Hydrogen Exchange Reveals a Stable and Expandable Core within the Aspartate Receptor Cytoplasmic Domain*

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Intensive study of bacterial chemoreceptors has not yet revealed how receptor methylation and ligand binding alter the interactions between the receptor cytoplasmic domain and the CheA kinase to control kinase activity. Both monomeric and dimeric forms of an Asp receptor cytoplasmic fragment have been shown to be highly dynamic, with a small core of slowly exchanging amide hydrogens (Seeley, S. K., Weis, R. M., and Thompson, L. K. (1996) Biochemistry 35, 5199–5206). Hydrogen exchange studies of the wild-type cytoplasmic fragment and an S461L mutant thought to mimic the kinase-inactivating state are used to investigate the relationship between the stable core and dimer dissociation. Our results establish that (i) decreasing pH stabilizes the dimeric state, (ii) the stable core is present also in the transition state for dissociation, and (iii) this core is expanded significantly by small changes in electrostatic and hydrophobic interactions. These kinase-inactivating changes stabilize both the monomeric and the dimeric states of the protein, which has interesting implications for the mechanism of kinase activation. We conclude that the cytoplasmic domain is a flexible region poised for stabilization by small changes in electrostatic and hydrophobic interactions such as those caused by methylation of glutamate residues and by ligand-induced conformational changes during signaling.

The bacterial chemotaxis system is a well studied system in which detailed investigations of signal transduction mechanisms can be conducted. Although the mechanism of transmembrane signaling by bacterial chemoreceptors is unknown, the functionality of chimeric receptor constructs suggests that the mechanism may be general. For example, ligand-mediated regulation of the kinase activity has been demonstrated in chimeric proteins containing the ligand binding domain of the aspartate receptor and the tyrosine kinase domain of the human insulin receptor (1).

The aspartate, serine, ribose/galactose, and dipeptide receptors of Escherichia coli comprise a family of homologous chemoreceptors that senses environmental conditions and transmits this information to the intracellular signaling proteins, which control the direction of rotation of the flagellar motor and thus cell swimming behavior (for recent reviews, see Refs. 2–6). The chemoreceptors are largely α-helical, homodimeric transmembrane proteins with an extracellular sensing domain, an intracellular signaling domain, and two transmembrane helices per subunit. The receptor regulates the activity of an associated histidine kinase, CheA, within a ternary complex consisting of receptor, CheA, and CheW (7–9). In addition, the receptor mediates temporal adaptation to ongoing stimuli via methylation of 4–5 Glu residues in the signaling domain of the receptor (10).

The mechanism of transmembrane signaling has been probed by comparing the properties of signaling states in both the intact receptor and receptor fragments. Small ligand-induced conformational changes in the periplasmic and transmembrane domains of the receptor have been revealed by crystallographic and solution state NMR studies of soluble periplasmic constructs (11–14) as well as site-directed disulfide cross-linking, EPR, and solid state NMR studies of the intact receptor (15–19). Similarly, studies of both intact receptor (20–24) and soluble cytoplasmic fragment constructs (25–28) have been used to investigate the structural changes in this domain that activate the CheA kinase. High resolution crystal structure information is available for a fully amidated cytoplasmic fragment from the serine receptor of E. coli consisting of residues 284–520 (the methylation sites are Gin residues in this construct, which mimics the fully methylated signaling state (29)). The domain is organized as a dimer in which each subunit has a coiled-coil hairpin structure that associates to form an extended four-helix bundle (30). Moreover, the subunit hairpins interact with two other dimers to form a trimer of dimers.

A number of studies suggests that structural rearrangements of the dimer interface are involved in signaling. Disulfide cross-links formed in the cytoplasmic domain of the intact receptor across the dimer interface perturb signaling (22, 24, 31). DimORIZATION of the cytoplasmic fragment in leucine zipper fusion proteins can activate the CheA kinase (26, 28, 32), and different degrees of activation can be achieved by altering the orientation of subunits at the zipper/domain junction and by varying the extent of covalent modification (25, 32).

Early approaches to investigating structural differences between signaling states compared properties of soluble cytoplasmic fragment proteins with single missense mutations (33), which locked the receptor into the smooth-swimming (kinase-inactivating) or tumble (kinase-activating) signaling states (34, 35). These proteins exhibited monomer/oligomer equilibria that correlated with the signaling state; kinase-inactivating mutants were found to form oligomers, whereas the wild-type and kinase-activating mutants remained monomeric (36). Studies of the association-dissociation kinetics of two oligomer-forming fragments demonstrated a high energy barrier to interconversion, which led us to suggest that oligomer dissociation/associ-

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EXPERIMENTAL PROCEDURES

Chemicals, Strain, and Plasmids—Nickel-nitrilotriacetic acid resin was purchased from Qiagen (Chatsworth, CA). Isopropyl-β-p-galactoside was purchased from Bachem (Torrance, CA). Isopropyl-β-p-galactoside was isolated from bacterial lysate by centrifugation at 30,000 × g for 60 min. The supernatant was then applied to a 10-ml nickel-nitrilotriacetic acid-Sepharose column, pre-equilibrated with buffer B at pH 6.0, 7.0, or 8.0. This separation served to remove the free monomer and dimer equilibrium before the exchange-out process was initiated. The column was then attached in-line to the HPLC and c-fragment eluted by monitoring of absorbance at 230 nm at a constant flow rate of 0.5 ml/min. Exchange-out proceeded by immediately incubating the eluted tritiated c-fragment at a concentration of 1.0–1.2 mg/ml in a variable temperature water bath at 18 °C. The volume of the protein eluent from the G-25 Sephadex column was diluted to 15 ml with 20 mM sodium phosphate, pH 7.0, 0.50 mM NaCl, and 1 mM EDTA (buffer C), again to remove free tritium. 1.25 ml of c-fragment eluent was monitored for absorbance at 280 nm and found to be reproducible in all studies performed (0.06–0.13 mg/ml).

The exchange-out process described above was begun after sample renaturation at 4 °C for 15 min after trace labeling of all exchangeable sites by thermal denaturation. The concentration of c-fragment was determined by absorbance at 280 nm and found to be reproducible in all studies performed (0.06–0.13 mg/ml).

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The number of unexchanged hydrogens per c-fragment subunit was ~0.07 mg/ml was then incubated in a water bath at 18 °C (Neslab RTE-211, Neslab Instruments, Newington, NH). The approach to equilibrium was monitored by analyzing 100-μl aliquots of the incubation mixture at various times by analytical GFC (equilibrated with buffer B at pH 7.0) to determine the fractions of dimer and monomer in the sample (determined from the absorbance at 280 nm, corresponding to monomer and dimer, using a Rainin Dynamax software package). Zero time was taken as the time when the dimer first eluted off the G-25 Sephadex buffer-exchange column (used to set the pH). Several samples of dimer (of similar concentration as measured by absorbance at 280 nm) were collected from the initial c-fragment stock to measure the time course of dissociation at each pH.

The fraction of dimer (f_d) was plotted as a function of time and fit according to the integrated rate equation describing a dimer dissociation process defined previously in Ref. 27. The curve fitting of the data was performed with Kaleidagraph software package 3.0.2 (Abelbeck Software) on an Apple G3 Power Macintosh computer using a macro describing the integrated rate equation to obtain values for the parameters f_d0 (fraction of dimer at time zero), f_d eq (fraction of dimer at equilibrium), and k_d (the dissociation rate constant). The uncertainties reported for k_d and f_d eq are the standard deviations resulting from the curve fitting.

The concentration of c-fragment in combination with the parameters f_d0 and k_d defined from the curve fitting of the data are used to derive the association rate constant, k_a, from the dimer dissociation process (27)

\[
2\Delta[M]_{\text{total}} = k_d(f_{d0} - f_{d eq}) (1 - f_{d eq})
\]

where \(f_{d0}\) is the fraction of dimer at equilibrium and \([M]_{\text{total}}\) is the total concentration of c-fragment. The equilibrium constant, \(K_d\), is calculated directly from the rate constants \(k_a\) and \(k_d\).

Hydrogen-Tritium Exchange—Exchange of backbone peptide hydrogens in the c-fragment was measured using a general method of hydrogen-tritium (HT) exchange with trace tritium labeling of protein and two stages of separation of tritiated solvent from tritiated protein on G25 Sephadex columns as described originally by Englander (39). To cause thermal denaturation of the c-fragment is >90% reversible at pH 7.0 (40), HT exchange experiments were initiated by labeling the c-fragment under denaturing conditions. A 1 ml exchange-in reaction volume contained 3.75 mL of tritiated water and ~2.5 mg of c-fragment in buffer B. The exchange-in sample was incubated at 80 °C for 1 h. Refolding occurred during subsequent incubation of the exchange-in sample at 4 °C for 15 min. A 1-ml aliquot was removed and diluted to 10 ml with buffer, from which six 1-ml aliquots were taken for scintillation counting to obtain the initial tritium level in the equilibration mixture. Exchange-out was initiated by applying 1 ml of sample onto a 1.5-ml void volume G-25 Sephadex column pre-equilibrated with buffer B at pH 6.0, 7.0, or 8.0. This separation served to remove the free tritium and reset the solvent to the desired pH for monitoring of the exchange-out process. The column was then attached in-line to the Rainin HPLC and c-fragment eluted by monitoring of absorbance at 230 nm at a constant flow rate of 0.5 ml/min. Exchange-out proceeded by immediately incubating the eluted tritiated c-fragment at a concentration of 1.0–1.2 mg/ml in a variable temperature water bath at 18 °C. The volume of the protein eluent from the G-25 Sephadex separation was diluted to 15 ml with 20 mM sodium phosphate, pH 7.0, 0.50 mM NaCl, and 1 mM EDTA (buffer C), again to remove free tritium. 1.25 mg of c-fragment eluent was collected from the second separation; 1 ml of sample was added to 5 ml of scintillation fluid and counted in a scintillation counter. The concentration of the c-fragment after the second column separation was determined by absorbance at 280 nm and found to be reproducible in all studies performed (0.06–0.13 mg/ml).

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Table I

| pH  | $T^{\text{den}}_{\text{m}}$ (°C) | $\Delta H^{\text{m}}_{\text{endo}}$ (kcal/mol) | $\Delta H^{\text{m}}_{\text{exo}}$ (kcal/mol) | $K_D$ (µM) | $k_f$ ($10^4$ sec$^{-1}$) | $k_s$ (M sec$^{-1}$) |
|-----|-------------------------------|-------------------------------------------|-------------------------------------------|------------|--------------------------|-------------------|
| 6.0 | 61.2                          | 118                                       | 54.2                                      | 0.85       | 4.2 ± 0.3                | 4 ± 0.4           |
| 7.0 | 58.6                          | 56.3                                      | 52.2                                      | 9.5        | 18 ± 1                   | 2.0 ± 0.1         |
| 7.0 | (59.9 ± 0.5)$^a$              | (61 ± 1)$^a$                               | (61 ± 4)$^a$                               | (4.9)$^a$  | (19 ± 2)$^a$             | (4.0)$^a$         |
| 8.0 | 58.3                          | 45.0                                      | 56.3                                      | 72         | 140 ± 10                 | 2.0 ± 0.2         |

$^a$ Thermal denaturation properties of the His-tagged S461L c-fragment measured by DSC. Denaturation transition temperature and van’t Hoff and denaturation enthalpies were calculated using a non-two-state curve fitting routine as reported previously (40).

$^b$ S461L dimer dissociation was monitored by GFC at 18 °C. The total monomer concentration of c-fragment was ~2.5 µM for all three studies. Uncertainties reported for $k_s$ are the standard deviations resulting from the curve fitting.

$^c$ Previously reported calorimetric properties for the high temperature transition of the S461L c-fragment at pH 7.0 (40).

$^d$ Previously published values for the S461L c-fragment at 18 °C and 2 µM monomer concentration (27).

determined from the measured tritium counts in the sample collected after the second Sephadex separation and the protein concentration according to the definition of Englander and Poulsen (41)

$$H/\text{Molecule} = \frac{111}{\text{H/Molecule}} \times \frac{C(t)}{C_\text{t}} \times \frac{V_\text{molar}}{V_\text{ aliquot}} \times 1.2 \times \text{[cl]} \quad \text{(Eq. 2)}$$

where $H/\text{Molecule}$ is the number of unexchanged sites per c-fragment subunit, 111 is the molar concentration of hydrogen in H$_2$O, $C(t)$ represents counts left on the protein at a particular time, $C_\text{t}$ represents the initial amount of tritium in the original 1-ml labeling mixture, $V_\text{molar}$ is the volume of the aliquot (typically 150 µl) used for the second G25 Sephadex separation step, $V_\text{ aliquot}$ is the total exchange-out sample volume (typically 2 ml), and [cl] is the molar concentration of c-fragment collected after the second Sephadex separation step. The factor 1.2 corrects for equilibrium isotope effects present in tritium-exchange experiments (41–43) in which an unstructured peptide group preferentially selects tritium over hydrogen by 20% in H$_2$O. The uncertainty in each experimental time point, $H/\text{Molecule}(t)$, is calculated from Equation 2 by error propagation using the standard deviations of $C(t)$ obtained from six separate samples and of $C(t)$ obtained from five repeated scintillation counts of the same 1-ml sample.

Hydrogen-Tritium Exchange Kinetics Analysis—For more than one class of exchangeable sites within a macromolecule, the exchange kinetics are the sum of multiple first order rate processes. The kinetics of S461L c-fragment HT exchange adequately fit to classes of exchangeable sites: a rapid class and a slow class. The exchange kinetics were therefore fitted according to

$$H/\text{Molecule} = n_1 e^{-kt} + n_2 e^{-kt} \quad \text{(Eq. 3)}$$

where $n_1$ defines the number of exchangeable hydrogen atoms in the fast class, $n_2$ defines the number of exchangeable hydrogen atoms in the slow class, and $k_1$ and $k_2$ define the rate constants for the fast and slow classes, respectively. The wild-type c-fragment data gave a reasonable fit for a single exponential decay defining only a single class of slowly exchanging hydrogens, because rapid exchange for the wild-type c-fragment was complete within the dead time of the method (15–20 min).

Curve fitting was performed with the Origin 6.0 software package (Microcal, Northampton, MA) using the uncertainties for $H/\text{Molecule}$ values estimated as described above. The uncertainties in each $k$ and $n$ are the standard deviations resulting from the curve fitting.

Differential Scanning Calorimetry—Differential scanning calorimetry (DSC) was performed on the S461L c-fragment as described previously by Wu et al. (40). A Micral VP differential scanning calorimeter was used for monitoring the unfolding of the S461L c-fragment at pH 6.0, 7.0, and 8.0. 1-ml aliquots of the S461L c-fragment stock solution in buffer B at pH 7.0 were shifted to the desired pH using G-25 Sephadex columns pre-equilibrated in buffer B at pH 6.0, 7.0, or 8.0. Buffer-exchanged c-fragment eluted at a concentration of ~1 mg/ml and was dialyzed against 4 liters of buffer B at the respective pH. 2 ml of dialyzed sample and 2 ml of dialysis buffer were used for sample and reference solutions, respectively. Scans were begun by bringing sample and reference cells to thermal equilibrium below 5 °C for 1 h. Specific heat capacity data were collected at 1 °C/min from 5–95 °C. This process was followed by low temperature re-equilibration and re-scanning.

One series of two scans was performed at pH 6.0, 7.0, and 8.0. A reference trace used to subtract from the data for the analysis was obtained with water in the reference and sample cells.

DSC Data Analysis—Data analysis of the specific heat capacity versus temperature trace was performed essentially as described previously (40) using the Origin 6.0 software package (Microcal). The normalized data for the dimer-forming S461L c-fragment was baseline-corrected using the reference trace and fit with a non-two-state model. As described previously (40), the high temperature transition (monomer to unfolded) was determined by excluding the baseline-corrected trace above 50 °C, which overlapped with the dimer to monomer transition. The $T_m$ for the high temperature transition and the associated van’t Hoff and calorimetric enthalpies were then estimated by curve fitting of the trace above 50 °C (40).

RESULTS AND DISCUSSION

Lower pH Stabilizes the S461L c-Fragment Dimer—GFC and DSC were used to demonstrate that the stability and monomer/dimer equilibrium of S461L is unaffected by the N-terminal His tag. The monomer and dimer GFC elution volumes for the His-tagged S461L c-fragment are the same as observed previously for S461L (36). Furthermore, the N-terminal His tag does not perturb the monomer/dimer equilibrium at 18 °C and pH 7.0 or the monomer unfolding transition, as summarized in Table I.

The S461L dimer dissociation rate, measured in GFC experiments, was found to be pH-dependent; both the rate of dissociation ($k_d$) and the equilibrium constant ($K_D$) decrease with pH. Fig. 1 shows dimer dissociation kinetics of the S461L c-fragment that were analyzed by a simple dimer dissociation model accounting for the approach to equilibrium (27). The curves in Fig. 1 are fits of the data to the model to obtain the parameters listed in Table I. Each pH decrease of 1 pH unit produces about a 10-fold decrease in the dissociation rate and equilibrium constants, with equilibrium constants of 72, 9.5, and 0.85 µM for pH 8.0, 7.0, and 6.0, respectively. Because the association rates are not affected by pH, the pH dependence of dissociation indicates that lower pH stabilizes the dimer state relative to the monomer and transition state as depicted in the reaction coordinate scheme of Fig. 2. Thus, the previously observed pH dependence of $K_D$ (36) is now shown to be caused by stabilization of the dimer relative to the monomer.

The Stable Slow Exchanging Core of S461L Is Present in the Monomer, Dimer, and Transition State—Hydrogen-tritium exchange experiments (Fig. 3 and Table II) demonstrate that the rate of very slow exchange of a stable core of amides, previously observed by NMR D$_2$O exchange (37), is unaffected by pH. All exchangeable protons in the c-fragment that were labeled uniformly with tritium (as described in Refs. 42 and 44) by complete exchange in the unfolded state at 80 °C for 1 h followed by refolding for 15–30 min at 4 °C. HT exchange was initiated by removal of the tritiated solvent by buffer exchange on a G-25 Sephadex column. After incubation of the labeled protein, aliquots were removed and applied to a second G-25 Sephadex column to remove any tritiated solvent before counting the remaining tritium bound to the protein. The number of unexchanged hydrogens per monomer are plotted versus time in...
As observed previously, the majority of amides undergo fast exchange, and a small core of amides undergo slow exchange. Thus, the data were fit to a bi-exponential decay to determine the size and exchange rate of the slow exchanging fraction. The wild-type c-fragment data in Fig. 3D were fit by a single exponential decay representing a single slowly exchanging population of \( ^2\text{H} \) hydrogens. The balance of the amide hydrogens in the wild-type c-fragment was assumed to exchange at a rate too fast to monitor, given the constraints of the experimental dead time of 15 min. The slow exchange rate for S461L was found to be independent of pH (\( k_2 = 6 \times 9 \times 10^{-7} \sec^{-1} \) for S461L at pH 6.0–8.0) and was essentially equivalent in the wild-type c-fragment (\( k_2 = 5 \times 10^{-7} \sec^{-1} \)).

The rate of slow exchange does not depend on the fraction of dimeric c-fragment. The wild-type protein is monomeric throughout the measurement of the amide exchange. GFC chromatograms (data not shown) indicate that the S461L protein is monomeric initially after tritium labeling and refolding. Simulation of the approach to equilibrium for each condition, using the measured rates (Table I), indicates that the monomer/dimer equilibrium is established quickly; the arrows in Fig. 3, A–C, indicate the time at which the fraction dimer reaches a value within 1% of its equilibrium value. Thus the slow exchange rate is being measured largely from data collected after the monomer/dimer equilibrium has been reached. Furthermore, the slow exchange time course and rate constants are essentially equivalent for a sample that was preincubated (3 days at 18°C) after refolding to establish monomer/dimer equilibrium before the exchange assay (see Fig. 3B). Thus, the slow exchange rates represent the rates for the equilibrium fractions of S461L monomer and dimer, which ranged from 48 to 88% dimer. We conclude that the S461L monomer and dimer and the wild-type monomer have equivalent slow exchange rates.

Previous characterization of the thermodynamics of the monomer/dimer interconversion suggested that the transition state resembled unfolded protein (27), which led to the idea that the slow exchange kinetics might parallel the dissociation kinetics. The current results indicate that this is not the case; the core of the slow exchanging amides is retained in the monomer and dimer. This conclusion is based on the fact that conditions that populate the transition state and monomer (S461L at high pH or wild type at pH 7.0) and conditions that populate the dimer (S461L at low pH) all retain a core of amides exchanging at equivalent rates.

For a more detailed analysis, we consider the reaction typically used to interpret hydrogen exchange in proteins (45, 46).

\[
\begin{align*}
\text{Cf}^7 \xrightarrow{k_{\text{open}}} \text{Cf}^7_{\text{open}} \xrightarrow{k_{\text{ex}}} \text{Cf}^7_{\text{closed}}
\end{align*}
\]

(Eq. 4)

where \( \text{Cf}^7 \) is a tritiated site on the c-fragment, \( \text{Cf}^7_{\text{open}} \) is the protonated site after exchange, \( k_{\text{open}} \) and \( k_{\text{class}} \) are the rate constants for the structural opening (deprotection) and closing
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Hydrogen-tritium exchange performed at 18 °C. Uncertainties reported for hydrogen-tritium exchange kinetics parameters are standard deviations resulting from the curve fitting.

Table II

| c-fragment sample | pH | Initial oligomeric state | $t = 0^a$ | Fraction of dimer at equilibrium | $n_1 + n_2$ | $k_1$ | $n_2$ | $k_2$ |
|-------------------|----|--------------------------|------|-----------------|-------|------|------|------|
|                   |    |                          |      |                 |       |      |      |      |
| S461L             | 6.0| Monomer                  | 0.88 | 431 ± 30'       | 2.0 ± 0.3 | 169 ± 9 | 7.5 ± 2.0 |
| S461L             | 7.0| Monomer                  | 0.69 | 276 ± 8         | 3.2 ± 0.2 | 93 ± 2  | 5.9 ± 0.8 |
| S461L             | 7.0| Monomer/dimer equilibrium | 0.68 | 340 ± 12'       | 1.8 ± 0.1 | 82 ± 4  | 8.1 ± 2.0 |
| S461L             | 8.0| Monomer                  | 0.48 | 90 ± 3          |           | 8.7 ± 1.4 |
| Wild type         | 7.0| Monomer                  | 0.00 | 39 ± 1          |           | 5.4 ± 1.0 |

$^a$ Oligomeric state quantified by GFC at $t = 0$ for the exchange-out incubation period.

$^b$ Fraction of dimer at equilibrium calculated with kinetic parameters reported in Table I and the concentration of c-fragment monomer (~30 μM) used in HT exchange experiments.

The labeling scheme assured complete labeling of backbone amides. The total number of hydrogens labeled shown indicates that under certain conditions more than the 300 backbone amides of the His-tagged S461L C-fragment were labeled. The complete labeling by denaturing produced conditions conducive to labeling side chain hydrogens exchanging with slower than diffusion-limited rates (47, 48, 51).

(1) In the monomer state, $k_{ex}$ is the intrinsic base-catalyzed solvent exchange rate calculated for unstructured poly-DL-alanine for HT exchange (43, 47, 48). Because $k_{ex}$ increases at least 10-fold per pH unit increase, the pH independence of the slow exchange in the c-fragment indicates that $k_{ex}$ is not the rate-determining step and predicts that the measured exchange rate $k_{ex} = k_{open}$. Because the pH-independent exchange kinetics ($k_{ex} = 6 \times 10^{-7}$ sec$^{-1}$) do not match the pH-dependent dissociation kinetics ($k_{dis} = 3 \times 10^{-6}$ sec$^{-1}$ for pH 6.0–8.0), the open state is not the monomeric protein. The exchange kinetics also do not match the association kinetics for the c-fragment at the concentrations (30 μM) at which exchange was measured; $k_{ex} \times [c] = 10^{-4}$ sec$^{-1}$ ($= 3 \times 10^{-6}$ sec$^{-1}$ × 30 × 10$^{-3}$ μM). This indicates the open state is not the dimeric protein. Thus, the open state is not the monomer, dimer, or transition state for the association-dissociation process. The unfolding that occurs during dimer dissociation retains a stable structure in the small core.

The open state for hydrogen exchange of the core is likely to be the globally unfolded protein. Exchange from the most slowly exchanging sites is thought to be exchange from unstructured protein via a global unfolding process rather than exchange within a fluctuating native structure (46, 49–53). Furthermore, the slow exchange seems to be in the EX1 limit (45, 46), $k_{close} \ll k_{ex}$, which predicts the rate-limiting step is the structural opening event, so $k_{ex} = k_{open}$ and is a pH-independent process. Although rarer, this mechanism is indicative of global unfolding to expose highly protected residues (54).

The enthalpy of the monomer unfolding transition ($\Delta H_{cal}^{mon}$) in Table I correlates with the core size for S461L, providing further support for the conclusion that core exchange occurs upon global unfolding of the protein. Decreasing the pH from 7.0 to 6.0 increases the core size (Table II) and $\Delta H_{cal}^{mon}$ (Table I) approximately 2-fold. Also, there is no significant change in either core size or $\Delta H_{cal}^{mon}$ between pH 7.0 and pH 8.0. It has been noted previously that $\Delta H_{cal}^{mon}$ (cal/g) and $\Delta G_{cal}^{mon}$ (cal/gdeg) for c-fragment denaturation at pH 7.0 are $\sim 1/4$ of the typical magnitude for protein denaturation (40). The discrepancy has been ascribed to the molten globule-like nature of the c-fragment but may indicate that the denaturation transition is largely monitoring denaturation of the core, which is $\sim 1/4$ of the S461L c-fragment at pH 7.0 (but only $\sim 1/4$ of the wild-type c-fragment).

The Stable, Slow Exchanging Core Is Significantly Expanded by Small Changes in Hydrophobic and Electrostatic Interactions—Changes in a few amino acids in the c-fragment via mutagenesis (S461L) or protonation of His residues (pH 7.0 to 6.0) cause $\sim 50$–130 amino acids to change from the fast exchanging to the very slow exchanging group of amides. S461L has a core size approximately double that of the wild type c-fragment. This point mutation is likely to change hydrophobic interactions in the protein. Another doubling of the stable core size occurs upon decreasing the pH from pH 7.0 to 6.0 in the S461L c-fragment. Because this pH change also shifts the equilibrium toward the dimer, this might suggest that the dimer has a larger core size than the monomer. However, because the core size is independent of pre-equilibrium at pH 7.0 (Fig. 3B), which alters the monomer fraction from about 1 to 0.3, monomeric and dimeric S461L have similar core sizes. Furthermore, the doubling of the core size from pH 7.0 to 6.0 is accompanied by a 2-fold increase in $\Delta H_{cal}^{mon}$ for monomer unfolding, suggesting that the core size in the monomer has doubled at pH 6.0. Thus, we conclude that both the monomeric and dimeric forms of S461L have a 2-fold larger core size at pH 6.0. This change may be caused by changes in electrostatic interactions resulting from protonation of His residues (see below). Thus, both changes in electrostatic and hydrophobic interactions localized within a very few amino acid residues can dramatically alter the hydrogen exchange properties of the c-fragment.

The pH effect on the core size of S461L is likely to involve His residues, because no other residues typically titrate in the pH 6.0–7.0 range (55). The increase in core size then occurs as histidine side chains change from neutral at pH 7.0 to $1+1$ at pH 6.0. There are three histidines in the c-fragment sequence and an additional six histidines in the N-terminal His tag. As discussed above, the His tag does not change the energetics of the S461L c-fragment dimer dissociation (same $K_d$ and $k_d$) or unfolding (same $T_{m}^{un}$) at pH 7.0. Furthermore, it has been noted in previous work on the non-His-tagged wild-type c-fragment that reducing the pH from 7.0 to 5.5 shifts the equilibrium toward monomerization (36). Further inspection of the published data indicate an $\sim 10$-fold decrease in $K_p$ from pH 7.0 to 5.5, which parallels our measured equilibrium constants (Fig. 1 and Table I) for the His-tagged S461L c-fragment at pH 6.0 and 7.0. Thus, the N-terminal His tag has no significant effect on the shift in equilibrium toward dimer at lower pH, and the stabilization of the dimer is likely to be caused by protonation of the three native His residues in the c-fragment sequence.

The S461L mutation, which expands the slow exchanging core, stabilizes both the monomer and dimer states of the c-fragment. The S461L mutation has been shown previously to increase the $T_{m}$ for monomer denaturation by 10 °C and also to shift the equilibrium toward the dimer state. In the pH 7.0 exchange experiments the greater stability of S461L versus wild type is evident in both the increased core size and the
slower rate for the fast exchanging amides (compare Fig. 3, B and D). If the fast exchanging amides are in the typical EX2 limit, then \( k_{ex} = K_{open} k_{eq} \). If \( K_{open} = K_w \) the equilibrium constant for unfolding, then the pH independence of \( T_m \) and \( \Delta H^{den}_{cal} \) predicts that \( K_{open} \) is independent of pH for S461L. Then the expected 10-fold increase in \( k_c \) per pH unit increase is the likely cause of the increased rate of fast exchange in S461L at pH 8.0 versus 7.0 (compare Fig. 3, C and D), although it is not clear why the fast exchange rates at pH 6.0 and 7.0 are similar. The \( K_\alpha \) difference between S461L and wild type c-fragment can be estimated from the \( T_m \) and \( \Delta H^{non}_{cal} \) values measured by DSC (40). \( K_\alpha \) is estimated to be 10\(^3\)-fold larger for the wild-type c-fragment, predicting a 10\(^3\)-fold faster fast exchange rate, consistent with the complete disappearance of this phase in the exchange data for the wild-type c-fragment (Fig. 3D). Finally, the slowed fast exchange rate in S461L occurs both for samples that are initially \( \sim 100\% \) monomer and \( \sim 30\% \) monomer (Fig. 3B), indicating that both monomer and dimer are stabilized by the S461L mutation.

**Insights from the cTsrQ Structure**—The results of this hydrogen exchange study on the *E. coli* Asp receptor S461L c-fragment can be interpreted in the context of the recent crystal structure of the fully amidated, dimeric cytoplasmic fragment of the Ser receptor (cTsrQ; ref. 30) to deduce the likely location of the core and the effect of protonating the histidine residues. The cTsrQ crystal structure of a Ser receptor c-fragment (residues 286–520) shows ordered structure for residues 294–520 (30), corresponding to residues 292–518 of the Asp receptor c-fragments (which contain residues 257–553) used in our studies. Because this is a highly conserved region of the cytoplasmic domain, with 84\% sequence identity between the Asp and Ser receptors, the cTsrQ structure is an excellent model for the c-fragments in the current study. One possible difference between the structures is caused by the differences in the methylation signaling state; the cTsrQ crystal structure represents the fully methylated state (mimicked by full amidation, QQQQ), whereas our c-fragments represent the intermediate, unmodified (QEQE) methylation state (33).

Based on the distribution of temperature factors observed in the cTsrQ structure, we propose that the stable core of the c-fragment corresponds to the tip of the helix hairpin. Fig. 4A shows the dimeric four-helix bundle structure of the cTsrQ fragment colored by temperature factors. Fig. 4, B and C, represents different side-view orientations of a monomer subunit with charge and hydrophobic content represented (see Fig. 4 legend). The high temperature factors and disordered side chains throughout much of the crystal structure provide further evidence for the highly dynamic nature of this domain, previously shown in studies of both the c-fragment (37) and intact receptor (23). The tip of the structure, residues 361–420 in the cTsrQ structure, has low T factors, is highly conserved among related proteins (56), and is important in contacting CheW and possibly CheA (57). This region of \( \sim 60 \) residues is proposed to contain the slow exchanging core of \( \sim 40 \) residues (Table I) in the wild-type c-fragment (box 1 in Fig. 4).

The S461L mutation increases the core size \( \sim 2\)-fold, possibly by stabilizing the structure in the methylation region near the mutation site (box 3 in Fig. 4). The corresponding residue in the cTsrQ structure, 463, contacts residues Val116 and Ala320 within the coiled-coil monomer and has contacts across the dimer interface. Our results indicate that this mutation stabilizes both the dimer (decreased \( K_\alpha \) and increased core size) and monomer (increased \( T_m^{den} \) and core size). Stabilization of both the monomer and dimer helix interfaces in a region represented by box 3 would double the core size in both the monomer and dimer, consistent with the \( \sim 80–90 \) residue core size observed in S461L at pH 7.0.

The increase in core size in S461L at pH 6.0 is proposed to be caused by protonation of two His residues that should stabilize the structure in the middle of the elongated c-fragment (box 2 in Fig. 4). His\(^{357}\) and His\(^{359}\) in the Asp receptor (which correspond to Arg\(^{238}\) and Arg\(^{240}\) in the cTsrQ structure) are colored green in the space fill view in Fig. 4 and lie in a stretch of acidic and basic residues on the surface of the monomer subunit. The third native His residue, His\(^{257}\), lies outside the cTsrQ structure near the N terminus of the c-fragment. In the box 2 region from \( \sim 320 \) to \( \sim 360 \), there is an excess charge of \( \sim 5 \) at pH 7.0, which is reduced to \( \sim 3 \) upon protonation of the two His side chains for the Asp receptor sequence (for the Ser receptor the charges are \( \sim 4\)–3 at pH 7.0/6.0). This decrease in electrostatic repulsion apparently stabilizes the structure sufficiently to increase the size of the core of slowly exchanging amides. Because both the dimer and monomer are stabilized (\( K_\alpha \) decreases and \( \Delta H^{non}_{cal} \) increases), both states are likely to have a larger core. Thus, the pH 6.0 S461L core of 169 residues is suggested to span boxes 1–3, which is most of the cTsrQ structure.

**Implications for Transmembrane Signaling**—Our hydrogen exchange results suggest that the helix hairpin tip of the dynamic cytoplasmic domain is a stable scaffold structure re-
tained in both monomeric and dimeric states of the c‐fragment and the transition between them. Furthermore this stable core is expandable by small changes in electrostatic and hydrophobic interactions such as the S461L mutation and a pH decrease from 7.0 to 6.0, which is likely to protonate three His residues. Interestingly, both of these changes are known to favor the smooth‐swimming or kinase‐inactivating signaling state; S461L was isolated as a smooth mutant (35), and decreasing pH has been shown to elicit a smooth response for the Asp receptor (58). The pH effect was localized to the cytoplasmic domain through construction of chimeric receptors and was suggested to be caused by protonation of the three His residues (59). Thus expansion of the slow exchanging core of amides seems to correlate with kinase inactivation.

We demonstrate that changes that expand the core stabilize both monomeric and dimeric forms of the c‐fragment. The pH decrease from 7.0 to 6.0 expands the core size (probably for both monomer and dimer states), increases \( \Delta H_{\text{ben}} \) (monomer stabilization) and decreases \( K_P \) and \( k_{\text{diss}} \) (dimer stabilization). The S461L mutation increases \( T_{\text{m}} \) (monomer stabilization), increases the core size, and decreases the rate of the fast amide exchange component. The latter two effects apply to both monomer and dimer states, because equivalent core sizes and exchange rates are observed in samples with different monomer/dimer ratios. Stabilization of both monomer and dimer states is consistent with the location of the S461L site in the cTsrQ structure, with potential contacts at the interfaces both within and between the monomer coiled‐coils that make the four‐helix bundle of the dimer.

We suggest that the flexible cytoplasmic domain is poised for intradimer stabilization (via intra‐ or intermonomer contacts that expand the stable core), which leads to the kinase‐inactivating state, and that strengthening of interactions between dimers leads to the kinase‐activating or tumble‐signaling state. This model is consistent with a number of conditions that stabilize different signaling states and with growing evidence for the importance of receptor clusters in chemotaxis. The kinase‐inactivating (smooth) state is stabilized by cytoplasmic mutations such as S461L, which stabilize contacts within the dimer unit, and by ligand binding at the dimer interface, which has been shown to decrease subunit exchange between dimers (60). The kinase‐activating state is stabilized by methylation, which should decrease electrostatic repulsion between dimers (61), because the methylation sites are found on the outer surface of the four‐helix bundle in the cTsrQ structure (30). The results of studies that fused leucine zippers to the c‐fragment support this idea; constructs that promoted dimerization at the methylation site interface were most active, and covalent modification further enhanced the activity (25, 32). Interestingly, the leucine zipper‐linked c‐fragments with the highest kinase activation show evidence of oligomerization beyond a dimer and are maximal for the QQQQ protein. A recent proposal for the structure of the ternary complex of receptor, CheA and CheW, places the CheA dimer at the interface between two receptor dimers (62). In such a model, dimer‐dimer interactions between receptors should play an important role in regulating trans‐phosphorylation. Finally, interaction between receptor dimers has been demonstrated experimentally in both methylation (63, 64) and phosphorylation (65, 66).

The c‐fragment hydrogen‐exchange results demonstrate that small changes have a dramatic effect on the size of the stable core in this flexible protein; a single amino acid mutation doubles the core, and protonation of three His residues doubles it again. These effects reveal that this domain may be a flexible region poised for stabilization by small changes in electrostatic and hydrophobic interactions. The former are known to occur during signaling upon methylation of glutamate residues during adaptation. The latter could result from the conformational changes induced by ligand binding. Thus, these properties of the cytoplasmic domain may play an important role in the mechanism of signaling.

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