Alkylisocyanide adducts of microsomal P450 exist in two interconvertible forms, each giving the Soret maximum around 430 or 455 nm. This is demonstrated with a rabbit liver P450 2B4. Resonance Raman spectra of the 430- and 455-nm forms were examined for typical P450s of the two types as well as for P450 2B4 because the 430-nm form of P450 2B4 is liable to change into P420. P450cam and P450nor were selected as a model of the 430- and 455-nm forms, respectively. For the n-butyl isocyanide (CNBu) adduct, the Fe(II)-CNBu stretching band was observed for the first time at 480/467 cm\(^{-1}\) for P450cam and at 471/459 cm\(^{-1}\) for P450nor with their \(^{13}\)CNBu/\(^{12}\)CNBu derivatives. For P450cam, but not P450nor, other \(^{13}\)C isotope-sensitive bands were observed at 412/402, 844/835, and 940/926 cm\(^{-1}\). The C-N stretching mode was identified by Fourier transform IR spectroscopy at 2116/2080 cm\(^{-1}\) for P450cam and at 2148/2108 cm\(^{-1}\) for P450nor for the \(^{12}\)C/\(^{13}\)C derivatives. These findings suggest that the binding geometry of isocyanide differs between the two forms—bent and linear structures for P450cam-CNBu and P450nor-CNBu, respectively. In contrast, in the ferric state, the Raman \(^{13}\)C isotopic frequency shifts, and the IR C-N stretching frequencies (2213/2170 and 2215/2172 cm\(^{-1}\)) were similar between P450cam and P450nor, suggesting similar bent structures for both.

Cytochromes P450 (P450s)\(^{1}\) belong to a superfamily of thio-late-coordinated heme proteins and are involved in the oxidative metabolism of various endogenous and exogenous organic compounds, catalyzing monoxygenase reactions. The X-ray crystallographic structures of five bacterial P450s currently available (1–5) suggest that the general features of a protein structure are essentially conserved in all P450s, although many specific sites vary within individual molecules (2–5). It is well known that alkyl and phenyl isocyanide adducts of ferrous P450 enzymes from several sources such as microsomes (6–10), mitochondria (11), and also certain fungi (12) exhibit two Soret bands around 430 and 455 nm. The relative intensity of these two bands has been found to be sensitive to pH. The 455 nm band increases with increasing pH at the expense of the 430 nm band. These observations have been interpreted to mean that the ferrous isocyanide derivatives of P450s exist in two interconvertible forms, the 430- and 455-nm forms hereafter, which are in a pH-dependent equilibrium (6–12).

Studies of ferrous isocyanide derivatives of P450s, mostly of the hepatic microsomal P450s, have revealed several aspects of the 430- and 455-nm forms. For instance, the 430-nm form gives \(\alpha\) and \(\beta\) bands around 560 and 530 nm, while the 455-nm form exhibits them around 580 and 555 nm (8, 9). It has also been found that the \(pK_a\) with respect to the equilibrium between the 430- and 455-nm forms varies over P450 species (9, 12).

The reality in the differences between the two interconvertible forms, however, remains elusive. This is partly due to the fact that ferrous isocyanide derivatives of many P450s undergo a gradual denaturation, particularly under acidic as well as alkaline conditions (6, 7). Therefore, observations on the derivatives have been successful in a pH range from 6 to 8 at which populations of the 430- and 455-nm forms in the equilibrium are in the same order of magnitude (6–9). Such a limitation on the experimental conditions makes it difficult to investigate each of these interconvertible forms individually.

Among P450 species so far studied, P450nor (CYP 55A1 in the systematic nomenclature system (13)) purified from denaturing fungus Fusarium oxysporum (14) exhibits the spectral properties characteristic of P450 (14–16) and belongs to the P450 superfamily (17). Nevertheless, this P450 has no monoxygenase activity. Instead P450nor catalyzes the reduction of NO to N\(_2\)O by accepting electrons directly from NADH (14). Especially interesting is that P450nor has the highest \(pK_a\) value for the equilibrium between the 430- and 455-nm forms of the ferrous isocyanide derivatives (12). Although the \(pK_a\) value was not determined exactly, it has been observed that ethyl, n-butyl, and t-butyl isocyanide adducts of ferrous P450nor are essentially in the 430-nm form in pH ranges below 7.25 (12). Therefore, the ferrous isocyanide derivatives of P450nor are capable of being studied as the best representative of the 430-nm form.

On the other hand, P450cam (CYP 101) from Pseudomonas putida is one of a few exceptions in the P450s with respect to the properties of the ferrous isocyanide derivatives described above. Derivatives such as ethyl, n-butyl, and t-butyl isocyanide adducts of ferrous P450cam present a single Soret band at 452/453 nm, not exhibiting any occurrence of species corre-
The methods for preparing ferric P450 2B4 from rabbit liver have been described elsewhere (21). The ferric sample thus prepared was dissolved in a potassium phosphate buffer (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913 (Kao-Atlas Co., Tokyo, Japan). For the preparation of the n-butyl isocyanide adduct of ferrous P450 2B4, the ferric sample was anaerobically reduced by sodium dithionite in the presence of n-butyl isocyanide. P450nor and P450cam were prepared from the transformed Escherichia coli cells as described previously (22, 23). Myoglobin (Sigma, catalog no. M0830) was dissolved in 50 mM sodium phosphate buffer (pH 7.4), oxidized by potassium ferricyanide, and subjected to a Sephadex G-25 column.

The 13C-labeled (13CNBu) and unlabeled n-butylisocyanide (CNBu) were synthesized using the carbamyl reaction (24) from n-butylamine and chloroform. The 13C-labeled chloroform (99 atom %) was obtained from Nihon Sanso Corp. A solution of n-butyl isocyanide, containing 1% (v/v) n-butyl isocyanide in the phosphate buffer, was prepared just before use. For measurements of resonance Raman spectra, a 30 µM solution of P450nor or P450cam in 100 mM phosphate buffer at pH 6.5 or 7.4, respectively, was prepared. To derive the P450-isocyanide adducts, the solution of n-butyl isocyanide was added under anaerobic conditions to the P450 solution to give a final isocyanide concentration of 2 mM. Sodium dithionite was used to reduce the samples.

Raman scattering from P450 2B4 was excited at 430 and 455 nm by using a dye laser (Coherent CR-599, with stilbene-420) pumped by an Ar laser (Coherent INNOVA 20). The sample was contained in a spinning cell (1800 rpm). The scattered light at right angles was dispersed with a single polychromator (Ritsu Oyokogaku, model DG-1000) and detected by a cooled charge-coupled device detector. To avoid photodissociation by the monitoring light, the sample was excited with the 441.6 nm line of a He-Cd laser (Kinmon Electrics, model CD4805R) or the 423.2 or 422.6 nm line of a diode laser (Hitachi Metals, model ICD-430). The laser power was made as low as possible to avoid photodissociation by the monitoring light. The sample was contained in a spinning cell (1800 rpm). The scattered light at right angles was dispersed with a single polychromator (Ritsu Oyokogaku, model DG-1000) and detected by a cooled charge-coupled device detector (Astromed). The slit width was 200 µm, and the temperature was 20 °C for all the measurements. The Raman shifts were calibrated with indene and CCl4. Infrared spectra were measured using a FTIR spectrometer (SPECTRUM 2000, PerkinElmer) as explained elsewhere (25).

**RESULTS AND DISCUSSION**

Fig. 1 shows the effects of pH on the absorption spectrum of the CNBu adduct of ferrous P450 2B4. Two Soret bands appear at 430 and 455 nm, and a single Q band appears at 553 nm at pH 8.0 (Fig. 1, *solid line*). Upon lowering the pH to 6.0, the 455 nm band decreased in intensity, whereas the 430 nm band was intensified with an isosbestic point in good agreement with the previous report (8, 9). The Q band region was also altered; the β band appeared at 534, and the α band was shifted to 559 nm. However, this P450 was unstable under an acidic condition. When the 430 nm sample was kept under the same pH condition for 0.5 h, the Soret band was shifted to 435 nm and intensified by 1.4-fold, indicating the conversion to P420-CNBu. The absorption spectrum of the P420-CNBu is compared with that of the 430-nm form in the inset of Fig. 1.

To take advantage of RR spectroscopy with regard to the selective enhancement, the excitation wavelength was chosen to coincide with an absorption band of individual chromophores of the n-butyl isocyanide adduct of ferrous P450 2B4 (P450 2B4-CNBu). In other words, the Raman scattering of P450 2B4-CNBu at pH 7.25 was excited at 430 and 455 nm with hopes that the former and the latter excitation selectively enhances RR bands for the 430- and 455-nm forms, respectively. Fig. 2 shows the RR spectra of P450 2B4-CNBu at pH 7.25 in the 1300–1700 cm⁻¹ region where traces A and B represent the spectra obtained with the 455 and 430 nm excitation, respectively. To avoid denaturation of the enzyme, the measurements were carried out within 5–6 min following preparation of the sample by reducing ferric P450 2B4 in the presence of n-butyl isocyanide. Nevertheless a certain amount of denatured product was recognized when the absorption spectra were recorded after the RR measurements (not shown). Such a denaturation is frequently seen for P450 enzymes (6, 7, 18). Therefore, an RR spectrum of the CNBu adduct of a fully denatured sample, i.e., P420 2B4-CNBu, was also recorded and depicted as trace C in Fig. 2 for which the excitation wavelength was 430 nm.

*Trace B* in Fig. 2 closely resembles *trace C*. It should be noted that the absorption spectrum of P450 2B4-CNBu, which is recognized as the V-band region in the RR spectra, shows a shoulder at 435 nm in the absence of isocyanide, as expected from Fig. 1, though this shoulder is not clearly visible in the spectrum of P420 2B4-CNBu.
recalled that P420 2B4-CNBu gives a Soret band around 435 nm and that Raman intensity is nearly proportional to the square of absorbance at the excitation wavelength. Therefore, the contribution from P420-CNBu to trace B, obtained by using the excitation wavelength at 430 nm, might be large. On the other hand, trace A, which was obtained with the 455 nm excitation, is distinct from traces B and C. For example, Raman bands at 1497 and 1367/1368 cm$^{-1}$ ($v_3$ and $v_4$, respectively) in traces B and C are downshifted to 1490 and 1362 cm$^{-1}$ in trace A. This feature indicates that the P420 form and also the 430-nm form scarcely contribute to trace A. Thus, trace A is attributable to the 455-nm form from which Raman scattering was selectively enhanced upon excitation at 455 nm. Based on the frequency of the $v_3$ mode (1490 cm$^{-1}$), which reflects the coordination and spin states of the heme (26), the 455-nm form of P450 2B4 is considered to adopt the six-coordinate low-spin state. For the 430-nm form, the coordination and spin states are unclear because of the involvement of the P420 form in the samples as described above. To overcome the difficulty in measuring the RR spectra of P450 2B4, we used other P450s that can serve as models of the typical 430- and 455-nm forms. In this context, RR spectra of P450cam (model of the 455-nm form) and P450nor (model of the 430-nm form) were measured. Absorption spectra of the n-butyl isocyanide adduct of ferrous P450cam and P450nor are shown in Fig. 3. Unlike other P450s, both samples have a single Soret peak. P450cam-isocyanide has the Soret band at 452 nm and Q bands at 548 and 575 nm, while P450nor-isocyanide has a more intense and sharp Soret band at 427 nm and Q bands at 527 and 558 nm. The relative intensity of $\alpha$ to $\beta$ bands and their separations (856 cm$^{-1}$ for P450cam and 1054 cm$^{-1}$ for P450nor) are distinctly different. The spectra shown in Fig. 3 resemble the absorption spectra of hepatic microsomal P450s shown in Fig. 1. It is noted, however, that the absorption spectrum of P450nor was obtained immediately after the addition of isocyanide, but it was 20 min later when the absorption spectrum of P450cam was completely changed.

Fig. 4 presents the Soret excited resonance Raman spectra in the 1300–1700 cm$^{-1}$ region of ferric P450cam (A), P450cam-n-butyl isocyanide (B), and ferrous P450cam (C) in the left panel (a) and ferric P450nor (D), P450nor-n-butyl isocyanide (E), and ferrous P450nor (F) in the right panel (b). Ferric and ferrous P450cam were measured in the presence of camphor, and therefore the high-spin state is dominant in trace A, while trace D suggests that ferric P450nor is a mixture of high- and low-spin species.

For the isocyanide adduct of reduced P450cam (Fig. 4, left), the $v_3$ and $v_4$ bands were observed at 1492 and 1362 cm$^{-1}$, respectively. These frequencies are close to those found for the 455-nm form of P450cam at 1490 ($v_3$) and 1362 ($v_4$) cm$^{-1}$ (Fig. 2, about A). Other bands of the isocyanide adduct of P450cam such as those at 1554 ($v_2$), 1582 ($v_2$), and 1619 (vinyl mode) cm$^{-1}$ are also close to those in the RR spectrum of the 455-nm form. As already described, the isocyanide adduct of P450cam is a uniform product with an absorption spectrum that is similar to those of the 455-nm form of other P450s (12, 18, 19). Such similarities not only in the absorption spectra but also in the RR spectra between the isocyanide adduct of P450cam and the 455-nm form of P450cam indicate that their active site structures are quite alike. This feature supports our idea that the isocyanide adduct of P450cam serves as a good model for the 455-nm form of other P450s, although the adduct of P450cam does not share the key property with others, i.e. it does not hold the pH-dependent equilibrium.

On the other hand, the isocyanide adduct of P450nor, which was essentially in the 430-nm form under the present experimental conditions (pH 6.5), was found to be notably more stable than the corresponding adduct of P450cam, which was readily

![Fig. 2. RR spectra of n-butyl isocyanide adducts of ferrous P450 2B4 (A and B) and ferrous P420 2B4 (C) at pH 7.25. A Raman band marked with an asterisk in A is due to glycerol present. Raman scattering was excited at either 455 nm (A) or 430 nm (B and C). Other conditions were as follows: laser power, 3–5 milliwatts; enzyme concentration, 30 millimolar; 4-coumarate, 30 millimolar; P450cam (model of the 455-nm form) and P450nor (model of the 430-nm form) were measured.](image-url)
denatured to the P420 form. In RR spectra of its isocyanide adduct (Fig. 4, right), the frequencies of the $v_4$ and $v_6$ modes were observed at 1365 and 1493 cm$^{-1}$. Since single $v_4$ bands were observed as a sharp single band at a middle frequency between those of the ferrous and the ferric unligated states, photodissociated species did not contribute to these Raman spectra, although Raman bands from ferrous unligated species were present in the case of Hb-isocyanide complex (27). These $v_4$ frequencies indicate that both P450-isocyanides adopt the six-coordinated low-spin state (26). The $v_4$ and $v_6$ frequencies are similar between P450cam and P450nor. However, a few distinctive differences are present between the two isocyanide adducts. The $v_{111}$ band is clearly observed at 1554 cm$^{-1}$ for P450cam but not for P450nor. The vinyl modes are seen at 1428 and 1619 cm$^{-1}$ for P450cam but only at 1622 cm$^{-1}$ for P450nor.

In general, alkyl isocyanide has the $\text{R-N}^+\equiv\text{C}^\cdot\cdot\cdot\text{N}$ structure and its stretching mode ($v_{\text{N-C}}$) is observed at 2000–2200 cm$^{-1}$, very similar to those of alkyl nitrile (2220–2260 cm$^{-1}$). We tried to detect isotope-sensitive Raman bands of P450-isocyanide using $^{13}$C-labeled compounds, but isotope difference spectra in the high frequency regions yielded no peak. The $v_{\text{N-C}}$ Raman band of the isocyanide complex is considered as weak as that of a cyanide complex that was detected for the first time by Hirota et al. (28). However, infrared measurements revealed the isotope-sensitive bands at 2213/2170 and 2215/2172 cm$^{-1}$ for the $^{13}$CNBu/$^{13}$CNBu derivatives of ferric P450cam and P450nor, respectively. The corresponding bands for the ferrous states were observed at 2116/2080 and 2148/2108 cm$^{-1}$ for P450cam and P450nor, respectively. The $v_{\text{N-C}}$ frequencies of the ferric and ferrous states are higher and lower than those of free BuN$^{13}$CNBu/N$^{13}$CNBu in an aqueous solution (2175/2134 cm$^{-1}$), respectively. It is to be stressed that the $v_{\text{N-C}}$ frequency is higher for P450nor than for P450cam in the ferrous state, but they are alike in the ferric state. This means that back donation of electrons from Fe(II) ion to an antibonding $\pi^*$ orbital of $\text{C$\equiv$N}$ is larger for P450cam than for P450nor.

Fig. 5 shows Raman spectra in the 200–1000 cm$^{-1}$ region of P450cam-12CNBu, P450nor-13CNBu, and their difference, respectively. For P450cam-12CNBu shown in a, several isotope-sensitive bands are observed around 791 and 820 cm$^{-1}$ for P450cam but not for P450nor.

Fig. 6 shows Raman spectra in the 200–1100 cm$^{-1}$ region of P450cam-n-butyl isocyanide excited at 441.6 nm and P450nor-n-butyl isocyanide excited at 422.6 nm. Traces A, B, and C in the left panel (a) present the spectra of P450cam-$^{12}$CNBu and P450cam-$^{13}$CNBu, and their difference, respectively. Traces D, E, and F in the right panel (b) denote the spectra of P450nor-$^{12}$CNBu, P450nor-$^{13}$CNBu, and their difference, respectively. The experimental conditions were as follows: excitation, 422.6 nm; laser power, 15 milliwatts at sample; accumulation time, 5 min.
of the anion (CN\textsuperscript{−}) adduct of ferric P450cam reported by Simianu and Kincaid (29).

The cyanide adduct of ferric heme proteins, which has the same number of electrons as a Fe(II)-CO adduct, adopts a linear geometry in the absence of any steric hindrance. In the case of the ethyl isocyanide adduct of reduced Mb, the side chain of the distal His swings away from the ligand to avoid steric congestion (29), but no \textsuperscript{13}C isotope-sensitive bands were observed for the isocyanide adduct of Mb in this study. For the ferric cyanide complex, if the Fe(III)-C-N group is linear and perpendicular to the heme plane, the Fe(III)-C-N bending (δ(FeCN)) fundamental mode should be Raman-inactive. In practice, the Fe-C-N unit in MbCN is linear but tilted according to the x-ray crystallographic (31) and NMR studies (32). In the linear Fe-C-N structure, the frequencies of the Fe-CN stretching (ν(Fe-C)) and δ(FeCN) modes should exhibit a linear and a zigzag pattern, respectively, upon the isotope substitution in the order of \textsuperscript{12}C\textsuperscript{14}N, \textsuperscript{13}C\textsuperscript{14}N, \textsuperscript{12}C\textsuperscript{15}N, and \textsuperscript{13}C\textsuperscript{15}N. The ν(Fe-C) and δ(FeCN) RR bands were first observed for methHb III isolated from Chironomus thummi thummi (methHb CTT III) at 453 and 412 cm\textsuperscript{−1} (33). The corresponding frequencies for human methHa (34) and sperm whale methMb (35) are similar to those of methHb CTT III. For peroxidases, however, the order of ν(Fe-C) and δ(FeCN) frequencies was reversed: ν(Fe-C) = 361 and δ(FeCN) = 454 cm\textsuperscript{−1} for myeloperoxidase (36), ν(Fe-C) = 360 and δ(FeCN) = 422 cm\textsuperscript{−1} for horseradish peroxidase (37), and ν(Fe-C) = 360 and δ(FeCN) = 453 cm\textsuperscript{−1} for lactoperoxidase (38). The ν(Fe-C) and δ(FeCN) RR bands of P450 have been observed at 413 and 387 cm\textsuperscript{−1}, respectively (29).

On the other hand, when the Fe-C-N unit adopts a bent geometry, the CN isotope-sensitive band appears around 310 cm\textsuperscript{−1} as reported for horseradish peroxidase (37), P450 (29), and lactoperoxidase (38). For a bent structure, the ν(Fe-C) and δ(FeCN) modes are mixed significantly. Simple normal coordinate calculations on a triatomic molecule suggested that the patterns of the isotopic frequency shifts of ν(Fe-C) and δ(FeCN) were reversed from those for the linear case when the Fe-CN angle is smaller than 160° (29). Consequently for an appreciably bent structure, both ν(Fe-C) and δ(FeCN) would exhibit appreciable amounts of \textsuperscript{13}C isotopic frequency shifts, although only δ(FeCN) is expected to show a large frequency shift for a linear structure.

Since CNBu binds to the Fe(II) heme, its geometry would be different from that of Fe(III)-CN\textsuperscript{−}. Furthermore, we have used only \textsuperscript{12}C\textsuperscript{14}NBu and \textsuperscript{13}C\textsuperscript{14}NBu in this experiment. Therefore, assignments of Raman bands to ν(Fe-C) and δ(FeCN) cannot be determined unequivocally. However, since the \textsuperscript{13}C isotopic frequency shift is fairly large for both the 480/467 (Δν = 13 cm\textsuperscript{−1}) and 412/402 cm\textsuperscript{−1} bands (Δν = 10 cm\textsuperscript{−1}), it is likely that the former and latter arise mainly from the ν(Fe-C) and δ(FeCN) modes of a bent structure, although the two modes may be mixed to a greater extent.

In the higher frequency region of Fig. 6, there are two additional isotope-sensitive bands at 844/835 and 940/926 cm\textsuperscript{−1} for \textsuperscript{12}CNBu/\textsuperscript{13}CNBu derivatives. Their isotopic frequency shifts (9 and 14 cm\textsuperscript{−1}, respectively) are less than or similar to those of the ν(Fe-C) and δ(FeCN) fundamentals. Therefore, these bands cannot be attributed to a combination or an overtone of ν(Fe-C) and δ(FeCN). Since there is no \textsuperscript{13}C isotope-sensitive mode in this frequency region among internal modes of CNBu, and there are many porphyrin modes around 420–460 cm\textsuperscript{−1} that are not always Raman-active, the high frequency \textsuperscript{13}C-isotope sensitive bands might arise from combinations of a Fe-CN vibration and a porphyrin vibration, but detailed assignments remain to be clarified.

In contrast, for P450nor, the \textsuperscript{12}CNBu – \textsuperscript{13}CNBu difference spectrum (Fig. 6F) showed only one isotope-sensitive band at 471/459 cm\textsuperscript{−1}. There are no other isotope-sensitive bands in the 800–1000 cm\textsuperscript{−1} region. To confirm the present results, we measured the Raman spectra of ferrous MbCNBu (not shown), which gives a Soret band at 430 nm, with the same excitation wavelength. There was no peak in the isotope difference spectrum, although the ν\textsubscript{d} and ν\textsubscript{a} bands (1365 and 1496 cm\textsuperscript{−1}, respectively) indicated that the observed Raman spectra arise from the isocyanide complex (not from a photodissociated species). Thus, P450nor-CNBu is distinct from MbCNBu for which swinging up of the distal His was noted from x-ray crystallographic analysis (30). It is natural to assign the 471/459 cm\textsuperscript{−1} bands of P450nor-CNBu to ν(Fe-C). Then the absolute value of ν(Fe-C) is slightly lower for P450nor than for P450cam, while the \textsuperscript{13}C isotopic frequency shifts are alike (Δν = 12 cm\textsuperscript{−1} for P450nor and Δν = 13 cm\textsuperscript{−1} P450cam). Furthermore, consideration of the presence of additional \textsuperscript{13}C isotope-sensitive bands in the high frequency region as well as in the low frequency region for P450cam leads us to conclude that isocyanide is bound to the Fe(II) ion in a linear geometry for P450nor but in a bent geometry for P450cam. Since the bent structure causes more vibrational coupling with porphyrin modes, the appearance of an increased number of \textsuperscript{13}C isotope-sensitive bands for P450cam is not unreasonable as noted for the Fe(III)-cyanide adduct of P450cam (29). If the bending direction is toward the
vinyl groups, their geometrical structure might be perturbed, and a possible difference between P450nor and P450cam may appear in the vinyl-related Raman bands.

X-ray crystallographic studies of both P450s support this conclusion (1, 5). The binding site of an external ligand of P450cam is surrounded with hydrophobic residues so tightly that substrate cannot be accommodated into the heme pocket in the presence of isocyanide (18). In fact, there was no difference between Raman spectra of P450cam-CNBu in the presence and absence of camphor. Thus, isocyanide on P450cam would be forced to adopt a bent geometry. For P450nor, on the other hand, a large cleft, presumably a NADH binding site, is present at the ligand binding site (5). Accordingly the Fe-C-N moiety of isocyanide on P450nor could adopt a linear geometry along the cleft. Recently x-ray crystallographic analysis was completed for both P450cam-CNBu and P450nor-CNBu by Shiro and co-workers (25), who have reported the coordination structure differences between P450cam(II)-CNBu (d(Fe-C) = 1.86 Å, <Fe-C-N = 159°) and P450nor(II)-CNBu (d(Fe-C) = 1.85 Å, <Fe-C-N = 175°) complexes.

n-Butyl isocyanide could also bind to ferric P450cam and P450nor, giving rise to a Soret peak similarly at 430 nm, and their absorption spectra closely resemble that of the cyanide adducts of P450. Prominent Raman bands of ferric P450-CNBu at 1371 and 1501 cm⁻¹ (data not shown), which are assignable to v₁ and v₃, respectively, were indicative of a six-coordinated low-spin heme and distinct from those of ferrous complexes. There was no isotopic-sensitive band in this frequency region. However, some isotopic-sensitive bands were observed in the lower frequency region.

Fig. 7 presents the Raman spectra in the 200–1100 cm⁻¹ region of P450cam(III)-¹³CNBu (A), P450cam(III)-¹²CNBu (B), and their difference (C) in the left panel (a) and P450nor(III)-¹³CNBu (D), P450nor(III)-¹²CNBu (E), and their difference (F) in the right panel (b). All the spectra were excited at 422.6 nm. The pattern of the isotopic difference spectrum for P450cam(III)-CNBu (Fig. 7C) is similar to that for P450cam(II)-CNBu (Fig. 6C), although all peaks are shifted to lower frequencies in the ferric state relative to the ferrous state. In contrast, the pattern of the isotopic difference spectrum of P450nor(III)-CNBu (Fig. 7F) was distinct from that of P450nor(II)-CNBu (Fig. 6F). There are two isotopic-sensitive bands at 924/914 and 840/829 cm⁻¹, although they are weak. This means that the structures of P450cam(III)-CNBu and P450nor(III)-CNBu are closer to each other than are the structures of P450cam(II)-CNBu and P450nor(II)-CNBu. This is consistent with the behavior of the IR ν(C-N) frequencies described above. The Raman band of P450nor(III)-¹³CNBu (Fig. 7D) at 409 cm⁻¹ is absent in the spectrum (Fig. 7E), suggesting that the 409 cm⁻¹ band arises from the Fe(III)-CNBu stretching mode. So, this frequency is substantially lower than that of the ferrous state. However, the isotopic difference spectrum shown by trace F (Fig. 7) gives another positive peak at 432 cm⁻¹. It is unclear why a negative peak does not appear strongly in the difference spectrum, even if there were vibrational coupling with porphyrin ring modes. Except for this negative peak feature, the difference spectra (Fig. 7, C and F) are alike. Consequently, n-butyl isocyanide binds to ferric P450cam and P450nor similarly in a bent geometry.

In conclusion, the two absorbing forms of alkyl isocyanide adducts of hepatic microsomal P450s would presumably arise from the presence of two geometrically different forms, that is, bent and linear structures about the Fe(II)-C-N moiety.

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Elucidation of the Differences between the 430- and 455-nm Absorbing Forms of P450-Isocyanide Adducts by Resonance Raman Spectroscopy
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