The Cytoplasmic Fragment of the Aspartate Receptor Displays Globally Dynamic Behavior†

Stacy K. Seeley, Robert M. Weis, and Lynmarie K. Thompson*

Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

Received October 19, 1995; Revised Manuscript Received February 21, 1996⊗

ABSTRACT: A number of cloned soluble fragments of the bacterial chemotaxis transmembrane receptors retain partial function. Prior studies of a fragment corresponding to the cytoplasmic domain (c-fragment) of the *Escherichia coli* aspartate receptor have correlated the signaling state of mutant receptors with the oligomerization state of the c-fragments: equilibria of smooth-swimming mutants are shifted toward oligomeric states; tumble mutants are shifted toward monomeric states [Long, D. G., & Weis, R. M. (1992) *Biochemistry 31,* 9904−9911]. We have applied several experimental probes of local and global structural flexibility to two signaling states, the wild-type (monomeric) and S461L smooth mutant (predominantly dimeric) c-fragments. Featureless near-UV CD spectra are observed, which indicate that the single Trp residue is in a symmetric environment (most likely averaged by fluctuations) and suggest that the C-termini of both proteins are highly mobile. Both proteins undergo extremely rapid proteolysis and enhance ANS fluorescence, which indicates that many sites are accessible to trypsin cleavage and hydrophobic sites are accessible to ANS binding. The global nature of the flexibility is demonstrated by ^{1}H NMR studies. Lack of chemical shift dispersion suggests that fluctuations average the environments of side chain and backbone protons. Rapid exchange of 90% of the observable amide protons suggests that these fluctuations give high solvent accessibility to nearly the entire backbone. This evidence indicates that both monomeric and dimeric c-fragments are globally flexible proteins, with properties similar to "molten-globule" states. The significance of this flexibility depends on whether it is retained in functioning receptors: the c-fragment structure may lack important tertiary contacts, protein−protein interactions, or topological constraints needed to stabilize a nondynamic native structure, or the cytoplasmic domain of the native receptor may retain flexibility which may be modulated in the mechanism of transmembrane signaling.

The aspartate receptor of *Escherichia coli* belongs to a family of homologous receptors that bind specific ligands in the initial step of the chemosensory signal transduction pathway that ultimately controls the motor apparatus of the bacterial cell [for recent reviews, see Bourret et al. (1991), Stock et al. (1991), Hazelbauer (1992), Hazelbauer et al. (1993), and Parkinson (1993)]. This receptor family is an excellent system for investigating the mechanism of transmembrane signaling. The receptors consist of a periplasmic ligand-binding domain, two transmembrane α-helices, and a cytoplasmic signaling domain. The cytoplasmic domain plays key roles in both the excitation and adaptation pathways of chemotaxis through interactions with several cytoplasmic signaling proteins. In conjunction with CheW, this domain modulates the rate of CheA kinase autophosphorylation. CheA transfers the phosphate group either to the response regulator CheY (which interacts with the motor in the excitation pathway) or to the receptor methylesterase CheB (of the adaptation pathway). As part of a negative feedback loop, the cytoplasmic domain is methylated at specific glutamic acid residues (Kehry & Dahlquist, 1982; Terwilliger & Koshland, 1984; Nowlin et al., 1987), which enables the receptor to mediate adaptation to sustained stimuli. The methylation level is controlled by interactions with CheR (a methyltransferase) and CheB. Thus, ligand binding to the receptor ultimately controls the level of phosphorylated CheY, to control the tumbling frequency of the cell, and the level of phosphorylated CheB, to control the extent of methylation of the receptor.

In spite of the central importance of the cytoplasmic domain, it is the least structurally understood region of the chemotaxis receptors. The X-ray crystal structure of the periplasmic ligand-binding domain fragment protein has been determined (Milburn et al., 1991; Scott et al., 1993; Yeh et al., 1993), resulting in a detailed understanding of the interactions between ligand and receptor. A structural model for the arrangement of the transmembrane segments in the receptor dimer has been determined from an extensive site-directed disulfide cross-linking study (Pakula & Simon, 1992a). In contrast, less is known about the structure of the cytoplasmic region. Residues which are methylated in sensory adaptation probably occur in α-helices (Krikos et al., 1983; Terwilliger & Koshland, 1984), and are predicted by sequence analysis to form coiled-coils (Lupas et al., 1991).

Chemical mutagenesis studies of the aspartate and serine receptors have generated a number of chemotaxis null mutations that localize to the cytoplasmic region, in which a single amino acid point mutation seems to lock the receptor into either one of two signalling states causing tumbling or smooth-swimming behavior (Mutoh et al., 1986; Ames &

† This research was supported by U.S. Public Health Service Grants GM47601 (L.K.T.) and GM42636 (R.M.W.), an award from Research Corp. (L.K.T.), an NSF Young Investigator Award (L.K.T.), and a Beckman Young Investigator Award (R.M.W.).

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

0006-2960/96/0435-5199$12.00/0

Parkinson, 1988). The cytoplasmic region of the aspartate receptor has been cloned and expressed as a 31 kDa soluble protein (c-fragment), and one of the mutations found for the intact receptor also produced some of the same behavioral effects when expressed in the c-fragment (Kaplan & Simon, 1988; Oosawa et al., 1988). The corresponding c-fragment and smaller fragments of the serine receptor have been constructed which alter flagellar rotation patterns *in vivo* and augment the autophosphorylation of CheA *in vitro* (Ames & Parkinson, 1994). These studies demonstrated that some soluble c-fragments retain partial function: they can interact with the cytoplasmic chemotaxis proteins to control the phosphorylation of CheA. Clustering equilibria of c-fragments have been observed and correlated with the behavioral phenotype of the mutation: mutations that mimic ligand binding (smooth mutants) shift the equilibrium toward the oligomeric state relative to the wild-type and tumble mutant c-fragments (Long & Weis, 1992). Several c-fragments have also been shown to adopt a predominantly α-helical secondary structure which unfolds cooperatively (Wu et al., 1995).

An understanding of the properties of the cytoplasmic domain in different signaling states is important for obtaining insight into its role in conformational change or clustering mechanisms of transmembrane signaling (Pakula & Simon, 1992b; Long & Weis, 1992). We have probed the structural flexibility of both aspartate receptor c-fragment signaling states: the monomeric wild-type protein and the dimeric S461L smooth mutant protein. A range of experiments using nuclear magnetic resonance (NMR),[1] near-UV circular dichroism (CD), proteolysis, and ANS fluorescence indicate that both the monomeric and dimeric forms are globally dynamic, which may have significant implications for the function of the receptor.

MATERIALS AND METHODS

Chemicals, Bacterial Strains, and Plasmids. Octyl β-D-glucopyranoside was obtained from Calbiochem (La Jolla, CA). Camphorsulfonic acid, chicken gizzard tropomyosin, ANS (1-anilinonaphthalene-8-sulfonate), and G-10 Sephadex were purchased from Sigma Chemical Co. (St. Louis, MO). The analytical Toso-Haas TSK-gel G3000SWXL GFC column was obtained from the NEST Group (Southborough, MA). Modified trypsin was purchased from Promega (Madison, WI). D_2O was obtained from Isotec (Miamisburg, OH). All other chemicals were reagent grade. JM103 (supE thi- Δ(lac-proAB), F′[traD36 proAB lacIq]) was the host strain used. The plasmid system for the wild-type form of the c-fragment is pNC189 (Kaplan & Simon, 1988). The dimer-forming smooth mutant serine-461 to leucine (S461L, Long & Weis, 1992) was used, where the residue number indicates the position in the intact receptor (553 amino acids).

Purification of the c-Fragment. The bacterial growth and purification of c-fragment were carried out essentially as described by Long and Weis (1992). An additional step before chromatography was added to exploit the reversibility of c-fragment denaturation (Wu et al., 1995). $(NH_4)_2SO_4$-precipitated c-fragment (derived from sonicated cell supernatants) was diluted into buffer (20 mM TRIS-HCl, pH 8.0, 1 mM EDTA) until a conductivity of 80 μS was reached. The sample was heated to 80 °C for 30 min and then rapidly cooled in an ice bath. Aggregated protein was removed by centrifugation for 60 min at 30000*g*. Samples purified with or without this heat denaturation step gave equivalent far-UV CD spectra, NMR spectra, and D_2O exchange properties, indicating that this purification step does not perturb the secondary or tertiary structure of the protein. Concentrations of purified protein were determined by the absorbance at 280 nm ($\epsilon_{280} = 7500\ M^{-1}\ cm^{-1}$; Long & Weis, 1992). All concentrations are reported as the total monomer subunit present. Typical yields of c-fragment were 60 mg from 1 L of cell, with a purity of ≈90% (visually estimated by SDS−PAGE with Coomassie blue staining).

Gel Filtration Chromatography. The distribution of c-fragment between dimeric and monomeric forms was determined by GFC as described previously (Long & Weis, 1992). GFC was also used to isolate different oligomer states of the c-fragment by injecting an aliquot (300−500 μL) of concentrated stock (∼160 μM) onto the column and collecting the eluent corresponding to the monomeric and dimeric forms. Isolated monomeric and dimeric forms of S461L were used in proteolysis and fluorescence studies.

Cleavage by Modified Trypsin. Reactions were carried out with 320 μg of c-fragment and 3.2 μg of modified trypsin in a total volume of 400 μL, using a freshly prepared trypsin stock solution in 20 mM sodium phosphate, pH 7.0, 50 mM NaCl, and 1 mM EDTA (buffer A). At various times, 40 μL aliquots were withdrawn, and proteolysis was quenched with 16 μL of 56 mM PMSF in 2-propanol. Aliquots were assayed by SDS−PAGE.

Proton Nuclear Magnetic Resonance Spectroscopy. NMR samples were 0.5−1.0 mM c-fragment in buffer A (without EDTA) with 10% D_2O. Data were acquired on 500 MHz Bruker AMX spectrometers at Brandeis University and the University of Massachusetts. Chemical shifts were calibrated with external standards of 3-(trimethylsilyl)-1-propanesulfonic acid or 3-(trimethylsilyl)propionate (methyl protons at 0 ppm). The water resonance was suppressed by a low-power presaturation pulse for 1 s prior to data acquisition. Typically, 128 free induction decays were added with a spectral width of 8 kHz and 4096 data points. Line broadening of 1 Hz was used. GFC was used to analyze the samples after the NMR experiments; no evidence of proteolysis was found.

Quantitation of the number of residues observed in NMR spectra of the c-fragment was made by integration of the amide and aromatic proton intensities (6.5−9.0 ppm) and normalization to the area of the single tryptophan indole proton (10.0−10.3 ppm) of the same spectrum. One potential issue is whether the recycle delay was long enough to allow full relaxation of the tryptophan residue, which is fairly mobile (see Results). The relaxation time of the indole proton of the amino acid tryptophan was measured to determine an upper limit (the highest possible mobility); $T_1 = 0.8$ s suggested a recycle time of 4 s ($5T_1$) would be sufficient. Proton NMR spectra were acquired as described above for the S461L smooth mutant c-fragment using a 5 s recycle time. Integration of the amide and aromatic proton intensities normalized to the tryptophan indole proton yielded similar results, indicating that the 1 s recycle time used above did not distort this measurement. Another potential issue is

[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; CD, circular dichroism; c-fragment, residues 257−553 of the *E. coli* aspartate receptor; GFC, gel filtration chromatography; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

differential saturation of the tryptophan indole proton *vs* the amide backbone protons from the presaturation pulse. The same ratio of Trp indole intensity to amide backbone intensity is obtained with 1 and 1.5 s presaturation pulses, indicating that differential saturation is also not likely to be a problem.

For hydrogen exchange experiments (pH 7.0), the NMR samples were spun through two consecutive G-10 Sephadex columns equilibrated with chilled D_2O buffer (99.993%). The sample was then immediately placed in the spectrometer where further exchange continued at 8 °C. After two columns, exchange was essentially complete: the water signal was 2% relative to the water signal of unexchanged samples, in spectra acquired without the water presaturation pulse. Proton NMR spectra for the exchanged samples were acquired as above, at various times after exchange. Samples exchanged back to H_2O buffer produced spectra which are identical to the original (unexchanged) spectra.

Near-UV Circular Dichroism. CD spectra were acquired on an Aviv 62DS spectrometer with thermostatically controlled cell holders as previously described (Wu et al., 1995). The instrument was calibrated with *d*-10-camphorsulfonic acid at 290 nm. All experiments were performed in a 5 mm path length rectangular cell at 4 °C. The wavelength scans were collected from 240 to 320 nm at 2 nm increments with a bandwidth of 0.5 nm, and an averaging time of 10 s for each point. The protein concentrations were 200 and 260 μM for the wild-type and S461L mutant, respectively, in buffer A. Each sample was checked by GFC to determine the fractions of dimer and monomer. Spectra were processed by averaging three scans, subtraction of the buffer scan, and smoothing with a five-parameter fast Fourier transform function (Origin, Microcal Software Inc., Northampton, MA). The results are expressed as the mean residue ellipticity, $[\theta]$, which was determined using the observed ellipticity in degrees; the total molar concentration of amino acid residues, c; and the path length in centimeters, l ($[\theta] = 100\theta_{obsd}/lc$).

ANS Fluorescence Studies. ANS fluorescence was measured at 4 °C with a Photon Technology International spectrofluorometer (South Brunswick, NJ). The excitation wavelength was 370 nm, and the emission wavelength was scanned from 400 to 600 nm. All spectra were acquired with 2 nm slitwidths, a scan rate of 0.2 nm/s, and a 0.1 s integration time, and were processed by subtracting the buffer spectrum and averaging three scans. c-Fragment concentrations ranged from 1 to 2.5 μM in buffer A; the ANS concentration was 50 μM.

RESULTS

Proteolysis of c-Fragments Is Extremely Fast. Most native proteins are resistant to cleavage by proteases. In general, the most susceptible regions display a high degree of flexibility and are often located between independently folded domains within the protein. Thus, proteolysis can be used to probe the solvent accessibility and flexibility of a protein (Fontana et al., 1986; Hubbard et al., 1994; Cohen et al., 1995). Previous studies have demonstrated that a similar c-fragment is highly susceptible to proteolysis in detergent solutions (Mowbray et al., 1985), but detergents are thought to perturb the tertiary structure of this protein (Wu et al., 1995). Figure 1 displays the time course of trypsin digestion of the c-fragment under nondenaturing conditions at 4 °C, monitored by SDS−PAGE. Under these conditions, the

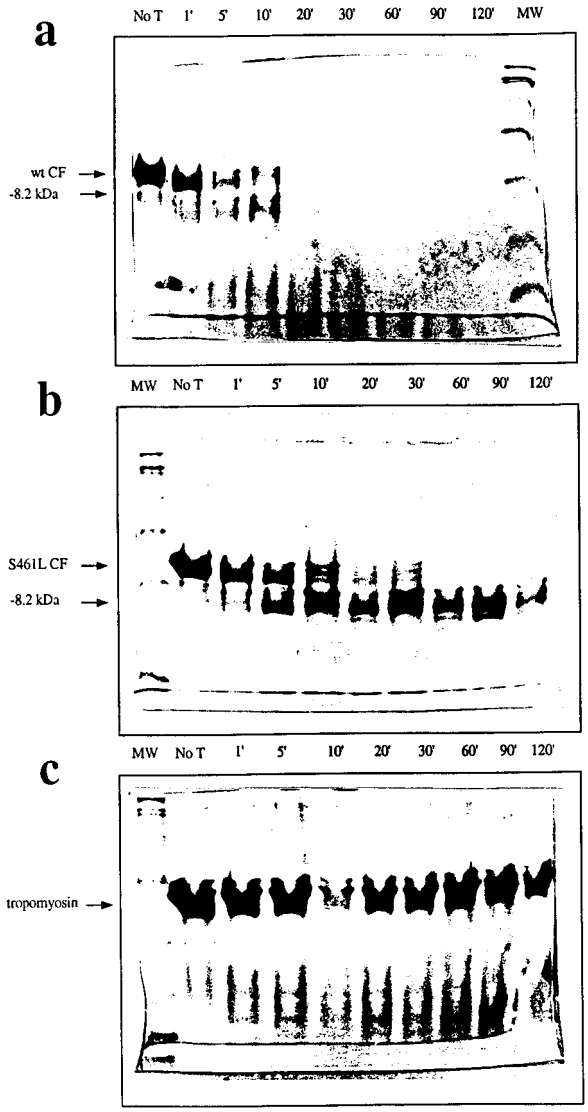

FIGURE 1: Proteolysis by modified trypsin. The time course of digestion at 4 °C was monitored by SDS−PAGE for (a) wild-type c-fragment, (b) S461L smooth mutant c-fragment, and (c) tropomyosin. Reaction volumes were 400 μL containing 320 μg of c-fragment and 3.2 μg of trypsin. Time profiles are as indicated above gels; "No T" indicates no trypsin was added.

wild-type protein is proteolyzed and no longer observable (pieces <7 kDa) in less than 20 min (Figure 1a). A major product corresponding to a loss of about 8 kDa is formed upon cleavage of each of the c-fragments. For the wild-type protein (100% monomer), this proteolysis product (intact minus 8.2 kDa) is further digested and is no longer observed after 20 min (Figure 1a). For the dimer-forming S461L c-fragment (60% dimer, 40% monomer), this proteolysis product is observable for over 2 h (Figure 1b). Digestion of isolated S461L c-fragment dimer (93% dimer, 7% monomer) and monomer (79% monomer, 21% dimer) is similar (data not shown). In addition, digestion of two other mutant c-fragments, A436V (monomeric) and S325L (oligomeric), is as rapid as digestion of the wild-type c-fragment (data not shown). These data indicate that the increased resistance of the S461L c-fragment to proteolysis is unique to this c-fragment, and reflects its increased thermal stability (its T_m is 6−10 °C higher than all other c-fragments studied; Wu et al., 1995). Thus, we conclude that both monomeric and oligomeric states of c-fragments are susceptible to rapid proteolysis.

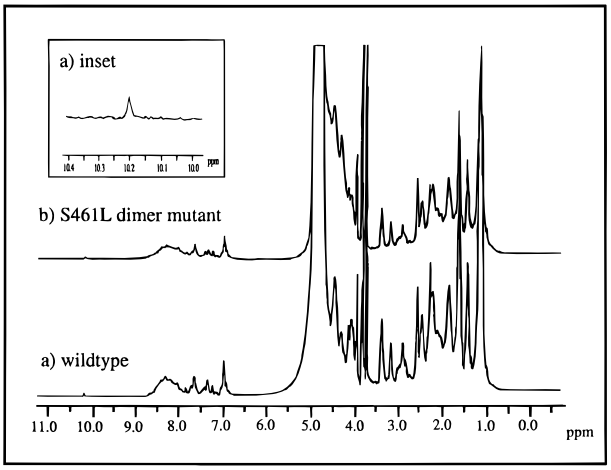

FIGURE 2: 500 MHz ^{1}H NMR spectra of (a) wild-type c-fragment (0.70 mM, 85% monomer) and (b) S461L smooth mutant c-fragment (0.63 mM, 90% dimer).

Proteolysis of native proteins by trypsin yields complete protein digestion in 3−30 min at 37 °C (Flannery et al., 1989). The above proteolysis reactions were also performed at 37 °C and observed to proceed very quickly at this temperature: wild-type c-fragment is completely digested to fragments smaller than 7 kDa in 3 min, and S461L digestion is complete in 20 min (data not shown). Thus, the wild-type c-fragment is on the extreme end of rapid proteolysis, indicating that it is very accessible to trypsin. The c-fragments have been suggested to be elongated because of their anomalous migration in GFC columns (Long & Weis, 1992) and their predicted coiled-coil structures (Lupas et al., 1991). For comparison, the coiled-coil protein tropomyosin was treated with trypsin (Figure 1c) and found to be completely resistant to cleavage by trypsin under the same conditions. Thus, the high accessibility of the c-fragment to trypsin proteolysis is not characteristic of all folded proteins, and indicates that the c-fragments have open, flexible structures. An alternate interpretation of the rapid proteolysis is that initial cleavage at a few accessible sites in the c-fragment could lead to unfolding that creates greater access to additional sites throughout the protein. Thus, it is important to apply independent approaches which probe global flexibility, as presented below.

Solution NMR Studies Indicate a Dynamic Structure. Solution NMR was used to investigate the properties of the monomeric wild-type and dimeric S461L smooth mutant c-fragments. Figure 2a presents the proton one-dimensional spectrum of the wild-type c-fragment (GFC indicates that this 0.7 mM NMR sample consists of 15% dimer and 85% monomer). This spectrum lacks the chemical shift dispersion typical of native proteins and resembles spectra observed for denatured proteins. In the amide and aromatic proton regions, the chemical shifts do not deviate significantly from those of a random coil (Wüthrich, 1986). In addition, the spectrum lacks signals upfield of 1 ppm which are typical of native proteins (due to ring-shifted methyl protons). Further evidence for random coil backbone chemical shifts was obtained in a TOCSY-HMQC spectrum of a uniformly ^{15}N-labeled sample of the wild-type c-fragment (data not shown): only 42 of the expected 289 NH/C_αH cross-peaks are resolved (presumably due to spectral overlap), and the proton chemical shifts are similar to random coil shifts (NH of 7.9−8.8 ppm and C_αH of 4.1−4.8 ppm).

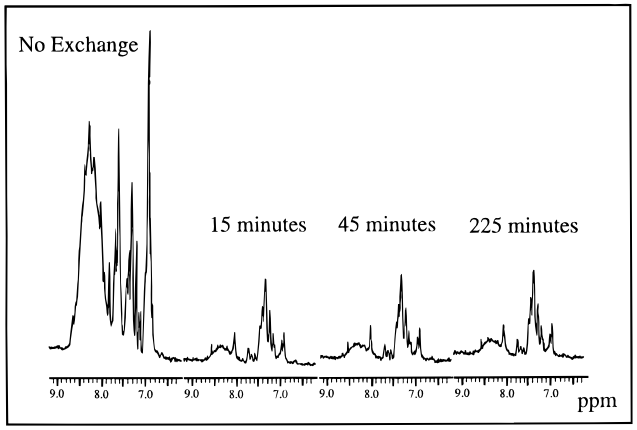

FIGURE 3: Amide hydrogen exchange spectra for the wild-type c-fragment. The amide backbone regions are shown for the wild-type c-fragment at the indicated times after exchange at 8 °C, pH 7.0.

A possible explanation for the random coil shifts and the unusual exchange behavior reported below is that we are observing a subset of the protein, such as residues located on the surface or in a mobile loop or tail. However, integration of the amide backbone region calibrated with the single tryptophan indole proton (Figure 2a, inset) indicates that the amide peaks correspond to greater than 90% of the expected intensity for the entire c-fragment backbone. Thus, observation of random coil chemical shifts indicates that the side chain and backbone protons of the wild-type c-fragment have a rapidly fluctuating environment.

The hydrogen exchange technique is commonly used to probe protein dynamics by measuring rates of exchange of solvent-accessible protons (Englander & Kallenbach, 1984; Roder et al., 1988; Udgaonkar & Baldwin, 1988). Protons involved in hydrogen bonds or buried within the protein display slower exchange than protons on the surface of the protein. D_2O exchange experiments on the wild-type c-fragment are displayed in Figure 3. The amide backbone region is shown before exchange and after 15, 45, and 225 min in deuterated buffer. In less than 15 min, approximately 90% of the amide protons exchange to deuterium. The remaining 10% of the c-fragment amide protons do not exchange for at least 12 h (data not shown). This remaining amide signal is not due to residual protons from the small fraction of H_2O (estimated to be only 2%) or to aggregated c-fragment (GFC after the NMR experiment estimated no more than 5% aggregated c-fragment). For comparison, only a fourth of the amide backbone protons of the coiled-coil GCN4 leucine zipper peptide exchange on a similar time scale (<15 min) under similar conditions (pH 7, 6 °C; Goodman & Kim, 1991). In addition, CD results indicate the c-fragments contain about 38−60% α-helix, depending on the mutant and the oligomerization state (unpublished results). Thus, the rapid exchange of 90% of the amide protons suggests that these secondary structures are both solvent-accessible and dynamic, suggesting the protein is globally flexible.

The dimeric S461L mutant c-fragment exhibits NMR properties very similar to the native c-fragment. Figure 2b shows the proton spectrum for the predominantly dimeric S461L protein (90% dimer and 10% monomer by GFC). Unexpectedly, the ^{1}H 1-D spectrum of the dimer-forming mutant protein looks nearly identical to the spectrum of the

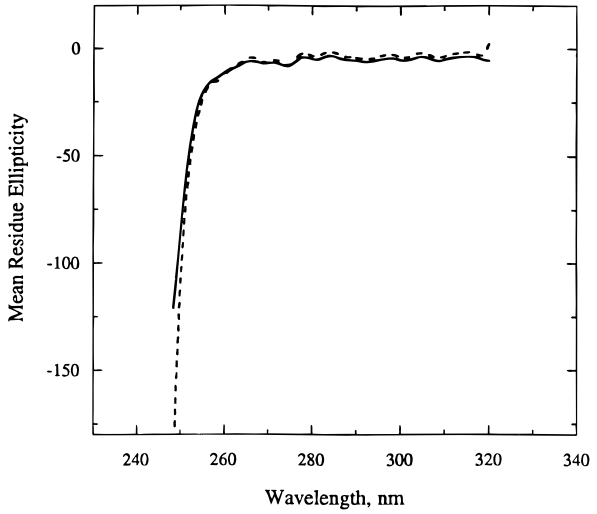

FIGURE 4: Near-UV CD spectra of wild-type (solid line, 200 μM) and S461L smooth mutant (dashed line, 260 μM) c-fragments at 4 °C.

wild-type (monomeric) protein. If we were observing only the 10% monomeric fraction of the S461L protein, the spectral intensity would be 10-fold smaller for this c-fragment. The similar spectral intensities observed for the two samples indicate that both the monomer and dimer are observed, and comparison of the integrated signal intensity of the amide *vs* indole proton again indicates that at least 90% of the S461L mutant protein amide backbone resonances are observed. The observation of similar line widths in the monomeric and dimeric states suggests that many protons have a greater mobility than that determined by the tumbling of the entire protein. The D_2O exchange properties for this predominantly dimeric S461L c-fragment are similar to those of the wild-type protein (data not shown). The slow interconversion of the monomer and dimer forms of S461L ($t_{1/2}$ of dissociation is 53 h at 9 °C) indicates that averaged chemical shifts would not be observed and rapid proton exchange does not require dissociation.

These NMR results indicate that both the monomeric and dimeric c-fragments are extremely dynamic, as demonstrated by the lack of chemical shift dispersion and the rapid proton exchange. In addition, a small number of protons (~10%) are well-protected from exchange in both the monomeric and dimeric proteins.

Near-UV CD Indicates a Symmetric Environment of Aromatic Groups. The lack of detectable dichroism in the near-UV CD spectrum of a protein indicates the aromatic group environments are symmetric due to averaging via intramolecular fluctuations; such data have often been used as evidence for lack of a stable tertiary structure (Ptitsyn, 1992). Figure 4 presents the near-UV CD spectra of high concentration samples (≈200 μM) of wild-type and S461L c-fragments, which each contain a single Trp residue. For comparison, a number of single Trp-containing carbonic anhydrase mutant proteins display detectable near-UV CD features at 10-fold lower concentration (Freskgard et al., 1994). Therefore, the absence of near-UV CD features in these c-fragment spectra can be attributed to a symmetric environment for tryptophan-550, the fourth amino acid from the c-terminus of the protein, and indicates that both monomeric and dimeric c-fragments have a highly mobile c-terminus. This conclusion is further supported by the narrow NMR line width (8 Hz) observable for the Trp indole proton (Figure 2a, inset). These results are in agreement with prior ^{19}F NMR evidence for rapid motion of Trp-550 in the c-fragment, which may be retained in the intact receptor (Falke et al., 1992).

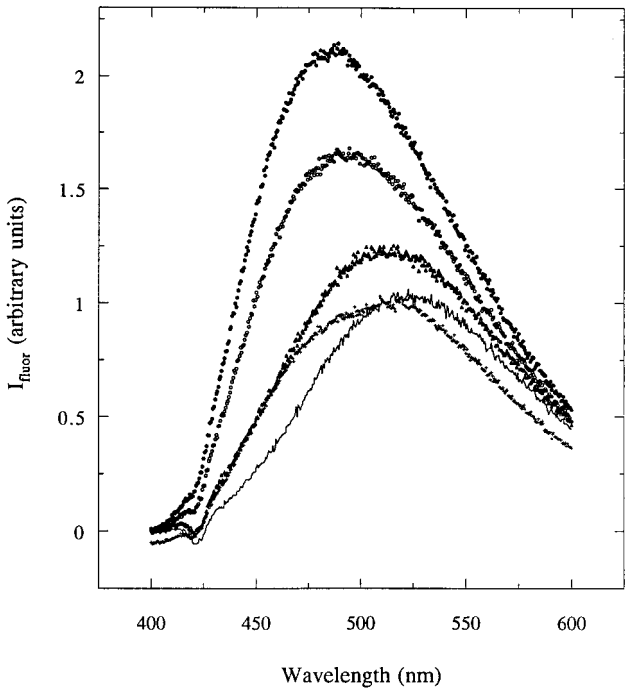

FIGURE 5: Fluorescence emission spectra of 50 μM ANS in the presence and absence of wild-type and S461L c-fragments (bottom to top): no additions (line); lysozyme control (2 μM, crosses); wild-type c-fragment (1 μM, 100% monomer, open triangles); isolated monomer of S461L c-fragment (2.4 μM, 93% monomer, open circles); and isolated dimer of S461L c-fragment (2.4 μM, 81% dimer, filled circles).

c-Fragments Bind the Hydrophobic Probe ANS. The fluorescent probe ANS has a low fluorescence yield in aqueous solution, which is enhanced upon binding to hydrophobic sites (Andley & Chakrabarti, 1981). Semisotnov et al. (1991) demonstrated that ANS binds more effectively to the partially folded "molten globule" state (see Discussion) than to either the native or the completely unfolded state of a protein: ANS is thought to bind to solvent-accessible clusters of nonpolar atoms which are less frequently observed in unfolded chains and native proteins. ANS binding enhances its fluorescence intensity and shifts its emission maximum to shorter wavelengths.

ANS fluorescence was used to probe the accessibility of nonpolar clusters in the c-fragments. Figure 5 plots the emission spectrum of 50 μM ANS in the presence and absence of c-fragments. 50 μM ANS has a λ_{max} of 523 nm and a fluorescence intensity of 1 (normalized). Addition of isolated monomeric S461L (2.4 μM; 93% monomer and 7% dimer by GFC) increases the ANS fluorescence to 1.6 and shifts the emission maximum to 495 nm. The same protein isolated in the dimeric form (81% dimer and 19% monomer by GFC) increases the ANS fluorescence to 2.0 and shifts the emission maximum to 489 nm. Addition of wild-type c-fragment (1 μM; 100% monomer) increases the ANS fluorescence to 1.2 and shifts the emission maximum to 511 nm. No fluorescence was detected in the absence of ANS. As a control, lysozyme (2 μM) added to ANS results in no

detectable increase in the fluorescence intensity and only a modest 2 nm blue shift.

The results of these ANS-binding experiments are similar to results obtained with proteins in the "molten globule" state at neutral pH, where small enhancements are typically observed [for example, the results of Semisotnov et al. (1991) suggest that the neutral molten globule state of bovine carbonic anhydrase would cause only a 2−5-fold increase in the fluorescence of ANS under conditions comparable to ours]. However, these modest enhancements are also consistent with observations of ANS binding to some native proteins (Mulqueen & Kronman, 1982; Goto & Fink, 1989). These results indicate that the c-fragments possess solvent-accessible hydrophobic clusters which can be observed in native proteins but are more typical of "molten globule" states.

DISCUSSION

c-Fragments Are Globally Flexible Proteins. A range of local and global experimental probes used in this study indicates that both monomeric and dimeric c-fragments are highly dynamic. The single tryptophan residue has both high mobility (yielding a narrow NMR line width) and a symmetric environment (yielding featureless near-UV CD spectra), which indicates the c-terminus of the protein is highly mobile. The c-fragments are highly accessible to trypsin proteolysis, hydrophobic sites are accessible to ANS binding, and 90% of the backbone is accessible to solvent for rapid amide proton exchange. Thus, three independent approaches suggest the c-fragment has an open structure with high accessibility. Our results also indicate this protein has a high degree of flexibility, based on the fact that most of the hydrogen bonds of the 40−60% α-helical structure exchange rapidly and that similar line widths are observed for monomeric and dimeric states of the protein. The lack of chemical shift dispersion also suggests that fluctuations average the environments of side chain and backbone protons. Thus, the accessibility of the c-fragment to trypsin, ANS, and solvent is likely to be due to a fluctuating structure. The fact that the NMR experiment probes at least 90% of the protein suggests that the observed flexibility is global. Finally, all of these experimental probes have demonstrated flexibility in both the monomeric wild-type and the predominantly dimeric S461L smooth mutant proteins (as well as two other mutant c-fragments, A436V and S325L; data not shown). Therefore, both monomeric and dimeric cytoplasmic fragments of the aspartate receptor are globally flexible proteins.

A number of other proteins have been found to display similar behavior intermediate between the characteristics of fully folded and unfolded states of a protein. These states, known as "molten globules" or "compact intermediates" [reviewed by Kuwajima (1989), Ptitsyn (1992), Haynie and Friere (1993), and Dobson (1994)], have been observed as short-lived intermediates during protein folding, and can also be stabilized by a range of nonphysiological solvent conditions, most typically at acid pH. Although a wide range of such states have been studied which are likely to differ in detail, the following properties are shared by the c-fragment and typical molten globule states. The c-fragment has a high content of secondary structure (we have estimated 38−60% α-helix, unpublished results) but shows significantly flexibility and appears to lack a well-defined tertiary structure. Another property which the c-fragment and molten globule states have in common is the tendency to form high molecular weight aggregates at intermediate to high protein concentrations (Jaenicke, 1987). Finally, molten globule states can display an enthalpy of unfolding, although it is not always measurable (Haynie & Friere, 1993). A cooperative, reversible denaturation has been observed for the c-fragment, with an enthalpy (54 kcal/mol) which is about 25% of that expected for a typical globular protein of 31 kDa (Wu et al., 1995), consistent with its being incompletely folded. Thus, the c-fragment shares a number of properties with the molten globule state.

However, in the case of the c-fragment, it is not known whether its dynamic behavior is due to its being partially unstructured or is characteristic of the native, functional receptor (see below). The dynamic behavior of the c-fragment differs from the typical molten globule in that it occurs in physiologically compatible buffers and the protein is at least partially functional. Other instances of molten globule like behavior under physiological conditions include a number of *incomplete proteins* which are missing ligands, portions of the protein sequence, or additional subunits (Feng et al., 1991; Moore & Lecomte, 1993; Eder et al., 1993; Tasayco & Carey, 1992; Lycksell et al., 1994). As in these cases, the dynamic behavior of the c-fragment may be due to the absence of other components of the system. On the other hand, the functionality of the c-fragment resembles proteins which are known to be *both flexible and functional* and in which changes in dynamics occur during function. For instance, a number of DNA-binding proteins undergo folding and oligomerization transitions upon binding DNA [reviewed by Spolar and Record (1994)]. Finally, another unique aspect of the dynamic behavior of the c-fragment is that *both monomers and dimers are molten globule like*, which presents the opportunity to characterize the thermodynamics of interconversion of two molten globule states (S. Seeley, G. Wittrock, L. Thompson, and R. Weis, unpublished observations).

Insights into Transmembrane Signaling. The functionality of c-fragment proteins has been demonstrated in studies of the equivalent (75% sequence identity) and smaller constructs of the serine receptor (Ames & Parkinson, 1994), which also appear to have dynamic behavior (F. Dahlquist and J. Parkinson, personal communication). Preliminary results indicate that the c-fragments used in this study can also modulate phosphorylation of CheA (G. Li and R. Weis, unpublished observations). One key question raised by these findings is whether the flexibility observed in the c-fragment is also a property of the functioning receptor. One possibility is that some of the missing components (the N-terminal half of the receptor, the membrane, and/or other cytoplasmic proteins) are needed to stabilize a nondynamic tertiary structure in the cytoplasmic domain, and that this is the functional state of the receptor. The other possibility is that the functional intact receptor retains dynamic behavior, which then may be involved in the mechanism of signaling. Thus, the globally dynamic nature of the c-fragment provides insight into either the structure or the mechanism of chemotaxis receptors, as discussed below.

Functional chemotaxis receptors may not retain the global mobility observed in the c-fragments. The c-fragment may not be fully native based on its lack of complete functional-

ity: methylation is extremely slow (A. Pautsch and R. M. Weis, unpublished results), and phosphorylation assays of similar constructs of the serine receptor c-fragment require long preincubation times (Ames & Parkinson, 1994). Although c-fragments retain partial function, since other proteins are involved in each function, protein interactions may stabilize the c-fragment to form a functional, nondynamic tertiary structure. This interpretation suggests that the c-fragment monomers and dimers lack significant tertiary contacts needed to stabilize the native structure. For instance, there is evidence that the receptor, CheA, and CheW form a long-lived complex (Gegner et al., 1992); these proteins may make a significant number of tertiary contacts with the c-fragment which are needed to stabilize its native tertiary fold. Another possibility is that the topological constraints of the membrane-bound receptor are important in maintaining a native dimeric state in the cytoplasmic domain. Such insight is important for the choice of conditions for structural studies of this domain of the receptor, which has so far eluded structure determination by crystallography or NMR. In addition, it is possible that the structure present in the c-fragment is partially non-native: although a number of studies have suggested that native secondary structure (Kuwajima, 1989; Ptitsyn, 1992; Haynie & Friere, 1993; Dobson, 1994) and even native-like tertiary folds (Peng & Kim, 1994) are preserved in molten globule states, non-native secondary structures have also been observed (Chaffotte et al., 1991). Thus structural studies of the c-fragment must be interpreted with caution, since it is possible that the c-fragment adopts a structure different from the functional form.

On the other hand, functional receptors may retain some of the dynamic properties observed in the c-fragment. With this interpretation, it seems likely that the global mobility would have functional significance. A key question would be whether ligand binding to the intact receptor can modulate the dynamics of the cytoplasmic domain. The "frozen dynamic dimer" model (Kim, 1994) proposes such a role for changes in dynamics, based on the observation that ligand binding yields a more compact structure in the periplasmic domain (Milburn et al., 1991; Scott et al., 1993; Yeh et al., 1993). In addition, ^{19}F NMR spectra of p-F-Phe-labeled periplasmic fragment are better resolved (indicating less dynamic heterogeneity) upon Asp binding or disulfide formation, both of which occur across the dimer interface (Danielson et al., 1994). Thus, it appears that ligand binding induces compactness and decreases mobility for the periplasmic fragment. Disulfide trapping experiments have also detected flexibility in the intact receptor (Falke & Koshland, 1987; Careaga & Falke, 1992; Chervitz et al., 1995), and have shown that ligand binding decreases subunit exchange (Milligan & Koshland, 1988). However, since these studies focused on cysteines in the periplasmic and transmembrane regions, it is not known whether the flexibility and ligand-induced "tightening" extend throughout the receptor. We have now demonstrated global mobility in the cytoplasmic domain, in mutants which mimic the ligand-bound and ligand-free signaling states, though we do not know whether this mobility is retained in the intact receptor. Experiments are in progress to determine the extent of dynamic behavior in more complete receptor systems and the effect of ligand binding on these dynamics.

There are a number of potential mechanistic roles for the unusual dynamics we have observed. Flexibility may be functionally important to facilitate protein−protein interactions between the cytoplasmic domain (only 31 kDa) and the five other proteins it must interact with (itself to form dimers, CheR, CheB, CheA, and CheW). A comparison of the surface areas involved in typical protein−protein interactions (800 Å^2 each, and larger for oligomerization; Janin & Chothia, 1990) with that of the cytoplasmic domain (10 000 Å^2, estimated from the dimensions observed in an electron microscopy study of the related ribose−galactose receptor; Barnakov et al., 1994) suggests that roughly half of the surface area would be needed for five nonoverlapping bindng interactions. Interface areas of 5−20% of the protein surface are more typical (Janin & Chothia, 1990). Flexibility in this receptor domain may serve to accelerate binding of some of these proteins (Pontius, 1993) or to interconvert the conformations needed for each of these binding interactions.

Finally, *changes in dynamics* may be involved in transmembrane signaling. The ligand-induced "tightening" of the periplasmic portion of the receptor (Kim, 1994) may extend to the cytoplasmic portion: a reduction in its dynamics could alter its interactions with cytoplasmic proteins, for example, by destabilizing the conformation needed to activate CheA phosphorylation. Conversely, interaction of some cytoplasmic ligands (CheB, CheR, CheA, CheW) with the receptor could rigidify its structure on both sides and thus modulate its interaction with periplasmic ligands (aspartate). As discussed above, a number of laboratories have noted dynamic behavior in chemotaxis receptors and its potential role in signaling. However, most currently proposed transmembrane signaling mechanisms (in chemotaxis and other fields) focus on changes in conformation or oligomerization state. It is important to add changes in dynamic behavior to these concepts, as well as to recognize the inter-relatedness of the three: stabilization of a compact dimer which is one of a set of interconverting conformations can be alternately pictured as a change in dynamics, conformation, or oligomerization.

ACKNOWLEDGMENT

We thank Dr. John Brandts and Dr. Lila Gierasch for helpful discussions. We also thank Dr. Anne-Frances Miller and Dr. Thomas Pochapsky for their guidance in preliminary NMR experiments as well as Dr. Susan Pochapsky at Bruker Instruments for performing the three-dimensional NMR experiments.

REFERENCES

Andley, U. P., & Chakrabarti, B. (1981) *Biochemistry 20*, 1687−1693.
Ames, P., & Parkinson, J. S. (1988) *Cell 55*, 817−826.
Ames, P., & Parkinson, J. S. (1994) *J. Bacteriol. 176*, 6340−6348.
Barnakov, A. N., Downing, K. H., & Hazelbauer, G. L. (1994) *J. Struct. Biol. 112*, 117−124.
Bourret, R. B., Borkovich, K. A., & Simon, M. I. (1991) *Annu. Rev. Biochem. 60*, 401−441.
Careaga, C. L., & Falke, J. J. (1992) *Biophys. J. 62*, 209−219.
Chaffotte, A., Guillou, Y., Delepierre, M., Hinz, H.-J., & Goldberg, M. E. (1991) *Biochemistry 30*, 8067−8074.
Chervitz, S. A., Lin, C. M., & Falke, J. J. (1995) *Biochemistry 34*, 9722−9733.

Cohen, S. L., Ferre-D'Amare, A. R., Burley, S. K., & Chait, B. T. (1995) *Protein Sci. 4*, 1088−1099.
Danielson, M. A., Biemann, H.-P., Koshland, D. E., Jr., & Falke, J. J. (1994) *Biochemistry 33*, 6100−6109.
Dobson, C. M. (1994) *Curr. Biol. 4*, 636−640.
Eder, J., Rheinnecker, M., & Fersht, A. R. (1993) *Biochemistry 32*, 18−26.
Englander, S. W., & Kallenbach, N. R. (1984) *Q. Rev. Biophys. 16*, 521−625.
Falke, J. J., & Koshland, D. E., Jr. (1987) *Science 237*, 1596−1600.
Falke, J. J., Luck, L. A., & Scherrer, J. (1992) *Biophys. J. 62*, 82−86.
Feng, Y., Wand, A. J., & Sligar, S. G. (1991) *Biochemistry 30*, 7711−7717.
Flannery, A. V., Beynon, R. J., & Bond, J. S. (1989) *Proteolytic Enzymes: A Practical Approach* (Beynon, R. J., & Bond, J. S., Eds.) pp 145−162, IRL Press, Oxford.
Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M., & Zambonin, M. (1986) *Biochemistry 25*, 1847−1851.
Freskgard, P.-O., Martensson, L.-G., Janasson, P., Jonsson, B.-H., & Carlsson, U. (1994) *Biochemistry 33*, 14281−14288.
Gegner, J. A., Graham, D. R., Roth, A. F., & Dahlquist, F. W. (1992) *Cell 70*, 975−982.
Goodman, E. M., & Kim, P. S. (1991) *Biochemistry 30*, 11615−11620.
Goto, Y., & Fink, A. L. (1989) *Biochemistry 28*, 945.
Haynie, D. T., & Freire, E. (1993) *Proteins: Struct., Funct., Genet. 16*, 115−140.
Hazelbauer, G. L. (1992) *Curr. Opin. Struct. Biol. 2*, 505−510.
Hazelbauer, G. L., Berg, H. C., & Matsumura, P. (1993) *Cell 73*, 15−22.
Hubbard, S. J., Eisenmenger, F., & Thornton, J. M. (1994) *Protein Sci. 3*, 757−768.
Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117−237.
Janin, J., & Chothia, C. (1990) *J. Biol. Chem. 265*, 16027−16030.
Kaplan, N., & Simon, M. I. (1988) *J. Bacteriol. 170*, 5134−5140.
Kehry, M. R., & Dahlquist, F. W. (1982) *J. Biol. Chem. 257*, 10378−10386.
Kim, S. H. (1994) *Protein Sci. 3*, 159−165.
Krikos, A., Mutoh, N., Boyd, A., & Simon, M. I. (1983) *Cell 33*, 615−622.
Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87−103.
Long, D. G., & Weis, R. M. (1992) *Biochemistry 31*, 9904−9911.
Lupas, A. N., Dyke, M. V., & Stock, J. B. (1991) *Science 252*, 1162−1164.
Lycksell, P.-O., Ingemarson, R., Davis, R., Graslund, A., & Thelander, L. (1994) *Biochemistry 33*, 2838−2842.
Milburn, M. V., Prive, G. G., Milligan, D. L., Scott, W. G., Yeh, J., Jancarik, J., Koshland, D. E., Jr., & Kim, S.-H. (1991) *Science 254*, 1342−1347.
Milligan, D. L., & Koshland, D. E., Jr. (1988) *J. Biol. Chem. 263*, 6268−6275.
Moore, C. D., & Lecomte, J. T. J. (1993) *Biochemistry 32*, 199−207.
Mowbray, S. L., Foster, D. L., & Koshland, D. E., Jr. (1985) *J. Biol. Chem. 260*, 11711−11718.
Mulqueen, P. M., & Kronman, M. J. (1982) *Arch. Biochem. Biophys. 215*, 28.
Mutoh, N., Oosawa, K., & Simon, M. I. (1986) *J. Bacteriol. 167*, 992−998.
Nowlin, D. M., Bollinger, J., & Hazelbauer, G. L. (1987) *J. Biol. Chem. 262*, 6039−6045.
Oosawa, K., Mutoh, N., & Simon, M. I. (1988) *J. Bacteriol. 170*, 2521−2526.
Pakula, A. A., & Simon, M. I. (1992a) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4144−4148.
Pakula, A., & Simon, M. (1992b) *Nature 355*, 496−497.
Parkinson, J. S. (1993) *Cell 73*, 857−871.
Peng, Z.-Y., & Kim, P. S. (1994) *Biochemistry 33*, 2136−2141.
Pontius, B. W. (1993) *Trends Biochem. Sci. 18*, 181−186.
Ptitsyn, O. B. (1992) *Protein Folding* (Creighton, T. E., Ed.) pp 243−300, Freeman, New York.
Roder, H., Elove, G. A., & Englander, S. W. (1988) *Nature 335*, 700−704.
Scott, W. G., Milligan, D. L., Milburn, M. V., Prive, G. G., Yeh, J., Koshland, D. E., Jr., & Kim, S.-H. (1993) *J. Mol. Biol. 232*, 555−573.
Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. I., & Gilmanshin, R. I. (1991) *Biopolymers 31*, 119−128.
Spolar, R. S., & Record, M. T., Jr. (1994) *Science 263*, 777−784.
Stock, J. B., Lukat, G. S., & Stock, A. M. (1991) *Annu. Rev. Biophys. Biophys Chem. 20*, 109−136.
Tasayco, M. L., & Carey, J. (1992) *Science 255*, 594−597.
Terwilliger, T. C., & Koshland, D. E., Jr. (1984) *J. Biol. Chem. 259*, 7719−7725.
Udgaonkar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 694−699.
Wu, J., Long, D. G., & Weis, R. M. (1995) *Biochemistry 34*, 3056−3065.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley-Interscience, New York.
Yeh, J. I., Biemann, H.-P., Pandit, J., Koshland, D. E., Jr., & Kim, S.-H. (1993) *J. Biol. Chem. 268*, 9787−9792.

BI9524979