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LOW- AND ULTRALOW-TEMPERATURE MAGNETIC CIRCULAR DICHROISM STUDIES OF REDUCED CYTOCHROMES P-450-LM_2 AND P-420-LM_2 AND OF PHOTOPRODUCTS OF THEIR CO-COMPLEXES
THE SPIN-STATE AND AXIAL LIGATION OF HEME IRON

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SUMMARY: MCD spectra of reduced cytochromes P-450 and P-420 have been recorded in the spectral region 350 - 800 nm at temperatures 4.2 - 290 K and were compared with the respective low-temperature photolysed CO-complexes at 4.2 K. The MCD data are consistent with the suggestions that: i) the heme iron is high-spin in the reduced proteins and in the photolysed species; ii) mercaptide is the protein-derived ligand of the heme iron in the reduced cytochrome P-450, as well as in its CO-complex; iii) imidazole of histidine is the fifth ligand of the heme iron both in the reduced P-420 and its CO-complex; iv) structural changes in the heme iron coordination sphere occur at CO-binding.

The catalytic peculiarity of cytochrome P-450 consists in its capability to activate molecular oxygen and after splitting to transfer one oxygen atom on an appropriate substrate. These properties are decisively determined by the axial heme iron ligands. Despite efforts to characterize the axial ligands only the proximal heme iron ligand has been identified as sulfur /1-3/. It has remained unclear which ligand occupies the 5. coordination position at conversion of P-450 to the enzymatically inactive P-420 as yet. Two possibilities are discussed: the protonation of the

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ABBREVIATIONS: cytochrome P-450-LM_2 = P-450; cytochrome P-420-LM_2 = P-420; magnetic circular dichroism = MCD.

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mercaptide to a mercaptan group or the displacement of the thiol-group by an imidazole group of a histidine concomitant with the conversion of P-450 to P-420 /4/. Studies of such kind are complicated in so far as P-420 is not a precisely defined state of the denatured P-450 /5-7/ but dependent on the reagent used. Moreover there is no experimentally proved evidence about possible changes of the 5th ligand at CO binding neither in P-450 nor in P-420. MCD measurements at low and ultralow temperatures have proved an appropriate tool to determine the active site structure of hemoproteins (e.g. spin state, axial ligands /8,9/) and to detect possible nonequilibrium state(s) /9,10/.

The results in the present paper are consistent with the suggestions (i) that the heme iron is high spin in the reduced proteins (P-450 and P-420) and in the corresponding photolysed species, (ii) that mercaptide is the protein derived ligand of the heme iron in the reduced P-450 and in its CO-complex, (iii) that imidazole of histidine is the fifth ligand of the heme iron both in the reduced P-420 and its CO-complex, (iv) that structural changes in the heme iron coordination sphere occur at CO binding. These structural changes were detected as differences in the MCD spectra between reduced P-450 and P-420 in the equilibrium (reduced by dithionite at 293 K, registered at 4.2 K) and in the nonequilibrium state (photolysed CO-complex at 4.2 K).

MATERIALS AND METHODS

Cytochrome P-450-LM_2 was isolated from liver microsomes of rabbits according to the method of Haugen and Coon /11/. The concentration of P-450 was determined by the method of Omura and Sato /12/. The specific content of P-450 amounts to 16.8 nmol heme per mg Protein The protein was solved in 0.1 M phosphate buffer at pH 7.4 in the presence of 20 % (V/V) glycerol. Low and ultralow temperature experiments were performed by adding glycerol in a 1:1 volume ratio to this solution. The oxidized enzyme was reduced by adding few crystals of dithionite ("Merck") at 293 K (equilibrium state). The CO-derivatives were obtained by incubation of the reduced P-450 in a CO-atmosphere during 30 min under occasionally shaking. The reduced and CO-derivative of P-420 were obtained as follows /6/. The CO-complex of P-450 was converted to the nonreactive P-420 form by adding KSCN-crystals to the solution. Titration was followed spectrophotometrically at 420 nm. The reduced P-420 was

obtained from its CO-derivative using irradiation by a Xenon lamp (power 150 W) under bubbling helium gas (equilibrium state). The nonequilibrium forms of the reduced P-450 ($P\text{-}450^{X}_{red}$) and P-420 ($P\text{-}420^{X}_{red}$) were formed by photolysis of their CO-complexes at 4.2 K. The irradiation was carried out during 10-15 min at the Soretband wavelength maximum. After registration of the MCD spectrum a second irradiation of the protein was performed by a xenon lamp (1000 W). The absence of spectral changes after repeated irradiation evidenced complete photodissocitaion of the corresponding CO-complex. The MCD spectra were registered on a magnetic dichrograph, equipped with a cryostat allowing measurements from room temperature to 2 K. Further details of the application of this method are described in /13/. Absorption spectra were registered on a "Beckman 26" spectrophotometer. For the absorption and MCD-spectra the same cuvette was used with an optical path length of 1.0 mm. The magnetic field strength equals 1.1 - 1.2 T. The MCD values $\Delta\mathcal{E}/H$ were related to a field strength of 1T = 10 000 Gauss and are given in units ($M^{-1}cm^{-1}T^{-1}$). Data processing was performed on a "Hewlett-Packard" HP-9830 computer.

RESULTS AND DISCUSSION

The MCD spectra of the reduced cytochromes P-450 and P-420 have been recorded in the temperature interval from room temperature (293 K) to 4.2 K. For illustration the MCD spectra of P-450 and P-420 at 290 K, 77 K and 4.2 K in the Soret region and at 4.2 K in the visible region are shown in figures 1 and 2. The spectra are extremly temperature dependent. On lowering the temperature from 290 K to 4.2 K the Soret MCD intensity of reduced P-450 shows a 6-fold and that of reduced P-420 a 60-fold increase. The strong dependence of MCD on temperature is typical only for paramagnetic molecules and can be used to distinguish paramagnetic complexes from diamagnetic ones /14/. Indeed, in contrast to the diamagnetic heme proteins all paramagnetic derivatives studied exhibit temperature-dependent MCD /8,15,16/. Based on magnetic susceptibility and Mössbauer investigation /17,18/ the paramagnetic high-spin (S=2) behaviour of reduced cytochrome $P\text{-}450_{cam}$ has been demonstrated previously. So far the magnetic state of reduced cytochrome P-420 has not been proved by a suitable physical method. The large temperature dependence of its MCD clearly evidences that the heme of this cytochrome is also paramagnetic,

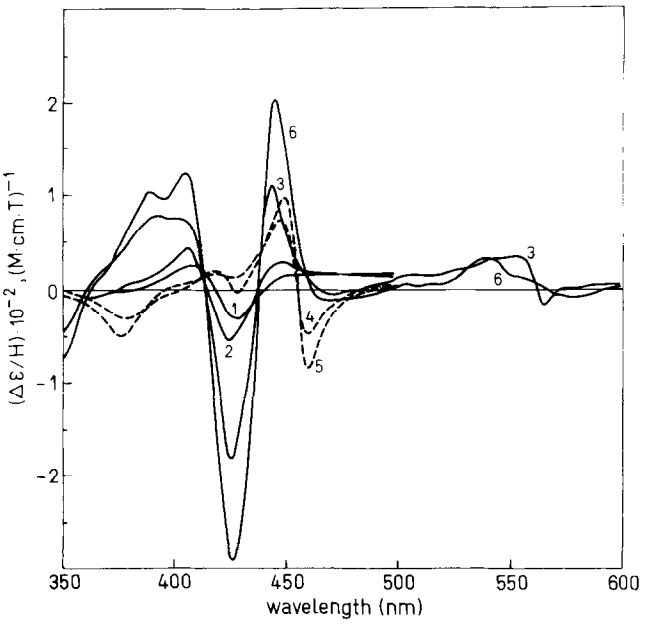

Fig. 1. MCD-spectra of the P-450-CO-complex, its photolysis product and of the reduced enzyme:

1- $P\text{-}450_{red}$ at 290 K; 2- $P\text{-}450_{red}$ at 77 K;
3- $P\text{-}450_{red}$ at 4.2 K; 4- P-450-CO at 290 K;
5- P-450-CO at 77 K, 6- $P\text{-}450^{x}_{red}$ (photolysis product of P-450-CO) at 4.2 K.

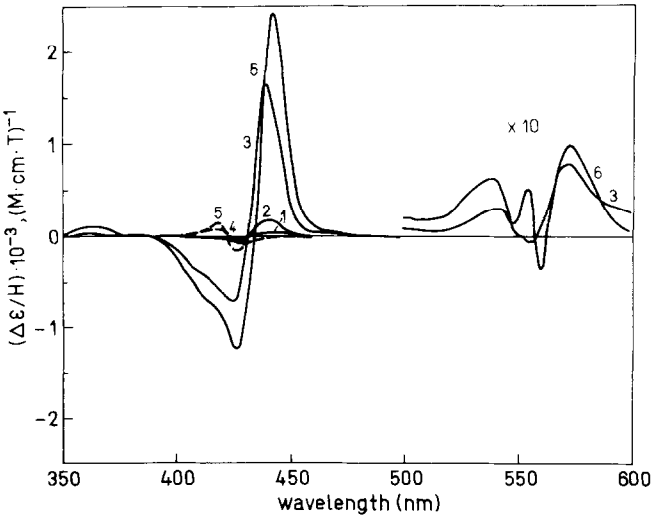

Fig. 2. MCD-spectra of the P-420-CO-complex, its photolysis product and of the reduced enzyme:

1- $P\text{-}420_{red}$ at 290 K; 2- $P\text{-}420_{red}$ at 77 K;
3- $P\text{-}420_{red}$ at 4.2 K; 4- P-420-CO at 290 K;
5- P-420-CO at 77 K; 6- $P\text{-}420^{x}_{red}$ (photolysis product of P-420-CO) at 4.2 K.

being presumably in the high-spin form, the only known paramagnetic form of ferrous heme proteins.

Figures 1 and 2 display the MCD spectra of CO-complexes of P-450 and P-420 as well as the spectra obtained after photolysis of these complexes at 4.2 K. Repeated photolysis did not produce any further changes in the MCD, indicating complete photodissociation. In accordance with diamagnetism of the CO-complexes their MCD spectra were temperature-independent below 77 K. The small changes observed between 290 K and 77 K could be ascribed to band narrowing in this temperature region. MCD spectra of reduced P-450 and P-420 and of their CO-complexes at room temperature (Figs. 1 and 2) are in agreement with those reported by Dawson et al. /19/.

Except differences in the intensity the similarity of the Soret MCD spectra of reduced proteins with those of the corresponding photoproduct is striking. Taking into account the extreme sensitivity and selectivity of MCD to the redox - and spin - states as well as to axial ligation of the heme iron /20/ the observed similarity strongly indicates the identity of the spin state and of the 5th axial ligand in the reduced and photodissociated species of a given protein. The paramagnetism of the photoproducts is further supported by the strong temperature dependence of their MCD in the temperature region where rebinding of CO-ligand did not occur. Due to paramagnetism of reduced and photolysed species their MCD have to be compared at the same temperature.

At the temperature of photolysis (4.2 K) the protein conformation is frozen and cannot change after displacement of the CO-ligand by light. Therefore the protein-derived ligand remains unchanged. In the CO-complex of reduced P-450 the protein-derived (5th) ligand of the heme iron has been shown to be mercaptide, presumably originating from a cystein /4,21-23/. Hence in the reduced P-450 the fifth heme iron ligand should be a thiolate anion, too.

In P-420 there is no reliable evidence as yet for the nature of the protein-derived ligand neither in the reduced state nor in the CO-complex, although imidazole of histidine or mercaptan of cysteine have been considered as the most likely candidates in the CO-complex /4,19/. The MCD data indicate that imidazole rather than mercaptan occupies the fifth position of the heme iron. The Soret-MCD of reduced and photolysed species of P-420 displays sign, shape and temperature behaviour which are typical for high-spin

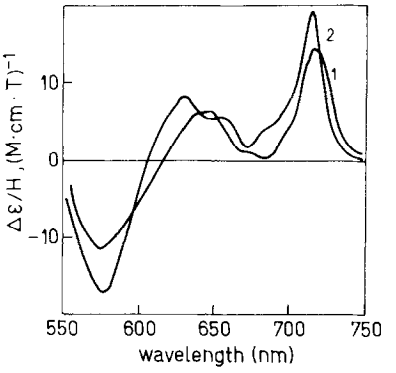

Fig. 3. MCD-spectra of the photolysis product of the P-420-CO-complex and the myoglobin-O_2-complex in the near infrared:
1- $P\text{-}420^{x}_{red}$ (photolysis product of P-420-CO) at 6 K;
2- Mb^{x}_{red} (photolysis product of Mb-O_2) at 15 K /9/.

ferrous heme proteins where histidine is known to be the axial ligand (deoxymyoglobin, deoxyhemoglobin, reduced horseradish peroxidase and reduced heme a_3 of cytochrome c oxidase) /10,24/.

To prove the validity of imidazole being the proximal axial heme iron ligand the MCD spectrum of the photolysed CO-complex of P-420 was recorded in the red and near infrared region. The result is shown in Fig. 3 together with the MCD of the photolysis product of oxymyoglobin for comparison (taken from Fig. 3 of /9/. High-spin ferrous heme proteins with histidine in the fifth position of the heme iron show characteristic MCD bands in this spectral region, a negative band at ca. 630 nm and positive ones at ca. 680 nm and ca. 760 nm /9/. The bands have been assigned by Seno et al. /25/ to iron-porphyrin and iron d - d transitions. Because the antibonding orbitals of iron are involved in the transitions the exact location of the two last bands is extremely dependent on the mode of bonding of the axial-iron ligand. The bands located at ca. 680 nm and ca. 760 nm show a red-shift up to 10-15 nm in the nonequilibrium protein conformation produced by photolysis of liganded (CO or O_2) ferrous forms of myoglobin, hemoglobin and horse radish peroxidase as compared to their positions in the equilibrium unliganded ferrous conformations /9,26/, although the protein-derived fifth heme iron ligand remains unchanged. Substitution of imidazole for another axial ligand is expected to be accompanied by more drastic alterations in the near infrared MCD. The similarity of the two MCD spectra shown in Fig. 3 gives strong evidence that imidazole is the protein-

derived 5th ligand in the CO-complex of P-420 and presumably in the reduced protein.

In contrast to the Soret band the visible MCD spectra of reduced (equilibrium state) and photodissociated (nonequilibrium state) species of a given cytochrome differ significantly (Figs. 1 and 2). The only difference between the photoproduct and the reduced protein is that in the first the protein conformation cannot relax after removal of the CO-ligand and corresponds to the conformation of the liganded (CO-bound) state. Therefore the MCD differences in the visible region together with variations of the Soret-MCD-intensity indicate a rearrangement in the coordination sphere of heme iron to occur at CO binding. The higher sensitivity of the MCD in the visible region to structural changes as compared with the Soret band is common to paramagnetic heme proteins /9,15,16/ and has been explained by the borrowing of intensity by the weak Q-band from the very strong B-band through interaction of π-electrons and d_{π}-electrons of the iron /16/.

Nonequilibrium conformational states in reduced P-450 were recently detected at reduction of the enzyme at 77 K /27/. Nonequilibrium conformational states of reduced P-450 (and P-420) could further originate at photolysis of the corresponding CO-complexes at 4.2 K as discussed above. The existence of these states in the reduced P-450 may explain differences in the CO-binding kinetics performed by means of stopped-flow and flash-photolysis techniques /28/.

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