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Substrate induced changes of the active site electronic states in reduced cytochrome $P450_{cam}$ and the photolysis product of its CO complex

Low-temperature magnetic circular dichroism data

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MCD spectra of camphor-free and camphor-bound reduced cytochrome $P450_{cam}$ have been recorded for the near UV and visible spectral regions at temperatures from 300K down to 2.1K and compared with those of the carbon monoxide photoproducts generated at 4.2K. In the absence of camphor, the reduced *P450* is spectroscopically different from the photoproduct. In the presence of camphor, however, the spectra of the reduced *P450* and of the photoproduct are almost similar and behave like the photoproduct of the camphor-free enzyme. This behavior indicates that substrate binding induces a higher active site rigidity. From the significant alteration of the temperature dependence of the MCD intensity for the reduced enzyme induced by camphor binding it is concluded that the near degeneracy of the electronic ground state in the substrate-free enzyme is removed by substrate binding.

Cytochrome *P450*; Magnetic circular dichroism; Low temperature; Flash photolysis

1. INTRODUCTION

It has been supposed that the structural dynamics of the protein moiety and its alteration by substrate binding may be important for the enzyme function [2–4]. Non-equilibrium conformational states in cytochrome $P450_{lm2}$ were detected in experiments on the low-temperature reduction of the oxidized enzyme by hydrated electrons [5] and on the photolysis of its CO complexes of the reduced enzyme [6]. For cytochrome $P450_{cam}$ there are indications from the X-ray crystal structure analysis [7,8], from flashphotolysis experiments [9] and from infrared studies of the CO-stretch vibration [10] at low temperatures that camphor binding has only a small effect on the static three-dimensional structure but does significantly change the temperature factors of active site amino acids, the CO rebinding rate and the population of conformational substates. In this paper we represent low-temperature MCD data for the reduced $P450_{cam}$ in the equilibrium and non-equilibrium conformations generated by the photolysis of its carbon monoxide complex at 4.2K. Temperature-dependent terms in the MCD spectra of paramagnetic hemoproteins are extremely sensitive to subtle changes in the heme environment [11] and may therefore be an appropriate tool to detect structural alterations of the active site in *P450* induced by substrate binding.

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Abbreviations: MCD, magnetic circular dichroism; HRP, horseradish peroxidase; $P450_{cam}$, bacterial cytochrome $P450_{cam}$ (CYP101 [1]); $P450_{LM2}$, microsomal cytochrome $P450_{LM2}$ (CYP2B2 [1]).

2. MATERIALS AND METHODS

Cytochrome $P450_{cam}$ from *Pseudomonas putida* isolated and purified according to the procedures described in [12] was a kind gift from Prof. I.C. Gunsalus from the Biochemistry Department of the University of Illinois, Urbana. Camphor was removed by dialysis against 50 mM Tris-HCl buffer, pH 7, 10 mM dithiotreitol, followed by a Sephadex G25 (medium) chromatography run in the same buffer. Finally, the protein was extensively dialyzed against 50 mM potassium phosphate buffer, pH 7.

The low-temperature measurements were performed in a glass-forming mixture of potassium phosphate buffer, pH 7, and glycerol with a volume ratio of 1:1.5, respectively. In the case of the substrate-bound enzyme a saturated solution of camphor was added to get a final camphor concentration of 400 μM. The oxidized enzyme was reduced in an atmosphere of gaseous helium by adding a few crystals of sodium dithionite (Merck) at room temperature. The CO complex was obtained by incubating the reduced *P450* in a CO atmosphere for 30 min with occasional shaking. As verified by recording the MCD and the absorption spectra a formation of significant amounts of cytochrome P420 formation was not detected. The photolysis of the CO ligand was achieved by irradiating the sample for 10–15 min as described elsewhere [13].

The MCD spectra were recorded at various temperatures down to

2K in a magnetic field of 1.45 T using a magnetic dichrograph equipped with a cryostat. The application of the MCD method, experimental techniques and details of low-temperature measurements are described in [11]. The same cell with an optical pathlength of 1 mm was used for recording the MCD and the absorption (Shimadzu UV-160A spectrophotometer) spectra.

3. RESULTS AND DISCUSSION

There are at least three lines of evidence to suggest that photodissociation of the CO ligand from hemoproteins at 4.2K keeps the coordination shell of the heme iron with the proximal protein-derived ligand and with the porphyrin nitrogen atoms in a mainly unrelaxed state and maintains the geometry of the ligated heme in a configuration similar to that existing before photolysis. First, Iizuka et al. [14] were the first to report that the charge transfer band at about 760 nm in reduced myoglobin is redshifted by about 10 nm from its position in equilibrium deoxymyoglobin after photodissociation of the CO complex at 4.2K. Photolysis experiments using the resonance Raman method for myoglobin at 4.2K revealed a shift of the Fe-Nε (proximal His) stretching mode in the low-frequency region of the resonance Raman spectrum between the CO photoproduct and the deoxymyoglobin [15]; such a shift had not been detected even in the 30 ps time-resolved experiments at room temperature [16]. Secondly, the MCD spectra recorded for the photoproducts of neutral and alkaline forms of CO-HRP at 4.2K are indistinguishable whereas there are large differences between the MCD spectra for the reduced enzyme at these pH values [17]. Moreover, the MCD spectra of both photoproducts are very similar to that of the reduced enzyme at alkaline pH. These findings were interpreted with the existence of large restrictions imposed by the protein moiety to alterations of the geometry of Fe–porphyrin and Fe–His bonds on the ligand binding or release at alkaline pH values [18]. Because of the high rigidity of the protein moiety at alkaline pH the iron coordination sphere is locked at a fixed geometry independent of whether the sixth ligand is bound or not bound. Thirdly, even for the protein-free heme–(2-methylimidazole) complex in a water/glycerol glass the iron coordination sphere remains unrelaxed after photodissociation of the CO ligand at low temperatures [17]. Unrelaxed conformations and iron-heme configurations as mentioned are also observed for $P450_{cam}$ as shown below.

Fig. 1 compares the MCD spectra of reduced $P450_{cam}$ with those of the metastable carbon monoxide photoproducts recorded at 4.2K for both the camphor-free and the camphor-bound enzyme. The effect of substrate binding on the MCD spectra of *P450* behaves very similar to that of the heme-linked ionization in HRP at different pH as discussed above. For both substrate-bound and substrate-free CO–$P450_{cam}$, photolysis of the CO ligand produces MCD spectra which are different from those of the reduced enzyme. While the difference between the reduced (equilibrium state) and the photolyzed (non-equilibrium state) samples in the presence of camphor at 4.2K is very small and only quantitative, qualitatively different spectra are observed for the camphor-free enzyme. Furthermore, the MCD spectra of the photolyzed samples and of the camphor-bound reduced *P450* are very similar supporting the conclusion drawn from the temperature dependence of the infrared CO stretching modes in the CO complex [10] and from thermal unfolding studies of $P450_{cam}$ [19] that camphor binding increases the rigidity of the active site structure. In the absence of camphor the protein has substantial freedom to move and to occupy a multitude of conformational substates [20] which are frozen out at low temperatures. In contrast, camphor binding seems to hinder such motions which keeps the protein in a restricted conformational space.

There are temperature-dependent and temperature-independent contributions to the MCD spectra of paramagnetic hemoproteins. The temperature behavior of the MCD intensity is a distinctive feature of the oxidation and the spin state of the heme iron and is determined by the Boltzmann redistribution between the split components of the electronic ground state multiplet [11]. For high-spin ferrous hemoproteins a saturation behavior for the MCD intensity at low temperatures is characteristic [11,21]. That should also be observed for *P450* because the heme iron is in the high-spin state as shown by Champion et al. [22] using magnetic susceptibility measurements in the temperature region between 1.7 and 4.5K. The temperature profile of the MCD intensity for *P450*, however, does not follow the typical saturation behavior. Fig. 2 shows the MCD spectra of reduced $P450_{cam}$ in the presence and the absence of camphor at different temperatures. The temperature profiles of the MCD intensity for the different samples studied are displayed in Figs. 3 and 4. Both substrate-free $P450_{cam}$ and $P450_{lm2}$ [23] reveal a linear increase of the MCD intensity at temperatures below 4.2K independent of the wavelength regarded (Fig. 3). Such temperature behavior can be explained either by the presence of a small amount of ferric low-spin form (model 1) [23] or by a negative value of the zero-field splitting parameter D with a nearly degenerate electronic ground state (Model 2). For Model 1 the experimental temperature profiles given in Fig. 3 were fitted using Eq. 1:

$$\Delta\varepsilon(T) = \alpha\Delta\varepsilon(Fe^{2+}_{hs})/H+(1-\alpha)c/kT \quad (1)$$

where

$$\Delta\varepsilon(Fe^{2+}_{hs}) = b_1+ b_2h(T) \quad (2)$$

$$h(T) = \frac{1}{3} \times \frac{8\exp[-4D/kT] + 28\exp[-D/kT] - 36}{2\exp[-4D/kT] + 2\exp[-D/kT] + 1} \quad (3)$$

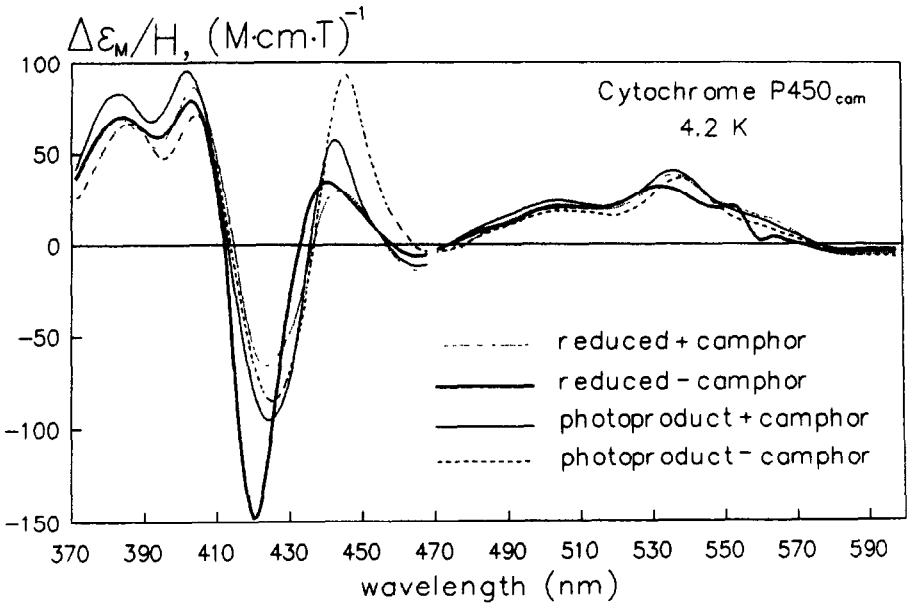

Fig. 1. Comparison of the MCD spectra of reduced $P450_{cam}$ and its carbon monoxide photoproduct recorded at 4.2K for the camphor-free and the camphor-bound enzyme in 50 mM potassium phosphate (pH 7.0) buffer-glycerol (1:1.5; v/v) solvent mixture without camphor and with 0.4 mM camphor, respectively. *P450* concentration: 0.15–0.26 mM, magnetic field: 1.45 T.

$\Delta\varepsilon(T)$ is the MCD intensity at temperature T. α indicates the fraction of ferrous high-spin state (Fe^{2+}_{hs}). H is the magnetic field strength.

The first term in the right hand side of Eq. 1 describes a contribution in the MCD of high-spin ferrous *P450* in an approximation given by Eq. 2 and 3 while the second term corresponds to the contribution of a small admixture of low-spin ferric enzyme whose MCD intensity is linear in $1/T$ for all measured temperatures in our experimental limit $\beta H \ll kT$.

The term b_1 in Eq. 2 is the temperature independent signal arising from B and/or A terms in MCD whose intensity is the same for excitation from all sublevels of the ground quintet. The second term in Eq. 2 corresponds to the signal originating from B terms in MCD whose intensity is different for excitation from various sublevels of the ground multiplet. This term is temperature dependent [11].

The temperature factor $h(T)$ given in Eq. 3 has been theoretically derived by Seno et al. [24] for high-spin

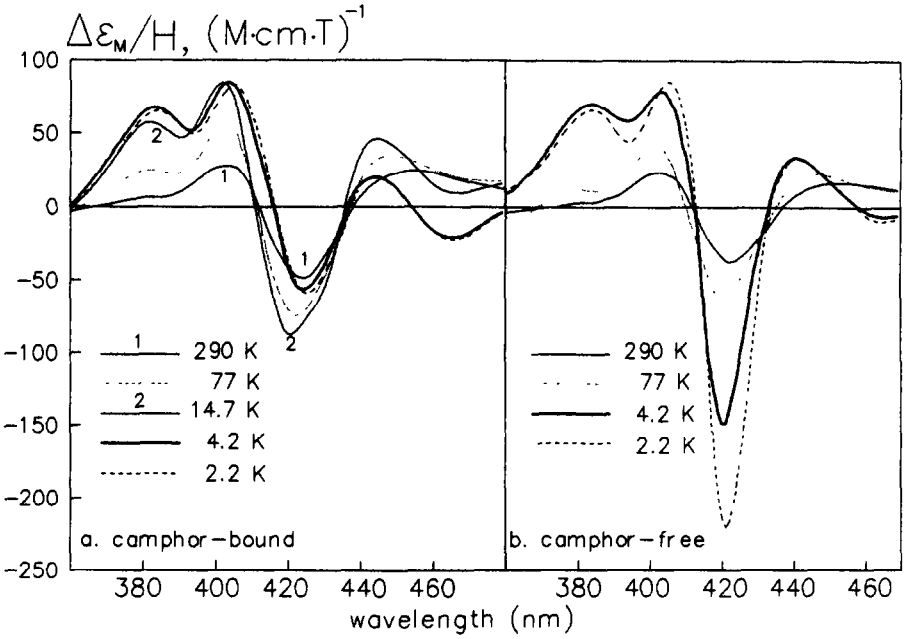

Fig. 2. MCD spectra for reduced $P450_{cam}$ in the presence (left) and the absence (right) of camphor for different temperatures.

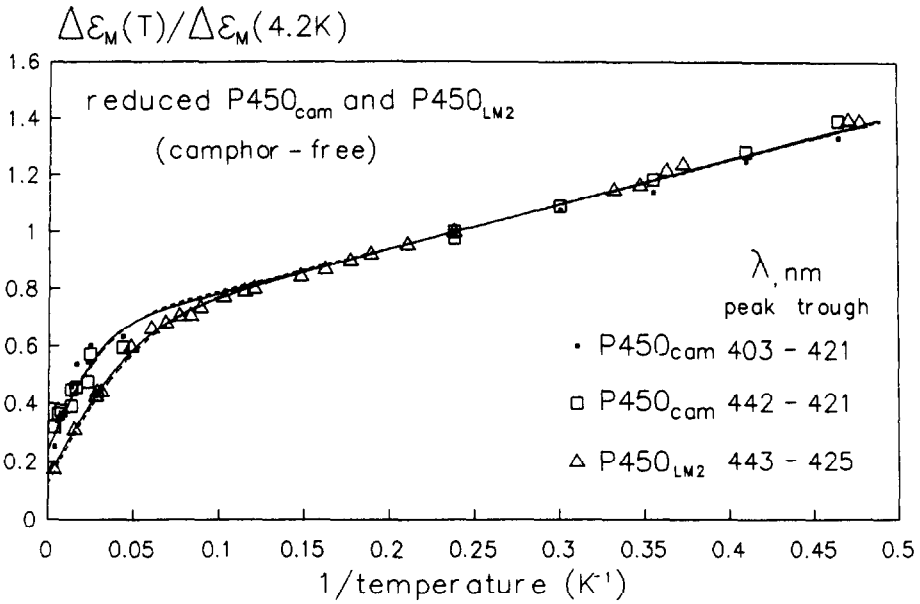

Fig. 3. Temperature dependence of the MCD intensity for substrate-free reduced $P450_{cam}$ and $P450_{lm2}$ represented as ratio of the peak-to-trough intensity at a given temperature to that at 4.2K; magnetic field: 1.45 T. Solid line (Model 1): $D = 31$ cm^{-1} ($P450_{lm2}$) and $D = 52$ cm^{-1} ($P450_{cam}$) and 5–10% of low-spin ferric form; dashed line (Model 2): $D = -5.2$ cm^{-1} ($P450_{lm2}$) and $D = -8.3$ cm^{-1} ($P450_{cam}$).

ferrous heme. The coefficients in the numerator of Eq. 3 show the relative contribution and sign of summed B terms for different sublevels.

For Model 1, the zero-field splitting parameter D was estimated by least-square fitting procedure to be equal (52 ± 8) cm^{-1} and (31 ± 5) cm^{-1} for $P450_{cam}$ and $P450_{lm2}$, respectively, assuming the admixture of about 5–10% of low-spin ferric *P450*. In Model 2, we assume that the parameter D is negative and a rhombic distortion at the heme iron is practically neglectable [25]. In this approximation the degenerate level with $S_z = \pm 2$ becomes the lowest one in energy. Consequently, a third term considering C terms and the $1/T$ dependence have to be added to Eq. 2:

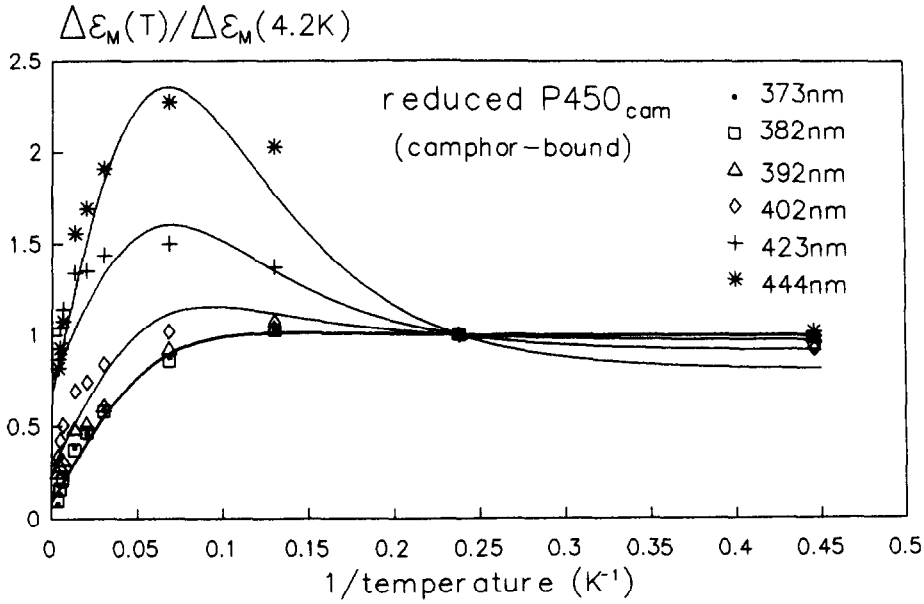

Fig. 4. Temperature dependence of the MCD intensity at different fixed wavelengths shown for camphor-bound reduced $P450_{cam}$. The MCD intensities are normalized as indicated in Fig. 3; magnetic field: 1.45 T. solid line: two co-existing ferrous high-spin forms with $D_1 = 12$ cm^{-1} and $D_2 = 28$ cm^{-1}.

$$\Delta\varepsilon(Fe^{2+}_{hs}) = b_1 + b_2 h(T) + c/kT\, h'(T) \quad (4)$$

with

$$h'(T) = \frac{2\exp[-4D/kT] + \exp[-D/kT]}{2\exp[-4D/kT] + 2\exp[-D/kT] + 1} \quad (5)$$

In Model 2, the zero-splitting parameters $-(8.3 \pm 3)$ cm^{-1} and $-(15 \pm 3)$ cm^{-1} were obtained for $P450_{cam}$ and $P450_{lm2}$, respectively.

Although both models fit the experimental data with the same quality we exclude the first model because (i) both $P450_{lm2}$ and $P450_{cam}$ show the same behavior and (ii) an excess of the reducing agent dithionite was present as indicated by its absorption in the ultraviolet region.

Camphor binding induces a changed temperature profile (Fig. 4) with a clear saturation behavior at low temperatures indicating that the energetic degeneracy of the ground state is practically removed. In addition, and in contrast to the substrate-free sample, the temperature profile becomes wavelength-dependent showing a maximum at about 15K for the long-wavelength side of the Soret band and an asymptotic behavior for the short-wavelength side of the Soret band. The latter is typically observed in other high-spin ferrous hemoproteins for all wavelengths [11]. Such an effect of the substrate binding on the MCD signal in *P450* is unexpected and has not been observed before. This unusual temperature profile, however, can be explained and fitted by the assumption that two high-spin forms coexist whose MCD temperature dependence are described in Eq. 2 but with different positive D values for the two components. The estimated D_1 and D_2 values are (12 ± 3) cm^{-1} and (28 ± 4) cm^{-1}, respectively. C-terms were omitted because Mössbauer studies [26] show that the ground state of camphor-bound $P450_{cam}$ is nondegenerate due to a strong rhombic distortion of the heme iron ligand field symmetry. The value for D_1 is in good agreement with the value of 13.9 cm^{-1} (20K) obtained from magnetic susceptibility measurements [22] and Mössbauer studies [26]. Until now, a second high-spin ferrous form with D_2 has not been detected by other methods. Interestingly, two conformers for the carbon monoxide complex of camphor-bound $P450_{cam}$ has been found by infrared studies [10] which are interpreted with conformational substates of the protein [20].

Summarizing the data we conclude that substrate binding does not only modify the active site and the electronic structure of the heme complex in the oxidized state of *P450* as extensively shown by many spectroscopic studies [27] but also in the reduced state. Two effects of the substrate can be differenciated: (i) Substrate binding alters the symmetry of the heme iron ligand field: the change from a nearly degenerate electronic ground state (negectable rhombic distortion) with a small negative zero-field splitting parameter in the substrate-free protein to the co-existence of two high-spin ferrous forms with larger positive zero-field splitting parameters and a strong rhombic distortion in the camphor-bound protein. The different sign of the zero-field splitting parameter indicates different ligand field strengths along the heme normal. (ii) Substrate binding increases the rigidity of the protein active center: no qualitative spectral difference between the reduced (equilibrium state) and the photolyzed (non-equilibrium state).

A structural assignment of these experimental findings requires further experimental as well as theoretical studies. The effect of the substrate on the heme iron coordination sphere and on the protein mobility of reduced *P450* protein may have important consequences for the dioxygen binding step following the first reduction of the heme iron in the reaction cycle of cytochrome *P450* [27].

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