Oligomerization of the Cytoplasmic Fragment from the Aspartate Receptor of *Escherichia coli*†

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ABSTRACT: We have observed that a 31-kDa cloned fragment from the *Escherichia coli* aspartate receptor exhibits a reversible monomer–oligomer reaction. The fragment, derived from the cytoplasmic region of the receptor (c-fragment), contains the signaling functions of the receptor. The wild-type and nine missense mutant fragments were analyzed. The latter were selected by the effect of the mutations on the signaling properties of the intact receptor, which induced either persistent smooth swimming or tumbling in bacteria [Mutoh, N., Oosawa, K., & Simon, M. I. (1986) *J. Bacteriol. 167*, 992–998]. In pH 7.0 buffer, the mutations caused five out of the six smooth mutant c-fragments to form oligomers, while neither the three tumble mutant nor wild-type fragments exhibited significant oligomer formation. At a lower pH (5.5), all of the fragments displayed some tendency to form oligomers. The equilibria between the monomer and the oligomers were monitored by gel permeation chromatography (GPC) which resolved two to three forms with apparent molecular weights between 110 000 and 270 000. The proportions of the different forms depended on concentration, indicating an association–dissociation reaction. Static light scattering (SLS) was used to demonstrate that the solution molecular mass of the wild-type c-fragment was 31 kDa and not 110 kDa as indicated by chromatography. One oligomer-forming c-fragment (S461L) eluted as the monomer and one other form, which was determined to be a dimer by SLS. The weight-average molecular weights, calculated from GPC data as a function of protein concentration, agreed well with the weight-average molecular weights obtained by SLS for this mutant. The GPC data indicate that the c-fragment forms oligomers of well-defined stoichiometry, and the anomalously large molecular weight estimates indicate that the fragment is extremely nonspherical in shape. The correlation between the behavioral phenotype and the tendency to form oligomers suggests that the mutations can indeed lock the receptor into well-defined signaling forms and that subunit interactions in the cytoplasmic region are stronger in the attractant-bound form of the receptor. The formation of the oligomers may have important implications for the mechanism of transmembrane signaling and is consistent with a mechanism that involves ligand-induced receptor clustering.

The aspartate receptor is one of four homologous, methylatable transmembrane receptors involved in the sensory response of *Escherichia coli* to environmental stimuli. The receptors, which bind specific ligands, are the first components of a signal transduction pathway that bias the tumble frequency of the cell. Increases in the concentration of attractants such as certain amino acids (like aspartate) and sugars lead to a transient suppression in tumbling and a larger persistence length in the direction of increasing attractant concentration. Conversely, sharp decreases in the attractant concentration result in an increase in the tumble frequency. In this manner the bacterium displays a net bias in swimming toward increasing concentrations of attractants. [For recent reviews of the chemotaxis system in *E. coli* and *Salmonella typhimurium*, see Bourret et al. (1991), Hazelbauer et al. (1990), Stewart and Dahlquist (1987), and Stock et al. (1991)]. All of these receptors consist of a single polypeptide chain with a molecular weight (MW)[1] of about 60 000, have two transmembrane segments, and are divided into a periplasmic ligand-binding region and a cytoplasmic signaling region. Sequence analyses (Russo & Koshland, 1983; Krikos et al., 1983) and biochemical studies (Foster et al., 1985; Mowbray et al., 1985) have revealed the membrane topology of the aspartate receptor and have provided information of the secondary structure and the domain structure. Chemical cross-linking and equilibrium centrifugation studies have provided evidence for the existence of a dimeric form of the receptor (Milligan & Koshland, 1988) and the arrangement of the transmembrane helices within the dimer (Falke & Koshland, 1987; Falke et al., 1988; Lynch & Koshland, 1991). A dimeric form of the receptor is also evident in the crystal structure of the ligand-binding domain from the *S. typhimurium* aspartate receptor, although this structure was solved using a dimeric, disulfide-cross-linked protein containing a single cysteine, which was introduced by site-directed mutagenesis (Milburn et al., 1991). Similar information about the arrangement of the polypeptide chain and organization of the receptor has been obtained through the detailed studies of mutants and their suppressors (Mutoh et al., 1986; Oosawa & Simon, 1986; Ames & Parkinson, 1988). An important result of these

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‡ Deceased. Tragically, David G. Long died on January 3, 1992, as a result of injuries received in an automobile accident. This paper is dedicated to his memory as a friend and colleague and as a promising young scientist whose full potential will never be realized.

[1] Abbreviations: GPC, gel permeation chromatography; MW, molecular weight; M, molecular mass; c-fragment, cytoplasmic c-terminal fragment; IPTG, isopropyl thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; R_θ, Rayleigh ratio; dn/dc, specific refractive index increment; $\bar{M}_w$, weight-average MW; SDS, sodium dodecyl sulfate; kb, kilobase; PAGE, polyacrylamide gel electrophoresis; SLS, static light scattering.

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mutagenesis studies was the isolation of receptors that were apparently locked into one signaling form or the other (Mutoh et al., 1986; Ames & Parkinson, 1988), where certain amino acid substitutions gave rise to receptors that generated only a smooth-swimming signal while other amino acid substitutions induced a persistent tumble signal. In spite of this determined effort, the basic events involved in signal transmission across the membrane are not understood.

Two models often put forward to explain transmembrane signaling are ligand-induced clustering and a conformational-change model, where cluster size does not change but allosteric regulation of the signaling domain is mediated by the ligand-binding domain across the membrane. Whereas the receptor clustering model has gained some experimental support in transmembrane signaling mediated by growth factor receptors [for reviews, see Schlessinger (1988) and Ullrich and Schlessinger (1990)], the conformational-change mechanism also has its proponents, especially in the case of the aspartate receptor (Milligan & Koshland, 1988, 1991; Milburn et al., 1991; Hazelbauer et al., 1990).

We have used a cloned fragment of the aspartate receptor cytoplasmic signaling region (Oosawa et al., 1988; Kaplan & Simon, 1988) to characterize further the properties of transmembrane signaling. Two results are reported here. First, as determined from its behavior on a HPLC gel permeation column, the cytoplasmic fragment (c-fragment) does not have the properties of a globular protein but has instead the properties of a molecule with an extremely elongated shape. Also, certain variants of the c-fragment were observed to participate in a reversible oligomerization reaction: those mutant forms which in the intact receptor generated a persistent smooth-swimming signal. The oligomers did not appear to be nonspecific aggregates but were well-defined. In one particular smooth mutant c-fragment, containing a serine to leucine substitution at position 461 of the intact receptor (S461L), dimers were found to be the major oligomeric form, but evidence of larger oligomers was detected in other variants. At pH 7.0 neither the wild-type c-fragment nor the tumble mutant c-fragments formed detectable levels of oligomer under our experimental conditions. The good correlation between the swimming behavior displayed by the mutant-receptor-containing cells (Mutoh et al., 1986) and oligomer formation of the c-fragment suggests that subunit interactions in the cytoplasmic domain of the receptor are stronger in the attractant-bound form of the receptor, and that this may play an important role in the mechanism of transmembrane signaling. [A preliminary account of this work has been published; see Long and Weis (1992).]

MATERIALS AND METHODS

Chemicals, Bacterial Strains, and DNA plasmids. pNC189, the expression vector for the wild-type form of the receptor c-fragment, and the point mutation variants A436V and S461L (Kaplan & Simon, 1988) were kindly provided by Mel Simon, as were the *tar* alleles 505, 506, 508, 513, 515, and 525 (Mutoh et al., 1986), all derivatives of the plasmid pAK101 (Krikos et al., 1985). The mutations in the c-fragment, the corresponding allele numbers, and the behavioral phenotypes of cells carrying the intact receptor are summarized in Table I. GM2929 (*dam-13*::Tn*9 dcm-6 hsdR2 refF143* McrA$^-$ McrB$^-$) was provided by Martin Marinus (University of Massachusetts School of Medicine, Worcester, MA). Restriction enzymes were obtained from New England Biolabs (NEB, Beverly, MA), Promega (Madison, WI), and Boehringer Mannheim (Indianapolis, IN). DNA fragments were isolated from agarose gel slices using QIAEX ion-exchange resin according to the manufacturer's specifications (Qiagen Corp., Sunset City, CA). Pharmacia DEAE Sepharose Fast Flow CL-6B was obtained from Sigma Chemical Co. (St. Louis, MO), and Affi-Gel Blue Gel (Cibachrome blue F3GA) was obtained from Bio-Rad Laboratories (Melville, NY). Analytical and preparative Toso-Haas TSK-gel G3000SW GPC columns were obtained from the Nest Group (Southborough, MA). Protein standards, used to calibrate the GPC columns, were obtained from Sigma. All other chemicals were reagent grade.

Table I: Mutant Forms of the Aspartate Receptor c-Fragment Analyzed for Oligomer Formation

allele[a]	residue no.[b]	amino acid		swimming behavior[a]	oligomer formation
		original	substituted		
505	301 (45)	glutamate	lysine	smooth	no
506	311 (55)	threonine	isoleucine	smooth	yes
508	325 (69)	serine	leucine	smooth	yes
510	346 (90)	valine	methionine	tumble	no
513	360 (104)	alanine	threonine	smooth	yes
515	370 (114)	alanine	valine	smooth	yes
525	433 (177)	valine	isoleucine	tumble	no
526	436 (180)	alanine	valine	tumble	no
529	461 (205)	serine	leucine	smooth	yes

[a] Allele numbers assigned and swimming behavior determined by Mutoh, Oosawa, and Simon (1986). [b] Numbered according to the position in the intact receptor (553 amino acids). Numbers in parentheses refer to position in the c-fragment (297 amino acids).

Plasmid Construction. All alleles were transferred to pNC189 by generating a gel-purified 570 bp insert from the pAK101 variants by digestion with either *Asp*718 or *Kpn*I and *Cla*I. This insert was ligated with the gel-purified 3.27-kb fragment resulting from double digestion of pNC189 with the corresponding enzymes. Since the *dam* methylation system blocked cleavage by *Cla*I, constructs were made from pNC189 and pAK101 plasmid preparations isolated from the *dam*$^-$ strain GM2929.

Purification of the c-Fragment. Growth of bacteria and induction of the expression of c-fragment were carried out essentially as described by Kaplan and Simon (1988). All variants of the c-fragment protein were purified according to the same protocol. LB cultures (2 mL) were inoculated with either JM103 cells that were freshly transformed with pNC189 or transformed cells derived from frozen (−80 °C) stocks. The cultures were grown in at 37 °C with 260 rpm shaking and were induced to express c-fragment at early log phase with 1 mM IPTG. Expression of c-fragment was determined by SDS–PAGE, and cultures with the highest expression levels were used to inoculate 1-L cultures. The 1-L cultures were grown to an OD_{600nm} equal to 0.5 from 20-μL inoculums, at which time IPTG was added to a final concentration of 1 mM. Cells were harvested after a 4-h induction period. The cell pellet was resuspended in 20 mM Tris–HCl, 1 mM EDTA, pH 8.0 buffer (buffer A) with 50 mM NaCl and recentrifuged. A concentrated slurry of bacteria ($\sim 10^{11}$ bacteria/mL) in buffer A with 50 mM NaCl and 1 mM PMSF (buffer B) was kept on ice and sonicated with a sonic dismembranator (Fisher Scientific, Pittsburgh, PA) at 300 W for 10 1-min intervals. To prevent the sample from overheating, an ice slurry of buffer B was added to maintain the sample temperature below 6 °C. The cell lysate was centrifuged for 60 min at 30000*g* to remove cell debri. The c-fragment was precipitated from the supernatant by the addition of solid $(NH_4)_2SO_4$ to 40% saturation.

The c-fragment was purified by a combination of ion-exchange, dye-binding and gel permeation chromatography. The $(NH_4)_2SO_4$ fraction was diluted with buffer A until the conductivity was less than the diluent containing 80 mM NaCl.

After filtering (0.2 μm), the solution was applied to an Affi-Gel column connected in series to a DEAE column, both 25 mm × 30 cm, at a flow rate of 1.5 mL/min. The columns were washed with buffer A until the A_{280} returned to baseline at which time the Affi-Gel column was taken offline and a gradient from 80 to 250 mM NaCl in 500 mL (1 mL/min flow rate) was applied to the DEAE column. The c-fragment eluted at ~125 mM NaCl. Fractions containing c-fragment (determined by SDS–PAGE) were pooled and precipitated with in 50% $(NH_4)_2SO_4$ solution. The precipitate was resuspended at a concentration of 10 mg/mL in 20 mM potassium phosphate buffer, 50 mM NaCl, pH 7.0, with 1 mM EDTA. A 3-mL aliquot of sample was loaded onto a TSK gel G3000SW column (21.5 mm i.d. × 60 cm long) and eluted at a flow rate of 3 mL/min. The most highly purified fractions of c-fragment were pooled and stored as 50% $(NH_4)_2SO_4$ precipitates at 4 °C until used.

Concentrations of the purified c-fragment for light scattering and analytical GPC studies were determined by the absorbance at 280 nm using an extinction coefficient, ϵ_{280} of 7500 M^{-1}·cm^{-1}. ϵ_{280} was determined both by the methods of dry weight and by amino acid analysis and yielded the same value for ϵ_{280} to within 5%.

Analytical Size Exclusion Chromatography. Analysis of the monomer–dimer equilibrium was carried using a TSK-gel G3000SW_{XL} GPC column (7.8 mm i.d. × 30 cm), to determine the distribution of material between the monomeric and oligomeric forms. Elutions were carried out in the same potassium phosphate buffer used during purification, and the flow rate was either 0.5 or 0.7 mL/min. Proteins were detected using absorbance at either 280 or 214 nm. Apparent MWs were estimated from calibration of the column using the following globular proteins as standards: β-amylase, 200 000; alcohol dehydrogenase, 150 000; transferrin, 78 000; ovalbumin, 45 000; carbonic anhydrase, 29 000; cytochrome *c*, 12 400.

Static Light Scattering. A Photal Model DLS700 photon-counting dynamic light-scattering instrument and a Photal Model RM-102 differential refractometer (Otsuka Electronics, Osaka, Japan) were used to determine the solution molecular weight of the wild-type form of the c-fragment and the weight-average molecular weight ($\bar{M}_w$) of the oligomer-forming S461L mutant. Both instruments were temperature-regulated at 21.5 ± 0.5 °C, and utilized 632.8-nm-wavelength light. The DLS700 was operated in the static mode and calibrated with benzene using a value of $R_{90} = 8.51 \times 10^{-6}$ cm^{-1} (Pike et al., 1975) at 22 °C. Excess scattering due to protein was determined at each angle by the subtraction of scattering due to buffer alone. The differential refractometer was calibrated with NaCl solutions of known refractive index. The refractive index of the buffer was determined to be 1.332 from the Δn between buffer and H_2O, using a value of n equal to 1.331 for H_2O (Angelides et al., 1979). The solution molecular weight of the wild-type c-fragment was determined by the standard double-extrapolation procedure: extrapolation to zero scattering angle gave the Rayleigh ratio (R_θ) at zero scattering angle, $R_0(c)$, at different protein concentrations, and extrapolation to zero protein concentration, $R_0(0)$. R_θ is given by the equation

$$R_\theta = (2\pi^2/N_0\lambda^4)n_0^2(dn/dc)^2M_w \quad (1)$$

where dn/dc is the specific refractive index increment of the solution, n_0 is the refractive index of the buffer, N_0 is Avogadro's number, c is the protein concentration in g/mL, λ is the wavelength of light in vacuum, and M_w is the molecular weight (Flory, 1953). Absolute molecular weight determination by eq 1 is most accurate in dilute solution limit, since excluded-volume effects, electrostatic effects, and aggregation phenomena can produce errors at large concentrations. The molecular weight of the wild-type c-fragment was calculated according to the equation

$$K^*c/R_0(c) = 1/M_w + 2Bc \quad (2)$$

where K^* is equal to a collection of constants, $(2\pi^2/N_0\lambda^4)$-$n_o^2(dn/dc)^2$ and B is the second virial coefficient. Extrapolation of $K^*c/R_0(c)$ to zero concentration yielded the reciprocal of the protein's molecular weight.

The monomer molecular weight of the dimer-forming c-fragment (S461L) could not be determined by extrapolation to zero protein concentration, since significant amounts of dimer were present at all of the concentrations studied. $\bar{M}_w$'s were calculated at each protein concentration by assuming that the concentration variation of K^* was small and that excluded-volume effects and charge effects (taken into account by B of eq 2) could be neglected. The concentration dependence of K^* has been described by Heller (1965). Under our conditions, corrections to K^* could be neglected since it did not amount to more than 0.5% for the most concentrated solutions (10 g/L). Excluded-volume and charge–charge effects were also estimated to be insignificant based on previous investigations of the properties of proteins under similar conditions of solution ionic strength and protein concentration (Tanford, 1961).

RESULTS

Correlation of c-Fragment Oligomerization with Mutant Phenotype. Figure 1a shows the elution profiles of the 10 different forms of the c-fragment (wild-type and nine mutants) from a TSK-gel G3000SW_{XL} GPC column in 20 mM phosphate buffer, with 50 mM NaCl, at pH 7.0 and 4 °C. As reported by Kaplan and Simon (1988), we found that the wild-type fragment and the mutant A436V eluted with an apparent M of 110 kDa and that the smooth mutant c-fragment S461L eluted as two peaks, 110 kDa and 220 kDa. A particularly striking feature arose from the analysis of additional mutant forms; only the smooth mutant c-fragments (T311I, S325L, A360T, A370V, S461L) eluted in more than one resolvable peak. (The amino acid substitutions are summarized in Table I.) Wild-type and the tumble mutant forms (V346M, V433I, A436V) exhibited a single form, which eluted with an apparent M of 110 kDa. Only one of the six smooth mutant forms that we analyzed did not display multiple forms in the HPLC elution profile (E301K) with these conditions. The specificity of oligomer formation observed at pH 7.0 was not apparent at lower pH's. When the buffer pH was decreased to pH 5.5, all of the c-fragments including the wild-type form, the anomalous smooth form E301K, and the tumble forms exhibited oligomer formation (Figure 1b).

The presence of more than one well-resolved peak on the sizing column in the purified samples of c-fragment could be explained by coexisting and slowly interconverting forms that differed either by their conformation or in the degree of oligomerization. To distinguish between these possibilities, we analyzed the proportion of high and low apparent molecular weight forms as a function of concentration, since only oligomerization would be expected to depend strongly on protein concentration. The results of such experiments are plotted in Figure 2. In every case the chromatograms of the c-fragments displayed a larger fraction of the total protein eluting as high molecular weight forms at high protein concentrations, demonstrating that the different peaks corresponded to different aggregate sizes, but the details of the equilibria were different for each of the variants.

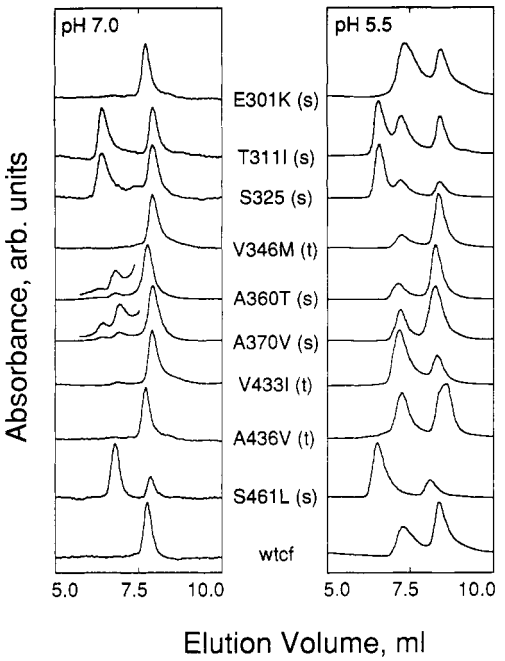

FIGURE 1: GPC of the aspartate receptor c-fragment at pH 7.0 (a) and pH 5.5 (b). The HPLC gel filtration elution profiles of the wild-type c-fragment and various mutants are offset for clarity. Amino acid mutations are indicated between the panels with the traces at pH 7.0 and pH 5.5, noting the position of the affected residue in the intact receptor and the phenotype induced by the mutation: either smooth swimming (s) or tumbling (t). Absorbances (not to scale) were monitored at either 280 or 214 nm. Material eluting at 7.8 mL had an apparent M of 110 kDa, according to globular protein standards, and protein eluting from 6.0 to 6.5 mL had apparent Ms of 270–220 kDa. Injection volumes were 100–120 μL. The flow rate was either 0.5 or 0.7 mL/min, and elutions were carried out at 4 °C. The concentrations of the c-fragments in the various samples were between 0.1 and 0.5 mg/mL except for the S461L mutant at pH 5.5, which was 20 μg/mL.

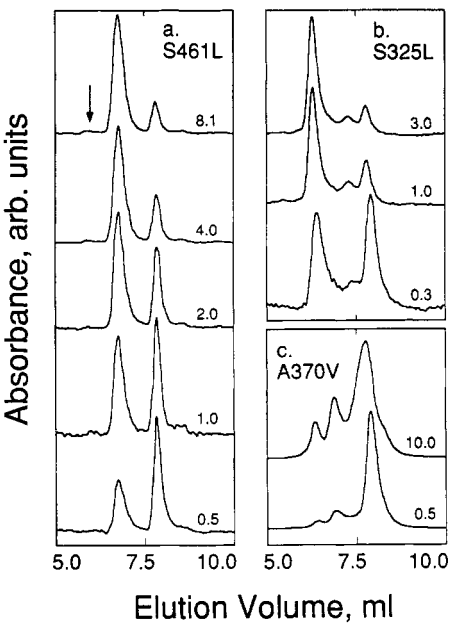

FIGURE 2: GPC of the oligomer-forming c-fragments at different protein concentrations. Aliquots from protein solutions with different total c-fragment concentration were resolved into different molecular weight forms. Protein concentrations (mg/mL) are noted adjacent to the GPC trace. Absorbances were measured either at 280 nm (a and b) or at 214 nm (c). Chromatograms are scaled so that the peak heights of the most abundant molecular weight form in each of the traces are equal. (a) The S461L fragment. Solutions were incubated and chromatographed at 20 °C and eluted with a flow rate of 0.7 mL/min. Magnification factors for the 0.5, 1.0, 2.0, and 4.0 mg/mL traces, relative to the 8.1 mg/mL trace, are 17.5, 10.6, 5.2, and 2.2, respectively. The arrow in the 8.1 mg/mL trace points to a high molecular weight form observed to be a minor component at high concentrations. (b) The S325L fragment. Solutions were incubated and chromatographed at 4 °C and eluted with a flow rate of 0.7 mL/min. Magnification factors for the 0.3 and 1.0 mg/mL traces, relative to the 3.0 mg/mL trace, are 22.0 and 3.4, respectively. (c) The A370V fragment. Solutions were incubated and chromatographed at 4 °C and at a flow rate of 0.5 mL/min. The 0.5 mg/mL trace is magnified 11.3 times relative to the 10 mg/mL trace.

Chromatograms of S461L (Figure 2a) displayed two forms, except at very high concentrations, where a small amount of a second high molecular weight form was detected (arrow). The S325L c-fragment displayed two major peaks, but also a minor component intermediate in its apparent molecular weight. This intermediate form was also considered to be c-fragment and not a protein impurity since coomasie-stained SDS gels of the column fractions did not indicate the presence of an impurity, and in preparative GPC runs, the protein was collected from a peak corresponding to the highest molecular weight form; so that any impurities in the molecular weight range of the intermediate peak should have been removed.

The elution profile of S325L and S461L differed in the apparent molecular weight of the high molecular weight form. The high molecular weight form of S325L eluted 1.53 mL earlier than the low molecular weight form, but the high molecular weight form of the S461L variant eluted only 1.02 mL earlier than the low molecular weight form. Clear evidence that more than two oligomeric forms were present came from chromatograms of the A370V oligomer-forming c-fragment, which clearly displayed three forms. The two high molecular weight forms eluted earlier than the low molecular weight form by 1.45 and 0.93 mL, differences in elution that are similar to those observed for S325L and S461L, respectively. That each of these forms was interconvertible was verified routinely by collecting protein from one peak and reinjecting it. Figure 2c depicts a representative result. Protein was collected from the middle peak, shown in the top chromatogram of Figure 2c, which upon reinjection produced the pattern displayed by the lower chromatogram. At this lower concentration of protein, the low molecular weight form was the predominant species although all three were detected, demonstrating that the forms were interconvertible.

For the S461L variant, the fractions of the protein in the low and high molecular weight forms were determined by incubating proteins at a series of concentrations. An aliquot was withdrawn from preequilibrated samples and injected onto the GPC column. To determine if the distribution between the two forms had altered over the course of elution, an aliquot of the protein eluting in the high molecular weight peak was reinjected immediately after elution. Over 90% of this reinjected material eluted from the column at the same molecular weight, even though it had been diluted significantly (~10-fold) during the first passage through the GPC column. Subsequent injections of protein collected from the high molecular weight peak showed that the fraction of monomer increased as a function time, due to dilution of the protein during the first pass through the GPC column. These observations were taken as evidence that over the time period during which the two species of the protein were in the process of being resolved on the column (approximately the first 5 min), no significant dissociation of the S461L c-fragment had taken place. Thus the peak areas of the high and low molecular weight forms could be taken to represent the equilibrium distributions for preequilibrated samples of this particular c-fragment.

Wild-Type c-Fragment: Monomer in Solution. The apparent M of 110 kDa exhibited by the low molecular weight form of the receptor c-fragment could be accounted for by

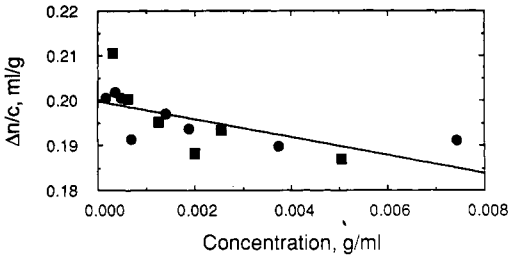

FIGURE 3: Change in solution refractive index as a function of protein concentration. The plot of $\Delta n/c$ for the wild-type c-fragment (●) and the tumble mutant form A436V (■) are plotted as a function of protein concentration, c (g/mL). The specific refractive index increment dn/dc was found to be 0.200 mL/g at the y-axis intercept using a least-squares line fit to the data.

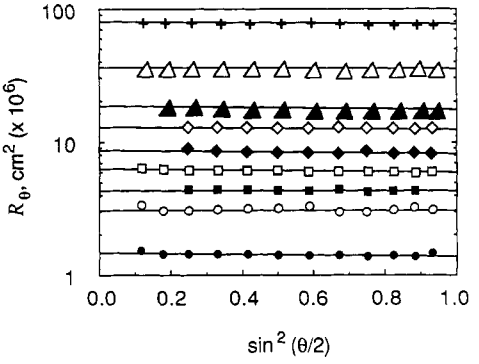

FIGURE 4: R_θ data for the wild-type c-fragment. The R_θ were calculated from background-subtracted scattering intensities and were obtained at different values of θ from 40 to 140° at each protein concentration. The concentrations of c-fragment (in mg/mL) were 0.17 (●), 0.35 (○), 0.46 (■), 0.70 (□), 0.93 (◆), 1.40 (◇), 1.86 (▲), 3.71 (△), 7.42 (+).

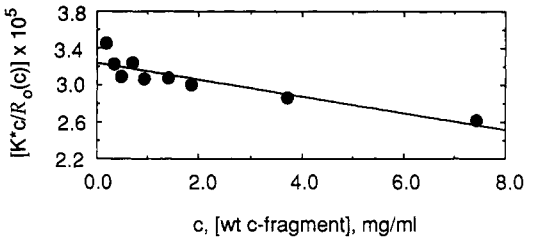

FIGURE 5: Debye plot of the wild-type c-fragment. The values of R_0 used in this plot were obtained by extrapolation of the R_θ data plotted in Figure 4 to zero scattering angle.

either a monomer, a dimer, a trimer, or possibly a tetramer. Sequence analysis predicted a monomer M of 31 190 Da (Oosawa et al., 1988). To establish the molecular nature of the association–dissociation reaction, the molecular weight of the protein was determined by static light scattering, which provided a shape-independent estimate of the molecular weight in solution. Since the determination of the protein molecular weight by light scattering was sensitive to the value of the specific refractive index increment, $\Delta n/c$ was measured for the c-fragment. dn/dc was found to be 0.200 mL/g by extrapolation to zero concentration, as shown in Figure 3. R_θ for the wild-type c-fragment was determined for a concentration series as a function of the scattering vector [$\sin^2(\theta/2)$, plotted in Figure 4] and was found to be essentially independent of θ, the scattering angle, as would be expected for a macromolecule in the large-wavelength limit (molecular length $<\lambda/20$). Values of $R_0(c)$ were found by extrapolation at each concentration. The $R_0(c)$'s were then extrapolated to zero protein concentration (c) as depicted in Figure 5. The reciprocal of the intercept at zero protein concentration yielded a M value for the protein of 31 000 Da, in excellent agreement with the value predicted from the sequence. A similar double-extrapolation procedure, where the $R_\theta(c)$ data were first

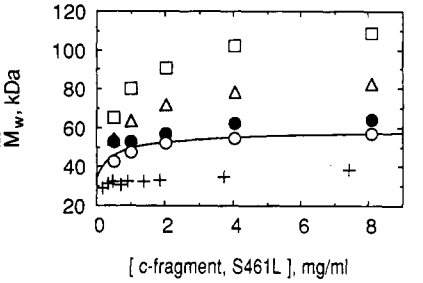

FIGURE 6: $\bar{M}_w$ of the oligomer-forming S461L c-fragment as a function of protein concentration. The monomer–oligomer ratio was determined from the proportions of low and high molecular weight peaks observed in the GPC traces of Figure 2a, and the $\bar{M}_w$ was determined by assuming either a monomer–dimer (○), monomer–trimer (△), or monomer–tetramer (□) equilibrium. Solid circles (●) are the $\bar{M}_w$'s determined from SLS data. The SLS data of the wild-type c-fragment (+) are plotted for comparison.

extrapolated to zero c for different values of θ and then the $R_\theta(0)$'s were extrapolated to $\theta = 0$, yielded the same value. This established that the low molecular weight form observed by GPC at pH 7.0 corresponded to a monomer of the c-fragment.

Participation of One Smooth Mutant c-Fragment in a Monomer–Dimer Reaction. To establish the oligomer size of the high molecular weight form, SLS was carried out with the S461L c-fragment, as a function of protein concentration. For each protein concentration where light scattering was measured, the sample was also analyzed by GPC to determine the fraction of the protein in each of the low and high molecular weight forms. The data from the GPC were then modeled in terms of simple monomer–dimer, monomer–trimer, and monomer–tetramer equilibria, and the $\bar{M}_w$ was calculated for each concentration. Multiple equilibria were not considered since essentially only two forms were detected by GPC for the S461L mutant. These data are plotted in Figure 6. The assumption was made that the variation with concentration of molecular weight as determined by SLS was due solely to the existence of a monomer–oligomer equilibrium. R_θ was used to calculate $\bar{M}_w$ at each concentration. The SLS results, plotted in Figure 6 as a function of S461L c-fragment concentration (and the wild-type c-fragment for comparison), were explained best by a monomer–dimer equilibrium. The curve in Figure 6 was generated from eq 3, where $\bar{M}_w$ is the

$$\bar{M}_w = 2M_1\sqrt{1 + 8[P]/K_d}/(1 + \sqrt{1 + 8[P]/K_d}) \quad (3)$$

weight-average molecular weight, K_d is the dissociation constant for the monomer–dimer reaction, M_1 is the molecular weight of the monomer form (taken to be 31 000), and [P] is the total molar concentration of protein monomer.

Dissociation constants for monomer–dimer equilibria estimated from the GPC data for the oligomer-forming mutant S461L at two temperatures are displayed in Figure 7, where the extent of dimer formation is plotted as a function of c-fragment concentration. At pH 7.0 and 4 °C, the K_d for S461L was found to be approximately 3 μM, and at 21 °C, the K_d was 22 μM. At 21 °C the different symbols represent separate sets of experiments. Some of the 21 °C GPC data in Figure 7 (black squares) were collected with the same samples that were used in the light scattering experiment, plotted in Figure 6.

DISCUSSION

The results presented here clearly demonstrate that the c-fragment has an extended, nonspherical shape and that it

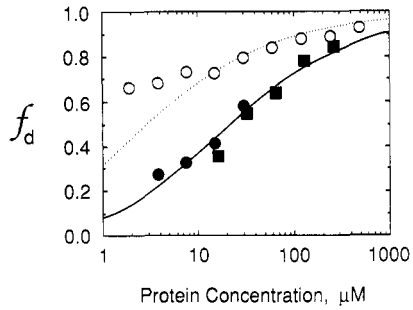

FIGURE 7: Extent of dimer formation as a function of protein concentration. The mass fraction of S461L dimer, f_d (determined by GPC), is plotted as a function of the total monomer concentration. Buffer conditions: 20 mM potassium phosphate, pH 7.0, 50 mM NaCl. Symbols are experimental points and curves are theoretical binding isotherms (22 μM, —; 3 μM, - - -) for a monomer–dimer reaction. Equilibrations were carried out at 21 (●, ■) and 4 °C (○).

can participate in monomer–oligomer equilibria. With one variant (S461L) this proved to be a monomer–dimer equilibrium; with other variants it appears to be an equilibrium that includes larger oligomers. Previous investigations of the c-fragment have detected these features without establishing their true nature. The analogous fragment from the *S. typhimurium* aspartate receptor was detected by GPC as two species with apparent *M*s of 135 and 225 kDa (Mowbray et al., 1985). Kaplan and Simon (1988) have also reported that the S461L c-fragment eluted from a GPC column as two species with apparent *M*s of 105 and 240 kDa. While Kaplan and Simon postulated that the two forms arose from different states of aggregation, they did not determine the aggregation number.

The c-Fragment: A Nonglobular Protein. It is well-known that the GPC elution volume of a protein is shape-dependent, so the molecular weight of nonglobular proteins cannot be reliably estimated with globular protein standards. For example the 64-kDa coiled-coil protein tropomyosin elutes with an apparent *M* of 330 kDa by GPC using the column and conditions described in Materials and Methods (D. G. Long, unpublished observations). Recently it has been predicted that the cytoplasmic region of the aspartate receptor contains a coiled-coil segment (Stock et al., 1991), which could account for the extended conformation of the c-fragment.

Static light scattering provides a means to determine the molecular weight of a monodisperse macromolecule in solution that is not influenced by macromolecule shape and leads to a value of 31 000 for the wild-type form of the c-fragment. Thus the wild-type c-fragment and presumably all of the tumble mutants are monomers in solution, since these were found to have the same elution volume by GPC. The GPC and SLS data obtained as a function of concentration for the S461L c-fragment were found to be most consistent with a monomer–dimer equilibrium. The appearance of forms of even larger apparent molecular weight in the GPC traces of the other oligomer-forming variants (T311I, S325L, A360T, and A370V) may be due to larger aggregates.

Properties of the c-Fragment Oligomerization. It is significant that the separate GPC peaks for monomers, dimers, and perhaps larger oligomers of the c-fragment can be resolved. This means that the kinetics of conversion must be slow compared to the time scale of a GPC experiment and is consistent with the idea that extensive conformational changes may occur during the association–dissociation process.

To detect an equilibrium experimentally, the difference in the standard chemical potential between the species in equilibrium must be smaller than ca. 2 kcal. With such a small energy difference it follows that the equilibrium should be sensitive to concentration, temperature, buffer conditions, and single amino acid mutations, as we have observed for the c-fragment. One of the most dramatic factors influencing oligomer formation is the amino acid composition. A single amino acid change is sufficient to shift the equilibrium significantly toward oligomer formation. Differences in the wild-type c-fragments from *E. coli* and *S. typhimurium* are also pronounced. The wild-type form of the c-fragment from *E. coli* showed a slight tendency to form oligomers at pH 7.0, and a more pronounced tendency only at pH 5.5. In contrast, the c-fragment from the *S. typhimurium* aspartate receptor appeared to have a relatively strong tendency to form an oligomer at pH 7.0 (Mowbray et al., 1985). The variation in amino acid composition between the two receptors could account for these observed differences.

Implications for the Mechanism of Transmembrane Signaling. The correlation between the behavioral phenotypes of bacteria harboring mutant receptors and the tendency of the c-fragment to form oligomers is striking and should provide some clues to the mechanism of transmembrane signaling. All of the mutations in the c-fragment were first isolated and characterized in the intact receptor (Mutoh et al., 1986). Depending on the mutation, the bacteria that carried a plasmid expressing the mutated *tar* gene either swam without tumbling or displayed a strong tumble bias. In both cases the cells were rendered nonchemotactic, because the mutations apparently locked the receptor into either the smooth or the tumble signaling state. From the in vitro CheA phosphorylation experiments of Borkovich and Simon (1990), it is apparent that a receptor locked into the smooth signaling state by a mutation resembles the attractant-bound wild-type receptor and that a receptor locked into the tumble state has CheA-activating properties similar to the wild-type receptor in the absence of aspartate. The correlation of the smooth signaling state of the receptor with the oligomerization of the c-fragment implies that attractant-bound receptors have stronger subunit interactions in the cytoplasmic domain. Conversely, the fact that none of the tumble variants of the c-fragments were found to form oligomers under our conditions at pH 7.0 suggests that the receptor without ligand has weaker subunit interactions in the cytoplasm.

The different models of transmembrane signaling are separable into the two broad categories, clustering and conformational change. Conformational-change mechanisms have been proposed to be the likely means of transmembrane signaling by the aspartate receptor, based primarily on data indicating that the intact receptor is dimeric under a variety of in vitro conditions (Milligan & Koshland, 1988) and from receptor methylation studies of disulfide-cross-linked receptors which show an increase in the rate of receptor methylation in response to aspartate binding (Lynch & Koshland, 1991; Milligan & Koshland, 1991). Nevertheless, these studies have not excluded the occurrence of clustering under physiological conditions and thus have not ruled out a role for clustering in transmembrane signaling.

As pointed out above, clustering equilibria can be influenced strongly by a number of factors, so that without the appropriate conditions an equilibrium will not be detected. When receptors are overproduced by genetic techniques, the receptor concentration in the membrane may exceed the normal physiological concentration by 100-fold or more, so that a clustering equilibrium which is balanced between the different forms near the physiological concentration will be shifted strongly toward the clustered form at elevated concentrations. In a monomer–dimer equilibrium ($2M = M_2$) with an association

constant K_a, the fraction of receptor as monomer (f_m) is given by

$$f_m = -\frac{1}{4K_aC} + \frac{1}{2}\sqrt{\left(\frac{1}{2K_aC}\right)^2 + \frac{2}{K_aC}} \qquad (4)$$

where C is the total receptor concentration. Assuming that f_m is equal to 0.5 at the physiological receptor concentration, C_0, then $K_aC = K_aC_0 = 1$. With a 100-fold overproduction, f_m will equal 0.07, making it difficult to detect the equilibrium.

In addition to the observed correlation between the receptor signaling state and the extent of c-fragment association, the stoichiometry of ligand binding provides additional evidence that is consistent with a clustering mechanism for transmembrane signaling. The crystal structure of the ligand-binding fragment from the *S. typhimurium* aspartate receptor shows a single molecule of aspartate bound to a receptor dimer with binding interactions contributed partly by each monomer (Milburn et al., 1991). Preliminary titration calorimetry experiments with L-aspartate and the *S. typhimurium* aspartate receptor, or its ligand-binding fragment [R. M. Weis and C. Bremicker, unpublished results, and in Brandts et al. (1990)], have also indicated a 1:2 ratio. Finally, it was reported that the rate of exchange of monomers between receptor dimers was diminished greatly upon aspartate binding (Milligan & Koshland, 1988). These data are consistent with a mechanism where ligand binding changes a monomer–dimer equilibrium constant in favor of the dimeric form.

Similar observations have been reported with other receptors. A 1:2 binding ratio was clearly evident in studies of the human growth hormone receptor where one hormone molecule was bound to two molecules of growth hormone binding protein (Cunningham et al., 1991; de Vos et al., 1992). In chemical cross-linking studies with the epidermal growth factor receptor, a cloned ligand-binding domain of the receptor formed dimers to a significant extent only in the presence of ligand (Hurwitz et al., 1991).

To transmit a signal across the membrane, the binding of external ligand must influence the properties of the intracellular signaling apparatus. Adaptation of the signaling system to a wide range of concentrations, as occurs with the aspartate receptor, suggests that the intracellular events may also somehow control the apparent aspartate binding constant. An attractive feature of the clustering mechanism is the straightforward means by which these processes can be accomplished (Brandts & Jacobson, 1983). The illustrative example in Figure 8 depicts a receptor in a monomer–dimer clustering process, which interacts with one dimer-favoring external ligand and one dimer-favoring internal ligand. Since both ligands act to shift the monomer–dimer equilibrium in the same direction, they will be in constant touch in a positively cooperative way. Calculations show (Brandts & Jacobson, 1983) that the binding of either ligand can be enhanced by orders of magnitude when the other ligand is bound as opposed to when it is not bound. If one ligand is dimer-favoring and one is monomer-favoring, then their binding will be linked through negative cooperativity. In a more general sense, perturbations other than ligand binding may also enter the picture. For example, if the receptor is chemically modified internally (e.g., methylation–demethylation, phosphorylation–dephosphorylation, mutations, etc.) in such a way as to favor either the monomer or the dimer, then this will have equivalent effects on binding of the external ligand which will then be superimposed on any other existing effects.

Proceeding on with this speculative mechanism as it might apply to the aspartate receptor, it is expected that the clustering equilibrium will be shifted in favor of the monomer in heavily

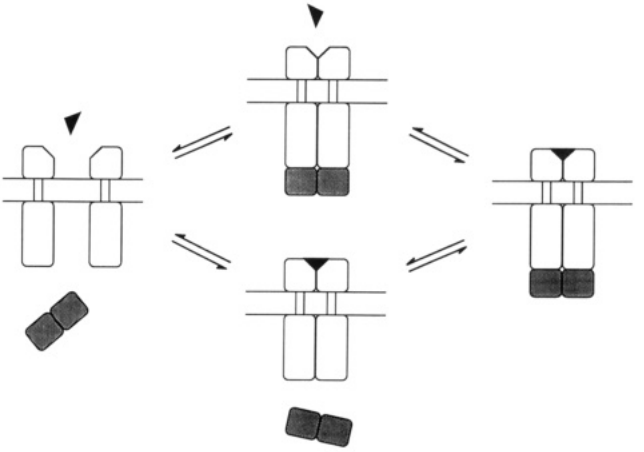

FIGURE 8: Clustering model for transmembrane signaling. As illustrated, a monomer–dimer equilibrium can be shifted toward dimer either by the binding of an external ligand (black triangle) to the external domain of a receptor or by the binding of an internal ligand (depicted here as a dimeric protein) to the receptor. In the case of positive cooperativity, the binding of either one of the two ligands will increase the apparent binding constant of the remaining ligand, through the effect on the monomer–dimer equilibrium. The monomer–dimer equilibrium in the absence of ligand is not shown.

methylated forms of the receptor and that ligand affinity decreases as the level of receptor methylation increases. Consistent with this model are the data of Yonekawa and Hayashi (1986), who have shown that the affinities of aspartate and serine for their respective receptors were significantly lower in membranes containing methylated receptors relative to membranes containing nonmethylated receptors. However, in a similar study by Dunten and Koshland (1991), only a small change in aspartate binding was found. An explanation for the discrepancy between these two studies may be a result of differences in the membrane preparations. While Yonekawa and Hayashi used membranes containing receptor expressed at wild-type (physiological) levels, Dunten and Koshland utilized membranes in which the aspartate receptor was overproduced. As pointed out previously in this section, the extent of clustering is sensitive to the receptor concentration, and thus it might not be possible to detect the effect of receptor modification on ligand-binding affinity in membranes where the receptor is overproduced.

The aspartate receptor modulates activity of an intracellular signaling pathway through its interactions with a 70-kDa autophosphorylating kinase (CheA), assisted by CheW (18 kDa), whose precise role is not yet known. Interestingly, CheA has been shown to be a dimer in solution (Gegner & Dahlquist, 1991). Thus, dimerization of receptors by aspartate could result in an enhancement in the affinity of CheA for the receptor, since CheA is dimeric and the attractant-bound form of the receptor is probably also (at least) a dimer.

Figure 8 utilizes dimers to illustrate the clustering model, and although the data cited above are most consistent with a monomer–dimer equilibrium, it is still possible that larger oligomers may play a role in signaling. In the membranes of intact *E. coli* cells, tetramers of the aspartate receptor and tetramers of the serine receptor were found in a chemical cross-linking study by Chelsky and Dahlquist (1980). Although the tetramers in that study could have been a nonspecifically cross-linked form of the receptors, it does provide a simple explanation for the high molecular weight forms of the S325L and A370V c-fragments observed by GPC (Figure 2b and c). A role for larger oligomers in signaling will be strengthened if some of the high molecular weight forms of the smooth mutant c-fragments prove to be oligomers

larger than dimers, and if they can be shown to be active in signaling.

Although a clustering mechanism is consistent with and supported by the data described above, they do not provide proof. It is our present opinion that neither the conformational-change mechanism nor the clustering model can be ruled out on the basis of the available data. A conformational-change mechanism may bear a strong resemblance to the clustering mechanism, particularly in view of the data presented here correlating c-fragment clustering and receptor signaling state. If receptor clustering transmits the excitatory response to attractants, then the association–dissociation process must be faster under physiological conditions than we have observed for the c-fragment, since it is known that the time interval between ligand binding and the chemotactic response is on the order of 0.2 s (Segall et al., 1982). It is possible that transmembrane signaling may utilize a combination of mechanisms. In the case of adaptation to stimulus, signaling may occur through a single subunit (Milligan & Koshland, 1991), whereas signaling via the phosphorylation cascade may occur through dimers, or by receptor clustering. Regardless of the mechanism of transmembrane signaling, the precise nature of the various oligomeric forms of the c-fragment, and their relationship to the signaling states of the intact receptor should contribute further to a detailed understanding of transmembrane signaling.

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