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Conference Paper

Circular Dichroism of Partially Purified Cytochrome *P450* from Rabbit Liver Microsomes*

K. Ruckpaul, H. Rein, G.-R. Jänig, W. Winkler, and O. Ristau

Department of Biocatalysis, Central Institute of Molecular Biology, Academy of Sciences of GDR, 1115 Berlin, GDR

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The heme-related circular dichroic bands of solubilized cytochrome *P450* from rabbit liver microsomes and some of its liganded derivatives were measured in the Soret region. All *P450* derivatives exhibit negative circular dichroic bands in the region of the Soret absorption. The wavelengths of the dichroic bands and their ellipticities vary with ligand substitution and the oxidation state of the iron. The results are compared with CD-data from other hemoproteins and discussed with respect to stereochemical conclusions concerning the geometry and the physicochemical character of the vicinity of the heme group with regard to results obtained from other studies.

INTRODUCTION

The different functions of the hemoproteins are all determined by specific interactions of the same prosthetic group with different protein moieties. By these interactions modified optical activities are induced into the heme chromophore, from which conclusions on the spatial arrangement and on the conformational changes of the polypeptide chain in the immediate environment of the heme chromophore may be drawn[1].

We have attempted to answer three questions concerning conformational changes and possible relationships between these changes and the catalyzing function of *P450*.

(1) Is substrate binding to *P450* connected with changes in optical activity?
(2) Is the reduction of the heme iron in *P450* connected with changes in optical activity?
(3) What conclusions can be drawn from the optical activity about the spatial arrangement of the immediate protein environment of the heme chromophore as compared with other hemoproteins?

RESULTS AND DISCUSSION

We used solubilized and partially purified cytochrome *P450* from phenobarbital induced male rabbits[2]. *P420* in our sample was too low to be measured, but cytochrome b_5 amounted up to 6% of the *P450* content. The Soret CD-band

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of cytochrome b_5 is, however, lower than that of *P450* by about one order of magnitude[3]. Therefore, it seems to be justified to neglect this contribution to the *P450* CD-values. In order to eliminate errors from preparation dependent scattering the data in our paper were obtained from the same preparation. Rotational strength, dipole-strength and anisotropy were calculated according to the following equations:

$$\text{Dipole-strength } (D) = 0.919 \cdot 10^{-2} \int_0^\infty E_M/\nu \cdot d\nu \; [Db^2]$$

$$\text{Rotational strength } (R) = 0.306 \cdot 10^{-3} \int_0^\infty \Theta_M/\nu \cdot d\nu \; [Db \cdot MB]$$

$$\text{Anisotropy} = R/D$$

At first, evidence was obtained of changes in optical activity occurring at substrate binding. In the Soret region the substrate-free oxidized form of the solubilized *P450* exhibits a negative Cotton-effect. Binding of type I substrates (benzphetamine-HCl) does not produce any significant changes in the amplitude of the CD-spectrum of the oxidized form of *P450*. Aniline, however, as a type II-substrate, causes a small decrease in ellipticity.

Typical electron donor ligands of the heme iron, such as cyanide, imidazole and also alkylisocyanides which, unlike hemoglobin, are bound not only to the reduced but also to the oxidized form[4,5] show a similar behaviour — but the decrease is more pronounced. The decrease in the negative ellipticity of the Soret-band of *P450* indicates altered interactions between the heme chromophore and the protein.

A significant change in the CD-spectrum of *P450* is caused by the reduction of the heme iron with dithionite forming a relatively broad negative CD-band with a maximum at 400 nm. Its ellipticity is diminished to about half of the the value of the oxidized form. A striking result with the reduced form is the strong displacement of the absorption maximum at 421 nm from the maximum of the CD-band at 400 nm. A discrepancy between the absorption- and CD-maximum like for the reduced form of *P450*LM was observed with *P450*CAM by Peterson[6], who found a broad CD-band with a maximum at 388 nm which significantly differs from the absorption maximum at 408 nm, indicating that the Soret absorption maximum is composed of at least two overlapping bands of which only one is optically active.

The binding of CO to the reduced form leads to a negative CD-band with a maximum at 454 nm which is slightly shifted towards higher wavelengths compared with the absorption maximum of *P450*LM. A striking fact is the existence of a second negative CD-band at 356 nm with nearly the same intensity as the band at 454 nm. The quantum-chemical calculations of Hanson *et al.*[7] as well as the preliminary ones of Ristau and Jung[8] have shown that the Soret band of the CO-complex of *P450* is split into 2 bands: one component is shifted to higher and the other to lower wavelengths.

The band at 356 nm obviously appears as a shoulder in the spectrum of the oxidized form with about the same wavelength. The alkylisocyanide--complex of the reduced as well as of the oxidized form so far investigated show the same CD-band at about 355 nm. Alkylisocyanide complexes with reduced *P450* show two CD-bands in the Soret region: a larger one with a maximum at 428 nm and a smaller one with a maximum at 455 nm in

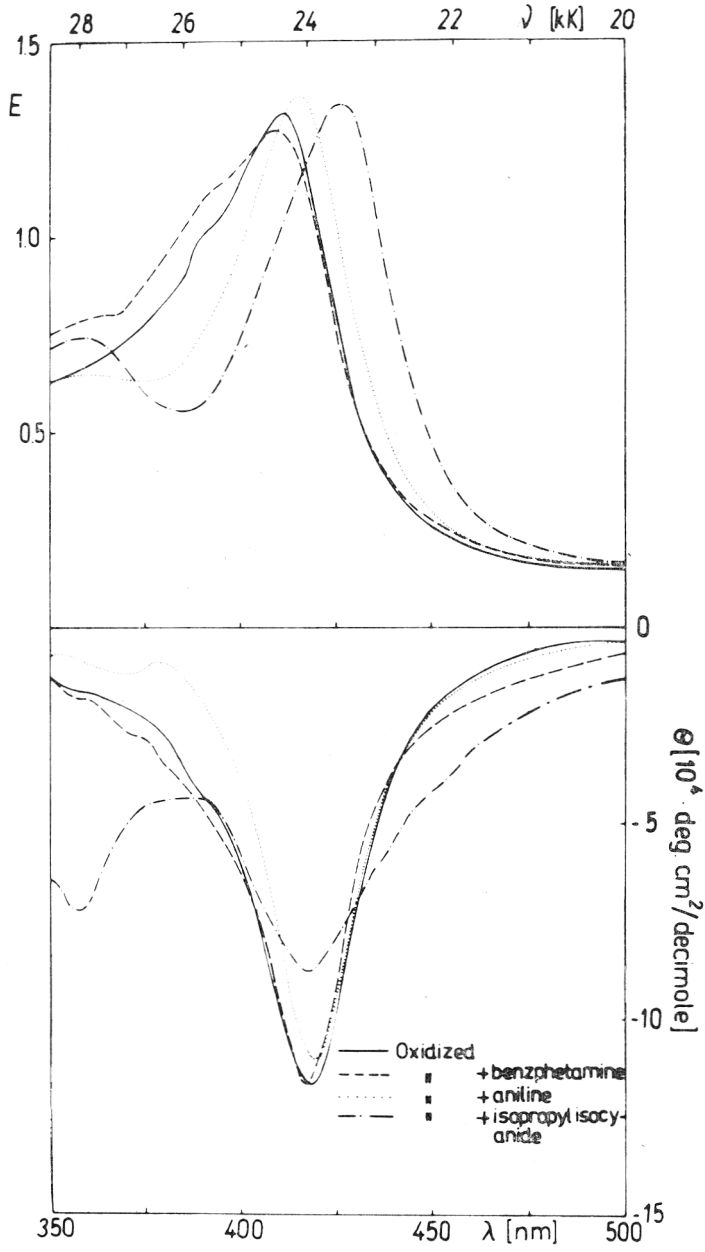

Figure 1. CD and absorption spectra of the oxidized form of *P450*LM (———) and in presence of benzphetamine-HCl (— — — —), aniline (. . . . . .) and isopropylisocyanide (— · — · — ·).

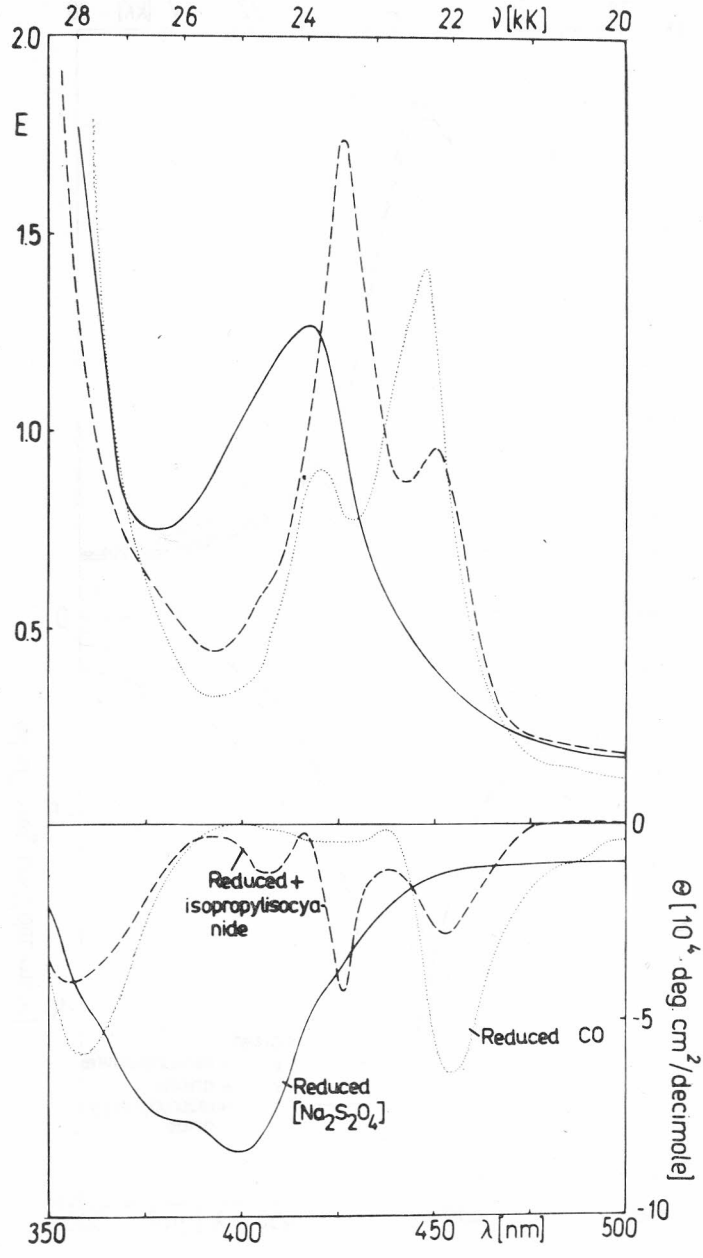

Figure 2. CD and absorption spectra of the reduced form of *P450*LM (———) and in presence of CO (. . . . . .) and of isopropylisocyanide (— — — —). Due to the absorption of dithionite the peak at about 370 nm in the absorption spectrum of the CO-complex is not well resolved.

good agreement with their absorption bands. The absolute values of the ellipticities of *P450* are in good agreement with the data of *P450*CAM published by Peterson[6] (Table I). The slightly higher values of the oxidized *P450* observed by Guengerich *et al.*[9] are probably due to the differences in the degree of purification and to different preparation procedures. This assumption is supported by the difference in the ellipticities of the Soret CD-band of *P450*LM which was observed in preliminary studies in dependence on the preparation.

Comparing different stages of purification the Soret CD-band of *P450*LM in the oxidized form becomes higher with increasing specific content of the heme enzyme, indicating that probably the micromilieu in some way interferes with optical activity. If we now look for the conclusions that can be drawn from these findings on the spatial arrangement of the immediate protein environment of the heme chromophore, we have to take into account the fact that the optical activity of the Soret band of hemoproteins is considered to arise predominantly from dipole-dipole interactions originating from interactions of the Soret transitions of the heme with the excited state of the nearby aromatic side chains of the protein. According to Hsu and Woody[10] the optically active Soret band is composed of two components which contribute to the rotational strength with opposite signs. The specific interaction of the two components of the Soret transition with the amino acid residues

TABLE I

*Wavelengths and ellipticities of CD-bands, and wavelengths of the absorption maxima of different derivatives of P450*LM *in the Soret region*

*P450*LM-derivatives	CD-spectrum		absorption spectrum
	band maximum wavelength/nm	ellipticity $\theta \cdot \lvert 10^4 \cdot deg \cdot cm^2 \cdot dmole^{-1} \rvert$	band maximum wavelength/nm
Oxidized	419	11.6	416
+ benzphetamine	417	11.6	414
+ metphenetamine	419	11.6	415
+ aniline	419	11.0	418
+ cyanide	427	5.5	433
+ imidazole	419	9.2	423
+ ethylisocyanide	418	7.7	423
	354	3.5	354
+ isopropylisocyanide	417	8.8	426
	358	7.2	356
Reduced	400	8.3	418
+ carbonmonoxide	454	6.3	449
	358	5.6	- *
+ ethylisocyanide	458	3.4	450
	428	6.2	425
	361	4.6	- *
+ isopropylisocyanide	453	2.8	450
	426	4.1	426
	356	4.0	- *

* Not resolved because of the high absorption of dithionite; a detailed analysis of this band is under investigation.

determines the shape, sign and the rotational strength of the CD-band. Hence, these properties are sensitive indicators of conformational changes as well as of ligand-induced changes in the electronic structure of the heme chromophore.

It is assumed that the binding of substrates to *P450*LM occurs near the prosthetic group. Therefore, conformational changes in the protein caused by the binding of substrates should be indicated by changes in the Soret CD-band. The slight changes in the band occurring at substrate binding may be explained by the relatively weak interactions between the enzyme and substrate. The binding takes place preferably by nonpolar interactions as has been shown by binding studies[11] as well as by analyzing the temperature dependence of substrate binding[12]. Therefore, the lack of a conformational change upon substrate binding is in line with these considerations.

A conformational change on reduction is plausible, because in the oxidized form the heme iron is linked by two axial ligands originating from the protein, resulting in a low spin complex. On reduction the binding of one of these ligands is split off forming a high spin complex with $S = 2$ [13]. The formation of the 5-coordinated high spin complex is connected with an increase in the distance between the heme iron and the separated ligand, resulting in a conformational change. In this process amino acid residues, which interact with the heme chromophore, can be involved. The decrease of the negative Soret CD-band at reduction may be therefore explained by disturbed interactions leading to an increase in the positive component of the Soret CD-band.

A striking result is the negative sign of the Soret CD-band of *P450*LM as it was also found for *P450*CAM[6]. In order to discuss this phenomenon it is to be noted that certain other hemoproteins also exhibit a negative Soret CD-band: chironomus-Hb[14], lamprey-Hb[15], leg-Hb[16,17] and catalase[18]. Human-Hb, myoglobin[1] and cytochrome c[19,20] however, exhibit a positive CD-band. Since in the intensities of the interactions between the prosthetic group and the protein moiety sterical arrangements are also involved, it is obvious that the properties of the Soret CD-band are in some way correlated with the geometry and the size of the heme pocket. In the case of chironomus-Hb a relatively broad heme pocket has been shown by x-ray analysis[21] as well as by titration studies using different alkylisocyanides[22]. A similar conclusion has been derived from a solvent proton magnetic relaxation study of leg-Hb[23]. Binding of alkylisocyanides to the reduced form of lamprey hemoglobin converts the Soret CD--band into a positive one[15]. This conversion was assumed to be produced by interactions of the hydrophobic side chains of the alkylisocyanides with the protein.

In order to make these correlations more precise we calculated the rotational and dipole strengths for myoglobin, human and lamprey hemoglobin and cytochrome *P450* after resolving the CD- and absorption-bands into gaussian curves (Table II).

From these data the anisotropy was evaluated. It contains information about the intensity of interactions between the prosthetic group and the protein environment.

If it is correct to assume that in the rotational strength structural information is also contained, then a relationship between anisotropy and the data

TABLE II

Comparison of anisotropy, rotational strength and dipole-strength of different hemoproteins

	Rotational strength Db•BM	Dipole--strength Db^2	Anisotropy BM/Db
Hb-A (pH 7.0)	4.3	243	$17.6 \cdot 10^{-3}$
Myoglobin (pH 7.0)	3.2	299	$10.8 \cdot 10^{-3}$
Lamprey-Hb	- 1.3	269	$- 4.7 \cdot 10^{-3}$
Cytochrome *P450*	-11.0	340	$-33 \cdot 10^{-3}$

containing structural information can be expected. In structural terms this correlation indicates a broadening of the heme pocket with decreasing rotational strength or anisotropy, respectively. Despite the fact that unlike hemoglobins cytochrome *P450* is in the low spin state, this result supports the notion, which is in agreement with proton magnetic resonance studies of the enzyme[24-26], that *P450* has a relatively open heme pocket.

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DISCUSSION

L. K. Hanson:

The CD spectrum of the high-spin ferrous *P450* is the only CD spectrum which differs in band shape from the corresponding absorption spectrum. Do you have an explanation for this? Does this also hold true for deoxy Hb/Mb?

K. Ruckpaul:

We carried out the CD-measurements of the reduced form of *P450* with an excess of dithionite. It is possible that additional reaction products or dithionite itself may interfere with the spectrum. An analysis with stoichiometric amounts of dithionite is now under way. We have never observed something like this with deoxyHb or deoxyMb.

H. Schleyer:

When one tries to analyze the reduced minus oxidized difference spectra of rat liver microsomes one obtains, after subtraction of the b_5 contribution, an unusually broad »trough«, with two maxima. λ-positions seem to be in agreement with Dr Ruckpaul's CD spectrum for (S=2) Fe^{2+}. We have no explanation for this phenomenon — nor has anybody else to my knowledge.

T. G. Traylor:

It would be helpful to be able to remove the excess dithionite to improve the spectra as you suggest. In principle, because dithionite is not only a reducing agent but a nucleophile, it should be possible to add substances active toward substitution. For example, iodoacetamide reacts with dithionite in the presence of our reduced models and while removing dithionite it leaves the reduced spectrum unchanged. It should be possible to get other reagents which are less reactive toward proteins than iodoacetamide but still react with dithionite.

K. Ruckpaul:

In the case of your model compounds the use of iodoacetamide seems to be an appropriate possibility of removing dithionite from the solution. In the case of a protein solution, however, I would expect additional reactions with functional groups of the proteins which may lead to serious modifications of the native structure and/or function.

SAŽETAK

Cirkularni dikroizam djelomično pročišćenog citokroma *P450* iz mikrosoma jetre zeca

K. Ruckpaul, H. Rein, G.-R. Jänig, W. Winkler i O. Ristau

Vrpce cirkularnog dikroizma hema u solubiliziranom citokromu *P450* iz mikrosoma zečje jetre i nekih njegovih derivata izmjerene su u Soretovu području. Svi derivati tog enzima imaju negativan cirkularni dikroizam, a valne duljine tih vrpci i njihova eliptičnost zavise o ligandu i o stanju željeznog iona. Rezultati se razmatraju u odnosu na CD-karakteristike drugih proteina s obzirom na stereokemiju i fizičko-kemijska svojstva okoline hema. Zaključak o otvorenosti džepa hema iz proučavanja drugim metodama može se dobro korelirati s podacima iz ovih CD-mjerenja.

DEPARTMENT OF BIOCATALYSIS
CENTRAL INSTITUTE OF MOLECULAR BIOLOGY
ACADEMY OF SCIENCES OF THE GDR
1115 BERLIN, GDR

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