the data under likelihood ratio tests; these are asymptotically equivalent to 95% confidence limits. Log L is the difference in natural log likelihood from that of the model with two classes of equal deleterious effects, which has the same likelihood as the one-equal-effect model with U_I variable. In the two-equal-effects model, a positive value for s_2 gave a higher likelihood than $s = 0$, but the difference in log likelihood was nonsignificant.
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A Piston Model for Transmembrane Signaling of the Aspartate Receptor

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To characterize the mechanism by which receptors propagate conformational changes across membranes, nitroxide spin labels were attached at strategic positions in the bacterial aspartate receptor. By collecting the electron paramagnetic resonance spectra of these labeled receptors in the presence and absence of the ligand aspartate, ligand binding was shown to generate an ~1 angstrom intrasubunit piston-type movement of one transmembrane helix downward relative to the other transmembrane helix. The receptor-associated phosphorylation cascade proteins CheA and CheW did not alter the ligand-induced movement. Because the piston movement is very small, the ability of receptors to produce large outcomes in response to stimuli is caused by the ability of the receptor-coupled enzymes to detect small changes in the conformation of the receptor.

Cells receive signals from the outside world by way of receptors that span the membrane. Although some receptors transmit information across the membrane by means of an ion channel that allows ions into the cell, most receptors do not transmit material across the membrane. Rather, these receptors undergo conformational changes induced by the ligand or stimulus that interacts with the exterior part of the receptor, and these conformational changes travel across the membrane to the cytoplasmic portion of the receptor. The types of conformational changes used by receptors to carry out transmembrane signaling are not known.

To distinguish among alternative models proposed for transmembrane signaling, we developed a strategy in which the distances between appropriately placed spin labels would give different results for different models (*1*) (Fig. 1). In this procedure, spin-labeling electron paramagnetic resonance (EPR) spectroscopy (*2*) was combined with the use of a spectroscopic ruler (*3, 4*). Spin labeling has been used to describe qualitative protein structures (*5, 6*) and has subsequently been developed for quantitative assessment of protein movements and applied to several proteins (*4*, *7*, *8*), including the aspartate receptor (*9*). In a hypothetical example (Fig. 1), a model of transmembrane signaling that depended on an association-dissociation, as well as one that required a scissors movement, would move residue a closer to b and c. A ligand-induced piston motion would move residue a closer to b, but further from c. A rotation mechanism would move a further from b, but closer to c. A see-saw motion would move b and c both further from a. Thus, judicious placement of spin labels allows an analysis that can distinguish among models.

This strategy was applied to the bacterial aspartate receptor, a receptor with a structure similar to that of many other receptors that contain one-two transmembrane domains per subunit and function as oligomers [such as the insulin receptor, the epidermal growth factor receptor (EGFR), and the cytokine receptors] (*10–12*). The aspartate receptor and its homologs are used by bacteria to navigate through spatial gradients of nutrients and toxic substances, using detection of temporal gradients to modulate swimming behavior (*13–17*). Although the aspartate receptor has been studied in detail, the mechanism of signal transduction has remained elusive. Disulfide cross-linking experiments have suggested that aspartate triggers global changes in the receptor (*18*). Several mechanistic models, postulated on the basis of fragments of either the ligand-binding domain (*19–22*) or the cytoplasmic domain (*23, 24*), have been proposed. Ligand binding does not affect the dimerization state of the aspartate receptor (*25*), and receptors that are disulfide cross-

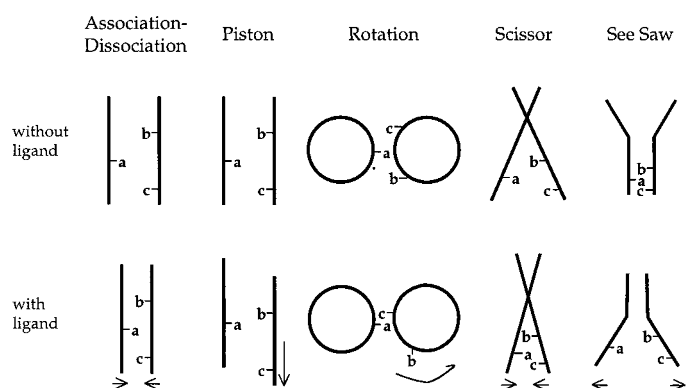

Fig. 1. Postulated models for transmembrane signaling. General schemes for possible ligand-generated movements in transmembrane receptors (*1*). The thick lines represent receptor transmembrane helices. The view is from the side, except in the rotation diagram where the view is from the end of the helices. Any of these motions could occur between two helices in separate subunits, or between two helices within one subunit. The letters represent amino acid side chains where labels could be attached. The rotation model could have results similar to those seen in the piston model, but the labels could be placed such that they would distinguish between models, as shown.

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linked across the dimer interface retain function (*18*), suggesting that transmembrane signaling occurs in the absence of any change in oligomerization. Aspartate receptors with only one functional cytoplasmic domain are able to signal, supporting the idea that transmembrane-signaling movements occur within one subunit (*26–28*). In corroboration, disulfide bonds between transmembrane-spanning helices within one subunit of the receptor can lock the receptor into a ligand-independent "off" or "on" signaling state (*29*). These cumulative results indicate that transmembrane signaling in the aspartate receptor occurs by way of an intrasubunit conformational change. By using the x-ray structure of the periplasmic domain of the aspartate receptor (*19*, *22*) and deduced transmembrane regions (*21*, *30*), it was possible to calculate where spin labels should be placed to allow the clearest discrimination among transmembrane signaling mechanisms (Fig. 2).

To incorporate the spin labels into the aspartate receptor, the codons of the identified residues were mutated to cysteines, and the resultant protein was solubilized with detergent and purified (*9*, *31*). The mutant protein was then reacted with the thiol-specific methanethiosulfonate spin label and reconstituted into purified *Escherichia coli* membranes; all EPR spectra were collected from receptors in membranes. Because previous work had suggested that transmembrane domain (TM) 2 moves relative to TM1 when aspartate is added to the receptor (*9*, *20*, *21*), we chose to monitor intrasubunit changes in spin-spin distances by placing one spin label in TM1 and the other in TM2 (Fig. 2). From the combination of views (B) and (D) (Fig. 2), a downward piston-type movement of TM2 would bring residue 207 closer to 10 and residue 210 and 215 further from 10, whereas a counterclockwise rotation would bring residues 207 and 210 closer to 10, and residue 215 further from 10 (Table 1). An association-dissociation, scissors, or seesaw motion would bring all the labels either closer together or further apart (Table 1). The spin-labeled aspartate receptors retained function as assessed by in vitro phosphorylation assays (*32*).

Spectra were obtained of receptors labeled at positions 10 and 207 (AR 10-207), 10 and 210 (AR 10-210), and 10 and 215 (AR 10-215) in the presence and absence of aspartate (*33*). Small but detectable spectral differences were seen when aspartate was added to AR 10-210 and AR 10-215, but none were seen in AR 10-207 (*34*). Each receptor is composed of two identical subunits and thus contains four spin labels; therefore, spin-spin interactions could result from several possible pairwise spin-spin dipolar couplings. To distinguish whether the observed spectral changes were the result of movements within one subunit or between the two subunits, spin-labeled aspartate receptor was mixed with an equimolar amount of unlabeled wild-type aspartate receptor, and the subunits were allowed to interchange. This diluted the doubly labeled aspartate receptor such that the final concentration of aspartate receptor with four spin labels was one-eighth the population of the receptor with two spin labels (*25, 35*). In the exchanged samples, aspartate caused spectral changes in AR 10-207 as well as AR 10-210 and AR 10-215 (Fig. 3). In AR 10-207, aspartate-induced changes were detected only when the spin labels were on one subunit. One explanation for this is that aspartate binding generated both inter- and intrasubunit movements (*9*), and at positions 10 and 207 these motions canceled each other so there was no apparent aspartate-induced spectral change unless the intrasubunit movements were isolated.

To characterize the ligand-induced changes in the aspartate receptor, we analyzed distance changes between the spin-label pairs in the exchanged receptors. Although both inter- and intrasubunit movements occur in the aspartate receptor, intrasubunit movements are sufficient for transmembrane signaling (*26–28*), and so we characterized only these aspartate-generated motions further. When spin-labeled samples are frozen, spin-spin dipolar coupling results in more broadening of the EPR spectrum when the spin labels are closer to each other; this can be used to determine the distance between two spin labels (*36*). Aspartate binding brought the spin labels in AR 10-207 closer to each other. In AR 10-210 and AR 10-215, in contrast, aspartate binding increased the distance between labels. Similar types of spectral changes were seen in both frozen and room temperature samples, suggesting that these changes are not artifacts of freezing. These movements, as well as the previously characterized change at positions 39 and 179 (*9*), are most consistent with a downward piston motion of TM2 relative to TM1 (Fig. 2 and Table 1). This type of motion was previously postulated for the aspartate receptor (*20, 21, 37*), although different types of movements have also been postulated (*19*, *38*).

With a Fourier transform analysis (*4*), the magnitudes of the aspartate-generated spin-spin distance changes were calculated. In all cases, aspartate binding caused movements on the order of 1 Å. This technique, however,

Table 1. Predicted and observed effect of different transmembrane-signaling models. Further, the spin labels would move further apart after ligand binding; Closer, the spin labels would move closer after ligand binding. The prediction for the piston and rotation models depends on the direction of the piston (in this case described for TM2 moving downward) or rotation (in this case described as a counterclockwise movement of TM2). The data for aspartate receptor with spin labels at positions 39 and 179 are from (9).

Location of spin-labeled residues	Predicted relative position of spin labels					Observed relative position of spin labels
	Assoc.-dissoc.	Scissor	Seesaw	Piston	Rotation	
10-207	Closer	Closer	Further	Closer	Closer	Closer
10-210	Closer	Closer	Further	Further	Closer	Further
10-215	Closer	Closer	Further	Further	Further	Further
39-179	Closer	Closer	Further	Further	Further	Further

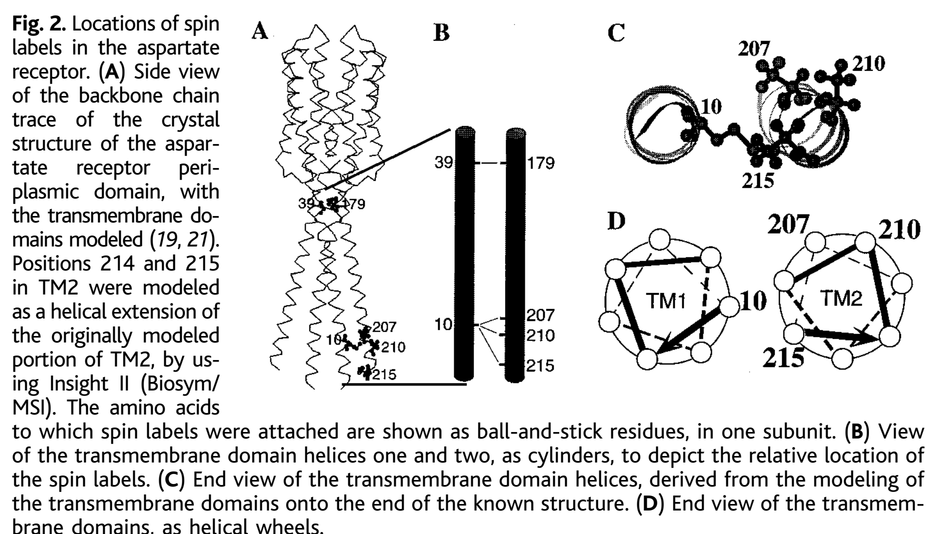

Fig. 2. Locations of spin labels in the aspartate receptor. (**A**) Side view of the backbone chain trace of the crystal structure of the aspartate receptor periplasmic domain, with the transmembrane domains modeled (*19*, *21*). Positions 214 and 215 in TM2 were modeled as a helical extension of the originally modeled portion of TM2, by using Insight II (Biosym/MSI). The amino acids to which spin labels were attached are shown as ball-and-stick residues, in one subunit. (**B**) View of the transmembrane domain helices one and two, as cylinders, to depict the relative location of the spin labels. (**C**) End view of the transmembrane domain helices, derived from the modeling of the transmembrane domains onto the end of the known structure. (**D**) End view of the transmembrane domains, as helical wheels.

measures the distance between nitroxide groups at the end of a 5 Å side chain (from the α-carbon), and because these side chains could be in multiple conformations, the actual net movement of the α-helix backbone could be up to 2.5 Å (*4*, *39*). The movement of 1 Å is similar to the distance change measured previously in the aspartate receptor periplasmic domain at positions 39 and 179 (*9*). This suggests that there is little or no signal amplification by the mechanism of conformational change as the signal travels from the binding pocket through the transmembrane domain. Aspartate binding to the receptor causes a large effect on the enzymes that carry out both the downstream kinase and methylation reactions (*16*, *17*, *40*). Thus, the signal amplification probably occurs because these enzymes can differentiate the small receptor conformational changes.

In order to characterize how the receptor-associated proteins affect the signaling properties of the receptor, we mixed purified CheW and CheA together with our spin-labeled receptors and collected the spectra again (*41*). In vivo, the aspartate receptor exists in a stable complex with the CheW and CheA proteins of the phosphorylation cascade; aspartate binding does not change the association constants of these proteins (*42*). In spin-labeled AR 10-207, as well as AR 10-210 and AR 10-215, the extent of electron spin-spin interaction decreased slightly in the presence of CheW and CheA, suggesting that these proteins drew the spin labels apart (Fig. 4). The receptors used in these experiments had spin labels on both receptor subunits, so the CheW-CheA effect could be caused by an increase in either the inter- or intrasubunit spin-spin distances. Exchange of the labeled aspartate receptor with wild-type aspartate receptor before adding CheW and CheA did not appreciably enhance the outcome of adding CheW and CheA, suggesting that both exchange and CheW-CheA binding had similar effects on spin-spin interaction in the receptor (*34*, *43*).

Even though CheW and CheA caused changes in the receptor transmembrane domain structure, these proteins did not change the quality of the aspartate-induced conformational changes in AR 10-207, 10-210, or 10-215 (Fig. 4). This indicates that an aspartate receptor in the ternary complex with CheW and CheA has the same pistonlike ligand-induced conformational change as the aspartate receptor alone.

By using spin-labeling EPR on an intact receptor in a membrane, we have been able to map and define a ligand-induced conformational change as it travels through the lipid bilayer. The measured changes are explained best by a piston movement of one TM helix downward relative to the other, within one subunit. Although our present results preclude an exclusive rotation model (*23, 44*), it is possible that a pistonlike movement with some small rotation, a screw-type motion, could fit the data. The piston model has a number of implications. α Helices are resistant to compression or extension and therefore would faithfully deliver a small conformational change over a long distance, as would be required for the aspartate receptor (~100 Å from the site of aspartate binding to the initial cytoplasmic portion of the receptor). This pistonlike movement, in which ligand binding causes one helix to shear past another, has been described in several soluble proteins (*45, 46*). Receptors in general, and the aspartate receptor in particular, must greatly amplify the energy of binding to generate the energy of response (bacterial swimming). The proposed piston mechanism would transmit a 1 Å conformational change in the periplasmic domain as a 1 Å change to the cytoplasmic domain. If the 1 Å change in the cytoplasmic domain can be perceived by the downstream methylation and phosphorylation enzymes, then amplification occurs. A small conformational change can certainly be detected by the discriminatory power of enzymes, as exemplified by isocitrate dehydrogenase (*47*) and restriction enzyme substrate recognition (*48*). Although it is possible that the 1 Å movement is structurally amplified further within the receptor cytoplasmic domain, this does not seem to be necessary for signal transduction.

Although it is difficult to predict how the piston-type movement would affect the aspartate receptor cytoplasmic domain because the structure has not been determined, the piston may push a portion of the cytoplasmic domain away from the membrane, thus decreasing an allosteric interaction between the receptor and the membrane. This allosteric action in turn would trigger the kinase and methylation cascades.

Mechanistic details of aspartate receptor signaling may be common to a larger class of receptors. The one-two transmembrane domains per subunit family of receptors, to which the aspartate receptor belongs, shares signaling techniques. For example, chimeras of the aspartate and insulin receptors (*49*) as well as ones of the insulin and EGF receptors (*50*) retain signaling ability. Although many members of this family signal by way of an association-dissociation mechanism (*11*), additional mechanistic steps are probably required. The insulin receptor, for example, is a disulfide-bonded dimer. Crystallographic evidence suggests that the erythropoietin receptor signals by way of a conformational rearrangement of a preexisting dimer (*51*). Antibodies that dimerize the EGFR result in about 60% of ligand-induced signaling activity, suggesting that there are further requirements for full activation such as additional ligand-caused conformational changes (*52*, *53*). Given the signaling similarities between these receptors, it is possible that the piston mechanism proposed may be used by this family of receptors, as well by others. Binding of a ligand to a seven–transmembrane domain GTP-binding protein (G protein)–coupled receptor could induce a piston-type motion in

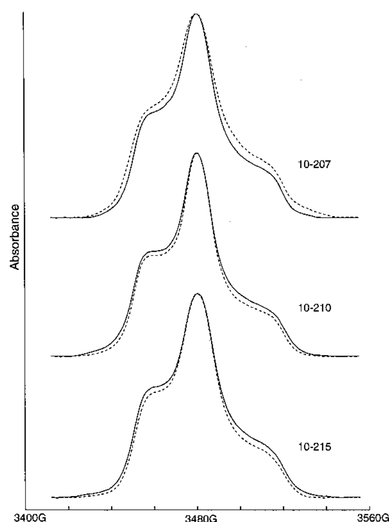

Fig. 3. Aspartate-induced changes in the absorbance EPR spectra of spin-labeled aspartate receptor. The EPR spectra were taken at 140 K in the presence (dotted line) or absence (solid line) of aspartate. The *x* axis is H (Gauss); the *y* axis is arbitrary absorbance units. Most aspartate receptors are composed of one doubly labeled and one unlabeled subunit. The spectral contribution from the noninteracting species as a result of incomplete labeling and unidentified background labeling (*9*) is subtracted from the EPR spectra. This noninteracting species amounts to 50% of the total number of spins. The Fourier deconvolution analysis (*4*) reveals that aspartate binding decreases the interspin distance of AR 10-207 from 11 to 10 Å; increases the interspin distance from 11.5 to 12.5 Å in AR 10-210; and increases the interspin distance in AR 10-215 from 10.5 to 11.5 Å.

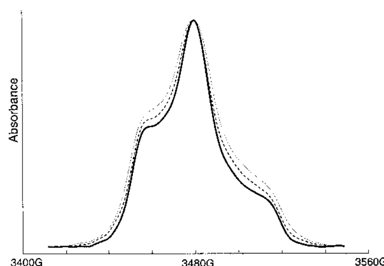

Fig. 4. The effect of CheA and CheW on the absorbance EPR spectra of spin-labeled AR 10-207 at low temperature. Spectra in the presence (thick solid line) and absence (dashed line) of aspartate when the aspartate receptor is mixed with equimolar amounts of CheA and CheW. These spectra are compared with the spectrum of aspartate receptor without CheA and CheW (thin solid line). The *x* axis is H (Gauss); the *y* axis is arbitrary absorbance units.

one of the transmembrane helices, thus altering the G-protein binding site on the cytoplasmic side of the membrane. The spin-labeling EPR strategy outlined here may well be applicable to these other receptors.

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31. The gene encoding the wild-type aspartate receptor (tar_s) was expressed from pCK2 (*54*) and mutated with QuikChange (Stratagene). The cysteine-containing aspartate receptor was expressed, purified, reacted with the methanethiosulfonate spin label, and reconstituted into *E. coli* membranes as described (*9*).
32. In order to verify that the spin-labeled aspartate receptors were still functional, an in vitro phosphorylation assay was carried out on labeled, reconstituted aspartate receptor mixed with purified CheW, CheA, and CheY, as described (*9*). AR 10-207, AR 10-210, and AR 10-215 decreased the amount of phospho-CheY in response to aspartate similarly to wild-type aspartate receptor, suggesting that spin labeling did not substantially alter the function of these receptors.
33. EPR spectra were collected with a Bruker ESP 300E X-band spectrometer equipped with a loop-gap resonator (Medical Advances). The microwave power was 8 μW and the modulation amplitude was 2 G. The protein concentration ranged from 50 to 150 μM; the concentration of aspartate was 8 mM. Addition of the amino acid serine to the same concentration as aspartate did not result in any spectral changes.
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35. Detergent-soluble spin-labeled aspartate receptor was mixed with an equimolar amount of unlabeled, detergent-soluble wild-type aspartate receptor. After time for subunit exchange (*25*), the samples were reconstituted into *E. coli* membranes as described (*9*). The comparison of the amount of protein with the spin concentration revealed that the cysteine at position 10 is approximately 50% labeled, whereas all positions on TM2 were 100% labeled. The spectral contribution from the noninteracting species as a result of incomplete labeling and unidentified background labeling (*9*) is subtracted from the EPR spectra.
36. The two nitroxide spin labels attached to a protein interact with each other through the electron-electron dipolar interaction (EED). The biradical spin-Hamiltonian H is expressed as
$$H = h\Omega_1 S_{1z} + h\Omega_2 S_{1z} + h^2\gamma_e^2 \times (1 - 3\cos^2\theta)(3S_{1z}S_{2z} - \mathbf{S}_1 \cdot \mathbf{S}_2)/2R^{-3}$$
where h is Planck's constant and γ_e is the gyromagnetic ratio of an electron. θ is the angle between the interspin vector and magnetic field z direction. Ω_1 and Ω_2 represent the resonance offsets for electron spins 1 and 2. S_{1z} and S_{2z} are the z direction components of spins S_1 and S_2, respectively. The first two terms give rise to the EPR spectrum in the absence of the EED, which is given as the third term of the equation. However, the existence of the EED perturbs the noninteracting EPR spectrum to give rise to an overall spectral broadening. The EED has R^{-3} dependence on the interspin distance R; the shorter R is, the more broadened the EPR spectrum. Recent developments of an EPR spectroscopic ruler and the Fourier spectral analysis method (*4*) allow us to accurately determine R from the analysis of the EED-broadened EPR spectrum.
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41. Purified CheW and CheA (*9*) were each added to reconstituted spin-labeled aspartate receptor, such that there was an equimolar amount of each. These were allowed to associate for at least 2 hours at room temperature, and EPR spectra were collected as for the receptor alone. Neither CheA or CheW added alone (at molar concentrations equal to or twice that of the receptor) caused spectral changes, suggesting that the changes are specific to the ternary complex. Similar CheW-CheA effects were seen with all spin-labeled aspartate receptors examined (spin labels at receptor residues 10-205, 10-211, 10-214, and 2-211).
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