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Biochemical and Biophysical Research Communications 312 (2003) 197–203
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Compressibility and uncoupling of cytochrome P450cam: high pressure FTIR and activity studies

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Received 24 September 2003

Abstract

The effect of the hydrostatic pressure on the CO ligand stretch vibration in cytochrome P450cam-CO bound with various substrates is studied by FTIR. The vibration frequency is linearily shifted to lower values with increasing pressure. The slope of the shift gives the isothermal compressibility of the heme pocket and is found to be related to the high-spin state content in an opposite direction to that previously observed from the pressure-induced shift of the Soret band. This opposite behaviour is explained by the dual effect of heme pocket water molecules both on the CO ligand and on electrostatic potentials produced by the protein at the distal side. The latter effect disturbs ligand-distal side contacts which are needed for a specific proton transfer in oxygen activation when dioxygen is the ligand. Their loss results in uncoupled H_2O_2 formation.

Keywords: Cytochrome P450; Uncoupling; FTIR; CO stretch mode; Compressibility

The cytochrome P450 (P450) reaction cycle reveals side reactions which lead to the production of cytotoxic oxygen species such as hydrogen peroxide or of water in the oxidase reaction [1] (Fig. 1). These so-called uncoupling processes have been observed in many cytochrome P450 systems [1,2]. Various observations suggest that the dynamics of the protein structure and in particular the accessibility of the active site for water molecules are very important for these side reactions [3,4]. The time-averaged population of water molecules at the sixth iron coordination site leads to the well-known high-spin/low-spin state equilibrium in the iron-oxidized form of P450 which can be monitored using the heme Soret band [5]. We previously found that such substrate-modulated water accessibility exists also when the enzyme is reduced and complexed with CO [6]. The position of the Soret band of P450cam-CO, as well as the slope of its pressure-induced shift, correlates with the high-spin state content which is maximally induced by the particular substrate [7,8]. The slope of the shift is interpreted as heme pocket compressibility, revealing that high-spin state complexes are more rigid than low-spin state complexes [9].

Besides the Soret band, the CO ligand stretch vibration measured by Fourier transform infrared spectroscopy (FTIR) is another spectroscopic probe for the active site. In the present paper we show that increasing hydrostatic pressure shifts the CO stretch mode frequency for various substrate complexes to lower values similar to that observed for the Soret band. However, the slope of the pressure-induced frequency shift of the CO stretch mode shows a linear relation to the high-spin state content in a manner opposite to that found for the Soret band. This would suggest that high-spin state complexes are more compressible than low-spin state complexes, which is in contradiction to the conclusion from the pressure studies of the Soret band. This contradiction is only apparent and is explained by the dual effect of heme pocket water molecules both on the CO

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doi:10.1016/j.bbrc.2003.09.164

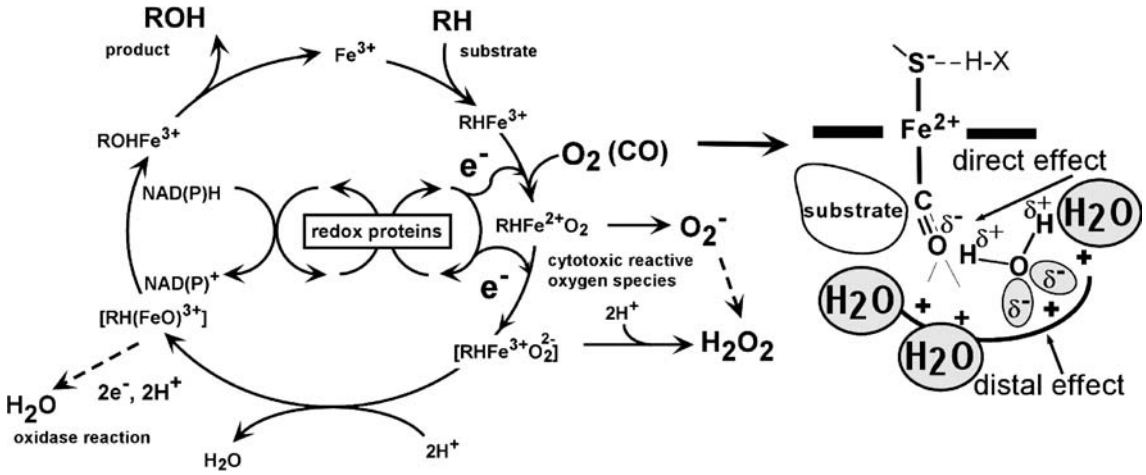

Fig. 1. Reaction cycle of cytochrome P450. Fe^{3+} (Fe^{2+}): ferric (ferrous) heme protein, RH (ROH): substrate (product) (left). Sketch of the heme pocket indicating the effect of water on the CO ligand (direct effect) and on the distal side amino acids of the protein (distal side effect) (right).

ligand and on electrostatic potentials produced by the protein at the distal side. Disrupting the electrostatic interaction between the ligand and the protein results in an increased uncoupled H_2O_2 formation.

Materials and methods

Materials. P450cam (CYP101) was expressed in *E. coli* TB1, purified and made free of substrate as described [10]. The final buffer was 50 mM K-phosphate, pH 7, 57% (v/v) glycerol. The concentrations of the substrates and of P450 were 10 and 0.5 mM, respectively. The high-spin state content for the IR samples was determined in the identical sample before reduction and CO binding using the fit procedure described earlier [5] [(1*R*-camphor (98–100%), norbornane (39%), fenchone (73%), substrate-free (2%), adamantanone (90%), tetramethylcylohexanone (58%), norcamphor (52%), adamantane (32%)]. Camphor analogues [(1*R*)-*endo*-borneol propyl ether (CEP), (1*R*)-*endo*-borneol allyl ether (CAL), (1*R*)-camphoroxime (COX), (1*R*)-isoborneol methyl ether (CIS), (1*R*)-dimethylallyl ether borneol (DAL), (1*R*)-camphor-*N*-methylimine (CIM)] were a gift from E. Gill (Dept. of Pharmacology, University of Oxford). (1*R*)-Camphor, adamantanone, adamantane, norcamphor, norbornane, fenchone, and tetramethylcyclohexanone were from Aldrich.

Enzyme activity. Activities were determined from the rate of NADH oxidation by monitoring the loss of absorbance at 340 nm ($\varepsilon = 6.22\,mM^{-1}\,cm^{-1}$) versus time. The assay mixtures contained P450cam (0.05 μM), putidaredoxin (10.0 μM), putidaredoxin reductase (4.0 μM), substrate (1.0 mM), NADH (0.5 mM), and KCl (200 mM) in 50 mM Tris–HCl buffer, pH 7.0 at 20 °C.

Oxygen consumption. O_2 consumption was measured using a Clark-type electrode (Hansatech oxygen electrode disc) in conjunction with a CB1-D3 control box connected to a computer. The concentrations of the proteins in the P450cam reconstituted system and substrates are as specified above. A full-scale deflection of approximated 280 μmol/ml was determined by the method of Robinson and Cooper [11]. NADH oxidation was simultaneously monitored by initiating a 3-ml reaction mixture by adding NADH in the presence of P450cam and in its absence as a control experiment. At least three determinations were made for each protein–substrate combination. The concentration of NADH was then plotted versus the concentration of oxygen, and the slope of the line was determined.

Hydrogen peroxide formation. H_2O_2 production was determined from the change in rate of O_2 consumption upon the addition of 3 μg (108 U) of catalase (Sigma) to the reaction mixture. Catalase had no effect on the rates of NADH oxidation itself. The percentage of H_2O_2 formation was then calculated from the difference between the rates of O_2 consumption in the absence and presence of catalase multiplied by a factor of 2.

FTIR experiments. FTIR measurements were performed on the IFS66 (BRUKER) Fourier transform infrared spectrometer equipped with the liquid-nitrogen-cooled MCT detector D316 (BRUKER) at $2\,cm^{-1}$ spectral resolution, and 1250 scans were accumulated. The interferogram was treated with the Blackman–Harris three-term apodization function and Mertz phase correction. The buffer intensity spectrum at the particular pressure was used as background The baseline correction and determination of the concrete frequency of the band maximum were performed by fit procedures as described [6]. All spectra were normalized to the total area under the CO stretch bands. The membrane-driven high-pressure cell with sapphire anvils and the procedure for determination of the in situ pressure between the anvils using the ruby R_1 luminescence band are described in [12].

Results

High-pressure FTIR studies

Fig. 2 shows the CO ligand stretch vibration infrared spectra for P450cam bound with selected substrates and of the substrate-free protein [13]. Cytochrome P420 formation is avoided by a glycerol content of approx 60% [7]. For all substrate complexes *i* the CO ligand stretch band is linearily shifted to lower frequencies ($\nu_{CO,i}$) with increasing pressure. The slope of the shift of the band maximum with the pressure P is interpreted as linear isothermal compressibility $\beta_{CO,i}$ (Eq. (1)) [9], which differs for the different P450cam complexes *i*. $\nu^0_{CO,i}$ is the frequency of the intercept of the linear dependence at $P = 0$ kbar. V is the volume and T the temperature.

$$\beta_{CO,i} = -\frac{1}{V}\left[\frac{\partial V}{\partial P}\right]_T \equiv \text{const}\cdot\frac{1}{\nu^0_{CO,i}}\left[\frac{\partial \nu_{CO,i}}{\partial P}\right]_T \qquad (1)$$

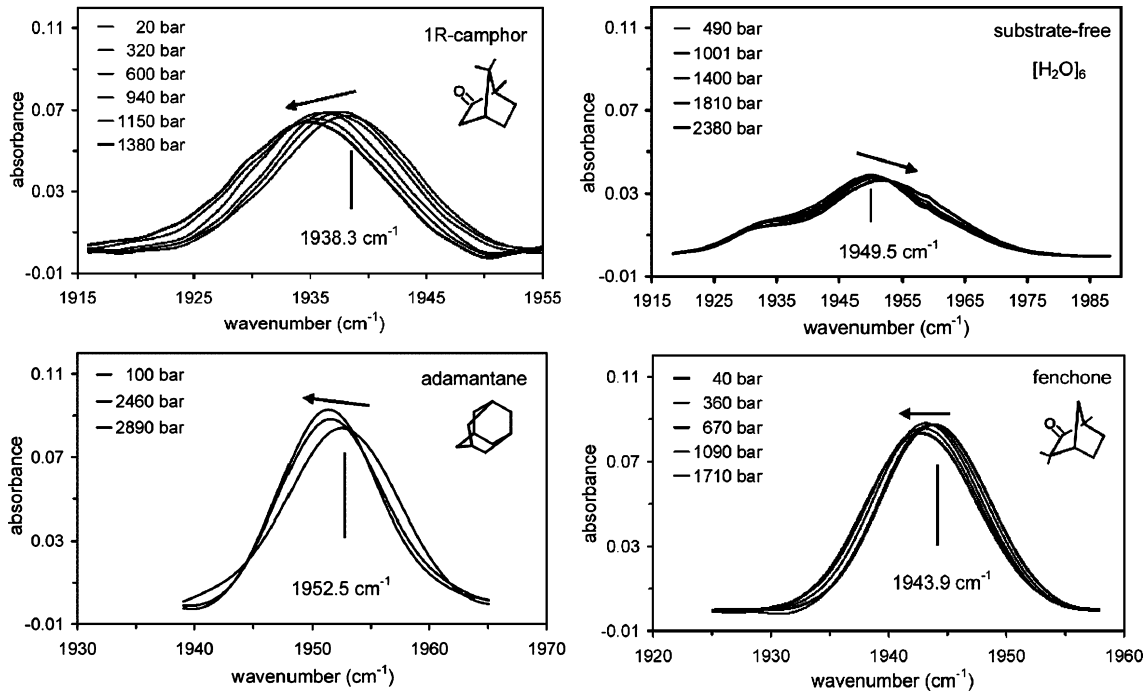

Fig. 2. Infrared spectra for the CO ligand stretch mode in P450cam-CO bound with selected substrates at different pressures, 300 K (290 K for substrate-free P450cam [13]).

The value of const is assumed to be −1 per definition [9] (see Discussion). The inset to Fig. 3 shows that the isothermal compressibility β_{CO} is linearly related to the high-spin state content (HS) produced by the different substrates:

$$\beta_{CO} = 0.000127 \cdot HS - 0.002947. \quad (2)$$

v^0_{CO} also correlates with HS (regression coefficients r^2: (2) 0.84; (3) 0.76)):

$$v^0_{CO} = -0.1395 \cdot HS + 1954.1. \quad (3)$$

Excluding the substrate-free complex, the resulting relation is $v^0_{CO} = -0.2072 \cdot HS + 1959.1$ ($r^2 = 0.96$), which is almost identical to the relation at 298 K and 0.001 kbar reported earlier [6].

Enzyme activity data

The ratio of the rates NADH/O_2 is found to be approximately one (Table 1), indicating that water formation in the oxidase reaction is not important and uncoupling leads only to H_2O_2 formation. In the case of a remarkable oxidase reaction this ratio should be larger than one and maximal of two [14]. CEP, CAL, and DAL show an unusually low NADH/O_2 ratio. The reason is not yet clear and we do not consider these data in the further discussion. The percentage H_2O_2 formation is calculated from the ratio of the rates H_2O_2/O_2 and is different for the substrate complexes with different high-spin state content.

Discussion

Isothermal compressibility determined from FTIR and UV-VIS

The position of the Soret band of P450cam-CO correlates with the initial high-spin state content (HS), which is maximally induced by the particular substrate as previously found ($v^{0.001\,kbar}_{Soret} = 0.71 \cdot HS + 22358$ [6], $v^0_{Soret} = 0.707 \cdot HS + 22351.9$ [7]). There is a relation between v^0_{CO} and v^0_{Soret}:

$$v_{CO} = -0.17 \cdot v_{Soret} + C, \quad (4)$$

with $C = 5681.5$ at 0.001 kbar and 298 K [6].

Our earlier study showed that the Soret band is red-shifted with increasing pressure [7–9]. The compressibility β_{Soret} determined from these data according to Eq. (1) with const = −1 gives

$$\beta_{Soret} = -0.000071 \cdot HS + 0.0147 \quad (5)$$

(regression coefficient of (5) r^2: 0.56; $\beta_{Soret} = -0.00010 \cdot HS + 0.0172$, excluding substrate-free P450, $r^2 = 0.63$).

These relations for the Soret band indicate that P450cam complexes with a large initial high-spin state content are less compressible than the low-spin complexes, which agrees with suggestions from various other experimental data [3,4]. The dependence of β_{CO} determined from the CO stretch mode shift on the high-spin state content (Eq. (2)) is opposite to that observed for β_{Soret} (Eq. (5)). Therefore, one would conclude from the

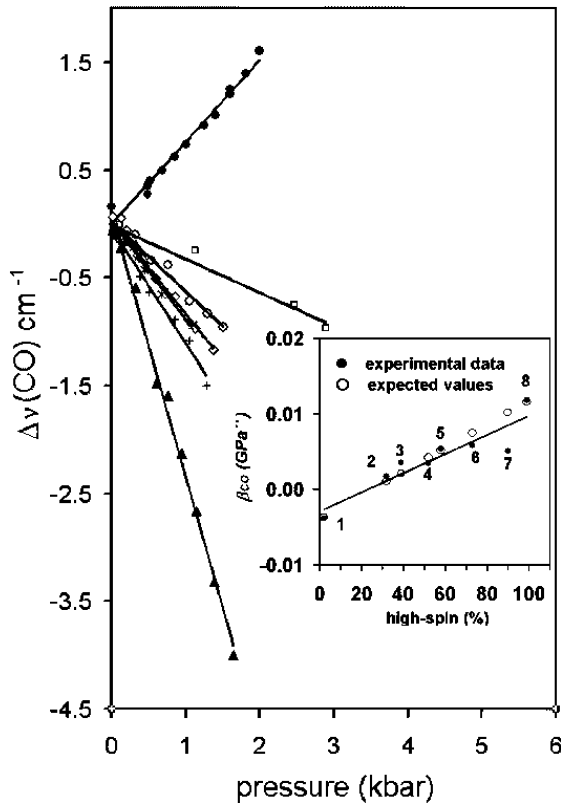

Fig. 3. Shift of the CO stretch mode frequency as function of the pressure. The shift is presented as difference to the intercept frequency at $P = 0$ kbar in the linear plot: ● (1) substrate-free (1949.54 cm^{-1}); □ (2) adamantane (1952.22 cm^{-1}); ○ (4) norcamphor (1946.62 cm^{-1}); △ (3) norbornane (1952.70 cm^{-1}); * (6) fenchone (1943.90 cm^{-1}); + (5) tetramethylcyclohexanone (1933.10 cm^{-1}); ◇ (7) adamantanone (1941.15 cm^{-1}); ▲ (8) (1*R*)-camphor (1938.20 cm^{-1}). Inset: β_{CO} in relation to the high-spin state content HS of different substrate complexes of P450cam (numbering see above) (1 bar = 0.1 MPa). (●) experimental data; (○) $\beta_{CO}^{expected}$ according to Eq. (7).

IR data that substrate complexes with a larger high-spin content, and following with a lower water content in the heme pocket, should be more compressible. This apparent contradiction between IR and UV-VIS data, however, is solved considering the earlier observed linear relation between ν_{Soret} and ν_{CO} at ambient conditions. Eq. (4) is reformed using Eq. (1):

$$\beta_{CO} = -0.17 \cdot \frac{\nu^0_{Soret}}{\nu^0_{CO}} \cdot \beta_{Soret} + \frac{C^0}{\nu^0_{CO}} \cdot \beta_C. \quad (6)$$

Using the HS-dependent equations for $\nu^0_{CO}, \beta_{Soret}$, and ν^0_{Soret} one gets for the expected compressibility $\beta_{CO}^{expected}$ as function of HS:

$$\beta_{CO}^{expected} = 1.576 \cdot 10^{-4} \cdot HS + 1.448 \cdot 10^{-8} \cdot HS^2 - 0.0286 + C', \quad (7)$$

with $C' = \frac{C_0}{\nu^0_{CO}} \cdot \beta_C \approx 0.02464$.

$\beta_{CO}^{expected}$ (empty circles in the inset to Fig. 3) shows the same dependence on HS as the experimentally observed β_{CO} (full circles) because the square-term of HS is negligibly small and the ratio of ν^0_{Soret} to ν^0_{CO} only slightly depends on HS. Neglecting these terms one gets

$$\beta_{CO} \approx -1.944 \cdot \beta_{Soret} + 0.02464. \quad (8)$$

Eq. (8) shows that the inverse behaviour of the compressibility as function of the high-spin state content determined from the Soret band and from the CO stretch mode frequency is indeed expected from the already earlier observed data at ambient conditions [8].

Role of internal water and heme pocket electric field for the pressure-induced frequency shifts

The relevant physical parameter for pressure-induced frequency shifts of absorption bands is the electric field, which acts on the chromophore, caused by the solvent or any surrounding molecules or atomic groups [15,16]. Because the heme as chromophore is deeply buried in the protein, the protein itself including internal water

Table 1
Activity of cytochrome P450cam with different substrates

Substrate	High spin (%)[a]	Rates (μM/min/μM P450)				Ratio of rates	
		NADH	O_2	O_2/catalase	H_2O_2	NADH/O_2	O_2/H_2O_2 (%)
(1*R*)-Camphor	95–100 (av. 98)	1467 ± 26	1471 ± 112	1471 ± 112	0	1.00	0
(1*S*)-Camphor	88–100 (av. 94)	1120 ± 10	1120 ± 32	1080 ± 65	80	1.00	7
Camphane	74–82 (av. 78)	81.5 ± 7	99 ± 5	73 ± 7.7	52	0.82	53
Norcamphor	46–68 (av. 57)	121.9 ± 7.7	122.6 ± 2.2	97.7 ± 3.6	49.8	0.99	41
Fenchone	73–82 (av. 78)	161 ± 12	156 ± 10	144 ± 7	24	1.03	15
COX	75	110.8 ± 10.6	115 ± 5	101 ± 5	28	0.96	24
CIM	58	214 ± 5	212 ± 6	204 ± 8	16	1.01	8
CIS	65	132 ± 12	136.8 ± 2	126 ± 6	21.6	0.96	16
CEP	35–85 (av. 60)	39 ± 1.5	89 ± 7	73 ± 12	32	0.44	36
CAL	77–96 (av. 87)	39 ± 6	87 ± 4	114 ± 15	nd	0.45	nd
DAL	nd	27 ± 9	42 ± 5	32 ± 5	20	0.64	48

[a] av., average value of the percentage of high-spin state content reported in different papers. The range of the values is given. nd, not determined.

molecules has to be regarded here as "solvent." Although the substrate in P450 is located in van der Waals distance to the CO ligand [17] a specific steric constraint inducing substrate-specific bending or tilting of the Fe-C-O group is not relevant for the observed shifts because structurally different substrates with a same high-spin state content show similar effects.

We recently adapted the microscopic theory for solute–solvent interaction and for reversible pressure effects on electronic absorption bands developed by Laid and Skinner [15] for P450 to explain the pressure-induced red-shift of the Soret band [7–9]. The same formalism can be used also for the IR CO stretch mode [9]. The absorption frequency $v_i(R,P)$ for a P450 substrate complex i is given by

$$v_i(R,P) = v_{\text{vac}} + v_{si}(R_0, P=0) + \alpha_i \cdot P, \qquad (9)$$

where R is the distance between the solvent and the solute molecule (chromophore) at the pressure P, v_{vac} is the absorption wavenumber in the absence of the solvent ("vacuum"), and $v_{si}(R_0, P=0)$ is the change of the absorption wavenumber caused by the interaction with the solvent molecules in a distance R_0 at the pressure $P = 0$ kbar ("solvent shift") for a particular substrate complex i. This "solvent shift" is related to the high-spin state content. $v_{\text{vac}} + v_{si}(R_0, P=0)$ corresponds to $v^0_{\text{Soret},i}$ (or $v^0_{\text{CO},i}$) for a particular P450 substrate complex i. α_i is the coefficient of the pressure dependence of the absorption frequency and is related to the compressibility ($\beta_{\text{Soret},i}$ or $\beta_{\text{CO},i}$) of the chromophore environment in the P450 substrate complex i. Based on published equations [6–9] one can show that the definition for the compressibility $\beta_{\text{Soret(CO)},i}$ in Eq. (1) for the Soret band (CO stretch mode) corresponds to

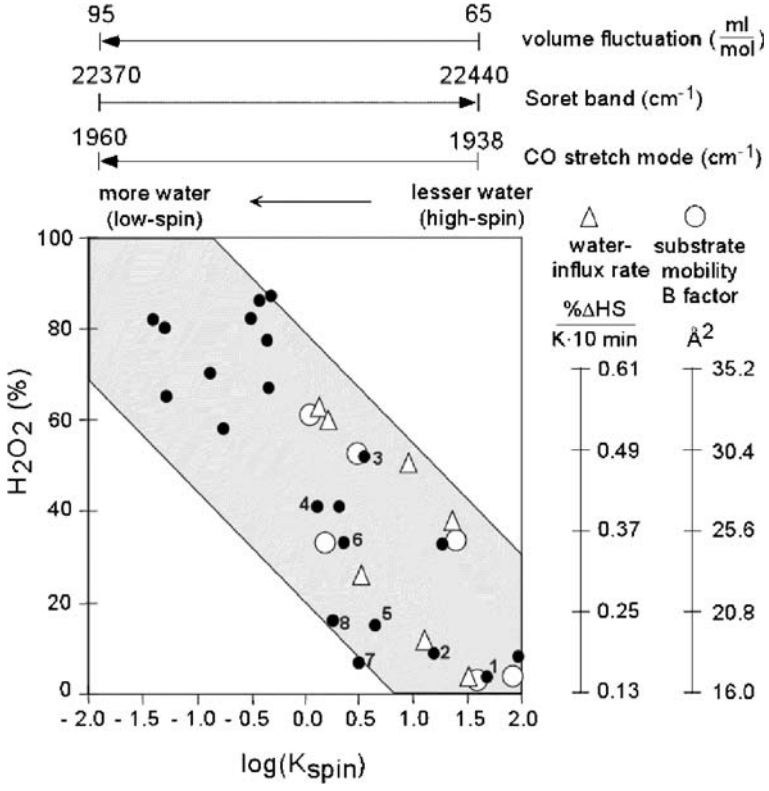

Fig. 4. Cross-correlation between physical parameters of P450cam bound with various substrates and H_2O_2 formation (Fig. 1). H_2O_2 (%): percentage of O_2 which is converted to H_2O_2; $K_{\text{spin}} = \text{HS}/(1 - \text{HS})$: spin-state equilibrium constant for a particular substrate complex of wild type or mutant P450cam producing the high-spin state content HS. The following complexes are included (●): (i) wild type P450cam: (1*R*)-camphor (1), (1*S*)-camphor (2), camphane (3), norcamphor (4), fenchone (5), (1*R*)-camphoroxime (COX) (6), (1*R*)-camphor *N*-methyl imine (CIM) (7), (1*R*)-isoborneol methyl ether (CIS) (8) (Table 1). Averaged values from data in Table 1 and from literature were taken for (1*R*)-camphor [19–21], (1*S*)-camphor [21]; ethylbenzene [20], hexachlorethane, pentachlorethane, 1,1,2-trichlorethane, 1,1,1-trichlorethane [22], (*R*)-2-ethylhexanol [19]; (ii) mutant P450cam: T101M + ethylbenzene, T185L + ethylbenzene, T185F + ethylbenzene, V247M + ethylbenzene, V295I + ethylbenzene, T101M/T185F/V247M + ethylbenzene [20], F87W+(1*R*)-camphor, F87W + (*R*)-2-ethylhexanol [19]. Water influx rate (△) is the amount of percentage of high-spin state content which is lost per one degree temperature decrease during 10 min in negative temperature jump experiments (inverse value of the rigidity factor) [3] corresponding to the formation rate of the low-spin state which reflects the access rate of the heme pocket for water molecules (wild type P450cam with: (1*R*)-camphor, (1*S*)-camphor, camphane, norcamphor, (1*R*)-camphorquinone, (1*S*)-camphorquinone, norbornane [3]. Substrate mobility factor (○) is the temperature factor of the substrate in the crystal structure of P450cam in the ferric state for the following substrates: (1*R*)-camphor (16.2 Å^2), adamantanone (16.5 Å^2), adamantane (24.7 Å^2), norcamphor (33.5 Å^2) [23], (1*S*)-camphor (25.1 Å^2) [24]. Volume fluctuation calculated from the compressibilities determined from the Soret band [7].

$$\beta_{\mathrm{Soret(CO)},i} = -\frac{v^0_{\mathrm{Soret(CO)},i}}{v_{\mathrm{Soret(CO)},si}} \cdot \frac{3}{n} \cdot \frac{1}{v^0_{\mathrm{Soret(CO)},i}} \cdot \left[\frac{\partial v_{\mathrm{Soret(CO)},i}(R,P)}{\partial P}\right]_T, \quad (10)$$

where n is the exponent in an inverse distance power dependence for the interaction between the solvent and the solute molecules and is 6 for long-range dispersive or higher-order electrostatic interactions [15] and $v_{\mathrm{Soret(CO)},si}$ is the pressure-independent solvent shift for the Soret (CO) band of the complex i at the distance R. It follows for Eq. (1) that const $= -\frac{v^0_{\mathrm{Soret(CO)},i}}{v_{\mathrm{Soret(CO)},si}} \cdot \frac{3}{n}$. The factor const is ~ -4 (for CO) and ~ -9.5 (for Soret) instead of -1. Considering these values, the absolute values for the compressibility fall in the range of compressibilities known for other proteins (0.02–0.15 GPa^{-1} [7]). For our purpose, relative values are only of interest.

Direct and distal side effect of water on ν(CO)

The partial negative net charge at the end-standing oxygen atom of the CO ligand leads to an electrostatic interaction with a positive electrostatic potential produced by the protein on the distal side resulting in a red-shift of the CO stretch mode band for complexes with 100% high-spin state content compared to a distal side without a positive electrostatic potential. With an increasing number of water molecules in the heme pocket this electrostatic interaction is disturbed due to compensation of the positive electrostatic potentials at the distal side by water molecules (Fig. 1). Consequently, the CO stretch mode frequency increases as observed [6]. In summary, $v^0_{\mathrm{CO},i}$ contains a "solvent shift" ($v^{0,\mathrm{direct}}_{\mathrm{CO},i}$) which should lead to a decrease of the frequency due to the direct polar interaction of water with the CO dipole (analogous to the effect on the Soret band) and a "solvent shift" ($v^{0,\mathrm{distal}}_{\mathrm{CO},i}$) which induces an increase of the frequency due to electrostatic compensation of the positive electrostatic potential on the distal side and loss of interaction to the CO ligand. The net compressibility is then given by $\beta_{\mathrm{CO},i} = \beta^{\mathrm{direct}}_{\mathrm{CO},i} - \beta^{\mathrm{distal}}_{\mathrm{CO},i}$. $\beta^{\mathrm{direct}}_{\mathrm{CO},i}$ should show the same trend with increasing high-spin state content as observed for $\beta_{\mathrm{Soret},i}$ because of the same physical effect on the chromophore. However, $\beta^{\mathrm{distal}}_{\mathrm{CO},i}$ seems to be larger (more pressure sensitive frequency shift) than $\beta^{\mathrm{direct}}_{\mathrm{CO},i}$, which leads to the opposite dependence of $\beta_{\mathrm{CO},i}$ on HS in comparison to $\beta_{\mathrm{Soret},i}$.

In conclusion, our data indicate that an increased heme pocket hydration would disturb ligand-distal side contacts. In the case of the dioxygen ligand such contacts are needed for a specific proton transfer in oxygen activation [18]. Disturbing these contacts may result in an unspecific protonation of the terminal oxygen atom of the dioxygen ligand, which should favor the formation of hydrogen peroxide (uncoupling) over the substrate hydroxylation (Fig. 1) [14]. Indeed, cross-correlation of different structural parameters and the fraction of H_2O_2 formation determined in this study for various substrate complexes and by other laboratories for different substrate-bound P450cam mutants support this conclusion (Fig. 4).

Acknowledgments

This paper is dedicated to I.C. Gunsalus in honor of his 90th birthday. C.J. appreciates very much his mentorship during her stays in his laboratory at the University of Illinois at Urbana–Champaign, where the present work has its scientific roots. G.H.B.H. expresses much gratitude to I.C. Gunsalus for initiating and encouraging him to study cytochrome P450 systems for 25 years. C.J. thanks Karel Heremans (Katholieke Universiteit Leuven, Belgium) for much advice for handling high-pressure anvil cells. The Deutsche Forschungsgemeinschaft (grants Ju229/1-1; Ju229/3-1; SK35/3.3-5) and the European Commission (grant BIO2-942060) are acknowledged for funding.

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