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Articles

A Single Hydrophobic to Hydrophobic Substitution in the Transmembrane Domain Impairs Aspartate Receptor Function†

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ABSTRACT: Many transmembrane receptors, such as the insulin, EGF, and bacterial chemotaxis receptors, have only one or a few transmembrane domains connecting an extracellular ligand-binding domain to a cytoplasmic signaling domain. The general belief is that the transmembrane domains in these receptors have no specific sequence requirements as long as they are hydrophobic and long enough to span the membrane as an α-helix. To test this model, we constructed mutants in the aspartate receptor. This receptor is a dimer with two transmembrane domains per subunit. Amino acid substitutions can be made at several positions in the second transmembrane domain, which connects the periplasmic aspartate-binding domain to the cytoplasmic signaling domain, and the receptor remains functional. However, a single substitution of one hydrophobic residue for another can impair receptor function in methylation and swarm plate assays. These results suggest that the second transmembrane domain may pack against the other transmembrane domains in the receptor and small changes in this packing can affect the function of the receptor.

The *Escherichia coli* aspartate receptor mediates the chemotactic response to aspartate. It functions as a dimer with a periplasmic ligand-binding domain, a cytoplasmic signaling domain, and two transmembrane domains per subunit (Figure 1) (Mowbray et al., 1985; Falke & Koshland, 1987; Milligan & Koshland, 1988). Our studies addressed the role of the transmembrane domains in the ligand-induced signaling pathway. A general belief is that for receptors with one or a few transmembrane domains, the specific sequence of each domain is not important as long as it is hydrophobic and of the correct length. In the tyrosine kinase receptor family, many mutant receptors with altered transmembrane domains have been shown to be functional, although activity was affected in some cases where the mutations involved the replacement of the whole transmembrane domain or the introduction of a charged residue (Carpenter et al., 1991; Kashles et al., 1988; Frattali et al., 1991; Yamada et al., 1992; Longo et al., 1992; Escobedo et al., 1988).

The EGF receptor mutants retained ligand-stimulated autophosphorylation activity and ligand-stimulated dimerization when the transmembrane domain was shortened by 3, 6, or 10 residues or lengthened by 3 residues. Other active mutants contained substitutions of prolines, charged residues (lysine, aspartate, glutamate, or arginine), or polar residues (cysteine or glutamine) in place of small, hydrophobic residues in the transmembrane domain (Carpenter et al., 1991; Kashles et al., 1988).

The insulin receptor was also tolerant of some mutations in its transmembrane domain (Frattali et al., 1991; Yamada et al., 1992). It retained insulin-stimulated kinase and autophosphorylation activity with the insertion of three residues or the deletion of one, two, four, or five residues. In addition, a mutant in which the whole transmembrane domain was replaced by the transmembrane helix from the PDGF receptor was still active. However, some transmembrane

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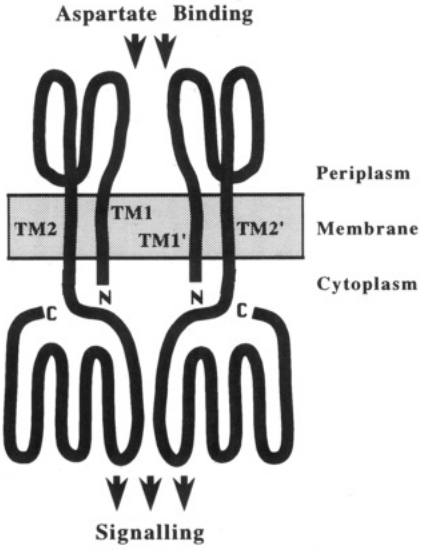

FIGURE 1: Schematic representation of aspartate receptor structure. The aspartate receptor has two transmembrane domains per subunit and is a dimer both in the presence and absence of aspartate. The first transmembrane domain, designated 1 and 1′, is located near the N-terminus of the protein sequence. The second transmembrane domain, designated 2 and 2′, connects the periplasmic aspartate-binding domain to the cytoplasmic signaling domain.

domain mutations caused altered receptor activity. When the insulin receptor transmembrane domain was replaced by the *neu* oncogene transmembrane domain, the mutant receptor had normal autophosphorylation and kinase activity *in vivo* but constitutive autophosphorylation and kinase activity *in vitro*. The insulin receptor also retained ligand-induced autophosphorylation and kinase activity with the introduction of an aspartate or glutamate residue at some positions in the transmembrane domain, but one report indicated a valine to aspartate substitution at position 938 caused constitutive autophosphorylation and cell growth (Longo et al., 1992).

Mutagenesis of the PDGF receptor indicated it does not tolerate substitution of the whole transmembrane domain with the transmembrane domain of another receptor. When the transmembrane domain was replaced with the transmembrane sequence from the *neu* oncogene, the cellular analogue of *neu* (c-*neu*), or the LDL receptor, the mutant receptors did not respond to PDGF as measured by tyrosine kinase activity, phosphatidyl inositol hydrolysis, or DNA synthesis. However, the PDGF-induced receptor internalization was normal for all three mutant receptors (Escobedo et al., 1988).

Previous mutagenesis of the aspartate receptor transmembrane domains demonstrated that the aspartate receptor can tolerate some changes in the sequence of its transmembrane domains (Lynch & Koshland, 1991; Jeffery & Koshland, in preparation). Acceptable substitutions included hydrophobic to polar residues, small to larger residues, and large to smaller residues. However, we found one mutant receptor that was not functional although it contained only hydrophobic to hydrophobic substitutions. Three of the substitutions were phenylalanine residues in place of smaller residues. We wanted to determine if just one of these substitutions could also cause a change in aspartate receptor function.

In this work, we constructed two sets of mutants in the second transmembrane domain of the aspartate receptor. This sequence connects the periplasmic ligand-binding domain to the cytoplasmic signaling domain. In the first set of mutants, we substituted a large, aromatic residue, phenylalanine, in place of a smaller residue (alanine, valine, leucine, or isoleucine) at 10 consecutive positions along the transmembrane helix. Nine of the 10 mutant receptors were functional in a swarm plate assay which measures *in vivo* activity of the receptor. However, the substitution of a phenylalanine for an isoleucine at position 204 caused a decrease in receptor activity. In the second set of mutants, this isoleucine was replaced by smaller residues (A or V), larger residues (F, Y, or W), and a residue of the same size (L). Mutant receptors containing these substitutions were characterized using an *in vivo* swarm plate assay and an *in vitro* methylation assay.

MATERIALS AND METHODS

Materials. The Sequenase kit was from United States Biochemical Corporation. The Mutagene kit was from BioRad. Aspartate and other chemicals were from Sigma unless otherwise noted. ^{125}I-labeled protein A was from Amersham. RP408 helper virus was from Stratagene. Oligonucleotide purification cartridges (OPC) were from Applied Biosystems.

Strains. Epicurian coli strain XL1-blue (*recA1 endA1 gurA96 thi hsdR17 supE44 relA1* [*F′ proAB laclqADM15 Tn10*(*tetR*)]) was purchased from Stratagene. *Escherichia coli* strain CJ236 (*dut ung thi relA1 pCJ105* (*F′, Cm*^{r})) was purchased from Biorad. *E. coli* strains RP5838 ((*tar-tap*)-*DE5201* (*tsr*)*DE7021 leuB6 his-4 metF*(*Am*)*159 srlC*::*Tn10-300 rpsL136* [*thi-1 ara-14 mtl-1 xyl-5 tonA31 tsx-78*]) and RP3808 ((*cheA-cheZ*)*DE cheW-tar-tap-cheR-cheB-cheY-tsr-1 leu- his- thi-*) were gifts from J. S. Parkinson.

Mutagenesis and Screening of Mutant Receptors. Single-stranded pMK650 plasmid was prepared in CJ236. Mutagenic oligonucleotides were made at the U. C. Berkeley DNA synthesis facility and purified using an OPC column. The mutagenic oligonucleotides were used in site-directed mutagenesis of pMK650 by the Kunkel method (Kunkel, 1985) using a Mutagene kit. XL1-Blue competent cells were transformed with an aliquot of the mutagenesis mix, and transformation mixes were spread on LB 100 μg/mL ampicillin plates. Alkaline lysis miniature plasmid preparations were prepared from overnight cultures of the transformants. The presence of each mutation was confirmed by double-stranded sequencing of the region around and including the second transmembrane domain using a Sequenase sequencing kit.

Swarm Plates. To test the *in vivo* chemotactic activity of the mutant receptors, an *E. coli* strain which initially lacked the receptor (RP5838) was transformed with the plasmids encoding each of the mutant receptors. Control transformants contained either a plasmid encoding the wild-type receptor (pMK650) or a vector alone (pEMBL19). The response of the transformants to aspartate was measured in a swarm plate assay. Minimal swarm plates contained 1% glycerol, Vogel–Bonner citrate, 0.3% agar, 100 μg/mL ampicillin, and 500 mg/L of each of the following: L-histidine, L-methionine, L-leucine, L-threonine, and thiamine. Aspartate swarm plates were supplemented with 100 μM aspartate. Plates were inoculated with a sterile wooden stick from a 2-mL overnight LB 100 μg/mL ampicillin culture. Swarm plates were incubated at 30 °C. Swarm diameters were measured five or six times over an 8–10-h period starting approximately 20–24 h after inoculation. Swarm rates were defined as the slope of a linear curve fit of the swarm diameters using Kaleidagraph.

Growth of Cultures for Membrane Preparations. A strain which lacks all the *che* genes, RP3808, was transformed with pMK650, pEMBL19, or a plasmid encoding a mutant receptor. Cultures of RP3808 transformants were grown as follows: 2 × 1-L cultures contained minimal media (Vogel–Bonner citrate, 1% glycerol, and 500 mg/L of each of the follwing:

L-histidine, L-methionine, L-leucine, L-threonine, and thiamine) with 100 μg/mL ampicillin. Each 1-L culture was inoculated from a 50-mL LB 100 μg/mL ampicillin overnight culture; 1-L cultures were grown with shaking for approximately 8 h at 30 °C. Cells were harvested by centrifugation and stored at −80 °C.

Membrane Preparations. Cell pellets were thawed in 10 mL of low-salt buffer (100 mM sodium phosphate, pH 7.0, 10% glycerol, 5 mM EDTA, 5 mM phenanthroline, and 1 mM PMSF) on ice. Cells were lysed by two rounds of French press. Unbroken cells were removed by low-speed centrifugation at 4 °C. The membranes were isolated by high-speed centrifugation (100K rpm, 10 min, TLA 100.3, 4 °C). The membrane pellets were resuspended in a total of 4 mL of high-salt buffer (20 mM sodium phosphate, pH 7.0, 2 M KCL, 10% glycerol, 5 mM EDTA, 1 mM phenanthroline, and 0.5 mM PMSF) and centrifuged again. The membrane pellets were resuspended in a total of 2 mL of high-salt buffer and centrifuged again. The membrane pellets were resuspended in a total of 1 mL of final buffer (10 mM Tris–HCl, pH 7.5, 10% glycerol, 5 mM phenanthroline, 1 mM PMSF, and 5 mM EDTA) and stored at −80 °C.

Estimation of Relative Receptor Concentration in Membrane Preparations. Membrane preparations were run on a 7.5% acrylamide SDS gel by the method of Laemmli (Laemmli, 1970). Proteins were transferred to nitrocellulose filter, and the filter was probed with an antiaspartate receptor polyclonal antibody (antibodies #9207 from H. P. Biemann). After washing, the blots were probed with ^{125}I-labeled protein A. After incubation and additional washing steps, the bound protein A was quantitated using a phosphoimager.

Methylation. Methylation buffer contained 10% glycerol, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 3H-*S*-adenosyl methionine (3H-SAM), and transferase. Reactions labeled as "+ aspartate" also contained 1 mM aspartate (final concentration). The time courses were started with the addition of an euqal volume of the methylation buffer to the membrane preparation sample. At time points, 10 λ of the reaction mixture was spotted onto a 1-cm^2 square of filter paper and dropped into 300 mL of 10% TCA (w/v). At the end of all the time points, the papers were washed twice with TCA and twice with methanol and then dried. The papers were placed in capless eppendorf tubes sitting in 7-mL scintillation vials containing 2.3 mL of scintillation fluid; 200 λ of 1 N NaOH was added to each paper, and the vials were immediately capped. The vials were incubated overnight at room temperature to allow hydrolysis of the methyl esters. The released methanol was quantitated by scintillation counting.

RESULTS

Construction of Phenylalanine Mutants. In previous mutagenesis studies, we discovered a mutant aspartate receptor, 7RII-96, that does not enable the cells to respond to aspartate in an *in vivo* chemotaxis assay although it had only hydrophobic to hydrophobic substitutions in the transmembrane domain (Jeffery & Koshland, submitted). The only outstanding feature of this mutant is the presence of three phenylalanine residues in place of smaller residues at positions V200, V202, and I204. We wanted to determine whether or not the introduction of a phenylalanine at a single position could also affect receptor signaling.

The transmembrane sequences of the aspartate receptor are probably α-helical. α-Helices are very stable structures in the low dielectric constant of the lipid bilayer since all the backbone hydrogen bonds can be satisfied. Structural determinations of the bacterial photoreaction center (Deisenhofer & Michel, 1989) and bacteriorhodopsin (Henderson et al., 1990) indicate that their transmembrane domains are composed of α-helices. In the aspartate receptor and the other chemotaxis receptors, the predicted first and second transmembrane sequences are the correct length to cross the membrane once as an α-helix.

Table 1: Aspartate Receptor Mutants with a Single Phenylalanine Substitution in the Second Transmembrane Domain

AR[a]	FAQWQLAVIALVVVLILLVAWYGI
L199F	F
V200F	F
V201F	F
V202F	F
L203F	F
I204F	F
L205F	F
L206F	F
V207F	F
A208F	F

[a] AR is the sequence of the second transmembrane domain in the wild-type receptor.

Cross-linking studies have indicated that in the dimer the first transmembrane domains are near to each other and the second transmembrane domains are near to their respective first transmembrane domains (Lynch & Koshland, 1991). It is possible that if the transmembrane helices are very close to each other in the bilayer, the large, bulky phenylalanine side chains could prevent correct packing of the second transmembrane domain against the first transmembrane domain in each subunit. This information would narrow down the ways in which the transmembrane helices can be arranged in the membrane.

We used site-directed mutagensis to construct three mutant aspartate receptors containing a phenylalanine in place of a smaller residue at one of the positions corresponding to a phenylalanine substitution in mutant 7RII-96. These three mutant receptors were tested for response to aspartate in an *in vivo* chemotaxis assay. After swarm plate assays indicated that the I204F mutant was not fully functional in chemotaxis, we constructed seven other phenylalanine mutants. Each of these phenylalanine mutations replaced a smaller residue, alanine, valine, or leucine. The sequence of the wild-type transmembrane domains and the location of each mutation are shown in Table 1. Together, these 10 mutant receptors contain a bulky residue around three turns of the transmembrane α-helix. The set of mutants include residues 199–208, almost half of the transmembrane sequence.

Swarm Rates of Phenylalanine Mutants. To test the *in vivo* chemotaxis activity of the mutant receptors, we performed a swarm plate assay. A strain which initially lacked the receptor was transformed with a plasmid encoding each of the mutant receptors, the wild-type receptor (pMK650), or the vector alone (pEMBL19). Swarm diameters were measured on minimal plates and on minimal plates supplemented with aspartate. The swarm rates for all 10 phenylalanine mutants are given in Figure 2. Cells expressing the wild-type aspartate receptor (pMK650) have a low rate of swarming on minimal

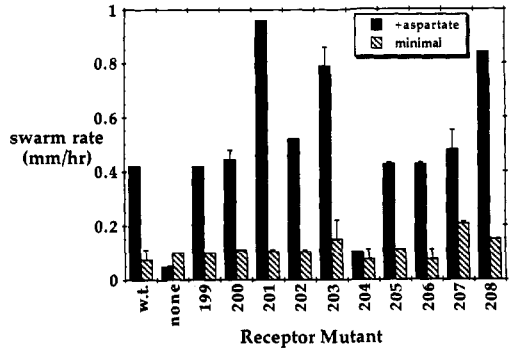

FIGURE 2: Swarm rates of cells expressing aspartate receptors with phenylalanine substitutions in the second transmembrane domain. Cells transformed with a plasmid encoding the wild-type receptor (pMK650) have a low rate of swarming on minimal swarm plates and a higher rate of swarming on plates which were supplemented with aspartate. Cells transformed with the vector alone (pEMBL19) have a low swarm rate both in the presence and absence of aspartate. Most of the mutant receptors which have a phenylalanine in place of a wild-type amino acid allowed the cells to have a much greater rate of swarming in the presence of aspartate than in the absence of aspartate. However, the mutation at position 204 caused a low level of swarming both in the presence and absence of aspartate.

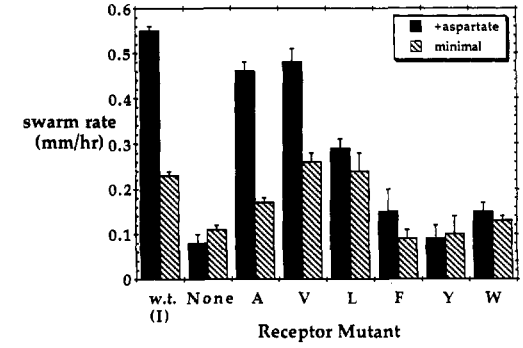

FIGURE 3: Swarm rates of cells expressing receptors with mutations at position 204. The receptors with alanine or valine at position 204 allowed the cells to form a large swarm in the presence of aspartate. The receptor with leucine at this position allowed a swarm rate that was higher than that of the negative control but lower than that of the wild-type control. The receptors with phenylalanine, tyrosine, or tryptophan at this position did not allow the cells to increase their swarm rate in response to aspartate.

swarm plates (an increase of swarm diameter under 0.25 mm/h) and a higher rate of swarming on plates which were supplemented with aspartate (an increase of swarm diameter of approximately 0.4 mm/h). Cells which do not express an aspartate receptor (pEMBL19) have a low rate of swarming on plates with or without added aspartate. Most of the mutants which have a phenylalanine in place of a wild-type amino acid allowed the cells to respond to aspartate like the wild-type receptor; they also had a low swarm rate in the absence of aspartate and a much higher swarm rate in the presence of aspartate. Three of the mutants, 201, 203, and 208, had unusually high rates of swarming in the presence of aspartate. However, the substitution of a phenylalanine for an isoleucine at position 204 caused a decrease in swarm rate both in the presence and absence of aspartate (the swarm rate was less than half the swarm rate of cells expressing the wild-type receptor under the same conditions). This rate of swarming was similar to the negative control rate, and the rate did not increase with the addition of aspartate. The single change from one hydrophobic residue to another prevented the normal function of the receptor.

Construction of Mutants at Position 204. The decreased chemotaxis could be due to steric hindrance from the larger, less flexible aromatic ring. To test that possibility, site-directed mutagenesis was used to construct a second set of mutant receptors in which the isoleucine at position 204 was substituted with residues which are larger (Y or W), smaller (A or V), or the same size (L).

Swarm Rates of Mutants. These receptors were tested for response to aspartate in the swarm plate assay. The receptors with alanine or valine at position 204 allowed the cells to respond to aspartate (Figure 3). They had a low swarm rate in the absence of aspartate and a higher swarm rate in the presence of aspartate like the wild-type receptor. The receptors with phenylalanine, tyrosine, ir tryptophan at this position did not allow the cells to respond to aspartate. They had very low swarm rates both in the presence and absence of aspartate like the negative control.

Cell expressing the receptor with leucine at position 204 had an intermediate swarm rate. It allowed a swarm rate that was higher than the negative control but lower than the wild type control, which indicates there was some receptor function, but that it was impaired. The appearance of this swarm was also unusual. In the presence of aspartate, cells expressing the wild type receptor, I204A, or I204V form large diffuse colonies referred to as swarms. The negative control transformants and cells expressing the I204F, I204Y, or I204W receptors form small, dense colonies that look like the colonies which form on plates that lack aspartate. The cells expressing the receptor with leucine at position 204 formed a colony that was larger than the negative control but smaller that the wild type swarms. Although this colony had a small response to aspartate, it did not form a diffuse swarm like the one that is formed by cells expressing the wild type receptor. Instead, the colony was dense like the ones formed by the negative control.

Methylation of Mutant Receptors. During chemotaxis, the receptor adapts to the current level of aspartate so it can respond to the next level it encounters. In order to do this, the receptor is methylated by the CheR methyltransferase. The function of the aspartate receptor can be tested *in vitro* by measuring the rate by which membrane preparations containing the receptor undergo methylation in the presence and absence of aspartate. Membranes containing the wild-type receptor undergo a low rate of methylation in the absence of aspartate. When aspartate is added, the rate of receptor methylation increases.

A strain which did not initially express an aspartate receptor, RP3808, was transformed with a plasmid encoding the wild-type receptor, the receptors with mutations at position 204, or the vector alone. Membrane preparations were prepared from the transformants. The amount of receptor in each membrane preparation was estimated with a quantitative Western blot using ^{125}I-labeled protein A. The relative amounts of ^{125}I bound to the membrane were determined with a phosphoimager. The relative amounts of mutant receptor were estimated to be within 2-fold of the concentration of the wild-type aspartate receptor and did not affect the rate of methylation since the receptor was in excess in the assay mixture.

In vitro methylation assays were performed as described in Materials and Methods. The methylation rates were normalized to the methylation rate of the wild-type aspartate receptor in the absence of aspartate and are indicated in Figure 4. Membranes from cells expressing the wild-type receptor had a significant rate of methylation in the absence of aspartate and a higher rate of methylation in the presence of aspartate. Membranes from cells transformed with the vector alone did

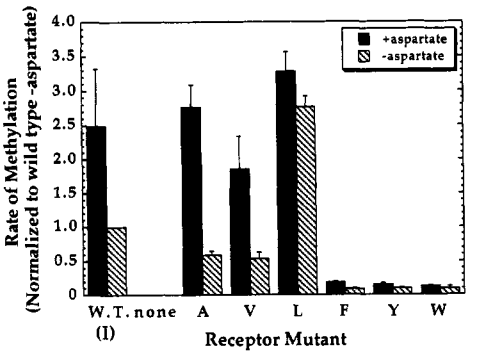

FIGURE 4: *In vitro* methylation assay of receptors with mutations at position 204. Wild-type receptors undergo a low rate of methylation in the absence of aspartate. When aspartate is added, the rate of methylation increases. The receptors with alanine or valine at position 204 had a significant rate of methylation in the absence of aspartate and a higher rate of methylation in the presence of aspartate, like the wild-type receptor. The receptor with leucine at position 204 had a high methylation rate both in the presence and absence of aspartate. Receptors with phenylalanine, tyrosine, or tryptophan at position 204 were methylated to a very low extent in both the presence and absence of aspartate.

not have a significant rate of methylation in the presence or absence of aspartate. Membranes from cells expressing the alanine or valine mutants had methylation rates that were similar to the wild-type rates both in the presence and absence of aspartate. Membranes from cells expressing the receptors with phenylalanine, tyrosine, or tryptophan at position 204 were methylated to a very low extent in both the presence and absence of aspartate. The basal rate of methylation for these three mutants was very low, and the methylation rate only increased a small amount with the addition of aspartate. As in the swarm plate assay, the receptor with leucine at position 204 did not behave like the wild-type receptor nor like the negative control. Membranes containing this receptor had a very high methylation rate in the absence of aspartate, and the rate increased with the addition of aspartate.

DISCUSSION

Effects of Single Mutations in the Second Transmembrane Domain. To study the effects of the transmembrane domain mutations on transmembrane signaling, site-directed mutations were constructed in the second transmembrane domain of the *E. coli* aspartate receptor. The substituion of a large, inflexible phenylalanine residue in place of a smaller residue at nine different positions allowed the receptor to respond to aspartate in the swarm plate assay, but the substitution of a phenylalanine residue for isoleucine at position 204 resulted in a receptor that did not respond to aspartate in either the swarm plate or methylation assays. To test if the large, inflexible phenylalanine side chain prevented normal receptor function, a second set of mutant receptors was constructed in which the isoleucine at position 204 was replaced by residues which were smaller (A or V), larger (F, Y, or W), or the same size (L). In both the swarm plate and methylation assays, the receptors in which the isoleucine was replaced by a smaller residue behaved in a similar fashion to the wild-type receptor and the receptors in which the isoleucine was replaced by a bulkier residue behaved more like the negative control. The mutant in which isoleucine was replaced by leucine also exhibited altered behavior. It allowed only a small amount of swarming in the presence of aspartate in the swarm plate assay and had an elevated level of methylation both in the presence and absence of aspartate in the methylation assay.

Since the second transmembrane domain tolerates many amino acid substitutions, and yet there is one position at which a single point mutation causes a loss of function, the sequence requirements for the second transmembrane domain are more than just hydrophobicity. While previous experiments have demonstrated that the introduction of a charged residue, a lysine, in the first transmembrane helix can affect receptor function (Oosawa & Simon, 1986), this is the first example that a small change in side chain structure can affect the function of the aspartate receptor.

Interactions between Transmembrane Helices. Studies of other transmembrane proteins have demonstrated specific interactions between the individual transmembrane α-helices. Bacteriorhodopsin, which has seven transmembrane domains, can be reconstituted from fragments that contain only a few transmembrane helices each. In one such experiment, bacteriorhodopsin was cleaved by chymotrypsin into two fragments which contained two and five helices. These fragments were able to assemble into a functional protein (Huang et al., 1981; Popot et al., 1987). In another experiment, the seven transmembrane helices were expressed as a five-transmembrane-helices fragment and two synthetic peptides (Kahn & Engelman, 1992). These fragments were also able to reassemble into a functional receptor. Similar reconstitution experiments were performed on lactose permease which contains 12 transmembrane sequences. In one experiment, it was reconstituted from separate cloned fragments containing 10 and 2 helices (Bibi & Kaback, 1990). In addition, some mutants containing deletions of two or more transmembrane helices were able to complement each other (Bibi & Kaback, 1992). These reconstitution experiments with bacteriorhodopsin and lactose permease show there are specific interactions between helices which enable the protein to fold even in the absence of a covalently connected polypeptide chain. It is possible that similar close interactions may also be present between the aspartate receptor transmembrane helices.

Side of Helix. The isoleucine at position 204 in the second transmembrane domain can be replaced with smaller residues, and the receptor remains functional, but bulky residues affect the receptor function. A similar pattern of bulky versus small residues was observed in studies of the dimerization of the glycophorin A transmembrane domain. When the glycophorin A transmembrane helix was fused to the C terminus of staphylococcal nuclease via a flexible linker, the resulting fusion protein formed dimers in solution (Lemmon et al., 1992). Since wild-type staphylococcal nuclease is a monomer, dimer formation was due only to interactions between the glycophorin A transmembrane helix. Site-directed mutagenesis of the transmembrane helix in the fusion protein showed that the introduction of a bulky residue on one side of the transmembrane helix can inhibit dimerization but the introduction of a bulky group on the opposite side of the helix does not inhibit dimer formation. The authors concluded that the decrease in dimer formation caused by the first mutation was due to the location of the bulky residue on the side of the helix which comes together during dimer formation and that the second mutation did not inhibit dimer formation because the second position was on the other side of the helix. When the valine at the second position was replaced with a smaller residue (glycine, alanine, leucine, or isoleucine), dimerization was somewhat disrupted, but replacement with a larger residue (tyrosine or tryptophan) was more disruptive.

In the aspartate receptor, nine of the phenylalanine mutants were functional, so it appears that the introduction of an aromatic side chain at many positions of the transmembrane domain does not adversely affect the interaction of the receptor

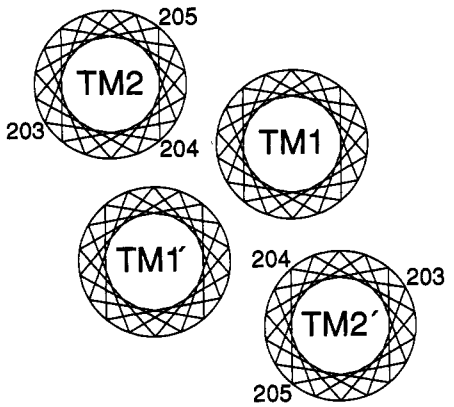

FIGURE 5: Helical wheel diagrams of the aspartate receptor transmembrane domains. From cross-linking experiments, the first transmembrane domains, 1 and 1′, are near to each other and the second transmembrane domains, 2 and 2′, are farther out (Lynch & Koshland, 1991; Pakula & Simon, 1992). Since nine of the phenylalanine mutants were functional, it appears that the introduction of an aromatic residue at several positions of the transmembrane domain does not adversely affect the interaction of the receptor with the lipid bilayer. This suggests that the side of the helix containing position 204 faces the first transmembrane domain helices.

with the lipid bilayer. This suggests that the side of the helix containing position 204 faces the first transmembrane domain helices.

Cross-linking studies of the aspartate receptor indicate that the first transmembrane domain in each subunit is located next to the first transmembrane domain of the other subunit (Lynch & Koshland, 1991). Each second transmembrane helix is near the first transmembrane helix in the same subunit. It has been suggested that these four helices form a "pseudo-four-helix bundle" (Milburn et al., 1991). The results from our mutagenesis studies suggest that the second transmembrane helix actually packs closely against the first transmembrane domains (Figure 5).

A model of the aspartate receptor transmembrane helices was constructed by extending the sequence of helices 1 and 4 in the four-helix bundle of each subunit of the *Salmonella typhimurium* aspartate receptor ligand-binding domain crystal structure (Milburn et al., 1991). Due to the high sequence conservation between the *E. coli* and S. *typhimurium* receptors, the crystal structure and the transmembrane structure are probably similar in the two receptors (Russo & Koshland, 1983; Krikos et al., 1985). In the transmembrane domain model, the side chain of isoleucine 204 in the second transmembrane domain faces the first transmembrane helix in the same subunit and the first transmembrane helix in the other subunit. The transmembrane model can be used to predict which residues in the first transmembrane helices are across from position 204 in the second transmembrane domain. In the *S. typhimurium* transmembrane structure model, position 204 is across from valine at position 14 in the same subunit and leucine at position 15 in the other subunit. These residues are conserved in the *E. coli* receptor. While there is less sequence conservation in the other three chemotaxis receptors (*tsr*, *tap*, and *trg*) (Boyd et al., 1983; Bollinger et al., 1984), the residues which are probably located at similar positions in the serine receptor transmembrane domains are also valine 14 and leucine 15.

Leucine Mutant. The decrease in swarm rate and the altered methylation levels of the I204L mutant receptor are especially interesting. Since small changes in side chain structure can cause a decrease in function, it suggests that the aspartate receptor transmembrane helices pack closely against each other and subtle disruptions of this packing can affect signaling. Such specificity of helix–helix interactions was observed in studies of oligomerization of transmembrane helices from glycophorin A and the FCγIIIA receptor. In both of these studies, the presence of a leucine in a specific position in the transmembrane domain resulted in a different amount of oligomerization than when an isoleucine was present at that position. In the glycophorin A/staphylococcal nuclease fusion protein experiments mentioned above, the substitution of an isoleucine in place of valine at position 84 allowed significant dimer formation but the substitution of leucine at that position allowed only "detectable" dimer formation (Lemmon et al., 1992). This observation that isoleucine and leucine can be distinguished in oligomerization of transmembrane helices was also observed in studies of the assembly of the Fcγ receptor (Kurosaki et al., 1991). Heterodimer formation between the α and ζ subunits in the endoplasmic reticulum results in surface expression of the FcgRIIIA complex. The interaction between these two subunits is due to noncovalent interactions in their transmembrane domains. The wild-type human ζ transmembrane helix contains a leucine at position 46 and has a high degree of affinity for the α subunit. Mutagenesis to an isoleucine caused a decrease in the interaction with the α subunit, resulting in 65% reduction in the surface expression of the complex. Conversely, in the mouse ζ transmembrane helix, an isoleucine to leucine substitution caused an increase in interaction with the human α subunit, resulting in a 3-fold increase in surface expression of the complex. Since the substitution of an isoleucine for a leucine (or the substitution of a leucine for an isoleucine) can alter transmembrane helix oligomerization in these proteins, it supports the model that transmembrane helices in a protein like the aspartate receptor may also make very specific interactions.

Transmembrane Signaling. The results of the mutagenesis studies of the aspartate receptor contrast with the apparently higher tolerance to mutagenesis of the transmembrane domain which was found with the EGF receptor. This may be due to differences in the mechanisms of transmembrane signaling in the two receptors. The EGF receptor, which retain ligand-stimulated kinase activity with mutations in its transmembrane domain, signals through association and dissociation. The transmembrane helix has a somewhat passive role in signaling and may not need to interact directly with other transmembrane helices. In contrast, the aspartate receptor is a dimer in both the presence and absence of aspartate and probably signals through a conformational change within the dimer (Milligan & Koshland, 1986).

The insulin receptor also has the same oligomeric state in the presence and absence of ligand due to disulfide cross-links between subunits (Ebina et al., 1985; Ullrich et al., 1985). It probably also signals through a conformational change in the heterotetramer, but this conformational change may not involve such a close packing of helices. The results of mutagenic experiments in the EGF and insulin receptors do not rule out the possibility that some conservative mutations may be found that affect receptor signaling. Not every possible mutation has been made in the transmembrane sequences of those receptors, and many of the mutations in the aspartate receptor still allowed the receptor to remain functional.

It is not clear why the PDGF receptor mutants were not fully functional. Several experiments indicate this receptor signals through association and dissociation like the EGF receptor. It is possible that the PDGF receptor transmembrane domains may play a part in oligomerization, like in the Fcγ

receptor and glycophorin A. It is also possible that replacing the whole transmembrane domain affected the folding of the extracellular or cytoplasmic domains so that the receptor was not active.

Models for transmembrane signaling in the aspartate receptor involve a change in the relative locations of the two subunits (such as in the "scissors" model), a conformational change within individual subunits (such as in the "piston" model), or a combination of intra- and intersubunit mechanisms. Each mechanism involves a shift in the position of the transmembrane helices. In either case, the introduction of a large, bulky group on the side of the second transmembrane helix that faces an adjacent helix could interfere with this movement. For example, in the piston model, the phenylalanine could prevent the sliding of the first transmembrane helix against the second transmembrane helix.

SUMMARY

We have found a position in the second transmembrane helix of the *E. coli* aspartate receptor at which the substitution of a hydrophobic residue in place of another hydrophobic residue can cause a decrease in the function of the receptor. These results support the model that the side of the second transmembrane helix containing position 204 faces the other transmembrane helices in the receptor and suggest that the second transmembrane helix packs closely against them.

REFERENCES

Bibi, E., & Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4325–4329.

Bibi, E., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1524–1528.

Bollinger, J. C., Park, C., Harayama, S., & Hazelbauer, G. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3287–3291.

Boyd, A., Kendall, K., & Simon, M. I. (1983) *Nature 301*, 623–626.

Carpenter, C. D., Ingraham, H. A., Cochet, C., Walton, G. M., Lazar, C. S., Sowadski, J. M., Rosenfeld, M. G., & Gill, G. N. (1991) *J. Biol. Chem. 266*, 5750–5755.

Deisenhofer, J., & Michel, H. (1989) *Science 245*, 1463–1473.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., & Rutter, W. J. (1985) *Cell 40*, 747–758.

Escobedo, J. A., Barr, P. J., & Williams, L. T. (1988) *Mol. Cell. Biol. 8*, 5126–5131.

Falke, J. J., & Koshland, D. E., Jr. (1987) *Science 237*, 1596–1600.

Frattali, A. L., Treadway, J. L., & Pessin, J. E. (1991) *J. Biol. Chem. 266*, 9829–9834.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.

Huang, K.-S., Bayley, H., Liao, M.-J., London, E., & Khorana, H. G. (1981) *J. Biol. Chem. 256*, 3802–3809.

Kahn, T. W., & Engelman, D. M. (1992) *Biochemistry 31*, 6144–6151.

Kashles, O., Szapary, D., Bellot, F., Ullrich, A., Schlessinger, J., & Schmidt, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9567–9571.

Krikos, A., Conley, M. P., Boyd, A., Berg, H. C., & Simon, M. I. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1326–1330.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–92.

Kurosaki, T., Gander, I., & Ravetch, J. V. (1991) *PNAS 88*, 3837–3841.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lemmon, M. A., Flanagan, J. M., Hunt, J. F., Adair, B. D., Bormann, B.-J., Dempsey, C. E., & Engelman, D. M. (1992) *J. Biol. Chem. 267*, 7683–7689.

Longo, N., Shuster, R. C., Griffin, L. D., Langley, S. D., & Elsas, L. J. (1992) *J. Biol. Chem. 267*, 12416–12419.

Lynch, B. A., & Koshland, D. E., Jr. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10402–10406.

Milburn, M. V., Prive, G. G., Milligan, D. L., Scott, W. G., Yeh, J., Jancarik, J., Koshland, D. E., Jr., & Kim, S.-H. (1991) *Science 254*, 1342–1347.

Milligan, D. L., & Koshland, D. E., Jr. (1988) *J. Biol. Chem. 263*, 6268–6275.

Mowbray, S. L., Foster, D. L., & Koshland, D. E., Jr. (1985) *J. Biol. Chem. 260*, 11711–11718.

Oosawa, K., & Simon, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6930–6934.

Pakula, A. A., & Simon, M. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4144–4148.

Popot, J.-L., Gerchman, S.-E., & Engelman, D. M. (1987) *J. Mol. Biol. 198*, 655–676.

Russo, A. F., & Koshland, D. E., Jr. (1983) *Science 220*, 1016–1020.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature 313*, 756–761.

Yamada, K., Goncalves, E., Kahn, C. R., & Shoelson, S. E. (1992) *J. Biol. Chem. 267*, 12452–12461.