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Conference Paper

The Structure and Mechanism of Cytochrome *P450**

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The unusual hemoprotein called cytochrome *P450* is now recognized as representing a variety of monooxygenases with entirely different substrate specificities. In comparison with heme-mercaptide models it can be concluded that the unusual spectral properties reside in a heme-mercaptide linkage to the protein. The sixth ligand in the ferric form could be a hydroxyl group which is absent in the enzyme-substrate complex. In the reaction cycle the enzyme-substrate complex is reduced and then reacts with dioxygen to form an oxy-complex. Further reduction is believed to yield an »oxenoid« complex of the structure $[FeO]^{3+}$, which transfers the oxygen atom to the substrate.

INTRODUCTION

The unusual hemoprotein called cytochrome *P450*[1] is now known to represent a rather heterogenous group of enzymes catalyzing the monooxygenation of a variety of structurally unrelated compounds. It occurs in bacteria, yeasts, plants, and in many tissues of higher organisms. Without doubt these hemoproteins are different enzymes with different substrate specificities and it is misleading to call them cytochromes. These heme-containing monooxygenases can accept electrons like cytochromes but transfer them to molecular oxygen, which is thereby activated and one oxygen atom is introduced into a substrate molecule. Since the heme represents the catalytic center of these monooxygenases, the unusual spectral properties of cytochrome *P450* seem to be intimately related to its function as a monooxygenase. Indeed, the conversion to cytochrome *P420* by detergents or acid treatment result in both the loss of the unusual spectra and of the monooxygenase activity.

All attempts to elucidate the mechanism of the monooxygenation reaction have to start with an investigation of the coordination sphere of the heme iron. Studies with the isolated hemoproteins have been only partially successful, but recently the nature of cytochrome *P450* enzymes has been clarified considerably by the synthesis of model systems. This paper reports the ligand spectra of heme-sulfur complexes and summarizes our current view on the mechanism of cytochrome *P450*-dependent monooxygenases.

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* Based on the lectures delivered by V. U. and H. H. R. at the end of the Summer School on Membrane-Bound Enzymes held from 2—6. October, 1976, in Primošten — Yugoslavia. (The discussion was not recorded.)

The Properties of Oxidized Cytochrome P450 Enzymes

Irrespective of the source of cytochrome *P450*, its spectral properties in the oxidized state are surprisingly similar. The Soret band around 418 nm and the α and β bands at 570 and 540 nm, respectively, are indicative of a low spin hemeprotein. This is confirmed by the ESR spectrum which shows g values at around 2.4, 2.25 and 1.91[2], and a little anisotropy of the g tensor compared to other low spin ferric hemoproteins. The first model heme complexes that exhibited similar ESR characteristics were reported by Röder *et al.*[3] and were obtained by adding thiols to heme compounds. This suggested the presence of a sulfhydryl or mercaptide ligand in the coordination sphere of cytochrome *P450*, and the low redox potential of around — 400 mV would be in agreement with this assumption. It could also explain the rapid disappearance of the characteristic spectra after oxidation, treatment with sulfhydryl reagents or after denaturation, which resulted in normal heme spectra.

Experiments with the pyridine derivative metyrapone showed that hemochrome-type spectra were obtained when stoichiometric amounts of this potential ligand were added to oxidized cytochrome *P450*[4]. The Soret absorption shifted to a longer wavelength, but the anisotropy of the ESR spectrum increased only slightly. Obviously, the sulfur ligand had remained and the sixth ligand position was occupied by a pyridine nitrogen.

This hypothesis prompted us to test other lipophilic organic compounds with heteroatoms which function as ligands. Thioethers[5] gave very similar results as nitrogenous bases, but thiols and phosphines behaved quite differently[6,7]. In the case of thiol and phosphine ligands a splitting of the Soret band was observed resulting in a far red-shifted band at around 460—470 nm and a band below 400 nm.

The absolute spectra obtained after addition of α-toluenethiol and diethylphenylphosphine to oxidized cytochrome *P450*CAM* are shown in Figures 1 and 2.

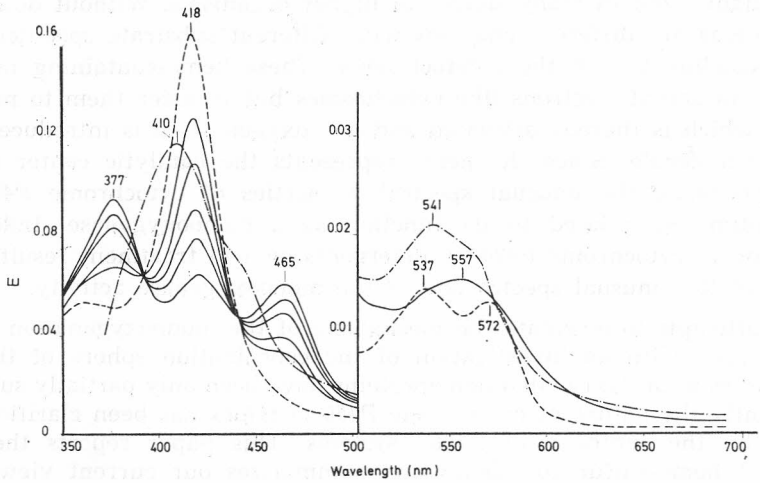


Figure 1. Ligand spectrum of cytochrome *P450*CAM with α-toluenethiol. To oxidized cytochrome *P450*CAM (dashed line) increasing concentrations (10^{-5} — 3×10^{-4} M of α-toluenethiol were added in methanol (solid lines). After addition of sodium dithionite the dashed-dotted line was recorded.

* We thank Dr. J. A. Peterson for a generous gift of purified cytochrome *P450* from *P. putida*.

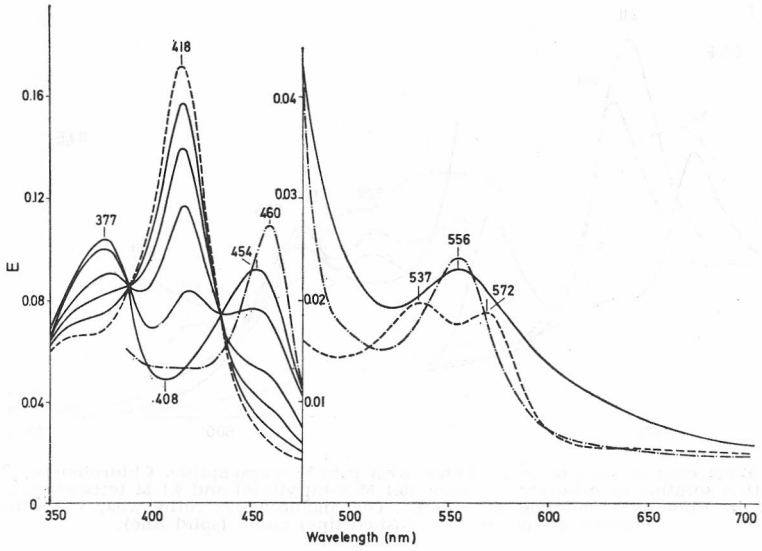


Figure 2. Ligand spectrum of cytochrome *P450*CAM with diethylphenylphosphine. To oxidized cytochrome *P450*CAM (dashed line) increasing concentrations (0.4, 1.0, 2.3, 7.3 and 150 μM of diethylphosphine were added in methanol (solid lines). Subsequent addition of sodium dithionite yielded the dashed-dotted spectrum.

The phosphine, thiol, tioether and nitrogen ligands showed competitive binding with substrates, and all could undergo ligand exchange reactions, indicating that in each case the heteroatom had occupied the sixth ligand position. This agreed with the ESR spectra, all of which showed low spin ferric heme with only moderate shifts in the g values.

The unusual optical properties of the phosphine and thiol ligand spectra challenged us to look for appropriate models. However, it soon turned out that hemin complexes in the presence of alkyl mercaptides and phosphines were always easily reduced to the ferrous state at room temperature. When the components were mixed together below — 40 °C this reduction was quenched. With a specially designed cuvette it was possible to record the optical and ESR spectra from the same sample at a low temperature.

When a Fe(III)-protoporphyrin complex was titrated with a solution of *n*-butyl mercaptide at — 60°C, a spectrum very similar to that of the cytochrome *P450*-mercaptide complex appeared and was fully developed at a greater than 2-fold excess of the ligand (Figure 3a). The corresponding ESR spectrum completely showed a low spin heme with the characteristic narrow splitting of the g values (Figure 3b). Under anaerobic conditions the same results were obtained. This model of a hemindimercaptide complex is in agreement with our interpretation of the cytochrome *P450*-thiol spectrum with the second mercaptide at the fifth coordination position being derived from a cysteinyl residue of the protein[6].

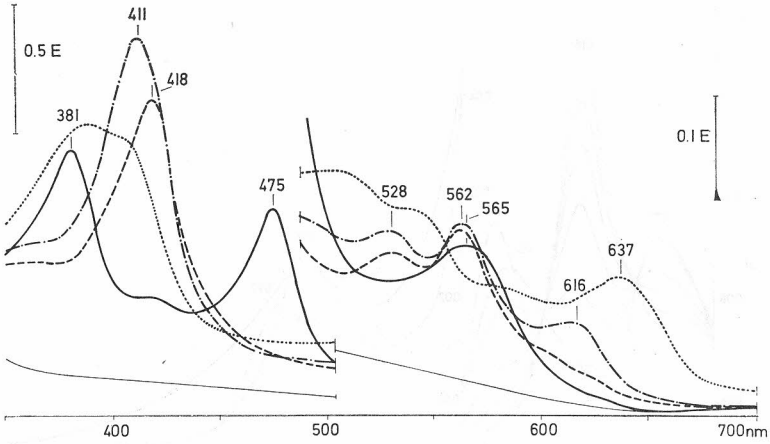


Figure 3a. Electronic spectra of ferric heme with *n*-butylmeracaptide. Chlorohemin (2 mM) was titrated with a solution of *n*-butylmercaptide (0.1 M *n*-butylthiol and 0.1 M tetramethylammonium hydroxide) in dimethylformamide at —60 °C. The ligand/heme ratio was: 0 (dotted line), 1 (dashed-dotted line), 2 (dashed line) and 5 (solid line).

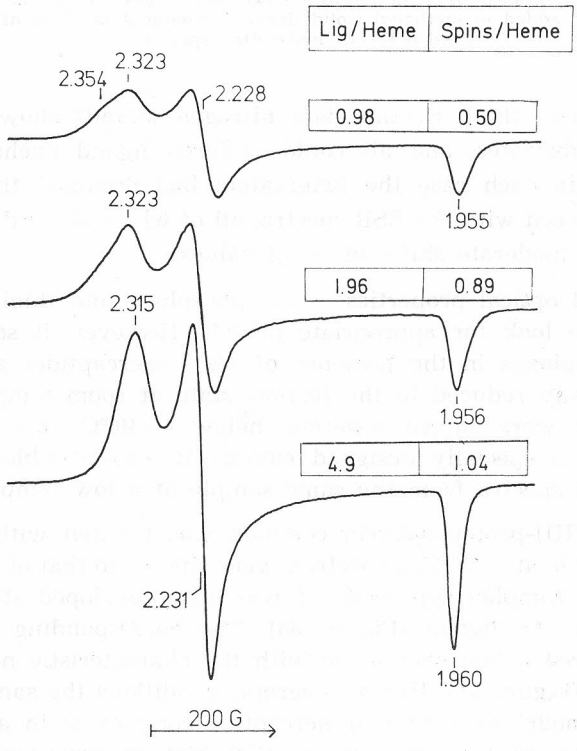


Figure 3b. ESR spectra of ferric heme with *n*-butylmercaptide. Conditions were as described in Figure 3a. ESR spectra were recorded at 100 K with a microwave power of 20 mW. The numbers indicate g values. The spin concentration was determined by double integration with a 1 mM Cu(II)-EDTA reference solution.

Chang and Dolphin[8] had obtained an almost identical spectrum after the addition of dioxygen to a ferrous heme-mercaptide complex and discussed it as the formation of an oxy-complex. According to our own results we suggest an alternative explanation, namely that the ferrous heme-mercaptide complex became oxidized by oxygen and subsequently formed the ferric heme complexed by two mercaptides, which showed the observed spectrum. This species remained stable until the addition of carbon monoxide or pyridine induced the reduction again.

Starting out with a hemin-dimercaptide complex, it was also possible to obtain a model from a ferric cytochrome *P450* phosphine complex by a ligand exchange reaction.

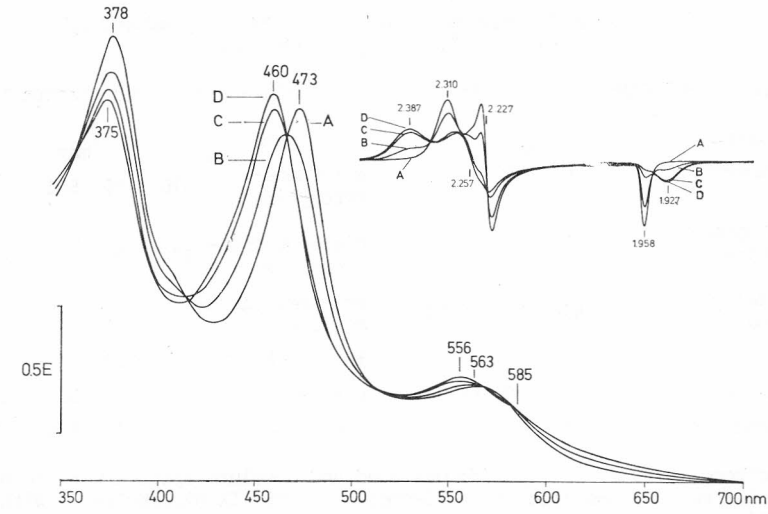


Figure 4. Electronic and ESR spectra (insert) of dimercaptide-hemin complexes. Spectrum A was recorded of a sample containing 0.8 mM Fe(III)-protoporhyrin IX dimethylester chloride, 13 mM sodium *n*-butylmercaptide and 6 mM dibenzo-18-crown-6 in methylene chloride. Spectra B, C and D were recorded after the addition of 1.6, 8 and 14 mM diethylphenylphosphine, respectively. The mixing temperature was —78 °C, the optical pathlength about 0.1 mM. The ESR-spectra were recorded as described in Figure 3b.

The typical spectrum with the Soret absorption at 460 nm and the narrow g tensor was obtained in a mixture of a mercaptide and a phosphine, but not with the phosphine alone. This also proved the interpretation of the cytochrome *P450*-phosphine spectrum in Figure 2 as a low spin ferric heme--mercaptide-phosphine complex. (H. H. Ruf, P. Wende and V. Ullrich, in preparation.)

Porphyrin spectra of this anomalous type, with a red-shifted Soret band and an intense band in the near uv, had been termed »hyperporphyrin« spectra[9]. The carbon monoxide complex of ferrous cytochrome *P450* also exhibits such a spectrum with bands at 363 and 446 nm. A theoretical interpretation was given by Hanson *et al.*[10] based on the assumption of a mercaptide ligand, which was supported by models of ferrous heme mercaptide com-

plexes with CO as the sixth ligand[11] (see also Figure 5). Using an extended Hückel calculation Hanson *et al.*[10] showed that the split Soret band is due to a charge transfer interaction of a lone-pair p^+-orbital of a mercaptide sulfur with the e_g (π^*) porphyrin orbital.

This interpretation also seems attractive for the spectra of our ferric heme-dimercaptide and ferric heme-mercaptide-phosphine complexes. In contrast, a second group of ligands, like amines and thioethers, produce normal porphyrin spectra with Soret bands between 425 and 435 nm. Interestingly, the hydroxyl group of ethanol yields a Soret band at 416 nm (Table I).

TABLE I

*Optical absorption maxima of cytochrome P450-ligand and of mercaptide-hemin ligand complexes**

cytochrome *P450*CAM-ligand spectra				mercaptide-heme-ligand spectra			
ligand	λ_{max}			ligand	λ_{max}		
α-toluenethiol	377	465	557	α-toluene-mercaptide	376	470	561
diethylphenyl-phosphine	377	454	556	diethylphenyl-phosphine	374	458	556 (585)
octylmethyl-sulfide		426	537 (580)	pentamethylene-sulfide		432	533 578
				imidazole		428	538 568
metyrapone[4]		421	536 (570)	pyridine		424	536 564
endogenous (?)		418	537 572	ethanol		416	534 559

*Cytochrome *P450* from *P. putida* was used and absolute spectra were recorded. The models consisted of FeIII-protoporphyrin IX dimethylester with the ligands added in organic solvents.

The ethanol-induced ligand spectrum is of relevance for our knowledge of the sixth coordination position in ferric low spin cytochrome *P450*. Since the Soret band of this form absorbs between 416 and 418 nm, depending on the source of enzyme, it seems very likely that, in addition to a mercaptide in the fifth position, a hydroxyl group is present at the sixth coordination positon near the substrate binding site.

There are now sufficient data indicating that the oxidized low spin state of the cytochrome *P450* enzymes represents the resting state. In the presence of their typical substrates the spectral characteristics change dramatically and a conversion to a high spin complex is observed[12]. The g values are at around 8, 4 and 2, indicating a highly rhombic coordination sphere, and the Soret band lies at 390 nm with an α band at 645 nm. Model heme complexes for this enzyme substrate complex have been reported[13] and clearly show that this high spin somplex is five-coordinated with a mercaptide ligand.

Hence, the sixth ligand must have been removed by the binding of the substrate to the active site. Since this binding process in some cases involves rather weak interactions, it must be assumed that the sixth ligand is not strongly bonded due to its chemical nature or to a strained configuration induced by the protein. Again, a hydroxyl group would fulfill the requirement for a weakly bonded ligand.

The Properties of Reduced Cytochrome P450 Enzymes

When a cytochrome *P450* monooxygenase has bound its substrate and is present in its high spin form, its redox potential is increased to about —160 mV[14]. This facilitates reduction by the reduced pyridine nucleotides which requires, however, an electron transport chain. In bacterial and mitochondrial systems a flavoprotein and an iron-sulfur protein are necessary, whereas in microsomal monooxygenases a NADPH-dependent flavoprotein serves as the electron transmitter. Sodium dithionite is usually applied as an artificial reductant. For the pure and homogenous cytochrome *P450* from camphor grown bacteria, clearly only one electron is required[15], but two electrons have been found necessary to completely reduce a preparation of microsomal cytochromes *P450*[16]. The bacterial cytochrome *P450* is high spin in its ferrous form[17] which leaves some doubt as to whether the mercaptide ligand is still present. The situation certainly is different when carbon monoxide or another strong field ligand occupies the sixth ligand position. In this case hyperporphyrin spectra are obtained, again indicating the participation of the mercaptide ligand. Model systems that simulate the characteristic 450 nm absorption band of the carbon monoxide complex require the presence of high mercaptide concentrations, which can either be achieved with strong bases[18] or with crown ethers in organic solvents as used in the experiment of Figure 5.

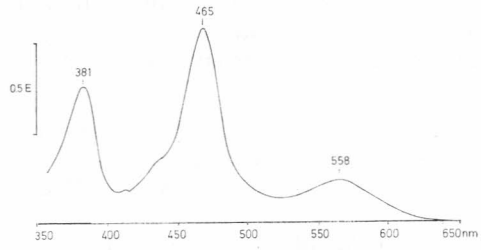


Figure 5. Electronic spectrum of a ferrous heme-mercaptide carbon monoxide complex. Fe(III)-protoporphyrin IX dimethylester chloride was mixed anaerobically under a CO-atmosphere with 0.6 M sodium *n*-butylmercaptide and 0.3 M dibenzo-18-crown-6 in dimethylacetamide at room temperature. The sample was frozen and measured as described in Figure 3. The complex did not show an ESR signal.

That the Soret band of the hemin-mercaptide-CO complex is shifted to a higher wavelength may be explained by the high polarity of the solvent, which influences the charge transfer from the mercaptide to the porphyrin ring.

The Reaction with Dioxygen

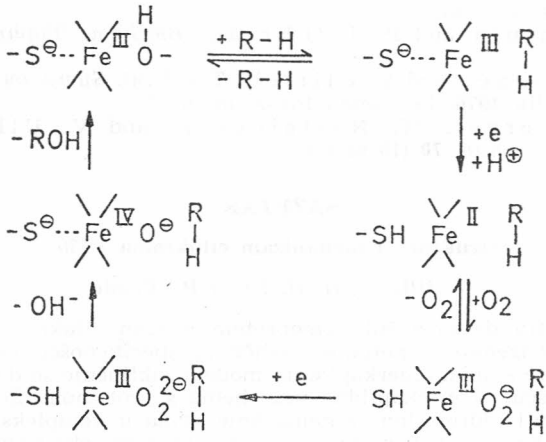
Under physiological conditions the reduced heme complex will immediately react with dioxygen to give an oxy form with a Soret absorption at 418 nm as shown for cytochrome *P450*$_{CAM}$[19]. This is the usual absorption band common to all oxygenated biological heme complexes, like oxyhemoglobin, oxymyoglobin, Compound III of peroxidase or the oxycomplex of tryptophan dioxygenase. It is therefore again unlikely that the mercaptide remains as the fifth ligand of the oxycomplex of cytochrome *P450* enzymes. The oxycomplex is stable in the presence of substrate. Taken together with its known weak oxidizing properties it is unlikely that it represents the active oxygen complex for the monooxygenation process. Transfer of a second electron to the oxygen is supposed to yield an »oxenoid« complex which is responsible for the introduction of an oxygen atom into the substrates. Two structures for the oxenoid complex have been proposed, one containing a peroxide ion (A) and a second in which an oxygen atom is bound to the ferric form of the heme complex (B)[20,21].

$$\underset{\text{A}}{[\text{Fe-O-O}]^{\oplus}} \quad , \quad \underset{\text{B}}{[\text{Fe O}]^{3\oplus}}$$

Recent results on the interaction of cumene hydroperoxide[22], peracids[23] or iodosobenzene[24] with the oxidized cytochrome favor the structure of an $[FeO]^{3+}$ complex as the active oxygen species, since it was possible to achieve monooxygenation reactions in the presence of these oxidants. In the case of iodosobenzene it was obvious that only one oxygen atom could be transferred to cytochrome *P450*. Addition of iodosobenzene to liver microsomes results in a transient Soret absorption at about 438 nm, which was also seen with the other oxidants[23]. Thus, we have tentatively concluded that the oxenoid complex exhibits an absorption band around 440 nm. In view of the high oxidizing potential of the assumed oxenoid complex it seems likely, again, that this complex contains the sulfur as a mercaptide ligand. We speculate that it may have been formed during reduction of the oxycomplex by breaking of the O—O bond and release of a hydroxyl ion, a process in which the thiol group could act as the proton donor (see summarizing scheme).

So far no heme enzyme with hydroxylating properties except cytochrome *P450* has been found. This suggests that sulfur may play an essential role in the formation and stabilization of the active oxygen. It is appealing to consider a mercaptide-iron(III) center by charge transfer as an Fe^{II} compound, which would explain why the O—O bond is unstable after a one-electron reduction of the oxycomplex. A negative charge at the $[FeO]^{3+}$ center would also stabilize the strongly electrophilic oxygen atom in trans position of the mercaptide ligand. It therefore seems to us that this ligand is an essential requirement for the oxygen activation process by iron containing monooxygenases. Because of these essential features of sulfur in these hemoproteins, it would be justified in our opinion to substitute the misleading terminology cytochrome *P450* by the term »heme-sulfur«-containing monooxygenases. Heme-sulfur proteins could be considered as another group of electron transfer proteins, like the iron-sulfur proteins, flavoproteins or cytochromes.

In summary, the mechanism of monooxygenation by heme-sulfur proteins and the concomitant changes in the coordination sphere are presented in the following scheme:



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SAŽETAK

Struktura i mehanizam citokroma *P450*

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Danas se smatra da neobični hemoprotein nazvan citokrom *P450* predstavlja raznovrsne monooksigenaze s potpuno različitom specifičnošću prema supstratima. Uspoređujući spektre s onima merkaptidnih modela zaključuje se da njihove neobične karakteristike potječu od merkaptidne veze hema s proteinom. Šesti ligand u feri-obliku mogla bi biti hidroksilna skupina, koje nema u kompleksu enzima sa supstratom. U reakcijskom ciklusu najprije se reducira kompleks enzima sa supstratom a onda reagira sa molekulom kisika stvarajući oksikompleks. Vjerojatno se u daljnjoj redukciji stvara »oksenoidni« kompleks FeO^{3+}, koji prenosi atom kisika na supstrat.

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