Hydrogen-Bonding Interactions in the Active Sites of Cytochrome P450cam and Its Site-Directed Mutants

Tian-jing Deng,[‡] Iain D. G. Macdonald,[§,||] Mihaela C. Simianu,[‡] Milan Sykora,[‡] James R. Kincaid,*[,‡] and Stephen G. Sligar*[,†]

Contribution from the Department of Chemistry, Marquette University, Wehr Chemistry Building, P.O. Box1881 (535 North 14th Street, 53233), Milwaukee, Wisconsin 53201-1881, Beckman Institute for Advanced Science and Technology and Department of Biochemistry, University of Illinois, Urbana, Illinois 61081

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Abstract: Resonance Raman spectroscopy is applied to the cyanide adducts of cytochrome P450cam and its T252A and D251N site-directed mutants, both in their substrate-free and camphor-bound forms, to probe active-site heme structure and, in particular, interactions of the FeCN fragment with potential active-site H-bond donors. In contrast to the ferrous CO and ferric NO adducts, which form only essentially linear (slightly distorted) FeXY fragments, the spectra of the ferric CN^- adducts provide clear evidence the for the existence of an additional, rather highly bent, conformer; that is, the cyanide complexes form both linear and bent conformers in both the substrate-free and substrate-bound forms. Formation of this bent conformer is most reasonably attributed to the presence of off-axis H-bond donors, which induce distortion on the FeCN fragment but not the FeCO and FeNO fragments, which are poorer H-bond acceptors. For all three proteins, the substrate-free form exhibits a complex spectral pattern which arises because one of the modes associated with the FeCN fragment is coupled with two heme macrocycle deformation modes. Significantly, no evidence for such coupling is observed in the spectra of the camphor-bound forms. While various unknown factors may possibly give rise to selective activation of such coupling in the substrate-free derivative, given the known facts about the active-site architecture of this enzyme, a plausible explanation is that the bent conformer is oriented toward the water-filled substrate-binding site in the substrate-free form, but oppositely, toward the proposed proton delivery shuttle, in the substrate-bound form. Sensitivity of the FeCN modes to H_2O/D_2O exchange in the two camphor-bound mutants, which is apparently absent for the camphor-bound native protein, is most reasonably attributed to the known presence of extra water in the active sites of these mutants.

Introduction

The monooxygenases of cytochrome P450 superfamily play important and diverse functional roles in a wide range of biological systems.[1,2] For more than 30 years cytochrome P450cam, which catalyzes the hydroxylation of camphor in the bacterium, *Pseudomonas putida*, has been the most extensively studied member of this class, owing to its ready availability, aqueous solubility, and relative ease of isolation and purification.[3−5] Indeed, the X-ray crystal structures of several forms of P450cam, including those of various substrate-bound derivatives and site-directed mutants, are now available.[6−11] Despite the accumulation of an impressive body of knowledge on this class of enzymes, mechanistic details of certain steps of the catalytic cycle remain somewhat obscure and continue to attract a great deal of attention.[3−5,12]

The early stages of the cycle are reasonably well-understood and documented. The binding of substrate displaces an active-site cluster of water molecules, one of which serves as an axial ligand to the ferric heme prosthetic group, inducing a spin state change of the heme from low- to high-spin which lowers its redox potential, thereby facilitating reduction to a high-spin ferrous species which is capable of binding molecular oxygen. The resulting quasi-stable oxygenated species is the last intermediate in the catalytic cycle to be observed and spectroscopically characterized.[13−16] Cleavage of the O−O bond in this

‡ Marquette University.
† University of Illinois.
§ University of Strathclyde, Glasgow, Scotland.
|| Previously at University of Illinois.

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system, eventually generating a powerful oxidizing species presumably analogous to the compound I intermediate of hydroperoxidases, apparently occurs by one-electron reduction and protonation of this species.[3]

In recent years, much attention has been focused on possible mechanisms for the required proton delivery to the active site. On the basis of extensive spectroscopic and kinetic studies of the native enzyme and several site-directed mutants,[11,17−22] it has been suggested that the Asp251 and Thr252 residues are important in constituting a controlled proton delivery pathway involving solvent water and providing an active-site H-bond donor, which may be a trapped water molecule rather than Thr252,[11,21] to stabilize the dioxygen adduct. Acquisition of direct experimental evidence for the presence of this active-site H-bond donor is clearly an important objective.

Resonance Raman spectroscopy is well established as an especially effective probe of the active-site structure of heme proteins, the spectra providing detailed structural information for the heme moiety and spectral signatures diagnostic of axial ligand disposition.[23,24] For the particular enzyme of interest here, the physiologically relevant oxygenated intermediate is generally quite reactive, though it can be stabilized under certain conditions.[13−16] Given this situation, attention was naturally turned to alternative diatomic ligands which form more stable adducts (e.g., CO, NO, and CN^-) and many RR studies of these have been reported (25−29). Of specific interest here is the use of RR spectroscopy to detect H-bonding interactions between the bound axial ligand and potential distal pocket proton donors and, as has been argued previously,[27] adducts of the negatively charged cyanide ligand, which possesses superior ability as an proton acceptor relative to CO and NO, offer an important advantage.

Many RR studies of various types of heme proteins have now been reported.[27−37] In the absence of any steric or off-axis electronic interactions which could destabilize the inherently preferred linear geometry of the Fe−CN^- fragment, only the Fe−C stretching mode is Raman active, and indeed, only this mode is observed in the RR spectra of the cyanide complexes of simple, protein-free, hemes.[31] However, within the varied distal pocket environments of heme proteins, there exist steric forces or off-axis H-bonding interactions which are apparently sufficient to distort the Fe−CN linkage so that both modes are active. For some proteins, such as the oxygen transport proteins, myoglobin and hemoglobin, only slight distortions are encountered, giving rise to a so-called "essentially linear" conformer. This conformer possesses a ν(Fe−C) stretching frequency between 410 and 450 cm^{-1}, which shifts to lower frequency in a monotonic fashion as the total mass of the CN^- isotopomer is increased.[30] The corresponding δ(FeCN) bending mode of this conformer is observed at slightly lower frequencies, its shift upon isotopic substitution of the CN^- ligand depending on the position of substitution, with significant frequency decreases occurring only for the ^{13}C isotopomers (i.e., $^{13}C^{14}N^-$ and $^{13}C^{15}N^-$). Such behavior (referred to as a so-called "zigzag" shift pattern, because the frequencies of the various isotopomers are usually listed in the order $^{12}C^{14}N^-$, $^{13}C^{14}N^-$, $^{12}C^{15}N^-$ and $^{13}C^{15}N^-$) is predicted for the bending mode of a (linear or near linear) geometry of the $FeCN^-$ fragment, wherein the iron-bound carbon atom experiences the largest displacements.[24]

In a few cases, such as the mammalian myeloperoxidase[33] and lactoperoxidase,[34] active-site perturbations are apparently of sufficient strength and appropriate orientation to induce a substantial degree of distortion, the resultant geometry of the $FeCN^-$ fragment being most accurately described as a bent conformation with vibrational behavior quite distinct from that of the essentially linear conformer. The distortion induces kinematic mixing of the stretching and bending coordinates and leads to a larger separation between the two modes of mixed character. This bent conformation is characterized by a mode appearing between about 420 and 450 cm^{-1}, exhibiting a "zigzag" shift pattern, and a lower frequency mode near 350 cm^{-1}, having a nearly monotonic shift pattern.

For many heme proteins,[32,35−37] including P450cam,[27] the active-site architecture is such that both the essentially linear and the bent conformers have been identified using RR spectroscopy. In the earlier work on P450cam,[27] both conformers were observed in the substrate-bound, as well as the substrate-free adducts, the latter being known from X-ray crystallography to possess an active-site cluster of water molecules. On the basis of observed sensitivity to H_2O/D_2O exchange of two modes ascribable to the FeCN fragment, both conformers of the substrate-free derivative were suggested to be involved in H-bonding interactions with different regions of the water cluster. In contrast, no convincing evidence of H_2O/D_2O sensitivity was obtained for either of the two conformers present in the substrate-bound derivative, though it must be emphasized that the lack of an observable shift does not exclude the possibility of such interactions (vide infra).

Evidence from intensive spectroscopic, crystallographic and kinetic studies of the two site-directed mutants of P450cam

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designed to perturb the proposed H-bonding network, which includes the two key residues (i.e., Thr252Ala and Asp251Asn), suggests that such alterations affect the proton delivery mechanism and may perturb H-bonding interactions which stabilize the bound dioxygen of the oxygenated intermediate.[17−19,21−22] In the present work, the cyanide adducts of these two proteins are studied by RR spectroscopy in an attempt to document any changes in the disposition of the FeCN fragment induced by these mutations. In contrast to the case of the wild-type protein,[27] clear evidence is obtained for the presence of H-bonding interactions in the substrate-bound forms of these mutant proteins.

Experimental Procedures

Materials and Methods. The P450cam mutants, T252A and D251N, were expressed in *Escherichia coli* and purified using previously published procedures.[10,21] For the preparation of the camphor-free mutant P450cam, the camphor-bound P450cam was passed successively over a G-25 column equilibrated with 50 mM MOPS buffer at 4 °C (pH 7.4). The substrate-free protein was exchanged with 20 mM potassium phosphate (pH 7.4) containing 100 mM KCl, using an appropriately equilibrated G-25 column.

The CO adducts of P450cam mutants were prepared by addition of a 40-fold excess of dithionite, followed by gentle bubbling with CO for several minutes. The UV−vis spectrum has been taken to confirm the absence of denatured product (P420). The final concentration of proteins in the solutions used for RR measurements was 0.1 mM.

The cyanide adducts of both the camphor-free and camphor-bound P450cam mutants were prepared by addition of a 0.1 M KCN buffered solution (final KCN concentration of 50 mM) to 0.1 mM protein solution. Both the KCN and protein solutions were buffered in 100mM phosphate solution with 100 mM KCl, pH = 7.4. The isotopomeric cyanide adducts were prepared in the same way and measured under identical instrumental conditions. The 99.9% isotopically labeled potassium cyanide samples ($K^{13}CN$, $KC^{15}N$, and $K^{13}C^{15}N$) were obtained from Isotech Inc. (Miamiburg, OH).

H_2O/D_2O buffer exchange was carried out in a microconcentrator (Centricon-10) at 4 °C. About 0.5 mL of protein solution was diluted to 2 mL with D_2O buffer containing 1 mM camphor and transferred to the concentrator. The volume of the solution was reduced to about 0.2 mL by centrifugation. This procedure was repeated five times, the total exchange time being ∼24 h.

Spectroscopic Measurements. The resonance Raman spectra were acquired with a Spex 1269 spectrometer equipped with a ICCD UV-enhanced detector (Princeton Instrument) using the 442 nm excitation line from a Liconix model 2420NB He-Cd laser. The Rayleigh scattered line was removed using a 442 nm notch filter (Kaiser Optical). The power at the sampling point was 15 mW. The samples were kept in sealed NMR tubes, and the tubes were spun during the measurement to avoid local heating effects on the protein. The spectra were typically accumulated for 5000−6000 s and calibrated by using the spectrum of fenchon. UV−vis spectra of each sample were collected before and after every RR measurement to ensure integrity of sample.

Computer Simulation Method. Owing to the overlap with of several heme vibrational modes in the low-frequency region, the modes of the Fe−CN fragment are not easily discerned in the absolute spectra of cyanide adducts of P450cam. Ideally, the difference spectra, generated by subtraction of various isotope labeled cyanide adducts, should reveal only those modes associated with the FeCN fragment, because the heme modes are expected to cancel in the subtraction. To obtain a more reliable estimate of the actual vibrational parameters of individual modes of the FeCN fragment, the six experimental difference spectra which can be generated from the four isotopically labeled cyanide adducts are fitted to simulated difference spectra using the method and program described previously.[36] As in the previous work, the simulated spectra shown here represent the best fit obtained by simultaneous fitting of all six experimental difference spectra. The digital subtraction of the absolute spectra was performed using SpectraCalc software. The subtraction was performed in such a way that the intense isolated heme modes (e.g., ν_7) were confirmed to be completely canceled.

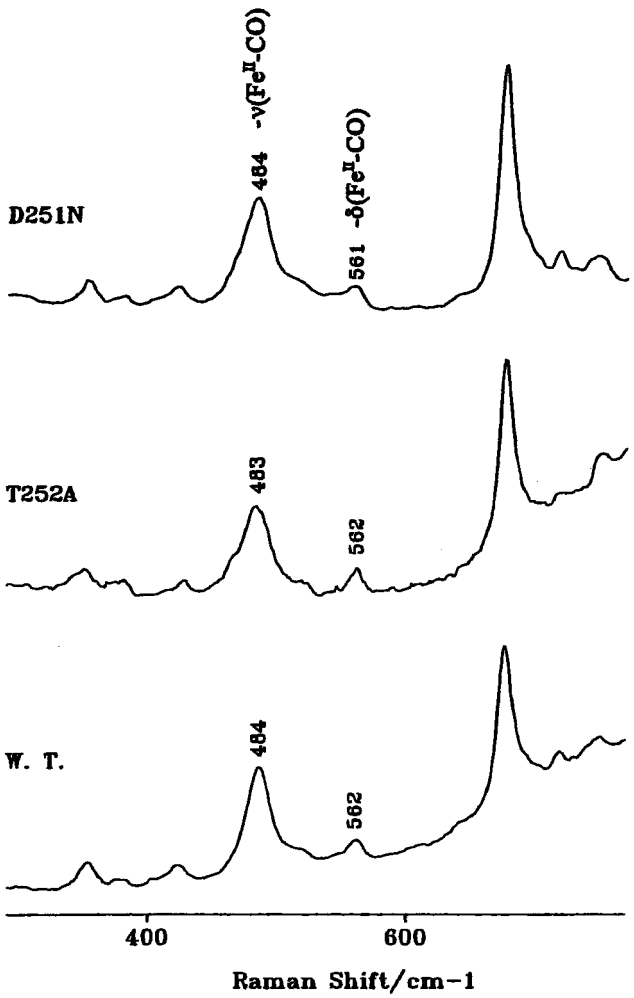

Figure 1. RR spectra of CO adduct of camphor-bound ferrous D251N, T252A, and wild-type P450cam. All spectra were obtained with 442 nm excitation line with a laser power less than 15 mW at the sample.

For the cyanide adducts of both camphor-bound D251N and T252A, difference spectra (H_2O − D_2O) are generated, and the simulation is conducted in the same manner described above, using the spectral parameters derived from the previous simulations of various isotopomers.

Results

1. CO Adducts of Ferrous T252A and D251N P450cam. Shown in Figure 1 are the RR spectra of the CO adducts of the camphor-bound derivatives of the ferrous forms of the two mutant proteins, along with those of the native enzyme. It is noted that there are no significant differences in the vibrational properties of the FeCO fragments among these three proteins.

2. Cyanide Adducts of Ferric T252A P450cam. a. Substrate-Free T252A P450cam. The absolute RR spectra of the cyanide adducts, in both the substrate-free and camphor-bound forms of all three proteins are given in Figure 2. From comparison of the spectra, it is obvious that substrate-binding induces changes in the low frequency modes. Elucidation of these changes for each of the mutants are dealt with below. The RR spectra of T252A P450cam ligated with the various cyanide isotopomers are shown in Figure 3. Owing to overlap with the relatively strongly enhanced heme deformation modes which occur in this region, difference spectra are needed to reveal the isotope

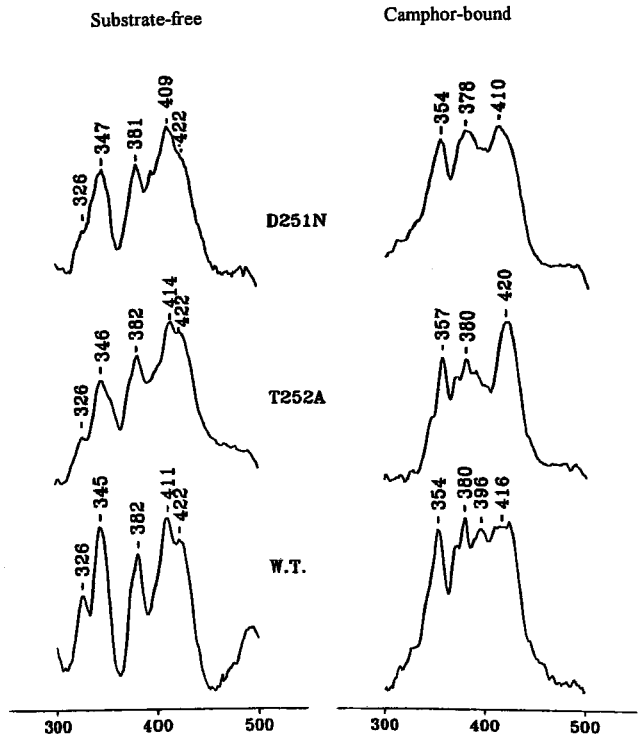

Figure 2. RR spectra of cyanide adduct of ferric D251N, T252A, and wild-type P450cam. Substrate-free forms are shown on left panel, and camphor-bound forms are shown on right.

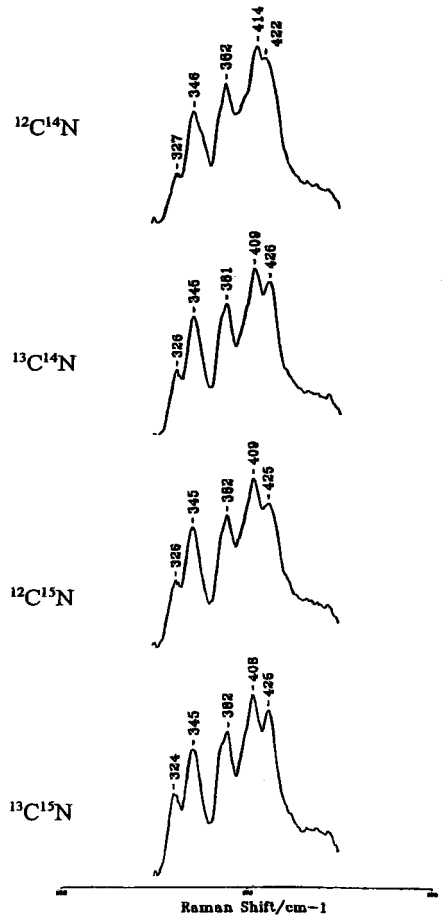

Figure 3. Low-frequency RR spectra of substrate-free T252A P450cam ligated with various cyanide isotopic forms.

sensitive modes associated with the FeCN fragment. The six separate difference spectra which can be generated by subtraction of various combinations of the four absolute spectra are shown in Figure 4, together with the simulated spectra. All the derived frequencies of the isotopic sensitive modes are listed in the Table 1. As can be seen by inspection of Figure 4, the experimental difference spectra are fairly well-reproduced by the set of derived parameters listed in Table 1. As was reported previously for the case of wild-type P450cam,[27] the inclusion of six modes is needed in order to adequately simulate the experimental data. The four modes associated with the FeCN fragments of the two expected FeCN conformers (the so-called "linear" and "bent" forms) exhibit relatively large isotopic shifts, while the two heme macrocycle modes located at 329 and 354 cm^{-1} (in the $^{12}C^{14}N$ isotopomer) exhibit rather small shifts. On the basis of the isotope shift patterns and the previous studies mentioned earlier, a monotonically shifting mode (414 cm^{-1}) is assigned as the stretching mode of the "essentially linear" conformer, and a "zigzag" shifting mode (388 cm^{-1}) is assigned to the bending mode of this conformer. The vibrational frequencies and isotopic shifts of the "linear" conformer of the T252A cyanide adduct are quite similar to those obtained for the wild-type cyanide adduct (413, 387 cm^{-1}), indicating a similar disposition of the "linear" conformers of both adducts. The "bent" conformer exhibits a monotonically shifting mode at 347 cm^{-1} and a "zigzag" shifting mode at a higher frequency ($\sim$433 cm^{-1}) (Table 2).

b. The Camphor-bound T252A. The RR spectra of the various isotopic cyanide complexes of camphor-bound T252A (not shown) give rise to the experimental and simulated difference spectra shown in Figure 5. The set of derived absolute frequencies, bandwidths, and relative intensities which yield these difference spectra are listed in Table 1. It is noted that, just as in the case of the wild-type enzyme,[27] a distinctly different spectral pattern is obtained upon binding of substrate. Thus, while six isotope-sensitive modes (including two heme core modes) are required to obtain an adequate fit for the substrate-free species, only four modes are required for the camphor-bound species.

In an attempt to probe potential H-bonding interactions within the active site, spectra were acquired for the camphor-bound derivative in both H_2O and D_2O buffers. As shown in the inset in Figure 5, the spectra in H_2O and D_2O indicate a shift of the 420 cm^{-1} mode to 424 cm^{-1} in D_2O. The actual shift is about 2 cm^{-1} (420 to 422 cm^{-1}) based on the derived parameters from stimulated spectra (not shown). As can be seen in the experimental difference pattern, the change to D_2O also causes an apparent decrease in the intensity of the mode near 358 cm^{-1}.

3. Cyanide Adducts of Ferric D251N. a. Substrate-Free D251N. The RR spectra of the various isotopic cyanide adducts of substrate-free D251N (not shown) give rise to the experimental and simulated difference patterns shown in Figure 6. Though it is apparent that the S/N ratios in these spectra are inferior to those obtained for the T252A and wild-type proteins,[27] it is noted that analyses for two separate data sets obtained with different samples yield the same set of derived vibrational parameters (both sets being listed in Table 1). As can be seen in Table 1, the derived frequencies for both modes of both conformers are quite similar to those previously derived for the wild-type protein.[27] As in the cases of the substrate-free complexes of wild-type and T252A proteins, it was necessary to include two heme core modes in the simulation in order to adequately fit the experimental difference patterns.

b. Camphor-Bound D251N. The RR spectra of the various isotopic cyanide adducts of camphor-bound D251N give rise

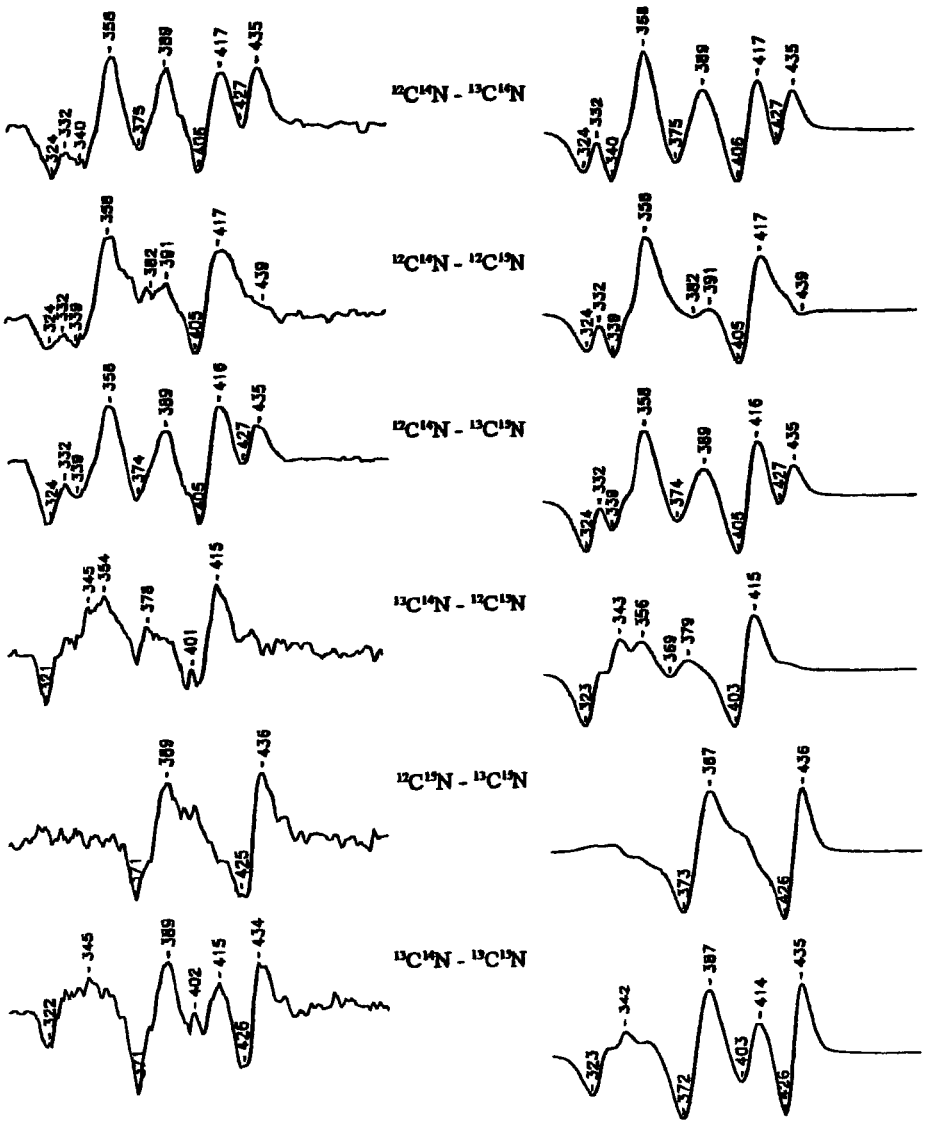

Figure 4. Experimental difference spectra for substrate-free T252A P450cam cyanide adduct, generated from Figure 2, and the simulated difference spectra.

to the experimental difference and the simulated spectra given in Figure 7. Again, only four modes are needed to adequately fit the experimental difference patterns and the derived parameters are listed in Table 1.

As shown in the inset in Figure 7, the H_2O/D_2O difference spectrum acquired for camphor-bound D251N cyanide complex provides clear evidence for sensitivity to H/D exchange, with two features exhibiting distinct shifts to higher frequency; that is, one near 411 cm^{-1} and one near 353 cm^{-1}. The parameter derived from the stimulated difference spectrum (not shown) shows a 4 cm^{-1} upshift; that is, from 414 cm^{-1} in H_2O to 418 cm^{-1} in D_2O.

Discussion

1. Origins and Relative Orientations of the Bent Conformers. The RR spectra acquired here and in the previous work[27] for the cyanide adducts of P450cam and its mutants document the presence of both linear and bent conformations of the FeCN fragment in both the substrate-free and substrate-bound derivatives. The essentially linear conformer (in both the substrate-free and -bound cases) exhibits a monotonically shifting mode near 415 cm^{-1} and a "zigzag" shifting mode near 390 cm^{-1}, the former showing slight shifts to higher frequency upon substrate binding. The bent conformer of the substrate-bound form gives rise to a characteristic vibrational pattern with a monotonically shifting mode near about 350 cm^{-1} and a weak mode near about 435 cm^{-1} which exhibits a "zigzag" isotopic shift pattern. The most notable observation for all three proteins (the native enzyme and both mutants) is the complicated vibrational pattern near 350 cm^{-1} which arises only for the substrate-free forms. Thus, as was pointed out in the previous section, to obtain satisfactory fits for the substrate-free derivatives it is necessary to include two heme modes (near 330 and 355 cm^{-1}) which apparently couple with the lower frequency internal mode of the bent FeCN conformer.

Evidence for coupling of internal FeCN modes (and for FeXY fragments, in general) with low-frequency heme deformation modes has been reported for several heme protein adducts with diatomic exogenous ligands,[27,34−40] with the most thoroughly

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Table 1. Derived Frequencies for Isotope Sensitive Modes from Computer Simulation

D251N	$^{12}C^{14}N$	329(328)	345(344)	354(354)	392(390)	413(414)	434(435)[c]
P450cam CN	$^{13}C^{14}N$	327(326)	340(339)	353(352)	374(376)	410(411)	429(429)
substrate-free	$^{12}C^{15}N$	328(327)	342(340)	353(352)	392(390)	410(410)	434(435)
	$^{13}C^{15}N$	326(326)	337(338)	352(351)	372(372)	407(406)	428(429)
	fwhm[a]	16		16	16	16	12
	RI[b]	3.7(4.4)	1.6(2.0)	3.5(5.0)	1.4(1.6)	2.2(2.2)	1.0(1.0)
T252A	$^{13}C^{14}N$	329	347	356	388	414	433
P450cam CN	$^{13}C^{14}N$	328	340	352	375	410	428
substrate-free	$^{12}C^{15}N$	328	340	352	386	409	434
	$^{13}C^{15}N$	327	338	351	373	407	427
	fwhm	16	16	16	16	16	12
	RI	6.3	2.3	4.2	1	3	1.3
D251N	$^{12}C^{14}N$		355	392	414	433	
P450cam CN	$^{13}C^{14}N$		351	375	409	417	
substrate-bound	$^{12}C^{15}N$		354	389	408	423	
	$^{13}C^{15}N$		350	374	405	415	
	whm		12	16	18	15	
	RI		1.2	3.1	3.7	1	
T252A	$^{13}C^{14}N$		360	393	420	436	
P450cam CN	$^{13}C^{14}N$		357	378	413	420	
substrate-bound	$^{12}C^{15}N$		358	392	417	432	
	$^{13}C^{15}N$		354	376	410	416	
	[a]fwhm		12	16	18	14	
	RI		2.6	4.5	4.5	1	

[a] Full width at half maxima. [b] Relative intensities. [c] Numbers in parentheses are derived from second sample.

Table 2. Frequencies of Fe−CN in Wild Type and Mutants of Cytochrome P450cam

	"essentially linear" Fe−CN		"bent" Fe−CN	
protein	ν(Fe−C)	δ(Fe−C−N)	ν(Fe−C)	δ(Fe−C−N)
w.t.[a,b]	413	387	342	434
camphor-bound w.t.[b]	416	393	359	424
admanatonone-bound w.t.[b]	423	387	357	437
T252A[c]	414	388	347	433
camphor-bound T252A[c]	420	393	360	436
D251N[c]	413	391	345	434
camphor-bound D251N[c]	414	392	355	432

[a] w.t.: Wild Type. [b] Reference 27. [c] This work.

studied cases being those of CN^- adducts[27,34−37] and the oxygen adducts of heme oxygenase[40] and P450cam.[16] In the case of the heme oxygenase adduct, effective arguments are made to suggest that such coupling of the δ(FeOO) bending mode with out-of-plane heme deformation modes occurs as a consequence of a highly bent conformation of the FeOO fragment; that is, the FeOO angle is estimated to be about 110°, and the terminal oxygen atom is suggested to be in close contact with a particular methine bridge carbon atom.[40] Such coupling of FeCN vibrational modes with out-of-plane heme modes was also observed for the cyanide adduct of lactoperoxidase, where an out-of-plane heme mode exhibits a small but distinct isotopic sensitivity; that is, 311 cm^{-1} for $^{12}C^{14}N$ and 309 cm^{-1} for $^{13}C^{15}N$.[34] Additionally, in the cyanide adduct of ferrous LPO this mode shows a similar isotopic shift. Such isotopic sensitivity of the heme out-of-plane mode is suggested to be the result of kinematic coupling with δ(FeCN) of a highly bent FeCN fragment in cyanide adducts of LPO.[34]

Significantly, though the inclusion of two heme modes is necessary to explain the RR spectra observed for the substrate-free derivatives of the three P450CN adducts studied here and previously,[27] such coupling is evidently absent for the corresponding substrate-bound derivatives.

One obvious explanation for this difference is that the bent conformer of the substrate-free form is more highly bent than that of the substrate-bound derivative. As was mentioned earlier, the separation between the frequencies of the two modes of a bent conformer increases for more highly bent configurations. It is true that the separation between these modes is apparently slightly greater for the substrate-free derivatives. For example, the T252A mutant yields a difference of 86 cm^{-1} for the substrate-free form (i.e., 433−347) while the corresponding difference for the camphor-bound form is 76 cm^{-1} (i.e., 436−360). The corresponding numbers for the native enzyme are 91 and 65 cm^{-1}, while the D251N mutant shows differences of 89 and 78 cm^{-1}. However, the adamantanone-bound derivative of the native enzyme exhibits a difference of 80 cm^{-1} (i.e., a value within 6 cm^{-1} of that shown by the substrate-free T252A mutant), yet shows no evidence of this coupling interaction,[27] suggesting it is unreasonable to ascribe the activation of this coupling solely to differences in the extent of bending of the FeCN fragment.

Although systematic experimental studies of structurally well-defined model systems, employing isotopically labeled hemes to solidify macrocycle mode assignments, supported by relatively high-level electronic structure calculations, would be necessary to elucidate the precise molecular mechanisms responsible for activation of these types of coupling interactions,[16,27,40] one factor which could be important is the orientation of the bent conformer relative to the heme macrocycle. Thus, while an essentially linear conformer obviously is nearly aligned with the normal to the plane of the heme macrocycle, it is important to consider the possibility that the bent conformers in the substrate-free and substrate-bound enzymes may adopt quite different relative orientations; that is, the lack of coupling of the substrate-bound form may result because the orientation of the bent FeCN conformer in the substrate-bound form is different from that of the substrate-free form. In fact, given the existing knowledge[6,7] of the active-site architectures of these proteins, this suggestion is plausible. Thus, the active-site water cluster present in the substrate-free form occupies the substrate binding pocket on the side of the heme opposite to the Asp251 and Thr252 residues of the postulated proton delivery shuttle.[11,21,41]

(41) Harris, D.; Loew, G. *J. Am. Chem. Soc*. **1996**, *18*, 6377−6387.

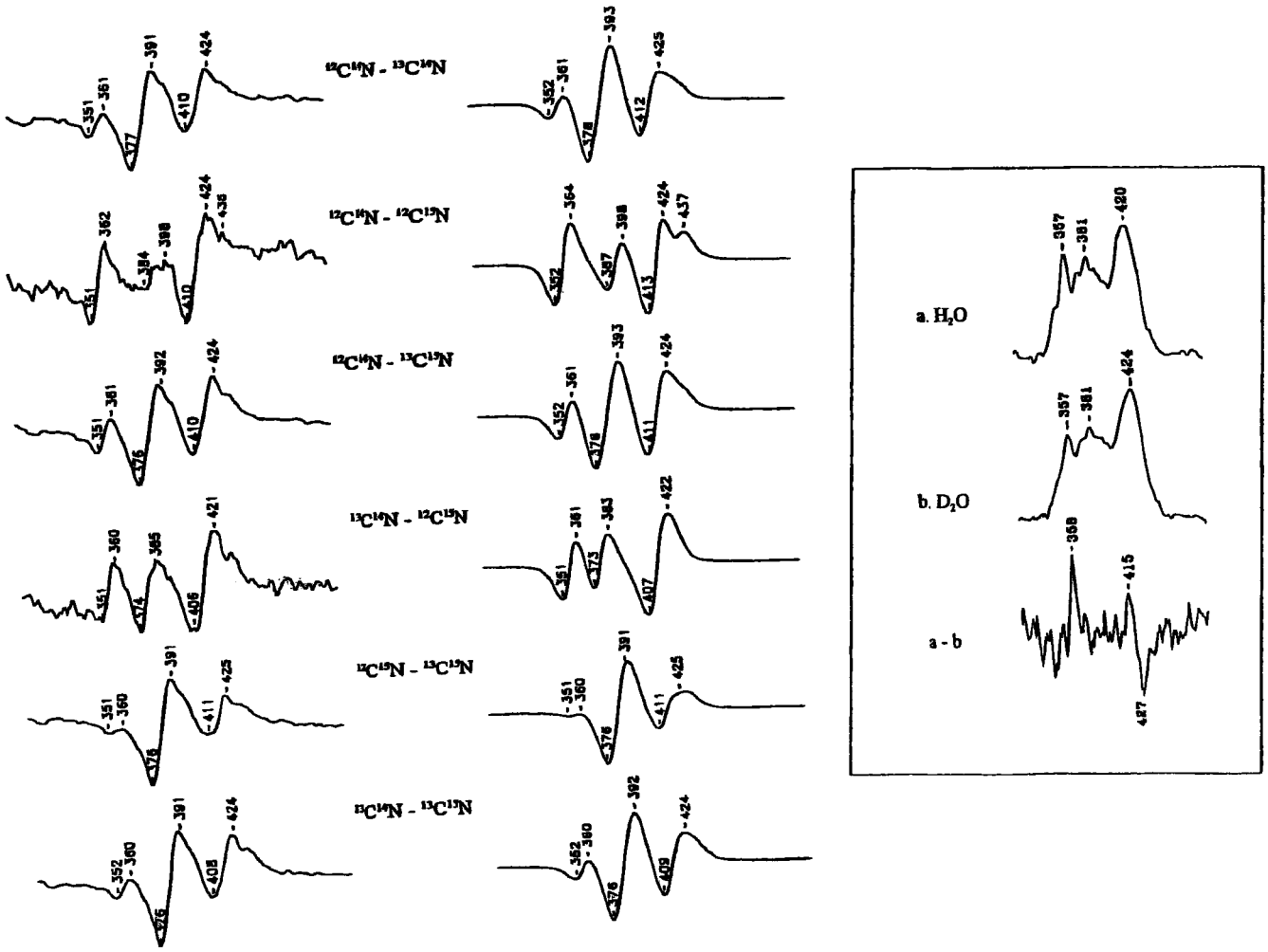

Figure 5. Experimental difference spectra for camphor-bound T252A P450cam cyanide adduct and the simulated difference spectra. The absolute spectra in H_2O and D_2O and the difference spectrum ($H_2O - D_2O$) are shown in the insert.

Based on the sensitivity to H_2O/D_2O exchange observed in the RR spectra of the substrate-free derivatives, it is reasonable to suggest that both conformers are involved in H-bonding interactions with (presumably different) regions of this water cluster and that the resultant orientation of the bent conformer is apparently favorably disposed to induce coupling with particular out-of-plane heme deformation modes.

Upon binding of substrate, the cluster is disrupted and water is apparently eliminated from this region of the active site, which is now occupied by camphor (or another substrate analogue).[7,10] The elimination of the active-site water cluster clearly obliterates the H-bonding interactions which had given rise to the bent conformer and had caused the slight distortion of the essentially linear conformer. Yet, both types of distortion persist in the substrate-bound form and it is necessary to consider what active-site structural features of the substrate-bound derivative could account for the existence of these two conformations.

The presence of the substrate provides sufficient steric pressure to cause slight distortion of inherently linear FeXY fragments, such as the ferrous CO (Figure 1) and ferric NO adducts, to the extent that the inactive δ(FeXY) bending modes are activated and the ν(FeXY) stretching modes are observed to shift.[25,28] The essentially linear conformer of the CN^- adduct behaves similarly, with its stretching mode shifting slightly, the magnitude of the shift depending on substrate size.[27] However, in contrast to the CO and ferric NO adducts, which adopt only a slightly distorted, essentially linear conformation, the FeCN fragment of the cyanide adduct may also adopt a bent conformation; that is, some force present in the active site, not operative for the former two adducts, can give rise to a second, more highly distorted FeCN conformer.

Given the greater tendency of the bound CN^- ligand, relative to CO and NO, to participate in H-bonding interactions,[33,35,42] it is most reasonable to suggest that an off-axis proton donor serves this function and the most attractive candidate for such a donor is the active-site terminus of the proposed proton delivery pathway involving the Thr252 and Asp251 residues, or possibly a trapped water molecule near this position which is not detected in the X-ray structure.[11] This suggestion can account for the lack of coupling of this bent conformer with out-of-plane heme modes, given the fact that this potential donor site is positioned on the opposite side of the heme from the substrate binding site (the site occupied by the water cluster in the substrate-free derivative). That is, the above considerations argue that the bent form of the substrate-free derivative is positioned toward one side of the heme (where it couples with certain out-of-plane heme deformation modes), but toward the opposite side of the heme in the substrate-bound form, apparently adopting an orientation wherein such coupling is not activated.

2. Effects of Mutation on Active-Site Structure. a. Substrate-Free Derivatives. The derived vibrational frequencies

(42) Evangelista-Kirkup, R.; Smulevich, G.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4420−4425.

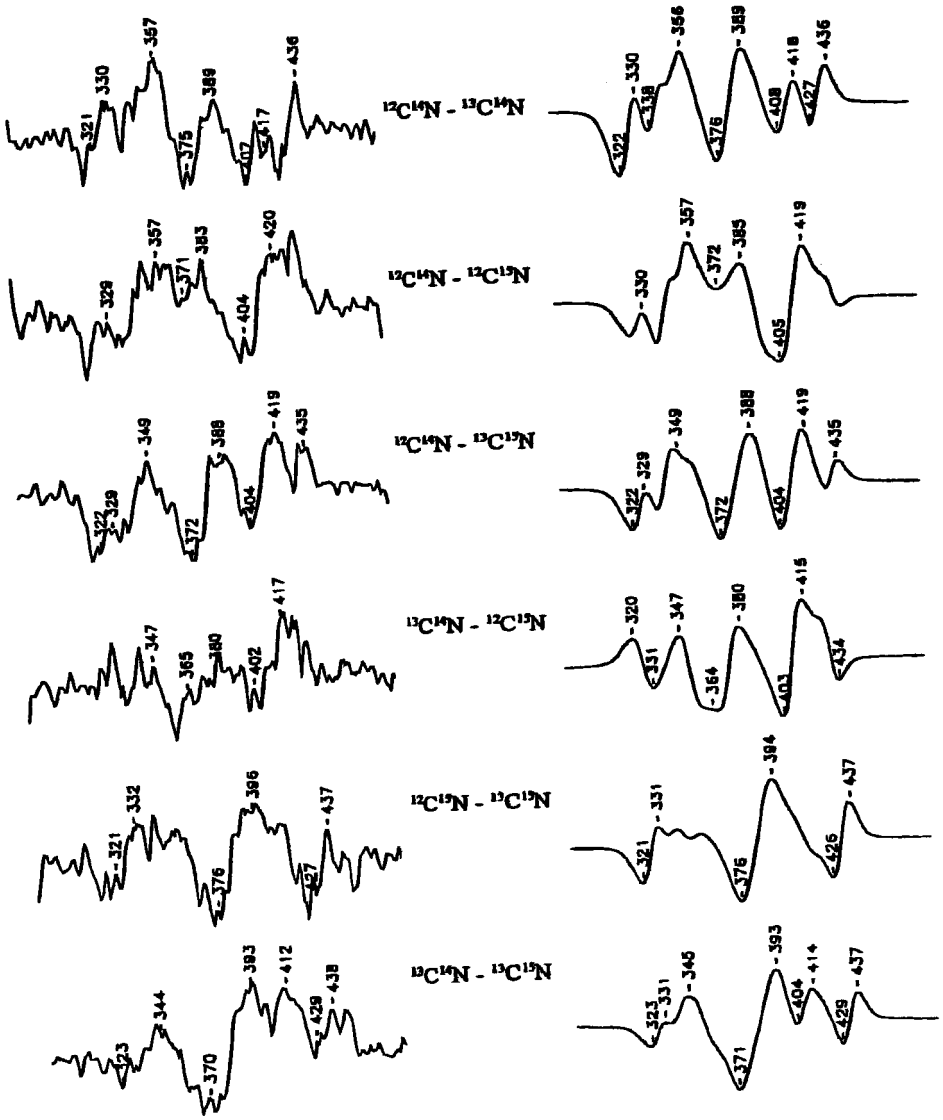

Figure 6. Experimental difference spectra for substrate-free D251N P450cam cyanide adduct and the simulated different spectra.

(Table 1) for both of the substrate-free mutants studied here are not substantially different from those observed previously for the substrate-free form of the wild-type protein.[27] This observation is not surprising, given the fact that the residues replaced in the mutant proteins are located on the opposite side of the heme from the water cluster present in the substrate-free active site; that is, little perturbation of the active-site cluster is expected.

b. Camphor-bound derivatives. As can be seen by inspection of Table 1, the vibrational parameters of the camphor-bound forms of both of the mutated proteins also are not dramatically different from those of the camphor-bound native enzyme, though, in general, the differences are greater than those seen for the substrate-free derivatives. The largest variations occur for the weak, high-frequency mode of the bent conformer, which occurs about 10 cm^{-1} higher (433 and 436 cm^{-1}) for the mutants than for the camphor-bound derivative of the native enzyme (424 cm^{-1}), although it is noted that the adamantanone-bound native protein also exhibits this mode at 437 cm^{-1}. For both of the mutant proteins, the other three modes all occur within 4 cm^{-1} of their positions in the native enzyme. Though experimentally significant, such shifts, taken alone, would seem to suggest only subtle differences in active-site architecture among the three proteins. However, consideration of the observed sensitivity to H_2O/D_2O exchange of the vibrational modes of the FeCN fragments in these three proteins reveals a rather dramatic difference in both of the mutant proteins, relative to native P450cam. Thus, in contrast to the camphor-bound and adamantanone-bound native enzyme, for which no evidence of H/D sensitivity could be detected,[27] both of the camphor-bound mutants exhibit clear evidence for shifts of the modes of the FeCN fragment in D_2O solution (Figures 6 and 7).

It is important to consider the possible origins of H_2O/D_2O sensitivity in the substrate-bound forms, because the mutant proteins were specifically designed to perturb the proposed H-bonding network comprising the proton delivery pathway.[10,11] In the native enzyme the H-bond donor to the bound dioxygen is suggested to be Thr252 or an associated H_2O molecule, held in place by H-bonding interactions with Thr252 and Asp251; although no such water molecule was detected in the X-ray structure of the camphor-bound native enzyme, its presence in solution cannot be ruled out.[11] It is not surprising that the modes of the essentially linear conformer of this derivative are not sensitive to D_2O exchange because, being directed along the normal to the heme plane, it is not properly oriented to interact with this off-axis donor. However, to the extent that the bent

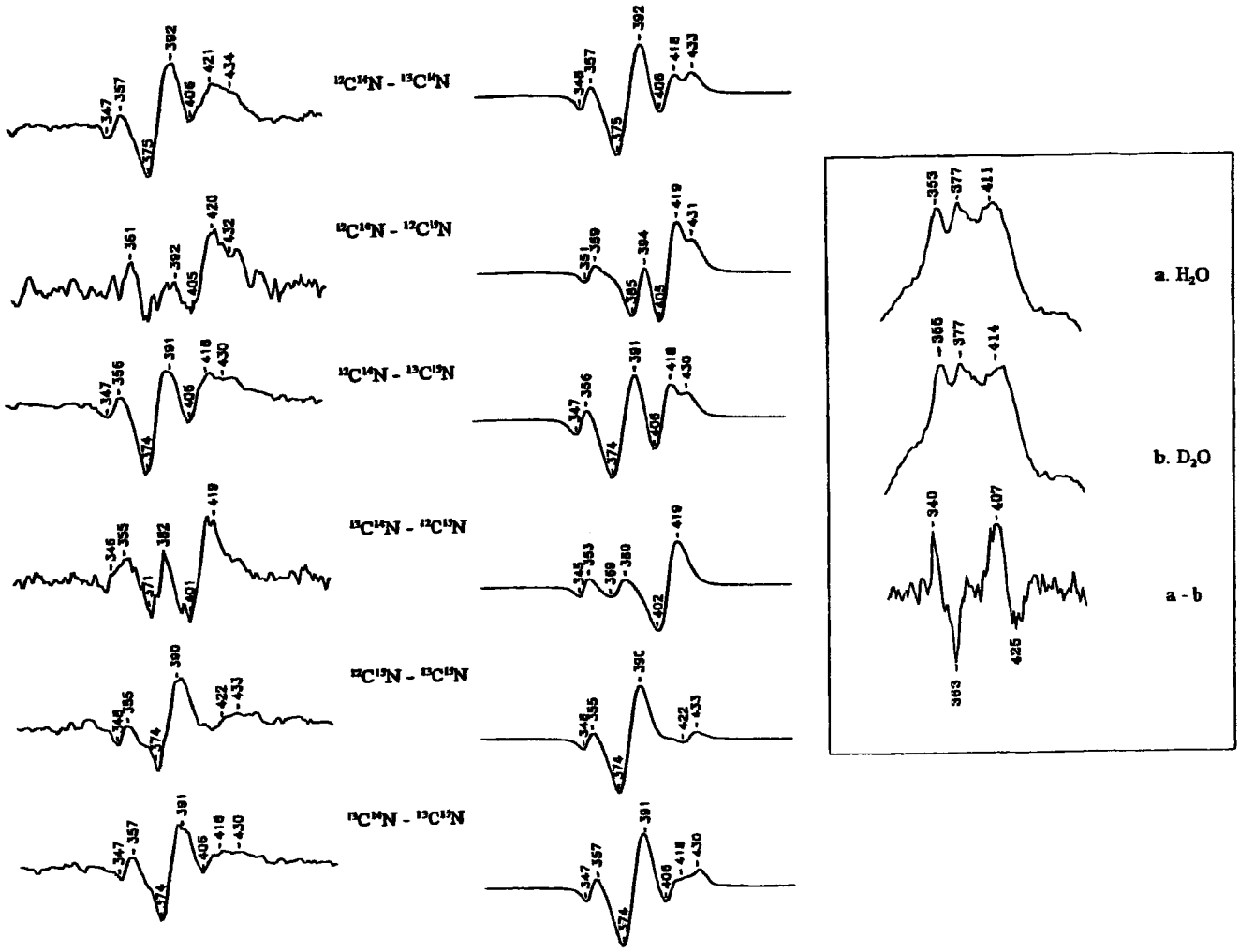

Figure 7. Experimental difference spectra for camphor-bound P450cam cyanide adduct and the simulated difference spectra. The absolute spectra in H_2O and D_2O and the difference spectrum ($H_2O - D_2O$) are shown in the insert.

conformer of the camphor-bound native enzyme arises from, and is thus oriented toward, this potential H-bond donor, it is expected that the modes of this conformer might, but not necessarily,[43] be sensitive to H/D exchange. The side chain of the Thr252 is expected to be a weaker H-bond donor than water and, in the absence of definitive evidence for the presence of a water molecule in the active site of the camphor-bound native enzyme, the lack of an H/D shift suggests that the bent conformer interacts with the off-axis Thr252.

In contrast to the native enzyme, the camphor-bound derivatives of both mutant proteins exhibit shifts for both conformers in D_2O. In the case of the D251N mutant, the observed H_2O/D_2O sensitivity of the modes of both conformers is quite reasonable to expect, given the fact that this particular replacement apparently leads to the inclusion of a larger number of water molecules in the active site of the camphor-bound form;[11] that is, the active site apparently contains a sufficient number of waters to account for interaction with both conformers. In the case of the T252A mutant, the crystal structure shows that two water molecules have direct access to the active site (W720 and W687 in ref 10). While the former molecule is apparently held in a position disposed to interact with a bent FeCN conformer (by association with Thr252), the latter lies at a distance too far removed to interact directly with a linear FeCN fragment. However, it is not unreasonable to suggest that the presence of the negatively charged axial cyanide ligand may attract this additional active-site water molecule such that it assumes a position sufficiently close to H-bond to the FeCN fragment, thereby accounting for the H/D sensitivity observed for the linear form.

Conclusions

The resonance Raman spectral data for the cyanide adducts cytochrome P450 cam and its T252A and D251N site-directed mutants reveal the presence of two structurally distinct species in both the substrate-free and camphor bound forms; one contains an essentially linear FeCN fragment, while the other exhibits a vibrational pattern characteristic of a highly bent configuration. Careful analysis of the difference patterns obtained upon subtraction of the spectra acquired for the adducts of various cyanide isotopomers demonstrates that, for the substrate-free adducts, the lower frequency internal mode of the FeCN fragment of the bent conformer is vibrationally coupled with internal modes of the heme macrocycle, while no such coupling is evidenced for the camphor-bound adducts. A plausible explanation for this behavior is the expected difference in the orientation of the FeCN fragment of the bent conformers in the two cases; that is, the FeCN fragment is oriented toward the Threonine-252 residue in the camphor-bound forms, but toward the opposite side of the heme in the substrate-free

(43) Jayarajah, S.; Proniewicz, L. M.; Bronder, H.; Kincaid, J. R. *J. Biol. Chem.* **1994**, *269*, 31047−31050.

adducts. While the site mutations have only small effects on the vibrational parameters of the FeCN fragments, in agreement with corresponding data obtained for the ferrous CO adducts, such mutations do affect the H_2O/ D_2O sensitivity of the vibrational modes of the FeCN fragments in the camphor-bound forms; that is, H_2O/ D_2O shifts are observed for the mutants, but not for the native protein. Such shifts are most reasonably interpreted to reflect the known increase in active-site water molecules in the mutants, relative to the native protein.

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