Aspartate Receptors of *Escherichia coli* and *Salmonella typhimurium* Bind Ligand with Negative and Half-of-the-Sites Cooperativity†

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ABSTRACT: The aspartate receptors of *Escherichia coli* and *Salmonella typhimurium* which mediate chemotactic responsiveness to aspartate have 79% amino acid sequence identity but exhibited apparently quite different aspartate binding plots. The Scatchard plot of the *Salmonella* receptor was concave upward whereas the *E. coli* receptor gave a straight line. Because the two binding sites in the *Salmonella* receptor lacking aspartate have a 2-fold crystallographic symmetry axis and do not overlap, the observation of more than one class of binding sites must be due to a ligand-induced conformational change giving negative cooperativity. The closely related *E. coli* receptor was found to bind with only one class of sites but with a stoichiometry of one aspartate per dimer. The *E. coli* receptor thus binds with half-of-sites reactivity, an extreme form of negative cooperativity in which the second ligand is not observed to bind at all. Comparison of the X-ray crystal structure of the ligand binding domain with and without bound aspartate revealed ligand-induced conformational changes that explain the two examples of negative cooperativity.

A large number of transmembrane receptors have been identified, yet high-resolution structural information has been obtained for portions of just a few. Thus far, none has had its cooperative binding characteristics related to its structure. For the aspartate receptor that mediates chemotaxis in bacteria the topology of the entire receptor (Krikos et al., 1983 Russo & Koshland, 1983; Mowbray et al., 1985) is known, the functional form has been shown to be a dimer, and the structure of the periplasmic ligand-binding domain has been established (Milburn et al., 1991). The transmembrane topology is shared with a class of eukaryotic and prokaryotic receptors which have one or two transmembrane domains such as the insulin receptor, growth factor receptors, and the interleukin receptors, etc. (Benton, 1991; Stoddard et al., 1992). Aspartate binds to the periplasmic domain of the receptor (Clarke & Koshland, 1979; Wang & Koshland, 1980; Mowbray et al., 1985) and transduces a signal through the transmembrane domains to the cytoplasmic domain. The crystal structure of the dimeric ligand-binding domain of the *Salmonella* receptor has revealed two identical binding sites in the absence of aspartate (Milburn et al., 1991).

The reported binding of aspartate to the receptor has several anomalous features that have direct relevance to regulatory phenomena and transmembrane signaling. The binding of aspartate to the *Escherichia coli* receptor follows Michaelis–Menten behavior (Mowbray & Koshland, 1987), indicating a single type of binding site, and it has been suggested that only one aspartate can bind the dimeric receptor (Borkovich et al., 1992). On the other hand, the periplasmic domain of the *Salmonella* receptor exhibits two unequal binding affinities (Milligan & Koshland, 1993). The crystal structure (Milburn et al., 1991) of the periplasmic domain in the presence of aspartate has one site fully occupied with aspartate and one empty site. The *E. coli* receptor, but not the *Salmonella* receptor, transduces signals by both aspartate and maltose, and the two stimuli act additively and independently (Mowbray & Koshland, 1987). Reconciliation of these varied and apparently contradictory results in two receptors with 79% identity in sequence is important for understanding general structure–function relationships in proteins as well as receptor behavior. When several affinities are observed for a purified protein, they can be explained (Levitzki & Koshland, 1969) by four possible mechanisms: (a) an asymmetric dimer that starts with two nonidentical sites, (b) a ligand-induced conformational change in a dimer that reduces or eliminates affinity for the ligand at the subsequent binding site (negative cooperativity), (c) initially identical sites becoming altered by denaturation during the purification process to create nonidentical sites, or (d) the presence of two or more distinct proteins that bind the same ligand.

We examined the stoichiometry and cooperativity of aspartate binding to the aspartate receptors of *E. coli* and *Salmonella typhimurium* in order to establish which of these four alternatives applied. We then studied conformational differences between the X-ray crystal structures with and without ligand in order to relate the structure of the receptor to its function.

MATERIALS AND METHODS

Plasmids and Strains. The receptors in this were expressed in *E. coli* from pDK124 (Dunten & Koshland, 1991), pMK650 (Mowbray & Koshland, 1987), pEMBLtar$_s$ (Falke & Koshland, 1987), and derivatives prepared by site-directed mutagensis under conditions in which all four cytoplasmic methylation sites are present as free glutamates. *E. coli* strain RP3080, which lacks methyltransferase and methylesterase activities, was used for expression of the *Salmonella* receptor without cysteines as well as for the *E. coli* receptor with Cys-36. RP4080, which lacks methyltransferase but has methylesterase activity, was used to express the other receptors.

Preparation of Membranes and Purified Receptors. Receptors were expressed in large scale fermentation culture, and membranes were prepared essentially as described previously (Foster et al., 1985). All manipulations and assays

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of membranes and receptors were at 4 °C in the presence of 40 mg/mL phenylmethanesulfonyl fluoride. Receptors were extracted from batches of membranes containing 100–300 mg of membrane protein by incubation with 1.25% β-octylglucoside (OG)[1] for 15 min and removal of insoluble material by centrifugation for 20 min at 300000*g* in Beckman TL 100.3 rotors. Extracts were brought to 17% (*E. coli*) or 30% (*Salmonella*) saturated ammonium sulfate, and the pH was neutralized with a Tris-HCl solution. Precipitates were isolated by centrifugation at 4000*g* and immediately dissolved in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.4% Zwittergent 3–12 (Calbiochem). Particulates were removed by centrifugation, and the supernatant was immediately loaded on a 10-mL aminooctyl agarose column (Sigma A5642) preequilibrated in the Zwittergent buffer. The column was washed with 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1.2% OG buffer, and receptors were eluted in this buffer with stepped increases of NaCl in the range of 0.1–0.3 M. Pooled fractions were immediately dialyzed against 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 0.9% OG. Receptors were concentrated to 1–4 mg/mL using Centricon 100 and/or Centriprep 100 units from Amicon. Cys-36 receptors were ordinarily prepared in the presence of 10 mM dithiothreitol.

Quantitation of Receptors. Purified preparations of receptors in OG were prepared for quantitative amino acid analysis by precipitation with 15% w/v trichloracetic acid, and the resulting pellets were washed with acetone. The mass of the protein in the precipitated sample was calculated from the amino acid composition. To control for the possibility of modification of the receptors during purification, receptors were also analyzed in cellular membrane preparations. For quantitation of receptors in membranes, Coomassie stained SDS–polyacrylamide gels containing membrane samples and known amounts of purified receptors were scanned with a laser densitometer, and relative band densities were determined from digitized data after background subtraction. Receptor concentration values for a given membrane preparation determined in different analyses with different standard preparations of receptors had standard deviations of about 10%.

Binding Assays of Purified Receptors. Purified receptors (40–90 μL in OG dialysis buffer) were included in 200-μL assays containing 50 mM sodium phosphate (pH 7.0), 0.1% Triton X-100, and 0.5–25 mM [^{3}H]-L-aspartate (1 Ci/mmol). After 5 min on ice, 95-μL aliquots were mixed with 1 μL of 100 mM L-aspartate or 1 mL of water, placed in a 30 000 M_r cutoff Ultrafree MC filtration unit (Millipore), and centrifuged until 12–15 μL of liquid had passed through the membrane. The concentration of aspartate in this filtrate was determined by scintillation counting. Specifically bound aspartate was calculated by subtracting the concentration of free aspartate, measured in the absence of excess cold ligand, from the total concentration of aspartate (not including nonspecifically bound), measured in the presence of excess cold ligand. Identical results were obtained when the assay was performed in 1% OG without Triton X-100 (not shown).

Binding Assays of Membrane-Bound Receptors. The procedure of Clarke and Koshland (1979) was followed with minor modifications. One hundred microliters of membrane preparations containing receptors was brought to 200 μL at final concentrations of 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM 1,10-phenanthroline, and 5% glycerol plus [^{3}H]-L-aspartate (1 Ci/mmol). After 5 min on ice, 95-μL aliquots of the binding reactions were mixed with 2 μL of 100 mM L-aspartate or 2 μL of water and membranes were pelleted in Beckman miniature ultracentrifuge tubes (no. 343775) for 6 min at 80 000 rpm in a TL-100 rotor. Supernatants were counted and bound and free aspartate concentrations calculated as above.

Data Analysis. Data were analyzed by fitting the curve of bound ligand vs free to the Hill equation (Hill, 1913) in the form:

$$\text{bound} = (\text{bound}_{max})[(\text{free})^{n}_{H}]/[k_D + (\text{free})^{n}_{H}]$$

and to the Scatchard Plot (Scatchard, 1949). Curve fitting was performed with Enzfitter and Kaleidagraph software and gave correlation coefficient (R) values exceeding 0.98. The concave upward Scatchard plots obtained with the *Salmonella* data precluded determination of the concentration of binding sites from the x intercept. As a result, data from fitting *Salmonella* data to the Hill equation were used for stoichiometry calculations in Table I. Hill equation fits and Scatchard intercept data are used for *E. coli* calculations in Table II.

Binding data were also fit to an equation for a protein with two ligand sites using a nonlinear least-squares algorithm on Enzfitter.

Binding Stoichiometry. We quantitated the stoichiometry of aspartate binding to the receptor in membranes and in detergent solution by comparing the concentration of binding sites measured from binding analyses with the concentration of receptor present in each assay (Tables I and II).

RESULTS

Aspartate Binding Analyses. To confront concerns about artifacts that might occur in binding measurements, observations were made on purified receptors and on receptors in membranes. Freshly prepared membranes are unlikely to have receptor denaturation, and purified receptors lack membrane vesicles that could complicate assays by compartmentalization. Agreement between the two complementary approaches should substantiate the conclusions. Receptors purified to homogeneity were obtained by detergent extraction from membranes as described under Materials and Methods. Equilibrium aspartate binding reactions were performed on both types of receptor preparations. Scatchard plots (Scatchard, 1949) of equilibrium binding data to *Salmonella* (Figure 1a) and *E. coli* receptors (Figure 1b) in membrane preparations are shown. The *Salmonella* receptor exhibited a concave-upward Scatchard plot and a Hill coefficient (Hill, 1913) of 0.7 ($R > 0.99$), indicating two types of binding sites. On the other hand, the *E. coli* receptor had only one class of binding sites with a straight Scatchard plot and a Hill coefficient of 1.0. The binding of aspartate to the purified receptors in detergent was seen to have the same characteristics as the binding of aspartate to membrane receptors. The purified *Salmonella* receptor (Figure 1c) appears to have more than one class of aspartate binding sites with a Hill coefficient of 0.7 indicating negative cooperativity. The purified *E. coli* receptor appears to have only one class of sites (Figure 1d) and a Hill coefficient of 1.0 ($R > 0.98$). The binding data for the *Salmonella* receptor fit best to a model for a two-site system with k_D's of approximately 0.1 and 2.0 μM. Nonlinear least-squares analysis gave χ^2 values of 4.8×10^{-3}. The *E. coli* data fit only to an equation for one class of sites with a k_D of approximately 1.2 μM.

[1] Abbreviations: OG, β-octylglucoside; n_H, Hill coefficient; SD, standard deviation.

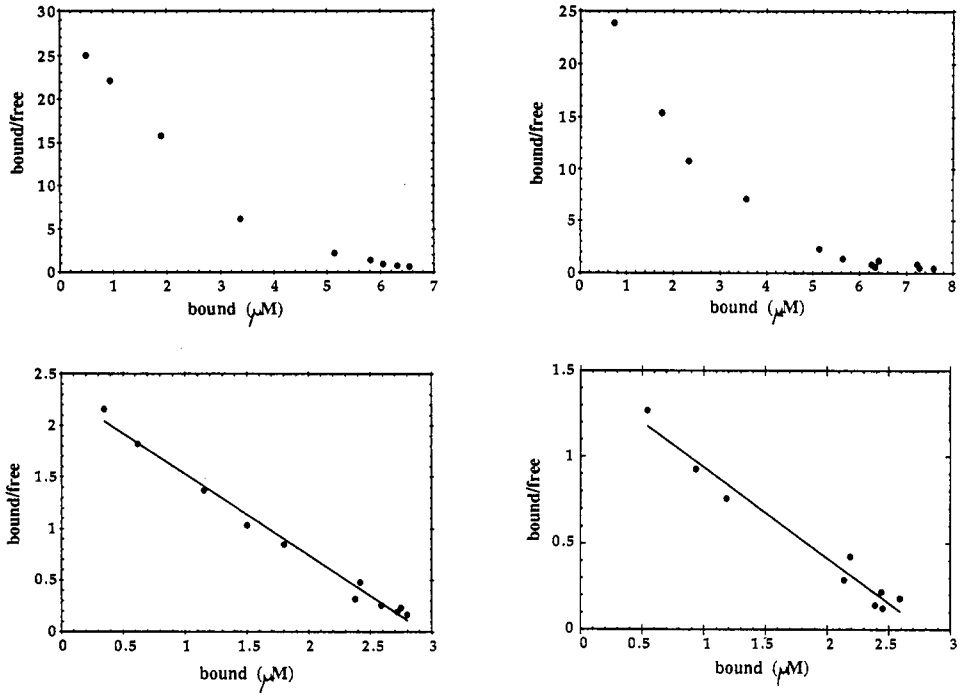

FIGURE 1: Scatchard plots of aspartate binding to purified receptors. (a, top left) *Salmonella* receptors in membranes; (b, bottom left) *E. coli* receptors in membranes; (c, top right) *Salmonella* receptors, purified; (d, bottom right) *E. coli* receptors, purified. *Salmonella* plots are curved suggesting more than one class of binding sites whereas *E. coli* plots are linear suggesting that all sites have the same affinity.

Table I: Aspartate Binding to the *Salmonella* Receptor[a]

receptor type	receptor dimer (μM)	aspartate bound (μM)	aspartates per receptor	Hill coefficient
wild type	4.1	9.0 ± 1.1	2.2	0.6 ± 0.1
wild type	3.3	7.1 ± 1.1	2.2	0.6 ± 0.2
Cys-36	4.3	7.7 ± 0.6	1.8	0.8 ± 0.1
Cys-36	4.2	8.7 ± 1.0	2.1	0.7 ± 0.1
Cys-36	4.2	8.1 ± 0.7	1.9	0.7 ± 0.1
Cys-36	4.3	8.4 ± 1.1	2.0	0.6 ± 0.1
membrane, wt	3.3	7.8 ± 0.7	2.4	0.6 ± 0.1

[a] *Salmonella* receptor is a dimer of two identical subunits. Concentration of receptor is recorded in micromolar (μM) dimer, i.e., two initially identical sites/receptor. Aspartate bound is calculated from fitting the binding data to the Hill equation.

Stoichiometry of Binding. In Table I data are presented from multiple analyses of several forms of the *Salmonella* receptor: purified wild-type receptor (assays 1 and 2), purified receptor with an Asn → Cys mutation at position 36 (assays 3–6), and membrane-bound wild-type receptor (assay 7). The ratio of the maximum concentration of aspartate bound in equilibrium bindind assays to the concentration of binding sites calculated from receptor protein revealed that the stoichiometry was two aspartates bound per dimer. The receptor cross-linked with a disulfide bond between cysteines introduced at position 36 in each subunit behaved identically to the wild-type receptor. Thus the cross-link did not change the binding stoichiometry from that of the wild type, consistent with the finding that this cross-link does not significantly interfere with transmembrane signaling (Falke & Koshland, 1987) or the conformation of the binding sites (Yeh et al., 1993).

The binding stoichiometry data for the *E. coli* receptor are shown in Table II. Four forms of the purified receptor were studied, and in all cases the observed stoichiometry values were consistent with only one aspartate bound per dimer. The Hill coefficients (n_H = 1) and the straight Scatchard plots (Figures 1B and 2B) indicate one class of sites. Analyses of membrane bound receptors agree. Receptors with cysteine substituted at position 36 had the same characteristics as wild type whether or not they were disulfide cross-linked.

Cooperativity and Conformational Changes. The apparent discrepancy in binding curves between the *E. coli* and the *Salmonella* receptor is intriguing since they share 67% amino acid sequence identity in the ligand-binding domain and the "contact" amino acids in the binding sites are completely conserved. Homology modeling and energy minimization studies (Stoddard & Koshland, 1992) had indicated that the three-dimensional structure of the *E. coli* receptor domain is very similar to the X-ray structure of the *Salmonella* counterpart and essentially identical for the contact amino acids at the binding sites. In view of the X-ray crystallographic data that the unbound form of the *Salmonella* receptor has two identical binding sites which do not overlap (Milburn et al., 1991), mechanism (a) outlined above in the introduction for multiple affinities is eliminated for both receptors. The fact that highly purified receptors were analyzed and that the results agree with membrane analyses rule out mechanisms (c) and (d). Therefore, the explanation for all these data is that both receptors exhibit mechanism (b), negative cooperativity, i.e., the binding of the first aspartate induces conformational changes that diminish the affinity for the second. To explain the difference in stoichiometry between the *Salmonella* and *E. coli* receptors, it would appear that the negative cooperativity of the *Salmonella* receptor is changed to the form of negative cooperativity called half-of-sites reactivity (Levitzki & Koshland, 1969; Long et al., 1970) in the *E. coli* receptor because of the amino acid sequence changes between the two receptors. The affinity at the first site on the *E. coli* receptor is found to be one-tenth that of the *Salmonella* receptor so that if the K_1/K_2 ratio is the same for both receptors, no binding at the second site of the *E. coli* receptor could be detected. No such binding was observed.

To understand the structural basis of the observed cooperativity, the structures of the *Salmonella* periplasmic domain in the presence and absence of aspartate (Milburn et al., 1991) were examined. Each subunit in the X-ray structure of the

Table II: Aspartate Binding to the *E. coli* Receptor[a]

receptor type	receptor dimer (μM)	aspartate bound Scatchard (μM)	aspartate bound HIll (μM)	aspartates per receptor (Scatchard)	aspartates per receptor (Hill)	Hill coefficient
wild type	2.8	2.8	2.7 ± 0.2	1.0	1.0	1.1 ± 0.2
wild type	4.5	4.0	4.0 ± 0.5	0.9	0.9	1.0 ± 0.3
wild type	3.8	4.3	4.7 ± 0.5	1.1	1.2	0.8 ± 0.2
Cys-103	5.4	4.4	4.4 ± 0.3	0.8	0.8	1.2 ± 0.3
Cys-36 (red)	1.4	1.4	1.7 ± 0.2	1.0	1.2	0.9 ± 0.1
Cys-36 (ox)	1.4	1.1	1.2 ± 0.1	0.8	0.9	1.0 ± 0.3
membranes, wt	3.9	2.9	3.0 ± 0.1	0.8	0.8	0.9 ± 0.1
membranes, wt	4.8	4.5	5.1 ± 0.4	0.9	1.1	0.7 ± 0.1

[a] *E. coli* receptor is a dimer of two identical subunits. Concentration of receptor is recorded in micromolar (μM) dimer, i.e., two initially identical sites/receptor. Aspartate bound is calculated either from the intercept of a Scatchard plot or from fitting the binding data to the Hill equation.

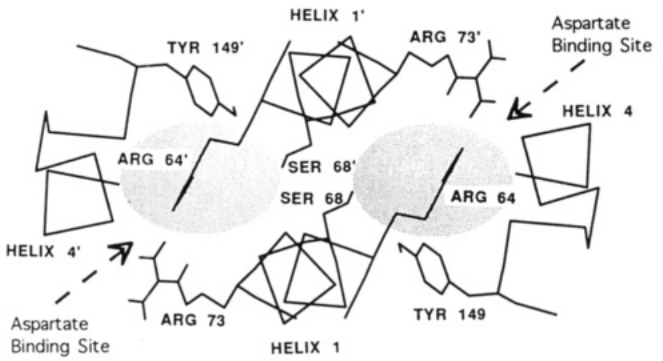

FIGURE 2: Top view of the binding sites in the *Salmonella* receptor lacking bound aspartate. Several amino acids involved in the binding sites are shown as well as portions of helices 1 and 4 in both subunits. Arginines 64 and 73 are contributed to each site by different subunits. Arginine 69 also lies in each site but is not shown. Serine 68 and tyrosine 149 have previously been shown to interact with the ligand via a bound water molecule. All of these side chains move when aspartate binds and the 2-fold symmetry between the sites is lost (see Figure 3 and Table III).

periplasmic domain is a four-helix bundle (Milburn et al., 1991). Helices 1 and 1′ and 4′ of the other subunit contribute to the two binding sites. Figure 2 shows several of the amino acids that form the two binding sites which are related by a 2-fold crystallographic symmetry axis in the absence of aspartate. Arginines 64, 73, and 69 (not shown) have multiple electrostatic interactions with the bound aspartate in the aspartate complex structure.

We observed small conformational changes at *both* the occupied and unoccupied binding sites as the result of aspartate binding at one site. The ligand-induced conformational changes include (a) a rigid body intersubunit motion causing helices 1′ and 4′ to shift away from the bound aspartate toward the second site; α-carbons 68–73 in helix 1′ move an average of 2.2 Å (SD = 0.2 Å); (b) a smaller intrasubunit bend of helix 1 occurs away from the bound aspartate causing α-carbons 68–73 in helix 1 to move 0.9 Å (SD = 0.2 Å). These motions reposition the three helices that form the second binding site, and thus the shape of that site changes.

The conformational changes in the unoccupied site include motions of the three arginines. Arginine 64 flips about 1 Å upward into the unoccupied binding cleft in the aspartate-bound structure so that it would interfere sterically with the γ-carboxyl of an aspartate in the binding site as shown in Figure 3. Serine 68 and tyrosine 149, which have been shown to interact with the ligand via a bound water molecule (Milburn et al., 1991), also move. Serine 68 protrudes farther into the unoccupied site, and tyrosine 149 shifts 1.9 Å laterally with respect to side chains across the site. Table III presents the results of these motions by comparing distances between several pairs of side chains that face each other across the empty binding site in the receptor with no ligands and the empty site of the receptor with one ligand bound. The height, width, and depth of the second binding site are reduced by about 1 Å so that an aspartate can no longer be accommodated sterically. These aspartate binding induced conformational changes at the unoccupied site explain the reduced affinity of the second site for aspartate observed after the first aspartate is bound (Figure 1).

DISCUSSION

This report has described distinct negatively cooperative binding patterns in two receptors with 79% amino acid sequence identity. In both receptors binding of the first aspartate diminishes the affinity of the second site. However, in the case of the *E. coli* receptor either because the first site has a lower affinity or because there is increased negative cooperativity between the sites, the second ligand does not bind detectably to the dimer, i.e., the half-of-the-sites phenomenon. The binding results were confirmed in both purified detergent-solubilized receptors and membrane receptor preparations to rule out potential artifacts of denaturation or compartmentalization. Because the binding sites in crystals formed in the absence of aspartate are identical and do not overlap, the conformational changes induced upon binding of the first aspartate ligand must alter the affinity of the second site. For the first time it has been possible to describe such conformational changes transduced between cooperative binding sites using X-ray data for an intermediate binding state. The negative cooperativity in this receptor allows crystallization of the protein with one site 100% occupied and the second site empty (Milburn et al., 1991). Previously, only positively cooperative proteins have been studied that can crystallize only in unoccupied and fully bound forms. Thus we have proposed a structural basis for allosteric communication between sites that bind with negative cooperativity. Conformational changes induced by the bindng of aspartate to the first site alter the initially equivalent binding site for the second aspartate in the *Salmonella* receptor as shown in Figure 3 and Table III.

Apparently, the differences in the amino acid sequence of the *Salmonella* protein relative to the *E. coli* protein convert the negative cooperativity of the *Salmonella* protein to the half-of-sites cooperativity of the *E. coli* protein even though none of the amino acids that differ between the two receptors are those in direct contact with the ligand. That amino acid changes in the subunit interfaces of proteins can cause dramatic changes in cooperativity or ligand binding without changes at the active site follows from the sequential theory of cooperativity (Koshland et al., 1966), in which changes in subunit interactions were shown to change apparent K_m's ($S_{0.5}$'s) and the degree of cooperativity without need to change any amino acids at the active site that are in con tact with the substrate.

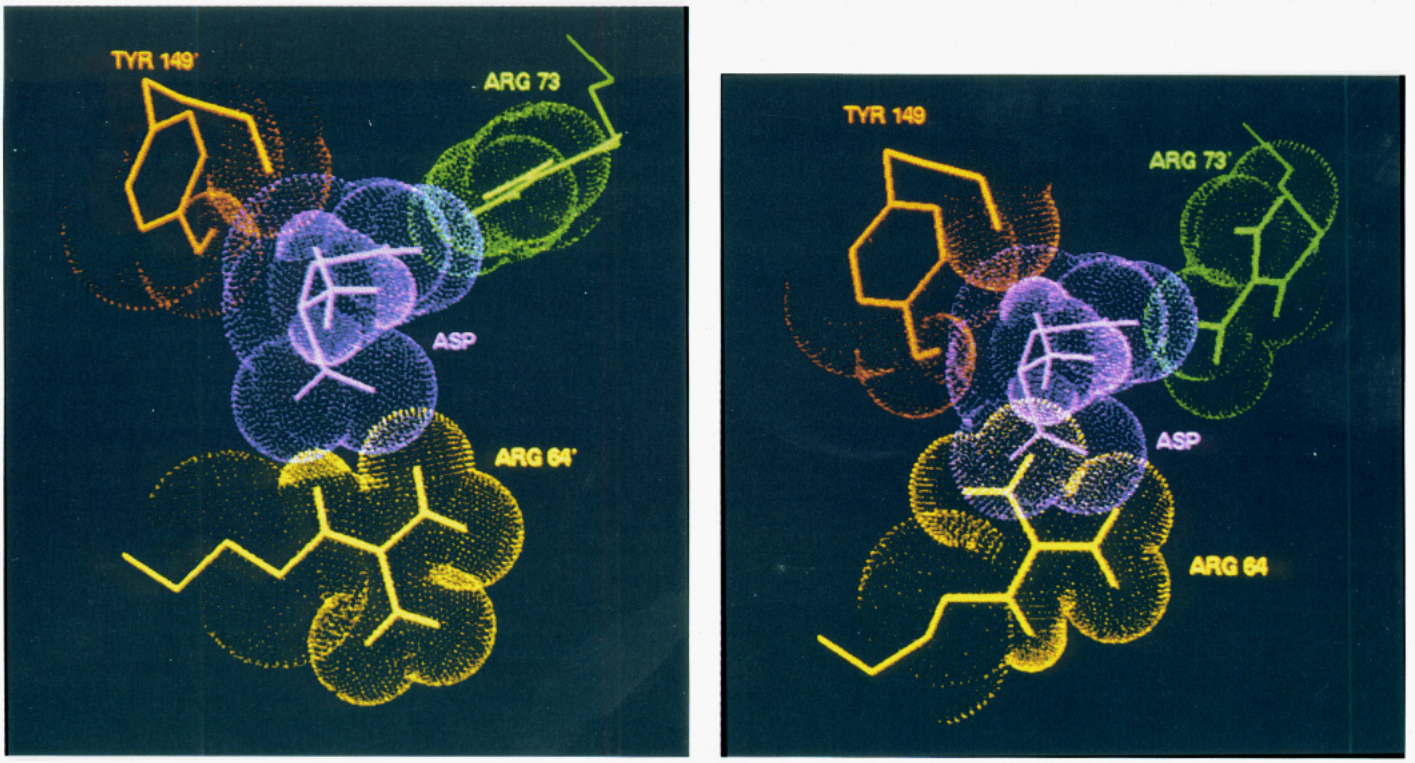

FIGURE 3: Conformational changes diminish the volume of the second aspartate binding site. (A, left) X-ray crystal structure of the bound aspartate with three major *Salmonella* receptor side chains in the binding site shown. Aspartate in purple binds to Arg-64 (yellow), arg-73 (green), and Tyr-149 (orange) with favorable van der Waals overlap. (B, right) Imposition of a hypothetical aspartate molecule at the empty site in the receptor which has one aspartate already bound predicts van der Waals repulsion. The X-ray structure of Arg-64, Arg-73, and Tyr-149 in the unoccupied site are shown revealing aspartate-induced conformational changes at the filled site that reduce the size of the cleft at the unoccupied site. The purple aspartate placed in the empty site is crowded significantly; repulsive van der Waals overlap is unavoidable, explaining the decreased aspartate affinity. Arginines 64 are stably anchored by interactions with a pair of conserved glutamines (Lynch & Koshland, 1992) in all structures. Structures were refined to 2.2-Å resolution and lower. The van der Waals radii shown are 90% of the radii calculated with the Quanta program (Polygen).

Table III: Distances between Side Chains in Binding Sites in the *Salmonella* Aspartate Receptor Ligand-Binding Domain

amino acids	separation (Å) in unbound receptor[a]	separation (Å) in empty site of Asp-bound receptor[a]	reduction in distance (Å)
Ser-68, Thr-154	8.9	8.1	0.8
Tyr-149, Arg-73	6.9	6.0	0.9
Tyr-149, Arg-64	4.1	3.2	0.9
Phe-150, Arg-73	4.8	3.5	1.3
Ser-68, Arg-69	7.4	6.6	0.8

[a] Distance between closest non-hydrogen atoms.

Curves with negative, positive, and no cooperativity (Michaelis–Menten) as well as half-of-sites binding and changes in affinity are predicted to be generated by altering the interface between unoccupied subunits (K_{AA}) as well as the interface between pairs of occupied subunits (K_{BB}) and occupied and unoccupied (K_{AB}). Examples supporting this theory have are now known in which single point mutations at the interface of subunits in aspartate transcarbamylase (Eisenstein et al., 1990) and glutathione reductase (Scrutton et al., 1992) have been shown to induce or abrogate cooperativity.

The subunit interface is highly conserved between the aspartate receptors of *Salmonella* and *E. coli* with one amino acid difference at the interface near the binding sites, that being alanine 72 in the *Salmonella* receptor, which becomes a valine in the *E. coli* receptor. Alanines 72 and 72′ face each other across the *Salmonella* subunit interface but do not touch. The valine side chains in the modeled structure of the *E. coli* receptor are capable of interacting and may be the reason for the difference in the subunit interactions which change negative cooperativity in the *Salmonella* receptor to half-of-sites reactivity in the *E. coli* receptor. That change may be caused either by the change in affinities (K_D in the *E. coli* receptor is 10 times the K_D of the *Salmonella* receptor) or by an increase in the negative cooperativity. Either or both changes must be caused by amino acids outside the outside the active site since all the aspartate bond contacts are the same in the two species.

The concept of modulating K_m or cooperativity by changes in subunit interactions, rather than the active site, has major evolutionary advantages. Active sites are difficult to design (e.g., a site which can bind O_2 but does not reduce the O_2, as in hemoglobin or a site highly specific for a substrate as in the case of an enzyme). Yet in different species or in different states of the same species the need for a different K_m or degree of cooperativity in a protein may arise. For instance, the tadpole and frog experience very different ambient oxygen concentrations. Redesigning the oxygen binding site in hemoglobin to produce a higher O_2 affinity for the tadpole would be very difficult considering the high degree of selection required to optimize the heme–O_2 interaction. The sequential theory (Koshland et al., 1966) indicates that alterations in amino acids at the subunit interface outside of the active site can alter the affinity of the site and optimize the characteristics of the protein for each organism. Evidently, this approach to modulating the basic affinity and cooperativity of a protein is used in this receptor to optimize its efficiency.

One possible rationale for half-of-the-sites aspartate binding by the *E. coli* receptor was put forth by Stoddard and Koshland (1992) in a paper that proposed half-of-the-sites binding of maltose binding protein to the receptor. Half-of-sites binding of both maltose binding protein and aspartate could explain

the surprising finding of independence of maltose and aspartate signaling through the same receptor (Mowbray & Koshland, 1987).

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