Unambiguous Heme Proton Hyperfine Resonance Assignments of a Monomeric Hemoglobin from *Glycera dibranchiata* Facilitated with a Completely Deuterated Protein†

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Abstract: One of the most challenging tasks facing those who work with low-spin ferriheme proteins is assigning all of the heme proton NMR resonances. In part this stems from the relatively large resonance line widths and rapid relaxation rates characteristic of hyperfine-shifted protons. Perhaps as important is that, depending upon the protein, approximately one-half of the hyperfine-shifted resonances are buried in a very dense spectral region. Identifying single hyperfine-shifted proton resonances in an extremely crowded spectrum imbues the resulting assignments with a degree of uncertainty. In an attempt to simplify this procedure, we have produced an extensively deuterated Component IV globin of *Glycera dibranchiata* in an expression system consisting of the *E. coli* strain BL21 (DE3) grown in a medium that was at least 98% deuterated. After purification, this globin was constituted with fully protonated hemin to form the recombinant deuterated monomer hemoglobin, which was subsequently studied by both 1D and 2D NMR methods. Complete deuteration makes comprehensive heme proton assignments rapid to achieve, straightforward, and unambiguous. All heme proton resonances are identifiable in partially relaxed 1D spectra, including those of the heme meso protons. Moreover, our experience with this completely deuterated protein offers insights to many general and potential uses of completely deuterated proteins.

Introduction

Proton NMR spectra of low-spin ferriheme proteins, such as cyanide-ligated metmyoglobins (MbCN) and peroxidases, exhibit highly shifted hyperfine resonances which originate from the heme protons and the protons of amino acids that line the heme pocket.[1−4] As an example, the proton resonance shift dispersion of sperm whale MbCN encompasses nearly 40 ppm, with many of the heme and neighboring amino acid protons shifted away from the very intense manifold of proton resonances found in the −2 to 11 ppm region.[2,4−6] Hyperfine-shifted proton resonances inherently contain a great deal of information about the active site structure and dynamics of these proteins, which makes achieving unambiguous hyperfine-shifted proton resonance assignments fundamental to studies on low-spin ferriheme proteins. However, the chemical shift correlations commonly used for diamagnetic samples cannot be applied to the proton hyperfine resonance shifts that result from the paramagnetism of the heme, making the task of arriving at their unambiguous assignments difficult. During the past five years, however, advances in two-dimensional methods have overcome complications due to the large line widths and extremely rapid relaxation rates of hyperfine-shifted protons and have led to increasing ease in making assignment-related correlations between hyperfine-shifted nuclei.[1,4,7−14]

A significant complexity in pursuing proton assignments of low-spin ferriheme proteins is a result of asymmetry in the heme paramagnetic electronic state that can cause as many as 65% of the potentially identifiable hyperfine-shifted proton resonances to reside under the intense envelope of protons between −2 and 11 ppm.[1−4,6,10,15] Depending upon the size of the protein being studied, this makes unambiguously identifying those resonances very difficult. About 30−60 significantly hyperfine-shifted proton resonances may occur in a low-spin ferriheme protein, but they are only a small fraction of the total number of protons in the protein. For molecules of interest to us, the total number of protons ranges from ~1100 protons for a protein the size of myoglobin to ~2400 protons for molecules the size of cytochrome *c* peroxidase. Approximately 95% of these proton resonances display shifts in the −2 to 11 ppm region

† Abbreviations used: Mb, myoglobin; MbCN, cyanide-ligated metmyoglobin; rec, "recombinant" or bacterially expressed protein; GMG4, *Glycera dibranchiata* native monomer Component IV globin; DrecGMG4, completely deuterated *Glycera dibranchiata* recombinant monomer Component IV globin; GMH4, *Glycera dibranchiata* native monomer Component IV hemoglobin; DrecGMH4, completely deuterated *Glycera dibranchiata* recombinant monomer Component IV hemoglobin; GMH4CN, native cyanide-ligated *Glycera dibranchiata* monomer Component IV methemoglobin; DrecGMH4CN, completely deuterated *Glycera dibranchiata* recombinant cyanide-ligated monomer Component IV methemoglobin.

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and overwhelm any hyperfine-shifted resonances that occur there. Whereas ^{15}N and ^{13}C isotope enrichments combined with heteronuclear multidimensional NMR methods have proven beneficial for assigning resonances in diamagnetic molecules, these methods have yet to be widely applied to strongly hyperfine-shifted resonances where nuclear T_1s may be shorter than 100 ms. Therefore, it would be advantageous to have a method for selectively improving the visibility and detectability of hyperfine-shifted resonances which appear in the crowded −2 to 11 ppm proton shift region. In this work, we describe the production of a fully deuterated myoglobin-like protein, the Component IV monomer hemoglobin from *Glycera dibranchiata*, and present initial results in its use to achieve spectral simplification for the purposes of assigning heme proton hyperfine resonances, improving assignment efficiency, and minimizing assignment ambiguities.

This monomer hemoglobin has been extensively studied in this laboratory[16−24] to the stage where recently we have reported its primary sequence and a modeled structure[17] and expression of the recombinant native protein.[24] In the work reported here, we have found not only that the recombinant Component IV globin expressed in highly deuterated media (DrecGMG4) has advantages for making proton resonance assignments of a reconstitutable prosthetic group, in this case heme b, but also that careful protein handling can lead to enhanced opportunities for making other proton assignments as well.

To place this work in context, it is important to realize that random fractional deuteration and selective deuteration of proteins have been used since 1968[25,26] to simplify spectra and facilitate assignments. Random fractional deuteration of proteins has been carried out to the extent of 25−75%,[27−30] and incorporating selectively deuterated amino acids into expressed proteins not only has aided making assignments but also has been used to resolve stereochemical questions.[26,28,31−34] However, there are fewer reports of fully deuterated proteins being employed in NMR studies.[34−37]

Experimental Section

Materials. The recombinant expression system for the production of recGMG4 in the *E. coli* strain BL21(DE3) pLysS pET3d:recGMG4 was previously described.[24] Fully deuterated recGMG4 was created by growing the bacteria and inducing protein expression in deuterated algal hydrolysate dissolved in 99.9% D_2O, purchased from Martek Corp. (Columbia, MD). The host bacteria were first adapted for growth in a deuterated environment before expression experiments were carried out. This was accomplished by growing the bacteria in four steps of increasing levels of deuteration in Martek's algal medium (50%, 70%, 90%, and 98%). At each step the bacteria were incubated in a liquid medium with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol at 37 °C, until the A_{600} was approximately 1, at which time an aliquot was diluted 100-fold into media of the next highest deuteration level. Growth was slowed by about 2-fold in the deuterated medium.

A 2 L culture of BL21(DE3) pLysS pET3d:recGMG4 was grown at 37 °C in deuterated algal hydrolysate (98% D Celtone; Martek Corp.) containing 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. Growth was initiated with a 1:100 dilution of a 98% deuterium-adapted bacterial starter culture. Expression of the DrecGMG4 (apoprotein) was induced with the addition of isopropyl β-D-thiogalactopyranoside (IPTG, from a sterile D_2O stock) to a final concentration of 0.4 mM, after the A_{600} reached 0.8. At this time the incubation temperature was lowered to 25 °C to increase production of soluble protein as previously discussed.[24] The cells were incubated at this temperature for 20 h to a final A_{600} of 3.0. Heme constitution and purification of the deuterated recGMH4 protein were identical to those of the protonated protein.[24]

NMR Spectroscopy. The cyanide-ligated form of the deuterated recGMH4 (DrecGMH4CN) was prepared as previously described[14] and was exchanged into the final 100 mM potassium phosphate, 100 mM potassium chloride, and 20 mM potassium cyanide buffer (99.99% D_2O from Isotec, pH′ = 6.42) using an ultrafiltration cell (Amicon). pH′ refers to the direct pH measurement in D_2O not taking into account the deuterium isotope effect. Due to a lower expression level of recGMH4 in the D_2O medium, the final DrecGMH4CN concentration in the NMR sample was approximately 260 μM.

All NMR spectra were collected on a Varian VXR500s spectrometer at temperatures ranging from 15 to 25 °C. 2D homonuclear magnitude and phase-sensitive 2D correlation spectra (MCOSY and PSCOSY)[38,39] were collected by averaging 512 transients for each of 256 t_1 increments over a 16 000 Hz sweep width. Each t_1 block consisted of 2048 complex points. Residual water was saturated with the decoupler during the relaxation delay period, and the experiment was recycled at a repetition rate of 4 s^{-1}.

Proton homonuclear phase-sensitive 2D NOE (NOESY)[40] spectra were collected over the same 16 000 Hz sweep width also using 2048 complex t_2 points for each of 256 t_1 blocks. In each t_1 block, 512 transients were averaged and quadrature detection along t_1 was achieved using the States−Haberkorn−Reuben method.[41] Mixing times at 25 °C were varied from 20 to 100 ms. NOESY spectra were collected over the 15−25 °C temperature range using a 40 ms mixing time to document temperature dependencies. A recycle rate of 5 s^{-1} was used for each spectrum. In all experiments the residual water resonance was saturated using the decoupler during the relaxation delay and mixing periods. SUPERWEFT spectra were also collected at each temperature as previously described.[16] A description of the data processing employed here has been outlined in our work on naturally protonated GMH4CN, where assignment strategies and results for the heme protons in the fully protonated form of the native GMH4CN have

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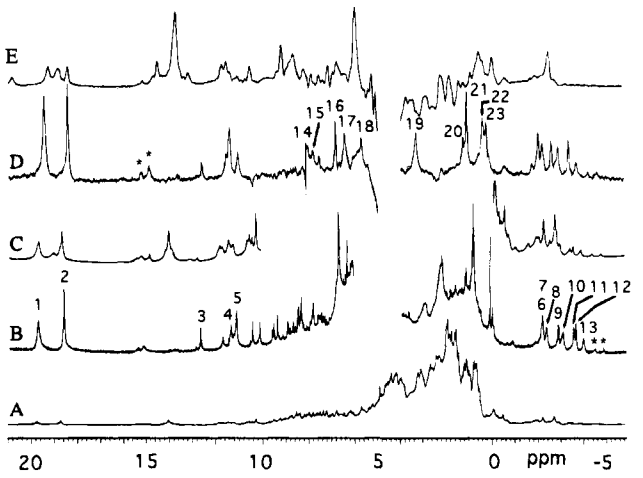

Figure 1. One-dimensional 500 MHz ^{1}H NMR comparison of native GMH4CN (^{1}H natural isotopic abundance) and DrecGMH4CN. (A) Entire one-dimensional spectrum of the the naturally protonated native GMH4CN recorded at 20 °C while buffered in 100 mM potassium phosphate, 100 mM potassium chloride, and 20 mM potassium cyanide in 99.99% D_2O (pH′ = 6.42). This was scaled so that the 0−2 ppm region had approximately the same intensity as the largest peak in part B. (B) DrecGMH4CN 1D spectrum taken at temperature and buffering conditions identical to those of A. (C) High- and low-frequency vertical scale expansions of the 1D NMR spectrum of A. (D) Partially relaxed spectrum of DrecGMH4CN recorded at 25 °C. (E) Partially relaxed spectrum of the naturally protonated native GMH4CN at 25 °C. All heme proton assignments are visible in the 1D spectra of the deuterated protein and are presented above parts B and D as follows: (1) $8CH_3$; (2) $3CH_3$; (3) $H90N_p$-H; (4) 4α; (5) 7α1; (6) 6β1; (7) 2βt; (8) 7β1; (9) 4βt; (10) 7β2; (11) 2βc; (12) 4βc; (13) 6α2; (14) 6α1; (15) 2α; (16) $5CH_3$; (17) β-meso; (18) 7α2; (19) δ-meso; (20) 6β2; (21) $1CH_3$; (22) α-meso; (23) γ-meso. Asterisks indicate minor heme isomer resonances. Individual peak assignment numbers correspond to the numbers in Table 1.

been discussed in detail.[16] All chemical shifts were referenced to the residual water resonance which was assigned the value of 4.7 ppm.

Results and Discussion

Brief Background. The *Glycera dibranchiata* monomer hemoglobins are a group of three major monomeric hemoglobins.[20] Component IV (GMH4) is one of these proteins, and a crystal structure of a related *Glycera dibranchiata* monomer hemoglobin has been published[42] which reveals the structural similarity of this class to myoglobin.[17] Despite this similarity and the fact that both sperm whale MbCN and GMH4CN are low-spin ferriheme proteins, the proton shift dispersion of the GMH4CN proton spectrum encompasses only ~60% of the dispersion of the sperm whale MbCN spectrum.[16] This further complicates making proton resonance assignments in GMH4CN because, compared to MbCN, a nearly identical number of hyperfine-shifted resonances are found in a much smaller chemical shift region.[16] Despite these complicating factors, we devoted great effort over the course of two years in order to obtain a self-consistent set of one- and two-dimensional homonuclear proton NMR assignments for the heme and axial ligand (His90) protons of GMH4CN.[16] At the completion of that work, we felt uncertain about only two assignments.

Such uncertainties present a problem for many heme proteins. We recognize that this is also potentially a problem for other proteins with noncovalently bound prosthetic groups for which NMR assignments for the prosthetic group are essential as a means of studying the active site. As one solution for removing assignment ambiguities, we have produced an extensively deuterated, recombinant Component IV globin (GMG4), then constituted it with heme b carrying the natural isotopic abundance of hydrogen in order to form the corresponding holoprotein, DrecGMH4.

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Table 1. ^{1}H NMR Chemical Shifts for the Heme Proton Assignments of Perdeuterated recGMH4CN in ^2H_2O

resonance	Figure 1 label	chemical shift (ppm)[a]
$1CH_3$	21	0.98
$3CH_3$	2	18.28
$5CH_3$	16	6.72
$8CH_3$	1	19.30
2α	15	7.71
2βc	11	−3.37[b]
2βt	7	−2.06[b]
4α	4	11.28
4βc	12	−3.37[b]
4βt	9	−2.71
6α1	14	7.94
6α2	13	−3.81
6β1	6	−2.04[b]
6β2	20	1.13
7α1	5	10.92
7α2	18	5.60
7β1	8	−2.32
7β2	10	−3.00
α-meso	22	0.28
β-meso	17	6.33
γ-meso	23	0.12
δ-meso	19	3.19
$H90N_p$-H	3	12.48

[a] Chemical shift in ppm measured at 25 °C in 99.99% D_2O buffered with 100 mM potassium phosphate and 100 mM potassium chloride at pH′ of 6.42. All chemical shifts were measured using the partially relaxed 1D spectrum of Figure 1D, unless indicated by a *b* superscript. [b] Chemical shifts (ppm) were measured using a 2D NOESY spectrum (Figure 4) when overlap was evident in the 1D ^{1}H spectrum (Figure 1).

One-Dimensional Proton NMR. One-dimensional proton NMR spectra comparing protonated GMH4CN (i.e., natural isotopic abundance hydrogen) and DrecGMH4CN in identical D_2O buffer solutions, at 20 °C, are shown in Figure 1. Figure 1A is a complete proton spectrum of GMH4CN, while Figure 1B shows the spectrum of DrecGMH4CN plotted so that the tallest resonance corresponds to the tallest intensity in Figure 1A. An appreciation of the extent of deuteration (we estimate ~90%) is found by comparing parts A and B. Figure 1C is a vertically expanded plot of the hyperfine shift regions of Figure 1A. Comparing parts B and C also indicates which hyperfine-shifted resonances correspond to the globin, as opposed to the heme, by their absence in Figure 1B.

Figure 1D presents the partially relaxed (i.e., SUPERWEFT) spectrum of DrecGMH4CN at 25 °C, and Figure 1E is the corresponding spectrum for GMH4CN. Clearly, several of the fastest relaxing resonances that dominate spectrum E for native GMH4CN are absent in the spectrum of the deuterated protein, indicating that they belong to protons of amino acids within the active site.

Assignments, indicated by numbers above the resonances in Figure 1B,D, are given in the caption to Figure 1 and in Table 1. Although there are the expected temperature-dependent differences between the spectra in parts B (20 °C) and D (25 °C), the resonance correspondence should be clear. These two spectra reveal that the extent of deuteration in DrecGMH4CN is suffiently large so that most of the heme proton resonances can be clearly identified in the absorption spectrum (Figure 1B). Noteworthy is the fact that all four heme meso proton resonances can be found in these spectra (peaks labeled 17, 19, 22, and 23 in Figure 1D), which to our knowledge is the first time direct

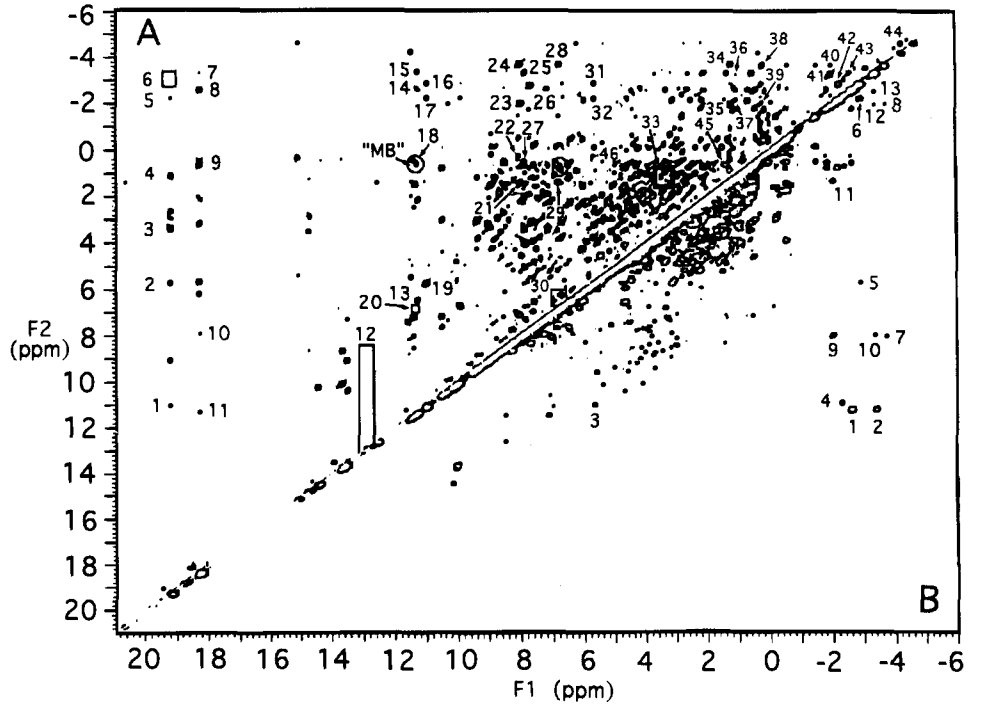

Figure 2. Summary of previous heme proton assignments of GMH4CN using the naturally protonated form of the native protein presented as a composite NOESY/COSY spectrum at 25 °C. (A) A homonuclear NOESY contour spectrum using a 40 ms mixing time and the same buffering conditions presented in Figure 1. All intra- and inter-pyrrole NOE connectivities used for heme assignments are presented above the diagonal. Note that the numbers used to identify the cross peak assignments in this figure do not correspond to the numbers of the individual proton resonances found in Figure 1 and Table 1. Cross peak assignments are as follows: (1) $8CH_3-7\alpha1$; (2) $8CH_3-7\alpha2$; (3) $8CH_3-\delta$-meso; (4) $8CH_3-1CH_3$; (5) $8CH_3-7\beta1$; (6) $8CH_3-7\beta2$; (7) $3CH_3-4\beta c$; (8) $3CH_3-4\beta t$; (9) $3CH_3-\alpha$-meso; (10) $3CH_3-2\alpha$; (11) $3CH_3-4\alpha$; (12) $H90N_p$-H−89,91 NH not previously observed; (13) $4\alpha-\beta$-meso; (14) $4\alpha-4\beta t$; (15) $4\alpha-4\beta c$; (16) $7\alpha1-7\beta2$; (17) $7\alpha1-7\beta1$; (18) $7\alpha1-\gamma$-meso; (19) $7\alpha1-7\alpha2$; (20) $4\alpha-5CH_3$; (21) $6\alpha1-6\beta2$; (22) $6\alpha1-\gamma$-meso; (23) $6\alpha1-6\beta1$; (24) $6\alpha1-6\alpha2$; (25) $2\alpha-2\beta c$; (26) $2\alpha-2\beta t$; (27) $2\alpha-\alpha$-meso; (28) $5CH_3-6\alpha2$; (29) $5CH_3-6\beta2$; (30) β-meso$-5CH_3$; (31) $7\beta2-7\alpha2$; (32) $7\beta1-7\alpha2$; (33) $1CH_3-\delta$-meso; (34) $6\alpha2-6\beta2$; (35) $6\beta1-6\beta2$; (36) $1CH_3-2\beta c$; (37) $1CH_3-2\beta t$; (38) γ-meso$-6\alpha2$; (39) γ-meso$-6\beta1$; (40) $6\alpha2-6\beta1$; (41) $2\beta c-2\beta t$; (42) $7\beta1-7\beta2$; (43) $4\beta c-4\beta t$; (44) minor $2\beta c-2\beta t$; (45) γ-meso$-6\beta2$; (46) γ-meso$-7\alpha2$. Boxes indicate NOES observed at lower contour levels, while circled cross peaks show connectivities that were originally used for the β-meso assignment (MB) (ref 16). (B) Homonuclear MCOSY contour spectrum of the protonated GMH4CN. Conditions identical to those in Figure 1A. All heme correlations are labeled below the diagonal. Cross peak assignments are as follows: (1) $4\alpha-4\beta t$; (2) $4\alpha-4\beta c$; (3) $7\alpha1-7\alpha2$; (4) $7\alpha1-7\beta1$; (5) $7\alpha2-7\beta2$; (6) $7\beta1-7\beta2$; (7) $6\alpha1-6\alpha2$; (8) $6\alpha2-6\beta1$; (9) $2\alpha-2\beta t$; (10) $2\alpha-2\beta c$; (11) $6\beta1-6\beta2$; (12) $2\beta t-2\beta c$; (13) $4\beta t-4\beta c$.

observation of all four of these resonances has been reported for a low-spin ferriheme protein.

Two-Dimensional Proton NMR. As a tool for simplifying the process of completely identifying prosthetic group resonances, the value of using an extensively deuterated protein must be judged in the context of the spectra given by the more complicated (i.e., naturally protonated) native protein. For this purpose we present the split-diagonal (proton homonuclear) NOESY/COSY contour plot for native GMH4CN (natural isotope abundance hydrogen) in Figure 2 and the corresponding NOESY/COSY contour plot of DrecGMH4CN in Figure 3. In Figures 2 and 3, the assignments for naturally protonated native GMH4CN[16] and the recombinant perdeuterated GMH4CN (DrecGMH4CN), respectively, are indicated by numbered cross peaks, for which the proton assignments are defined in each figure caption. Note that there is no correlation between the numbers used to identify individual resonances shown in Figure 1 and Table 1 and the numbers used to identify the cross peaks in Figures 2 and 3.

The reduction of cross peak density demonstrated in Figure 3 is dramatic in comparison to that of Figure 2. Heme proton resonance connectivities are immediately obvious in Figure 3, free from the uncertainties and complications caused by the dense crosspeak regions of the native protein (Figure 2). In this case, making unambiguous heme hyperfine proton resonance assignments is exceedingly straightforward and efficient. All heme proton assignments were apparent with 24 h of NMR time using DrecGMH4CN. Although demonstrating the utility of using a deuterated protein is the main goal of this work, this work has confirmed all of the previous assignments,[16] except for the resonance of the β-meso proton, which this work has revealed was previously misassigned.

Conclusions

Our results demonstrate the utility of extensively deuterated proteins for making rapid, unambiguous prosthetic group assignments. Although we have specifically applied this to a paramagnetic heme protein, in principle, this approach could be employed for any protein with a removable prosthetic group or for an enzyme−substrate (or substrate analog) complex.[35,37]

Production of this extensively deuterated globin was straightforward but rather expensive. We are refining this procedure, and it appears that we will be able to optimize production of normal NMR quantities of completely deuterated protein for about one-third the cost of producing this sample ($3600). Economy may also be achieved by producing proteins with lower deuteration levels, and experiments are in progress to determine optimum levels of isotope incorporation.

There is obvious reduction in the dynamic range of the completely deuterated sample (Figure 1B). Dynamic range limitation is a problem for studies of the broad, fast relaxing hyperfine-shifted resonances of paramagnetic proteins. The problem in detectability lies not in the peak-integrated intensity

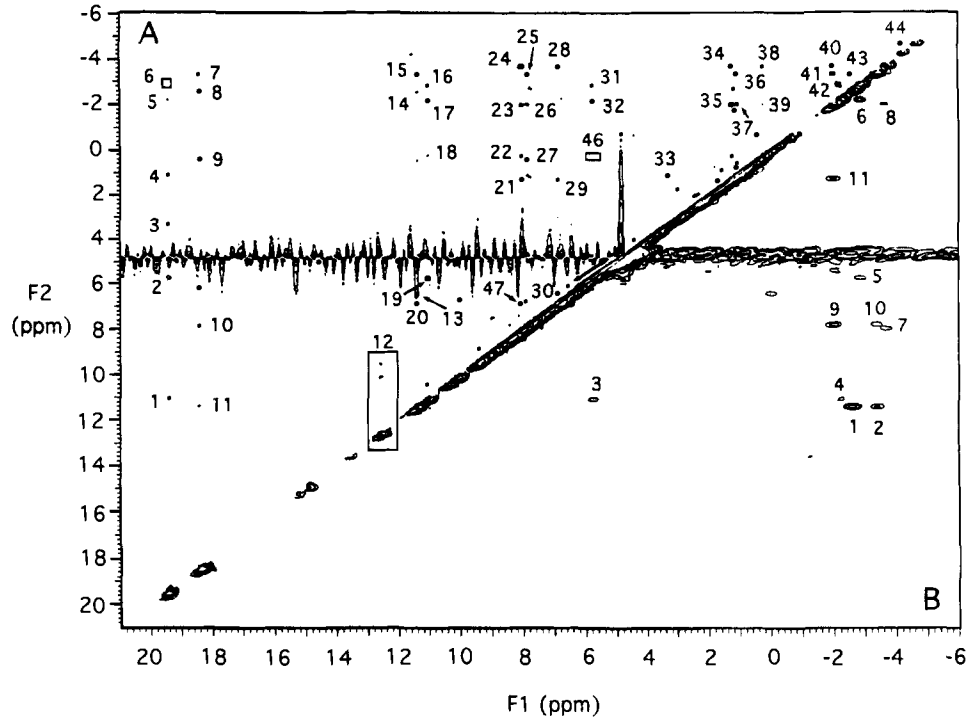

Figure 3. Composite NOESY/COSY contour plot (unsymmetrized) for a 260 μM solution of DrecGMH4CN at conditions identical to Figure 2. (A) Homonuclear $^1H-^1H$ NOESY contour plot of DrecGMH4CN, using a 100 ms mixing time. All intra- and inter-pyrrole NOES are labeled identically to those depicted in Figure 2A. The following additional cross peaks (with assignments) appear in this spectrum: (12) $H90N_p$-H−89-NH and −91-NH, not previously observed; (47) 6α2−5CH_3. (B) Homonuclear $^1H-^1H$ MCOSY contour plot (unsymmetrized) of DrecGMH4CN. All correlations are numbered identically to Figure 2B, with correlations 12 and 13 being observed at low contour levels. Note that the numbers used to identify the cross peak assignments in both sections of this figure do correspond to those in Figure 2 but do not correspond to the numbers of the individual proton resonances found in Figure 1 and Table 1.

but in the peak height/baseline noise ratio. This ratio is significantly lower in the broad hyperfine-shifted proton resonances than it is in narrower, diamagnetic resonances.[8]

This method could be used effectively with lower-field (hence less costly) NMR spectrometers. These initial results also show that using an extensively deuterated heme protein will be advantageous for making heme proton resonance assignments in larger proteins with larger paramagnetic moments, such as hemoglobin (~64 kD) and the heme peroxidases (~34−50 kD). In these cases, the Curie spin relaxation mechanism[3] severely broadens hyperfine resonances at higher fields so that these proteins are often optimally studied at lower fields, with accompanying lower proton resonance shift dispersion.

We believe that extensively deuterated proteins may also find use in protein folding studies. Our optimism in this case stems from Figure 1B where one can see many individual sharp resonances in the amide proton region (11−6.5 ppm). Initially we were puzzled by this residual proton intensity given that the protein was maintained in a 99.99% D_2O buffer solution for NMR experiments. However, in our purification procedure, the recombinant deuterated globin was initially dialyzed in H_2O solutions prior to heme reconstitution, purification, and transfer into the 99.99% D_2O buffer solution for NMR spectroscopy. We have concluded that residual amide proton intensity occurs because those amide protons were originally fast-exchanging while the partially unfolded globin was dialyzed in a H_2O buffer solution, and so the amide protons were exchanged with solvent protons. However, these same protons evidently became slow-exchanging in the refolded holoprotein and were not subsequently exchanged with deuterons when the protein was maintained in the D_2O buffer solution for NMR experiments. Extensively deuterated protein may thus be useful in NMR/pulsed hydrogen exchange investigations of protein folding.[43-45]

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