although the results of the spin-diffusion experiments shown in Figures 9 and 10 fit exactly with the (known) connectivities in the structure, some caution must be exercised in their interpretation in terms of three-dimensional connectivities in the structure. The theory of spin diffusion between dilute nuclei has been described by Ernst[25] and by McDowell,[26] Clayden,[27] and Henrichs et al.[28] and discussed in terms of its use in probing internuclear distances by VanderHart.[19a] In the recent paper by McDowell and Kubo, the interactions involved in spin diffusion under MAS conditions are discussed in detail.[26b] The rate of spin diffusion is strongly dependent on internuclear distance ($\alpha l/r^6$), but it is also dependent on the frequency separations of the isotropic chemical shifts and the magnitudes and relative orientations of the shift anisotropy patterns of the nuclei involved. Since the latter element will in general be undefined, its contribution to the spin-diffusion process will be unknown. Because all of the silicon nuclei are in tetrahedral environments and have relatively small shift anisotropies, it is probably unlikely that this effect will dominate the spin-diffusion process, but studies of a number of additional systems will be necessary to confirm this at least on an empirical basis.

Conclusions

For the sample of ZSM-39 investigated, 2D NMR experiments based both on scalar couplings and on spin diffusion yield information on $^{29}Si/O/^{29}Si$ connectivities in exact agreement with the known crystal structure and suggest that these techniques may be of general use in the determination of zeolite crystal structures. Further, experiments based on scalar couplings will be best carried out on samples that exhibit the narrowest resonances possible (and correspondingly long T_2 and T_2^* values) in order to permit the longest possible evolution times for the small scalar couplings involved, although positive results could be obtained for resonances of up to 2 ppm. Spin-diffusion experiments yielded the correct connectivities for the lattice structure investigated, but work on other systems will be needed in order to establish more clearly the reliability of these experiments in this context due to unknown contributions of non-distance-dependent factors. Further work based on these conclusions is currently in progress. Although, in general, ^{29}Si enrichment facilitates the experiments, the expense involved obviously limits their general application. By paying careful attention to the importance of the T_2 or T_2^* values in these experiments, we have been able in preliminary work to apply these techniques to natural-abundance samples in the form of COSY and INADEQUATE experiments and to observe the $^{29}Si/^{29}Si$ couplings directly.[29] Although the experiments are very demanding and time consuming, in terms of both sample preparation and the spectroscopy involved, the wealth of information potentially available makes them attractive additions to the techniques currently available for investigating three-dimensional lattice structures.

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^{15}N NMR Study on Cyanide ($C^{15}N^-$) Complex of Cytochrome P-450$_{cam}$. Effects of *d*-Camphor and Putidaredoxin on the Iron–Ligand Structure

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Abstract: The cyanide ($C^{15}N^-$) complex of *Pseudomonas putida* cytochrome P-450 (P-450$_{cam}$) exhibited well-resolved and hyperfine-shifted ^{15}N NMR resonances arising from the iron-bound $C^{15}N^-$ at 423 and 500 ppm in the absence and presence of the substrate, *d*-camphor, respectively. The values were smaller than those for cyanide complexes of myoglobin and hemoglobin (~1000 ppm) but fell into the same range as those for the cyanide complexes of peroxidases (~500 ppm). The ^{15}N shift values of P-450$_{cam}$ were not incompatible with the existence of anionic ligand, such as cysteinyl thiolate anion, at the fifth coordination site of heme iron. The difference in the ^{15}N chemical shift values between camphor-free and -bound enzymes was inferred by the increase in the steric constraint to the Fe–C–N bond upon substrate binding. When putidaredoxin was added to the $C^{15}N^-$ complex of camphor-bound P-450$_{cam}$, the ^{15}N NMR signal changed from 500 to 477 ppm. The spectral changes were interpreted in terms of the structural changes in the vicinity of iron-bound ligand and discussed in relation to the functional properties of P-450$_{cam}$.

Cytochrome P-450 is a generic name given for b-type cytochrome, which exhibits Soret absorption at 450 nm upon complex formation with CO. The enzyme P-450 acts as a terminal oxidase in the monooxygenation reaction and cleaves dioxygen into a water and a single oxygen atom, which is inserted into a hydrocarbon bond. Recent X-ray crystallographic studies[1] of *Pseudomonas putida* cytochrome P-450 (P-450$_{cam}$) clearly showed that the substrate molecule, *d*-camphor, is buried in an internal pocket just

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above the heme distal surface adjacent to the oxygen-binding site and is held in place through hydrogen bonding with Tyr-96 and hydrophobic interactions with some amino acid residues. Many spectroscopic studies revealed that the binding of camphor causes drastic changes in the heme environment and its electronic structure. For example, upon camphor binding to resting ferric P-450, the sixth axial ligand, most probably O-ligand, is expelled from the coordination site, and the pentacoordinate heme iron is produced, thereby causing the iron spin change from ferric low spin ($S = {}^1/_2$) to high spin ($S = {}^5/_2$) states.[2] Thus, one of the most important problems for the evaluation of the structure–function relationship of P-450 is analyses of an interaction of the bound-substrate molecule with the ligand coordinated to the heme iron. The second problem of P-450 study is the interaction profile of the iron–sulfur protein, putidaredoxin, with P-450$_{cam}$, which offers two electrons for the cleavage of the coordinated molecular oxygen O–O bond. Although putidaredoxin is known to form a stable complex with P-450$_{cam}$,[3] there is rare evidence for its binding effect on the structure at the ligand coordination site of P-450$_{cam}$. Evaluation of these two problems would provide us a clue to unveil the structure–function relationship of P-450.

In order to characterize the structural and the electronic environments of the prosthetic group, small-molecule axial ligands potentially can serve as useful NMR probes. In these regards, the ^{15}N NMR signal of the iron-coordinated cyanide ion ($C^{15}N^-$) has proved to be most sensitive to the nature of the fifth amino acid ligand and to subtle change in the heme steric and electronic environments of the ferric hemoproteins.[4,5] The cyanide complex in the ferric state is invariably in the low-spin state ($S = {}^1/_2$), and the spin state is especially attractive for study by ^{15}N NMR methods in that paramagnetic line broadening is not excessive. The chemical shift values reported among several hemoproteins, such as cytochrome *c*, myoglobin (Mb), hemoglobin (Hb), and peroxidases,[4,5] are widely distributed from 400 to 1200 ppm. These characteristic shifts have been interpreted in relation to the heme surrounding structure such as iron–ligand bond nature and interaction of the ligand with the amino acid residues.

In the present study, we examined ^{15}N NMR for $C^{15}N^-$ bound to the ferric heme iron in P-450$_{cam}$ with special emphasis on the nature of the iron fifth ligand, the effect of *d*-camphor, and putidaredoxin binding on the heme environmental structure. The results serve as the first demonstration of the $C^{15}N^-$ signal for oxygenase types of hemoproteins. Being in a region around 500 ppm, the ^{15}N NMR signal lies in the same chemical shift region as those of some peroxidases[5] but deviates from the region for those of oxygen-carrying (Mb and Hb) or electron-transferring (cytochromes) hemoproteins.[4] The ^{15}N NMR spectrum is remarkably altered upon the camphor binding at the catalytic site or upon the putidaredoxin binding to P-450$_{cam}$. These observations will be related to the structural and electronic changes at the iron–ligand bond and then be discussed with relevance to the P-450$_{cam}$ catalytic mechanism.

Materials and Methods

Enzyme. Cytochrome P-450$_{cam}$ was isolated from cells of *P. putida* (ATCC 17453), which were cultivated in the presence of *d*-camphor as a sole carbon source, and was purified according to the methods of Gunsalus and Wagner with slight modifications.[6] The highly purified enzyme preparations with 1.55–1.6 RZ value (A_{392}/A_{280}) were employed for the experiments. Camphor-free form of cytochrome P-450$_{cam}$ was prepared by passage through a Sephadex G-25 column equilibrated with 0.1 M Tris-HCl buffer, pH 7.5. Cyanide complexes of these proteins were prepared by an addition of about 10 molar excess of $KC^{15}N$, which was purchased from Prochem (96.7 atm % ^{15}N). Putidaredoxin was prepared according to the method of Gunsalus and Wagner.[6]

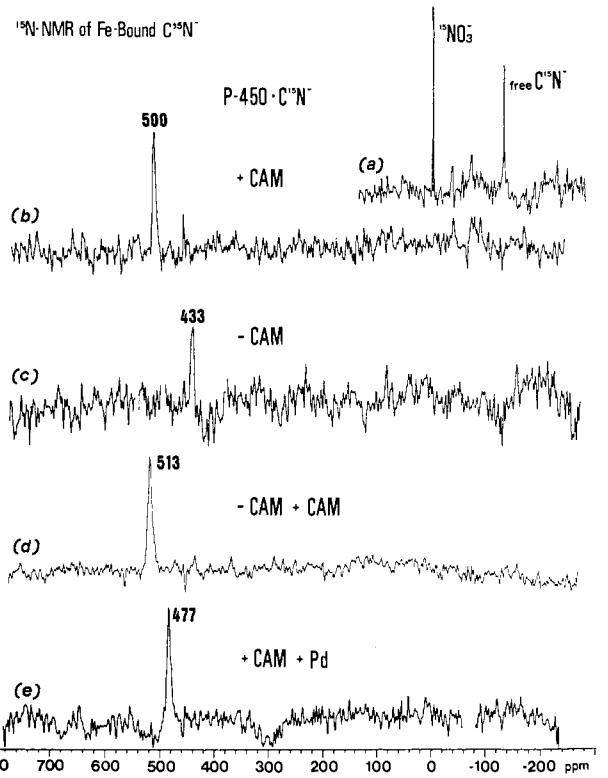

Figure 1. (a) ^{15}N NMR spectra of $KC^{15}N$ and $Na^{15}NO_3$ solution. ^{15}N NMR spectra of cyanide ($C^{15}N^-$) complexes of cytochrome P-450$_{cam}$ (b) in the presence of *d*-camphor and (c) in the absence of *d*-camphor. Upon an addition of *d*-camphor to the camphor-free enzyme, the spectrum (d) was obtained. (e) The spectrum of P-450$_{cam}$–$C^{15}N^-$ in the presence of *d*-camphor and oxidized putidaredoxin (Pd; 3 mM). Enzyme concentration for all samples is 2 mM. Spectra (a), (b), and (e) were measured at pH 7.4 (0.1 M phosphate buffer) and 22 °C. Spectra (c) and (d) were at pH 7.4 (50 mM Tris-HCl buffer containing 5% glycerol) and 13 °C.

Measurements. ^{15}N NMR spectra of the $C^{15}N^-$ complexes were taken at 30.41 MHz on a Nicolet NT-300 spectrometer equipped with a 1280 computer system, using 8K data points and a 20-μs pulse over 60000-Hz bandwidth. Typical spectra of the enzymes consisted of about 1×10^6–2×10^6 transients (12–24 h). Chemical shifts are given in ppm with reference to the resonance of external $^{15}NO_3^-$, assigning a positive value for downfield resonances.

Results

The ^{15}N NMR spectrum of camphor-bound cytochrome P-450$_{cam}$ was measured at 22 °C in the presence of excess cyanide ion ($C^{15}N^-$). With a slow repetition time (500 ms) of pulse irradiation, two ^{15}N signals were observed at −129 and +500 ppm. In Figure 1a is shown the signal at −129 ppm in an upfield region from the signal of $^{15}NO_3^-$ as an external reference. The signal at −129 ppm is already assigned to be that of free $HC^{15}N$. By contrast, in the measurement with a fast repetition time (500 μs), the signal of free $HC^{15}N$ was undetectable due to the signal saturation, but the broadened signal at 500 ppm was still observed, as shown in Figure 1b. This means that the relaxation time of ^{15}N nuclear spin for the downfield-shifted signal is excessively shortened due to the effect of paramagnetic ferric iron ($S = {}^1/_2$), in contrast with that of the free cyanide ^{15}N signal. Thus, it is concluded that the resonance at 500 ppm is assignable to the cyanide ion coordinated to the heme iron.

Convincing evidence for this assignment is found in the temperature-dependent behavior of the ^{15}N NMR signal for camphor-bound P-450$_{cam}$–$C^{15}N^-$. In Figure 2 is illustrated the temperature dependence of the ^{15}N NMR signal of the iron-coordinated $C^{15}N^-$ in the form of a Curie plot. Its linear dependence on inverse temperature ($1/T$), in spite of the narrow

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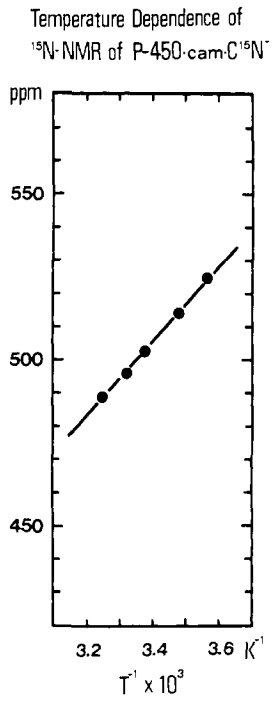

Figure 2. Temperature dependence of ^{15}N NMR signal of camphor-bound P-450$_{cam}$–C^{15}N$^-$ in the form of a Curie plot. The chemical shifts were plotted against the inverse of absolute temperature.

temperature range (7–35 °C), evidently showed the iron paramagnetic effect on the downfield shifted ^{15}N signal.

To analyze the effect of camphor binding on the heme environment, the ^{15}N NMR measurement of the camphor-free form of P-450$_{cam}$ was carried out. To avoid a denaturation of unstable camphor-free enzyme, the measurement was done at 13 °C. The cyanide complex of camphor-free P-450$_{cam}$ gave a hyperfine-shifted ^{15}N NMR resonance at 433 ppm (Figure 1c). The addition of *d*-camphor caused again a shift of the signal to 513 ppm, indicating that the effect of *d*-camphor binding was fully reversible (Figure 1d). Although the optical absorption spectrum of the cyanide complex does not show a significant difference between camphor-bound and -free forms of P-450$_{cam}$, the difference in the ^{15}N NMR signal positions between these two forms clearly indicates that the heme environment of P-450$_{cam}$–CN$^-$ is altered upon the *d*-camphor binding.

Figure 1e shows the ^{15}N NMR spectrum of camphor-bound P-450$_{cam}$–C^{15}N$^-$ in the presence of putidaredoxin, which was measured at 22 °C. The iron-coordinated C^{15}N$^-$ afforded an ^{15}N NMR signal at 477 ppm. The resonance position is distinct from that for either camphor-bound or -free enzyme, showing that putidaredoxin combines with P-450$_{cam}$ and substantially affects the structure at the active site.

The values of ^{15}N shift obtained in this study were corrected to the values at 22 °C in order to compare with those for other hemoproteins so far investigated (Figure 3).

Discussion

Characteristic Shift of ^{15}N NMR Signal for P-450$_{cam}$·C^{15}N$^-$. Comparing previous ^{15}N NMR data for C^{15}N$^-$ complexes of hemoproteins, the chemical shift values can be classified into two groups (Figure 3). One is the group for oxygen-carrying and electron-transferring hemoproteins such as Mb, Hb, and cyt *c*,[4] which afford the C^{15}N$^-$ ^{15}N NMR signals around 1000 ppm from the reference $^{15}NO_3^-$ signal. The other is a peroxidase group including horseradish peroxidase and lacto- and chloroperoxidases,[5] whose ^{15}N signals are located around 500 ppm. The difference in C^{15}N$^-$ resonance values between these two groups reflects functional diversity for the hemoproteins; the former group is relatively stable in a ferrous (Fe^{2+}) state in nature, while the peroxidase group plays in a ferric (Fe^{3+}) state for scission of the O–O bond of peroxide and stabilizes a heme in higher oxidation states (Fe^{4+} or formally Fe^{5+}) as a catalytic intermediate. It must

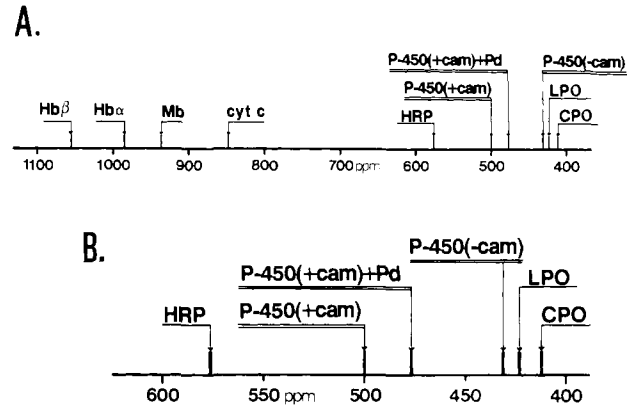

Figure 3. (A) Stick diagram of ^{15}N NMR signals for cyanide complexes of various hemoproteins reported so far.[4,5] (B) The region of P-450 group in (A) was expanded.

be also noted that the Fe^{3+}/Fe^{2+} redox potentials (−250 to −100 mV) of the latter group are much lower than those (+260 to +50 mV) for the former group.

P-450$_{cam}$, which has a lower redox potential (−140 mV),[7,8] is believed to form a reaction intermediate in the higher oxidation state of heme iron in its enzymatic cycle, like peroxidases. In the ^{15}N NMR spectra of P-450$_{cam}$, the iron-coordinated C^{15}N$^-$ of three states of P-450$_{cam}$ expectedly gave smaller hyperfine-shifted ^{15}N signal than Hb group, and the position fell into the similar range to that of the peroxidase group (Figure 3). Then, the ^{15}N resonance position of the iron-coordinated C^{15}N$^-$ is considered to reflect the structural characteristics in the heme environment of P-450$_{cam}$ such as a chemical nature of the fifth ligand, an iron–cyanide bond, or interaction of the cyanide with the surrounding peptide, which are biologically relevant to the enzymatic function.

Before discussing structural factors relevant to the unique ^{15}N NMR shift of P-450$_{cam}$, we estimate the dipolar shift contribution to the apparent downfield shift of the bound C^{15}N$^-$, according to the method by Morishima and Inubushi.[4e] The dipolar shift in the hyperfine shift might be significant in evaluating the structural characteristics in the heme environment from the ^{15}N NMR spectra. Available ESR data of P-450$_{cam}$–CN$^-$[9] allow us to estimate this dipolar shift as about 30 ppm, which is about 5% of the ^{15}N hyperfine shift of P-450$_{cam}$–C^{15}N$^-$. Due to the low anisotropy of the *g*-values, the value of the dipolar shift for P-450$_{cam}$–C^{15}N$^-$ is smaller than that of Mb–C^{15}N$^-$ (~100 ppm). It then follows that the observed ^{15}N NMR shift of P-450$_{cam}$–C^{15}N$^-$ is predominately attributable to the contact contribution arising from the positive spin density induced on the nitrogen.

Among the factors that might affect the ^{15}N NMR hyperfine shift for cyanide (C^{15}N$^-$) complexes of hemoproteins, contributions from either the heme proximal side or the distal side or combination of both have been thought to be important. Comprehensive ^{15}N NMR studies for cyanohemoproteins and their model compounds by Morishima et al.[4] have suggested that a nature of the heme fifth ligand and a hydrogen bonding of the cyanide with the distal residues are important factors in determining the cyanide ^{15}N NMR signal position. In particular, a basicity of the fifth ligand plays a major role in dictating the C^{15}N$^-$ signal position. Indeed, Goff et al.[5] well simulated a smaller shift (~500 ppm) of the iron-coordinated C^{15}N$^-$ signal for horseradish peroxidase and lactoperoxidase by using of iron(III) porphyrin model complexes. The C^{15}N signal shift value of the imidazolate complex was substantially smaller than that of the imidazole one. Thereby,

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Table I. ^{15}N NMR, IR, and Raman Spectroscopic Parameters of P-450$_{cam}$

	$C^{15}N^-$ complex: ^{15}N shift,[a] ppm	CO complex	
		ν_{C-O},[b] cm^{-1}	ν_{Fe-C},[c] cm^{-1}
-CAM	423	1963	464
+CAM	500	1940	481
+CAM + Pd	477	1932	481

[a] Present work. [b] Reference 12. [c] Reference 16.

the smaller ^{15}N shifts of the cyanoperoxidases, compared with the Hb group, were predominantly attributed to the anionic nature of their proximal histidyl imidazole.

A binding of the anionic fifth ligand to the heme iron, of which binding is stronger than that of the neutral fifth ligand, makes an unpaired electron spin transfer from the ferric iron onto the cyanide nitrogen atom less effective. Along with this line, the small ^{15}N shift of cyanochloroperoxidase was also explained as due to the binding of thiolate anion as the proximal ligand.[5] Furthermore, a recent X-ray absorption spectral (EXAFS) study[10] revealed that the Fe–S bond length of P-450$_{cam}$ is the same as that of chloroperoxidase. It is thus reasonable to conclude that the smaller shift of ^{15}N NMR resonance for P-450$_{cam}$, compared with those for Hb group, is due to the binding of the proximal thiolate anion ligand to the heme iron.

In spite of structural similarity for the iron coordinations between P-450$_{cam}$ and chloroperoxidase, there is a slight but significant difference in the $C^{15}N^-$ resonance position between these two enzymes (Figure 3). This observation is presumably related to the structural differences at the heme distal side between them. It has been suggested for chloroperoxidase that the distal histidyl imidazole is located adjacent to the heme sixth coordination site[11] and that there exists the hydrogen bond between the distal imidazole and the iron coordinated ligand. On the other hand, in the case of P-450$_{cam}$, no amino acid residue responsible for the formation of hydrogen bonding is located in the heme distal moiety, as was visualized by X-ray crystallographic analysis.[1] Therefore, the hydrogen-bonding effect may contribute to the ^{15}N signal position for chloroperoxidase, while not for P-450$_{cam}$–$C^{15}N^-$. Indeed, previous results on model compounds clearly showed that the hydrogen bonding with the iron-coordinated $C^{15}N^-$ lowers the shift value of the $C^{15}N^-$ NMR signal.[4] Goff et al.[5] also recognized this effect on the ^{15}N NMR signal position of cyanoperoxidases.

Camphor-Binding Effect on the ^{15}N NMR Signal of P-450$_{cam}$–$C^{15}N$ Complex. As demonstrated above, the ^{15}N NMR resonance of the iron-coordinated $C^{15}N^-$ serves as a sensitive probe for the heme distal and proximal structural characteristics of P-450$_{cam}$. Standing on this view, it is of interest to see that the ^{15}N NMR signal of camphor-free P-450$_{cam}$–$C^{15}N^-$ showed a smaller shift than that of the camphor-bound form. The change in going from camphor-free to -bound enzyme is indicative of an increment of unpaired spin density at the cyanide nitrogen atom. We discuss the change in relation to the structural change in the heme vicinity associated with the substrate binding.

Implications for the heme environmental structural changes of P-450$_{cam}$ upon *d*-camphor binding have been accumulated by some spectroscopic studies. Infrared spectroscopic studies for CO complexes of ferrous P-450$_{cam}$ by O'Keefe et al.[12] indicated that the C–O stretching frequencies are located at 1963 and 1940 cm^{-1} for camphor-free and -bound P-450$_{cam}$–CO, respectively (see Table I). The low-frequency shift of the heme-bound carbonyl for camphor-bound form has been suggested to result from the Fe–C–O bond becoming more bent, presumably due to the steric hindrance from the bound camphor. This causes the increase in π-bonding character of Fe–C bond and consequently results in strengthening the Fe–CO bond and weakening the C–O bond. This suggestion was recently confirmed by resonance Raman spectral studies,[13] in which the Fe–CO stretching vibration is moved from 464 to 481 cm^{-1} upon *d*-camphor binding to camphor-free P-450$_{cam}$–CO. Corresponding to these spectroscopic observations, the quantum yield of photodissociation of the iron-coordinated CO is smaller in camphor-free enzyme than in camphor-bound one.[14]

No available information about the Fe–C–N bond nature of P-450$_{cam}$ has been yet accumulated by IR and Raman spectral studies. However, the linear configuration of the Fe–C–N bond seems to be favorable for camphor-free cyanoenzyme since the distal site at the heme crevice is widely opened for P-450$_{cam}$.[1] On the basis of the results for the CO complex, the binding of camphor near the sixth coordination site would cause a steric hindrance to the Fe–C–N bond in such a manner that it forces the Fe–C–N angle to be bent or tilted away from 180°. A bent or tilt structure of the Fe–C–N bond may favor the overlap between the iron dπ and ligand pπ orbital, thereby increasing in a Fe–CN π-bonding character. Since the unpaired electron spin of the ferric iron is transferred to the axial cyanide through a σ and/or π bond of the Fe–C–N by the spin-polarization mechanism,[4c] the geometrical change in the Fe–C–N bond may make the spin transmission more effective to increase the level of the spin density at the cyanide nitrogen atom.

Previously, we found that the ^1H NMR spectra of P-450$_{cam}$–CN^- were different between camphor-bound and -free forms, where the heme peripheral methyl proton signal is observed at 23 ppm in the presence of *d*-camphor and at 26 and 17 ppm in the absence of *d*-camphor.[14,15] On the basis of the upfield shift of the hyperfine-shifted heme methyl proton resonances, it was suggested that the electron spin density in the iron center is enhanced upon the binding of *d*-camphor to the enzyme. Present ^{15}N NMR study together with the ^1H NMR results shows that the electron spin density at the iron increased by the substrate binding is further delocalized on the axial ligand atoms.

Interaction of Putidaredoxin with P-450$_{cam}$. In the P-450 catalytic cycle subsequent to the formation of oxy P-450$_{cam}$, one electron is offered to the enzyme from the electron donor, putidaredoxin, followed by the O–O bond cleavage and presumably the formation of a reaction intermediate. Our understanding of P-450$_{cam}$–putidaredoxin complex is not well advanced since the structure of putidaredoxin or of the complex has been unknown. However, Ishimura and his co-workers[16] recently measured IR and resonance Raman spectra of P-450$_{cam}$–CO in the presence or absence of putidaredoxin and found that the stretching vibration of C–O (ν_{C-O}) is changed from 1940 to 1932 cm^{-1} upon the binding of putidaredoxin, while that of Fe–C (ν_{Fe-C}) is virtually unchanged (see Table I). These results suggested that the binding of putidaredoxin to P-450$_{cam}$ weakens the iron-bound C–O bond. Combining the present ^{15}N NMR results with these IR and Raman data, we speculate the interaction of the enzyme with putidaredoxin as follows.

The significant change of the ^{15}N NMR spectrum of P-450$_{cam}$–$C^{15}N^-$ (Figure 1) clearly indicates that the binding of putidaredoxin to the camphor-bound enzyme alters the electronic state of the cyanide ^{15}N atom. The decrease in the ^{15}N shift value (from 500 to 477 ppm) is indicative of the decrease of electron spin density on the ^{15}N atom upon the electron donor binding. On the basis of the Raman results for the CO complex, we assume

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that the Fe–CN bond strength is unchanged upon putidaredoxin binding, while the iron-bound C–N bond is weakened under the situation. The weakened iron-bound C–N bond likely makes a less efficient electron spin delocalization from the iron to the ^{15}N atom. Here, we note that the binding of putidaredoxin to the enzyme regulates the electron spin transfer from the iron to the ligand. If the oxy form (Fe^{3+}–O–O^-) of P-450_{cam} is assumed to be similar to the cyano form (Fe^{3+}–C–N^-), this electronic configuration may be favorable to a heterolytic scission of the O–O bond when one more electron is given from the electron donor to the heme in the oxy form.[17]

Concerning the electronic and structural changes in the heme vicinity upon putidaredoxin binding, three possibilities were considered: (1) an increase in the Fe–S^-(Cys) bond strength, (2) a decrease of the steric restriction to the Fe–C–N bond, and (3) a formation of hydrogen bonding of cyanide N atom with nearby amino acid residues. As stated in the preceding sections, any of these effects serve to change the cyanide ^{15}N signal position to upfield region. Recent X-ray studies[1] revealed that the heme of P-450_{cam} is deeply buried in the peptide, and the closest approach to the protein surface occurs at its proximal face, at a distance of about 8 Å. So, if putidaredoxin binds to the protein surface of P-450_{cam} at the proximal side of the heme, it is likely that its binding alters the Fe–S bond strength, supporting the first possibility. Further, when the conformational change at the heme distal side would be induced upon the putidaredoxin binding, the second and/or third possibilities are also likely. Further studies will be required to reveal this problem.

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Distortion in Six-Membered Saturated Rings by Natural Abundance Deuterium NMR

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Abstract: Distortions in six-membered aliphatic ring systems were examined using deuterium chemical shifts and multiple linear regression analysis. A new set of regression parameters has been developed that successfully interprets not only spectra for those methylcyclohexanes with normal chair conformations but also spectra for *cis*- and *trans*-decalin and for three highly distorted trimethylcyclohexanes with skew-boat type distortions to their equilibrium conformations. The origin of deformations in the cyclohexane ring and the effect of distortions on ring hydrogen/deuterium chemical shifts are discussed in terms of gauche interactions between vicinal C–C and C–D bonds. The fits for 115 deuterium shifts in these cyclic alkanes exhibited a multiple $R = 0.9816$ and $\sigma = 0.0623$ ppm for an overall shift range of about 1.5 ppm. As the 14 structural parameters obtained in this study are very sensitive to distortions and conformational features, the results indicate that ^2H NMR methods should be very powerful in those cyclic paraffins in which highly second-order banding is observed in the corresponding ^1H NMR spectrum.

I. Introduction

The use of very high field spectrometers for ^2H NMR has been shown to be valuable in extracting structural information not readily available from complex ^1H spectra.[1] Similar to ^{13}C spectra, proton-decoupled, natural abundance ^2H spectra exhibit single resonances for chemically distinct sites. The absence of scalar couplings in these ^2H spectra produces a simple spectrum, which is extremely helpful in determining chemical shifts when the ^1H spectrum is either banded or exhibits overlapping multiplets. Compounds containing six-membered saturated rings usually suffer severe banding in their ^1H spectra and hence are particularly suitable candidates for study by ^2H NMR.

It is well-known[2,3] that the chair conformation of cyclohexane is energetically more stable than the boat conformation and that cyclohexane interconverts rapidly between degenerate chair conformations at room temperature.[4] Methylcyclohexane preferentially assumes the chair conformation in which the methyl group is equatorial,[5-7] since the strain energy associated with an axial methyl is ~1.8 kcal/mol higher than that for an equatorial methyl. In multiple-substituted methylcyclohexanes there are four different conformational cases: case 1, two equal energy conformations exist and the compound interconverts between them (e.g., 1,1-dimethylcyclohexane); case 2, one chair conformation is energetically favored (>99%) over the other (e.g., 1,*cis*-3-dimethylcyclohexane); case 3, one chair conformation is only slightly favored over the other (e.g., 1,*trans*-2,*cis*-4-trimethylcyclohexane); case 4, substantial distortion to the ring occurs due to extensive steric crowding of substituents (e.g., 1,*cis*-2,*trans*-3-trimethylcyclohexane).

In the first three cases it has been possible to make $^2H/^1H$ NMR spectral assignments very successfully using substitution parameters established by multiple linear regression.[1] In the last case, where some contribution from skew-boat type conformations has been proposed, it has not proved possible to predict or to interpret spectra satisfactorily using the parameters developed from compounds exhibiting "pure" chair conformations. This work develops a parameter set that successfully characterizes the

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