The chemoreceptor Tcp of *Salmonella enterica* serovar Typhimurium can sense citrate and a metal-citrate complex as distinct attractants. In this study, we tried to investigate the molecular mechanism of this discrimination. That citrate binds directly to Tcp was verified by the site-specific thiol modification assays using membrane fractions prepared from *Escherichia coli* cells expressing the mutant Tcp receptors in which single Cys residues were introduced at positions in the putative ligand-binding pocket. To determine the region responsible for the ligand discrimination, we screened for mutations defective in taxis to magnesium in the presence of citrate. All of the isolated mutants from random mutagenesis with hydroxylamine were defective in both citrate and metal-citrate sensing, and the mutated residues are located in or near the α1-α2 and α3-α4 loops within the periplasmic domain. Further analyses with site-directed replacements around these regions demonstrated that the residue Asn67, which is presumed to lie at the subunit interface of the Tcp homodimer, plays a critical role in the discrimination of the metal-citrate complex from citrate or the presence of citrate. Although wild-type *E. coli* has no citrate transport system and cannot respond to citrate, *E. coli* cells expressing Tcp as a sole chemoreceptor respond to citrate (13, 14). This suggests that the response to citrate does not require citrate transport or a periplasmic binding protein, unlike *E. coli* taxis to maltose, which requires maltose-binding protein (MBP) in addition to the chemoreceptor Tar (15–17), although there is no direct evidence that Tcp directly binds citrate. A remarkable feature of Tcp is that it can sense a divalent cation, including magnesium and calcium ions, as an attractant only in the presence of citrate, even after Tcp adapts to citrate. Therefore, Tcp has been considered to recognize a metal ion in the presence of citrate or a metal-citrate complex as an attractant distinct from citrate (hereafter, this attractant will be referred to as metal-citrate for simplicity).

Among all of the homologous chemoreceptors, Tar (for aspartate) and Tsr (for serine) have been studied extensively with respect to ligand recognition sites (18–30). The chemoreceptors are homodimeric proteins with a subunit molecular mass of ~60 kDa. Each subunit consists of an amino-terminal cytoplasmic region, a first transmembrane segment (TM1), a periplasmic ligand-binding domain, a second transmembrane segment (TM2), and a carboxyl-terminal cytoplasmic signaling/adaptation domain (5, 8, 10–12). The residues of Tar and Tsr involved in ligand binding are located around the apex of the two helices (α1 and α4) spanning the cytoplasmic membrane as TM1 and TM2, respectively (Fig. 1 and Refs. 27 and 28). In the previous study, we substituted Cys for the four conserved basic or polar residues, as well as three other basic ones, in the Tcp periplasmic domain, which is otherwise devoid of Cys (14). The results demonstrated that the five residues (Arg63, Arg68, Arg72, Lys75, and Tyr150) are involved in the recognition of citrate. However, none of the residues mutated seemed to be involved in the specific recognition of divalent cations in the presence of citrate. Therefore, the molecular mechanism of metal-citrate recognition by Tcp remained unknown.

In this study, we addressed this question. First, we demonstrated direct binding of citrate to the Cys-replaced Tcp proteins by examining protection of Cys by the ligand from thiol modification (31). Next, we isolated and characterized mutants deficient in taxis toward magnesium in the presence of citrate. Considering the results obtained, we then performed site-directed mutagenesis. The substitution of Cys, Ala, or Ser for Asn67 deteriorated metal-citrate sensing without impairing citrate sensing, suggesting that the Asn residue is important for discrimi-
nating metal-citrate from citrate alone. Based on these results, we propose models for the ligand discrimination that involves cooperative interaction of the two subunits of the Tcp homodimer.

EXPERIMENTAL PROCEDURES

Chemicals—Citric acid and 5'-iodoacetamido-}flourescein (IAF) were purchased from Nacalai Tesque Inc. (Kyoto, Japan) and Molecular Probes, Inc. (Eugene, OR), respectively. Enzymes for DNA manipulation were the products of Takara Shuzo Co. (Otsu, Japan).

Bacterial Strains and Plasmids—The E. coli strain HCB339 (Δtsr-7021 Δ(tar-tap)5201 trg::Tn10 thr leu his rpsL136) (32), which lacks all four chemoreceptors (Tar, Tsr, Trg, and Tap), was used as a plasmid host in chemotaxis assays. Strain HCB436 (Δtsr-7021 Δ(tar-cheB)2234 Δtrg-100 zbd::Tn5 thr leu his rpsL136) (33), which lacks CheB and

FIGURE 1. Structures of ligand-binding sites of the bacterial chemoreceptors. A, alignment of the amino acid sequences around the ligand-binding site of the bacterial chemoreceptors of Salmonella (indicated with subscript S) and E. coli (indicated with subscript E). Bold letters indicate the residues involved in recognition of citrate that have been studied in the previous work (14). Underlined letters of Tcp indicate residues involved in the ligand recognition, which was demonstrated by random mutagenesis in this study. Closed circles indicate the residues replaced by site-directed mutagenesis in this study. Numbering of residues is for Tcp. B, three-dimensional structure near the aspartate-binding site of Salmonella Tar (Protein Data Bank code 2LIG) with different shades of gray for the subunits. Bound aspartate is shown as a ball-and-stick representation. The residues corresponding to those involved in ligand recognition in Tcp are represented by Corey-Pauling-K representations of Cα atom. The indicated number is for Tcp.
Figure 2. Labeling of the Cys-replaced Tcp receptors with the thiol-specific fluorescent dye IAF. Membrane fractions prepared from HCB436 cells expressing Cys-replaced Tcp were incubated with 50 μM IAF for 5 min at 19 °C in the presence or absence of 100 mM citrate. The denaturation treatment with heat and SDS were performed prior to the termination of the reactions. The samples were subjected to SDS-PAGE (15% acrylamide). Bands labeled with IAF were visualized with a lumino-image analyzer. The bands corresponding to Tcp proteins are indicated by an arrow. WT, wild type.

### RESULTS

Citrate Binds Directly to Tcp—It has not been unambiguously determined whether citrate binds directly to the Tcp protein like aspartate to Tar or through a certain binding protein like maltose to Tar through MBP. To test these possibilities, we employed the site-specific thiol modification assay, in which a ligand competes with a thiol-modifying reagent for a unique Cys residue in the ligand-binding pocket (24–26, 31). In the previous study (14), we introduced a Cys residue at various positions (R63C, R68C, R72C, K75C, R78C, Y150C, and K157C) of the periplasmic domain of Tcp, which is otherwise devoid of Cys, and examined the citrate sensing capabilities of the resulting mutant Tcp receptors; the R78C and K157C receptors mediate essentially wild-type responses to citrate, but the other mutant receptors mediate weaker responses. These mutant receptors, as well as wild-type Tcp, were expressed in strain HCB436 (33), which lacks all four chemoreceptors, the methyltransferase CheR, and the methyltransferase CheB. Membranes of the resulting strains were prepared and subjected to the modification of thiol-specific fluorescent dye (IAF) in the presence or absence of various concentrations of citrate (Fig. 2). In the absence of citrate, bands specific to the R72C, K75C, and K78C receptors were detected clearly, whereas no corresponding band was observed for wild-type Tcp. The R68C, Y150C, and K157C receptors gave only faint bands, whose intensities did not increase upon treatment with SDS and heat. The faster migration of the R63C protein than the other mutant Tcp proteins in the gel (14) made it impossible to discriminate the receptor band from nonreceptor bands. The addition of 0.1M citrate significantly decreased the intensity of the band of the R78C receptor, but not those of the R72C and K75C receptors, demonstrating that citrate competes with IAF for the thiol group of Cys.

### Site-directed Mutagenesis of tcp

—Site-directed mutagenesis was performed by a two-step PCR (35) as described previously (36). The PCR products were cloned into the pSU18-based plasmid pSN19 carrying the tcp gene, in which cytosine at 702 is replaced by adenine to introduce a BglII restriction site in the middle of the gene. The resulting plasmids were transformed into the E. coli strain HCB436 expressing wild-type Tcp. The nucleotide sequences were determined whether citrate binds directly to the Tcp protein like aspartate to Tar or through a certain binding protein like maltose to Tar through MBP. To test these possibilities, we employed the site-specific thiol modification assay, in which a ligand competes with a thiol-modifying reagent for a unique Cys residue in the ligand-binding pocket (24–26, 31). In the previous study (14), we introduced a Cys residue at various positions (R63C, R68C, R72C, K75C, R78C, Y150C, and K157C) of the periplasmic domain of Tcp, which is otherwise devoid of Cys, and examined the citrate sensing capabilities of the resulting mutant Tcp receptors; the R78C and K157C receptors mediate essentially wild-type responses to citrate, but the other mutant receptors mediate weaker responses. These mutant receptors, as well as wild-type Tcp, were expressed in strain HCB436 (33), which lacks all four chemoreceptors, the methyltransferase CheR, and the methyltransferase CheB. Membranes of the resulting strains were prepared and subjected to the modification of thiol-specific fluorescent dye (IAF) in the presence or absence of various concentrations of citrate (Fig. 2). In the absence of citrate, bands specific to the R72C, K75C, and K78C receptors were detected clearly, whereas no corresponding band was observed for wild-type Tcp. The R68C, Y150C, and K157C receptors gave only faint bands, whose intensities did not increase upon treatment with SDS and heat. The faster migration of the R63C protein than the other mutant Tcp proteins in the gel (14) made it impossible to discriminate the receptor band from nonreceptor bands. The addition of 0.1M citrate significantly decreased the intensity of the band of the R78C receptor, but not those of the R72C and K75C receptors, demonstrating that citrate competes with IAF for the thiol group of Cys. These results indicate that Tcp binds to citrate directly and that Arg is located in the citrate-binding pocket of Tcp.

| TCP Mutant | Citrate Response |
|------------|------------------|
| WT         | + + + + + + + + + + |
| R63C       | + + + + + + + + + + |
| R68C       | + + + + + + + + + + |
| R72C       | + + + + + + + + + + |
| K75C       | + + + + + + + + + + |
| K78C       | + + + + + + + + + + |
| Y150C      | + + + + + + + + + + |
| K157C      | + + + + + + + + + + |

### Assays of Chemotactic Responses

HC8339 cells expressing Tcp were grown at 30 °C in TGM medium (1% tryptone, 0.5% NaCl, 0.5% glycerol) supplemented with 25 μg/ml chloramphenicol. The cells were harvested in the late exponential phase, washed with KP motility medium (10 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 10 mM sodium di-lactate, 0.1 mM methionine), and resuspended in motility medium. If necessary, MOPS motility medium (20 mM MOPS-NaOH buffer, pH 7.0, 0.1 mM EDTA, 10 mM sodium di-lactate, 0.1 mM methionine) was used instead of KP motility medium. Temporal stimulation assays were carried out as described previously (37). Capillary accumulation assays were performed according to the method of Adler (38). When the responses to magnesium were examined, the cells were treated with citrate prior to the assays.

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3 H. Sakamoto, H. Tajima, D. Shiomi, T. Iwama, M. Homma, and I. Kawagishi, unpublished data.
Metal-Citrate Recognition of the Citrate Chemoreceptor

In the previous work (14), we studied the sensing abilities of the Cys-replaced Tcp receptors toward citrate. Here, we first examined the metal-citrate sensing abilities of these mutant receptors. Each mutant Tcp protein was expressed in the receptorless E. coli strain HCB339 (ΔMCP), and then the resulting cells were examined for the abilities of responding to citrate alone and magnesium in the presence of citrate by the capillary accumulation assay (Fig. 3). The results with citrate alone were consistent with those of the temporal stimulation assay (14). The magnesium-citrate sensing abilities of these mutant receptors were consistent with those of the temporal stimulation assay (14). The theoretically, a mutant receptor would mediate responses to both citrate and magnesium-citrate, as shown in Fig. 3. The responses almost exactly to their citrate sensing abilities. The clones that swarmed much more strongly in the presence of citrate by the capillary accumulation assay (Fig. 3). The results with citrate alone were consistent with those of the temporal stimulation assay (14). The magnesium-citrate sensing abilities of the mutant receptors corresponded almost exactly to their citrate sensing abilities. Theoretically, a given mutant receptor would mediate responses to both citrate and metal-citrate (this phenotype will be referred to as Cit⁺ Mec⁻), citrate only (Cit⁺ Mec⁺), metal-citrate only (Cit⁻ Mec⁺), or none (Cit⁻ Mec⁻). However, no Cys-replaced mutant showed Cit⁺ Mec⁻ or Cit⁻ Mec⁺ phenotype. These results suggest that the residues Arg⁶¹, Arg⁶⁸, Arg⁷², Lys⁷⁵, and Tyr¹⁵⁰ are important for the recognition of both attractants but not involved in the discrimination between them.

Random Mutagenesis with Hydroxylamine and Isolation of Mutants Deficient in Chemotaxis toward MgCl₂—To obtain an insight into the ligand discrimination mechanism, we carried out random mutagenesis of the tcp gene to isolate mutant(s) specifically defective in the recognition of metal-citrate. We first developed the following screening system. A slip of filter paper containing 1 mM MgCl₂ was placed in the middle of a minimal semisolid agar plate supplemented with 25 μg of chloramphenicol/ml, and then the plate was incubated at 30 °C. In minimal semisolid agar, a slip of filter paper containing citrate or MgCl₂ was placed in the middle of the plate prior to inoculation. Then HCB339 cells transformed with a library of randomly mutared tcp-carrying plasmids were inoculated at both sides of the filter with sterile toothpicks (Fig. 4C). Cells expressing wild-type Tcp swam toward higher concentrations of magnesium, forming a colony of biased shape. Cells lacking Tcp formed a small condensed colony, whereas cells with the R68C receptor formed a more diffused round-shaped colony presumably because their run/tumble bias was better suited to spread out in an agar matrix than the former cells, which cannot tumble (Fig. 4C). In the absence of citrate, cells with any Tcp receptor showed a small diffused round-shaped colony (data not shown). E. coli cells consume various nutrients in their environments and produce wastes, resulting in generation of their gradients around growing colonies. However, no gradient of citrate can be formed because E. coli cannot utilize citrate. Therefore, only a gradient of magnesium or a magnesium-citrate complex can be generated by diffusion from the slip of filter paper. In tryptone semisolid agar, cells expressing the R68C receptor or wild-type Tcp swam in response to repellents and unidentified chemoattractant(s) that are produced during growth (Fig. 4A and Ref. 14). Therefore, it is considered that the difference in colony shape between cells expressing the R68C receptor and those expressing wild-type Tcp may be related to the ability of sensing magnesium or magnesium-citrate. Thus, we concluded that this assay can be applicable to screening for mutants defective in the recognition of magnesium-citrate.

The plasmid carrying the tcp gene was treated with 0.4 M hydroxylamine and introduced into the receptorless E. coli strain HCB339. About 14,000 transformants were screened for the defect in swarming toward Mg²⁺ in the presence of citrate. The candidates were further subjected to swarming assays with tryptone semisolid agar in the absence of citrate to examine whether the mutants retained general receptor functions such as repellent sensing, transmembrane signaling, and adaptation. The clones that swarmed much more slowly than the parental strain (expressing wild-type Tcp) were excluded. Consequently, 25 independent clones were obtained as mutants defective in chemotaxis to magnesium-citrate.

The nucleotide sequences of the mutant tcp genes revealed that all of the nucleotide changes were GC to AT transitions, which are expected for random mutagenesis with hydroxylamine (39). Among them, one clone had a single amino acid substitution (G339E) in the cytoplasmic domain of Tcp that causes a tumbling behavior (i.e. the activation of CheA kinase), and therefore was not studied further. It should be noted that Gly⁵⁸⁰ of Tar, which is a positional equivalent of Gly⁵⁸⁰ of Tcp, has互协
Metal-Citrate Recognition of the Citrate Chemoreceptor

Mutants deficient in chemotaxis toward magnesium in the presence of citrate
Mutants were screened using a minimal semisolid agar with a gradient of attractant concentration. A mutant (G339E) that conferred constantly tumbling behavior to the cells was excluded from the table. Silent mutations are shown in parentheses. These mutants may not be independent.

| Mutant | Codon number | Codon change | Mutant number |
|--------|--------------|--------------|---------------|
| Periplasmic domain | | | |
| R63C | 63 | CGC → TGC | 745, 515 |
| R63H | 63 | CGC → CAC | *768, *769, 799 |
| R63H/G279S | 63, 279 | CGC → CAC, GGC → AGC | 782 |
| T62I/R63C | 62, 63 | ACC → ATC, GGC → TGT | 805 |
| R68C | 68 | CGC → TGC | 727, 643 |
| R68H | 68 | CGC → CAC | 557 |
| R68H/A119T | 68, 119 | CGC → CAC, GGC → ACG | 806 |
| A70V | 70 | GGC → GTG | 743 |
| G151D | 151 | GGC → GAC | 697, 725, 819 |
| G151N/L203F | 151, 203 | GGC → AAC, CTC → TTC | 795 |
| Transmembrane domain | | | |
| G25R | 25 | GGA → AGA | 751, 761, 593 |
| G25R/Ala70 | 25 | GGA → AGA, (GCC → GCA) | 772 |
| Cytoplasmic domain | | | |
| S261L | 261 | TCG → TTG | 460 |
| S261L/Ala289 | 261 | TCG → TTG, (GCC → GCT) | 774 |
| S261L/Leu296, Ser280 | 261 | TCG → TTG (CTC → CTT, AGC → AGT) | 543 |

* The cells were incubated for 5 h in the presence of 10 mM citrate to adapt it to prior to stimulation with MgCl2.

been demonstrated to play a key role in on-off switching of CheA kinase (40). Sixteen clones had missense mutations within the periplasmic domain, and twelve of them carried single mutations (Table 1). Many of them cause substitutions at Arg68 and Arg68, both of which are responsible for the recognition of citrate as demonstrated in the previous work (14). In addition, two novel mutations (A70V and G151D) were identified. These were further examined for their ligand sensing abilities by the temporal stimulation assay. Neither mutation affected the swimming pattern in the absence of any chemotactic stimulus or the repellent response to glycerol. However, the G151D mutation abolished the ability of magnesium-citrate sensing, whereas the A70V mutation caused a milder defect (Table 2). Similar results were obtained for the citrate sensing ability (Table 2). These results indicate that the two residues (Ala70 and Gly151) are involved in the recognition of both citrate and metal-citrate by Tcp.

Thus, we could not identify residue(s) responsible for the discrimination between the two attractants in this screening. However, all of the mutations obtained here are mapped in or near the α1-α2 and α3-α4 loops. In the crystal structures of the aspartate chemoreceptor Tar of Salmonella (27, 28) and E. coli (29), the corresponding regions are located near the apex of the receptor dimer and constitute two symmetrical aspartate-binding pockets. Therefore, we reasoned that, if citrate and metal-citrate share essentially the same binding pocket in Tcp, the α1-α2 and α3-α4 loops must contain residue(s) that interact specifically with metal-citrate. Alternatively, if a magnesium ion binds to a site outside of the citrate-binding pockets that is created upon a conformational change induced by citrate binding, the α1-α2 and α3-α4 loops must not be directly involved in the metal recognition, although they are essential for the citrate recognition.

The replacement of residues Gly25 and Ser261 caused a defect in taxis toward magnesium-citrate (Table 1). However, these residues are presumed to be located in the transmembrane segment and the cytoplasmic domain, respectively, and therefore, they are unlikely to be directly involved in the ligand recognition. In fact, the replacement of the cytoplasmic domain (residues 257–547) of Tcp by the corresponding sequence of Tar did not affect citrate and magnesium-citrate sensing abilities (data not shown).

Site-directed Mutagenesis of the α1-α2 and α3-α4 Loops—Next, we systematically substituted 14 polar, acidic, or basic residues in the α1-α2 and α3-α4 regions (Fig. 1 and Table 3). All of the resulting mutant Tcp receptors were expressed in HCB339 cells. Their expression levels were almost the same as that of wild-type Tcp as judged from immunoblotting with anti-receptor serum (data not shown). Moreover, disulfide-cross-linked dimers were detected in three (N67C, N76C, and N146C) of the six Cys-replaced Tcp receptors in nonreducing SDS-PAGE (data not shown); the N67C and N146C receptors gave small amounts of cross-linked dimers, and approximately half of the total amount of the N76C receptor was cross-linked. However, cross-linking at position 76 did not affect the receptor function (see below).

We examined general receptor functions of the mutated Tcp proteins by the swarm assay (Table 3). HCB339 cells expressing any mutant Tcp receptor other than the N146C receptor swarmed as fast as cells expressing wild-type Tcp. Cells expressing the N146C receptor formed a smaller swarm, which is at least partly accounted for by their slower growth rate (data not shown).

We next examined the citrate sensing capabilities of the mutant Tcp receptors by the temporal stimulation assay (Table 3). In the absence of any stimulus, cells expressing any mutant Tcp receptor swarm smoothly. When 12.5% glycerol (a repellent) was added, they showed tumbling responses similar to those of cells expressing wild-type Tcp, indicating that all of these proteins retain normal repellent sensing abilities. When 10 mM citrate were added together with 12.5% glycerol, cells expressing the E61A, N67C, N76C, D79N, H140A, K143A, D145A, N152C, or D160N/D161N receptor showed similar smooth swimming responses to those of cells expressing wild-type Tcp (i.e., they showed Cit− phenotypes). However, the T62I, N146C, Y147C, and Q156C receptors mediated significantly weaker responses (i.e., they showed Cit− phenotypes).
Metal-Citrate Recognition of the Citrate Chemoreceptor

TABLE 3
Sensing abilities of the mutated Tcp receptors

| Receptor | Tryptone swarming ability | Behavior of cells expressing the receptor |
|----------|--------------------------|------------------------------------------|
|          |                          | Response                                  | Response*                        |
|          |                          | 12.5% Glycerol | 10 mM Citrate | 12.5% Glycerol | 10 mM MgCl₂ |
| Wild type | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| E61A     | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| T62I     | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| N67C     | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| N76C     | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| D79N     | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| H140A    | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| K143A    | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| D145A    | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| N146C    | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| Y147C    | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| N152C    | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| Q156C    | ++ ++ ++ ++               | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |
| D160N/D161N | ++ ++ ++ ++       | ++ ++ ++ ++ | ++ ++ ++ ++ | ++ ++ ++ ++ |

* The cells were incubated for 5 h in the presence of 10 mM citrate to adapt to it prior to stimulation.

These results suggest that the residues Thr<sup>62</sup>, Asn<sup>146</sup>, Tyr<sup>147</sup>, and Gln<sup>156</sup> are involved in citrate sensing.

We then examined the abilities of the mutant Tcp receptors to sense magnesium-citrate (Table 3). Cells expressing each mutant Tcp receptor were allowed to adapt to 10 mM citrate and were then stimulated with 12.5% glycerol. Cells expressing any mutant Tcp receptor showed tumbling responses similar to those of cells expressing wild-type Tcp. When 10 mM MgCl₂ were added simultaneously with 12.5% glycerol after pretreatment by 10 mM citrate, the T62I, N67C, N146C, Y147C, and Q156C receptors mediated significantly weaker smooth swimming responses than those mediated by wild-type Tcp (i.e. they showed Mec<sup>−</sup> phenotypes), whereas the other receptors mediated essentially wild-type responses (i.e. they showed Mec<sup>+</sup> phenotypes). These results suggest that the residues Thr<sup>62</sup>, Asn<sup>146</sup>, Tyr<sup>147</sup>, and Gln<sup>156</sup> are involved in magnesium-citrate sensing. Among them, Asn<sup>67</sup> is suggested to be involved in discriminating magnesium-citrate from citrate alone because the N67C substitution did not affect the citrate sensing ability.

In the temporal stimulation assay, the ability of metal-citrate sensing was difficult to examine precisely because glycerol could not cause a sufficient repellent response because of the presence of a high concentration (10 molar) of citrate. To circumvent this difficulty, we carried out the capillary accumulation assay (38) to further examine chemotactic responses of cells expressing each mutant Tcp receptor. The results of the capillary assay with citrate agreed well with those of the temporal stimulation assay (Fig. 5A), except that cells expressing the N76C receptor showed a slightly weaker chemotaxis toward citrate. In contrast, the N67C and N146C receptors showed severe defects in the chemotaxis toward magnesium in the presence of citrate (Fig. 5B). The T62I, N76C, and N152C receptors showed weaker chemotaxis toward magnesium in the presence of citrate. The Y147C and Q156C receptors showed almost the same accumulations at high concentrations (above 10 mM) of magnesium in the presence of citrate as those expressing wild-type Tcp but exhibited greatly reduced accumulations at magnesium concentrations below 1 mM. In the absence of citrate, the cells expressing wild-type or any mutant Tcp did not accumulate into capillaries containing magnesium alone (data not shown). These results confirmed that residue Asn<sup>67</sup> is important for discriminating magnesium-citrate from citrate alone.

Asn<sup>67</sup> of Tcp corresponds to Ser<sup>68</sup> of *Salmonella* Tar, which has been identified as an allosteric switch (41). The latter residue is located at the subunit interface of the Tar homodimer and mediates the cooperativity through the contact with the same residue in the partner subunit. Substitutions at this position alter the type and degree of ligand binding cooperativity of the two ligand-binding sites, and the side chain size seems most critical (41). If Asn<sup>67</sup> of Tcp plays a similar role, the cooperativity of the two ligand-binding sites might be involved in the discrimination between citrate and metal-citrate. So, we examined how various amino acids at this position affect the citrate and metal-citrate sensing abilities. In the swarm assay, HCB339 cells expressing any mutant Tcp receptor swarmed as fast as cells expressing wild-type Tcp (data not shown). The capillary assay demonstrated that substitutions at position 67 affect the citrate and metal-citrate sensing abilities in differential manners. The results can be summarized as follows. The mutated Tcp receptors fell into either of the four possible
phenotypes: (i) Cit\(^+\) Mec\(^+\) (N67Q, N67T, N67V, N67L, and N67I; Fig. 6, A–C); (ii) Cit\(^-\) Mec\(^-\) (N67A, N67C, and N67S; Fig. 6, D–F); (iii) Cit\(^-\) Mec\(^+\) (N67D and N67E; Fig. 6, G–I); and (iv) Cit\(^-\) Mec\(^-\) (N67K, N67R, N67F, and N67Y; Fig. 6, G–I). Defects in either citrate or metal-citrate sensing are characteristic to substitutions at this position. These results suggest that Asn67 plays a critical role in the discrimination between citrate and metal-citrate.

**DISCUSSION**

The protection of the Tcp-R78C protein from thiol modification by citrate demonstrates unambiguously that citrate binds to Tcp directly, not through a periplasmic binding protein. This also showed that residue Arg78 is located in the citrate-binding pockets, but the replacement of Arg78 by Cys does not influence the citrate sensing ability at all (14). Therefore, this residue is considered not to interact directly with citrate but just sits underneath it in the citrate-binding site.

We introduced various mutations into Tcp by using random mutagenesis and site-directed mutagenesis. Most of the mutations isolated, i.e. those at the residues Thr62, Arg63, Arg68, Ala70, Arg72, Lys75, Asn76, Asn146, Tyr147, Tyr150, Gly151, and Gln156, cause Cit\(^+\) Mec\(^-\) phenotypes, indicating that these residues are involved in the sensing of both citrate and metal-citrate and therefore responsible for the recognition of citrate.

By contrast, the substitutions of Cys for Asn67 showed a Cit\(^-\) Mec\(^-\) phenotype. This is the first demonstration that the ability to sense these two attractants can be genetically dissected, and the residue Asn67 is supposed to be critical for the discrimination between metal-citrate and citrate alone. A simple explanation is that this residue interacts specifically with the metal moiety of the metal-citrate complex but not with citrate itself.

Asn67 of Tcp corresponds to Ser68 of Tar, which lies at the subunit interface of the homodimer and interacts with aspartate via a water
molecule (28). It has been considered that the interaction of the partner subunits at this position is responsible for the ligand binding cooperativity and hence allosteric switching (41). In *E. coli* Tar, the half-of-the-sites cooperativity has been considered to contribute to differential sensing of aspartate and the maltose-MBP complex (43). The fact that the N67C receptor forms a disulfide cross-linked dimer suggests that Asn67 of Tcp is located at or near the subunit interface. Introduction of various substitutions at this position variously altered citrate and magnesium-citrate sensing; the Ala, Cys, and Ser substitutions resulted in Cit$^+$/H11001 Mec$^-$/H11002 phenotypes, and the Gln, Thr, Val, Leu, and Ile substitutions resulted in Cit$^+$/H11001 Mec$^+$/H11001 phenotypes. In Tar, it has been shown that the size (or volume) of the side chain at residue 68 correlates with Hill coefficient; positive cooperativity increases with an increase in the side chain volume (41). In our results, substitutions of amino acids with small side chains result in Cit$^+$ Mec$^-$ phenotypes. Thus, changes at position 67 of Tcp might disrupt the normal cooperative interaction directly or through adjacent residues. If so, Asn$^{67}$ may not interact directly with the metal moiety of the metal-citrate complex. Indeed, the Val, Leu, and Ile receptors mediated normal responses to magnesium-citrate, suggesting that the polar functional group at position 67 is not required for the metal-citrate sensing.

The mutant receptors with acidic amino acids at position 67 showed Cit$^-$ Mec$^+$ phenotypes. This agrees well with the results with Tar (41); the replacement with Asp or Glu decreases the aspartate-binding affinity of Tar presumably because of the electrostatic repulsion. In Tcp, however, such an electrostatic repulsion, if any, must be compensated by a metal ion such as Mg$^{2+}$ because the N67D and N67E receptors mediated normal responses to magnesium-citrate, although they could not sense Mg$^{2+}$ in the absence of citrate (data not shown). Therefore, it is suggested that Tcp recognizes a metal ion as a complex with citrate.

If Asn$^{67}$ of Tcp is involved in cooperative interaction, how does it contribute to the discrimination of citrate and metal-citrate? In Tar, binding of an aspartate molecule to one of the two symmetrical binding pockets induces a slight conformational change of the other binding pocket to reduce its volume so that another aspartate molecule cannot be accommodated (42). We propose that in Tcp, a similar conformational change might create a binding site for metal-citrate. Namely, citrate binding to one of the two binding sites would alter the shape of the other binding pocket, the induced conformation of which accommodates metal-citrate but not citrate alone (Fig. 7, model A).

It is also possible that a metal ion binds to citrate that is already bound to one of the two binding sites to modify the conformation of the bound citrate, which then modifies the conformation of the ligand-binding domain. Through the intersubunit interaction, this leads to the conformational change of subunit 2 to elicit a signal distinct from that produced upon binding citrate alone (Fig. 7, model B). Tcp can sense metal-citrate even in the presence of a saturating concentration of citrate and the citrate-adapted (methylated) Tcp receptor can mediate a considerable attractant response to metal-citrate and adapt to it. It is therefore reasonable to assume that the metal ion binding to the bound citrate would impose a conformational change distinct from that of the citrate-bound Tcp dimer. As in the

**FIGURE 7. Two models for the mechanism of differential sensing by Tcp.** The periplasmic ligand-binding domain of the Tcp homodimer is shown. When Tcp binds citrate at one of the two sites, it transmits the signal into the cytoplasm through subunit 1 (panel II). Concomitantly, a conformational change of the opposing site is induced to prevent an additional binding of citrate (negative cooperativity). Model A postulates that the metal-citrate complex can bind to this site (panel IIIa), and the signal is generated through subunit 2 (panel IVa). In contrast, model B postulates that a metal ion binds to the citrate molecule that is already bound to Tcp (panel IIIb), triggering a conformational change of the bound citrate, which then modifies the conformation of the ligand-binding domain. Through the intersubunit interaction, this leads to the conformational change of subunit 2 to elicit a signal distinct from that produced upon binding citrate alone (panel IVb).
first model, metal-citrate signaling is proposed to involve cooperative conformational change(s) of the Tcp dimer. Namely, metal binding to the bound citrate would generate a signal through the subunit of the Tcp dimer other than the one that has already committed signal generation triggered by the bound citrate (Fig. 7, model B).

In either case, it should be noted that the replacements of Arg\textsuperscript{72}, Lys\textsuperscript{75}, Tyr\textsuperscript{147}, and Gln\textsuperscript{156} impair responses to metal-citrate more drastically than to metal-citrate. Thus, these residues may be more important for citrate sensing than metal-citrate sensing. This may also imply that the configuration of citrate in complex with metal ion is slightly different from that of citrate alone when they are bound to Tcp. The metal-citrate-binding site may be located slightly closer to the cytoplasmic membrane than that of citrate because residues Arg\textsuperscript{72}, Lys\textsuperscript{75}, and Tyr\textsuperscript{147} are assumed to lie at positions more distal from the cytoplasmic membrane than the residues Arg\textsuperscript{63} and Arg\textsuperscript{68}, the substitutions of which cause severe defects in sensing of metal-citrate as well as citrate alone.

Interestingly, in Tar, aspartate binding is considered to trigger a displacement of the residue Arg\textsuperscript{73} of Tar, corresponding to Arg\textsuperscript{72} of Tcp, so that its side chain covers the unoccupied aspartate-binding site (44). In Tcp, Arg\textsuperscript{72} within the unoccupied site might block the additional binding of the residue Arg\textsuperscript{73} of Tar, so that its side chain does not cover the unoccupied aspartate-binding site (44). In any case, structural analyses of Tcp are required to test this model.

In brief, we propose that discrimination of the two attractants, citrate and metal-citrate, a remarkable feature of Tcp, is based on cooperative interactions of the two substrates of the Tcp homodimer, such as allosteric switching of the two citrate-binding sites. To our knowledge, this type of ligand recognition mechanism has not been reported. However, binding of two different ligands to opposite sides of the homodimer is reminiscent of E. coli Tar; aspartate and the maltose-MBP complex can bind simultaneously to opposite sides of the Tar homodimer (43). This allows Tar, even when saturated with one ligand, to use the remaining subunit for signal output. The citrate-occupied Tcp homodimer might use similar mechanism. In any case, structural analyses of Tcp are required to test this model.

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