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Multidimensional Solid-State NMR of a Functional Multiprotein Chemoreceptor Array

Michael J. Harris[†], Jochem O. Struppe[‡], Benjamin J. Wylie[#], Ann E. McDermott[#], and Lynmarie K. Thompson^{†,*}

[†]Department of Chemistry, University of Massachusetts, 710 N Pleasant St, Amherst, Massachusetts 01003, USA

[‡]Bruker BioSpin Corporation, 15 Fortune Drive, Billerica, MA 01821, USA

[#]Department of Chemistry, Columbia University, 3000 Broadway, New York, NY 10027, USA

Abstract

The bacterial chemoreceptor complex governs signal detection and the upstream elements of chemotactic behavior, but the detailed molecular mechanism is still unclear. We have assembled native-like functional arrays of an aspartate receptor cytoplasmic fragment (CF) with its two cytoplasmic protein partners (CheA and CheW) for solid-state NMR studies of structural changes involved in signaling. In this initial study of the uniformly ¹³C,¹⁵N-enriched CF in these >13.8 MDa size arrays, residue-type assignments are made for amino acids that together make up 90% of the protein. We demonstrate that homo and heteronuclear two-dimensional spectra are consistent with structure-based chemical shift predictions: a number of major assignable correlations are consistent with the predominantly alpha helical secondary structure and minor correlations are consistent with the disordered C-terminal tail. Sub-ppm linewidths and spectral changes on sample freezing suggest these arrays are structurally homogeneous and sufficiently immobilized for efficient solid-state NMR.

Graphical Abstract

*Department of Chemistry, 122 LGRT, 710 N Pleasant St., University of Massachusetts Amherst, Amherst, MA 01003 USA. Telephone: +1 (413) 545-0827. thompson@chem.umass.edu.

Supporting Information

Comparison of ¹³C-¹³C DARR spectra of the CF alone and in arrays (Figure S1); comparison of direct polarization and cross polarization ¹³C spectra of CF₄Q arrays (Figure S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Understanding the physiological mechanisms of many proteins requires investigating them within the large, multi-protein complexes they form in the cell. Bacterial chemotaxis receptors are one such system that operates in the cell in large arrays (~200 nm diameter, > 100 MDa) of transmembrane receptors complexed with two cytoplasmic proteins, a coupling protein CheW and a histidine kinase CheA (see Figure 1). These arrays enable bacteria to detect attractants and repellents in the environment and adjust their swimming direction accordingly. Models of the arrays have been constructed by docking high-resolution crystallographic or NMR structures of protein fragments and complexes of protein fragments into low-resolution electron density maps from electron cryotomography of receptor arrays in whole cells.^{1–3} The array (Fig 1B right) consists of hexagons formed by trimers of receptor dimers (gray) surrounding rings of alternating CheA (blue) and CheW (cyan). The minimum functional unit (Fig 1A right, which corresponds to the proteins encompassed by the black oval in Fig 1B right) is thought to be a pair of pair of receptor trimer of dimers, bridged by a CheA dimer and two CheW.

Bacterial chemotaxis receptors have been studied extensively⁴ in an effort to understand the molecular details of the mechanism of transmembrane signaling. Receptor arrays (with CheA and CheW) activate the kinase CheA, which leads to cell tumbling. Attractant ligands bind to the periplasmic domain of the chemoreceptor (not shown in Figure 1), which shifts the receptor to a kinase-off state and turns off tumbling. Basal tumbling rates are restored when the receptor adapts to ongoing stimuli, through methylation of several glutamic acid residues in the cytoplasmic domain. This control of tumbling biases the cell to move toward attractants. The chemoreceptor signaling mechanism begins with a ligand-induced ~ 2 Å piston motion of a periplasmic-transmembrane helix towards the cytoplasm.^{5,6} However, it is not clear what changes then propagate an additional ~200 Å through the attached cytoplasmic domain (the part shown in Fig 1) to inhibit the kinase CheA bound at the membrane-distal cytoplasmic tip of the receptor, nor how methylation shifts the ligand-bound receptor back to the kinase-on state. High-resolution measurements of local structure of the cytoplasmic domain in the kinase-on and kinase-off signaling states are needed to determine the structural basis of the signaling mechanism. However even in a simplified system where the intact receptor is reduced to a cytoplasmic fragment as shown in Figure 1, the minimum functional unit is ~575 kDa, and it has not been crystallized with this native architecture and in defined signaling states.

Solid-state NMR enables the investigation of large macromolecular complexes with the necessary specificity and resolution to reveal structural changes between signaling states of this receptor. Solid-state NMR has shown tremendous progress over the last 15 years in applications to biological samples, from resonance assignments⁷ and structure determination of small soluble proteins⁸ to structure determination of moderately sized membrane proteins such as a GPCR in lipid bilayers.⁹ Hybrid approaches combining solid-state NMR with cryoelectron microscopy and Rosetta modeling have yielded atomic models of macromolecular assemblies such as the Type III secretion needle.^{10,11} Technical advances have also facilitated the study of sensitive biological samples, including static and magic angle spinning (MAS) NMR probes with reduced sample heating of high ionic strength

samples,^{12,13} allowing for the investigation of samples under physiologically relevant conditions. Such probes are essential to the study of unfrozen samples of functional chemoreceptor arrays, which require moderate ionic strength (~150 mM) for assembly. NMR studies of such arrays in previous generation NMR probes were conducted with sample freezing¹⁴ to avoid the substantial heating (over 50°C at these conditions)¹² that would have damaged the samples.

To investigate the structural basis of signaling in the cytoplasmic domain of bacterial chemotaxis receptors, we assemble native-like arrays of defined signaling states of a 33 kDa cytoplasmic fragment (CF) of the aspartate receptor with CheA and CheW for solid-state NMR measurements (Figure 1). We previously used molecular crowding agents (PEG 8000) to mediate assembly of highly functional, kinase-activating ternary complexes of CF_{4Q}, CheA, and CheW for site-directed ¹³C{¹⁹F} REDOR distance measurements¹⁵ (CF_{4Q} is a construct that incorporates glutamine at all 4 methylation sites to mimic the fully methylated receptor).¹⁶ Subsequent electron cryotomography has shown that these conditions generate large, well-ordered hexagonal arrays (Figure 1B, left), with 12 nm center-to-center spacing and subunit organization comparable to native arrays observed in whole cells.^{17,18} These PEG-mediated native-like arrays of CF_{4Q}, CheA, and CheW have multiple significant advantages for NMR experiments: (1) CF arrays are more homogeneous than complexes with CheA and CheW assembled from intact receptors in membranes¹⁸ or nanodiscs,¹⁹ (2) CF arrays preserve native architecture without the non-native contacts observed in crystal structures thus far,^{1,20} (3) CF arrays can be prepared in defined kinase-on and kinase-off signaling states, and (4) the NMR experiment is simplified by reducing the subunit size from the 60 kDa intact receptor to its 33 kDa CF. However, proteins of this size are typically studied with specific labeling at a site of interest; the majority of solid-state NMR studies of uniformly or extensively labeled proteins have focused on proteins with individual subunit sizes < 20 kDa, with some notable exceptions such as the 21 kDa DsbA,²¹ 28 kDa sensory rhodopsin,²² 29 kDa aquaporin,²³ 32 kDa VDAC beta barrel,²⁴ a 34 kDa GPCR,⁹ and 144 kDa cytochrome bo3 oxidase.²⁵ A further complication for this study of CF arrays of functional complexes with CheA and CheW, is that CF is at most ~70% of the protein mass (Figure 1A: 396 kDa total CF represents 69% of 574 kDa total complex, but excess CheA and CheW are used to drive array assembly). Thus, PEG-mediated functional arrays of the chemoreceptor CF represent a challenging target for solid-state NMR.

Despite these challenges, the application of solid-state NMR to these native-like, homogeneous arrays in defined signaling states is a promising approach for determining what structural changes in the CF mediate signaling. Here we report initial two-dimensional solid-state NMR spectra of U-¹³C, ¹⁵N-CF_{4Q} incorporated with CheA and CheW into PEG-mediated arrays in the kinase-on signaling state. These spectra provide a global overview of structure and dynamics of the receptor cytoplasmic fragment: (1) the spectra are consistent with the known CF structure (primarily helical with a random coil C-terminal tail) and (2) the spectra demonstrate favorable properties (structural homogeneity and limited dynamics) that set the stage for future NMR studies combining extensive labeling with measurements of local structure to probe the signaling mechanism. Such applications represent an exciting step forward in terms of size and complexity for biological applications of solid state NMR.

Materials and Methods

CF₄Q is a kinase-on construct of the cytoplasmic fragment of the *E. coli* aspartate receptor; constructs with glutamine (Q) at all 4 methylation sites have been shown to mimic the fully methylated receptor.¹⁶ CF₄Q was expressed in BL21 (DE3) co-transformed with pRWCF₄Q (encoding CF₄Q with an N-terminal His tag and ampicillin resistance)²⁶ and pCF430 (encoding lacI^q and tetracycline resistance) in M9 minimal media using U-¹³C-glucose and (¹⁵NH₄)₂SO₄ as the carbon and nitrogen sources, respectively. A small LB starter culture (~3 mL) was started from a single colony on an LB/agar plate containing ampicillin and tetracycline, and grown to OD₆₀₀ ~ 0.6 at 37° C with 200 rpm shaking. This was used to inoculate a 1 L volume of minimal media, which was typically grown overnight at 30° C with 200 rpm shaking, before inducing the following day for 5 hours at 25° C with a final concentration of 1 mM IPTG. The His-tagged CF₄Q was purified by nickel affinity chromatography, according to published procedures.¹⁵ Protein concentrations were measured by BCA assay (Thermo Fisher/Pierce) and purity was determined by SDS-PAGE. Isotope incorporation was measured to be > 95% using ESI-MS of natural abundance and isotopically enriched CF₄Q, and incorporation was calculated using the *E. coli* Asp receptor CF₄Q primary sequence and the Scripps Protein Calculator (<http://protcalc.sourceforge.net/>).

CheW, CheA, and CheY were expressed and purified as previously described.¹⁵

Each NMR sample assembly involved an overnight incubation of 5 ml (3.2 mm rotor experiments) to 10 ml (4 mm rotor experiments) of the protein mixture at 25° C in a circulating water bath. The protein mixture consisted of 12 uM CheA, 20 uM CheW, and 50 uM U-¹³C/¹⁵N-CF₄Q with 7.5% (w/v) PEG 8000 and 4% (w/v) D-(+)-trehalose in kinase buffer (50 mM potassium phosphate, 50 mM KCl, 5 mM MgCl₂, pH 7.5). Both PEG 8000 and D-(+)-trehalose were prepared as 40% (w/v) stock solutions in Milli-Q water and passed through a 0.22 µm sterile filter. An aliquot of the sample was subjected to an enzyme-linked spectrophotometric assay to measure CheA phosphorylation rates and a sedimentation assay to measure array formation.²⁶ Results indicated complete (> 98%) sedimentation of the sample and a specific activity of 6 s⁻¹. The samples were pelleted by ultracentrifugation for 2 hours at 25° C at 25,000 rpm in a Beckman Ti70 rotor, and then packed into a suitable NMR rotor (4 mm Bruker rotor for homonuclear experiments, 3.2 mm Varian rotor for heteronuclear experiments). Approximately 7.5 mg (240 nmol) of isotopically enriched CF₄Q was present in the 3.2 mm rotor (filling entire 22 uL active volume of rotor) for heteronuclear experiments, and 10 mg (300 nmol) in the 4 mm rotor (filling ~ 45 ul out of 50 ul active volume) for the homonuclear experiments. Excess CheA and CheW are used to assemble CF arrays because these conditions have been shown to drive assembly of the CF₄Q into native-like arrays^{17,18} with maximal kinase activity.¹⁵ Although the unlabeled excess CheA and CheW will be invisible in the NMR experiment, their presence reduces the CF to 58% of the protein mass in the sample. The CF-only sample (~ 20 mg) was precipitated using 10% PEG 8000 with 4% (w/v) trehalose in kinase buffer.

Homonuclear correlation experiments were performed on a 16.5 T Bruker Avance III spectrometer (¹H = 700 MHz, ¹³C = 176 MHz) using a 4 mm E-free HCN probe. A 2D sequence using cross polarization and dipolar-assisted rotational resonance (DARR) for

homonuclear mixing was used with 12.5 kHz MAS, mixing times of 25 ms, and swept-frequency TPPM (SWf-TPPM) decoupling at 75 kHz. The $\pi/2$ pulses used were 2.4 μ s and 4 μ s for ^1H and ^{13}C , respectively. ^1H - ^{13}C cross polarization was performed with a 2 ms contact time, ^{13}C field strength of 56.25 kHz, and ^1H ramped from 58.5 to 79.1 kHz (30% ramp centered on +1 condition). Each 2D homonuclear experiment consisted of 600 points in the indirect dimension, for a spectral width of 50 kHz and a total experiment time of approximately 40 hours. Heteronuclear experiments were performed on a 14.1 T Varian InfinityPlus spectrometer (^1H = 600 MHz, ^{13}C = 150 MHz, ^{15}N = 60 MHz) using a 3.2 mm BioMAS HCN probe at 12.5 kHz MAS, and field strengths of 111 kHz (^1H), 21.6 kHz (^{13}C), and 33.5 kHz (^{15}N), with SPINAL-64 decoupling at 75 kHz. These spectra were acquired in ~ 3.5 hour blocks, with each experiment taking ~ 48 hours. The SPECIFIC CP²⁷ sequence was used for selective magnetization transfer from the amide nitrogen to the intra-residue C α for the NCa and NcaCX experiments; DARR mixing (25 ms) was used for the carbon-carbon mixing period. Contact times of 1 ms (^1H to ^{15}N) and 4 ms (^{15}N to ^{13}C) were used for the heteronuclear experiments. For the 1D CP/MAS and DP/MAS experiments, a 16.5 T Bruker Avance III spectrometer (^1H = 700 MHz, ^{13}C = 176 MHz) with 4 mm E-free HCN probe was used, with ^1H and ^{13}C field strengths and decoupling identical to the 2D experiments. A contact time of 1 ms and recycle delay of 2 seconds was used for CP/MAS, while a 15 second recycle delay was used for DP/MAS. Reported temperatures are sample temperatures that were estimated using either the spin-lattice relaxation time of an external KBr sample (under the same MAS and gas flow conditions) or the proton chemical shift of TmDOTP in an aqueous sacrificial sample¹⁴ with 150 mM KCl, 7.5% (w/v) PEG 8000 and 4% (w/v) D-(+)-trehalose in D₂O.

^{13}C chemical shifts were referenced to DSS at 0 ppm, using external referencing through adamantane (adamantane at 40.5 ppm relative to DSS at 0 ppm).²⁸ NMRPipe (NIH)²⁹ and Sparky (UCSF) were used for NMR data processing and analysis, respectively. For the 2D homonuclear & heteronuclear spectra, sinebell apodization and zero-filling were applied in each dimension prior to Fourier transformation. For the 1D CP and DP spectra, 10 Hz of exponential linebroadening were used prior to Fourier transformation. Peak linewidths were calculated with the full-width half maximum after apodization. For all of the figures, the lowest contour threshold was set to 10 times the estimated noise (calculated in Sparky with 2000 points). However because the CF only sample (Figure S1 yellow) contained about twice the amount of protein, its lowest contour level was set 2-fold higher than this. The reported peak volumes (Table 1) were estimated using the Sparky integration method, fitting each peak to a Gaussian, starting from a measured value for the linewidth.

Structure-based chemical shift predictions for homonuclear and heteronuclear correlation experiments were generated using SHIFTX2³⁰ (one-bond correlations only) and Peakr (<http://www.peakr.org/>) with an aspartate receptor cytoplasmic fragment (CF) homology model. The homology model (see Figure 4D) was constructed using the aspartate receptor CF_{4Q} primary sequence and the crystallographic structure of the serine receptor cytoplasmic domain (PDB ID 1QU7). The appended N terminal residues were modeled as an alpha helix. The appended C-terminal residues are known to be unstructured,³¹ forming a flexible tail that binds the CheR methyltransferase for methylation of sites in the same and nearby CF

subunits.³² Database values for random coil chemical shifts³³ were used for these C terminal residues in the final predicted spectra.

Results & Discussion

Homo and Heteronuclear Correlation Spectra Are Consistent with Predicted Structure

Homonuclear (DARR) ^{13}C - ^{13}C correlation spectra were obtained for native-like arrays of U- ^{13}C , ^{15}N -CF_{4Q} in kinase-on ternary complexes with CheA and CheW (Figure 2). These experimental spectra are compared to structure-based chemical shift predictions that were generated with SHIFTX2 using an Asp receptor CF homology model, with predicted resonances shown as black dots in Figure 2. The predicted chemical shifts are in very good agreement with the spectra, and a number of distinctive chemical shifts permit the identification of resonances for 13 of the 19 residue types in CF. These residue-type assigned resonances (Table 1) generally correspond to the most numerous amino acids, ranging from 52 Ala to 8 Lys, which together correspond to 279 (90%) of the 310 residues in the CF_{4Q} sequence. The dashed and dotted lines in Figure 2A show the full side chain spin systems for isoleucine and valine in the ^{13}C - ^{13}C spectra. Additional predicted Ca-Cb correlations (not labeled in Figure 2A) are ~56,38 ppm (for 12 total Asn), ~58,40 ppm (strong for 33 total Asp, Leu), ~59,33 ppm (for 15 total Met, Lys), and ~59,30 ppm (strong for 74 total Gln, Glu, Arg, His). Weak resonances are also observed at predicted aromatic positions for the 9 His (Cb-Cg, Cg-Cd, and Ca-Cg; data not shown), but these are less interesting as they are likely dominated by the 6 non-native His of the N-terminal His tag. Not yet identified, due to a combination of lower signal intensity and less distinct chemical shifts, are the resonances of the 9 Pro, 7 Met, 4 Phe, 1 Tyr, and 1 Trp.

The predicted Ca chemical shifts that do not overlies experimental chemical shifts are upfield of the measured chemical shifts (smaller chemical shift value) and are consistent with beta-sheet or coil dihedral geometries. These predicted shifts arise from the c-terminus. The resonances corresponding to this region are likely not detected because of dynamics reducing the efficiency of cross polarization. Note also that the agreement between the predicted and observed spectra in Fig 2A and 2C is best for strongly overlapped peaks, perhaps because the outliers are too weak for our current detection. For Figure 2B, the correlations that are observed at positions not predicted by SHIFTX2 can all be accounted for by two bond correlations between the carbonyl and the strongest Cb resonances in Fig 2A for Ala ~18 ppm, Thr ~21 ppm, Val plus others at ~30 ppm, and Ile + others at ~40 ppm. The similarity between the experimental and predicted spectra suggests that the majority of the protein is observed (see discussion of mobility below), and that these spectra can provide an overview of the structure and dynamics of the CF in native-like arrays.

A heteronuclear correlation spectrum of the frozen array also exhibits qualitative agreement with the structure-based chemical shift predictions (Figure 3). A limited number of spin system assignments are possible, due to the strongly overlapping resonances expected for an alpha-helical protein, especially in the ^{15}N dimension. Although linewidths would likely be reduced in unfrozen spectra (see below), further deconvolution of these congested spectra would likely require higher-dimensional experiments for sequential assignments, as seen

recently with the helical membrane protein KcsA,³⁴ which is approximately half the size of the Asp receptor CF.

These spectra of the uniformly labeled CF_{4Q} are clearly consistent with its predominantly alpha helical structure, as shown in Figure 4, which compares the experimental spectra (black) with predicted resonances for residues with distinctive chemical shifts in alpha helical (green), beta strand (blue) and random coil (red) conformations.³³ Crystal structures of CF proteins alone^{35,36} or in complexes with CheW and CheA fragments^{1,20} indicate that its structure (see Figure 4D) is predominantly alpha helical, other than its membrane-distal hairpin turn and its C-terminal tail. Correlations observed in the DARR spectra for Ala, Val, and Ile Cb-Ca (Figure 4A), for Ala, Ser, and Gly Ca-CO (Figure 4B), and for Gly N-Ca and Ala N-Cb (Figure 4C) are all clearly most consistent with an alpha helical conformation. Chemical shifts of Leu Cb and Thr Cb (see Table 1) are also consistent with an alpha helical structure.

The 38-residue C-terminal tail, which is missing in the crystal structures and is highly mobile based on EPR evidence,³¹ is thought to be a flexible arm for binding the methyltransferase CheR (at the 5 terminal residues) that methylates specific Glu residues on the same and nearby CF dimers.³² The most common residue in the flexible tail (other than Pro which we did not identify in the ¹³C-¹³C spectra and would not be detected due to poor cross polarization of the unprotonated N for the ¹⁵N-¹³C spectra) is Ala, so we looked for possible resonances of the 5 tail Ala residues at non-helical chemical shifts. There are minor Cb-Ca and Ca-CO crosspeaks with shifts characteristic of Ala in a random coil conformation (arrows in Figure 4A and B). The intensity of the minor Cb-Ca peak (about 6% of the bulk Ala resonance), would correspond to 3 Ala residues, but these intensities cannot be compared quantitatively as the tail residues may be significantly more mobile which would reduce the efficiency of cross polarization. These minor Ala correlations suggest that the disordered C-terminal tail of the CF is observable in these spectra and is in a random coil conformation.

Comparison of ¹³C-¹³C DARR spectra of the U-¹³C,¹⁵N-CF_{4Q} in native-like arrays with CheA and CheW with spectra of a PEG precipitate of CF_{4Q} alone does not reveal any significant chemical shift perturbations (Figure S1), consistent with current views that the receptor fragment does not exhibit any gross structural changes when assembled into complexes with CheA and CheW. Solution NMR experiments on a related receptor fragment have shown small (< 1 ppm) shifts in ¹³C methyl peaks at its binding interface with CheA³⁷ and in ¹⁵N backbone shifts at its binding interface with CheW.³⁸ Such subtle changes in single carbon and nitrogen resonances would likely not be detectable with the resolution and sensitivity of these initial spectra, but may be seen after further optimization.

Interestingly, the Ala Ca-Cb correlations show a similar pattern in both CF arrays and CF alone, with a major 55 ppm/18 ppm correlation corresponding to an alpha helical conformation and a minor 52 ppm/19 ppm correlation (with comparable relative intensity) corresponding to a random coil conformation (Figure S1). The similar appearance in both spectra suggests that in both precipitated CF and PEG-mediated CF arrays the C terminal

tail has a similar structure, with limited dynamics (making it observable in these spectra – see below) and a random coil conformation.

Dynamics of the Receptor in Native-like Arrays Appear Favorable for NMR Studies

Comparison of one-dimensional ^{13}C spectra obtained with direct polarization (DP, which excites all carbons equally) and cross polarization (CP, which excites immobile carbons with strong dipolar couplings to protons) can be used to provide insight into mobility within a protein. $\text{CF}_{4\text{Q}}$ in unfrozen functional arrays exhibits similar CP and DP spectra (Figure S2), but with ~2-fold greater intensity per scan for protonated carbons in the CP spectrum. For an immobile system, CP spectra can theoretically provide a maximum of 4-fold signal enhancement. Observation of similar spectra with only 2-fold CP enhancement of the $\text{CF}_{4\text{Q}}$ signal suggests that there is limited mobility across the majority of the $\text{CF}_{4\text{Q}}$, which averages dipolar couplings to somewhat reduce CP efficiency. The further reduced CP intensity of the carbonyl resonances is expected because of the weaker CH dipolar couplings of carbons with no directly bonded protons. The resolved Glu side chain carbonyl carbon at 183.5 ppm has comparable relative intensity in CP and DP spectra, suggesting side chain and backbone have comparable dipolar couplings and therefore similar mobility. Thus the $\text{CF}_{4\text{Q}}$ is largely but not completely immobilized in the native-like arrays.

Differences between spectra of frozen and unfrozen arrays provide further insight into the mobility of the $\text{CF}_{4\text{Q}}$ in these samples and the best conditions for NMR studies. All peaks observed in the two-dimensional ^{13}C - ^{13}C DARR spectra of frozen $\text{CF}_{4\text{Q}}$ arrays are preserved in spectra of unfrozen arrays, but with unfrozen peak volumes reduced to about 20–30% of the frozen peak volumes (tabulated for the assigned spin systems in Table 1). A 50% reduction is expected due to reduced CP efficiency, based on the above comparisons of one-dimensional spectra. The additional 2-fold reduction in peak volumes to ~25% is likely due to reduced magnetization transfer efficiency during the DARR mixing period, again due to motional averaging of the dipolar couplings. The similarity of the 1D CP vs DP spectra (Figure S2), of the 2D DARR frozen vs unfrozen spectra (Figure 5), and of the 2D DARR vs predicted spectra (Figure 1) all suggest that the majority of the $\text{CF}_{4\text{Q}}$ has limited mobility, which makes it observable in spectra of unfrozen $\text{CF}_{4\text{Q}}$ arrays.

These first spectra of unfrozen functional arrays, which were previously not possible due to substantial predicted heating of these high ionic strength samples in the solenoid coil of our MAS NMR probe,¹⁵ are also promising indicators of the feasibility of solid-state NMR studies to compare CF in functional signaling states. As shown in Figure 5, in spectra of unfrozen arrays there is significant narrowing of a number of peaks and the appearance of additional resonances, especially in the more congested regions of the spectrum. This narrowing is large enough to result in a net increase in the signal to noise for most of the resonances in the 2D DARR spectra (see Table 1), in spite of the reduced CP efficiency and magnetization transfer. The observed spectral changes upon sample freezing are consistent with line broadening due to static disorder in the frozen state, with enhanced polarization and magnetization transfer due to the additional rigidity of the sample. Interestingly, the minor Ala resonance (asterisk in Table 1) with random coil chemical shift values shows both a greater narrowing and greater signal to noise increase than any other backbone or nonpolar

side chain resonance. This is consistent with an unstructured C terminal tail that likely freezes in multiple conformations, increasing the chemical shift dispersion more than observed for other backbone and nonpolar carbon resonances. Overall, these spectra demonstrate favorable NMR properties of the unfrozen PEG-mediated CF₄Q arrays with NMR probes that minimize sample heating.

Polar residues exhibit the most dramatic narrowing and increase in signal-to-noise in the unfrozen spectra, in some cases emerging as a resolvable peak. The side chain carbonyl-aliphatic correlations of glutamine, glutamate, asparagine, and aspartate (Figure 5A), and the correlations of Lys C β -C γ and Arg C γ -C δ (Figure 5C) are all too broad to be observed in the spectrum of the frozen sample (blue) but are clearly visible in the spectrum of the unfrozen sample (red). Furthermore, for peaks observed in both frozen and unfrozen spectra, Glu C β -C γ , Gln C β -C γ , and Lys C δ -C ϵ showed the greatest narrowing, to 10–20% of their frozen linewidths (Table 1). In contrast, the backbone and nonpolar side chain resonances narrow much less, to ~ 50 to 90% of the frozen linewidths. The fact that significant linewidth increases upon freezing are observed primarily for the most polar side chains that have the greatest surface exposure in proteins (Arg, Asn, Asp, Gln, Glu, and Lys, but not Thr) suggests that freezing traps polar side chains with a range of solvent interactions that cause a range of chemical shifts and broaden the resonance. The absence of this large change for the backbone and nonpolar side chains suggests that freezing does not perturb the core protein structure: significant static disorder in frozen CF₄Q arrays is likely limited to surface residues.

Finally, linewidths ~ 1 ppm are observed for some resonances corresponding to multiple residues (0.9–1.2 ppm for 16 Ile, 24 Val, 52 Ala) in the unfrozen arrays, which indicates very limited chemical shift dispersion among the multiple residues. This also suggests that single residues may have sub-ppm linewidths and that CF₄Q is structurally homogeneous in these native-like arrays. The limited chemical shift dispersion would be a challenge to studies requiring complete or extensive assignments, and suggests that the most promising NMR approaches will likely employ selective isotopic labeling and/or NMR detection schemes to probe structure in a region of interest. This should provide a powerful complement to other methods for testing existing models for the structure and mechanism.

Summary

We have applied two-dimensional solid-state NMR spectroscopic methods in combination with uniform isotopic labeling for an initial global look at the structure and dynamics of the aspartate receptor CF in PEG-mediated kinase-on native-like arrays with CheA and CheW. Experimental homonuclear and heteronuclear correlation spectra indicate good agreement with structure-based chemical shift predictions, and have permitted the spin system assignment of resonances from 13 of 19 residue types in the receptor fragment, which correspond to 90% of the primary sequence. This agreement, combined with the similarity of frozen vs unfrozen and CP vs DP spectra, indicates that the majority of the CF₄Q is observed in NMR spectra of unfrozen samples. Furthermore, NMR results demonstrate the feasibility and promise of future studies of unfrozen arrays, which are characterized by increased signal to noise and decreased ¹³C linewidths, with some in the range of ~ 1 ppm.

The favorable NMR properties of these homogeneous, functional, native-like arrays that can be prepared in defined signaling states indicate that solid-state NMR is a promising means to reveal differences in local structure and dynamics and provide insight into the signaling mechanism. Such an approach will be applicable to the study of other systems to understand the mechanisms of the many multi-protein complexes critical to fundamental cellular processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCA	bicinchonic acid
CF	cytoplasmic fragment of <i>Escherichia coli</i> aspartate chemotaxis receptor
CP	cross polarization
DP	direct polarization
DARR	dipolar-assisted rotational resonance
ESI-MS	electrospray ionization mass spectrometry
GPCR	G protein coupled receptor
IPTG	isopropyl β -D-1-thiogalactopyranoside
LB	Luria-Bertani broth
MAS	magic angle spinning
PEG	polyethylene glycol 8000
REDOR	rotational echo double resonance
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Figure 1.

Electron cryotomograms (left) and corresponding models (right) of PEG-mediated native-like functional arrays¹⁷ of aspartate receptor cytoplasmic fragment (CF_{4Q}, gray), CheW (cyan) and the histidine kinase CheA (blue). (A) Sideview of sandwich arrays (left, scale bar 50 nm) and cartoon/model showing architecture of one half of sandwich (right). The arrows at the top indicate where the intact receptor continues to the transmembrane and periplasmic domains, and where the PEG-mediated arrays have an overlap with CF termini of another array to form a sandwich between planes of the CheA/CheW rings. The cartoon and model (right) depict the minimum functional unit for kinase activity, a pair of receptor trimer of dimers (CF₂)₃ that are bridged by a CheA dimer (CheA₂) and also bind two CheW. (B) Top view of array (left, scale bar 50 nm); red boxes show subvolume average (scale bar 5 nm) for a single hexagon and corresponding portion of array model. Receptor trimers of dimers are organized at the vertices of hexagons surrounding a ring of alternating CheA and CheW. This array model has a stoichiometry of 6 receptor: 1 CheA: 1 CheW.¹ A hexagon of hexagons (right) is about 20 nm across and 6.9 MDa, which becomes 13.8 MDa for the double layer sandwich formed in PEG-mediated CF arrays. The cryotomograms (left) show the CF arrays to be larger than this, with dimensions ~50 nm as compared to the ~200 nm arrays observed in bacteria.

Figure 2.

^{13}C - ^{13}C DARR spectra of the unfrozen U- ^{13}C , ^{15}N -CF $_4$ Q in native-like arrays with CheA and CheW, with spin system assignments (labels) and SHIFTX2-predicted resonances (black and colored dots). (A) Intra-residue aliphatic correlations of receptor side chains, with complete side chain spin systems indicated for isoleucine (dashed lines) and valine (dotted lines). (B) Aliphatic-CO correlations of polar side chains and glycine backbone and their predicted resonances. (C) Backbone correlations, with assignments for distinctive Gly, Ala, and Ser resonances. For clarity, some labels and dots are shown in corresponding colors, and only the strongest predicted correlations are shown (for one-bond distances). Additional carbonyl correlations observed in (B) are consistent with two-bond correlations that are not included in the predicted spectrum, primarily for the most numerous beta carbons (those with the strongest signals in (A) ~18 ppm (Ala), ~21 ppm (Thr), ~30 ppm (Val + others) and ~40 ppm (Ile + others). Sample temperature ~275 K.

Figure 3. ^{15}N - ^{13}C SPECIFIC CP/DARR NcaCX spectra of the frozen $\text{U-}^{13}\text{C}$, ^{15}N -CF₄Q in native-like arrays with CheA and CheW, with spin system assignments (labels) and SHIFTX2-predicted resonances (dots). Top: Side chain correlations. Bottom: N-Cα correlations. For clarity, some labels and dots are shown in corresponding colors and only predicted one-bond correlations are shown. Sample temperature ~265 K.

Figure 4.

Backbone chemical shifts are consistent with predominantly alpha helical structure. Experimental spectra are shown in black for Ca-Cb (A) and Ca-CO (B) regions of DARR spectrum (unfrozen sample spectrum shown in figure 2), and for the N-Ca region of SPECIFIC CP/DARR NcaCX spectrum (C, frozen sample spectrum shown in figure 3). Colored ellipse outlines indicate predicted chemical shifts for alpha helical (green), beta strand (blue), and random coil (RC, red) backbone conformations; ellipses are shown at the average shift $\pm$ SD for these secondary structures.³³ Arrows identify Ala resonances with nonhelical chemical shifts. (D) Asp receptor CF homology model shows the largely helical structure, based on the homologous Ser receptor CF crystal structure (1qu7). The added N terminal residues (light green) are modeled as an alpha helix; the added C terminal residues shown in an extended conformation (red) form a flexible tail that allows the CheR methyltransferase to access methylation sites (magenta) on this and nearby CF subunits in the array.

Figure 5.

Temperature dependence of the ^{13}C - ^{13}C DARR spectrum of the U- ^{13}C , ^{15}N -CF₄Q in native-like arrays with CheA and CheW. Overlaid spectra for frozen arrays (blue, sample temperature ~265 K) and unfrozen arrays (red, sample temperature ~275 K). (A) Aliphatic-CO correlations of polar side chains and glycine backbone. (B) Ca-Cb correlations, (C) Side chain correlations.

Table 1

Spectral properties of assigned residue-type resonances.

Peak # in CF ₄ Q1	Peak volume decrease (U/F) ²	Signal to noise increase (U/F) ²	Linewidth decrease (U/F) ²	$\omega 1$ (ppm)	$\omega 2$ (ppm) α , β , RC ³
Backbone					
Ala CO-Ca (52)	0.23	1.25	0.74	55.1	180.3
Ala Ca-Cb (47a)	0.31	1.72	0.92	17.6	54.9
Ala Ca-Cb minor* (5 RC)		2.4*	0.37*	19.2	52.3
Gly CO-Ca (17)	0.31	0.85	0.53	47	176.2
Ile Ca-Cb (16)	0.19	0.90	0.71	37.8	65.6
Ser CO-Ca (31)	0.31	1.59	0.53	62.3	177.2
Val Ca-Cb (24)	0.28	1.47	0.62	31.4	66.2
Least polar side chains					
Ile Cb-Cg1 (16)	0.17	1.16	0.49	29.7	37.6
Ile Cb-Cg2 (16)	0.29	1.92	0.59	17.3	37.6
Ile Cg1-Cd1 (16)	0.27	1.61	0.62	13.8	29.7
Leu Cg-Cb (17)	0.22	1.96	0.72	26.7	41.7 41, 44, 42
Thr Cb-Cg (21)	0.32	1.67	0.72	21.9	68.2 69, 71, 70
Val Cb-Cg (24)	0.29	1.72	0.77	22.1	31.3
Most polar side chains					
Arg Cg-Cd (19)	N/A	N/A	N/A	27.6	42.9
Asn Cb-Cg (12)	N/A	N/A	N/A	37.7	175.3
Asp Cb-Cg (16)	N/A	N/A	N/A	40.1	178.6
Gln Cg-Cd (25)	N/A	N/A	N/A	33.7	179.8
Gln Cb-Cg (25)	N/A	4	0.09	28.1	33.5
Glu Cg-Cd (21)	N/A	N/A	N/A	35.9	183.2
Glu Cg-Cb (21)	N/A	4.55	0.16	29	35.7
Lys Cd-Ce (8)	N/A	6.25	0.19	29.3	41.7
Lys Cb-Cg (8)	N/A	N/A	N/A	25	32.2

* Number of residues of this type in CF₄Q.

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² Change in the unfrozen spectrum: U/F = Unfrozen/Frozen.

³ Predicted chemical shift for each secondary structure given in corresponding colors.

* Minor Ala resonance has 6% of the intensity of the major Ala resonance.