Mutations That Affect Ligand Binding to the *Escherichia coli* Aspartate Receptor

**IMPLICATIONS FOR TRANSMEMBRANE SIGNALING***

A. Malin Björkman‡, Pete Dunten§, Mats O. J. Sandgren¶, Vangipuram N. Dwarakanath***, and Sherryl L. Mowbray‡ ‡‡

From the ‡Department of Molecular Biology, Swedish University of Agricultural Sciences, Box 590, Biomedical Center, S-751 24 Uppsala, the ¶Department of Cell and Molecular Biology, Uppsala University, Box 596, S-751 24 Uppsala, Sweden, and **Howard Hughes Medical Institute, University of Texas, Southwestern Medical Center, Dallas, Texas 75235-9050

Three arginine residues of the binding site of the *Escherichia coli* aspartate receptor contribute to its high affinity for aspartate ($K_d \sim 3 \mu M$). Site-directed mutations at residue 64 had the greatest effect on aspartate binding. No residue could substitute for the native arginine; all changes resulted in an apparent $K_d$ of $\sim 35 \mu M$. These mutations had little impact on maltose responses. At residue Arg-69, a lysine substitution was least disruptive, conferring an apparent $K_d$ of $0.3 \mu M$ for aspartate. Results obtained for an alanine mutant were similar to those with cysteine and histidine mutants ($K_d \sim 5 \mu M$) indicating that side chain size was not an important factor here. Proline and aspartate caused more severe defects, presumably for reasons related to conformation and charge. The impact of residue 69 mutations on the maltose response was small. Mutations at Arg-73 had similar effects on aspartate binding ($K_d 0.3-7 \mu M$) but more severe consequences for maltose responses. Larger side chains resulted in the best aspartate binding, implying steric considerations are important here. Signaling in the mutant proteins was surprisingly robust. Given aspartate binding, signaling occurred with essentially wild-type efficiency. These results were evaluated in the context of available structural data.

Like all living creatures, bacteria need to keep track of things happening around them and to respond appropriately to important events. The proteins of the chemotaxis system allow the sensing of chemical signals and the use of that information to direct appropriate swimming behavior (for recent reviews see Refs. 1 and 2). Signal reception is the task of receptors found in the cytoplasmic membrane that either recognize an attractant or repellant (3). Although the *Tar* receptors from *Escherichia coli* and *Salmonella typhimurium* have 78% sequence identity, only *E. coli* Tar can recognize the complex between maltose-binding protein (MBP) and maltose (5). It has been shown that aspartate and maltose responses are largely independent, i.e. both attractants can be recognized simultaneously or sequentially (6–8).

The structure of intact Tar is not yet known, although many studies have combined to give a rather comprehensive picture of its structure. The sequence (3) first suggested its functional organization. The receptor has a short N-terminal segment (residues 1–6) on the periplasmic side of the inner membrane. A short hydrophobic segment (residues 7–30) then crosses to the periplasm, where a soluble domain (residues 31–188) capable of binding aspartate is located. A second membrane-spanning sequence (residues 189–212) brings the receptor back to the cytoplasm. The remainder of the protein (amino acids 213–552) comprises a soluble cytoplasmic unit that possesses the signaling and adaptation properties of the receptor. That the smallest functional unit is a dimer was first established by cross-linking of mutant cysteine-bearing receptors (9) and was later confirmed by the x-ray structure of the ligand-binding domain of the receptor (Fig. 1A (10)). The four-helix bundle of each periplasmic domain is connected to the two transmembrane segments through extensions of $\alpha_1$ and $\alpha_4$. As found for the periplasmic regions, the two $\alpha_1$ extensions of the dimer appear to be in close proximity in the membrane, whereas the $\alpha_4$ extensions are further apart (11). The structure of the greater part of the cytoplasmic domain of the related serine receptor has been shown to consist of a very long coiled coil in which two antiparallel helices from each subunit are packed onto each other in a four-helix bundle: in this portion of the receptor it is the extensions of $\alpha_4$ that form the basis of inter-subunit contacts (12).

There are two sites for aspartate binding in the dimer, each incorporating residues from both subunits (Fig. 1). Binding of aspartate to one of the sites hinders binding of the second effector molecule through negative cooperativity (13). The effect is modest in the case of *Salmonella* Tar, but the affinity at the second site of *E. coli* Tar is reduced at least 50-fold (14). Responses resulting from aspartate binding require the action of a set of cytoplasmic and flagellar proteins that control swimming. Adaptation is associated with the reversible methylation

---

* This work was supported by grants from the Swedish Natural Science Research Council and the Howard Hughes Medical Institutes (to S. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address: Roche Research Center, Hoffmann-La Roche, Inc., Nutley, NJ 07110.

§ Current address: Dept. of Pediatric Nephrology, Southwestern Medical Center, Dallas, TX 75390.

‡‡ To whom correspondence should be addressed. Tel.: 46 18 471 49 90; Fax: 46 18 53 69 71; E-mail: mowbray@xray.bmc.uu.se.

1 The abbreviations used are: Tar, aspartate receptor from *E. coli*; MBP, maltose/maltodextrin-binding protein; AdoMet, S-adenosylmethionine; PMSF, phenylmethylsulfonyl fluoride.
Fig. 1. The Tar periplasmic domain and its binding site for aspartate. A, ribbon drawing showing the domain as observed in the 2LIG structure. Major helices (a1, residues 33–75; a2, residues 89–109; a3, residues 117–143; and a4, residues 154–178), with different coloring for the two subunits. The short helix from residues 145 to 152 is shown as a coil, as are the loops connecting the longer helices. The A molecule is that with aspartate bound to Arg-64 and Thr-154, and the B molecule contributes residues Arg-69 and Arg-73. Bound aspartate is shown as a ball-and-stick representation with atomic colors. The figure was prepared using Molscript (53). B, hydrogen-bonding interactions in the aspartate-binding site, using the same coloring scheme for the residues as in part A. Interactions requiring the side chains of Arg-64, Arg-69, and Arg-73 (studied here) are shown in magenta, with other interactions in red (if they have been tested elsewhere) or green (those that have not yet been tested).

of specific glutamates on the cytoplasmic domain of the receptor itself (15, 16).

Although more is presently known about this signaling system than any other, many mysteries remain, including the means by which the receptor conveys information about ligand binding into the cell. Tar is a dimer both in the presence and absence of ligand (9), precluding monomer-dimer equilibria as a mechanism for transmembrane signaling. Key changes within an existing dimer could include relative motions of the two subunits, as well as motions within a subunit. Most structural studies suggest the former (10, 17), whereas other evidence seems to support the latter (summarized in Refs. 18–20).

In the present paper, we describe a study of site-directed mutations in the binding site of E. coli Tar. The residues chosen for this work are Arg-64, Arg-69, and Arg-73, which are conserved in all receptors that bind amino acids (21) and so are presumably central to receptor function. We explore the significance of the results in the light of available data on receptor structure and biology.

Experimental Procedures

Reagents, Strains, and Plasmids—Maltose (PM grade) was obtained from Merck, and aspartate (ultragrade) was from the Sigma. Unlabeled Ado-Met (Sigma grade I, iodide salt) was purified using an anion exchange resin (Dowex AG 1 × 8) to remove S-adenosylhomocysteine, an inhibitor of methyltransferase, and then stored at −80 °C. Radiolabeled AdoMet (15 Ci/mmol) was obtained from Amersham Pharmacia Bio-tech. Strains RP437 (F− thr leu his met(氨) eda strA), RP4080 (F− cheR227, thr leu his gal lac ara xyl mtl strA recA nAlA), and RP4372 (F− tar8216 strA-tap15201 thr leu his met(氨) eda strA) (22) were provided by J. S. Parkison at the University of Utah. Plasmid pME43 (23) was obtained from Jeff Stock (Princeton University) and transformed into RP4080 to provide a source of methyltransferase (prepared as described earlier (24)). The final buffer used in dialysis contained 1 mM 1,10-phenanthroline, 1 mM PMSE. Expression of mutant receptors was evaluated by SDS gel electrophoresis and Western blots. In vitro methylation/binding assays (16) were performed in a buffer containing 20 mM sodium phosphate, pH 7.0, 1 mM 1,10-o-phenanthroline, and 1 mM PMSF. Aspartate concentrations were chosen where possible to represent multiple points both above and below the $K_d$ of the particular receptor (as determined from preliminary experiments), with the highest concentration always at least 60 mM. Samples were taken at 15 and 30 min to observe both early and late stages of the modification process. The methylation obtained for RP4080pEMBL19 was subtracted from each value to account for methylation due to chromosomal receptor expression (less than 10% of the total).

Analysis of Binding Data—Methylation data were analyzed in terms of two aspartate-binding constants in the program Mathomatic (Wolfram Research), using Equation 1,

$$C_b = c(K_{Asp} + 2K_d)(K_{Asp}^2 + 1) + K_d^2$$

where $C_b$ is the difference between the counts/min obtained in the sample containing aspartate and that for the corresponding sample without aspartate; $c$ is equal to half the maximum number of counts expected at aspartate saturation (included in the fitting); $K_d$ is the measured association constant for the first aspartate site, and $K_{Asp}$ is the measured association constant for the apparent second site; the association constants were subsequently converted to $K_a$ values for presentation. It was assumed that the concentration of free aspartate was the same as that added, except for the wild-type Tar, where $K_a$ was similar to the receptor concentration (4 μM), and so a correction was needed.

Structural Comparisons—Table I lists the available x-ray structures of the periplasmic effector-binding region of Tar. Coordinates for most were obtained from the Brookhaven Protein Data Bank (27) and are identified here by their entry codes. Those for a form of E. coli Tar without bound ligands (28) were kindly provided by Prof. Sung-Hou Kim (University of California, Berkeley). All aspartate-bound structures have a dimer in the asymmetric unit. As all aspartate-free structures contain only a single subunit in the asymmetric unit, the second subunit of the dimer was first generated using crystallographic symmetry. The dimeric structures were compared with the $eq$ options of the
Overproduction of Mutant Tar—With the exception of R64P (which produced no detectable Tar), most mutant receptors were expressed at least half as well as the wild-type protein (Table II) and so represented 5–10% of the total membrane protein.

Swarm Assays—The behavior of bacteria can be assessed qualitatively with an in vivo swarm assay, in which cells are inoculated into a semi-solid agar medium with or without attractant (at concentrations of 0.1 or 1 mM aspartate, or 0.1 mM maltose, as indicated). As the cells take up the components of the medium, excrete wastes, and multiply, gradients are built up which cause chemotaxis (32). For the present study, RP4372 (tar-, tap-, tsr-, cheB-, cheR-) was transformed with pMR650 (containing wild-type Tar), with mutated versions of the same plasmid, or tested by itself as a control.

Representative swarm results are presented in Fig. 2. Although differences in the levels of receptor expression, as well as any in transport or metabolism, may make quantitative assessments of receptor function unwise, a number of patterns are clear. The background strain alone (RP4372) lacked Tar and exhibited no chemotaxis to either aspartate or maltose; the rate of swarming in the absence of added attractant is also low, due to the inherent smooth-swimming bias of the strain. Overexpression of the wild-type receptor (with pMR650) in the same strain improved swarming somewhat, presumably by reducing that bias. The overproduced receptor also enabled aspartate and maltose responses, with a characteristic pattern of saturation of the response at higher aspartate concentrations. Most of the mutant receptors also brought about a similar improvement of the unstimulated rate, as well as allowing aspartate and maltose. R69K was the least disruptive change; this mutant even showed saturation at the higher aspartate concentration, as observed for the wild-type Tar. Mutations at residue 73 gave rise to receptors with a relatively effective aspartate response but significantly impaired chemotaxis to maltose. The notable exception here was R73D, which had no apparent response to either ligand. These patterns confirm and extend the results of earlier studies (26, 33).

**TABLE I**

Available structures of Tar periplasmic domains

| PDB code | Description (Ref.) | Bound effector | Other ligands | Resolution | Space group | Phase solution |
|----------|--------------------|----------------|---------------|------------|-------------|----------------|
| 1LIH     | *Salmonella*, cross-linked with disulfide bonds (10, 37) | 1 Asp | Phenanthroline | 2.2 | P6/5/22 | MIR |
| 2LIG     | *Salmonella*, cross-linked with disulfide bonds (10) | | Phenanthroline, SO_4 | 2.0 | P3/2/21 | MIR |
| 1WAS     | *Salmonella*, wild type (17) | 1 Asp | Phenanthroline, SO_4 | 2.7 | I(1) | MR |
| 1WAT     | *Salmonella*, wild type (17) | 1 Asp | Phenanthroline, SO_4 | 3.0 | I(1) | MR |
| 1VLS     | *Salmonella*, wild type (54) | 1 Asp | Phenanthroline, SO_4 | 1.85 | I(1) | MR |
| 1VLT     | *Salmonella*, wild type (54) | 2 Asp | Phenanthroline, SO_4 | 2.2 | P2(1)2/12(1) | MR |
| 2ASR     | *E. coli*, wild type (55) | | Phenanthroline, SO_4 | 2.3 | P4(1)2/12/2 | MIR |
| NA*      | *E. coli*, wild type (28) | | Phenanthroline, SO_4 | 2.3 | C222(1) | MR |

NA, not applicable.

**RESULTS**

Methylation/Binding Assays—Because of problems inherent in measuring aspartate affinity to membrane-bound receptor when the binding is weak, an indirect method (26) was used, which exploits the proportional relationship between ligand binding and the modification of a receptor by the chemotaxis methyltransferase (15). This method has the additional advantage that it gives some measure of the signaling/adaptation properties of the receptor. The results were first analyzed with Scatchard plots, which consistently showed a concave biphasic shape indicative of negative cooperativity. Nonlinear curve fitting of the original data was then applied, as this method is considered to be less vulnerable to distortions from systematic experimental errors (34). Two sites were ultimately included in the fitting of both wild-type and mutant receptors, as a single site did not adequately reproduce the shape of the curves (despite suggesting a very similar $K_{d}$; Fig. 3). The assumption that aspartate binding to the second site results in the same amount of methylation as binding to the first site is implicit in this treatment, although no molecular model for this exists. Assuming that a different amount of methylation arose from binding at the second site did not improve the fit, and so could not be justified, although it remains a very plausible physical situation. The Hill equation (35) with $n$ of the order of 0.8 (i.e. negative cooperativity) performed as well as the two-site model. The values obtained from the two-site fitting are sum-

...
TABLE II

Mutant receptor expression and binding/signaling characteristics

| Receptor type | Level of Tar | % of possible methylation at highest [Asp] tested | Predicted maximum methylation, % of WT | Maximum aspartate coupling observed | $K_{d1}$ | $K_{d2}$ |
|---------------|--------------|-----------------------------------------------|----------------------------------------|-------------------------------------|---------|---------|
| WT            | ++           | 62                                           | 100                                    | 2.2 ± 0.3                           | 0.002 ± 0.002 | 0.9 ± 1.0 |
| RP4080pMK650, French press | ++           | 86                                           | 100$^a$                               | 1.4 ± 0.2                           | 0.005 ± 0.005 | 11 ± 13  |
| RP4372pMK650, sonicated       | ++           | 66                                           | 75$^b$                                | 1.3 ± 0.1                           | 0.004 ± 0.002 | 2.3 ± 2.0 |

Residue 64

| Residue 69 | ++           | 64                                           | 77                                    | 2.0 ± 0.0                           | 7.8 ± 1.6  | 155 ± 134 |
| R64C$^b$   | ++           | 85                                           | 95                                    | 1.9 ± 0.2                           | 1.6 ± 1    | 40 ± 5    |
| R64D       | ++           | 84                                           | 83                                    | 2.3 ± 0.0                           | 2.0 ± 0.3  | 650 ± 100 |
| R69H       | ++           | 65                                           | ND                                    | 1.5 ± 0.0                           | 4.9 ± 2.1  | 94 ± 11   |
| R69K       | ++           | 65                                           | 77                                    | 1.7 ± 0.0                           | 0.3 ± 0.2  | 35 ± 22   |
| R69P       | ++           | 65                                           | 77                                    | 2.7 ± 0.0                           | 39 ± 3     | 103 ± 7   |

Residue 73

| Residue 73 | ++           | 74                                           | 115                                   | 1.9 ± 0.0                           | 6.9 ± 1.3  | 60 ± 65   |
| R73D       | ++           | ND                                           | ND                                    | 2.2 ± 0.1                           | 0.4 ± 0.1  | 26 ± 1    |
| R73K       | ++           | 88                                           | 100                                   | 2.2 ± 0.2                           | 0.5 ± 0.1  | 140 ± 200 |
| R73Q$^b$   | ++           | 79                                           | 64                                    | 1.9 ± 0.0                           | 7.3 ± 0.9  | 56 ± 62   |
| R73W$^b$   | ++           | 99                                           | 87                                    | 2.7 ± 0.2                           | 0.3 ± 0.1  | 300 ± 400 |

$^a$Predicted by two-site model.

$^b$The actual level of methylation was lower in this pair (about 40% that obtained when using fresh S-AdoMet).

$^c$Asterisks indicate previous results with RP4372 membranes (26) that were re-analyzed in the curve-fitting; the previously reported values are given in parentheses. Maximum aspartate coupling refers to the stimulation of methylation at the highest aspartate concentration tested. WT, wild type.

$^d$ND, not determined.

Fig. 2. Effects of Tar mutations on swarm behavior. The results are reported as relative swarm rates, where the swarm rate observed for each receptor in the absence of attractants (given numerically for each case) was subtracted from those measured in their presence. At left are rates with the addition of 0.1 or 1 mM aspartate; at right are rates with 0.1 mM maltose. For comparison, the rates with the wild-type strain RP437 were 0.51, 1.63, 0.98, and 1.07 mm/h for unstimulated, 0.1 mM aspartate, 1 mM aspartate, and 0.1 mM maltose trials, respectively. Approximate errors in swarm rates are ±20%.

Fig. 3. Coupling of receptor methylation to aspartate binding. Methylation/binding data are shown for the R64K mutant, 90-min time points. The solid curve is that suggested by the two-site nonlinear fitting. The dashed line represents a curve when only a single site is considered, with a $K_d$ the same as the $K_{d2}$ fit with the two-site model. Additional factors (including the properties of the different mutants), and so the estimated $K_{d2}$ values are given only for the sake of completeness, as their physical meaning is still unclear. To allow comparison of the various receptors, extrapolation to the maximum methylation expected appeared to be justified, as the methylation generally covered a significant portion of the binding curve. The largest degree of coupling observed in each case, i.e., the increase in methylation found at the highest concentration of aspartate tested, is also reported, although the size of this quantity varied with the specific methyltransferase and AdoMet preparations. It should be noted that the given values for coupling are underestimates of the maximum, since the receptors were not usually fully saturated with aspartate.
The estimated affinity of the first site of the wild-type receptor for aspartate (2 μM) agrees well with earlier data obtained using a variety of methods (summarized in Ref. 2). Binding to the second site of E. coli Tar has previously been shown to be disfavored by negative cooperativity (14), although to an unknown extent. Our results suggest that the affinity of the second site is reduced at least 500-fold.

The strain RP4080, which has chromosomal expression levels of Tar and methyltransferase (CheB) but lacks methyltransferase (CheR), was used as the background for the methylation studies here instead of RP4372 (tar−, cheB+, cheR+) as used previously (26). During preparation of most membranes, cells were broken in a French press, and previous results were obtained with membranes prepared by sonication. To be certain that no systematic errors arose from these changes, aspartate binding to wild-type Tar was compared for membrane preparations that differed in these respects. The results obtained were very similar. The pair of experiments shown in Table II represents an extreme case, using older radioactive AdoMet (2 years after purchase); the results demonstrate that although the observed methylation and coupling under these circumstances are lower, there is little impact on estimates of binding to the first site. The Kd values (4–5 μM) are again in good agreement with previously published estimates. As a further test, membranes for the mutant R69C were also prepared using the RP4080 background and French press. The Kd results were indistinguishable from ones obtained with sonicated RP4372 membranes (Table II). Other possible effects due, for instance, to changes in ionic strength at high aspartate concentrations were ruled out by the fact that assays in which the 20 mM sodium phosphate buffer was replaced with 100 mM Tris-HCl or 100 mM phosphate gave equivalent results.

The Kd estimates for the different mutant proteins are shown in Table II, along with those derived using earlier data for mutants R64C, R69C, R69H, R73Q, and R73W overproduced in RP4372 (26), reprocessed with the nonlinear curve fitting. The values reported previously for the mutants are given for comparison and are consistent with those obtained here. As for the swarm assays, the results clearly fall into classes that are related to the site of mutation. The Kd of all of the residue 64 mutations was observed to be 20 mM or greater, with R64C having no detectable binding at aspartate concentrations as high as 60 mM. Residue 69 mutations had smaller effects on aspartate binding, with Kd values in the range of 2–8 mM; binding to the proline mutant receptor was 10-fold weaker and that to the lysine mutant was a factor of 10 stronger. At residue 73, replacing arginine with a large residue (glutamine, lysine, or tryptophan) was better than introducing a smaller one (cysteine or serine), by roughly a factor of 10, with Kd values clustered near 0.5 mM for the large residues versus 7 mM for the smaller ones. All of the mutant proteins gave methylation similar to that of the wild-type protein when saturated with aspartate, showing that once aspartate was bound all were competent for signaling.

Conformational Changes Caused by Aspartate Binding—Interpretation of the mutational results must utilize available structural data on the mechanism of aspartate binding/signaling. As shown in Table I, structures of the periplasmic domain of the E. coli Tar are available only for the aspartate-free form. It is thus necessary to use the aspartate-bound and aspartate-free structures of the equivalent domain of the Salmonella protein (66% sequence identity in the periplasmic region). Three such pairs are presently available, of which only 1LIH/2LIG and 1VLS/1VLT were solved at sufficient resolution to study conformational changes with confidence. The 1LIH/2LIG pair represents Tar in which residue 36 is mutated to a cysteine, with the consequent formation of an inter-subunit disulfide bond. Such cross-linked receptors are still functional in aspartate binding (including negative cooperativity) and signaling (13, 14, 36); 2LIG has a single molecule of aspartate bound. Both members of the pair have a phenanthroline molecule located at the subunit interface; numerous studies, including this one, have shown that Tar responds normally to aspartate in the presence of phenanthroline. The 1VLS/1VLT pair represents the wild-type Salmonella receptor, without the disulfide cross-link and without bound phenanthroline. 1VLT has two molecules of aspartate bound. Both aspartate-free structures (1LIH and 1VLS) consist of two identical subunits, i.e. the dimer is symmetrical in the absence of bound aspartate. This symmetry is lost when the first molecule of aspartate enters 2LIG and is not regained when a second is bound to 1VLT.

The portions of the periplasmic domain that are most relevant to transmembrane signaling are obviously those connected to the membrane-spanning segments, i.e. α1 and α4 of each subunit. Possible signaling mechanisms include intra-subunit (α1/α4 or α1'/α4') and inter-subunit (α1/α1' or α4/α4') changes. In Fig. 4, a simplified distance-diagonal plot illustrates the observed movements of these helices with respect to each other in the two pairs of structures. Although somewhat cumbersome to use, these plots have the advantage that they do not make use of any prior model of what is actually equivalent, i.e. which motions occur.

In the 1LIH/2LIG pair, movement of α4 of subunit A (that bearing aspartate bound to Arg-64) with respect to the rest of the dimer is clearly the motion with the most potential significance for signaling (Fig. 4, A and B). When the two structures are aligned as described under “Experimental Procedures,” i.e. starting from α1 of the A subunit, 136 C-o atoms (residues 51–76 of subunit A and residues 37–67, 92–134, 137–148, and 150–173 of subunit B) match within a 0.6 Å cut-off, giving a
root mean square difference of 0.31 Å. α4 is seen to move about 1.5 Å toward the membrane relative to α1 and tilt slightly in a “swinging piston” motion (18, 19). Smaller changes in α2 and α3 accompany the movement of α4. The dimer interface, mainly a function of α1/α1’ interactions, and the structure of the second subunit are quite static. Negative cooperativity is explained by motions in and near α2 which appear to make the second binding site smaller when the first is occupied (14). The structure of this receptor domain in the absence of phenan-throline is changed only locally (37).

For the 1VLS/1VLT pair, more complicated changes are observed in the distance diagonal plots (Fig. 4, C and D). A distinct rotation of the two subunits with respect to each other is seen as α1/α1’ movements. Motions of each α4 with respect to α1 of the same subunit are found as well, although with different characteristics from that found in the 1LIH/2LIG pair. Both of the α4s, however, appear to move in the same manner within their respective subunits. When the structures of the two dimers were aligned as described under “Experimental Procedures,” starting from α1 of the A subunit, the root mean square difference was 0.32 Å with 83 atoms matching (residues 55–72, 89–104, 121–127, 130–140, and 142–166 from the A subunit and 50–55 from the B subunit). When α1 of the B subunit was the starting point for the alignment, it was 0.34 Å with 66 atoms matching (residues 54–74, 95–105, 116–123, 126–134 and 153–168, all from the B subunit). These results confirm that more changes are found in this pair compared with the LIH/2LIG case, and that α1/α1’ motions are largest; changes within the individual subunits are much smaller. The changes in both α1 and α4 are most appreciable at the ends that would normally be attached to the transmembrane segments (Fig. 1).

The two E. coli apo structures have additional, and different, arrangements of the subunits, from each other, and from the Salmonella receptors. As there exist no structures for the aspartate-bound forms of this Tar, a meaningful analysis of conformational changes could not be carried out.

DISCUSSION

Arg-64 is clearly established as most important for aspartate binding. Mutations here gave rise to a remarkably constant apparent \( K_d \) of −35 μM. Although some changes (alanine, aspartate, and serine) reduced the expression of the protein (and presumably folding/stability), the binding result for each was the same. The only exception was a R64C mutant (26). Although it formed a stable receptor, R64C was totally defective in aspartate binding/signaling; since this could be explained by formation of an inter-subunit disulfide bond, the significance of this result as yet uncertain. Interestingly, the aspartate mutation (which might repel the ligand for electrostatic reasons) was not significantly worse than other substitutions. Nor did the lysine mutation, which should give a better match for the size and charge of the native arginine, result in better aspartate binding. The demands on this position in aspartate binding must, therefore, be so stringent that only arginine can fulfill them. Effects on unstimulated or maltose-induced swarming were not severe, indicating that most other aspects of Tar structure/function were intact.

Changes at residue 69 usually resulted in an apparent \( K_d \) of 2–8 μM. Binding to the proline mutant was 10-fold weaker, perhaps because Arg-73 (on the same helix) was also compromised. Binding to the R69D mutant was also poorer, which could be due to repulsion of the ligand by the negatively charged side chain or to the formation of a salt link with one of the remaining arginines. The swarm results, however, suggest that more general effects on receptor signaling are an issue for R69D; although its unstimulated swarm rate is normal, the maltose response for this mutant is weak. The relative efficiency of the lysine replacement at this position is interesting, given the inability of lysine to replace arginine at residue 64. In this respect, too, the role of Arg-69 appears to be less central for aspartate recognition. Effects on the maltose response were minimal for the residue 69 mutants.

Mutations at Arg-73 fell into two classes, apparently related to the size of the side chain introduced. Larger ones (regardless of their charge or hydrogen-bonding characteristics) gave rise to an apparent \( K_d \) of −0.5 μM, whereas smaller ones resulted in a value of −7 μM. Lysine was not significantly more effective than any other substitution. Thus, the interactions made by Arg-73 seem to be less critical than those of the other two arginine residues, provided that some space-filling role is satisfied. In contrast to the other sites, mutations at position 73 had a strong effect on the maltose response. The only exception to this pattern is the R73D mutant; the combined results suggest that although this receptor folds, it is defective in some aspects of signaling function.

Our results confirm earlier studies (21, 26, 33) and broaden the available binding data sufficiently to allow conclusions to be drawn from the patterns observed. The measured \( K_d \) of the wild-type receptor for aspartate is −3 μM. Binding was thus −10,000, 1500, and 100–1500 weaker on mutating arginines 64, 69, and 73, respectively. As each arginine residue has bidentate interactions with the aspartate ligand, hydrogen bonding alone does not offer an explanation for why their relative contributions to affinity are different. The reasons must lie in the structural context of each residue, including the nature of the aspartate-induced conformational change. Unfortunately, the available crystallographic results contradict each other in some respects. The wild-type structures show a large relative movement of the two subunits on aspartate binding, and the structures of Tar cross-linked at residues 36 suggest a motion of α4 within a single subunit as the significant change for transmembrane signaling. A number of studies have shown that receptors with cross-links across the α1/α1’ interface are still functional in signaling (reviewed in Ref. 19); these included cross-links between residues 36 (36), as well as a case where two disulfide bonds were introduced (18). As residues 36 are −20 Å apart in the 1VLS structure, the relationship between this view of the conformational change and that of the intact receptor is unclear. A large number of genetic and spectroscopic studies (reviewed in Refs. 19, 20, and 38) have also suggested that intra-subunit (α1/α4) changes are most important for receptor signaling. However, the undeniable fact remains that inter-subunit changes are observed in the wild-type structures. Although such differences could be due to crystal packing in the different space groups or to the loss of the other portions of the molecule, all new data should be measured against both sets of structures.

Some aspects of the bound/free structures are consistent, notably the nature of the interactions in the aspartate-binding sites (Fig. 1B). Arg-64 makes charge-charge interactions with the oxygen atoms of the α-carboxyl group of aspartate. This side chain appears to be held in position by hydrogen bonds to those of Gln-155 and Gln-156 in α4 of the same subunit, even before the introduction of ligand, and so may be presumed to lose little entropy on binding. It should be noted that arginine is the only residue that could make all of these interactions. As Arg-64 is relatively buried, mutations here may also disturb the local structure. In contrast, Arg-69 and Arg-73 interact with the side chain carboxyl group of the aspartate ligand and are apparently not as central to its binding. These residues are more exposed and so less constrained, so entropy may partly explain why they contribute less. The effects of residue size at
Arg-73 are probably related to the observation that the loop just before α2 (near Ser-84) folds down over this residue in occupied sites; this is the same change that is thought to be the basis of negative cooperativity. Smaller residues would leave a “hole” at this position of the aspartate-bound form and so should destabilize it. The observed affinities of residue 69 and 73 mutants for aspartate are at least as good as that of wild-type Tar for glutamate (K_a > 5 mM (4)), suggesting that either Arg-69 or Arg-73 (or both) is not optimally used when glutamate is bound.

Perhaps the most surprising result is that the three arginine residues contribute so much to aspartate binding but so little to signaling. Each of the mutant receptors generates a chemotactic signal, once aspartate is bound. Apparently, none of the interactions contributed by these residues (magenta lines in Fig. 1B) is essential to that process. Therefore, the roles of the remaining interactions in binding and signaling must be inspected closely. Some have been explored elsewhere (Fig. 1B, red dashed lines). Mutations of Ser-68 generally have little effect on the affinity of Tar for aspartate, although they can change the cooperativity greatly (39); maltose responses were apparently not tested. Mutations of Thr-154 have only modest effects (10–100 fold) on aspartate binding with no impact on signaling (40); weaker binding was observed for a proline mutant, most likely resulting from changes in local main chain conformation. There is little disturbance of the maltose response. Other workers showed a role for Tyr-149 in signaling to both aspartate and maltose (33), concluding that some common signaling mechanism might be impaired. The effects on aspartate affinity specifically were not measured, but a Tyr-149 mutant did show some aspartate-induced swelling, suggesting that binding/signaling occurred at high enough concentrations. More detailed studies of the aspartate affinity and signaling competence of Tyr-149 and Thr-154 mutants of Tar would be helpful. It is also clear that in these and a number of other cases mentioned here, structural data for the mutant receptors would be a real asset in the interpretation.

The interactions of the binding sites that have not been explicitly tested are shown as green dashed lines in Fig. 1B. Two things seem to be significant about them. First, they are all main chain and water-mediated interactions (that cannot be investigated by mutagenesis). Second, they represent links between the backbones of helices α1 and α4 of the same subunit that are made via the aspartate ligand. The presumably critical connection involving the main chain oxygen atom of Arg-64 and water would be expected to be altered in the R64P mutation, but unfortunately this mutant receptor could not be expressed and tested. Any of the many available Ser-68, Arg-69, or Arg-73 mutations would be expected to weaken links between α1 and α1’, and yet such mutants are able to signal effectively once ligand is bound. The fact that the side chain of Arg-64, which also mediates α1/α4 connections, is so important for aspartate binding would also seem consistent with the idea that α4 motions are involved in signaling, as is the fact that mutation of Tyr-149 affects both aspartate and maltose responses. Thus, the mutational results appear to be more consistent with a model of signaling in which the relative movement of α4 within a subunit is most critical. Important links in the chain of interactions whereby aspartate links α1 and α4 include ordered waters in the crystal structures. That such waters can have as large a contribution as protein atoms is supported by a number of structural studies (e.g. Refs. 41 and 42). An alternate interpretation is that none of the interactions is by itself essential, but rather that aspartate fills some appropriate space and so positions the important helices correctly. This argument would seem to be weakened by the fact that the “appropriate space” has a very different size and shape in the various mutant receptors, and yet signaling occurs with a similar final efficiency for each.

Also pertinent are recent studies showing that under physiological conditions, receptors form a noncovalent complex with the intracellular signaling proteins CheA and CheW (43, 44); together they form large aggregates that are necessary for maximal activity of CheA (45). It has been demonstrated further (46, 47) that the affinity of Tar, as well as of the related serine receptor, is modulated by its interactions with CheA and CheW (and in that complex by the state of receptor methylation). The feedback effects of receptor methylation on ligand binding appear to be small (e.g. Refs. 4 and 48) when CheA and CheW concentrations are low (discussed in Ref. 46). Both receptor overproduction and the high-salt washes used in the membrane preparation here would effectively reduce the amounts CheA and CheW. The methyltransferase preparation, although not highly purified, is also overproduced and thus will not add large amounts of CheA and CheW. Although the binding studies reported here can only claim to address intrinsic properties of the receptor itself, these must underlie the behavior of the receptor in the larger complexes with CheA and CheW.

Signaling of E. coli Tar in response to maltose is, in agreement with earlier studies (21, 33, 49), affected by changes at Arg-73 but not generally by ones at Arg-64 and Arg-69. Interactions with maltose-bound MBP seem to be dependent on having an arginine at position 73 of Tar, as no other amino acid, even lysine, could replace it efficiently. As this residue is not important to the structural integrity of Tar, direct involvement in interactions with MBP must be suspected. A model for the MBP/Tar interaction has been proposed (50) in which MBP interacts simultaneously and asymmetrically with the two subunits of a Tar dimer. Inspection of this model suggests to us the possibility of an interaction between residue Asp-41 or Glu-45 of MBP and Arg-73 of Tar; mutations of these MBP residues have both been reported to affect chemotaxis (50, 51). Although the loss of a single charge-charge interaction (even a bidentate one such as arginine can form with acidic side chains) would not ordinarily destroy function, the binding of MBP and Tar is already weak (52), and it may be very sensitive to such disruptions. The location of these residues of MBP just above α4 of Tar in the proposed complex may also be significant. A movement of α4 downward toward the membrane, as suggested above for the aspartate signal, could bring this portion of MBP into a more optimal position for interactions with Arg-73 of Tar and so support its role in signaling. The second aspartate-binding site would appear to be free to accept ligand, in a relatively independent signaling event (8). Our results suggest that the second site of Tar is indeed active under appropriate conditions.

Acknowledgments—We thank Quang A. Vu and Vahid Feiz for invaluable experimental assistance, Sandy Parkinson for strains, Jeff Stock for plasmids, Andrew Kolodziej and Steve Chervitz for helpful discussions, Sung-Hou Kim for coordinates, and Mike Manson and Paul Gardina for the model of the Tar MBP interaction and as well as other valuable help.

REFERENCES

1. Djordjevic, S., and Stock, A. M. (1998) J. Struct. Biol. 124, 189–200
2. Mowbray, S. L., and Sandgren, M. O. J. (1998) J. Struct. Biol. 124, 257–275
3. Russo, A. F., and Koshland, D. E., Jr. (1983) Science 220, 1016–1020
4. Clarke, S., and Koshland, D. E., Jr. (1979) J. Biol. Chem. 254, 9695–9702
5. Mizuno, T., Mutoh, N., Panasenko, S. M., and Imae, Y. (1986) J. Bacteriol. 163, 890–895
6. Wolff, C. (1983) Genetic and Biochemical Studies of Maltose Chemotaxis in Escherichia coli. M.Sc. thesis, University of Konstanz, Germany
7. Mowbray, S. L., and Koshland, D. E., Jr. (1987) Cell 50, 171–180
8. Gardina, P. J., Bormans, A. F., and Manson, M. D. (1998) Mol. Microbiol. 29, 1147–1154
9. Milligan, D. L., and Koshland, D. E., Jr. (1988) J. Biol. Chem. 263, 6268–6275

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
