The N-terminal Cytoplasmic Tail of the Aspartate Receptor Is Not Essential in Signal Transduction of Bacterial Chemotaxis

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To determine the role in transmembrane signaling of the N-terminal peptide of the first transmembrane region of the aspartate receptor, it was subjected to extensive mutagenesis. Drastic changes did not alter the chemotactic ability of the receptor to aspartate significantly. Thus the cytoplasmic N terminus of the first transmembrane region does not play an essential role in transmembrane signaling, and the entire signal that is transmitted to the cytoplasmic domain must be sent through the second transmembrane region. This eliminates the models requiring an interaction of this N-terminal peptide with the remaining cytoplasmic portion of the receptor.

Receptor-mediated transmembrane signaling is important in all living organisms from bacteria to human beings. Most receptors function as either homodimers or heterodimers (for recent reviews see Refs. 1 and 2). The dimerization process is sometimes induced by ligand binding and sometimes totally independent of it. The human growth hormone receptors belong to the first category (3). Others, such as human insulin receptor (4) and the bacterial chemotactic receptors (5), do not change their oligomeric states upon ligand binding. These receptor families share similar transmembrane topology (one or two single transmembrane segments connecting an extracellular ligand binding domain and an intracellular signal domain in each subunit). They are believed to transmit signals by a common mechanism, because chimera receptors containing the ligand binding domain of one receptor and the signaling portion of another are still capable of transmembrane signaling (6, 7).

Determination of the role in transmembrane signaling of the cytoplasmic N-terminal peptide of the first transmembrane region of the aspartate receptor shows that the signal is a conformational change of the receptor.

**Materials and Methods**

Chemicals and Media—N-Ethylmaleimide, N-octyl-β-D-glucopyranoside (OG), S-adenosyl-L-methionine, ω-aminooctylagarose gel, thiain, and the amino acids L-histidine, L-methionine, L-leucine, and L-tryptophan were from Sigma. L-Aspartic acid, Zwittergent 3–12, and isopropylthio-β-D-galactoside were from Calbiochem. S-adenosyl-L-[3H]methionine, L-[2,3]-[3H]aspartic acid, and 35S-protein A were purchased from Amersham Corp. Dithiothreitol was from Boehringer Mannheim. Mutagenic oligodeoxynucleotides were either from Operon Technologies (Alameda, CA) or from the DNA Synthesis Facility of the University of California at Berkeley. The Centriprep-30 ultrafiltration tubes were from Amicon. The T4 Mutagene kit, the protein assay kit, and the Affi-Protein A MAPS II kit were from Bio-Rad. The fmo1 DNA sequencing system was from Promega.

The minimal medium contains Vogel-Bonner citrate salt (16), 1% glycerol, 100 μg/ml ampicillin, and 500 μg/ml of each of the following: L-histidine, L-methionine, L-leucine, and L-tryptophan. The Luria broth contains 1% tryptone, 0.5% yeast extract, and 1% NaCl. The minimal plates have 0.3% agar in minimal medium. The aspartate plates are the same as the minimal ones except that aspartate was added to 100 μM. The tryptone plates have 1.3% tryptone, 0.6% NaCl, 0.3% agar, and 100 μg/ml ampicillin.

**Mutant Construction**—Site-directed mutagenesis was carried out according to Kunkel (17). For technical reasons, one set of the oligodeoxynucleotides contain an EcoRI restriction site right upstream of the start codon of the tar gene, in addition to the desired mutations. A second set has only the individual mutations in the oligodeoxynucleotides. The single-stranded pX90 plasmid contains the wild type tar-gene, as an insert in the pEMBL18 vector. Single-stranded pX90 DNA template was prepared from C. J. 236. The mutagenesis reactions were performed using a Mutagen...
kit according to the user’s manual. The mutagenic products were used to transform XL1-Blue or XL2-Blue cells. Plasmids were prepared by standard alkaline lysis method, and the mutants were confirmed by double-stranded DNA sequencing. The class A plasmids are pEMBL18-based. The receptor gene in this class was expressed under the control of its natural promoter. Class B plasmids were constructed by ligating the small EcoRI– HindIII restriction fragments of class A plasmids into the pBTac2 vector cleaved with the same restriction enzymes. The receptor genes in this class were expressed under the control of the tac promoter.

Swarm Assay—The plasmids listed in Table I were used to transform E. coli strain RP861, which has none of the four chemotactic receptor genes. Cells were grown from single colonies in a 1-ml Luria broth with 100 μg/ml ampicillin (LB + Amp) at 30 °C overnight. After overnight incubation at 30 °C, the cells were harvested when A<sub>600</sub> reached 1.0–1.6. Cells containing the class A plasmids were grown in 50 ml of LB + Amp and allowed to continue growing at 30 °C for 8–10 h before harvesting. Cells containing the class C plasmids were grown in 10 ml of LB + Amp overnight at 37 °C. The overnight culture was transferred into 1 liter of minimal medium and grown at 30 °C. Cells were harvested when A<sub>600</sub> reached 0.5–1.0. Cells containing the class A plasmids were grown in 50 ml of LB + Amp and allowed to continue growing at 30 °C overnight. 23 ml of the overnight culture was transferred into 1 liter fresh LB + Amp and allowed to continue growing at 30 °C for 8–10 h before harvesting. Cells containing the class C plasmids were grown in 10 ml of LB + Amp overnight at 37 °C. The overnight culture was transferred into 1 liter of LB + Amp and grown at 30 °C. Isopropylthio-β-d-galactoside was added to 0.5 mM when A<sub>600</sub> reached 0.5–1.0. Cells were harvested 3 h later. The harvested cells were frozen immediately in liquid nitrogen and stored at −80 °C until the membrane preparation.

Membrane Preparation—All steps were carried out at 4 °C. Frozen cells were thawed on ice and low salt buffer (100 mM NaPi, pH 7.0, 10% glycerol, 5 mM EDTA, and 1 mM PMSF) was added to 6–12 ml/liter culture. Cells were broken by sonication. The lysed cells were centrifuged at 95,000 rpm for 10 min (TLA100.3 rotor). Ammonium sulfate was added to 30% (w/v) to the supernatant fraction. The precipitated membrane sample was thawed on ice. The detergent OG was added to 1.4% and dialyzed against OG buffer overnight. The sample was left at −20 °C. Protein concentration was determined by Bradford protein assay (18) using the Bio-Rad protein assay kit.

Aspartate Binding Assay—The binding assays were done as published (Refs. 19 and 20 and references therein) with minor modifications as follows. Membrane samples were mixed with various amounts of aspartate in a final volume of 200 μl. After incubating on ice for more than 10 min, 90 μl of the mixture was transferred into a TLA100 ultracentrifuge tube containing 1 μl of either water or 100 mM cold aspartate. Membrane was pelleted by a 10-min centrifugation at 95,000 rpm. Triplicates of 20 μl of the supernatant fraction were added to a scintillation vial containing 150 μl of water. Then 2 ml of scintillation mixture was added, and radioactivity was counted. Binding data were analyzed (using the Kaleidagraph 3.0 program, Abelbeck Software) by fitting the curve of bound aspartate concentration versus free aspartate concentration to the Hill equation, where

\[
B = \frac{B_{\text{max}} F^H}{K_d + F^H} \quad (\text{Eq. 1})
\]

where B and F are the concentrations of bound and free aspartate, respectively, B<sub>max</sub> is the maximal bound aspartate concentration, K<sub>d</sub> is the dissociation constant, and n<sub>H</sub> is the Hill coefficient.

Methylation Assays—Methylation assays were performed either in the membrane vesicle system or in the reconstituted system. The methylation transferase was prepared according to Shapiro and Koshland (21). For membrane methylation assays, receptor-containing membranes were mixed with an equal volume of the methylation mixture (100 μCi of S-adenosyl-L-[<sup>3H</sup>]methionine, 150 mM NaPi, pH 7.0, 30 mM EDTA, 1 mM PMSF, and 4–10 μl of methyl-transferase/ml of mixture) with or without 2 mM aspartate. At various times, part of the reaction mixture was removed and dotted on a piece of 3M filter paper, and the paper immediately dropped in 10% trichloroacetic acid with stirring. After the final time point was taken, the filter papers were left in 10% trichloroacetic acid and twice with methanol for 10 min each. Finally the filter papers were air-dried and added to a vial containing 3 ml of scintillation fluid for counting.

### Table I

Comparison of the N-terminal amino acid sequences of some chemotactic receptors

| Receptor                  | Amino acid sequence       |
|---------------------------|---------------------------|
| E. coli aspartate receptor| MINIR                    |
| S. typhimurium aspartate receptor| MFNRIR                |
| E. coli dipeptide receptor| MINIR                    |
| E. coli serine receptor   | MINIEK                   |

### Table II

Mutations and plasmids used in this study

| Receptor         | N-terminal sequence | Nature of mutation | Class A | Class B | Class C |
|------------------|---------------------|--------------------|---------|---------|---------|
| Wild type        | MFNRIR              |                    |         |         |         |
| Null             | MFNRIR              | deletion           |         |         |         |
| ΔR4              | MFNRIR              | deletion           |         |         |         |
| ΔR–5             | MR                  | deletion           |         |         |         |
| ΔR–6             | MFNRIR              | deletion           |         |         |         |
| R4G              | MFNGIR              | helix breaker      |         |         |         |
| R4E              | MFNEIR              | positive to negative charge |         |         |         |
| R4K              | MFNKIR              | positive to positive charge |         |         |         |
| R4C              | MFPCIR              | positive charge to polar, potential disulfide |         |         |         |
| ICys2            | MFNCIR              | insertion, potential disulfide |         |         |         |
The radioactivity was counted. The counts/minute values were plotted against time. The methylation rate was reported as the slope of the linear fitting of the time points.

For the methylation of purified receptors in a reconstituted system, partially purified receptor sample was added into the reconstitution buffer (50 mM NaPi, pH 7.0, 40% glycerol, 1/10 volume of null membrane, 0.5% OG, and 1 mM PMSF) and incubated at room temperature for 45 min and then the procedure above was followed.

Cross-linking of the Cysteine-containing Mutant Receptor—The membrane samples were spun down to remove an excess amount of dithiorthreitol and resuspended in the final buffer. After pre-incubation at 37 °C for 5 min, the catalyst copper-(phenanthroline)3 (9) was then added to a final concentration of 1 mM to start the reaction. At various time points, an aliquot of the reaction mixture was removed, added to a tube containing 3 × SDS sample buffer with 10 mM EDTA (to chelate Cu2+) and 10 mM N-ethylmaleimide (to quench the free thiols), and frozen immediately in liquid nitrogen. The samples were then subjected to SDS-polyacrylamide gel electrophoresis, and the regions containing the dimeric and monomeric form of the receptor were excised. The content of radioactive methyl groups in the gel slice was then determined by a previously described methanol diffusion assay (22).

RESULTS

Several mutants within the N-terminal cytoplasmic tail region of the Salmonella typhimurium aspartate receptor were made (Table II). Three of them were deletion mutants: Arg4 deleted (ΔR4), four residues deleted from Phe2 to Ile5 (Δ2–5), and Ile5 and Arg6 deleted (Δ5–6). Four of them were point mutations that converted Arg4 to Gly (R4G), Glu (R4E), Lys (R4K), and Cys (R4C), respectively. The last one (ICys2) was an insertion of a cysteine residue between Met1 and Phe2. Two cysteine-containing mutants, R4C and ICys2, allowed us to cross-link the receptor molecules with disulfides.

FIG. 1. Swarm rates of the N-terminal mutants on minimal and aspartate plates. Swarm assays were performed at 30 °C on the minimal medium plate with or without attractant aspartate. Swarm rates (mm/h) were reported as the slopes of the linear fits of the time-course data points. For each receptor, the column on the left is the swarm rate in the absence of aspartate and the column on the right is that in the presence of aspartate. Top, class A mutants (see "Results" for details); middle, class B mutants; bottom, class C mutants. wt, wild type.

FIG. 2. Swarm rates of the N-terminal mutants on tryptone plates. Swarm assays were performed at 30 °C on the tryptone plates. Swarm rates (mm/h) were reported as the slopes of the linear fits of the time-course data points. Top, class A mutants (see "Results" for details); middle, class B mutants; bottom, class C mutants. wt, wild type.
In order to optimize the overexpression of the aspartate receptor, an EcoRI restriction site was placed immediately upstream of the tar start codon so that the tar gene could be subcloned into another vector that has a strong promoter. It was designed in the same oligonucleotide carrying each of the several N-terminal mutations (class B mutants). This approach also made the screening easier. Unfortunately, this EcoRI site altered the putative ribosome binding site for the receptor synthesis (23). It indeed lowered the expression level of the receptor (data not shown). These mutants were eventually subcloned into the plasmid pBTac2, and the expression of these class C receptor mutants were much better (data not shown). The oligonucleotides for the rest of the mutants (class A) only contained the desired mutations, and tar gene is under the control of its natural promoter (Table II).

We performed in vivo swarm assays to test the effects of the mutations on chemotaxis. As shown in Fig. 1, cells harboring each of the mutant receptor genes showed normal responses toward aspartate in the medium compared with those with wild type receptor genes, and the cells containing no receptor gene showed the same swarm rates in the presence and absence of aspartate. Fig. 2 shows the swarm rates of the mutants on tryptone plates. The results agree with those in Fig. 1. Thus it seems that a wide variety of mutations at the N-terminal tail region do not impair the chemotactic ability of the cells significantly.

The exceptions were the R4G mutant of class B (Figs. 1 and 2) and the R4C mutant of class A (Figs. 1 and 2). The R4G mutant in class B was very poorly expressed. However, when it was subcloned into pBTac2, the expression level was comparable with other mutants, and it swarmed normally (Figs. 1 and 2). The R4C mutant showed lower swarm rates than most of the others on both aspartate and tryptone plates, but its rates were still higher than those of the negative controls. For each swarm assay, we did immunoblotting analysis to monitor the expression level of the receptor. The differences in swarm rates that did occur on minimal and aspartate plates of different classes could be traced to the differences in expression level (data not shown).

Because swarm assay is only a semiquantitative assay to measure chemotaxis and because several factors could affect swarm rates, we also did some in vitro biochemical studies on the membrane samples and found that the mutant receptors still associate with the membrane after high salt washes (data not shown), indicating that the mutated N-terminal tail region could still function as a leader peptide.

| Table III: Aspartate binding parameters |
|----------------------------------------|
| Mutant | $n_H$ | $K_d$ |
| Wild type | $0.59 \pm 0.14$ | $1.09 \pm 0.45$ |
| ΔR4 | $0.61 \pm 0.11$ | $1.09 \pm 0.28$ |
| R4E | $0.86 \pm 0.10$ | $0.65 \pm 0.11$ |
| R4K | $0.98 \pm 0.17$ | $0.60 \pm 0.21$ |
| Δ2-5 | $0.70 \pm 0.01$ | $0.84 \pm 0.03$ |
| Δ5-6 | $0.53 \pm 0.12$ | $1.91 \pm 0.77$ |

**Fig. 3.** Methylation rate ratios of the N-terminal mutants in a membrane vesicle system. Methylation assays were performed at 37°C in the presence and the absence of aspartate. The methylation rates were the slopes of the linear fits of the time-course data points. The ratio of each receptor was the value of the methylation rate in the presence of aspartate divided by that in the absence of aspartate. wt, wild type.

**Fig. 4.** Methylation rates of the N-terminal mutants in a reconstituted system. Partially purified receptor samples were incubated with the null membrane at room temperature for at least 45 min prior to the methylation assays, which were performed at 37°C in the presence and the absence of aspartate. The methylation rates were the slopes of the linear fits of the time-course data points. The rates were normalized to the methylation rate of the wild type receptor in the absence of aspartate. For each receptor, the column on the left is the rate in the presence of aspartate, and the column on the right is that in the absence of aspartate.

**Fig. 5.** Cross-linking time-courses of the ICys2 mutant. Cross-linking reactions were performed in the presence of 1 mM copper-phenanthroline at 37°C. Aliquots were removed at various times, and the reaction was quenched to yield the reaction coordinate, the fraction of the receptor that was cross-linked (cpm of the dimeric receptor divided by the sum of cpm of the dimeric and monomeric receptor). The filled circles were the points in the presence of aspartate, and the open circles were those in the absence of aspartate. The time-courses of the R4C mutant looked similar to those of the ICys2 mutant.
To determine whether the mutations caused any significant change in the tertiary and quartenary structures of the receptor, the aspartate binding affinities of the mutants were tested. Table III listed the binding parameters of the wild type and some of the mutants. The dissociation constants ($K_d$) were within a 2-fold range of that of the wild type. The mutations at the N-terminal cytoplasmic region did not alter the binding affinity for aspartate of the receptor significantly. The index for cooperatively (the Hill coefficient ($n_h$)) of each mutant tested (except for R4K) was within the previously published range, 0.6–0.8 (20), indicating that the binding behavior was similar to that of the wild type.

We then used methylation assays as a test of signal transduction in vitro. As shown in Figs. 3 and 4 for the wild type and each of the mutants tested, aspartate increased the methylation rates in a similar manner to wild type. In the reconstituted system (Fig. 4), the addition of aspartate increased the methylation rate of the wild type by 1.8-fold. For the ΔR4 mutant and Δ2–5 mutant, the increase was 1.9- and 1.7-fold, respectively. The reactions with no purified receptor showed minimal levels of methylation in the presence and the absence of aspartate.

To probe the possible motion of TM 1 within each subunit (either a piston-like motion or a rotation), we compared the rates of disulfide bond formation of two cysteine mutants in the presence and the absence of aspartate. They looked virtually identical for each mutant, as shown in Fig. 5. Based on a previous cross-linking study (24), residues 4 and 4' are at the dimer interface, with the closest distance among the N-terminal tail residues. The inserted cysteine residue of the ICys2 mutant is located at the position for methionine residue in the wild type receptor. The side chain of this residue faces the second transmembrane segment within the same subunit.

**DISCUSSION**

The results reported herein indicate that major changes can be made in the N-terminal peptide projecting into cytoplasm with very minor effects on either transmembrane signaling or aspartate binding ability. The mutants did not interfere with insertion into the membrane or the folding of the cytoplasmic domain.

Previous studies showed that a TM 1-less receptor (first 30 amino acid residues deleted) was not functional as determined by both enzyme assays and methylation assays (25). However, we found that deletion of four residues at the N-terminal end did not affect chemotaxis significantly. Thus the TM 1 transmembrane region is important, but the peptide extending from the TM 1 region into the cytoplasm is not. This suggests that the transmembrane helices (TM 1 and TM 1') are important in maintaining the structural integrity of the whole receptor and probably the transmembrane signaling. A distortion of these interactions would lead to abolished function of the receptor, as shown by the A19K mutant in TM 1 (11) and the 204 mutants in TM 2 (26).

It has also been found that when TM 1 and 1' were cross-linked by a Cys$^4$-Cys$^4$ (24), Cys$^{18}$-Cys$^{18}$ (27), or Cys$^{36}$-Cys$^{36}$ (9) disulfide bond, the receptor could still signal as determined by methylation. This observation leads to the conclusion that TM 1 and TM 1' do not change position relative to each other during transmembrane signaling, and it is confirmed by similar reactions of a Cys$^4$-Cys$^4$/Cys$^{9}$-Cys$^{9}$ double cross-linked receptor (28).

If the interaction of the N-terminal hexapeptide with the cytoplasmic signaling domain is eliminated as a source of the indicated transmembrane conformational change, then the entire transmission of the signal that is delivered to the cytoplasmic domain must go through TM 2. Clearly that severely limits the mechanism for such transmembrane signaling. A rotation model, as suggested by Maruyama et al. (29), seems unlikely as a sole contributor to the transmembrane signaling. Rotation of the two cytoplasmic subunits relative to each other would be excluded as a transmembrane signaling option by the results of Milligan and Koshland (10), in which the cytoplasmic portion of one subunit could be eliminated with only a minor effect on signaling. Thus a piston model (30), in which the cytoplasmic domain moves relative to the membrane, or a model involving relative motion of transmembrane segments TM 2 and TM 2' seems indicated. Such a model could also explain the transmembrane signaling of the epidermal growth hormone and low density lipoprotein receptors, which have one transmembrane region per receptor subunit.

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