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Multichannel circular dichroism investigations of the structural stability of bacterial cytochrome *P*-450

Bengt Nölting [a], Christiane Jung [a] and Günther Snatzke [b]

[a] *Max-Delbrück-Center for Molecular Medicine, Berlin (Germany) and* [b] *University of Bochum, Department of Structural Chemistry, Bochum (Germany)*

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The thermal unfolding of cytochrome *P*-450 LIN and *P*-450 CAM measured in presence and absence of their specific substrates was analyzed by circular dichroism (CD) and the α-helix content was estimated. Both proteins show, independent of the presence or absence of the substrates, nearly the same amount of loss of the CD in the peptide region. The comparison of the half transition temperatures determined from different chromophores and different methods indicates a non-two-state transition of the thermal unfolding. For such analysis we developed a new spectrometer, which is capable of measuring the CD simultaneously at all wavelengths in a limited wavelength region.

Introduction

The cytochrome *P*-450 proteins play an important role in the biochemistry of higher and microorganisms [1–3]. The common chemical reaction is the insertion of an oxygen atom into a hydrogen-carbon bond of the substrates.

In spite of this knowledge the origin of the substrate specificity is not well understood until now. A great progress was stimulated by the recent crystal structure analysis of the bacterial camphor hydroxylating cytochrome *P*-450 CAM and its complexes with camphor and camphor analogues [4–6]. Several physico-chemical studies on the bacterial proteins *P*-450 CAM (abbreviated by CAM and CAM(S) in absence and presence of camphor, respectively) and the linalool hydroxylating *P*-450 LIN (abbreviated by LIN and LIN(S) in absence and presence of linalool, respectively) let us suppose that not only the static geometrical arrangement of the amino acid residues and of the substrate in the heme pocket, but also the dynamics of the protein structure may be important for the catalysis. Jung et al. [7,8] and Hui Bon Hoa et al. [9] concluded that both proteins differ in the flexibility or rigidity of their structure. The higher rigidity of the *P*-450 LIN structure compared to *P*-450 CAM seems to be also the origin of the higher stability against temperature [10] elevation. In *P*-450 CAM thermally induced conformational changes take place with less cooperativity and substrate binding has a stronger influence on the stability compared to the first one. Comparative differential scanning calorimetric and temperature difference absorption studies [10] indicate that the thermal unfolding of *P*-450 CAM and *P*-450 LIN is not a two-state transition but a transition within two or three folding units. These findings describe a behavior of the *P*-450 proteins of which the structural origin is not clear until now.

To get a deeper insight into the structural behavior we carried out circular dichroism measurements on *P*-450 CAM and *P*-450 LIN in presence and absence of substrates and did also analyze the temperature dependence of the α-helix content.

For the measurements we used a new detection technique [11] for the circular dichroism (CD) spectroscopy which allows a faster recording of the spectra in a relatively broad spectral region with a higher signal-to-noise ratio than that of our JASCO J-600 spectropolarimeter (University of Bochum, Department of Structural Chemistry). In contrast to the commercial CD-instruments, this new spectrometer avoids systematical errors of the curve shape in the case of an alteration of the CD-amplitude during the measurements. This is especially important for measurements of the peptide chromophore spectrum, which is used for estimation of the α-helix content.

Correspondence: C. Jung, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, D-O-1115 Berlin, Germany.

Materials and Methods

Cytochrome *P*-450 CAM (CYP101 according to the new nomenclature [12]) and cytochrome *P*-450 LIN, isolated from the bacteria *Pseudomonas putida* and *Pseudomonas incognita*, respectively, and purified according to the methods described in Refs. 2 and 13, were a kind gift from Prof. I.C. Gunsalus, University of Illinois, Urbana, USA. The purification procedure included DEAE anion exchanger and Bio-Gel P100 chromatography steps. The proteins were purified to an absorbance ratio A_{392nm}/A_{280nm} of 1.5. The substrates were removed from the proteins by dialysis against 50 mM Tris-HCl buffer (pH 7), followed by a Sephadex G25 (medium) chromatography run in the same buffer. Finally, the protein was dialysed against 50 mM potassium phosphate buffer (pH 7). D-Camphor (Sigma, crystalline; approx. 99%) and racematic linalool (Aldrich, approx. 97%) were used without further purification.

The *P*-450 concentrations were determined spectrophotometrically at 22°C (pH 7) in potassium phosphate buffer by using the molar absorption coefficient of $1.15 \cdot 10^5$ M^{-1} cm^{-1} at 417 nm in the substrate-free protein and of $1.02 \cdot 10^5$ M^{-1} cm^{-1} at 392 nm in the substrate-bound protein [2]. For the measurements of the substrate-bound proteins 4% of a saturated aqueous solution of camphor (final concentration 400 μM) and linalool (final concentration 200 μM), respectively, were added to the buffer. The CD- and absorption-baselines, measured with the buffers were subtracted from the spectra of the samples.

For the measurements in the wavelength region from 206 nm to 260 nm we build up a new spectrometer [11] (Fig. 1), which is capable of measuring the CD and the absorption simultaneously at all wavelengths in a limited wavelength region of about 80 nm. Like in commercial CD-instruments the light of the light source (So), a deuterium lamp, is elliptically polarized by a polarizer (P) and a modulator (M), but the detection is done like in diode array absorption spectrometers with a multichannel detector (D), which is illuminated by a concave grating (G) without an exit slit. In contrast to the constant correction factor of 0.79 [14,15] which describes the deviation of the elliptical polarization state from the circular polarization state in commercial CD-instruments, in this spectrometer [11], which has a slight wavelength dependent polarization state, we used a wavelength dependent correction factor between 0.81 (206 nm) and 0.85 (260 nm).

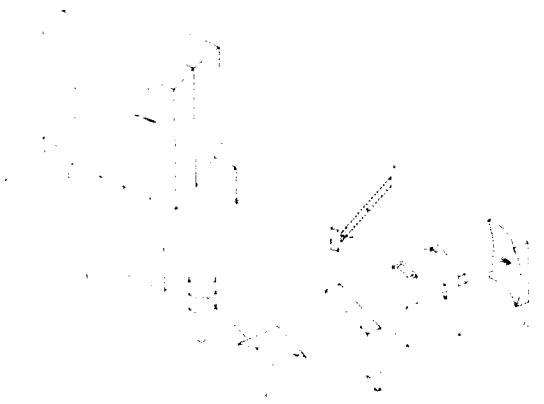

Fig. 1. Spectrometer for the simultaneous measurement of CD and absorption, simultaneously at all wavelengths in a limited spectral range. So, deuterium lamp; P, total reflecting prism, which acts as polarizer; M, electrooptical modulator, which produces alternatively left and right elliptically polarized light; C, sample cell; S, slit; G, concave grating; D, multichannel detector; E, electronics.

Measuring conditions. Concentration of the proteins: 3 μM in 50 mM phosphate buffer (pH 7), 1 mm path length, 2.4 min measuring time for one spectrum, 1.2 nm spectral width of one measuring point, approx. 2 nm spectral resolution.

For comparison with a commercial CD-instrument we made measurements in the region of the peptide chromophore at 22°C with a JASCO J-600 (Bochum) under the same conditions, but with 0.4 nm spectral step width and a higher measuring time of 10 min for one spectrum.

The measurements in the wavelength region from 250 to 500 nm were carried out with the JASCO J-600 (Bochum). Because of the high noise a curve smoothing was necessary.

Conditions. Concentration of the proteins: 70 μM in 50 mM potassium phosphate buffer (pH 7), 1 mm path length, 10 min measuring time for one spectrum, 0.4 nm spectral step width, 2 nm spectral resolution.

To get a further insight, what the structural differences between *P*-450 CAM and *P*-450 LIN are, and what the effect of the temperature might be, we estimated the α-helix content by using the method of Chang et al. [16]. For this analysis we assumed, that the basis curves are valid for all temperatures studied. This assumption had to be proved by further investigations. Nowadays factor analysis is preferred [17,18].

Results

Fig. 2 shows the CD spectrum for *P*-450 CAM and *P*-450 LIN in presence and absence of the substrates for the peptide chromophore absorption at 22°C taken with our new spectrometer. Compared with the spectra recorded using the JASCO J-600 (Bochum) (Fig. 3) the new technique gives a slightly better signal-to-noise ratio. There are no significant differences between these measurements with the two instruments.

In the following only the results obtained in the peptide chromophore region with the new technique

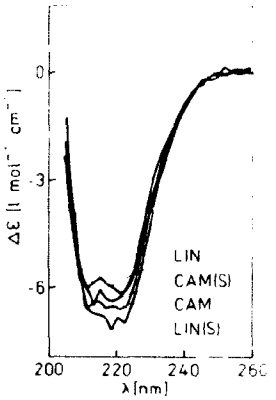

Fig. 2. Mean residual CD $\Delta\epsilon$ (l mol^{-1} cm^{-1}) at 22°C in the region of the peptide chromophor.

will be discussed. The shape of the CD curves is qualitatively similar for the four *P*-450 complexes (Fig. 2). Quantitatively, however, we observe significant differences between the linalool-bound (larger CD) and the linalool-free (smaller CD) *P*-450 LIN. In contrast, no remarkable difference could be detected between camphor-bound and camphor-free *P*-450 CAM. Temperature elevation up to 70°C leads to an approx. 50%

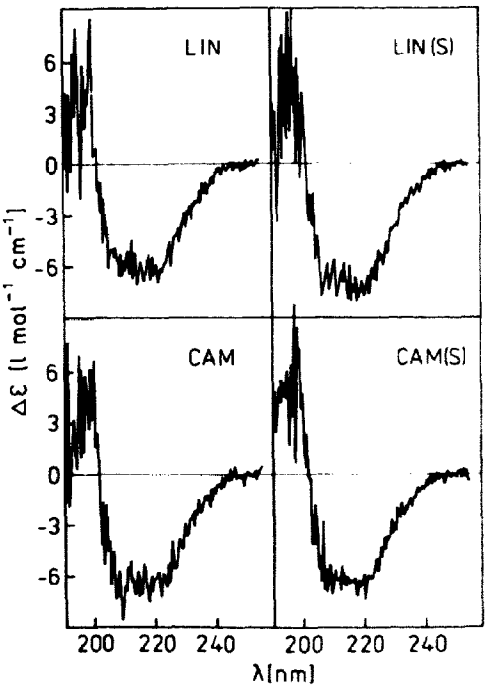

Fig. 3. Mean residual CD $\Delta\epsilon$ (l mol^{-1} cm^{-1}) at 22°C in the region of the peptide chromophor measured with a JASCO J-600 (Bochum).

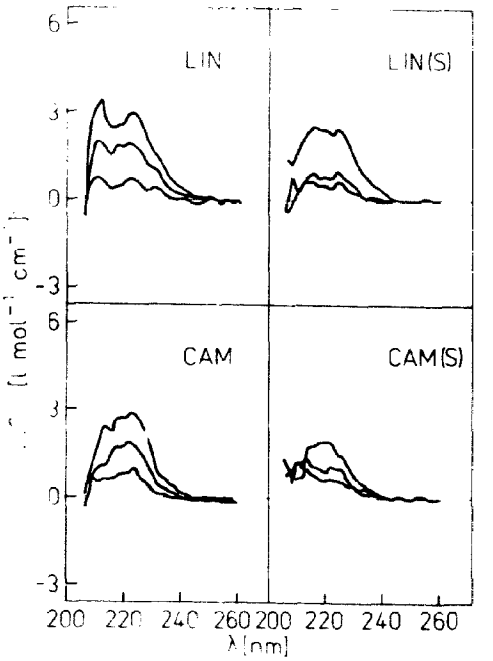

Fig. 4. Differences of the mean residual CD $\Delta\epsilon$ (l mol^{-1} cm^{-1}) at the selected temperatures of 40°C, 45°C and 50°C, respectively. (in each case lower curve: 40°C, middle curve: 45°C, and upper curve: 50°C) to 22°C in the peptide absorption region.

decrease of the CD in all four *P*-450 complexes (Fig. 4, Table I). As already shown in Ref. 10 the temperature induced unfolding is not reversible. The half transition temperatures ($K_T = 0.5$) are 43°C for the substrate-free proteins. Binding of the substrate induces an increase of the half transition temperature to 47°C for *P*-450 LIN and to 50°C for *P*-450 CAM (Fig. 5, Table II). The temperature induced change of the α-helix content is shown in Fig. 6. At 22°C the *P*-450 complexes reveal an α-helix content of about 40–50%. During heating the samples up to 70°C the α-helix content of all four complexes decreases to approx. 10–20%.

TABLE I

Mean residual circular dichroism $\Delta\epsilon$ (l mol^{-1} cm^{-1}) at 22°C and 70°C at 220 nm

LIN, *P*-450 LIN without substrate; LIN(S), *P*-450 LIN with substrate; CAM, *P*-450 CAM without substrate; CAM(S), *P*-450 CAM with substrate.

Sample	$\Delta\epsilon$ (l mol^{-1} cm^{-1})	
	22°C	70°C
LIN	6.1	3.0
LIN(S)	7.3	3.7
CAM	6.7	3.3
CAM(S)	6.3	3.1

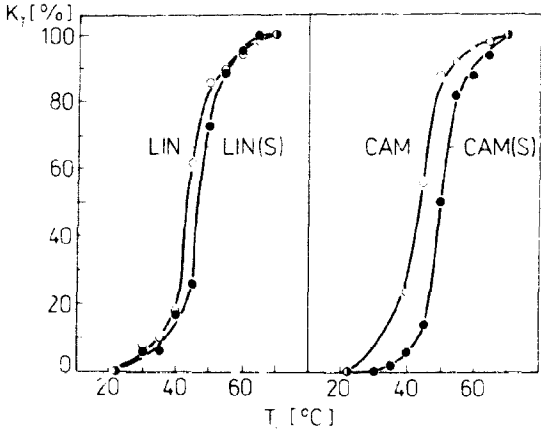

Fig. 5. Temperature induced alteration of the CD monitored in the region of the peptide chromophore (220 nm), expressed in K_T. K_T = (CD(22°C) − CD(T))/(CD(22°C) − CD(70°C)); T, temperature (°C).

The CD spectra in the region between 250 nm and 500 nm are presented in Fig. 7. The qualitatively different CD spectra between the substrate-free and the substrate-bound proteins reflect the different optical absorption spectra for the iron high-spin (substrate-bound) and the iron low-spin (substrate-free) state [19]. The Soret band of LIN shows a significantly higher CD than that of CAM. Heating the samples to 70°C leads to a complete loss of the CD of the Soret band for CAM and CAM(S). Because of protein precipation at higher concentrations we could not follow the complete unfolding of the LIN and LIN(S) using the Soret band, but we assume that also for these two complexes the CD is completely lost at high temperatures. The half transition temperatures estimated from the 50% loss of the CD effect of the Soret band match well with

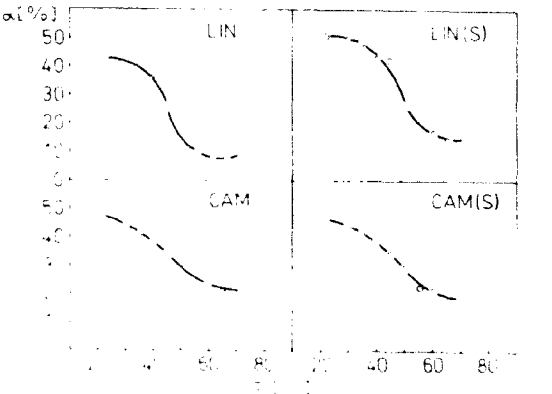

Fig. 6. Temperature dependence of the α-helix-content, calculated by the method of Chang et al. [16]. α, α-helix-content; T, temperature.

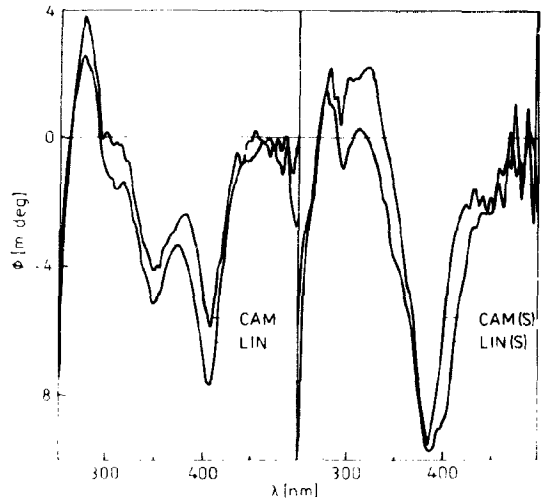

Fig. 7. Ellipticity ϕ (mdeg) at 22°C in the wavelength region from 250 to 500 nm.

the values obtained from the peptide chromophore absorption (Fig. 8, Table II).

Discussion

The advantage of our new spectrometer was to measure all CD values with the same time characteristics. As shown in Fig. 4, the CD amplitude does sensitively change within a narrow temperature range of only a few K. A drift of the temperature in this region during measuring with a scanning technique would lead to a significant distortion of the curve shape. A distortion would also be the result if the slow kinetics of conformational changes would interfere with the scanning time. In these cases the secondary structure elements (helix, sheet, turn) could not be deter-

TABLE II

Half transition temperatures $T_{1/2}$ (°C) determined by CD-measurements (this paper) compared with the temperature difference absorption investigations and scanning calorimetric studies by Jung et al. [10]

The wavelength in the Soret band was 392 nm for the substate-bound and 417 nm for the substrate-free protein. LIN, *P*-450 LIN without substrate; LIN(S), *P*-450 LIN with substrate; CAM, *P*-450 CAM without substrate; CAM(S), *P*-450 CAM with substrate.

	CD at 220 nm (°C)	CD at 392 nm/ 417 nm (°C)	Absorption at 392 nm/ 417 nm (°C)	Calorimetric (°C)
$T_{1/2}$: LIN	43	42	47.3	55.5
$T_{1/2}$: LIN(S)	47	46	48.4	61.7
$T_{1/2}$: CAM	43	41	39.6	53.3
$T_{1/2}$: CAM(S)	50	49	54.7	63.7

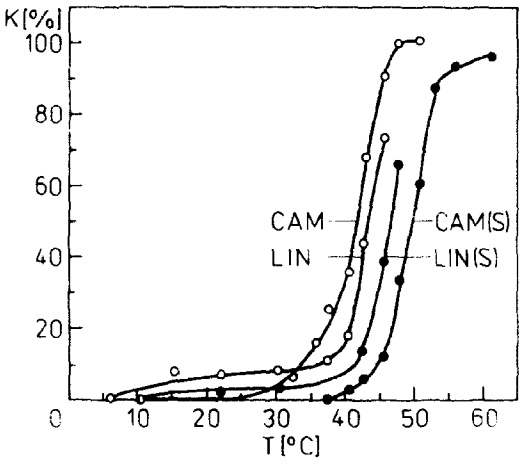

Fig. 8. Temperature induced alteration of the CD monitored in the Soret region (418 nm for the substrate-free proteins, 387 nm for the substrate-bound proteins), expressed in K. K = (CD(5°C) − CD(T))/(CD(5°C)); T, temperature (°C).

mined from the spectra. With our new detection technique, however, always a linear superposition (accumulation) of spectra is produced even if the CD varies, so that the accumulated spectra correspond to a temperature- or time-avaraged content for each structure element that mostly (for monoton functions) lies between the content obtained from the bordervalue measurements. In our studies, however, we did not benefit much from this advantage, because the temperature was stable during measuring within ±1 K and time dependent structural changes were not observed within 10 min.

The CD data obtained with the new spectrometer, presented in this paper, are in general agreement with that of earlier investigations of other *P*-450 proteins by Peterson [20] (*P*-450 CAM), by Chiang and Coon [21] (*P*-450 CAM) and by Shimizu et al. [22] (*P*-450 LM2 (CYP2B4 [12]) and *P*-450 LM4 (CYP1A2 [12])): they observed a negative Cotton effect in the Soret region and estimated an α-helix content of 40–50% from the CD effect in the peptide chromophore absorption which is also seen in our studies on the bacterial *P*-450 CAM. The same characteristics we also observed for *P*-450 LIN, which is investigated by CD for the first time.

Three results may be pointed out which are important:

(1) The estimated α-helix content of the native structure is within the experimental error similar in both bacterial proteins *P*-450 CAM and *P*-450 LIN. This finding is in contrast to our original expectation because the *P*-450 LIN structure is supposed to be more rigid compared to the *P*-450 CAM structure [10]. A higher α-helix content would explain such higher rigidity as it is known from myoglobin showing an α-helix content of 78% [23]. Obviously, other non-helical interactions must be responsible for the higher rigidity in *P*-450 LIN.

(2) The thermal unfolding of the protein structure of both *P*-450 proteins independent of the presence or absence of the substrates is accompanied by nearly the same amount of loss of the CD in the peptide region (approx. 50% at 220 nm) and in the α-helix content (approx. 30%). That may indicate that a similar secondary structure with distinct labile helices exists in *P*-450 CAM and *P*-450 LIN.

(3) The half transition temperatures for the thermal unfolding estimated from the CD spectral changes in the peptide chromophore and in the heme absorption are equal within the experimental error. This result lets us suppose that the labile helix parts are in interaction with the heme. The electronic coupling between the aromatic amino acids localized in the labile helices and the heme π-system which is responsible for the heme CD effect [24] may be strongly diminished by breaking the helical structure during the thermal unfolding. It should be noted here that the half transition temperatures observed in the CD studies do not agree with the values obtained from temperature difference absorption investigations nor from scanning calorimetric studies (Table II). We interpret this result as a further indication for a non-two-state transition during the thermal unfolding, as suggested in Ref. 10.

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