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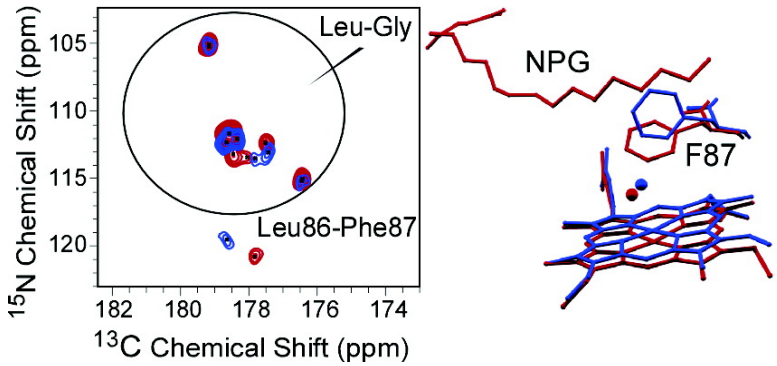

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Observation of Ligand Binding to Cytochrome P450 BM-3 by Means of Solid-State NMR Spectroscopy

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Abstract: A broad understanding of the binding modes of ligands and inhibitors to cytochrome P450 is vital for the development of new drugs. We investigated ligand binding in a site-specific fashion on cytochrome P450 BM-3 from *Bacillus megaterium*, a 119 kDa paramagnetic enzyme, using solid-state magic angle spinning nuclear magnetic resonance methods. Selective labeling and longitudinal relaxation effects were utilized to identify the peaks in a site-specific fashion and to provide evidence for binding. Well-resolved one-dimensional and two-dimensional NMR spectra of cytochrome P450 BM-3 reveal shifts upon binding of its substrate, *N*-palmitoylglycine. These data are consistent with the crystallographic result that a biochemically important amino acid residue, Phe87, moves upon ligation. This experimental scheme provides a tool for probing ligand binding for complex systems.

Introduction

Cytochrome P450 enzymes[1,2] carry out stereoselective and regioselective hydroxylation[3−5] in the course of biosynthesis and biodegradation of xenobiotics, including pharmaceuticals. Despite a wealth of biophysical studies, prediction or explanation of the product profiles based on a structural and mechanistic basis remains an elusive but important goal. P450 enzymes constitute one of the largest superfamilies of hemoproteins, and bacterial enzymes that serve as convenient model systems for eukaryotic P450 enzymes have been identified. We focused on cytochrome P450 BM-3 from *Bacillus megaterium*, a monooxygenase that acts selectively on saturated and unsaturated long-chain fatty acids and their derivatives. This enzyme obtains redox equivalents directly from oxygen and NADPH using an internal FAD/FMN domain as a redox mediator; this domain is homologous to the redox partners seen in microsomal eukaryotic systems. In P450 BM-3 the fact that the reductase domain is fused together with the catalytic heme domain leads to unusually high turnover rates.[6,7]

Although many X-ray structures of drug P450 complexes are beginning to appear, the binding sites of the ligands are often unexpected: in many cases, the site of attack on the substrate is far from the heme, so much so that a structural rearrangement must occur before commencing the reaction. Perhaps the binding sites distant from the heme offer insights into function. For example, it is known that multiple substrates bind simultaneously for some P450 enzymes.[8,9] Distant binding modes are conceivably important for activation or regulation of the enzyme. Clearly it is interesting to provide evidence for binding and to be able to identify the binding site for a variety of ligands under physiologically relevant conditions.

We have studied ligand binding in this system using high-resolution NMR to obtain site-specific evidence of binding.[10] When a ligand binds to a protein, multiple effects are seen in the NMR spectra, and these effects can give insights about the binding. Changes in chemical shifts observed upon addition of a small molecule to a biological target suggest that those atoms are involved in the binding surface. Alternatively, the changes in chemical shift could be due to conformational changes, and the binding might occur at a site distant from the perturbed NMR signal. These two possibilities can sometimes be distinguished.[11] The use of such shifts to identify ligands and classify them as to whether they share a binding pocket[10,12] has had an important impact on drug development. Structural activity relationships by nuclear magnetic resonance (SAR by NMR) experiments have been carried out for a broad range of systems. New solution NMR methods have been aimed at studying larger and more complex systems; many of the techniques require protein deuteration[15] or selective labeling.[16] For example, solvent-

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exposed amides with transverse-relaxation optimized spectroscopy (SEA-TROSY)[14] relies on protein deuteration, and selective labeling of a target with ^{13}C can additionally improve the sensitivity of the measurements for large proteins.[13] Recent studies demonstrate the use of solid-state NMR to carry out analogous probes of ligand binding,[17] which paves the way for binding studies on intrinsic membrane proteins and on low-temperature trapped species.

In this work, we demonstrate a new extension of the SAR by NMR studies of ligand binding by carrying out high-resolution solid-state NMR studies of binding of a substrate to a large paramagnetic protein. The presence of unpaired electrons leads to relaxation and temperature-dependent shifts, both of which have been utilized for structural insights extensively in solution NMR studies,[18] and to a limited extent in solid-state NMR studies.[19,20] The high molecular weight of P450 BM-3, and the difficulty of crystallizing the whole enzyme, makes it a logical target for high-resolution solid-state NMR studies. Thus, the present study serves to illustrate some simple ligand screening methods for large and paramagnetic proteins of broad potential applicability.

Materials and Methods

Preparation of the Samples for NMR Experiments. Two kinds of selectively labeled cytochrome P450 BM-3 samples were prepared for NMR measurements using a modification of an existing protocol[21] as described in detail elsewhere.[22] One sample was enriched with $^{13}CO-$Phe, $^{13}C\alpha-$Ala, and $^{15}N-$Gly, and the other was enriched with $^{13}CO-$Leu, $^{15}N-$Phe, and $^{15}N-$Gly; these enrichments were carried out by growth in a defined medium where the exclusive source of the amino acid was the isotopically enriched form. The purity[23] and activity[24] of the enzymes were confirmed. Approximately 80 mg of protein was obtained from a liter of growth culture. ω-^{13}C-labeled *N*-palmitoylglycine (NPG) and ^{13}C-labeled NPG from U$-^{13}C$ palmitic acid were synthesized in two steps from commercially available ω-^{13}C palmitic acid (99.2% ^{13}C enriched, Sigma) in 70% yield and U$-^{13}C$ palmitic acid (99% uniformly ^{13}C enriched, Sigma) in 55% yield, respectively, by the method described by Lapidot et al.[25] The products were recrystallized from ethanol. For ω-^{13}C-labeled NPG: ^1H NMR (300 MHz, CD_3OD) δ 3.79 (s, 2H), 2.15 (t, $J = 7.48$ Hz, 2H), 1.52 (br m2H), 1.19 (br m, 24H), 0.80 (br m, $J = 124.3$ Hz, 3H); ^{13}C NMR (300 MHz, CD_3OD) δ 14.4; LRMS (APCI) *m*/*z*: calculated 315 for $[M]^{+1}$, found 315. For ^{13}C-labeled NPG obtained from U$-^{13}C$ palmitic acid: ^1H NMR (300 MHz, CD_3OD) δ 3.79 (d, $J = 3.9$ Hz, 2H), 2.15 (br m, $J = 127$ Hz, 2H), 1.57 (br m, $J = 104$ Hz, 2H), 1.21 (br m, $J = 115$ Hz, 24H), 0.80 (br m, $J = 119$ Hz, 3H); ^{13}C NMR (300 MHz, CD_3OD) δ 177.1, 176.4, 37.4, 36.9, 36.7, 36.3, 33.6, 33.1, 32.7, 30.7, 27.3, 26.8, 26.4, 24.2, 23.7, 23.3, 15.0, 14.7, 14.4, 14.2, 13.9; LRMS (APCI) *m*/*z*: calculated 330 for $[M]^{+1}$, found 330. The water peak was used as an internal reference δ 4.78 ppm (TMS) for ^1H solution NMR, and methanol-d_4 resonance at 49 ppm was used for ^{13}C NMR. Determination of the dissociation constant and the turnover number of NPG and cytochrome P450 BM-3 was done as previously described.[22] For ω-^{13}C-labeled NPG, a dissociation constant of 0.29 ± 0.06 μM and a turnover number of 2650 mol/(min·mol enzyme) were obtained. For ^{13}C-labeled NPG from U$-^{13}C$ palmitic acid, a dissociation constant of 0.28 ± 0.03 μM and a turnover number of 2600 mol/(min·mol enzyme) were obtained. Acquired values are in good agreement with published results for unlabeled NPG and P450 BM-3.[26]

The samples were precipitated for magic angle spinning (MAS) studies according to the following protocol. Cytochrome P450 BM-3 in 50 mM MOPS, pH 7.4, with either 20% glycerol ($^{13}CO-$Leu, $^{15}N-$Phe, $^{15}N-$Gly labeled) or 30% glycerol ($^{13}CO-$Phe, $^{13}C\alpha-$Ala, $^{15}N-$Gly labeled) was concentrated to 200 mg/mL. The substrate was bound by direct addition of 1 equiv of 20 mM ω-^{13}C-labeled NPG ($^{13}CO-$Phe, $^{13}C\alpha-$Ala, $^{15}N-$Gly labeling scheme) or ^{13}C-labeled NPG from U$-^{13}C$ palmitic acid ($^{13}CO-$Leu, $^{15}N-$Phe, $^{15}N-$Gly labeling scheme) in 50 mM K_2CO_3. Saturated binding was confirmed with UV−vis spectrometry both before and after the NMR experiment. The enzyme was precipitated by mixing equal volumes of the enzyme and 40% (w/v) PEG 8000 in 25 mM $MgSO_4$, 50 mM MOPS pH 7.4 ($^{13}CO-$Leu, $^{15}N-$Phe, $^{15}N-$Gly labeling scheme), or 40% (w/v) PEG 8000 in 25 mM $MgSO_4$, 50 mM MOPS pH 7.4, 30% glycerol ($^{13}CO-$Phe, $^{13}C\alpha-$Ala, $^{15}N-$Gly labeling scheme). The precipitated enzyme was easily resuspended in the original buffer, and the ratio of the Soret and 280 nm peaks' absorbances were identical to their values before NMR measurement. The activity of precipitated enzyme was confirmed.[22] All UV−vis spectra were taken on a Shimadzu UV-1601 UV−visible spectrophotometer at 22 °C.

NMR Experiments. NMR spectra were recorded using a Varian/Chemagnetics Infinity Plus 600 MHz spectrometer, operating at Larmor frequencies of 599.3 MHz for proton, 150.7 MHz for carbon, and 60.7 MHz for nitrogen. Temperatures were corrected for heating caused by spinning and decoupling (by adding 20 °C to the temperature of the variable-temperature flow gas, approximately calibrated for these conditions using external samples). The two-dimensional (2D) $^{15}N^{13}$-CO spectrum of the substrate-free $^{13}CO-$Leu, $^{15}N-$Gly, and $^{15}N-$Phe-labeled cytochrome P450 BM-3 was acquired with a 3.2-mm rotor using T3 HFXY MAS probe (Varian Instruments) in $^1H^{13}C^{15}N$ mode with a sample temperature of 5 ± 5 °C and a spinning frequency of $13\,000 \pm 5$ Hz. All other spectra were acquired with a 4-mm triple resonance T3 MAS probe (Varian Instruments) in $^1H^{13}C^{15}N$ mode with a sample spinning frequency of $13\,000 \pm 5$ Hz. Temperatures during the acquisition were 0 ± 5 °C and -30 ± 5 °C for the $^{13}CO-$Leu, $^{15}N-$Gly, $^{15}N-$Phe-labeled and $^{13}CO-$Phe, $^{13}C\alpha-$Ala, $^{15}N-$Gly-labeled samples, respectively. Approximately 8 mg of $^{13}CO-$Leu, $^{15}N-$Gly, and $^{15}N-$Phe-labeled cytochrome P450 BM-3 in the resting state was used for acquiring 2D NMR experiments. In all other cases, approximately 15 mg of sample was used as judged by UV−vis spectra.

A simple pulse sequence with 90° excitation and detection on ^{13}C with background suppression[27] and two-pulse phase-modulated (TPPM) proton decoupling[28] (50.6 kHz) was employed. The carbon 90° excitation pulse width was 4.6 μs, and the spectral width was 156.3 kHz. Approximately 60 000 scans were recorded with a delay of 0.5 s between experiments for recovery. The acquisition time was 6.5 ms.

The one-dimensional (1D) ^{13}C cross-polarization (CP) experiments were carried out using a ramped[29] radio frequency (RF) field on ^{13}C, with an average field strength of 33.7 kHz, a linear downward-going

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ramp of amplitude 10.5 kHz, and a contact time of approximately 2 ms. TPPM proton decoupling of 50.6 kHz[28] was applied during the 16.9-ms acquisition. The proton and the carbon 90° pulse widths were 3.2 and 4.6 μs, respectively. A spectral window of 60.6 kHz was used with a pulse delay of 2 s. The experiment time was less than 50 min.

1D spin−lattice (T_1) measurements (data not shown) were carried out using the method proposed by Torchia[30] with the same CP condition as described above. Because of the rapid repetition rate, it was useful to employ a complete 64-phase cycle. The proton and the carbon 90° pulse widths were 3.2 and 4.6 μs, respectively. The field strength of the carbon excitation pulse was 58.3 kHz. A spectral window of 60.6 kHz was used with pulse delay of 3 s. The total time of the experiment for each τ value was 5.6 h.

A double cross-polarization (DCP) sequence[31] with a selective[32] ^{15}N−^{13}CO transfer (the spectrally induced filtering in combination with cross-polarization, or SPECIFIC CP) was used for acquiring the 2D ^{15}N^{13}CO spectrum of ^{13}CO−Leu, ^{15}N−Gly, and ^{15}N−Phe-labeled cytochrome P450 BM-3. ^{1}H−^{15}N cross-polarization was achieved using a ramped[29] RF field on ^{15}N, with an average field strength of 42.9 kHz (for the unligated form) or 35.2 kHz (for the NPG bound form), a linear downward-going ramp of amplitude approximately 7 kHz, and a contact time of 2.2 ms (for the unligated form) or 0.75 ms (for the NPG bound form). The ^{15}N−^{13}CO polarization transfer for the unligated form was achieved with an ^{15}N excitation at 119.9 ppm, with a CP field strength of 31.0 kHz, and a downward-going linear ramp for the carbon excitation at 179 ppm, with an average field strength of 41.5 kHz. The ^{15}N−^{13}CO polarization transfer for the NPG bound form was achieved with an ^{15}N excitation at 124.9 ppm, with a CP field strength of 21.4 kHz, and a downward-going linear ramp for the carbon excitation at 176 ppm, with an average field strength of 34.3 kHz. In both cases, the ramp amplitude of 2.5 kHz was used during a contact time of 3.5 ms. During detection, 50.4 kHz TPPM decoupling was applied on the proton channel, and between 50.4 and 56 kHz continuous wave decoupling was applied during ^{15}N−^{13}CO transfer. There were 1500 points in the direct dimension for both samples and 32 points (protein in the resting state) or 64 points (NPG bound protein) in the indirect dimension. The number of scans per t_1 point was 2496 for the unligated form and 480 for the NPG bound form. Using a recycle delay of 3 s, we found that the total experiment time was between 26 and 97 h. Spectral widths of 52.219 kHz in the ^{13}C dimension and 3.262 kHz in the ^{15}N dimension were used for the unligated form, while spectral widths of 78.125 kHz in the ^{13}C dimension and 3.250 kHz in the ^{15}N dimension were used for the NPG bound form.

The 2D T_1 ^{15}N filter pulse program was implemented using the DCP sequence[31] with selective[32] ^{15}N−^{13}CO transfer and two 90° pulses on the nitrogen channel with variable delay time, τ. SPECIFIC CP transfer conditions used for the experiment were identical to ones used before for the NPG bound, ^{13}CO−Leu, ^{15}N−Gly, ^{15}N−Phe-labeled sample. The number of scans per t_1 point was 496 for $\tau = 1$ s. The total experimental time was around 22 h.

The 2D dipolar-assisted rotational resonance (DARR)[33] spectra were acquired on ^{13}CO−Phe, ^{13}Cα−Ala, ^{15}N−Gly-labeled cytochrome P450 BM-3. ^{1}H−^{13}C cross-polarization was accomplished with proton excitation at 6.7 ppm and ramped carbon excitation at 114.5 ppm, with an average field strength of 44.2 kHz, and a linear downward-going ramp of amplitude approximately 10 kHz.[29] Contact times of approximately 2 ms were used. A recoupling proton pulse with a field strength of 13.4 kHz was applied during the 450-ms (ligated sample) or 131-ms (unligated sample) mixing period. During detection, 44.2 or 49.5 kHz TPPM decoupling was applied on the proton channel. The spectral sweep widths were 78 kHz in the direct and 39 kHz in the indirect dimension. The number of points collected in the direct and indirect dimensions was 1024 and 512, respectively. The number of scans per t_1 increment was 120 for the unligated sample and 80 for the ligated sample; the total experimental time was approximately 68 h.

Processing and Referencing of NMR Data. The 1D ^{13}C CP spectra were processed using the program Spinsight V 4.3.2 (Varian Inc., Fort Collins, CO) with zero filling to 8192 points. One-pulse spectra with background suppression were processed using a sine-bell apodization function and zero filling to 8192 points; initial data points were removed from the free induction decay (FID) so that the FID started from the first rotor echo. The difference T_1 ^{13}C spectra were zero-filled to 8192 points and processed with exponential broadening of 30 Hz for the unligated form or 10 Hz for the NPG bound form. The standard deviation of the baseline outside the carbonyl region of the spectrum was taken as the noise level. T_1 values and errors for NPG carbons were obtained by exponential fitting using Mathematica 5.0 (Wolfram research).

Two-dimensional spectra were processed with NMRPipe[34] and analyzed with Sparky 3.100 (Goddard, T. D. and Kneller, D. G., University of California, San Francisco). For phase-sensitive detection, the time proportional phase incrementation (TPPI) method[35] was utilized. For processing of the ^{15}N^{13}CO spectra, cosine-bell and sine-bell (81° phase shift) apodization functions were applied in the direct and the indirect dimensions, respectively. Spectra were zero-filled to 8192 points in the carbon dimension, and exponential line broadening of 25 Hz was applied for SPECIFIC CP experiments and 50 Hz for 2D T_1^{15}N experiment. The indirect dimension was zero-filled to 512 points.

For the DARR spectra, the direct dimension was zero-filled to 8192 points. A polynomial baseline correction and an exponential line broadening of 200 Hz (for the unligated sample) or an exponential line broadening of 30 Hz and cosine-bell apodization (for the ligated sample) were applied in the direct dimension. The indirect dimension was zero-filled to 4096 points, and a cosine-bell window function and polynomial baseline correction were applied.

All ^{13}C spectra were referenced using the ^{13}C adamantane methylene peak at 38.56 ppm as external reference. Adjustment of 1.7 ppm to DSS was then applied to give a final value for the chemical shift. The ^{15}N chemical shift axis was externally referenced to ammonium chloride using the value of 39.27 ppm relative to liquid ammonia at 25 °C.

Results and Discussion

To demonstrate binding using NMR spectral changes, it is useful to have site-specific markers for binding. In absence of full assignments of the spectra and in view of the complexity of the full-length P450 BM-3 (1048 residues), we elected to carry out selective experiments, wherein key regions of the protein were identified efficiently. For example, for P450 BM-3, Phe87 undergoes a significant conformational change upon binding of substrates as seen in Figure 1A. In fact, this residue with its bulky side chain appears to function as a "gatekeeper", controlling the access of substrates to the active site. To probe this amino acid specifically, we took advantage of the fact that it is found in the only Leu-Phe sequential pair in the full-length enzyme. In these experiments, specific sequential pairs of amino acids were detected through selective isotope enrichment. When the protein was prepared with the sole ^{13}C enrichment being in carbonyl-labeled leucine residues and the sole ^{15}N enrichment being in the backbone amides of glycine and phenylalanine

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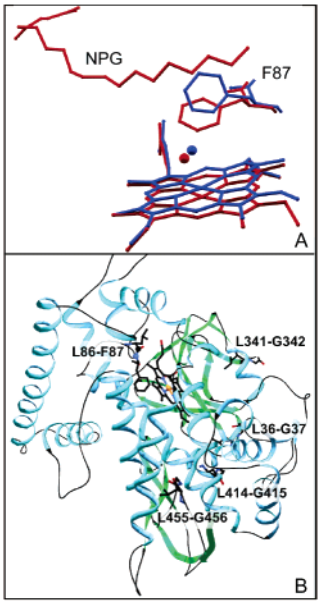

Figure 1. (A) Shift in the conformation of Phe87 residue and shift of the water ligand (red and blue balls) upon substrate binding; the PDB files 1BU7[36] (the resting state of the protein, in blue) and 1JPZ[26] (the NPG bound state of the protein, in red) were overlaid using 432 backbone atoms within conserved helices D, E, I, L, J, and K, employing Swiss PDB Viewer (SPDBV).[53] The RMS deviation for the backbone heavy atoms in this superposition was 0.60 Å. (B) Positions of the Leu-Phe and four Leu-Gly pairs in the heme domain of cytochrome P450 BM-3.[36] The unique Leu86-Phe87 pair is close to the heme iron and is involved in the binding pocket, but the Leu-Gly pairs are not. The figures were prepared with programs POV Ray (http://www.povray.org) and SPDBV.[53]

residues, the unique Leu-Phe sequential pair and the six Leu-Gly sequential pairs would provide the only directly bound $^{15}N-^{13}C$ pairs, and they were selectively detected using a 2D $^{15}N-^{13}C$ correlation experiment.[31] The Leu-Phe unique pair serves as a reporter of conformational changes at the active site, while the spectrally distinct Leu-Gly pairs are distant from the active site and serve as controls for nonspecific changes in the sample. In Figure 1B, on the basis of the PDB entry 1BVY,[36] we show the locations of the Leu-Phe and Leu-Gly amino acid pairs that are found in the heme and FMN domains. The 2D $^{15}N^{13}CO$ spectrum of $^{13}CO-$Leu, $^{15}N-$Gly, $^{15}N-$Phe-labeled cytochrome P450 BM-3 was measured using the SPECIFIC CP sequence[32] at 0 °C. Using this approach, we acquired the NMR data displayed in Figures 2, 4B, and 5A,B. All six Leu-Gly pairs (36−37, 341−342, 414−415, 455−456, 700−701, 927−928) and a single Leu-Phe pair (86−87) are resolved in the 2D $^{15}N-^{13}CO$ spectra and are selectively detected out of 1048 residues. We detected a single form of the enzyme for the unligated sample and a different but again single form for the ligated sample. On the basis of the chemical shift of the ^{15}N amidic nitrogen, we can identify the peak associated with Leu86-Phe87. Upon binding of NPG to form the substrate−enzyme complex, the Leu86-Phe87 peak shifts significantly in both dimensions, from 178.7 to 177.9 ppm in the carbon dimension, and from 119.8 to 121.1 ppm in the nitrogen dimension.

Some consideration of the sizes of the shifts is warranted. On the basis of the reproducibility of peaks in a previous SSNMR study of a different protein, shifts of the order of 0.1 ppm in homonuclear ^{13}C experiments were

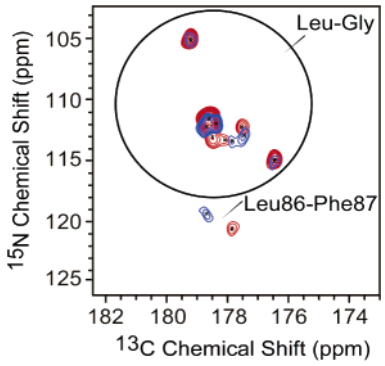

Figure 2. 2D solid-state NMR $^{15}N^{13}CO$ SPECIFIC CP spectra of $^{13}CO-$Leu, $^{15}N-$Gly, $^{15}N-$Phe-labeled cytochrome P450 BM-3 in the resting state (in blue), and in the NPG bound form (in red). The resonance of the $^{15}N^{13}CO$ backbone pair associated with the Leu86-Phe87 pair in the protein exhibits an ^{15}N amide chemical shift at approximately 120 ppm. The peaks with the amide chemical shifts of 105−115 ppm are circled, and they are associated with the six unassigned Leu-Gly pairs in the protein. The pronounced shift of the Leu86-Phe87 pair is presumably due to the conformational change, contact with the ligand, and the heme spin-state change upon substrate binding.

considered to be meaningful for the detection of ligand binding using SSNMR.[17] An extensive body of heteronuclear single quantum correlation (HSQC) solution NMR spectra suggests that perturbations are meaningful when the changes in chemical shifts for at least two peaks are $\sqrt{[\delta(^1\mathrm{H,ppm})_{\mathrm{free}}-\delta(^1\mathrm{H,ppm})_{\mathrm{bound}}]^2+0.04[\delta(^{15}\mathrm{N,ppm})_{\mathrm{free}}-\delta(^{15}\mathrm{N,ppm})_{\mathrm{bound}}]^2} > 0.1$ ppm.[37] Thus, when samples are prepared in a similar fashion except for ligand addition, rather small changes can be indicative of structural/environmental perturbations. On the other hand, comparison of solution and solid-state values for chemical shifts[38] indicated differences of the order of 2 ppm for ^{15}N and 0.6 ppm for ^{13}CO, which are as of yet not well understood. In the experiments reported here, since the samples with and without ligand were prepared in a similar fashion, we considered the binding-induced shift in the Leu86-Phe87 peak to be significant. We note that three of the LG peaks are shifted slightly (<0.2 ppm in the carbon and <0.6 ppm in the nitrogen dimension) and therefore might be experiencing conformational effects.

For a paramagnetic system, we must consider not only the effects of conformational change and binding surfaces, but also the effects of the through-space pseudocontact shifts (PCS).[18,39] If ligand binding induces a change in the spin state, then all residues within some radius of the iron will experience a shift. Thus, these measurements can probe spin-state changes in the protein as well as probing conformational effects and ligand contact. Since the carbonyls and amides of all Leu-Gly pairs available from the PDB[26] are more than 20 Å away from the heme iron, the effects of the paramagnetism are expected to be unimportant. The carbonyl carbon of Leu86 and the nitrogen of Phe87 are 9.3 and 8.7 Å away, respectively, from the heme iron in the resting state of the protein.[36,40−42] Upon binding of

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substrates (NPG and palmitoleic acid), their respective distances from iron shift slightly to 9.1 and 8.4 Å.[26,43] For atoms at this distance from the paramagnetic center, it is important to consider the shifts quantitatively; PCS can be appreciable up to a radius of approximately 10 Å from the heme iron and are often used as structural constraints in solution NMR in this distance range.[44] For low-spin (LS) ferric P450 BM-3, the PCS can be approximated based on g values reported by EPR[45] and crystal structure coordinates[36] assuming a uniaxial magnetic susceptibility tensor with the z-axis perpendicular to the heme plane:[39]

$$\delta_{PC} = \frac{1}{12\pi r^3}(0.5 + 1.5\cos 2\theta)(\chi_{||} - \chi_{\perp});$$

$$\chi_i = \mu_0 \mu_B^2 g_i^2 \frac{S(S+1)}{3k_B T};$$

Here, μ_0 is the vacuum permeability ($4\pi \times 10^{-7}$ kg ·m ·s^{-2} A^{-2}), μ_B is the Bohr magneton (9.27×10^{-24} J·T^{-1}), S is the electron spin state, k_B is Boltzmann's constant (1.38×10^{-23} J·K^{-1}), T is the temperature, r is the distance between the Fe and the nucleus, θ is the polar angle of the nucleus paramagnetic center vector with respect to the major susceptibility axis, and χ_i and g_i represent the magnetic susceptibility and the g tensor components along any given axis. For the carbonyl ^{13}C of Leu86 and the amidic ^{15}N of Phe87, the PCS values are both estimated to be 0.3 ppm at 0 °C in the LS state. These estimates are comparable to estimates obtained using magnetic susceptibility anisotropy values measured for other heme proteins in the LS state. The data on magnetic susceptibility tensors for high-spin (HS) ferric proteins are relatively more scarce. Using magnetic susceptibility anisotropy values reported for metmyoglobin[46] and P450 crystal structure coordinates[26] and assuming that the major axis of the susceptibility tensor is perpendicular to the heme plane, we found that the values predicted for the PCS for the carbonyl ^{13}C of Leu86 are between −0.1 and 1.6 ppm, while for the amidic ^{15}N of Phe87 they ranged from −0.3 to 1.7 ppm, depending on the azimuthal angle. On the basis of these estimates, it is conceivable that the shift from the LS to the HS iron can contribute to the change of the Leu86-Phe87 resonance in these 2D spectra. However, the contribution due to the PCS is expected to increase the chemical shift of the amidic ^{15}N and carbonyl ^{13}C upon binding, whereas in fact on binding we observe the reverse for the carbon dimension, namely that carbonyl ^{13}C decreases. Therefore, local conformational effects are also likely to contribute in this case. Indeed, since NPG binds to cytochrome P450 BM-3 in a pocket near residues Leu86 and Phe87, a contribution due to actual contact with the ligand would not be surprising (the carbons on the phenyl ring of Phe87 and ω-1 to ω-5 carbons of the substrate are, on average, 4 Å apart as suggested by the X-ray structure).[26]

We also probed ligand binding at a site that is more distant to the heme but is still in contact with the ligand in the X-ray structure, namely Ala82. We utilized another labeling scheme in which Phe residues were ^{13}CO-labeled, Ala residues were ^{13}Cα-labeled, and Gly residues were ^{15}N-labeled and 2D ^{13}C−^{13}C DARR[33] experiments were performed. Four Phe-Ala pairs were expected to contribute to the ^{13}CO−^{13}Cα correlation experiment; these locations involve pairs of atoms separated by two bonds, or 2.45 Å:[26,36] Phe81-Ala82, Phe275-Ala276, Phe405-Ala406, and Phe509-Ala510. The five Ala-Phe pairs are not expected to be seen in the spectrum at the same intensity, since the distance between them is around 5 Å.[26] All four expected peaks are present in the spectrum, although one is rather weak; these data are displayed in Figure 3. The most striking feature is a large shift of only one peak in the spectrum. The shift in this peak upon binding suggests that a substrate-induced conformational change can be observed with SSNMR for a large paramagnetic protein.

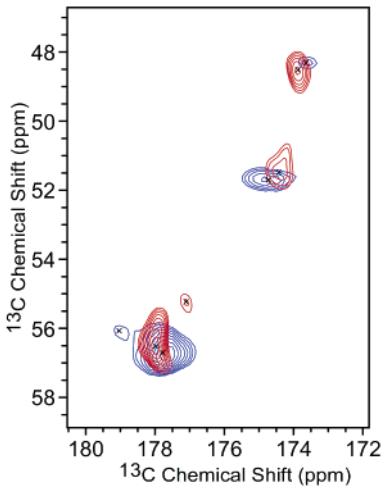

Figure 3. ^{13}Cα and ^{13}CO cross-peak region of the 2D DARR spectrum of ^{13}CO−Phe; ^{13}Cα−Ala, ^{15}N−Gly-labeled cytochrome P450 BM-3 at −30 °C in the resting state (in blue) and in the NPG bound form (in red). Four expected Phe-Ala pairs[26] are detected for both enzyme forms. Among these, Phe81-Ala82 is of interest because it is in close proximity to the ω end of NPG but not to the heme. A clear change in the spectrum upon binding is observed (one peak shifts from 179.0 ppm to 177.1 ppm in the carbonyl value and from 56.1 to 55.3 in the Cα value), further confirming that conformational changes due to ligation can be monitored with SSNMR using selectively labeled proteins.

We exploited the paramagnetism of the heme, via T_1 effects, to obtain additional confirmation for binding of the ligand. For NPG-bound, ^{13}CO−Leu, ^{15}N−Gly, ^{15}N−Phe-labeled cytochrome P450 BM-3, a 2D T_1 filtering approach was carried out, using the pulse sequence presented in Figure 4A. This experiment is intended to identify peaks closest to the paramagnetic center. Nuclei of the order of 9 Å or less from the paramagnetic center should have substantial additional contributions to their spin lattice relaxation rates. The Solomon−Bloembergen equation[47−50] (omitting hydrodynamic tumbling and the associated Curie term) was used to estimate the spin lattice relaxation time. Assuming an electron correlation time of the order of $\tau_e \approx 2 \times 10^{-10}$, as has been reported for HS ferric heme moieties at high magnetic fields,[51,52] we found that the estimated relaxation contribution is approximately 3 s^{-1} for ^{15}N and 20

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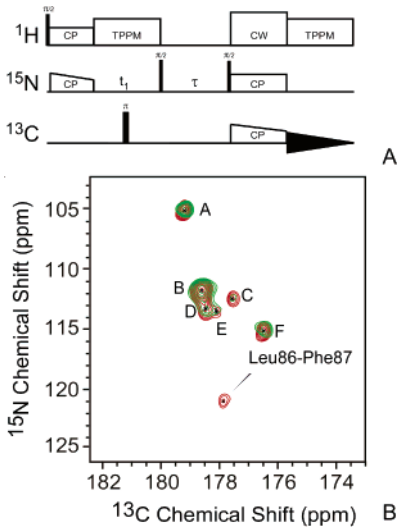

Figure 4. Pulse sequence (A), and 2D T_1 ^{15}N measurements on substrate-bound form of ^{13}CO−Leu, ^{15}N−Gly, ^{15}N−Phe (B). The SPECIFIC CP spectrum is in red, and the 2D T_1 ^{15}N filtered experiment for $\tau = 1$ s is in green. The Leu86-Phe87 peak is much reduced in the filtered experiment, due to its more efficient relaxation.

s^{-1} for ^{13}C for a distance of 9 Å. In Figure 4B, the 2D ^{15}N^{13}CO spectrum is presented in red, and the 2D T_1 ^{15}N filtered experiment for a relaxation delay time, $\tau = 1$ s, is shown in green. Spectra obtained with a relaxation delay of $\tau = 1$ s show that the Leu86-Phe87 peak is missing, while all others (unassigned Leu-Gly pairs, A−F peaks in the picture) are still present. The disappearance of the Lue86-Phe87 peak is likely to be governed by the T_1 effects influenced by the presence of the paramagnetic site and possibly other relaxation effects. This experiment provides supportive evidence of the proximity of these residues to the paramagnetic center.

Although the 2D spectra described above can be informative, the experiments are time-consuming. Binding and conformational change can also be evidenced based on 1D ^{13}C spectroscopy, which is potentially useful since 1D experiments take significantly less instrument time. Although the 1D spectra lose the selectivity of the transfer sequence and therefore might not have site-specific resolution, we utilized enhanced relaxation due to the heme as a filter for residues near the active site. In Figure 5, 1D ^{13}C CP (A) and one-pulse spectra with background suppression (B) of bound and unbound species of ^{13}CO−Leu, ^{15}N−Gly, ^{15}N−Phe-labeled BM-3, together with their difference spectra are presented. A clear change in positions of some peaks upon substrate binding is shown. The position of the stars is likely to represent the chemical shifts of Leu86, based on the 2D spectra. The one-pulse spectrum with background suppression is much broader than the CP spectrum due to inefficient decoupling and significant line broadening used to process the

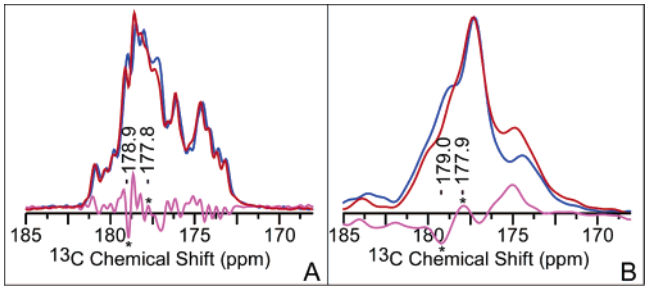

Figure 5. 1D ^{13}C CP (A) and one-pulse with background suppression (B) solid-state MAS NMR experiments on ^{13}CO−Leu, ^{15}N−Gly, ^{15}N−Phe-labeled cytochrome P450 BM-3. The 1D ^{13}C spectra of cytochrome P450 BM-3 in the resting state are presented in blue, while the substrate bound form is in red for both CP and one-pulse with background suppression experiments. For both pulse sequences used, the difference spectra (bound minus unbound) are shown in purple. The likely positions of Leu86 in the substrate free form (trough in the difference spectrum) and substrate bound form (peak in the difference spectrum) are labeled with the asterisk; the values anticipated on the basis of the 2D spectra described above are 178.7 and 177.9 ppm, respectively. All ^{13}CO-labeled Leu residues are likely to give resonances in the 1D ^{13}C CP experiment, leading to the significant overlap in the spectrum. However, the difference spectrum (bound minus unbound) can identify the Leu residues that experience changes due to ligation. In addition to the shift of the Leu86 residue upon binding (consistent with the 2D experiment), other changes are observed in the difference spectrum, and they are presumably due to Leu residues not followed by Gly or Phe, which are either close to the heme or involved in long-range conformational change. Furthermore, the one-pulse with background suppression sequence, by virtue of the relatively rapid recycle delay of 0.5 s, simplifies the spectra by means of a T_1 filter, allowing complete relaxation only of the signals close to the heme. The difference spectrum shows fewer peaks, one of them consistent with the shift of Leu86. These spectra show that conformational and spin-state change upon binding can be observed with 1D spectra, although site-specific identification of the residues involved in binding surface would not be possible.

spectrum. However, the difference spectrum much more clearly shows the features that can be attributed to binding related changes in the peak associated with Leu86, presumably because the rapid repetition of the experiment (0.5 s) effected a T_1 filtering effect. Such an approach could be a useful way to assess binding before embarking on complex multidimensional experiments.

Conclusions

We demonstrate that solid-state NMR spectra of a large paramagnetic protein can be used to provide evidence of ligand binding. Cytochrome P450 BM-3 was studied using selective labeling schemes and relaxation filters to obtain site-specific assignments for some peaks. We believe that these methods have promise for identifying changes in binding modes during the catalytic cycle.

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