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Deuterium NMR of Proteins in Solution, in Membranes, and in the Crystalline Solid-State

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Introduction

The overall aim of our research is to probe the structures, both static and dynamic, of systems that are not amenable to investigation using the powerful techniques of X-ray crystallography. Proteins in solution or in membranes fall into this category, as do soluble proteins which for one reason or another have not yet been obtained in forms suitable for crystallographic study. In addition, our methods may also complement X-ray studies of dynamics in crystals which are suitable for X-ray work[1,2] by (in principle) providing detailed information about the rates and types of motion of individual residues, on a wide-variety of timescales—from literally picoseconds to many hours!

We first discuss results on solution NMR of ^{2}H-labelled proteins which indicate that measurement of both the spin-lattice (T_1) and spin-spin (T_2) relaxation times (or linewidths) of irrotationally bound ^{2}H nuclei in macromolecules undergoing isotropic rotational motion outside of the extreme narrowing limit (i.e. for the case $\omega_o^2\tau_R^2 \gg 1$) permits determination of both the rotational correlation time (τ_R) of the macromoleule *and* the electric quadrupole coupling constant (e^2qQ/h) of the ^{2}H label.[3] The technique has the advantage over ^{13}C NMR that no assumptions about bond lengths (which appear to the sixth power in ^{13}C relaxation studies) need be made, and in addition relaxation will always be quadrupolar, even for aromatic residues at high-field. We show that rotational correlation times (τ_R) obtained using *only* solution T_1 and T_2 data (which give both τ_R *and* e^2qQ/h) are in good agreement with those obtained previously using ^{13}C NMR and inelastic light scattering.

Second, we discuss results on the first deuterium (^{2}H) NMR studies of a variety of individual types of amino-acid residue in the membrane protein, bacteriorhodopsin, in the purple membrane of *Halobacterium halobium* R_1, together with results on ^{2}H-labelled *E. coli* cell membranes. We show that high-field Fourier transform operation permits rapid data acquisition on intact membranes, including measurement of relaxation times, and at some temperatures high quality spectra can be obtained in <1 second.[4]

where e^2qQ/h in the deuteron quadrupole coupling constant and η the asymmetry parameter. There are thus three unknowns: e^2qQ/h, η and τ_R. Fortunately, however, η values only emcompass the range $\sim 0 - 0.06$ in almost all aliphatic or aromatic species to be found in proteins,[20,21] so η value uncertainties can therefore only introduce in general at most a $\sim 0.1\%$ error in T_1 a quite insignificant error. In addition, as shown below, both η and e^2qQ/h may in any case be determined directly in separate solid-state NMR experiments.

For proteins with correlation times $\gtrsim 5$ nsec, Equations 1 and 2 may be recast in simpler form, since $\omega_o^2\tau_R^2 \gg 1$ and $\eta^2/3 \to 0$, as follows:

$$\frac{1}{T_1} = \frac{3}{5}\left(\frac{e^2qQ\pi}{h\omega_o}\right)^2 \frac{1}{\tau_R} \tag{3}$$

$$W = \frac{1}{\pi T_2} = \frac{9\pi}{20}\left(\frac{e^2qQ}{h}\right)^2 \tau_R \tag{4}$$

Thus, τ_R is directly proportional to the linewidth ($W = 1/\pi T_2$) and T_1. Measurement of T_1 and W of a suitably irrotationally bound ^{2}H-labelled site in a protein in solution thus leads directly to determination of both e^2qQ/h and τ_R. For example, for [ϵ-^{2}H$_1$]His-15 labelled lysozyme (EC 3.2.1.17), pH 7.2, 13 mM, 40°C, at 34.1

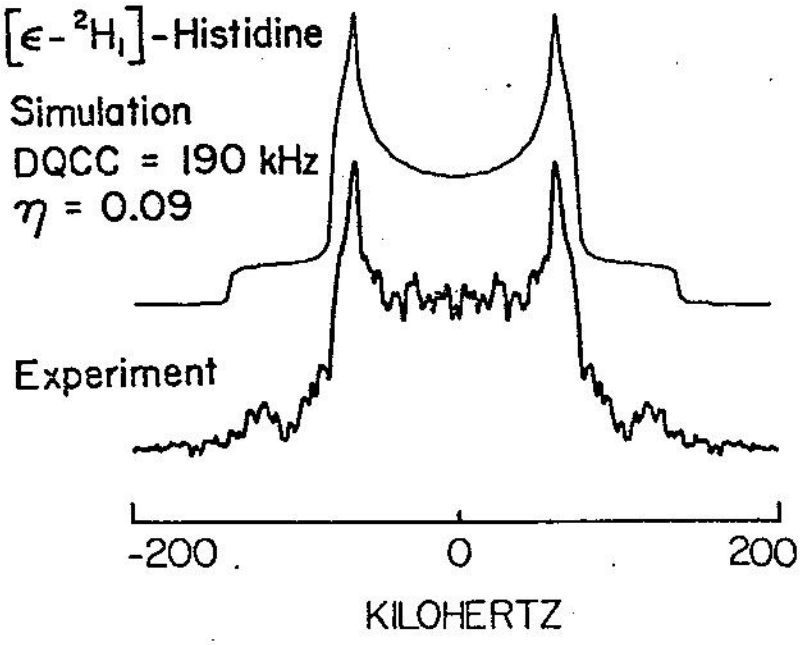

Figure 1. Computer simulation and 55.3 MHz ^{2}H NMR spectrum of polycrystalline [ϵ-^{2}H$_1$]histidine at 25°C.

where θ and ψ define the orientation of the principal axis of the electric field gradient tensor (usually the C-D bond vector) with respect to the laboratory coordinates. For rigid polycrystalline solids all values of θ are possible and one obtains a so-called "powder pattern", Figure 1. Spectral simulation of the results of Figure 2A (solid [$^2H_5$]Trp, 25°C) using the lineshape equations of Bloembergen and Rowland[34] and Cohen and Reif[30] indicate that the best fit of the experimental spectrum of [$^2H_5$]Trp, Figure 2A, is obtained using $e^2qQ/h = 183 \pm 3$ kHz and $\eta = 0.05 \pm 0.02$. The observed quadrupole coupling constant for [$^2H_5$]Trp, Figure 2A, is considerably in excess of the 167 kHz found in aliphatic C-^{2}H systems[20] using NMR methods, but this result is consistent with the e^2qQ/h values found in a variety of aromatic compounds,[20,33] the observed trends for electric field gradient (EFG) values for C-D bonds[35] being $sp > sp^2 > sp^3$, the average value for naphthalene and anthracene,perhaps the most reasonable models for [$^2H_5$]Trp, being ~ 184 kHz.[35-38] In addition, it is well known that C-D bonds in aromatic systems may have non-zero asymmetry parameters, η,[39] in those aromatic systems where asymmetry parameters have been investiqated, η values $= 0.053 \pm 0.015$ having been determined.[20] The

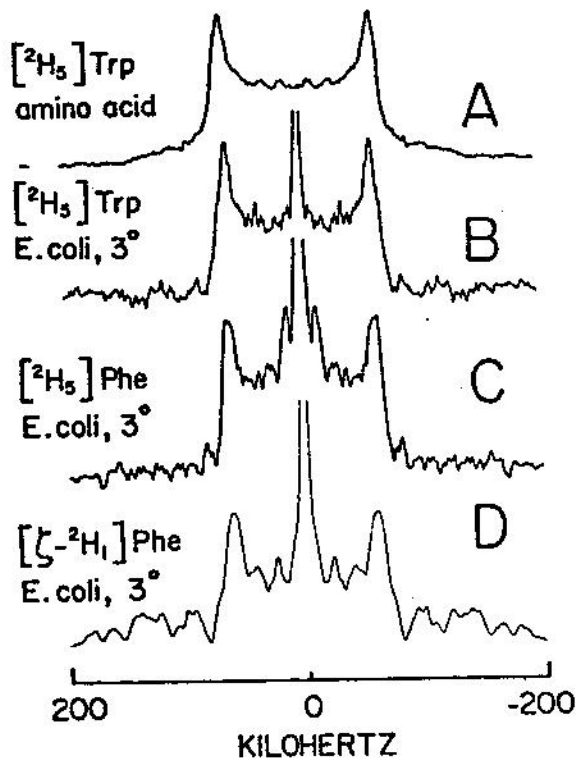

Figure 2. Deuterium NMR spectrum, obtained by the Fourier transform method at 55.3 MHz, of *A.* [δ_1, ϵ_3, ζ_2, ζ_3, η_1-$^2H_5$]tryptophan, 25°C; *B.* [$^2H_5$]tryptophan-labelled *Escherichia coli* cell membranes enriched with oleate, at 3°C; *C.* [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine labelled *E. coli* cell membranes enriched with oleate, at 3°C; *D.* [ζ-$^2H_1$]phenylalanine labelled *E. coli* cell membranes enriched with oleate, at 3°C.

the central doublet component seen in the spectrum of Figure 2C originates from *ortho* and *meta* deuterons in flipping phenylalanine rings, rather than from small particles, surface residues, or rigid body or "rocking" motions of a small population of Phe groups.

With bacteriorhodopsin in the photosynthetic purple membrane of *Halobacterium halobium* R_1, even more prounounced effects in the ^2H NMR spectra, due to phenyl group rotations, are seen, Figure 3A-C, especially at 37°, the growth temperature of the microorganism. For example, the results of Figure 3A,B, obtained with [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine and [ϵ_1, ϵ_2-$^2H_2$]tyrosine labelled purple membranes clearly indicate that amost all Phe and Tyr residues of the purple membrane of *H. halobium* R_1 are undergoing fast two-fold flipping motions at the growth temperature, assuming that the model described above is applicable. As with the *E. coli* membrane, experiments with [ζ-$^2H_1$]phenylalanine membranes rule out the alternatives listed above, for Phe residues. Unfortunately however, tyrosine does not have a ζ-^2H nucleus. We have therefore incorporated [β-$^2H_2$]tyrosine into bacteriorhodopsin in the purple membrane of *H. halobium* R_1, as shown in Figure 3C. The spectrum of the [β-^{2} $^2H_2$]tyrosine labelled membrane is essentially axially symmetric ($\eta \sim 0.05$) and has the full rigid-lattice breadth. The reduction in the ^2H spectral breadth of the [ϵ_1, ϵ_2-$^2H_2$]tyrosine labelled samples cannot therefore originate from anything other than Tyr-ring flipping, since there is no amino-acid breakdown.[44]

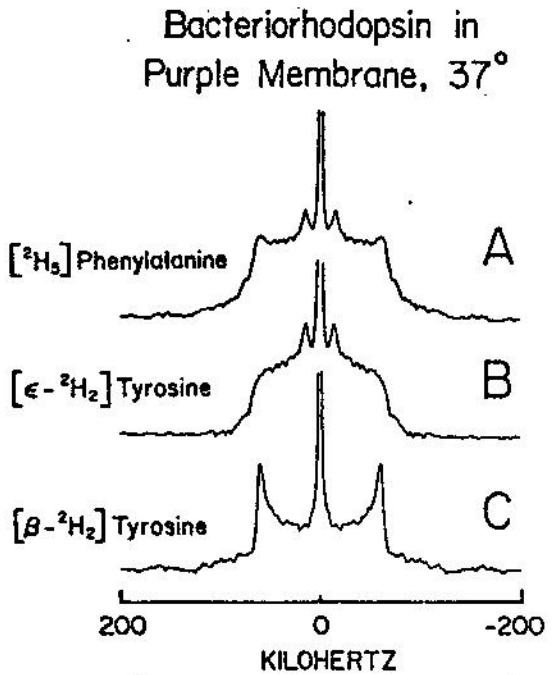

Figure 3. 55.3 MHz deuterium Fourier transform NMR spectra of aromatic ^2H-labelled *Halobacterium halobium* R_1 purple membranes at 37°C, the temperature of growth. The labelled amino-acids were as follows: *A*, [δ_1, δ_2, ϵ_1, ϵ_2, ζ-$^2H_5$]phenylalanine: *B*, [ϵ_1, ϵ_2-$^2H_2$]tyrosine and *C*, [β-$^2H_2$]tyrosine.

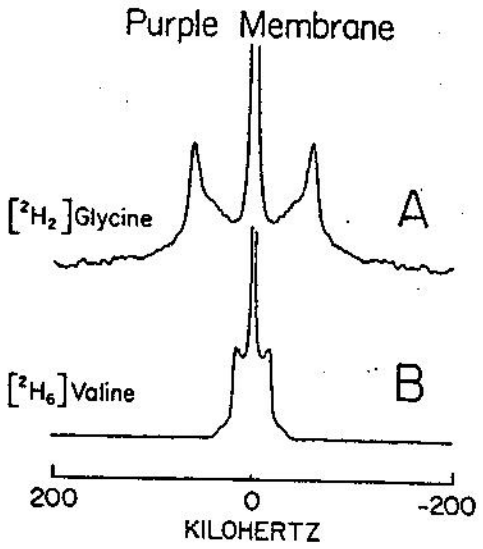

Figure 5. 55.3 MHz deuterium Fourier transform NMR spectra of aliphatic ²H-labelled *H. halobium* R_1 purple membranes at 37°C, the temperature of growth. The labelled amino-acids were as follows: *A*, [α-$^2H_2$]glycine and *B*, [γ-$^2H_6$]valine.

We show in Figure 5 representative results with aliphatic ²H-labelled amino-acids incorporated into the purple membrane of *H. halobium*. Membranes enriched with [α-$^2H_2$]glycine, Figure 5A, give spectra essentially indistinguishable from those of the solid amino-acid, indicating a "rigid" polypeptide backbone (on the timescale of $\sim 10^{-5}$sec). By contrast, spectra of *methyl*-²H labelled species, such as [γ-$^2H_6$]valine, give very narrow spectra, having $\Delta\nu_Q \sim 40$ kHz. For [γ-$^2H_6$]valine, this and additional experiments with [α-$^2H_1$] and [β-$^2H_1$]valine labelled cells[44,47] indicate that the only fast large amplitude motion in the valine side-chain is methyl group rotation,[4,44,47] which occurs on a tens of picoseconds timescale.[4] As with the case of the aromatic amino-acids, there is no fast large-amplitude backbone or C^α-C^β motion. Also, the above results rule out rotational diffusion of the entire bacteriorhodopsin molecule as a factor to be considered in interpreting such ²H NMR spectra of purple membrane samples, although we cannot rule out a very small population of small particles contributing to the central "isotropic" components seen in some spectra.

A summary of the rates and types of motions we have seen in a variety of amino-acids in bacteriorhodopsin in the purple membrane of *H. halobium* R_1 is shown in Figure 6.

We believe that the way is now open to examining a whole new area in membrane molecular biology by focussing on the active species, the membrane enzymes, rather than solely observing the membrane lipids, which for technical reasons have been the most attractive species to study, by NMR spectroscopy, during the last 10 years. Clearly, spectral sensitivity is now sufficient to permit extremely detailed investigation of the rates and types of motion of amino-acid residues in membrane proteins, including the effects of e.g. cholesterol and membrane lipid "fluidity" on protein structure, and even time-resolved studies.

in a field of intensity H is

$$E = -\tfrac{1}{2}H \cdot \chi \cdot H \tag{7}$$

while for a similar particle having a dipole moment μ in an electric field of intensity F the energy is

$$E = -\mu \cdot F. \tag{8}$$

Back of the envelope calculations using typical protein dipole moments of say 400 Debyes, and magnetic susceptibilities (χ) corresponding to ESR g-values of say $g_{max} = 3, g_{min} = 1$, led us to believe that for even the smallest protein microcrystals ($\sim 10^{-3}$ cm dimensions) that it should be possible to make both the electric and magnetic interaction energies considerably in excess of the thermal energy kT (per particle), in which case it should be possible to obtain highly ordered samples. Theoretically, and experimentally, the situation is more complex however, since it is not a simple matter to write an exact expression for the torque in terms of the g-tensor components, as suggested by our back-of-the-envelope calculations, since the high spin ferric iron is a Kramers ion, and the populations of the various energy levels, at room temperature, are not accurately known. Similarly, the actual field shapes and strengths in (cross-linked) protein crystals in an electric field are quite difficult to calculate, and sample heating effects in the electric-ordering experiment are generally quite severe.

Nevertheless, we have recently shown that microcrystals of a variety of paramagnetic heme proteins, suspended in ~ 90% saturated $(NH_4)_2SO_4$, may be perfectly aligned by an intense static external magnetic field, H_o, due to the large anisotropy in the magnetic susceptibility of the protein caused by the paramagnetic center. In addition, we have also utilized gravity to obtain oriented samples, which in combination with magnetic ordering appear to give arrays ordered in two-dimensions.[7] Flow systems (mechanical ordering) should also give quite ordered samples, but concentrations may be rather too low for NMR spectroscopy.

A photomicrograph of a typical oriented sample (metaquomyoglobin in ~ 90%saturated $(NH_4)_2SO_4$) is shown in Figure 7. For NMR spectroscopy we isotopically enriched myoglobin from sperm whale *(Physeter catodon)* at the C^{ϵ} methyl groups of methionine residues 55 and 131 with either ^{13}C or ^{2}H, and studied the labelled protein in the crystalline solid state by ^{2}H-quadrupole-echo and ^{13}C Fourier transform nuclear magnetic resonance spectroscopy.[5-7] We have found that suspensions of both high ($S = 5/2$) and low ($S = 1/2$) spin ferric forms of the labelled protein are ordered at fields as low as ~ 3 kG, the axis of ordering being approximately perpendicular to the low-temperature minimum g-tensor valve, even though upper Kramer's levels are populated at room temperature. The paramagnetic Co^{II} derivative "coboglobin" shows similar ordering behavior, but diamagnetic carboxymyoglobin is unaffected. The magnetic ordering method therefore permits the recording of "single-crystal" NMR spectra from microcrystalline arrays of proteins which

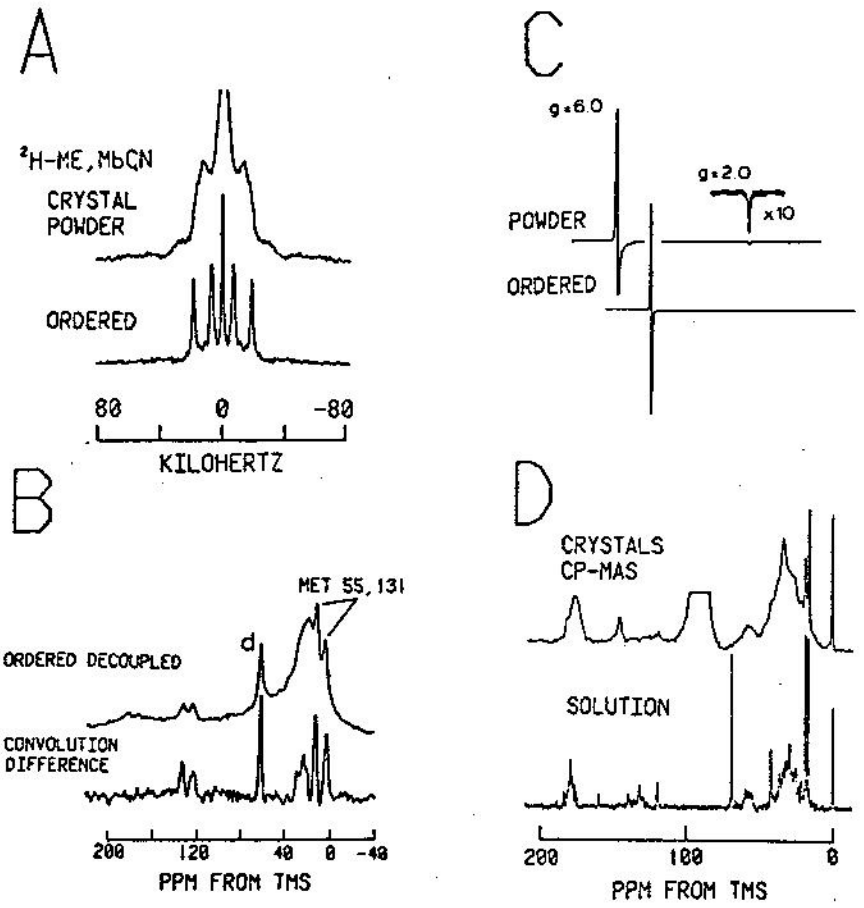

Figure 8. ^{1}H and ^{13}C nuclear magnetic resonance and electron spin resonance spectra of ferric myoglobin. *A*, 55 MHz ^{2}H NMR of [*methyl*-$^2H_3$]methionine labelled cyanometmyoglobin as a random powder and as a magnetically ordered array. *B*, ^{13}C NMR of [*methyl*-^{13}C]methionine aquometmyoglobin. *C*, as in *B* but 9 GHz ESR. *D*, 37.7 MHz proton decoupled ^{13}C NMR of [*methyl*-^{13}C]methionine labelled myoglobin in the solid state (solid microcrystalline powder hydrated with ~ 90% $(NH_4)_2SO_4$) obtained using magic angle sample spinning. Solution spectrum is of the same protein at 62.9 MHz, 0.65 mM, pH 7.8, 30°C.

and ^{13}C NMR, together with 9 GHz electron spin resonance spectra of random powder and magnetically ordered metaquomyoglobin microcrystals. For MbH_2O, the ^{2}H NMR results give fairly unambiguously values for θ', the angle between the S^{δ}-C^{ϵ} bond vector and H_o (apparently along c^* in MbH_2O) of 17.5° and 54.7°. Using the crystallographic results[52] we calculate $\theta' = 16.6°$ for Met-55 and 53.4° for Met-131.[7] Clearly then, the magnetic ordering method gives structural information of a type that cannot be obtained from more conventional high-resolution nuclear magnetic resonance spectroscopic methods, such as solution ^{13}C NMR, or by means of magic-angle sample spinning methods (Figure 8D). Such methods are primarily useful for resolution-enhancement and dynamics studies.

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