Solid-State REDOR NMR Distance Measurements at the Ligand Site of a Bacterial Chemotaxis Membrane Receptor†

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ABSTRACT: The *Escherichia coli* serine receptor senses serine levels in the environment and transmits this information across the bacterial inner membrane to modulate a protein phosphorylation cascade which controls swimming behavior. Solid-state nuclear magnetic resonance (NMR) has been used to characterize specific structural features of the ligand binding site interactions in the *intact, membrane-bound* Ser receptor. Rotational-echo double-resonance (REDOR) experiments on [^{15}N]Ser bound to a [1-^{13}C]Phe-receptor preparation are used to measure distances between the ligand amino group and the carbonyls of two phenylalanine residues in the ligand binding pocket. The results indicate two 4.0 ± 0.2 Å distances, in excellent agreement with the X-ray crystal structure of a soluble fragment of the homologous aspartate receptor [Milburn et al. (1991) *Science 254*, 1342−1347]. These results confirm the similarity of the binding sites of the Asp and Ser receptors, and demonstrate the feasibility of using solid-state NMR measurements to obtain specific structural information on the 120 kDa *intact* receptor for probing transmembrane signaling mechanisms.

Membrane receptors are thought to transfer information across the membrane via the broadly defined mechanisms of ligand-induced clustering or conformational changes. Unfortunately, the detailed mechanisms of this fundamental process are poorly understood, in part because of the challenging nature of structural studies of membrane proteins. The difficulty of obtaining high-quality crystals of membrane proteins has limited X-ray diffraction studies, and the slow tumbling of these large receptor proteins (particularly when bound to vesicles to mimic the native state) prevents complete structure determination by solution nuclear magnetic resonance (NMR). Although the structures of a number of soluble membrane receptor fragments have provided important insights (Milburn et al., 1991; de Vos et al., 1992; Hubbard et al., 1994; Livnah et al., 1996), the missing portions of the receptors are critical to understanding how ligand binding information is transferred between the external and internal domains. Solid-state NMR can provide complementary information through measurements of *local* structure in *intact*, membrane-bound receptors, to gain insight into the signaling mechanism.

The membrane receptors of bacterial chemotaxis are an excellent system for probing the mechanism of information transfer by transmembrane proteins, since they are relatively simple and well-studied. The four homologous receptors are 120 kDa homodimers, with each subunit consisting of a periplasmic ligand binding domain, two membrane-spanning α-helices, and a cytoplasmic domain. These receptors bind ligands which diffuse into the periplasmic space, and relay this information across the inner membrane to modulate interactions between cytoplasmic proteins which ultimately control the swimming behavior of the cell. Thus, the chemotaxis system enables bacteria to swim toward attractants such as amino acids and away from repellents.

Current understanding of the ligand binding site of these receptors is based principally on the crystal structure of a soluble, cross-linked, periplasmic domain fragment of the aspartate receptor (Figure 1), which was solved both with and without ligand bound (Milburn et al., 1991; Scott et al., 1993; Yeh et al., 1993, 1996). Since the fragment protein binds Asp with similar affinity, the ligand binding site in the crystal structure is thought to be similar to that of the intact receptor. In addition, the 39% sequence identity between the serine and the aspartate receptor ligand binding domains and the preservation of most of the amino acids of the ligand binding site (see Figure 1) predict a similar ligand binding site structure in the Ser receptor (Jeffery & Koshland, 1993). In this paper, we report the use of solid-state NMR to directly probe ligand to receptor interactions in the intact, membrane-bound *Escherichia coli* serine receptor.

NMR experiments on isotropic samples achieve narrow, resolved resonances either via rapid tumbling of molecules in solution or via the magic angle spinning (MAS) technique used in many solid-state NMR experiments. Although this narrowing enhances resolution and sensitivity, it also sacrifices information by averaging various orientation-dependent spin interactions such as the dipolar coupling, which contains distance information. A number of techniques have emerged in recent years for the measurement of homonuclear and heteronuclear dipolar couplings in MAS NMR experiments [reviewed in Garbow and Gullion (1995), Bennett et al. (1994), and Griffiths and Griffin (1993)]. The REDOR method developed by Schaefer and co-workers (Gullion & Schaefer, 1989b) employs a train of π pulses, synchronized

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[1] Abbreviations: CPMAS, cross-polarization magic angle spinning; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl thio-β-D-galactoside; NMR, nuclear magnetic resonance; REDOR, rotational-echo double-resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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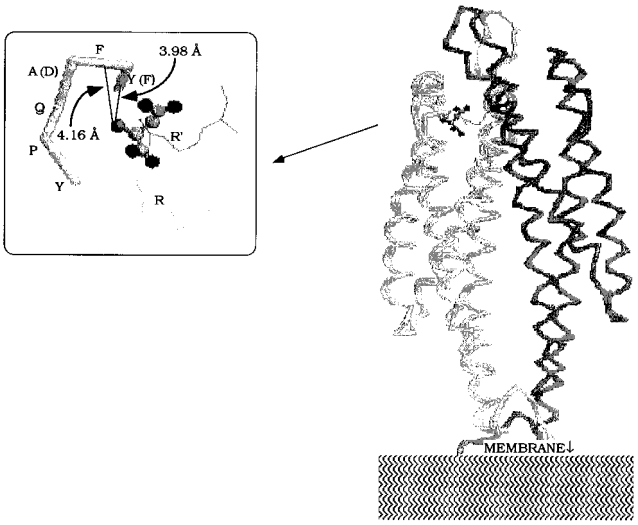

FIGURE 1: Structure of the Asp receptor periplasmic domain fragment and ligand binding site. The backbone of the complex (coordinates obtained from Brookhaven protein data base, entry 2lig) is shown with each monomer in a different shade, and the Asp ligand bound at the dimer interface. The expanded view of the ligand site identifies the amino acids which form the binding pocket in the Asp receptor; only two of these residues differ in the Ser receptor (Ser receptor residues in parentheses). The two receptor ^{13}CO-Phe to ligand [^{15}N]Ser distances in the intact Ser receptor measured with REDOR in this study correspond to the two crystal structure distances indicated in the expanded view.

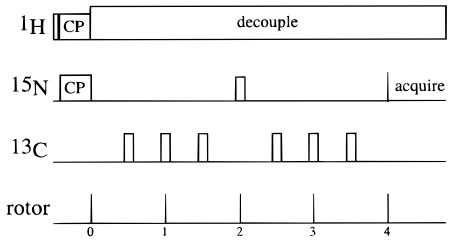

FIGURE 2: REDOR pulse sequence. ^{1}H nuclei are polarized by a 90° pulse, and polarization is transferred to the ^{15}N nuclei by the matched cross-polarization pulses. A four-rotor cycle dephasing period is shown for simplicity, during which ^{13}C 180° pulses invert the dipolar evolution of coupled ^{15}N nuclei, which interferes with the MAS averaging of the dipolar coupling. The ^{15}N 180° pulse produces an echo at the start of the acquisition period.

with the MAS, to interfere with the averaging of the heteronuclear dipolar coupling. For instance, in an ^{15}N-observed, ^{13}C-dephased REDOR experiment (Figure 2), the train of π pulses applied to the ^{13}C nucleus causes a dephasing of any ^{15}N nuclei which are dipolar-coupled to ^{13}C; this dephasing is observed as an intensity decrease in these ^{15}N resonances. Thus, in an appropriately labeled sample, specific ^{15}N to ^{13}C distances can be measured on the basis of the r^{-3} distance dependence of dipolar coupling; ^{15}N to ^{13}C distances of up to 6.3 Å have been measured in Gln binding protein (Hing et al., 1994).

We have applied ^{15}N-observed, ^{13}C-dephased REDOR experiments to samples of [^{15}N]Ser bound to the intact, membrane-bound L-[1-^{13}C]phenylalanine Ser receptor to measure the distance between the ligand and two Phe residues in the ligand binding pocket.

MATERIALS AND METHODS

Chemicals, Bacterial Strains, and Plasmids. IPTG was obtained from BaChem (Torrance, CA); bicinchoninic acid was obtained from Pierce Chemical Co. (Rockford, IL). L-[^{15}N]Serine (^{15}N, 95−99%), L-[1-^{13}C]phenylalanine (^{13}C, 99%), and [^{15}N]indole-L-Trp (≥98%) were purchased from Cambridge Isotope Laboratories (Andover, MA). All other chemicals were reagent grade. Efficient overexpression of the serine receptor was achieved with the plasmid pHSe5.tsr (Muchmore et al., 1989; Gegner et al., 1992), a gift of F. W. Dahlquist, University of Oregon. The plasmid was expressed in *E. coli* strain DL39 (*aspC13, fnr-25, ilvE12, tyrB507*), a multiauxotrophic strain (Phe$^-$, Tyr$^-$, Asp$^-$, Leu$^-$, Ile$^-$) obtained from the *E. coli* Genetic Stock Center (Yale University).

Bacterial Growth and Purification of Intact Serine Receptor. Preparation of the intact serine receptor followed the method of Gegner et al. (1992). Bacterial cells (DL39/pHSe5.tsr) were grown at 30 °C in defined media (Muchmore et al., 1989) containing L-[1-^{13}C]phenylalanine as the sole source of phenylalanine, and 100 μg/mL ampicillin, until the OD_{600} reached 0.2−0.4. Expression of the serine receptor was induced by addition of 0.5 mM IPTG; after 3 h, the cells were harvested by centrifugation for 15 min at 4000 rpm (Sorvall HG-4L rotor) and 4 °C. The cell pellet was resuspended to $^1/_{20}$th the original volume in 30% (w/w) sucrose and 10 mM Tris, pH 8. Within 10 min, hen egg white lysozyme was added to a final concentration of 100 μg/mL and EDTA to a final concentration of 5 mM. After incubation on ice for 40 min, the cells were slowly (10 min using either a separatory funnel or a small pump) diluted with 2 volumes of water containing 1 mM 1,10-phenanthroline, 5 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. Osmotic lysis was induced by rapid dilution into 4 volumes of ddH_2O. Lysed cells were stirred for 10 min, and membrane vesicles were pelleted by centrifugation at 10 000 rpm (Beckman JA 20 rotor) for 3 h. The pellet was homogenized with 30% sucrose, 10 mM Tris, pH 8, and 5 mM EDTA. Outer and inner membrane vesicles were separated on a sucrose gradient of 55%, 50%, 40% (w/w) sucrose, all with 5 mM EDTA, spun overnight at 28 000 rpm (Beckman SW28 rotor). The middle band was collected and dialyzed overnight against 20 mM sodium phosphate buffer, pH 7.4, to remove residual sucrose. The protein concentration was determined by the bicinchoninic acid assay (Smith et al., 1985). Yields were ∼30 mg of Ser receptor per liter of cells grown.

A detergent solubilization assay was used to screen for abnormal protein aggregation in these highly overexpressed receptor preparations; 1% β-octyl glucoside was added to 2−5 mg/mL protein; after 30 min incubation on ice, the sample was pelleted 1 h in an airfuge at 24 psi (∼90000*g*), and SDS−PAGE was used to compare unspun, supernatant, and pellet fractions. Receptor solubility was typically around 85%. The small fraction that was not solubilized was considered aggregated and inaccessible to ligand, and thus unlikely to interfere with the ligand-observed experiments of this study.

Ligand Binding Assay. A radioactive displacement ligand binding assay (Biemann & Koshland, 1994; Clarke & Koshland, 1979) using [^{3}H]Ser was used to assess the ligand binding activity and accessibility of the receptor vesicle preparations. The receptor preparations have native ligand binding properties: the measured K_d range of 5−20 μM is in agreement with previous measurements (Lin et al., 1994). Comparable values of K_d are obtained after lyophilization

and resuspension, indicating that lyophilization does not irreversibly alter the ligand binding site interactions. The fraction of solvent-accessible ligand binding sites in the vesicle preparations was determined by measuring the increase in binding upon β-octyl glucoside solubilization of the receptor vesicles.

NMR Sample Preparation. Studies of membrane proteins in their native membrane environments present additional challenges. The presence of the membrane (and other protein impurities) reduces the maximum amount of receptor which can be packed into the NMR rotor, relative to a purified, lyophilized protein for instance. An additional complication in this study of the interactions of a bound ligand is the accessibility of the binding site in vesicle preparations.

The ligand−receptor sample used for the REDOR experiments contained a mixture of bound and free ligand and thus required correction of the REDOR signal intensities in the analysis. The complex was prepared by incubating 90 mg of [1-^{13}C]Phe Ser receptor (3.15 mg/mL) with 750 μM [^{15}N]-Ser; thus, after centrifugation the ≈1 mL pellet contained 750 μM receptor dimer. The sample was pelleted at 35 000 rpm (Beckman 70 Ti rotor) for 1 h, and the pellet was lyophilized overnight. After grinding to a fine powder, about 41 mg was packed into the rotor. Based on the purity of the sample preparation, the fraction of the sample which was soluble in 1% β-octyl glucoside, and the receptor accessibility to ligand based on the ligand binding assay, we estimate that 56% of the serine ligand added is bound in the final sample.

Nuclear Magnetic Resonance Spectroscopy. Cross-polarization, magic-angle spinning ^{15}N solid-state NMR spectra were obtained on a Bruker ASX300 spectrometer. The samples were packed in 7 mm zirconia Bruker rotors fitted with Kel-F end caps for magic angle spinning at 4000 Hz. The magic angle spinning speed controller keeps spinning rate fluctuations to less than ±0.1%. REDOR experiments were performed on a Bruker triple resonance probe with a ^{13}C^{15}N insert. The tuning frequencies were 300.13 MHz for ^{1}H, 75.47 MHz for ^{13}C, and 30.41 MHz for ^{15}N. Cross-polarization (2 ms contact times) and proton decoupling were performed with 50 kHz B_1 fields; the power was increased to provide 80 kHz decoupling fields during the REDOR dephasing periods. ^{15}N chemical shifts were referenced to 5.6 M NH_4Cl (using an external Gly sample at 8.1 ppm). Extended periods of signal averaging with sample cooling were achieved by using dry air (dried with a Balston Instrument air dryer) for the spinning and cooling gases, cooled in a bath containing a CC-100II immersion cooler (NESLAB Instruments). Spectra were collected at 4 °C with a 1 s recycle delay.

The REDOR pulse sequence, depicted in Figure 2, employs a train of dephasing ^{13}C π pulses at half and completion of each rotor period, and a single refocusing ^{15}N π pulse. XY alternation of the dephasing pulses was used to suppress resonance offset effects (Gullion & Schaefer, 1991); the refocusing pulse was phase-cycled according to the scheme of Rance and Byrd (1983). Small blocks of data (32 scans) were collected alternately with and without dephasing pulses to correct for any long-term instrumental drift errors.

Because measurements of long distances with REDOR require long dephasing times, pulse imperfections can lead to cumulative errors which distort the distance measurement [typically longer distances are observed for increasing numbers of rotor cycles (McDowell et al., 1993)]. Such problems cannot be diagnosed on small molecules with short r_{CN} (1−2 bond separations) because complete dephasing occurs within 8−32 rotor cycles. We chose indole-[^{15}N]-Trp as a long-distance standard: the crystal structure indicates a 3.71 Å distance to the 1.1% natural-abundance $^{13}C_{\beta}$, which is conformationally independent. REDOR experiments with 32, 48, 64, and 96 rotor cycles yielded r_{CN} of 3.82, 3.88, 3.84, and 3.73 Å, respectively, indicating the REDOR experiment is accurate for long distances in this range.

REDOR dephasing data were analyzed on an Apple Power Macintosh 7600. REDOR dephasing of an isolated 2-spin system was analyzed using proFit software (Cherwell Scientific) by fitting the data to the equation:

$$\frac{\Delta S}{S} = 1 - \frac{1}{2\pi}\int_{\alpha=0}^{2\pi}\int_{\beta=0}^{\pi/2} \cos\left[DN_c T_r 2\sqrt{2}\,\sin(2\beta)\,\sin\alpha\right]\sin\beta\,d\beta\,d\alpha$$

where D is the dipolar coupling, N_c is the number of rotor cycles, and T_r is the rotor period, and α and β are the angles of the powder average (Gullion & Schaefer, 1989a). The 3-spin dephasing behavior expected in the Ser receptor ligand site was assumed to be equal to the powder average of the product of two independent 2-spin (^{13}C/^{15}N) dephasing interactions (McDowell et al., 1996), since the 4 kHz MAS is sufficient to average the ≈300 Hz ^{13}C/^{13}C dipolar coupling. Simulations of 3-spin dephasing curves were calculated using a Macintosh Pascal program kindly provided by Jacob Schaefer.

RESULTS

If the Ser receptor ligand site structure is similar to that of the Asp receptor, the crystal structure of the Asp receptor periplasmic fragment predicts there will be two Phe carbonyls nearly equidistant from the Ser ligand nitrogen (r_{CN} of 3.98 and 4.16 Å, see Figure 1). This prediction can be tested in the *intact*, *membrane-bound* Ser receptor with REDOR measurements of these distances in a complex of [^{15}N]Ser bound to [1-^{13}C]Phe receptor. ^{15}N-observed REDOR is used to take advantage of the unique ligand label, but the ^{13}C-observed REDOR experiment may also be feasible, if properly corrected for the presence of 13 (unresolved) Phe residues.

The ^{15}N-observed, ^{13}C-dephased REDOR experiment measures dipolar dephasing by comparing signal intensities in ^{15}N spectra collected with and without ^{13}C-dephasing π pulses. Two such ^{15}N spectra of the Ser receptor complex are shown in Figure 3: (a) is the full echo spectrum (S_0) collected without ^{13}C pulses; (b) is the dephased spectrum (S) collected with ^{13}C pulses. The narrow peak near 8 ppm corresponds to bound serine ligand; the broad signal at around 95 ppm is due to the natural-abundance ^{15}N in the peptide backbone of the 551 residues of the receptor. Such pairs of spectra are acquired for different dephasing periods (dephasing period = rotor period × number of rotor cycles = T_rN_c). Each pair of spectra are analyzed to determine $\Delta S = S_0 - S$ and $(\Delta S/S_0)$* for the Ser resonance, which is then corrected as outlined below and in Table 1.

The corrected $\Delta S/S_0$ values are plotted *vs* the dephasing time in Figure 4 along with several simulated 3-spin REDOR

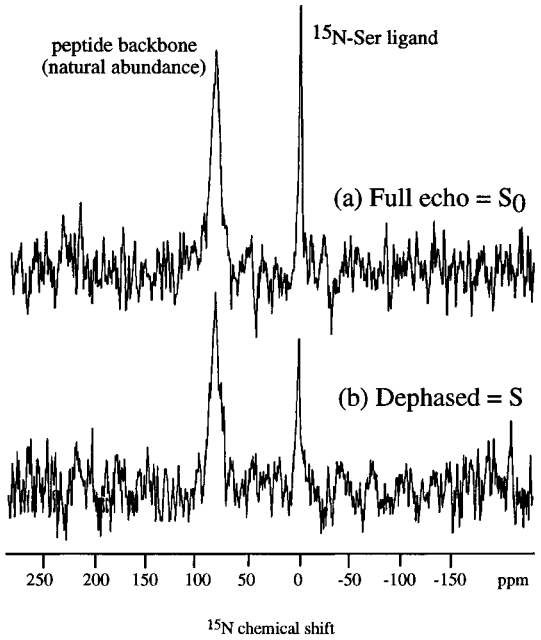

FIGURE 3: ^{15}N-observed, ^{13}C-dephased REDOR spectra. Each spectrum is the average of 80 000 scans using 4 kHz spinning speed and a 48-rotor cycle dephasing period on a 41 mg sample of lyophilized [1-^{13}C]Phe Ser receptor complexed with [^{15}N]Ser. The difference in the resonance intensities in the (a) full echo spectrum (S_0, without ^{13}C 180° pulses) and the (b) dephased spectrum (S) is used for the REDOR analysis: $\Delta S = S_0 - S$.

Table 1: Summary of REDOR Data and Analysis[a]

rotor cycles of dephasing	no. of scans	$(\Delta S/S_0)^*$ [b]	$\Delta S/S_0$	D (Hz)	r_{CN} (Å)
32	30000	0.156	0.207	41	4.2
48	80000	0.439	0.611	52	3.9
64	45000	0.464	0.647	41	4.2

[a] In each case, the indicated number of scans were collected for each of the full echo and the dephased spectra, with 4 kHz MAS. [b] Measured value. The data were corrected for natural-abundance ^{13}C and the estimated 70% of the resonance due to bound Ser: $\Delta S/S_0 = [(\Delta S/S_0)^* - 0.011]/0.7$.

dephasing curves. The dephasing depends on the two distances, r_{CN}, and the angle between the CN dipolar vectors, θ (the angle at the nitrogen in Figure 1). The data were initially analyzed (solid lines) by assuming, based on the crystal structure of the related Asp receptor fragment, that the two Phe carbons are equidistant from the nitrogen, forming a 40° angle. The boldface line indicates that with these assumptions the data are best fit by 4 Å distances; the extreme curves which fit the scatter in the data suggest an uncertainty of ±0.2 Å. The 4.0 Å ligand ^{15}N to receptor Phe ^{13}C distances measured by REDOR in the intact Ser receptor are in excellent agreement with the values predicted by the crystal structure of the related Asp receptor fragment (r_{CN} = 3.98 and 4.16 Å, θ = 40°).

The possibility of different angles or of nonequidistant Phe residues was considered with further simulations. For the short dephasing times which are experimentally feasible (longer dephasing times suffer from poor signal to noise due to T_2 relaxation), the dephasing curves are essentially indistinguishable for angles ranging from 30 to 150°. The dashed curve in Figure 4 plots the dephasing for 4 Å, 30° (or 150°) to demonstrate the largest deviation from the 40° curve among the 30−150° angle range. The covalent constraints of the two sequential Phe limit the possible ^{13}C

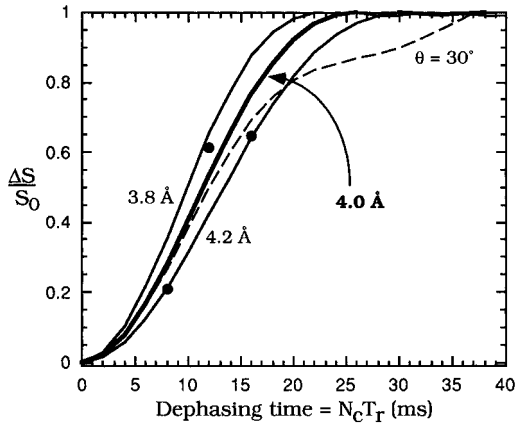

FIGURE 4: REDOR data and simulated dephasing curves. Data are for 32, 48, and 64 rotor cycles with 4 kHz spinning speed. Calculated curves are for a 3-spin system with three parameters, the two dipolar couplings and the angle between the two dipolar vectors. Solid lines are calculated with an angle of 40° (as observed in the crystal structure): the best fit (boldface line) indicates r_{CN} = 4.0; the other two curves indicate that the scatter in the data yields an uncertainty in the distance of ±0.2 Å. Similar dephasing is predicted for a large range of angles (30° ≤ θ ≤ 150°): the 30° curve (dashed), which represents the largest deviation from the 40° curve, is also consistent with the data. Since other angles are ruled out by the sequential connectivity of Phe150 and Phe151 (see text), the angle dependence does not introduce any uncertainty into this distance measurement.

to ^{13}C distances, which in turn constrains the angles which are possible for particular ^{13}C to ^{15}N distances (see Figure 1). Angles of less than 30° or greater than 150° would require r_{CN} which are too long or short to be compatible with the REDOR data. Thus, the angle dependence of 3-spin dephasing behavior does not introduce any ambiguity into the REDOR distance measurement for this case. Reasonable fits can also be obtained for a single Phe at 3.7 Å, with the second Phe at a distance ≥4.5 Å having little effect on the dephasing curve.

The REDOR $\Delta S/S_0$ data were corrected as follows (see Table 1). (1) Natural-abundance correction: A part of the REDOR difference signal, ΔS, is due to dipolar coupling between the labeled nitrogen and natural abundance ^{13}C nearby; 1.1% of the [^{15}N]Ser contains a directly bonded ^{13}C which, for the long dephasing times used in these experiments, will completely dephase the ^{15}N signal ($\Delta S/S_0$ = 1). To correct for this, 0.011 is subtracted from $(\Delta S/S_0)^*$. The small magnitude of the effect of this closest carbon (correction alters the final distance by only 0.03 Å) indicates that any dephasing due to more distant natural-abundance ^{13}C nuclei is negligible. (2) Fraction of ligand bound correction: The ligand accessibility assays and 1:1 ligand to receptor dimer ratio used indicate approximately 56% of the Ser ligand was bound in the REDOR samples. However, we observe nearly complete dephasing of the ligand signal, arguing that a larger fraction of the observed ligand is bound. The residual signal observed in long dephasing time spectra (128 rotor cycles) corresponds to free Ser (^{15}N with no nearby [^{13}C]Phe to dephase it) and to incompletely dephased bound Ser. About 30% of the full echo Ser resonance remains, indicating that at most 30% of the observed Ser is free and at least 70% of the observed Ser is bound. This estimate was used to correct the REDOR data for the fraction of the resonance due to bound ligand by multiplying S_0 by 0.7. To put the magnitude of this correction in perspective, the

maximum possible range of the fraction of the resonance due to bound Ser (56−100%) yields a distance range of 3.8−4.3 Å, which is comparable to the ±0.2 Å uncertainty inherent in the scatter in the data.

DISCUSSION

We have used solid-state NMR methods to probe the ligand environment in the intact Ser receptor. REDOR measurements indicate 4.0 ± 0.2 Å distances between the Ser ligand nitrogen and two Phe carbonyl carbons of the receptor. These data are consistent with the geometry and interactions of the ligand binding site predicted for the Ser receptor based on the crystal structure of the Asp receptor periplasmic fragment. This indicates that (1) the ligand binding site of the *fragment* resembles that of the intact receptor, as expected from their similar ligand affinities, and (2) the ligand interactions of Asp and Ser with their respective receptors are similar for the conserved amino group portion of these ligands, consistent with the high degree of sequence identity between the binding site residues of these receptors.

The ability to measure the geometry and ligand interactions at the binding site of *intact* chemotaxis receptors may prove useful in future experiments probing the mechanism of adaptation. Although methylation of specific residues in the cytoplasmic domains of these receptors enables the chemotaxis machinery to adapt to constant stimuli, it is still not clear whether this process involves a methylation-induced change in the ligand affinity of the receptor (Lin et al., 1994). Understanding the structural basis for such an affinity change would require studies of the intact receptor. Changes at the ligand site due to methylation could be probed with solid-state NMR studies similar to those presented here.

Finally, this investigation of a single site within a 120 kDa protein is one of the largest systems studied by REDOR to date, and represents, to the best of our knowledge, the first application of REDOR to a large membrane-bound protein. The application of REDOR to measure selected distances in membrane proteins promises to provide important mechanistic information which is unavailable by other methods. In particular, this demonstration of the feasibility of distance measurements in the membrane-bound, intact Ser receptor dimer sets the stage for distance measurements elsewhere in the receptor to characterize differences between signaling states and to test proposed models of transmembrane signaling.

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