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MERCAPTIDE-ANION AS A TRANS-LIGAND IN OXYCYTOCHROME P450

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Summary. Oxygenation of heme-mercaptide as well as spectroscopic characteristics of the dioxygen complex formed have been studied. Absorption and magnetic circular dichroism spectra of the O_2 complex support the retention of mercaptide in the heme fifth position. A release of O_2^- in the decomposition of the oxygenated complex and an independent formation of the latter from hemine-dimercaptide and O_2^- together with electron paramagnetic resonance and Mössbauer data support the O_2 presence in the heme coordination sphere. The similarity of optical and magnetic circular dichroism spectra and the closeness of the K_{CO}/K_{O_2} ratio for oxy-heme-mercaptide and oxycytochrome P450 unequivocally confirm the presence of an axial cystein mercaptide ligand in oxycytochrome P450.

Cytochrome P450 is an enzyme catalyzing a monooxygenation reaction (Fig.1) in tissues of aerobic organisms (1). Ferroprotoporphyrin IX (FeP), or heme, the prosthetic group of P450 serves as a binding locus for O_2 in analogy with hemoglobin (Hb) and mioglobin (Mb) (2). However, while Hb and Mb only bind and release O_2, P450 activates it and transfers an oxygen atom to the substrate molecule. Such behaviour is evidently caused by a difference between axial ligands (L_1) in the fifth coordination site of iron in P450 and Hb (Mb) (Fig.1).

It has been long known (2) that imidazole (Im) of histidine is the fifth axial ligand in Hb (Mb). Using physical techniques it has been established that L_1 is a cystein mercaptide (RS^-)

Abbreviations: FeP ferroprotoporphyrin IX, DMF dimethylformamide, DMSO dimethylsulfoxide, BuS^- n-butyl mercaptide, RS^- cystein mercaptide, Hb hemoglobin, Mb mioglobin, P450 cytochrom MCD magnetic circular dichroism, EPR electron paramagnetic resonance.

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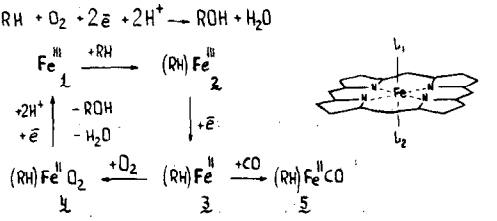


Fig.1. A scheme of monooxygenase reaction of cyt. P450 (indicated is only the change in the iron oxidation state), to the right - iron coordination environment in the active centre of cyt. P450.

(1,3) in states 1, 2, 3 and 5 of cyt. P 450 (Fig.1). These conclusions are confirmed by receiving the respective model complexes (4). Mercaptide is assumed to be a fifth ligand in state 4 as well (Fig.1). However, the physical techniques for oxycytochrome P450 led to a contradiction: some data reported Im as a 5-th ligand (5), the others - RS^- (6).

The aim of the present work was to obtain the dioxygen complex of heme-mercaptide and to compare it with oxycytochrome P450.

Materials and methods. All the operations were carried out in vacuum or under dry argon. Sodium n-butyl mercaptide (BuSNa) was obtained by dissolving Na wire in mercaptan diluted by toluene. Protohemin IX was twice recrystallized. KO_2 was obtained by reaction of O_2 with K in liquid NH_3. Dimethylformamide (DMF) prior to use was double-distilled in vacuum at first over potash. Toluene was double-distilled over potassium. Electron spectra were recorded on "Specord UV-VIS" spectrophotometer (GDR) equipped with thermostated cell compartment. EPR spectra were recorded on "Varian E-104A" spectrometer (USA) at 77 K. MCD spectra at -60°C were determined on a dichrograph developed at the Institute of Molecular Biology of the USSR Academy of Sciences (7) Mössbauer spectra were recorded on an electrodynamic-type spectrometer (Hungary) in the regime of constant acceleration with sodium nitro-prusside as a reference material at 77K, the source of γ-radiation being ^{57}Co. The O_2 and CO binding constants were found from spectrophotometric measurements of $P_{1/2}$ (the pressure required to convert 50% of the heme to the respective complex) and chromatographically determined solubility of these gases in DMF.

Results. On the addition of 200-fold excess of BuSNa to 1.10^{-4}M protohemin in DMF at -50°C an optical spectrum of six-coordina-

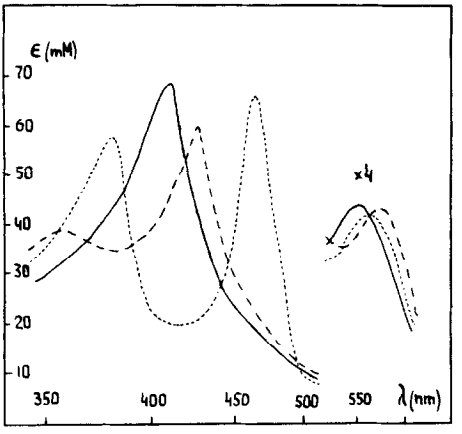


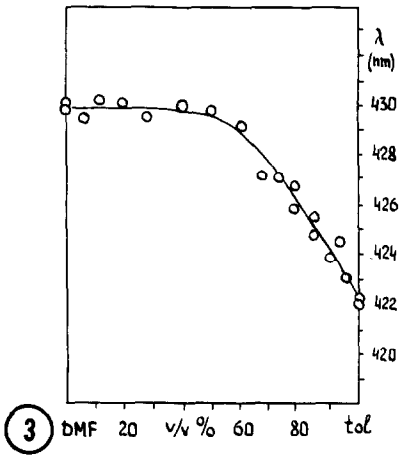
Fig.2. Absorption spectra of heme-mercaptide complexes: Fe^{II}(—), $Fe^{II}O_2$ (---), $Fe^{II}CO$(...), DMF, -50°C.

ted dimercaptide complex, $Fe^{III}P(BuS^-)_2$ (8-10) was observed. At heating to the room temperature mercaptide reduces iron to give a five-coordinated (4) heme-mercaptide complex, $Fe^{II}P(BuS^-)$, with λ_{max} 412 nm. To check up the ability of this system to serve as a spectral model of cytochrome P450 the CO binding was studied. The obtained values of λ_{max} and K_{CO} were in agreement with literature data for the model described earlier and P450 but were different from the data for Hb (Table).

Oxygenation of the heme-mercaptide complex in DMF at -50°C by dioxygen or air resulted in Soret band shifting from 412 nm to 430 nm and in a new band appearing in the region of 360 nm (Fig.2). A carbonyl complex formation was observed after O_2 evacuation and CO addition, which means that O_2 binding was reversible:

$$Fe^{II}P(BuS^-) + O_2 \rightleftharpoons Fe^{II}P(BuS^-)(O_2) \xrightarrow[-O_2]{+CO} Fe^{II}P(BuS)(CO)$$

A freshly prepared oxygen complex produces no EPR. The value of Soret λ_{max} depends on medium polarity and in toluene (ε = =2.38) used instead of DMF (ε =36.7) it falles from 430 nm to



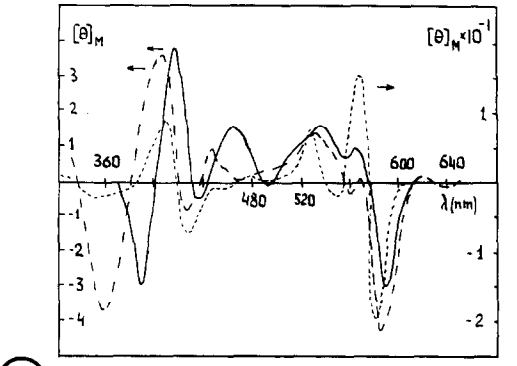


Fig.3. The relation of λ_{max} Soret from solvent polarity for $Fe^{II}P(BuS^-)O_2$, -50°C.

Fig.4. MCD spectra of oxy-heme complexes: $P450.O_2$(---), $Mb.O_2$(...) (6) and $Fe^{II}P(BuS^-)O_2$(—) (this work).

422 nm (Fig.3), the value close to λ_{max} for different oxycytochromes P450: 418 nm (11), 420 nm (12), 422 nm (13).

MCD spectra of $FeP(BuS^-)O_2$, $P-450-O_2$ and $Mb-O_2$ (if dependence on the medium is take into account) revealed a greater similarity of the first two spectra in comparison with the third one (Fig.4).

The obtained complex has Mössbauer parameters (mm/s: δ =0,56, ΔE_Q =1,75) close to the respective values for HbO_2 and $P450-O_2$ (14).

Heme-mercaptide also bound O_2 at 20°C. However, the complex produced was unstable under these conditions and was reduced to the initial complex by mercaptide excess.

The stability of the dioxygen complex increases with an increase of solvent polarity. While in toluene at 20°C the complex failed to be fixed, in DMF the period of half-conversion (determined by the disappearance of the band at 430 nm) was

equal to 150 s, and in the mixture of DMF-DMCO (1:9) it increased up to 200 s.

We investigated the decomposition of the model complex in DMF at -30°C by means of spectral analysis. In the visible region there was observed the appearance of absorption bands of Fe^{III}-$P(BuS^-)_2$ and the disappearance of the 430 nm band of the dioxygen complex. The EPR spectrum simultaneously with the signals of low-spin iron (g_1=2.30, g_2=2.22, g_3=1.96) displayed the signals of about the same intensity characteristic of O_2^- (g_1= =2.01, $g_{||}$ =2.11). The replacement of O_2^- by BuS^- was enhanced on addition of proton solvents (water, alcohols). This fact could elucidate the reason for $Fe^{III}P(BuS^-)_2$ formation in (9) instead of $Fe^{II}P(BuS^-)(O_2)$, since the authors (9) have used the mixture of dimethyl acetamide with water. On addition of the solution of KO_2/18-crown-6 in DMF to the solution of $Fe^{III}P$-$(BuS^-)_2$ at -30°C a reverse reaction of BuS^- replacement by O_2^- was observed to yield the oxygen complex, i.e. the process is reversible:

$$Fe^{II}P(BuS^-)(O_2) + BuS^- \underset{}{\overset{K}{\rightleftharpoons}} Fe^{III}P(BuS^-)_2 + O_2^- \quad K=\frac{K_{O_2^-}}{K_{BuS^-}}=0.13 \ (-30^\circ)$$

Discussion. Optical, MCD, EPR and Mössbauer spectra as well as the data on the formation and decomposition - all support the identification of the adduct obtained as a reversible dioxygen complex of heme-mercaptide. The optical (Table) and MCD (Fig.4) spectra which are sensitive to the nature of the fifth ligand are essentially different from the respective spectra of imidazole heme complexes. The observation of the splitted Soret and unresolved α β bands in the optical spectrum and the great negative ellipticity of the MCD spectrum in the Soret region for the dioxygen complex are the confirmation for the retention of

Table

Absorption band maxima and binding constants for the cytochrome P450, Hb and model complexes

System	T°C	L_1	L_2	λ max, nm		K_{L_2}, M^{-1}	$\frac{K_{CO}}{K_{O_2}}$
				Soret	Visible		
$FePL_1L_2$, DMF	-50	BuS^-	CO	384 465	558	$1.3.10^5$	3.5
			O_2	356 430	566	$3.7.10^4$	
P450, pH 7 (11)	0	RS^-	CO	365 450	550	$6.6.10^5$	3.0
			O_2	350 420	555	$2.2.10^5$	
Hb, pH 7.3 (16)	20	Im	CO	419	540 569	7.10^8	2000
			O_2	415	541 577	$1-6.10^5$	
$FePL_1L_2$, DMF/H_2O (16)	22	Im	CO	420	540 569	6.10^8	1200
			O_2	414	543 575	5.10^5	

BuS^- in oxygenation. Like the other FeO_2 complexes the adduct does not display any EPR signal in contrast to Fe(III)P.

The Mössbauer spectra are insensitive to the nature of the fifth ligand and bear much resemblance for $P450.O_2$ and HbO_2 (14). The Mössbauer spectrum of the adduct obtained is characteristic of O_2-heme complexes (15). The release of O_2^- in decomposition of the dioxygen adduct and an independent formation of the latter from $Fe^{III}P(BuS^-)_2$ and O_2^- testifies for the O_2 presence in the heme coordination sphere:

$$Fe^{II}P(BuS^-) + O_2 \rightleftharpoons Fe^{II}P(BuS^-)(O_2) \leftrightarrow$$
$$\leftrightarrow Fe^{III}P(BuS^-)(O_2^-) \rightleftharpoons Fe^{III}P(BuS^-) + O_2^-$$

The essential difference between P450 and Hb(Mb) is the difference in the relative affinity to O_2 and CO, which is determined by the ratio of K_{CO}/K_{O_2} (Table). Hb (Mb) and their models

bind CO stronger than O_2, while for cytochrome the binding of CO and O_2 is approximately of the same strength.

Besides, the spectra of oxy- and carboxy-Hb(Mb) as well as those of the respective model heme-imidazole complexes are characterized by the presence of well-resolved α and β bands in the region of 540-580 nm, whereas in the spectra of O_2 and CO-complexes of P450 and of $FeP(BuS^-)(O_2)$ (the present work) and $FeP(BuS^-)(CO)$ (4) these bands fuse into one, with a splitted band being observed in the Soret region (hyperspectrum) (Table).

The similarity of optical and MCD spectra of O_2 complexes and closeness of the ratio of K_{CO}/K_{O_2} for $FeP(BuS^-)$ and cytochrome P450 support unequivocally the presence of an axial cysteine mercaptide ligand in oxycytochrome P450.

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