^{19}F nuclear magnetic resonance studies of aqueous and transmembrane receptors

Examples from the *Escherichia coli* chemosensory pathway

Joseph J. Falke, Linda A. Luck, and Jon Scherrer
Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215 USA

INTRODUCTION

In general, protein conformational changes are best studied in the appropriate native environment, whether it be aqueous solution or a biological membrane. ^{19}F nuclear magnetic resonance (NMR) provides a valuable tool with which to carry out such studies, even for a variety of proteins too large or insoluble for multidimensional NMR techniques. Recombinant DNA techniques significantly extend the usefulness of ^{19}F NMR, both by facilitating resonance assignments, and by enabling separation of multidomain structures into smaller, more accessible units. The present study illustrates the type of information provided by this combined approach using two receptors from the *E. coli* chemosensory pathway as examples: the periplasmic D-galactose and D-glucose receptor and the transmembrane aspartate transducer.

THE ^{19}F NMR METHOD

The usefulness of ^{19}F NMR stems from several unique features of the ^{19}F nucleus. This spin one-half nucleus exhibits high sensitivity (0.94 relative to ^{1}H) and a relatively large frequency range (10^2-fold larger than ^{1}H). The ^{19}F NMR frequency is controlled largely by the fluorine lone-pair electrons, which provide a large antidiamagnetic term in the shielding formula. As a result the NMR frequency is exquisitely sensitive to changes in the van der Waals packing around the nucleus, whereas aromatic fluorines are further sensitive to changes in the electron distribution of the adjacent π system. Proteins expressed in *E. coli* can be routinely fluorine labeled by biosynthetic incorporation of tryptophan, phenylalanine, or tyrosine substituted at one ring position with fluorine. A number of studies have shown that the effects of such "single atom mutagenesis" on protein structure and activity are generally small or undetectable (reviewed in references 1–5). When biosynthetic labeling is impractical, alkylation of cysteine or lysine with a fluorine-labeled reagent provides another incorporation method (6, 7).

RESULTS AND DISCUSSION

D-galactose and D-glucose receptor

The D-galactose and D-glucose receptor is a 32 kDa periplasmic binding protein whose function is to sequester a sugar molecule, then dock to and activate one of two proteins spanning the cytoplasmic membrane: a sugar transporter or a sensory transducer. The structure of the receptor containing bound D-glucose has been solved to 1.9 Å resolution, and the protein is known to undergo a large conformational change upon glucose binding in solution (8, 9). The crystal structure reveals two domains, each composed of an α/β motif, connected by three strands of polypeptide proposed to comprise a flexible hinge. The bound sugar molecule is trapped in the closed cleft between the two domains. A plausible model for the conformational change proposes that bound sugar stabilizes the closed cleft, whereas the empty cleft has, on average, a more open conformation (9).

To test this model, we have biosynthetically incorporated 5-fluorotryptophan (5F-trp) into the five tryptophan positions of the receptor (65 ± 10% incorporation). In separate experiments, 3-fluorophenylalanine (3F-phe) has been incorporated into the seven phenylalanine positions (20 ± 10% incorporation). Fig. 1 shows the positions of the labeled residues. Trp 183 and phe 16 are in van der Waals contact with bound sugar, yet the fluorine-containing molecules in the labeled population are observed to have D-galactose affinities only slightly lower than that of the unlabeled molecule: 2.2- to 3.0-fold lower for 5F-trp, and 1.5- to 4.0-fold lower for 3F-phe. The receptor also possesses an EF-handlike Ca(II) binding site: fluorine labeling causes still smaller perturbations at this site (3).

The 5F-trp resonances were assigned by use of oligonucleotide-directed mutagenesis to replace a single tryptophan with tyrosine (or in one case, phenylalanine), thereby eliminating the associated resonance from the ^{19}F NMR spectrum. Fig. 2 illustrates the assigned spectra for the sugar-empty and sugar-occupied conformers. The 5F-trp 127 and 133 residues give rise to double resonances, indicating two stable structures in this

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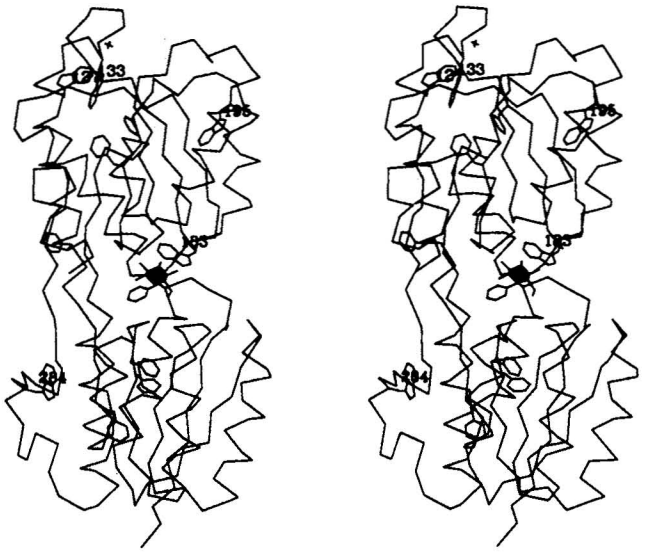

FIGURE 1 Stereo α-carbon backbone structure of the *E. coli* D-galactose and D-glucose receptor (8). The solid ring indicates the bound D-glucose molecule in the sugar cleft, and the cross denotes the Ca(II) ion in the metal site. Also shown are the five trp and seven phe positions which serve as ^{19}F-labeling sites.

region on the NMR timescale (7 ms), perhaps due to *cis-trans* isomerization of nearby proline 231. This structural heterogeneity is highly localized because it causes no detectable effects within the sugar and Ca(II) sites (3).

A simple test of the hinged cleft model is to monitor the solution exposure of 5F-trp 183, which is sequestered in the closed cleft, but should become accessible to the bulk solution if the cleft opens substantially. The trivalent lanthanide Gd(III) ($S = 7/2$) is a suitable paramagnetic probe for this purpose because it significantly broadens the NMR resonance of a nucleus it approaches, in proportion to the inverse sixth power of the separation (r):

$$\Delta\Delta_{1/2} = r^{-6}[\gamma_I^2 g^2 \beta^2 S(S+1)][4\tau_c + 3\tau_c/(1+\omega_I^2\tau_c^2)]/(15\,\pi). \quad (1)$$

Here $\Delta\Delta_{1/2}$ is the paramagnetic linebroadening, γ_I is the magnetogyric ratio for the nuclear spin, g is the Landé g-factor for a free electron spin, β is the Bohr magneton, ω_I is the nuclear resonance frequency in radians s^{-1}, and the correlation time τ_c of this magnetic dipole–dipole interaction contains contributions from the electron spin-lattice relaxation, the rotation of the interdipole

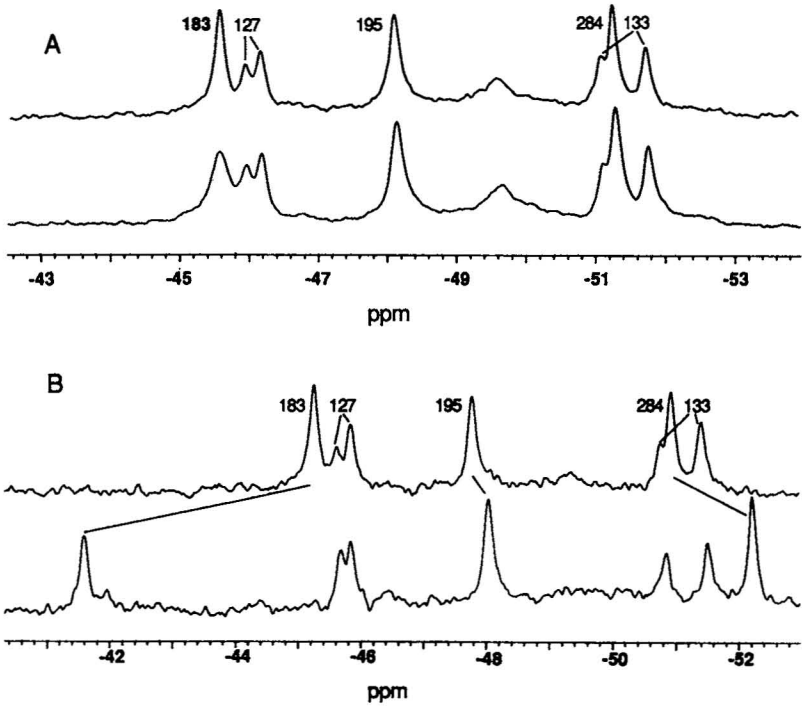

FIGURE 2 470 MHz ^{19}F NMR spectra of the D-galactose and D-glucose receptor. (*A*) Effect of Gd(III) · $EDTA^-$ on the 5F-trp labeled receptor in the absence of sugar. (*Upper traces*) no Gd(III) · $EDTA^-$ (*Lower traces*) 5.6 mM Gd(III) · $EDTA^-$. (*B*) Effect of D-galactose on the 5F-trp labeled receptor. (*Upper traces*) no sugar. (*Lower traces*) 1.0 mM D-galactose. *A and B*) Spectra were obtained at 25° on a Varian VXR500. Samples contained: 100 mM KCl, 10 mM pH 7.1 Tris, 0.5–5.0 mM $CaCl_2$, 10% D_2O for lock, and 75 μM 3F-phe as an internal frequency reference. Modified from references 3 and 5.

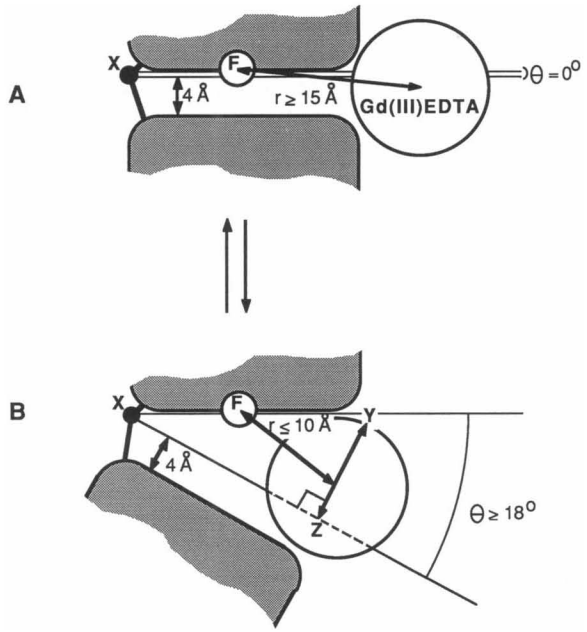

FIGURE 3 Schematic hinged-cleft of the D-galactose and D-glucose receptor. Dimensions are from the known structure of the closed cleft (8); the unhydrated diameter of the paramagnetic probe Gd(III) · $EDTA^-$ is 10 Å (5). (*A*) The ^{19}F NMR data indicate that the distance of closest approach between the probe and the observe ^{19}F nucleus (5F-trp 183, F) is $r \geq 15$ Å in the sugar occupied cleft. (*B*) In the empty cleft the distance of closest approach is $r \leq 10$ Å, requiring the cleft to open by an angle of $\theta \geq 18°$ to accomodate the probe. Modified from reference five.

vector, and the exchange time of the paramagnet in the site. In the present application the probe complex Gd(III) · $EDTA^-$ is used for its large size (least squares diameter = 9.7 Å), to ensure that the paramagnet cannot enter the closed cleft.

Fig. 2*A* illustrates the dramatic broadening of the 5F-trp 183 resonance caused by addition of Gd(III) · $EDTA^-$ to the empty cleft. The broadening of this resonance is more than twofold larger than any other, and increases linearly with the probe concentration, such that $\Delta\Delta_{1/2} = 130 \pm 10$ Hz when [Gd(III) · $EDTA^-$] = 9.3 mM. In contrast, as predicted by the crystal structure, no broadening of the 5F-trp 183 resonance is observed in the presence of 1.0 mM D-glucose, indicating that the sugar-occupied cleft remains closed, on average, in solution (5). These results confirm the predictions of the hinged-cleft model, and enable calculation of the angle of cleft opening. Using the model cleft illustrated in Fig. 3, together with Eq. 1 and the appropriate constants (5), the distance of closest approach of the probe complex to the nucleus is found to be $r \leq 10$ Å. For the probe to penetrate this deeply into the protein, the cleft must open $\geq 18°$.

A key question is whether this large hinged cleft motion perturbs the structure of the two domains. Alternatively, the domains could move as rigid bodies. This issue is central to receptor function because activation of the receptor must produce surface structure changes, which in turn regulate receptor docking to its target protein. To address this question, the effect of sugar binding on the 12 NMR positions in the receptor has been tested. Examination of the ^{19}F NMR frequencies of the five 5F-trp residues and seven 3F-phe residues indicates that 9/12 of these positions in the structure are perturbed by sugar binding, whereas 3/12 are not perturbed (3). For example, Fig. 2*B* illustrates the frequency shifts observed for the 5F-trp 183, 195, and 284 resonances; the latter two residues are distant from the sugar cleft. Together the data indicate that sugar binding triggers widespread structural changes in both domains. The only region unaffected is the Ca(II) site, which appears to be conformationally isolated from the rest of the receptor (4).

In short, the D-galactose and D-glucose receptor possesses a hinged substrate cleft which regulates an allosteric structural change in most regions of the molecule. Examination of the backbone structure suggests a simple mechanism for this allostery. Sixteen secondary structure elements terminate in the cleft (8 α-helices and 8 β-strands), and the closing of the cleft will shift many of these elements. The resulting small movements will be transmitted along the entire length of each perturbed element, out to the protein surface. (For a full account of these ^{19}F NMR studies of the D-galactose and D-glucose receptor, see references 3–5).

The transmembrane aspartate transducer

The aspartate transducer is a transmembrane homodimer of 60 kD monomers. Fig. 4 presents a hypothetical structure consistent with the available biochemical and structural evidence (10–12, 15). This receptor is activated by aspartate binding to its periplasmic domain, which in turn induces a transmembrane conformational change that regulates the cytoplasmic signaling domain. The transducer appears to be mechanistically related to a large class of eukaryotic receptors for insulin, EGF and other growth hormones; thus, when the kinase domain of the human insulin receptor is substituted for the cytoplasmic signaling domain, the resulting chimera is an aspartate-activated kinase (13).

A large, membrane-bound protein is often considered inaccessible to solution NMR techniques because the

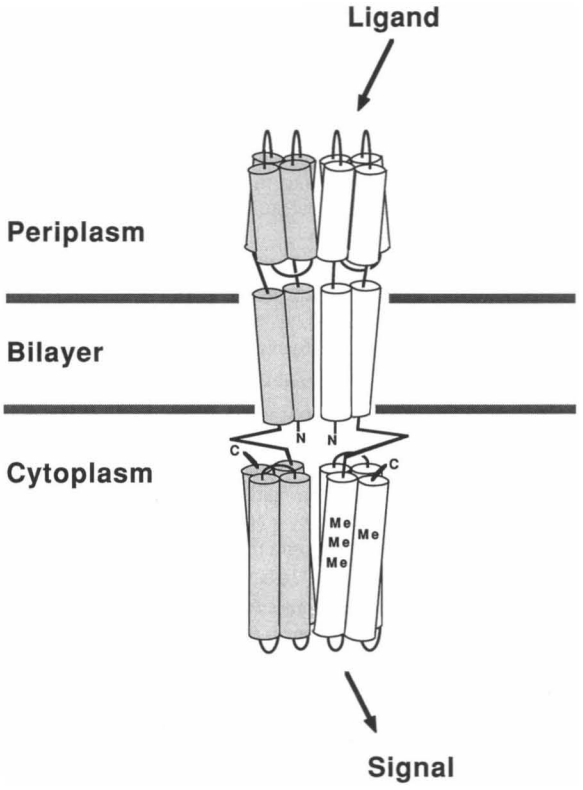

FIGURE 4 Hypothetical structure of the transmembrane aspartate transducer of *E. coli* (10–12, 15). Shown are the three domains of each monomer in the homodimer, each proposed to be dominated by α-helices. (*Upper*) the periplasmic aspartate binding domain of each monomer is a four-helix bundle. (*Middle*) the two predicted transmembrane helices of each monomer. (*Lower*) the signaling domain, which activates the cytoplasmic phosphorylation pathway.

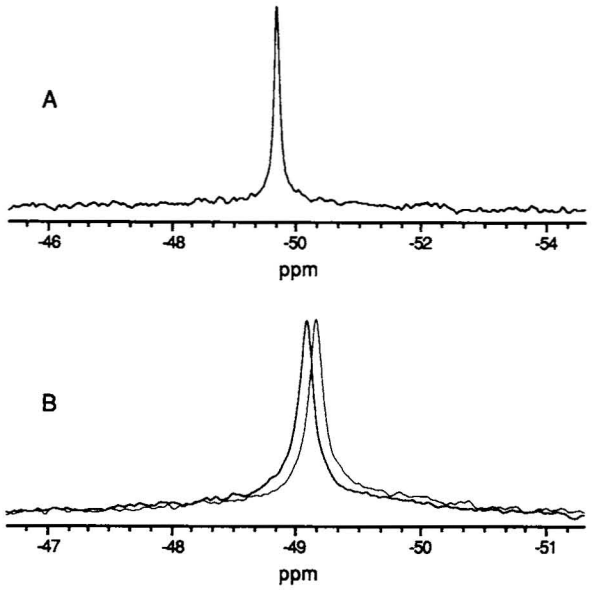

FIGURE 5 470 MHz ^{19}F NMR spectra of the 5F-trp labeled aspartate transducer. (*A*) Spectrum of the isolated cytoplasmic domain, showing the 5F-trp 550 resonance. (*B*) Spectra of the full-length transducer in the isolated native membrane, illustrating the frequency shift induced by aspartate binding (*bold spectrum*). Spectra were obtained at 25°. Samples contained: 50 mM NaCl, 20 mM pH 8.0 Tris, 10% glycerol, 10% D_2O for lock, and 50 μM 3F-phe as an internal frequency reference.

slow molecular tumbling will yield large spin–spin relaxation rates for most protein nuclei, such that the associated resonances are too broad to detect. However, if a probe nucleus is placed at a site which experiences sufficiently rapid and isotropic local motion, its NMR resonance can be detected irregardless of the molecular size.

To demonstrate this approach, we have biosynthetically incorporated 5F-trp into the intact transducer in the native, isolated membrane (mol wt > 10^6 kD per membrane), as well as the transducer's water soluble cytoplasmic domain (mol wt = 64 kD per homodimer). (Plasmids overexpressing each *E. coli* protein were graciously provided by Dr. M. Simon, Caltech [14]). The full-length transducer monomer contains four tryptophans, whereas the cytoplasmic domain contains one.

The ^{19}F NMR spectrum of the water-soluble cytoplasmic fragment is shown in Fig. 5 *A*, revealing a single sharp resonance for 5F-trp 550, which lies just three residues from the COOH-terminus. This result indicates that the two monomers within the dimer are equivalent, and suggests that the 5F-trp probe has significant local mobility on the relevant NMR timescale (10^{-7} s). Additional evidence for this picture is provided by the ^{19}F NMR spectrum of the full-length, membrane-bound transducer (Fig. 5 *B*). A single sharp resonance is again observed, presumed to be from the same 5F-trp 550 position in the COOH-terminal region, whereas the other three tryptophan positions are rendered invisible by the slow tumbling of the transducer-membrane complex. No resonances are observed for otherwise identical membranes lacking the transducer. When aspartate is added to generate the activated conformation, the resulting transmembrane conformational change yields detectable NMR frequency shift at the probe position (Fig. 5 *B*).

Together these results suggest that the ^{19}F NMR approach can be applied even to large, relatively immobile macromolecular systems. When extending this approach to a particular system of interest, the key challenge is to find suitable labeling sites which provide the needed local mobility. One approach which appears particularly promising is the chemical labeling of engineered surface cysteine residues.

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