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An $^1H-^{13}C-^{13}C$-Edited ^1H NMR Experiment for Making Resonance Assignments in the Active Site of Heme Proteins

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In paramagnetic heme proteins, it is often problematic to make proton resonance assignments for heme substituents that do not have large isotropic shifts and consequently lie under the large envelope of polypeptide resonances. Furthermore, assignments that would normally be performed with the aid of HMBC experiments in diamagnetic molecules can prove difficult in the active site of paramagnetic heme proteins if $T_2^{-1} > {}^2J_{CH}$. To circumvent this problem, a new method is presented to selectively detect ^1H in $^1H_n-^{13}C-^{13}C$ fragments biosynthetically introduced into the active site of heme proteins. The pulse sequence combines well-known building blocks such as INEPT to transfer ^1H spin magnetization to bonded ^{13}C nuclei, followed by INADEQUATE to generate $^{13}C-^{13}C$ double-quantum coherence that is selected with pulsed field gradients, and finally reverse-INEPT to transfer magnetization back to ^1H nuclei for subsequent observation. The new $^1H_n-^{13}C-^{13}C$ edited experiment takes advantage of the relatively large values of $^1J_{CH}$ and $^1J_{CC}$, avoiding the long interpulse delays in HMBC that compromise the detectability of rapidly relaxing nuclei. The potential applicability of the pulse sequence is demonstrated by its contribution to the unambiguous assignment of the carbonyl carbons in the heme propionates of ferricytochrome b_5.
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NMR spectroscopy is now well established as a powerful technique for the study of structure–function relationships of proteins in solution. In the case of paramagnetic heme proteins, NMR is able to provide unique structural information on residues near the heme active site because of the large hyperfine shifts that result from unpaired electron density (*1–4*). Nevertheless, ^1H NMR spectroscopy of paramagnetic proteins has some fundamental limitations. (a) As a result of heme axial ligation, unpaired electron density is asymmetrically distributed on the porphyrin macrocycle, resulting in large isotropic shifts for some of the heme substituents but small-to-negligible isotropic shifts for others (*5*). Those heme substituents displaying small isotropic shifts have resonances that are buried under the large envelope of resonances arising from the polypeptide, therefore making their assignments difficult. (b) Heme substituents in the reduced (usually diamagnetic) state lack isotropic shifts and are therefore difficult to examine by NMR spectroscopy. Observation of these resonances is important in order to study structure–function relationships in electron-transfer heme proteins, which function by shuttling their heme iron between ferric and ferrous oxidation states. (c) Efficient spin–spin relaxation often makes through-bond proton–proton correlations in COSY or TOCSY experiments unobservable ($T_2^{-1} > J_{HH}$) (*2, 6*). In fact, it has been proposed that the COSY cross peaks observed in paramagnetic systems arise from dipolar coupling and Curie spin–nuclear spin relaxation (*7, 8*).

One strategy designed to circumvent some of these problems involves the selective ^{13}C enrichment of heme substituents and their subsequent observation by ^{13}C NMR spectroscopy. We have recently reported a biosynthetic method for the expression of ^{13}C-labeled heme cytochrome b_5 (*9*), thus facilitating NMR experiments such as HMQC and HSQC (*10–14*) that rely on the heteronuclear through-bond connectivity and larger scalar coupling constant $^1J_{CH}$. However, assignment of the carbonyl carbon resonances in heme propionates, which would ordinarily be made using an HMBC experiment (*15*), can be stymied if $T_2^{-1} > {}^2J_{CH}$. As will be detailed below, the constant-time HCACO experiment (*16, 17*) may not offer an efficient alternative for making these assignments. In this report, a new method for selective ^1H detection of $^1H_n-^{13}C-^{13}C$ fragments from among a large number of overlapping protein resonances is illustrated for a test mixture of $H_3{}^{13}C{}^{13}COO^-$ with sucrose and for a sample of ferricytochrome b_5 in which $^{13}CH_2{}^{13}COO^-$ fragments were introduced biosynthetically into the heme propionates. The utility of the experiment for establishing resonance assignments of the heme propionate carbonyl carbons is also discussed.

As shown in Fig. 1, the pulse sequence for the one-dimensional $^1H_n-^{13}C_1-^{13}C_2$-edited experiment combines the well-known INEPT sequence (*18*) to transfer ^1H spin magnetization to bonded ^{13}C nuclei, INADEQUATE to generate ^{13}C double-quantum coherence between directly bound ^{13}C's (*19*), and reverse INEPT to detect the results through the

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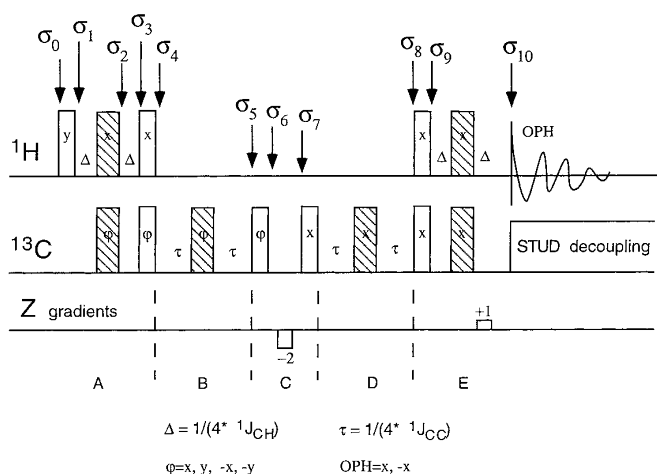


FIG. 1. Pulse sequence used for selective detection of $^{1}H_{n}-^{13}C-^{13}C$ fragments, including INEPT (A) and reverse-INEPT (E) polarization transfer steps, evolution of J_{CC} and refocusing of J_{CH} (B) and (D), and excitation of ^{13}C double-quantum coherence and conversion to observable single-quantum coherence (C). Pulse spacings of $\Delta = 1.5$ ms and $\tau = 5.0$ ms were set by assuming values for J_{CH} and J_{CC} of 167 and 50 Hz, respectively. The 500-μs pulsed field gradient in the middle of the sequence was typically preceded (2 μs) and followed (50 μs) by 90° pulses on ^{13}C. The spin density operators (σ_i) calculated at the indicated time points are available from the authors.

sensitive ^{1}H nuclei. Inspired by the *t*riple-*r*esonance *i*sotope-*ed*ited (TRIED) strategy described recently for NMR of $^{1}H-^{13}C-^{15}N$ fragments in dual-labeled metabolites within complex plant cell extracts (*20*), our 1D double-resonance experiment (DRIED) also draws upon the precedents of several related 2D and 3D INEPT–INADEQUATE experiments (*21–29*).

After an INEPT transfer of polarization from each ^{1}H to its bonded $^{13}C_1$, a delay of $2\tau = 1/2J_{CC}$ permits the development of antiphase ^{13}C coherence with respect to the directly bonded $^{13}C_2$, and midway through this period a 180° pulse on ^{13}C refocuses the one-bond CH scalar couplings. The central (INADEQUATE) section of the pulse sequence uses a 90° pulse on ^{13}C to excite double-quantum (DQ) and zero-quantum coherence, a pulsed field gradient (PFG) to dephase all coherences, and a second 90° pulse on ^{13}C to convert the (dephased) DQ to single-quantum coherence. The first two portions of the scheme are then executed in reverse, as the antiphase ^{13}C coherence is rephased, one-bond CH scalar couplings are again refocused, and a reverse-INEPT sequence transfers the ^{13}C magnetization back to ^{1}H for subsequent detection. Since the final PFG has opposite sign and half the magnitude of the first one, it rephases only the ^{13}C double-quantum coherence created at the beginning of the INADEQUATE sequence (*28*).

The new $^{1}H_{n}-^{13}C-^{13}C$-edited experiment provides a more generally useful alternative to HMBC experiments on heme proteins, since it relies solely on the larger coupling constants $^{1}J_{CH}$ and $^{1}J_{CC}$ for coherence transfer rather than on the smaller two-bond coupling $^{2}J_{CH}$ that may exceed T_2^{-1}. If a mixture of HCC, H_2CC, and H_3CC fragments is to be examined, use of the same editing sequence with a compromise setting of the time period Δ should yield nearly optimal signal intensities (*30*). By using PFGs to select only the desired DQ coherences arising from $^{13}C-^{13}C$ pairs, it is also possible to avoid the incomplete cancellation and dynamic range limitations encountered when phase cycling is used to remove large unwanted signals from ^{1}H's attached to ^{12}C, albeit with a $\sqrt{2}$ loss in sensitivity (*13*).

The DRIED experiment resembles a recently demonstrated 2D NMR scheme for ^{1}H-detected $^{13}C-^{13}C$ double-quantum coherence (*26*), with several modifications tailored to structural investigations of paramagnetic species. First, the evolution period (and refocusing π pulse on protons) is omitted: our 1D experiment requires no measures to spread the signals along an additional ^{13}C chemical shift axis, and incorporation of an evolution period might lead to signal losses for a chemical system with rapid transverse relaxation. For applications to heme proteins such as the biosynthetically labeled material described below, the samples are typically dilute solutions containing a limited number of spectrally resolved HCC fragments, allowing us to obtain the desired spectral information efficiently with a 1D version of the experiment. Second, improved capabilities for gradient performance make it possible to defocus the double-quantum and zero-quantum coherences using a single PFG rather than an oppositely signed pair separated by a 180° pulse on ^{13}C. Optimal implementation of this PFG scheme is achieved when the probe has short gradient recovery times (≤ 50 μs), minimizing relaxation-related losses and chemical shift evolution.

The pulse sequence was tested initially with a solution consisting of 5 mM sodium [1,2-^{13}C]acetate and 1 M sucrose, as shown in Fig. 2. The NMR experiments were carried out on a Varian Unity*plus* spectrometer (Varian Instruments, Palo Alto, CA) operating at a ^{1}H frequency of 600 MHz. Broadband ^{13}C decoupling was accomplished using an optimized STUD adiabatic pulse sequence (*31*). Although methyl protons from the doubly labeled acetate were barely visible among the numerous strong sucrose signals using traditional ^{1}H NMR methods, editing with the $^{1}H-^{13}C-^{13}C$ double-quantum sequence produced a simple spectrum in which only the methyl group of $^{13}CH_3{}^{13}COO^-$ was visible. Since the natural-abundance sucrose spins were not being detected, it was possible to adjust the spectrometer gain to detect the acetate signals at maximum sensitivity. If imperfections in the pulses and PFGs were negligible, it would be possible in principle to observe the acetate signals selectively at dilutions down to 1 part in ~8000; at this level the natural-abundance $^{13}C-^{13}C$ spin pairs would become comparable in number to the isotopically labeled species.

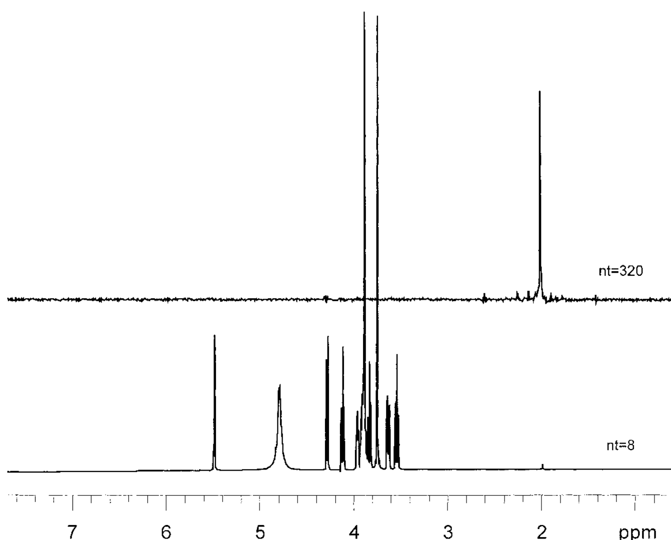


FIG. 2. 600-MHz ^{1}H NMR spectra obtained at 30°C for a test mixture of 5 mM $^{13}CH_3{}^{13}COO^{-}Na^{+}$ (99.3 at.% ^{13}C from Merck Isotopes) in 1 M sucrose (from Aldrich) obtained with a Varian Unity*plus* spectrometer and a 5-mm Varian triple-resonance probe running with *Z* gradients of 33 G/cm. (Bottom) Traditional one-pulse ^{1}H spectrum. (Top) ^{1}H–^{13}C–^{13}C-edited ^{1}H spectrum. Both spectra are referenced to the HOD peak at 4.8 ppm.

A more practical demonstration of the DRIED experiment used ^{13}C-labeled heme ferricytochrome b_5, as shown in Fig. 3. In this latter application, biosynthetic procedures (*9*) were used to produce $^{13}CH_3$–, $^{13}CH_2$=CH–, and $^{13}CH_2{}^{13}COO$– labeled moieties within each heme (>85% labeled). These NMR experiments were carried out both at 600 MHz on a Varian Unity*plus* instrument and at 400 MHz on a Varian UnityINOVA spectrometer. The conventional ^{1}H spectrum displayed a number of broad resonances outside the customary diamagnetic envelope (0–10 ppm), a well-known consequence of unpaired electron density originating on the heme iron (*1–4*). Nevertheless, some resonances from the heme substituents did not have large isotropic shifts and were thus buried under the large envelope of signals from protons of the polypeptide. The DRIED sequence was employed to focus exclusively on those resonances arising from the heme propionate β-methylene protons within $^{13}CH_2{}^{13}COO$ fragments, excluding singly labeled $^{13}CH_3$ or $^{13}CH_2{=}^{12}CH$ groups and numerous ^{12}C-containing molecular entities. Without this ^{1}H–^{13}C–^{13}C-editing procedure, protons in the heme active site that resonate between 0 and 1.6 ppm would be unresolved from the remainder of the protein spectrum and thus difficult to assign. As detailed below, the more customary HSQC and HMBC experiments also fail to provide all of the connectivity information needed to complete the assignments in the heme active site.

In the present work, our goal was to determine the role played by outer mitochondrial (OM) cytochrome b_5 heme propionates in electrostatic binding to cytochrome c (*32*). To this end, it was necessary to assign the ^{13}C resonances from the heme propionate carbonyl carbons in the ferric (paramagnetic) oxidation state of cytochrome b_5. Two isomers A and B had to be considered, since the heme in cytochrome b_5 is bound to the polypeptide in either of two orientations related to one another by a 180° rotation about the porphyrin α,γ-meso axis (Fig. 4). For rat liver OM cytochrome b_5, the ratio of these orientations is 1:1 and thus most resonances that arise from the heme are doubled (*33*).

Since the A6α and A7α protons had been assigned previously (*33, 34*), an HSQC experiment on cytochrome b_5 labeled at the heme propionate α-carbons established that the ^{13}C resonances from methylenes A7α and B6α occurred at −18 and −22 ppm (Fig. 5a). Each of these α-carbons was shown to be directly bound to two diastereotopic protons that resonate near 16 and −2 ppm. An HMBC experiment conducted on the same sample correlated these carbons to ^{1}H resonances A7Hβ and B6Hβ at 1.50 and 1.63 ppm (Fig. 5b). Similarly, HSQC methods confirmed the assignment of carbons A6α and B7α at −46 ppm, but HMBC failed to reveal correlations with A6Hβ or B7Hβ.

Additional information was obtained from a sample of ferricytochrome b_5 labeled at both the heme propionate carbonyl and the heme propionate β-carbons, as shown in Fig. 6. An HSQC experiment (Fig. 6a) correlated the aforementioned resonances at 1.50 and 1.63 ppm (A7Hβ and B6Hβ) to carbons resonating at 92 and 97 ppm, thus identifying them as A7β and B6β. It is noteworthy, how-

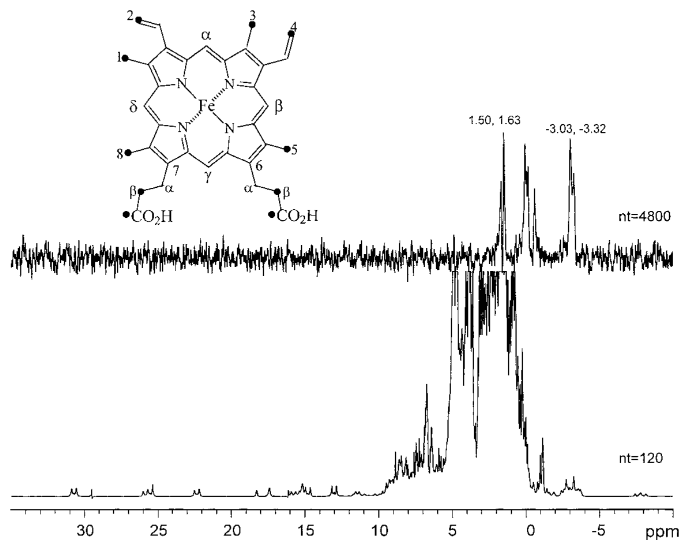


FIG. 3. 400-MHz ^{1}H NMR spectra obtained at 27°C for 3 mM >85% ^{13}C-labeled mitochondrial heme cytochrome b_5, obtained with a Varian UnityINOVA spectrometer and a 5-mm Nalorac inverse-detection probe running with *Z* gradients of 30 G/cm. The positions of the ^{13}C labels are highlighted (●). (Bottom) Traditional one-pulse ^{1}H spectrum, obtained with an acquisition time of 400 ms and a relaxation delay of 700 ms. (Top) ^{1}H–^{13}C–^{13}C-edited ^{1}H spectrum, obtained with an acquisition time of 400 ms and a relaxation delay of 400 ms. Both spectra are referenced to the HOD peak at 4.8 ppm.

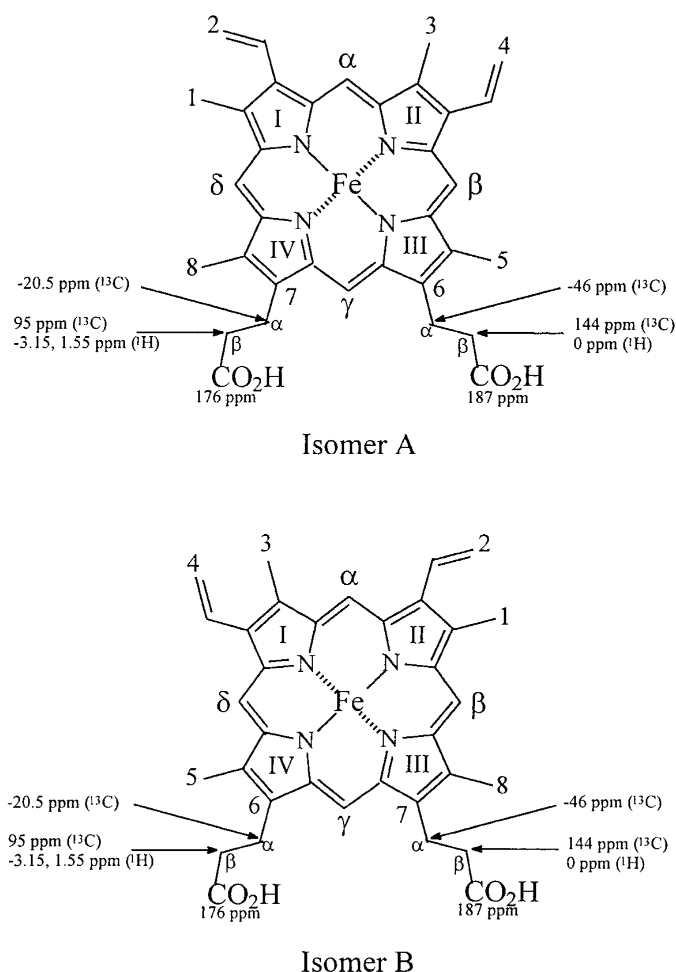
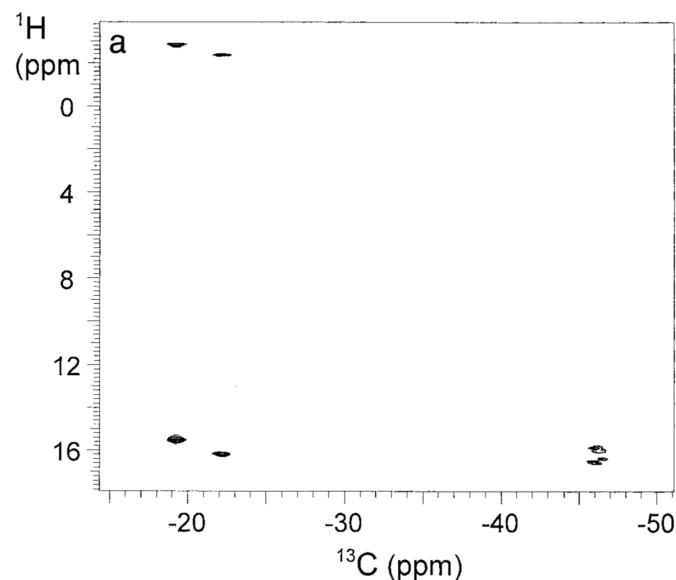


FIG. 4. Isomers corresponding to two heme orientations for cytochrome b_5 in solution. The chemical shift assignments, made as described in the text, are indicated for carbon nuclei and for two sets of diastereotopic protons. For cases such as A7α and B6α, which correspond to -22 and -18 ppm but cannot be distinguished further, average values of the chemical shift are quoted for both nuclei.

ever, that the HSQC spectrum shown in Fig. 6a displayed an additional set of cross peaks at -3.03 and -3.32 ppm. Thus it appeared that the A7Hβ and B6Hβ protons of the doubly labeled heme displayed HSQC cross peaks which were absent from HMBC spectra of ferricytochrome b_5 labeled at the heme propionate α-carbons (Fig. 5b). Furthermore, HMBC spectra of ferricytochrome b_5 labeled at the carbonyl and β-carbons of the heme propionate did not show long-range correlations. In light of the relatively long interpulse delays required for HMBC experiments ($1/[2*{}^nJ_{CH}]$) as compared with the rapid spin–spin relaxation rates of isotropically shifted heme resonances, such results were in fact reasonable.

In order to resolve these ambiguities and corroborate the full set of resonance assignments, the DRIED experiment was used to selectively detect ${}^1H_2$–${}^{13}C$–${}^{13}C$ fragments from among the envelope of overlapping ${}^1H$ resonances. The observation in Fig. 3 of the pairs of diastereotopic ${}^1H$ resonances at (1.63, -3.32 ppm) and (1.50, -3.03, ppm) confirmed directly that these signals correspond to heme propionate β-hydrogens, linked by HSQC to the A7 and B6 positions. The set of peaks centered at -3.15 ppm was broader than the set at 1.55 ppm, indicating a shorter value of T_2 and therefore providing a plausible explanation for their absence in the HMBC experiment (Fig. 5b). The DRIED results then allowed for definitive assignment of the



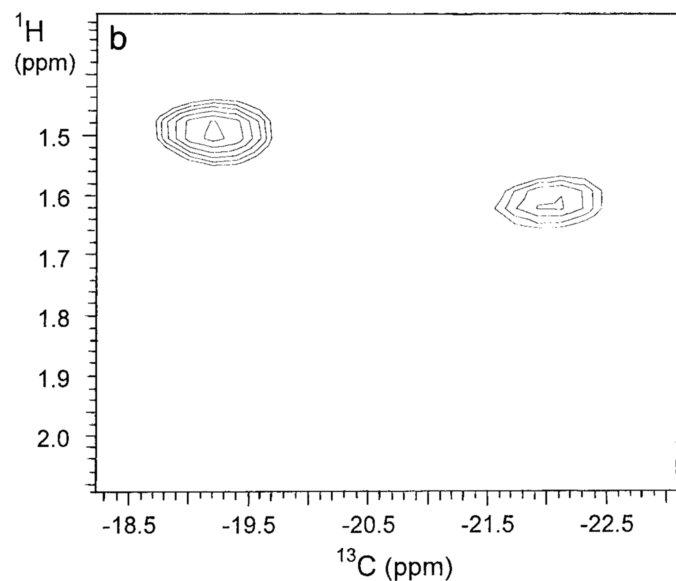


FIG. 5. ${}^1H$-detected through-bond correlated spectra for cytochrome b_5 labeled at the heme propionate α-carbons. (a) Portion of the HSQC spectrum for directly bonded ${}^1H$ and ${}^{13}C$ nuclei. (b) Portion of the HMBC spectrum for ${}^1H$ and ${}^{13}C$ separated by multiple bonds. The ${}^{13}C$ chemical shifts are referenced to the methyl groups of acetone set to 30.2 ppm.

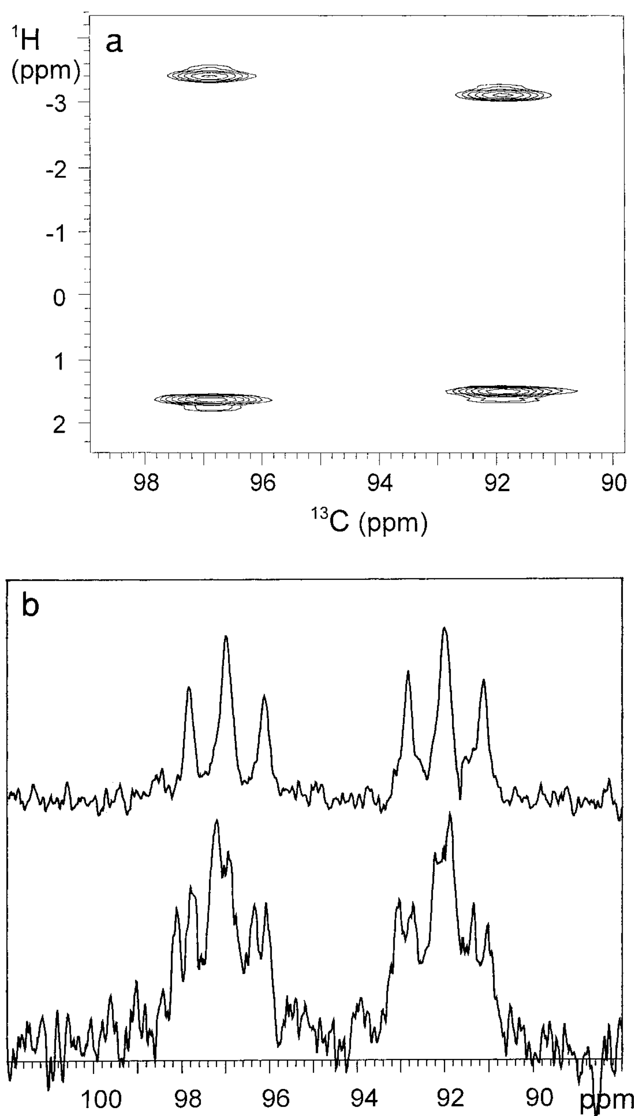


FIG. 6. Through-bond correlations and homodecoupling experiments for cytochrome b_5 labeled at both the heme propionate carbonyl and the heme propionate β-carbons. (a) HSQC. (b) ^{13}C irradiation of the carbonyls at 176 ppm simplifies the spectral patterns for the β-methylene carbon signals centered at 95 ppm.

resonances at 92 and 97 ppm to heme propionate β-methylene carbons; selective ^{13}C homodecoupling also established their connections to heme propionate carbonyls A7CO and B6CO at 176 ppm (Fig. 6b).

Also visible in the DRIED experiment were several peaks near 0 ppm, corresponding to methylene protons A6Hβ and B7Hβ. It is noteworthy that these protons were not detected with HMBC experiments. The A6Hβ and B7Hβ protons were then linked to methylene carbons A6β and B7β at 144 ppm via HSQC data, and to the carbonyl carbons A6CO and B7CO at 187 ppm by ^{13}C homodecoupling experiments (data not shown).

As illustrated previously with the TRIED NMR experiment (*20*), isotope-editing protocols can streamline the analytical characterization of low-level agricultural or pharmaceutical metabolites, avoiding laborious separation procedures, allowing functional group identification, and often achieving detection at submicrogram levels. In the current work with ^{1}H–^{13}C–^{13}C fragments, DRIED spectral editing permits high-sensitivity detection of doubly labeled heme propionates in the paramagnetic oxidation state of cytochrome b_5 and reveals spectral features that would otherwise be obscured by a forest of natural-abundance ^{1}H NMR signals. The lowest observable *absolute* concentration is determined as usual by overall spectrometer performance and the patience of the investigator. The lowest observable *relative* concentration of doubly ^{13}C-labeled species should be one part in 8000 if pulse errors are negligible, though this expectation will be compromised when the heme moieties of interest exhibit shorter T_2's (broader lines) than protons distant from the protein active site.

The scope and limitations of the DRIED experiment depend upon the spin-relaxation characteristics for the protons of interest in a particular paramagnetic heme protein. The spin–lattice relaxation rate is related to the spin state (S) of the metal, the proton–metal distance $(r_M)^{-6}$, and the electron spin-relaxation time (*35, 36*). For instance, for myoglobin with $S = \frac{1}{2}$ and protons that experience negligible contact shift, four zones have been defined (*2*): (i) $T_1 < 2$ ms if $r_M < 3.3$ A, (ii) 2 ms $< T_1 <$ 20 ms in the shell up to $r_M = 4.9$ A, (iii) 20 ms $< T_1 <$ 200 ms in the shell up to $r_M = 7.0$ A, and (iv) $T_1 > 200$ ms if $r_M > 7.0$ A. Larger paramagnetic contributions to spin–spin relaxation will be observed for proteins in which $S = \frac{5}{2}$. Since the typical time protons spend in the transverse plane during the DRIED experiment is ~27 ms, it is expected that protons may be observed in $S = \frac{1}{2}$ paramagnetic proteins if they are located in regions ii–iv and have T_2 values longer than 15 ms.

An alternative assignment protocol could be devised with 2D versions of the constant-time HCACO experiment (*16, 37*), though shortening the pulse delays to accommodate the short T_2's of typical heme proteins would result in very limited resolution for the CA and CO dimensions. The present work demonstrates the ease with which 85%-labeled HCC fragments may be observed and allows us to anticipate straightforward acquisition of DRIED spectra for isotopically labeled materials at the 10–40% levels typical of many biosynthetic procedures. Future uses of the DRIED experiment could also include drug metabolic studies in plant and animal systems (*38*), mechanistic investigations of bacterial biosynthesis (*25, 39, 40*), and molecular structure determinations for protective plant polymers (*41, 42*).

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