).

If Thr-156 is in the serine-binding site, excess serine should protect Tsr-T156C from SH modification. NEM inactivation of Tsr-T156C was delayed drastically by the prior addition of 1 mM serine (Fig. 3). Even after 60 min of incubation, more than one-half of the cells showed serine responses. Such a protective effect was not observed with 1 mM aspartate. Furthermore, serine, but not aspartate, had a similar protective effect on DTNB modification of Tsr-T156C (data not shown). Since Tsr has no other Cys residue (4), these results suggest that Cys-156 is located in the serine-binding site.

More than 200 μM DTNB is required for complete inactivation within 30 min of the serine-sensing ability of Tsr-T156C, whereas 5 μM DTNB is enough to inactivate the aspartate-sensing ability of Tar-T154C (8). On the other hand, nearly equal concentrations of NEM inhibit the attractant-sensing abilities of these two receptors. The ligand-binding pocket of
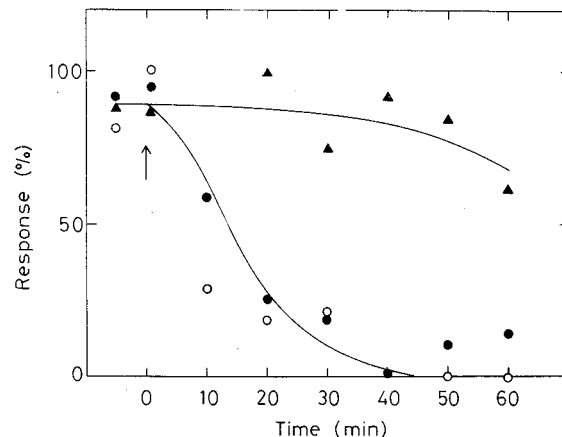
Tar may be a little larger than that of Tsr, reflecting their ligand sizes. If so, DTNB, a bulkier reagent than NEM, might have easier access to the Cys residue of Tar-T154C than to that of Tsr-T156C.

The lower the concentration of NEM or DTNB, the longer the duration before there is a detectable decrease in the serine response (Fig. 2). Therefore, the cell's full response to serine may require many fewer Tsr molecules than those expressed from multicopy tsr genes. Whether this is the case in the wild type, which contains about 2,000 molecules of Tsr per cell (35), may be examined by radioactive labeling.

Specific binding of radioactive NEM to Tsr-T156C. We constructed plasmid pGAN1, which contains tsr-T156C under the control of the tac promoter and the lacI^q gene, by recombining the tsr-T156C-carrying plasmid pJUN11 and the tsr-overproducing plasmid pCS20 (32). HCB339(pGAN1) and HCB339 (pCS20) cells produced comparable sizes of swarm in tryptone semisolid agar (data not shown). However, in the presence of 200 μM DTNB, HCB339(pGAN1) produced a smaller swarm than did HCB339(pCS20) (data not shown), indicating that Tsr-T156C was expressed from pGAN1.

We examined the receptor proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction. To avoid receptor methylation, which would have caused multiple receptor bands on the gel, plasmids were transferred into strain CP553 (5), which lacks CheB and CheR, the chemoreceptor-modifying enzymes. Fresh overnight cultures of cells that carried pCS20 or pGAN1 were inoculated (1:100 dilution) into LB medium (17) supplemented with 200 μg of ampicillin per ml and grown at 30°C. After 3 h, 1 mM IPTG was added to each culture and cells were further incubated for 4 h. When whole-cell extracts of both CP553(pGAN1) and CP553(pCS20) were analyzed, the intensity of the Tsr band increased drastically after 4 h of induction, while those of the other bands remained at the same levels (Fig. 4A). We have concluded that cells that carry pGAN1 overproduce Tsr-T156C in the presence of IPTG.

We then examined NEM binding to Tsr-T156C. After full induction, cells were suspended in motility medium (20) and incubated with 50 μM [3H]NEM (0.3 Ci/mmol; Dupont, NEN Research Products) for 30 min at room temperature. The
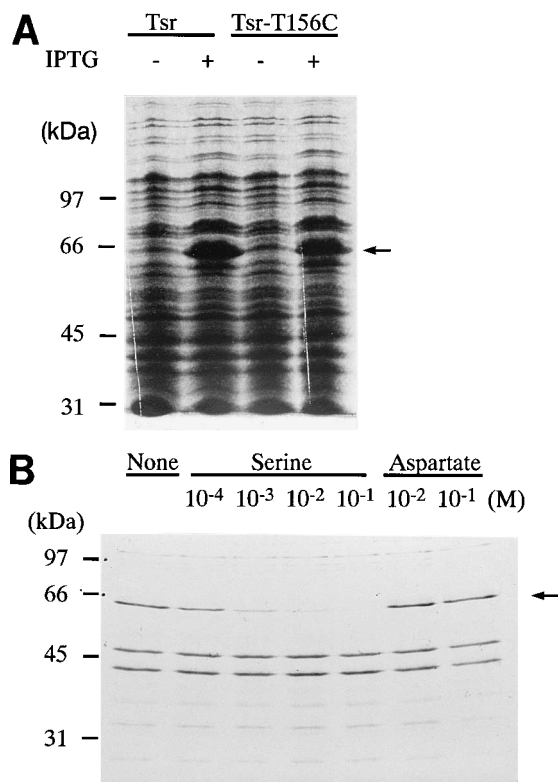
A

B

FIG. 4. Binding of radioactive NEM to Tsr-T156C and protective effect of serine. (A) Overproduction of Tsr-T156C. CP553 cells that carried pCS20 (Tsr) or pGAN1 (Tsr-T156C) were grown in LB medium at 30°C for 3 h (up to early exponential phase) and further incubated for 4 h in the presence (+) or absence (−) of 1 mM IPTG. Total cell extracts were analyzed by SDS-PAGE. Tsr protein is indicated by an arrow in each panel. (B) Binding of radioactive NEM to Tsr-T156C. CP553 cells that overproduced Tsr-T156C were suspended in motility medium supplemented with various concentrations of serine or aspartate and incubated with 50 μM [^{3}H]NEM at 25°C for 30 min. Samples were subjected to SDS-PAGE and fluorography.

reaction was terminated with 5% trichloroacetic acid, and then precipitates were subjected to SDS-PAGE and fluorography (37). The band with an apparent molecular mass of about 60 kDa, i.e., Tsr-T156C, was labeled strongly in the samples of cells that expressed Tsr-T156C (Fig. 4B), whereas no corresponding band was detected in the samples of cells that expressed Tsr (data not shown). The intensity of the NEM-labeled 60-kDa band (Tsr-T156C) decreased as the serine concentration increased (Fig. 4B). In contrast, 100 mM aspartate and 1 M glycerol had no effect. The intensity of the 45-kDa band, which was observed in all samples, was not affected by the addition of serine. Thus, serine competes with NEM only for Tsr-T156C. These results are consistent with the effect of NEM on serine-sensing ability and therefore indicate that Cys-156 (hence, Thr-156 in wild-type Tsr) is located in the serine-binding site.

Interestingly, disulfide bond formation in FlgI, the subunit protein of the flagellar basal body P ring, is essential for flagellation (6). The periplasmic protein DsbA (2, 16) and the cytoplasmic membrane protein DsbB (1, 27) are responsible for this process (6). Therefore, critical Cys residues in FlgI, DsbA, and DsbB may not be readily exposed to DTNB attack. On the other hand, NEM inhibits cell growth (8). We have shown that NEM binds to a considerable number of proteins
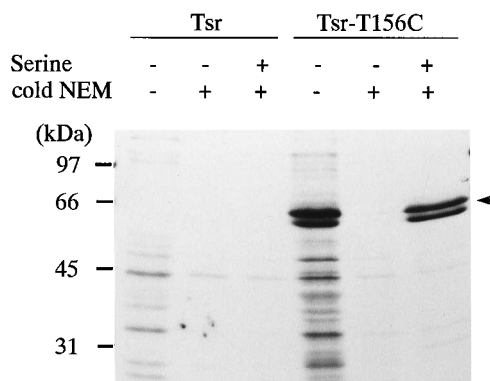

FIG. 5. Selective labeling of Tsr-T156C with radioactive NEM. CP553 cells that overproduced wild-type Tsr or Tsr-T156C were pretreated with 50 μM cold NEM in the presence (+) or absence (−) of 10 mM serine at 25°C for 30 min. After serine and NEM were washed out, cells were incubated with 50 μM [^{3}H]NEM at 25°C for 30 min. Tsr, CP553(pCS20); Tsr-T156C, CP553(pGAN1).

(Fig. 4B and 5). It would be interesting to compare NEM-binding proteins and DTNB-binding proteins.

Selective modification of Tsr-T156C with radioactive NEM. Using the serine protection effect, we selectively labeled Tsr-T156C among all of the membrane proteins in intact cells. Cells were incubated for 30 min with cold NEM in the presence of 10 mM serine. After being washed thoroughly, cells were incubated with radioactive NEM. A protein with an apparent molecular mass of 60 kDa (Tsr-T156C) was labeled strongly (Fig. 5). The only other labeled protein is one just below the 60-kDa band, which is probably a proteolytic product of Tsr-T156C, because it also was protected from NEM modification by serine and its intensity increased upon prolonged incubation. Thus, Tsr-T156C was selectively labeled in vivo with radioactive NEM.

This should provide the basis for a simple purification procedure for the chemoreceptor protein by SH-specific affinity chromatography. The ligand-binding ability of the purified protein could be assayed by competition between the ligand and radioactive NEM, as is the case in vivo. Its further characterization would provide invaluable information about ligand binding and other aspects of the information processing of this receptor.

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