

Role of Threonine Residue 154 in Ligand Recognition of the Tar Chemoreceptor in *Escherichia coli*

LAN LEE AND YASUO IMAE*

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

Received 3 July 1989/Accepted 11 October 1989

The Tar chemoreceptor of *Escherichia coli* mediates attractant responses to aspartate, maltose, and phenol, repellent responses to Ni²⁺ and Co²⁺, and thermoresponses. To understand the role of threonine residue 154, which is located in the ligand-binding domain of Tar, we replaced the residue with serine, isoleucine, and proline by site-directed mutagenesis. The replacements caused reductions in aspartate sensing but had only a small effect on maltose sensing and almost no effect on phenol sensing, repellent sensing, and thermosensing. These results indicate that Thr-154 of Tar is rather specifically involved in aspartate sensing. The reductions in the response threshold for aspartate by the replacements with serine, isoleucine, and proline were less than 1, about 2, and more than 5 orders of magnitude, respectively. When the corresponding threonine residue in the Tsr chemoreceptor was replaced with the same amino acids, roughly similar reductions in the response threshold for serine resulted. Thus, these threonine residues seem to have a common role in detecting the aspartate and serine attractant families. A mechanism by which these chemoreceptors detect the amino acid attractants is discussed.

Bacteria possess chemoreceptors that detect various chemical stimuli in their environments and that generate sensory signals inside the cell which elicit behavioral responses. Four receptors, Tar, Tsr, Trg, and Tap, in *Escherichia coli* have been identified, and their structural genes have been cloned and sequenced (for reviews, see references 9 and 19). Structural analysis of these receptors indicates that they are transmembrane proteins composed of a cytoplasmic and a periplasmic domain connected by a membrane-spanning segment (6, 10). The cytoplasmic domains of all four receptors are very similar, suggesting that the receptors generate a common sensory signal. However, their periplasmic domains differ in sequence, reflecting their ligand-binding specificities.

Among these receptors, Tar and Tsr are the major species and have been studied most extensively. Tar mediates chemotaxis toward attractants such as aspartate and maltose and away from repellents such as Ni²⁺ and glycerol, whereas Tsr mediates chemotaxis toward serine and away from leucine and glycerol (16, 18). Krikos et al. (5) showed that the cytoplasmic domains of Tar and Tsr could be exchanged without affecting their chemoeffector responses, indicating that ligand specificity is determined solely by the periplasmic domain.

To understand the mechanism of ligand recognition by Tar and Tsr, mutants with specific defects in ligand recognition have been analyzed. Wolff and Parkinson (23) reported that mutants with amino acid replacements at three closely spaced arginine residues in the ligand-binding domains of Tar—Arg-64, Arg-69, and Arg-73—showed alterations in aspartate recognition. Lee et al. (8) analyzed the serine-sensing mutants isolated by Hedblom and Adler (2) and showed that some of them contained amino acid replacements at Arg-64 in the ligand-binding domain of Tsr. The amino acid sequences around these arginine residues are similar in Tar and Tsr (6), suggesting that the positive charges of the residues might be involved in detecting a

common part of the aspartate and serine attractant families (8, 23).

The mutant analysis done by Lee et al. (8) showed that besides Arg-64, Thr-156 of Tsr is also involved in the specific recognition of serine. This portion of Tsr is quite similar to that around Thr-154 of Tar (8, 10), which raises the possibility that Thr-154 of Tar is involved in aspartate recognition. In this study, we constructed *tar* mutants with amino acid replacements at Thr-154 by site-directed mutagenesis and found that they indeed had rather specific defects in aspartate sensing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. All bacterial strains used in this work are derivatives of *E. coli* K-12. AB1200 [F⁻ *thr leu his met eda rps* Δ(*tar-tap*)5201 Δ*tsr*-7028] was obtained from M. I. Simon of the California Institute of Technology, Pasadena (5). HCB339 [Δ*tsr*-7021 Δ(*tar-tap*)5201 *trg::Tn10 thr leu his met rpsL136*] was obtained from H. C. Berg of Harvard University, Cambridge, Mass. (22). JM101 for a phage host (12) was obtained from N. Mutoh of Aichi Prefectural Colony Research Institute, Kasugai, Japan. CJ236 (*dut ung*) for the preparation of uracil-containing phages was purchased from Bio-Rad Laboratories (Richmond, Calif.).

A *tar*-carrying plasmid, pAK101 (5), and two types of *tar* (chimeric gene of *tar* and *tas*)-carrying plasmids, pAB157 and pAB160 (5), were obtained from M. I. Simon. A *tsr*-carrying plasmid, pJFG5 (10), was obtained from J. Gebert of the University of Konstanz, Federal Republic of Germany. Phage vectors M13mp18 and M13mp19 were purchased from Nippon Gene Co., Ltd. (Tokyo, Japan).

DNA manipulations and sequencing. Routine DNA manipulations and sequencing were carried out as described previously (8). Restriction enzymes were purchased from Nippon Gene Co., Ltd. A ligation kit and an M13 sequencing kit (Takara Shuzo Co., Kyoto, Japan) were used for all ligation reactions and DNA sequencing. [α-³²P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was used for dideoxy DNA sequencing (12).

* Corresponding author.

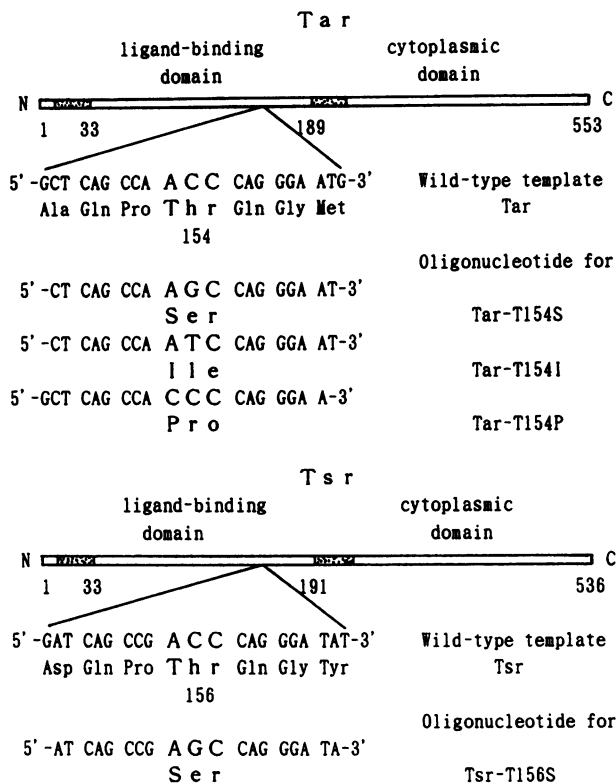


FIG. 1. Oligonucleotides used for site-directed mutagenesis at Thr-154 of Tar and Thr-156 of Tsr. Amino acids are numbered from the N-terminal residue. Putative membrane-spanning regions are indicated (▭).

Site-directed mutagenesis. For the amino acid replacement at Thr-154 in Tar, the *EcoRI-KpnI* fragment of the *tar* gene, which contains the coding sequence around Thr-154 (6), was isolated from pAK101 and subcloned in M13mp19. For replacements at Thr-156 in Tsr, the *HindIII-SalI* fragment of pJFG5, which contains the whole coding region of Tsr (1), was isolated and subcloned in M13mp18. Site-directed mutagenesis was performed by the method of Kunkel et al. (7), as described in the manual for the MUTA-GENE kit (Bio-Rad). Oligonucleotides for the mutagenesis were synthesized at the Center for Gene Research at Nagoya University (Fig. 1). Mutations were verified by sequencing the subcloned fragments as described previously (8). The mutant Tar proteins with amino acid replacements at Thr-154 for Ser, Ile, and Pro were designated Tar-T154S, Tar-T154I, and Tar-T154P, respectively. Similarly, the mutant Tsr protein with Ser at Thr-156 was designated Tsr-T156S.

Each of the *EcoRI-KpnI* fragments of the mutant *tar* genes was then exchanged with the corresponding fragment of the wild-type *tar* gene in pAK101, and the resultant plasmids were named pLAN1, pLAN2, and pLAN4, which encode Tar-T154P, Tar-T154I, and Tar-T154S, respectively. Similarly, the *HindIII-SalI* fragment of the mutant *tsr* gene for Tsr-T156S was exchanged with that of the wild-type *tsr* gene in pJFG5, and the resultant plasmid was named pLSN5.

The plasmids with wild-type and mutant *tar* genes were then transferred into HCB339, which lacks all four chemoreceptors. However, pLSN5 was transferred into AB1200, which lacks *tsr*, *tar*, and *tap*, since the plasmids with other mutant *tsr* genes were in this host (8).

Chemotaxis assays. For routine swarm assays, tryptone

swarm agar (1% tryptone, 0.5% NaCl, 0.35% agar) was used. For the quantitative measurement of the swarming ability of cells with mutant receptors, we used minimal swarm agar (2) consisting of 10 mM potassium phosphate buffer (pH 7.0); 1 mM $(\text{NH}_4)_2\text{SO}_4$; 1 mM MgSO_4 ; 1 mM glycerol; 0.1 mM each threonine, leucine, histidine, and methionine; and 0.3% agar supplemented with 0.1 mM aspartate or maltose. After $2 \mu\text{l}$ of cells (about 4×10^6 cells) was spotted onto the agar, the plates were incubated at 30°C for about 12 h.

For the repellent chemotaxis assays, the chemical-in-plug method of Tso and Adler (20) was used. Cells were suspended in motility medium (pH 7.0) consisting of 10 mM potassium phosphate buffer, 0.1 mM EDTA, and 10 mM lactate supplemented with 0.1 mM methionine and 0.2% agar at 45°C , and the suspension was poured into a plate. Agar plugs containing various concentrations of repellents were inserted into the cell suspension, and the plate was incubated for about 30 min.

Temporal stimulation assays. Temporal stimulation assays for chemoresponses and thermoresponses were carried out as described previously (8). The inverted thermoresponse was measured after the cells were preincubated with various concentrations of aspartate for 20 min at 30°C (13).

For all behavior analyses, cells were grown at 30°C in tryptone broth containing 0.5% glycerol. When necessary, ampicillin (50 $\mu\text{g}/\text{ml}$) was added to the growth medium. Cells were harvested at the late log phase and washed with motility medium as described previously (8). Unless otherwise noted, the motility medium was supplemented with 0.1 mM methionine and the medium pH was 7.0.

Analysis of methylation patterns of receptors. Receptor methylation patterns were measured as described by Oosawa and Imae (16). In brief, cells in motility medium (pH 7.0) supplemented with 200 μg of chloramphenicol per ml were incubated with 3.3 μM [*methyl*- ^3H]methionine (12 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) at 30°C . After 30 min of incubation, aspartate or maltose was added to a final concentration of 10 mM, and the cells were incubated for another 30 min. In the case of stimulation by a repellent, cells were incubated with [^3H]methionine for 58 min, NiSO_4 was added to a final concentration of 0.5 mM, and the incubation was continued for another 2 min. The methylation reaction was terminated by adding trichloroacetic acid to 5%. The methyl-labeled proteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Slonczewski et al. (17).

RESULTS

Mutant preparation. To analyze the role of Thr-154 in the receptor function of Tar, we wanted to introduce amino acid replacements at that position. However, it was difficult to predict at the outset which replacements would prove most informative. In the case of Tsr, the replacements at Thr-156 with Ile, Pro, and Ser altered serine-sensing ability without affecting other receptor functions (8; see below). On the basis of this finding, we replaced Thr-154 of Tar with Ile, Pro, and Ser.

The amino acid replacements were made by site-directed mutagenesis (Fig. 1). The resultant mutant Tar receptors were designated Tar-T154I, Tar-T154P, and Tar-T154S, corresponding to the replacement at Thr-154 with Ile, Pro, and Ser, respectively. The plasmids encoding these mutant receptors were then introduced into strain HCB339, which has all the genes necessary for chemotaxis but which lacks all four chemoreceptor genes (*tar*, *tsr*, *tap*, and *trg*).

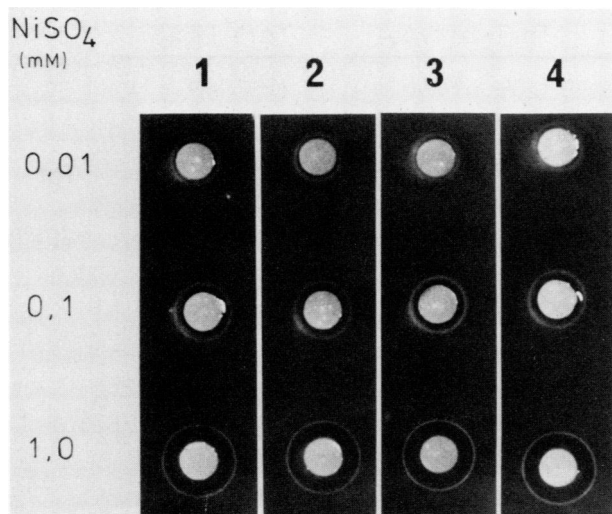


FIG. 2. Repellent chemotaxis in HCB339 cells with various mutant Tar proteins. The chemical-in-plug method was used, and the plugs contained NiSO_4 at concentrations of 0.01, 0.1, or 1.0 mM. Plates were photographed after incubation at 25°C for 20 min. HCB339 cells contained wild-type Tar (panel 1), Tar-T154S (panel 2), Tar-T154I (panel 3), or Tar-T154P (panel 4).

Repellent sensing by mutant Tar. To investigate whether or not the amino acid replacements at Thr-154 of Tar caused the nonspecific alterations in Tar function, we examined the repellent-sensing abilities of mutant Tar by the chemical-in-plug method with Ni^{2+} , a Tar-specific repellent (18). No detectable differences in the sizes of the clearing zones around Ni^{2+} -containing plugs (0.01 to 1.0 mM) were observed among the HCB339 cells with wild-type and mutant Tar receptors (Fig. 2). Similar results were obtained by using plugs with glycerol or Co^{2+} , both of which are also Tar-mediated repellents (16, 18). These results indicate that the replacements at Thr-154 in Tar do not alter the repellent-sensing ability of the chemoreceptor, demonstrating that the mutant receptors retain the ability to generate flagellum-controlling signals.

Aspartate and maltose sensing by mutant Tar. The aspartate- and maltose-sensing abilities of mutant Tar were first analyzed on a swarm plate. It has been shown that aspartate binds directly to Tar, whereas maltose interacts indirectly with Tar via maltose-binding protein (11, 21).

On a swarm plate containing 0.1 mM aspartate, HCB339 cells with wild-type Tar or Tar-T154S produced swarms of similar sizes (Fig. 3A). However, the cells with Tar-T154I produced a smaller swarm, and the cells with Tar-T154P produced swarms comparable in size to those of Tsr control cells, which have no aspartate-sensing ability (Fig. 3A). Thus, the amino acid replacements at Thr-154 of Tar resulted in clear alterations in aspartate sensing.

On a swarm plate containing 0.3 mM maltose, HCB339 cells with wild-type or mutant Tar produced swarms with sharp edges, whereas the control cells with Tsr produced diffuse swarms (Fig. 3B). The swarm produced by the cells with Tar-T154P or Tar-T154S was comparable to that of the wild-type control and was clearly larger than that of the cells with Tsr. Interestingly, the swarm produced by the cells with Tar-T154I was significantly larger than the others. Thus, these amino acid replacements at Thr-154 do not seem to reduce the maltose-sensing ability of Tar.

Temporal assays were used to quantify the aspartate and

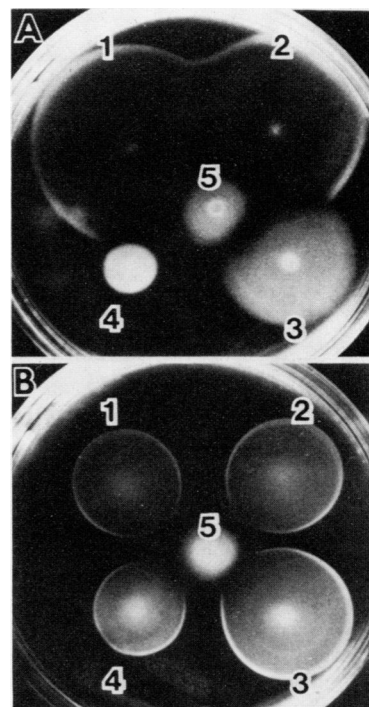


FIG. 3. Swarming abilities of HCB339 cells with various mutant Tar proteins in a swarm plate containing 0.1 mM aspartate (A) or 0.1 mM maltose (B). Plates were incubated for 12 h at 30°C. MCB339 cells contained wild-type Tar (swarm 1), Tar-T154S (swarm 2), Tar-T154I (swarm 3), Tar-T154P (swarm 4), or wild-type Tsr (swarm 5).

maltose responses in these mutant Tar strains. Figure 4 shows the results with aspartate. In the case of HCB339 cells with wild-type Tar, about 0.2 μM aspartate was sufficient to induce the attractant response in about 50% of the cells. In contrast, HCB339 cells with Tar-T154S, Tar-T154I, or Tar-T154P required about 1 μM , 200 μM , or 100 mM for the response in 50% of the cells, respectively. Thus, the different amino acid replacements at Thr-154 exhibited different detection thresholds for aspartate.

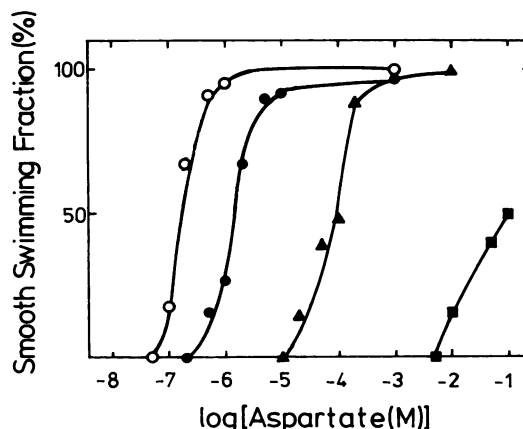


FIG. 4. Aspartate-sensing abilities of various mutant Tar proteins. Cells were stimulated at 25°C with various concentrations of aspartate, and the changes in the fraction of smooth-swimming cells after 30 s were measured. HCB339 cells contained wild-type Tar (○), Tar-T154S (●), Tar-T154I (▲), and Tar-T154P (■).

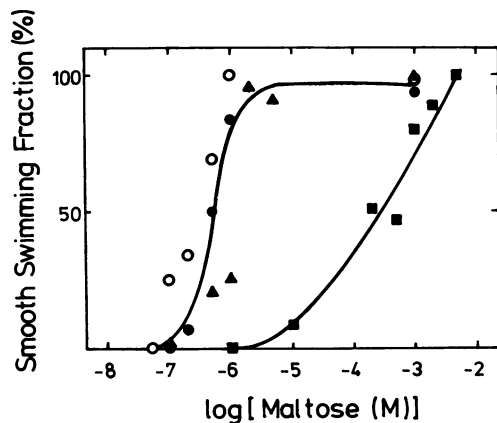


FIG. 5. Maltose-sensing abilities of various mutant Tar proteins. The fraction of smooth-swimming cells was measured as described in the legend for Fig. 4, except that maltose was used instead of aspartate. HCB339 cells contained wild-type Tar (○), Tar-T154S (●), Tar-T154I (▲), and Tar-T154P (■).

Figure 5 shows the results with maltose. HCB339 cells with wild-type Tar, Tar-T154S, or Tar-T154I showed similar response thresholds to maltose, although the overall response times were somewhat different (for 1 μ M maltose, the response times were 150, 120, and 60 s, respectively). In the case of the cells with Tar-T154P, only a small response was observed, even with 1 mM maltose. Thus, the amino acid replacements at Thr-154 also caused alterations in the maltose-sensing ability of Tar, but these defects were much less pronounced than those in aspartate sensing.

Phenol sensing by mutant Tar. Imae et al. (3) reported that Tar mediates the attractant response to phenol. The use of chimeras of Tar and Tsr constructed by Krikos et al. (5) revealed that the ligand-binding domain of Tar is responsible for phenol sensing (data not shown). We have therefore examined the attractant response to phenol in HCB339 cells with wild-type and mutant Tar receptors. All the strains had almost the same response threshold to phenol (about 0.1 mM). Furthermore, for 0.5 mM phenol, the response times of the cells with wild-type Tar, Tar-T154S, Tar-T154I, or Tar-T154P were 58, 57, 50, or 54 s, respectively. Thus, these replacements at Thr-154 of Tar had almost no effect on the phenol-sensing ability of Tar.

Methylation pattern of mutant Tar. Tar undergoes reversible and multiple methylation, and on sodium dodecyl sulfate-polyacrylamide gels it migrates as a set of bands whose positions reflect the various methylation states of the molecules (9, 19). Figure 6 shows the banding patterns of wild-type and mutant Tar. In the absence of any stimulation, all the receptors showed essentially the same pattern, and when the cells were stimulated by 0.5 mM Ni^{2+} , a clear demethylation occurred in both mutant and wild-type Tar. Stimulation by 10 mM aspartate or maltose caused clear increases in the methylation levels of wild-type and mutant Tar, except Tar-T154P. These results are consistent with the behavioral responses to aspartate in the mutants.

Thermosensing by mutant Tar. Thermosensing ability is another important function of Tar (13). HCB339 cells with mutant Tar receptors showed a clear increase in tumbling after a temperature decrease from 35 to 25°C, as in the case of the cells with wild-type Tar, and the time course of the thermoresponse was indistinguishable from that of the wild-type control (data not shown). Thus, neither the excitation

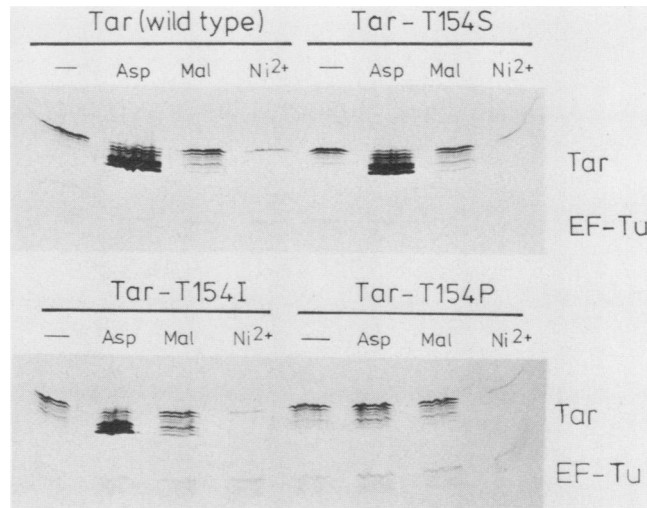


FIG. 6. Methylation pattern of wild-type and various mutant Tar proteins. HCB339 cells with various Tar proteins were incubated with [^3H]methionine at 30°C for 30 min, and then aspartate (Asp), maltose (Mal), or NiSO_4 (Ni^{2+}) was added to a final concentration of 10, 10, or 0.5 mM, respectively. As a control, an equal volume of distilled water (—) was added. The samples were withdrawn after incubation (30 min for attractants or 2 min for the repellent) and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The bands at the lower molecular weight (ca. 43,000) are from elongation factor Tu (EF-Tu).

nor the adaptation ability of Tar in response to the thermal stimulus was affected by these mutations.

The thermosensing ability of Tar is known to be modified by prior adaptation to 0.1 mM or higher concentrations of aspartate, in which case the cells show an inverted thermoresponse (13). Although HCB339 cells with wild-type Tar, Tar-T154S, or Tar-T154I showed the inverted thermoresponse, the minimal aspartate concentrations required for inducing the inverted thermoresponse in these cells were 1×10^{-5} , 5×10^{-5} , and 1×10^{-3} M, respectively. The cells with Tar-T154P failed to show an inverted thermoresponse, even after adaptation to 100 mM aspartate. Thus, the aspartate concentration needed to invert the thermoresponse in the mutants is roughly parallel to the decrease in the aspartate-sensing ability of mutant Tar.

Serine sensing by Tsr-T156S. In a previous report (8), we showed that the amino acid replacements at Thr-156 in Tsr with Ile or Pro caused about 100-fold or more than 10^5 -fold reductions in serine-sensing ability, respectively. We prepared Tsr-T156S, which has a serine replacement at Thr-156, to keep the hydroxyl group at this amino acid position, and we examined its serine-sensing ability. The serine concentration required for inducing the smooth-swimming response in 50% of AB1200 cells with wild-type Tsr or Tsr-T156S was 2×10^{-6} or 6×10^{-6} M, respectively. Furthermore, the ability of Tsr-T156S to sense repellents (acetate [pH 6.0] or glycerol) and temperature were normal (data not shown). Thus, the replacement at Thr-156 with Ser had only a small effect on the functions of Tsr, as in the case of the same substitution at Thr-154 of Tar.

DISCUSSION

The amino acid replacements at Thr-154 of Tar with Ser, Ile, and Pro resulted in various levels of alterations in

aspartate sensing but caused almost no alterations in repellent and phenol sensing. By using chimeras of Tar and Tsr, it was shown that the ligand-binding domain of Tar is responsible not only for aspartate sensing but also for repellent and phenol sensing (6; this paper). Thus, we can conclude that Thr-154 in the ligand-binding domain of Tar is rather specifically required for aspartate sensing. Perhaps Thr-154 of Tar is an important constituent of the aspartate-binding site and is directly involved in detecting aspartate molecules.

Wolff and Parkinson (23) reported that Arg-64 of Tar also has a rather specific role in aspartate sensing. In a previous paper (8), we showed that Arg-64 and Thr-156 in the ligand-binding domain of Tsr are required specifically for the recognition of serine. Thus, it is striking that two common amino acid residues located distantly in the amino acid sequence of the ligand-binding domain of Tar and Tsr are involved in the specific recognition of amino acid attractants. According to a model of the tertiary structure of the ligand-binding domain of Tar proposed by Moe and Koshland (14), a pair of peptide loops extrudes from the cytoplasmic membrane, with Arg-64 and Thr-154 located near the top of each loop. Therefore, as we proposed for serine recognition by Tsr (8), the recognition of aspartate by Tar may also be carried out by interposing aspartate between these two loops, although there is no direct evidence yet that either receptor really has this structure.

The replacements with Ser, Ile, and Pro at Thr-154 resulted in the reduction of aspartate-sensing ability of Tar by less than 1, about 2, and more than 5 orders of magnitude, respectively (Fig. 4). Interestingly, the same amino acid replacements at Thr-156 of Tsr caused similar levels of reduction in serine-sensing ability (8; this paper). The amino acid sequence around Thr-154 of Tar (residues 152 to 156) is exactly the same as that around Thr-156 of Tsr (residues 154 to 158) (Fig. 1). Therefore, as suggested for Arg-64 of Tar and Tsr (8, 23), the threonine residues in question seem to have a common role in detecting the aspartate and serine attractant families. Compared with other substitutions, the substitution for serine at the threonine residue had only a small effect on the receptor functions in Tar and Tsr. The idea that the hydroxyl group at this amino acid position is involved in ligand recognition, perhaps through hydrogen bonding, is an attractive one. Further amino acid replacements at these threonine residues may clarify these points.

In the case of maltose sensing, the amino acid replacements at Arg-64 or Thr-154 had little effect on the response threshold to maltose, except for the substitution of Thr-154 for Pro (23; Fig. 5). These results are consistent with the finding that the response threshold for maltose is mainly determined not by Tar but by the maltose-binding protein (11). On maltose swarm plates, however, cells with any of these mutant receptors formed near-normal swarms (4, 23; Fig. 3B), suggesting that neither Arg-64 nor Thr-154 of Tar is directly involved in interacting with maltose-binding protein. This is consistent with the independence of aspartate and maltose responses (15).

Thermosensing is one of the characteristic properties of Tar (13). This ability was not affected by the amino acid substitutions at Thr-154, indicating that Thr-154 has no role in inducing a proper conformational change of Tar after temperature changes for the thermoresponse. However, for inducing the inverted thermoresponse, the cells with mutant Tar required higher aspartate concentrations than the cells with wild-type Tar. This result is consistent with our previous idea that the increase in the methylation level of Tar

after adaptation to aspartate is responsible for causing the inverted thermoresponse (13).

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