Role of the Polarity of the Heme Environment for the CO Stretch Modes in Cytochrome P-450cam-CO†

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ABSTRACT: The CO stretch mode of various substrate complexes of cytochrome P-450cam-CO was measured using FT infrared spectroscopy. At room temperature most of the complexes show a single, but often asymmetric infrared band. The representative wavenumber of this band for the various complexes increases when the high-spin content, induced by the substrates in the oxidized protein, decreases. Additionally, the increase of the CO stretch wavenumber (1939 to 1956 cm^{-1}) correlates with the decrease of the Soret band wavenumber (22 440 to 22 373 cm^{-1}). It is suggested that the polarity of the heme pocket is modulated by the substrates due to changed accessibility of the heme environment for water molecules. The increased water content compensates positive electrostatic potentials near the CO ligand, which results in loosening the contact of CO to the I helix.

The CO stretch mode of the carbon monoxide ligand in hemoproteins is extensively used as spectroscopic probe for the heme pocket. Depending on the kind of information required to obtain about the structural behavior of the protein, the CO stretch mode is investigated in its function of physical parameters (temperature, pressure), solvent conditions (pH, cosolvent, salt effect), or interaction with other components (proteins, substrates). To date, the most detailed knowledge about the physical and chemical parameters determining the CO stretch mode is obtained for diverse hemoglobins and wild type and mutated myoglobins. Two phenomena are generally observed: (i) the appearance of several CO stretch modes with differently pronounced contribution of the single bands to the infrared spectrum and (ii) a different CO stretch mode wavenumber for different proteins and their mutants. The former has been attributed to the equilibrium of conformational substates of the proteins (Frauenfelder et al., 1991) while the latter is explained by the different local structure near the CO ligand in the different substates or different proteins. Very recently, many studies on myoglobin mutants indicated that the wavenumber of the CO stretch mode is predominantly determined by the polarity of the amino acid residues on the distal side (Li et al., 1994; Ray et al., 1994; Springer et al., 1994) rather than the Fe−C−O angle (Ormos et al., 1988; Li & Spiro, 1988). In contrast to hemoglobins and myoglobins, for cytochrome P-450 an additional influence has to be considered; that is the binding of substrates. Substrate binding does significantly change the CO stretch infrared spectrum as we have shown for cytochrome P-450cam (P-450cam)[1] in several papers (Jung & Marlow, 1987; Jung et al., 1992, 1996; Schulze et al., 1994, 1996; Contzen et al., 1996) and Tsubaki et al. (1992) for cytochrome P-450scc. For substrate-free P-450cam we have concluded that water molecules in the heme pocket and in the hydration shell of the protein play an important role for the substate equilibrium and the CO stretch mode wavenumber (Jung et al., 1992, 1996). Changed accessibility of the heme pocket for water molecules by substrate binding and disorder of the substrate in the heme pocket changes the substate equilibrium (Jung et al., 1992). In the present paper we demonstrate that at room temperature the wavenumber of the CO stretch mode in P-450cam-CO bound with various substrates correlates with the high-spin content induced by the substrates in the oxidized protein. Correlations were also found with the wavenumber of the Soret band in P-450cam-CO and for few complexes with the redox potential which are available in the literature. Using additional correlations we have already published (Jung, 1983; Legrand et al., 1995; Jung et al., 1995), we are able to get an insight how substrate binding changes the electronic structure of the carbon monoxide heme complex. In agreement with the studies on myoglobins we demonstrate that also for P-450cam the polarity of the heme pocket is the physical parameter which determines the wavenumber of the CO stretch mode.

MATERIALS AND METHODS

Cytochrome P-450cam from *Pseudomonas putida* expressed in *Escherichia coli* TB1 was isolated and purified according to Jung et al. (1992) until an absorbance ratio 392 nm/280 nm of approximately 1.0−1.3 was obtained. Substrate removal was performed using Sephadex G-25 (me-

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[1] Abbreviations: P-450cam, cytochrome P-450cam, soluble hemoprotein from *Pseudomonas putida* that catalyzes the hydroxylation of (1*R*)-camphor when utilized as the sole carbon source (EC 1.14.15.1) (CYP 101) (Nelson et al., 1993); ν(CO), wavenumber of the carbon monoxide stretch vibration; ν(Soret), wavenumber of the Soret band in the electronic absorption spectrum.

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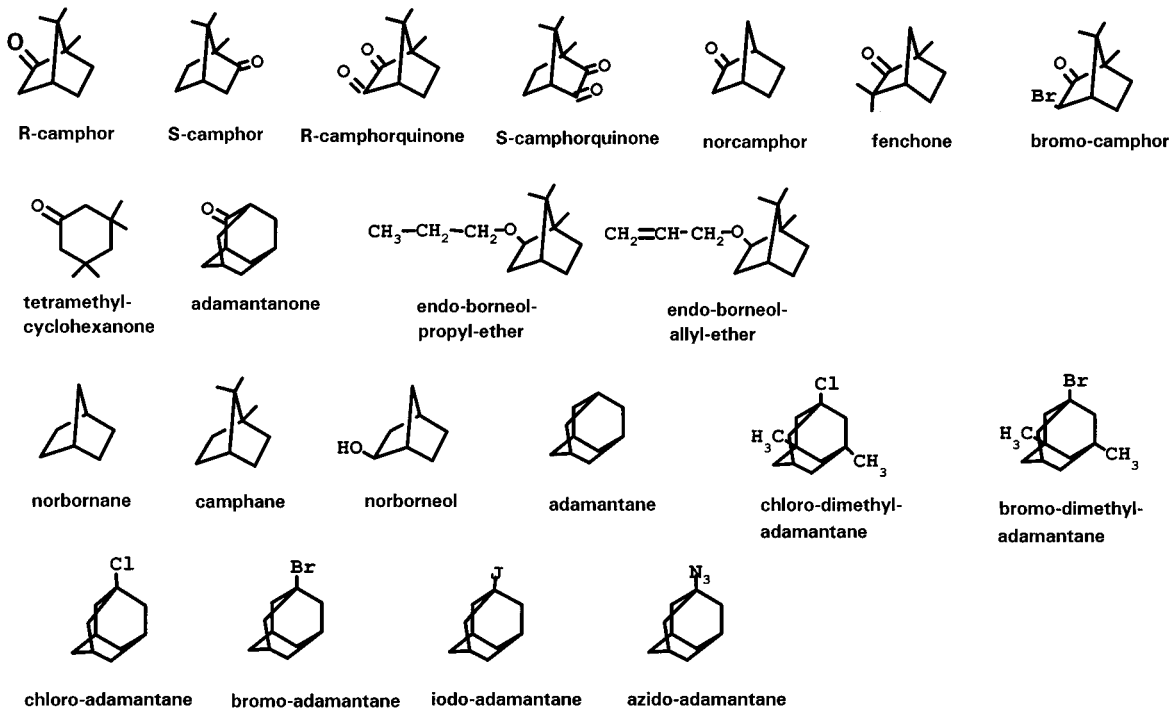

FIGURE 1: Structure of the substrates.

dium) and dialysis as described by Jung et al. (1992). Substrate analogues were added to substrate-free P-450cam (1−1.5 mM) in a few microliters of an ethanolic stock solution (700 mM) as described in Jung et al. (1992, 1995). The structure of the substrates included in the present study is shown in Figure 1.

(1*R*)-Camphor and (1*S*)-camphor were purchased from Sigma and Merck, respectively. (1*R*)-Camphorquinone, (1*S*)-camphorquinone, adamantane, 1-azidoadamantane, 1-chloroadamantane, 1-bromoadamantane, 1-iodo-adamantane, 1-chloro-3,5-dimethyl-adamantane, 1-bromo-3,5-dimethyladamantane, 3-*endo*-borneol, adamantanone, (1*R*)-fenchone, (±)-norcamphor, and norbornane were from Aldrich. Camphane, (1*S*)-*endo*-(−)-borneol *O*-allyl ether (CAL), and (1*S*)-(−)-*endo*-borneol *O*-propyl ether (CEP) were synthesized by E. Gill as described by Jung et al. (1995) and by Helms et al. (1996).

The sample preparation for the infrared studies followed the procedures published in previous papers (Jung et al., 1992, Schulze et al. 1994). All samples were studied in 0.1 M potassium phosphate buffer, pH = 7, 60% (by mass) glycerol, as used for low-temperature studies (Schulze et al., 1994; Jung et al., 1996; Contzen et al., 1996). The CEP complex was in 0.1 M Tris-HCl, pH 7, 20% (by mass) glycerol. The P-450 concentration was in the range of 1−1.5 mM in all studies. After adding the ethanolic substrate solution (700 mM, except for camphane and adamantane 100 mM), the final substrate concentration was about 33 mM, except for norbornane 64 mM and adamantane 9 mM. The ethanol content in the sample was less than 2−4%. To bind norcamphor, the protein was dialyzed against the buffer containing 85 mM water-soluble norcamphor. Complete substrate binding was reached in all samples, because the substrate concentrations are 100−500 times larger than their substrate dissociation constants.

The infrared spectra were recorded on a Bruker IFS66 Fourier transform infrared spectrometer at 2 cm^{-1} resolution in the double-sided/forward-backward acquisition mode. The spectra were taken at 296 K. A liquid nitrogen cooled midband MCT detector was used. Fourier transformation of the interferogram was performed with the Happ-Genzel apodization function and a zero filling factor of 2. A total of 200 interferograms were accumulated and coadded. The infrared cell consisted of two calcium fluoride windows separated by a 0.1 mm thick Teflon spacer.

Infrared absorption spectra were obtained from the ratio of the intensity spectrum for the CO complex to the intensity spectrum of the buffer. The infrared absorption spectra of the sample were base line corrected by substracting interactively the absorption spectrum of the buffer (with air as background) until a sufficiently good base line was visible. In most cases, however, a completely flat base line could not be obtained. Therefore, a final base line correction was made by fitting the left and the right sides of the spectrum, where no CO bands appear, using a cubic polynomial function. For a precise determination of the wavenumber of the experimental infrared band of the CO stretch mode the spectrum was fitted with the log-normal distribution function (Schulze et al., 1994).

$$E = (A/X)\exp[(\ln X)[1 - [(\ln 2)/(\ln^2 a)](\ln X)]];$$
$$X = [(\nu - \nu_0)(a^2 - 1) + \Delta\nu_{1/2}a]/(\Delta\nu_{1/2}a)$$

where a is the asymmetry parameter. For $a < 1$ the band is asymmetric at the lower-energy side while $a > 1$ indicates the asymmetry at the higher-energy side of the absorption band. $a = 1$ represents the symmetric Gaussian band.

Spectra which clearly indicated overlap of several bands were additionally decomposed with Gaussians using a least-squares fitting program.

The electronic absorption spectra of the CO complex as well as of the oxidized P-450cam were measured directly in the infrared cell on the Shimadzu PC2101 spectrophotometer. The high-spin content of the P-450 substrate complexes was

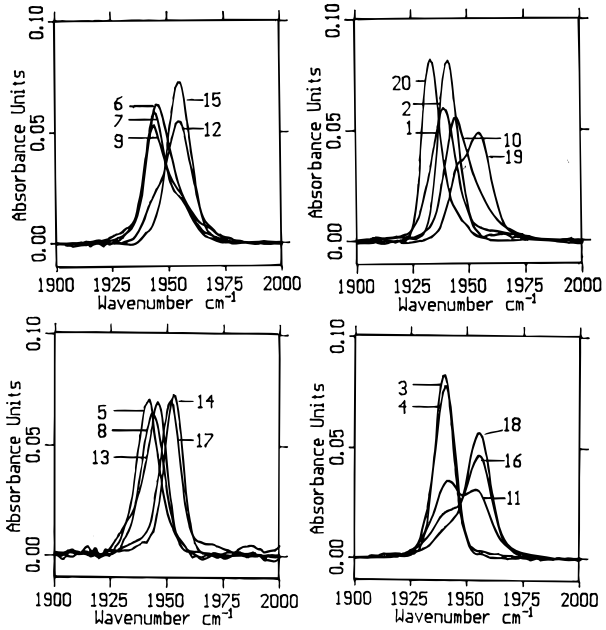

FIGURE 2: CO stretch vibration infrared spectra of cytochrome P-450cam-CO in the presence of various substrate analogues, in 0.1 M potassium phosphate buffer, pH 7, 60% (by mass) glycerol; all spectra are normalized to the total area under the infrared bands; the substrate complexes are indicated by the numbers given in Table 2.

determined by fitting the electronic absorption spectrum with the spectra of the pure spin states as described by Jung et al. (1991). The precise wavenumber of the Soret band in the carbon monoxide complex was obtained by fitting the Soret band maximum with a cubic polynomial.

RESULTS

Most of the substrate-bound P-450cam-CO complexes investigated here show at room temperature a single, but often asymmetric CO stretch vibration band which can be fitted with a log-normal distribution function (Schulze et al., 1994) (Figure 2). The wavenumber obtained by the fitting is taken as the representative CO stretch mode wavenumber (ν'(CO)) for the substrate complex. As shown earlier by Jung and Marlow (1987), Schulze et al. (1994), and Jung et al. (1996), most of the bands of the substrate complexes investigated in this paper split in several bands at low temperatures (<200 K), indicating conformational substates. In this paper we will discuss only the room temperature spectra. For those spectra that show a shoulder already at room temperature, the representative CO stretch wavenumber was determined by the sum of the area-weighted wavenumbers of the overlapping bands obtained by decomposing the experimental spectra into Gaussians. A similar intensity averaging procedure was used by Li et al. (1994) for the CO stretch mode of various myoglobin mutants.

Table 1 summarizes the parameters of the CO stretch mode infrared bands. In Table 2 the representative CO stretch wavenumbers are given together with the wavenumber of the Soret band for the carbon monoxide complex of P-450cam bound with the various substrates. The high-spin content (HS) for the oxidized state determined from the same sample before reduction and CO binding is also given. The representative CO stretch mode wavenumber (ν'(CO)) and the position of the Soret band (ν(Soret)) in P-450cam-CO both are correlated with the high-spin content (Figure 3A,B) and can be correlated with the free energy for the spin transition $\Delta G°_{spin} = -RT \ln [HS/(100 - HS)]$ at 296 K. The correlation coefficients (r) are as follows:

$$\nu'(CO) = (-0.21 \pm 0.02)HS + (1962.2 \pm 1.3); \quad r = 0.89$$

$$\nu'(CO) = (0.85 \pm 0.11)\Delta G°_{spin} + (1950.7 \pm 0.7); \quad r = 0.79$$

$$\nu(\text{Soret}) = (0.71 \pm 0.16)HS + (22358.3 \pm 11.6); \quad r = 0.56$$

$$\nu(\text{Soret}) = (-3.24 \pm 0.77)\Delta G°_{spin} + (22393.4 \pm 5.6); \quad r = 0.56$$

Therefore, a correlation between the wavenumber of the CO stretch mode and the Soret band exists (Figure 3C).

$$\nu'(CO) = (-0.17 \pm 0.03)\nu(\text{Soret}) + (5689.5 \pm 753.5); \quad r = 0.64$$

The coefficient for the correlation of ν(Soret) with the high-spin content is lower than for the correlation of the CO stretch mode with the high-spin content because the Soret band shift is rather small compared to the large absolute value of ν(Soret). In independent experiments of the pressure-induced shift of the Soret band of P-450cam-CO, we found the same correlation between ν(Soret), extrapolated to the pressure at 0 MPa, with the high-spin content. The correlation coefficient was 0.663 (Jung et al., 1995).

Three P-450cam-CO complexes do not fit into the correlation of ν'(CO) with the high-spin content. ν'(CO) for substrate-free P-450cam-CO is too low considering that the high-spin content is only 5%. On the other hand, ν'(CO) for the complexes with tetramethylcyclohexanone and 3-bromocamphor as substrates has almost the same value of approximately 1933−1934 cm^{-1} although the high-spin contents are very different and too low for these low stretch vibration wavenumbers (57.9% and 88.9%, respectively).

No correlation between the width of the CO stretch band and the high-spin content was found. Furthermore, there is no relation between the wavenumber and the width of the CO stretch mode infrared band in the various substrate complexes.

DISCUSSION

As derived from the crystal structure of substrate-free oxidized P-450cam which exists in the low-spin state and has a cluster of 6 water molecules in the heme pocket (Poulos et al., 1986), it is concluded that the low-spin state can be attributed to a higher water content in the heme pocket or, generally, in the protein structure. The crystal structures of P-450cam bound with the substrate analogues camphane, norcamphor, adamantane, and thiocamphor which induce a mixture of high-spin state and low-spin state population show a single water molecule in the heme pocket and, probably, disordered solvent molecules in the protein structure (Raag et al., 1991). Therefore, it is justified to assume that an increase of the low-spin state population or a decrease of the high-spin content indicates a more polar heme environment. This is also supported by studies of the second-derivative spectrum of the aromatic amino acids in wild type and Tyr96 mutant P-450cam (Atkins & Sligar, 1990).

Table 1: Infrared Absorption Band Parameters of the CO Stretch Modes in P-450cam-CO Bound with Various Substrates[a]

substrates	ν(CO) (cm^{-1}); width (cm^{-1}); asymmetry parameter (population) II	III	IV	V	ν'(CO) (cm^{-1})
(1*R*)-camphor		1939.8; 12.7; 0.93 (1.00)			1939.3
(1*S*)-camphor		1940.7; 9.5; 1.10 (1.00)			1940.7
(1*S*)-camphor quinone		1939.6; 9.1; 1.05 (1.00)			1939.6
(1*R*)-camphor quinone		1940.5; 10.6; 0.89 (1.00)			1940.5
adamantanone		1941.5; 9.0; 1.04 (1.00)			1941.5
1-azidoadamantane		1945.5; 13.2; 1.37 (1.00)			1945.5
1-chloroadamantane		1943.8; 8.3; 1.00 (0.88)	1952.6, 10.0; 1.00 (0.12)		1944.9
fenchone		1944.5; 13.8; 1.56 (1.00)			1944.5
1-bromoadamantane		1943.1; 8.4; 1.00 (0.60)	1950.8; 13.1; 1.00 (0.40)		1946.2
endo-borneol allyl ether		1944.0; 10.5; 1.00 (0.74)	1952.2; 12.4; 1.00 (0.26)		1946.5
camphane		1941.5; 15.7; 1.00 (0.60)	1955.3; 10.8; 1.00 (0.27)	1961.9; 21.0; 1.00 (0.13)	1947.9
1-iodoadamantane		1943.2; 9.9; 1.00 (0.16)	1954.6; 11.6; 1.00 (0.84)		1952.8
norcamphor		1946.1; 10.3; 0.98 (1.00)			1946.1
norbornane			1953.0; 10.0; 0.96 (1.00)		1953.0
adamantane			1955.0; 11.7; 0.90 (1.00)		1955.5
1-bromodimethyladamantane		1941.6; 11.1; 1.00 (0.20)	1955.2; 10.9; 1.00 (0.80)		1952.5
3-*endo*-norborneol			1951.5; 9.0; 0.96 (1.00)		1951.6
1-chlorodimethyladamantane		1943.4; 12.2; 1.00 (0.14)	1955.3; 10.9; 1.00 (0.86)		1953.6
endo-borneol propyl ether (20%)		1944.7; 8.0; 1.00 (0.20)	1954.7; 12.0; 1.00 (0.70)	1965.0; 12.6; 1.00 (0.10)	1953.7
substrate free, type 1		1939.8; 12.0; 1.00 (0.52)	1949.4; 10.4; 1.00 (0.07)	1955.3; 19.2; 1.00 (0.41)	1946.8
substrate free (20%), type 1		1940.4; 14.1; 1.00 (0.61)	1952.9; 11.2; 1.00 (0.19)	1962.4; 11.0; 1.00 (0.20)	1947.2
tetramethylcyclohexanone	1933.3; 10.9; 1.16 (1.00)				1933.3
tetramethylcyclohexanone (20%)	1934.2; 10.0; 1.00 (1.00)				1934.2
3-bromocamphor (20%)	1933.7; 10.6; 1.00 (1.00)				1934.0

[a] All samples in 0.1 M potassium phosphate buffer, pH 7, 60% (by mass) glycerol, except additionally indicated in 20% (by mass) glycerol. Band assignment to II, III, IV, and V according to Jung et al. (1996). Fit of the experimental spectra with the log-normal distribution function in the case of an apparent single band (asymmetry parameter deviates from 1) and with Gaussians for spectra with several bands (asymmetry parameter equal to 1). ν'(CO) = representative CO stretch wavenumber, population weighted in the case of several bands; data for substrate-free P-450cam from Jung et al. (1996); data for 3-bromocamphor complex from Jung et al. (1992).

Corresponding to this assignment, the correlations of the wavenumbers of the CO stretch mode and of the Soret band with the high-spin content (Figures 3A,B) suggest that the more polar heme environment in the oxidized form of P-450 is also present in the CO complex and might be the reason for the increased CO stretch mode wavenumber and the decreased wavenumber for the Soret band. The decrease of the Soret band wavenumber in a more polar heme environment is in agreement with our recent studies on the pressure-induced red shift of the Soret band for various substrate analogue P-450cam-CO complexes (Jung et al., 1995). Substrates that cause a larger low-spin state population in the oxidized P-450 protein show a stronger Soret band red shift due to the pressure-induced shift of water molecules to the heme environment.

Unexpected is the observed increase of the CO stretch wavenumber and the missing systematic change of the bandwidth when the heme environment becomes more polar. Usually, for protein-free metal carbonyl complexes, as well as heme carbonyl complexes the inverse behavior, is observed. A polar solvent induces a decrease of the CO stretch mode wavenumber and simultaneously a broadening of the band (Beck & Lotters, 1964) due to a direct interaction between the solvent molecules and the CO ligand. This seems not to be the case for P-450cam-CO. The polarity of the heme surrounding must have an indirect effect on the CO ligand in P-450cam.

The effect of the environmental polarity near the CO ligand, corresponding to an electrostatic field acting on the CO dipole, has recently been attributed to formal electronic resonance structures for the Fe−C−O group (Li et al., 1994).

Table 2: Spectral and Physicochemical Parameters of Cytochrome P-450cam Bound with Various Substrates at 296 K[a]

no.	substrates	HS (%)	$\Delta G°_{spin}$ (kJ/mol)	ν'(CO) (cm^{-1})	ν(Soret) (cm^{-1})	E_0 (mV)
1	(1*R*)-camphor	99.9	−17.108	1939.3	22 440.4	−170
2	(1*S*)-camphor	99.8	−15.389	1940.7	22 441.9	
3	(1*S*)-camphor quinone	98.8	−10.920	1939.6	22 433.0	
4	(1*R*)-camphor quinone	98.8	−10.920	1940.5	22 435.9	−183
5	adamantanone	92.8	−6.334	1941.5		−175
6	1-azidoadamantane	88.5	−5.056	1945.5	22 404.1	
7	1-chloroadamantane	84.5	−4.202	1944.9	22 387.3	
8	fenchone	82.2	−3.791	1944.5		−208
9	1-bromoadamantane	78.9	−3.268	1946.2	22 388.9	
10	*endo*-borneol allyl ether	77.5	−3.064	1946.5	22 443.3	
11	camphane	74.2	−2.617	1947.9	22 427.3	
12	1-iodoadamantane	62.7	−1.286	1952.8	22 377.7	
13	norcamphor	60.6	−1.067	1946.1	22 417.0	−206
14	norbornane	45.4	0.457	1953.0		
15	adamantane	44.5	0.547	1955.5	22 380.1	
16	1-bromodimethyl-adamantane	43.0	0.698	1952.5	22 373.4	
17	3-*endo*-norborneol	38.4	1.171	1951.6	22 400.0	
18	1-chlorodimethyl-adamantane	35.7	1.458	1953.6	22 376.1	
19	*endo*-borneol propyl ether (20%)	35.2	1.512	1953.7	22 393.6	
	substrate-free	5.0	7.295	1946.8	22 369.9	−303
	substrate-free (20%)			1947.2		
20	tetramethylcyclohexanone	57.9	−0.790	1933.3	22 442.6	−242
	tetramethylcyclohexanone (20%)			1934.2		
	3-bromocamphor (20%)	88.9	−5.155	1934.0	22 414.3	

[a] All samples in 0.1 M potassium phosphate buffer, pH 7, 60% (by mass) glycerol, except additionally indicated in 20% (by mass) glycerol; HS: high-spin content; $\Delta G°_{spin} = -RT \ln [HS/(100 - HS)]$; ν'(CO) = representative CO stretch wavenumber; redox potentials E_o from Fisher and Sligar (1985).

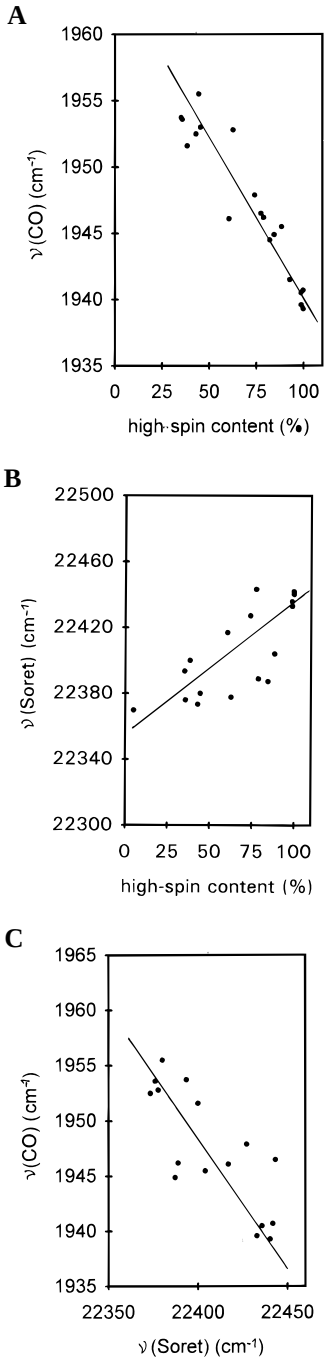

FIGURE 3: Correlation of the wavenumber of the CO stretch vibration ν'(CO) (A) and of the Soret band ν(Soret) (B) with the high-spin content in cytochrome P-450cam-CO bound with various substrate analogues, and correlation of ν(Soret) with ν'(CO) (C).

A positive electrostatic potential near the oxygen atom of the CO ligand should stabilize the partial negative charge at the oxygen atom and therefore favor the formal resonance structure $Fe^{\delta+}{=}C{=}O^{\delta-}$. The CO stretch mode wavenumber should decrease. In contrast, a negative electrostatic potential should show the inverse effect and should stabilize the formal resonance structure $Fe^{\delta-}{-}CO^{\delta+}$ which would result in an increase of the CO stretch wavenumber. Quantum chemical calculations show that the former resonance structure is valid for the carbonyl heme complexes with imidazole (model for myoglobin and hemoglobins) as well as cysteinate (model for P-450) as proximal iron ligand, however, to a different extent. The net charge distribution for the P-450 model is as follows: porphyrin (−0.858), Fe (+0.746), C (−0.026), O (−0.197) (Jung, 1980). This charge distribution results from the synergistic effect of CO ligand σ-donation and iron π-back-donation (Yu, 1986) which is modulated by the proximal ligand. A possible positive electrostatic potential at the distal side would therefore further stabilize this charge distribution and lower the CO stretch wavenumber. Indeed, a recent quantum chemical study (INDO) for a porphyrin−iron−CO complex with a pyridine as fifth ligand shows that a positive point charge which approaches the CO ligand along a pathway perpendicularly to the porphyrin plane induces a decrease of the CO stretch wavenumber from approximately 1970 to 1910 cm^{-1} by changing the point charge to porphyrin distance from 7 to 3 Å (Kushkuley & Stavrov, 1996). A negative point charge causes the opposite effect, which means an increase of the CO stretch wavenumber. This study also shows that the electrostatic influence is more important for the CO stretch wavenumber than the bending and tilting of the Fe−C−O group or the porphyrin ring deformation.

Thus for P-450cam, the increase of the CO stretch wavenumber indicates a loss of positive contributions to the electrostatic potential or the generation of a negative electrostatic potential when the water content becomes higher, which is reflected in a lower high-spin content. We suggest that the higher water content compensates the positive electrostatic potential. This may result in a weaker contact between the CO ligand and the protein, presumably Thr252 in the I-helix. However, there seems to be no direct contact between the CO ligand and the water molecules because the width of the infrared bands does not correlate with the high-spin content.

In general, a change of the CO stretch mode wavenumber can also be caused by a change of the charge of the proximal cysteinate ligand. A more negative proximal ligand should induce a stronger charge donation to the CO ligand, as can be shown by quantum chemical calculations (Jung, 1983), and it should therefore decrease the CO stretch wavenumber. For model complexes, this expected decrease is also observed (Collman et al., 1976). In hemoproteins, however, the polarity and the interaction between the CO ligand and the amino acids on the distal side mask this proximal effect. There is a possibility to separate the proximal and the distal effects. Recently, we have shown that for various substrate analogue P-450cam-CO complexes the ^{13}C-NMR chemical shift of the CO ligand correlates with the CO stretch wavenumber (Legrand et al., 1995). A similar correlation was found for hemoglobins and myoglobins (Park et al., 1991). The hemoglobin complexes, however, form another line because of the different proximal ligand. Thus, one could conclude that all complexes along one line have the same proximal ligand and the changes within this line result from distal effects. A similar explanation has been given for the different lines for the correlation between the Fe−C stretch mode and C−O stretch mode in hemoglobins and P-450 (Li & Spiro, 1988; Legrand et al., 1995).

It is discussed by Poulos et al. (1986) that the low-spin state shows a more negative redox potential (−303 meV) because of the polar water cluster in the heme pocket, while substrate binding shifts P-450 to the high-spin state and the redox potential becomes less negative (−173 meV). Fisher and Sligar (1985) have found a correlation between the redox potential and the high-spin content. According to these observations and our correlations (Figure 3A), a relation

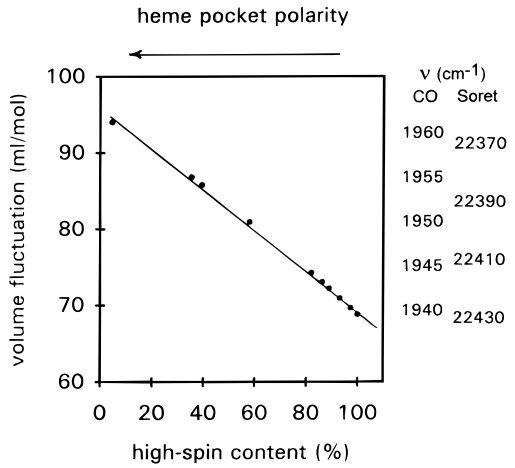

FIGURE 4: Cross correlation of the volume fluctuation, high-spin content, ν'(CO) and ν(Soret) for various substrate complexes of P-450cam-CO at 296 K (substrates: norcamphor, dimethylallylborneol ether, adamantane, fenchone, camphane, bromocamphor, *endo*-borneol allyl ether, (1*R*)-camphor, (1*S*)-camphor, adamantanone; volume fluctuation from Jung et al. (1995).

between the CO stretch mode wavenumber and the redox potential can be expected. Indeed, this is observed for the few substrate complexes for which redox potentials are available from the literature (Table 2).

The various correlations observed for P-450cam in this study and by Legrand et al. (1995) demonstrate the influence of the substrate on the electronic structure of the heme complex which is mediated by the substrate-induced accessibility of the heme environment for water molecules. One might expect that this higher accessibility of the protein structure for water molecules is caused by a higher flexibility of the protein in the presence of low-spin state inducing substrates. This higher protein flexibility might be connected with a higher mobility of the substrate in the heme pocket. Indeed, our recent study on the pressure-induced red shift of the Soret band in P-450cam-CO bound with various substrates shows that the low-spin state is more compressible than the high-spin state (Jung et al., 1995). From the compressibility β determined for the different P-450cam-CO substrate complexes (Jung et al., 1995) we calculated the volume fluctuation ΔV from $\Delta V/V = 100\ (\mathrm{RT}\beta/V)^{1/2}$ (%). V is the volume of P-450cam (33 729 mL/mol (Jung et al., 1995)). Figure 4 shows ΔV as function of the high-spin content. With the decrease of the high-spin content and the increase of the low-spin content, respectively, the volume fluctuation increases. Simultaneously, the CO stretch vibration wavenumber increases and the Soret band wavenumber decreases (Figure 4). We conclude that this larger volume fluctuation reflects the higher accessibility of the protein structure for water molecules. The substrate analogues used in this study seem to have less optimal substrate−protein contacts compared to the natural substrate camphor. Camphor is held in the optimal orientation in the active site by the hydrogen bond of its quinone group and the hydroxyl group of Tyr96 and by hydrophobic contacts of its methyl groups C-8 and C-9 to Val295 and Asp297 in the β_3 sheet and of the methyl group C-10 to Val247 in the I-helix and Thr185 in the F-helix (Poulos et al., 1987). Recently, we have shown by a comparative study of the (1*S*)-camphor and the (1*R*)-camphor P-450cam complexes that disturbed contacts especially of the methyl group C-10 to the protein lead to a larger substrate mobility and a larger water influx into

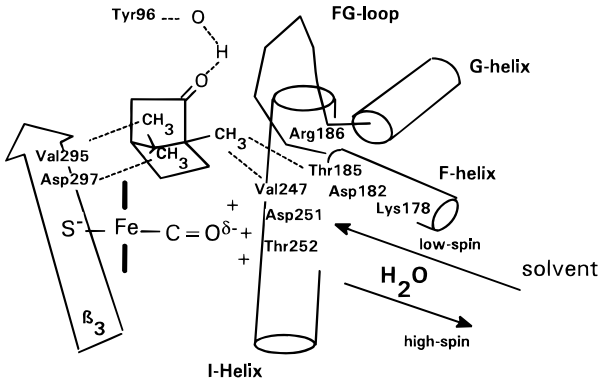

FIGURE 5: Sketch of the active site and the proposed electrostatic model for the heme environment of P-450cam-CO.

the protein structure (Schulze et al., 1996; Contzen et al., 1996). This might increase the flexibility in the I-helix groove near Thr252 and of the salt links formed by Lys178, Asp182, Arg186, and Asp251, allowing a better access of this region for water molecules (Figure 5). The electrostatic field of the water molecules present in the low-spin state might partially compensate the supposed positive electrostatic potential near the CO ligand, resulting in loosening the contact between the CO ligand and the I-helix. To date, it is not clear where the water molecules are located in the protein structure and how the compensation takes place. Further studies are required to support our model.

In addition, studies are also necessary to clarify why the P-450 complexes bound with tetramethylcyclohexanone and 3-bromocamphor do not fit in the correlation of ν'(CO) with the high-spin content. In contrast, for substrate-free P-450cam-CO one could explain the failed correlation by a possible network of hydrogen bonds between differently packed water molecules in the heme pocket which fills the space, usually occupied by the substrate, and to the CO ligand. This might also cause the multiple CO stretch bands in the absence of substrates (Jung et al., 1996).

Summarizing, we conclude that electrostatic potentials in the heme pocket due to different substrate-modulated accessibilities of the heme environment for water molecules are the relevant parameter for the CO stretch wavenumber in P-450cam-CO.

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