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Synthetic Active Site Analogues of Heme-Thiolate Proteins

Catalysis and Identification of Elusive Intermediates

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Abstract: Intermediates of the catalytic cycles of chloroperoxidase and cytochrome P450cam are identified and characterized by means of synthetic enzyme models.

Keywords: chloroperoxidase · chlorination · cytochrome P450 **.** enzyme model · EPR-ENDOR-spectroscopy **.** heme-thiolate protein · iron porphyrin

Introduction

The *heme-thiolate* proteins comprise a large number of important enzymes such as cytochromes P450 [1], the more recently discovered NO-synthase [2] and chloroperoxidase [3]. The inherent reactivity of these enzymes is attributed to an iron protoporphyrin IX complex which is bound to the protein via hydrogen bridges of the two propionate side chains, and most significantly through a thiolate ligand coordinating to the iron. The latter is delivered by a cysteine residue in a highly conserved area of the protein, such that the S- is placed at the face of the porphyrin opposite to the binding site of oxygen and the substrate. The thiolate ligand plays a crucial role concerning the reactivity of the heme group [4] and triggers the redoxpotential of the iron porphyrin [5]. X-ray structures of different forms of cytochrome P450cam [6] and chloroperoxidase [7] revealed that the S- is hydrogen-bonded to two peptide amide groups in addition to its coordi-nation to the iron.

Despite of considerable efforts over the past 30 years using different isoforms of P450 and pure enzyme preparations of chloroperoxidase (CPO) from *Caldariomyces fumago* [8], as well as employing a large number of synthetic model

compunds [9] we still lack a complete understanding of the catalytic cycles of both P450 and CPO.

In this communication we wish to address certain significant problems and present some recent results from our laboratory.

Next chapter: **The Reaction Mechanism of Chloroperoxidase (CPO)**

References

Comments

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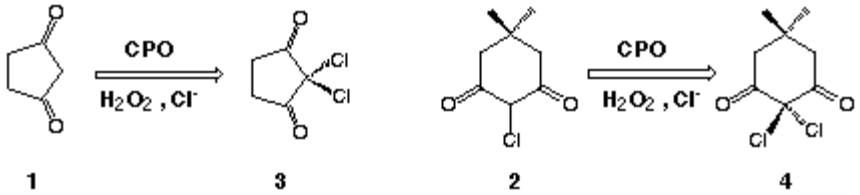
The Reaction Mechanism of Chloroperoxidase (CPO)

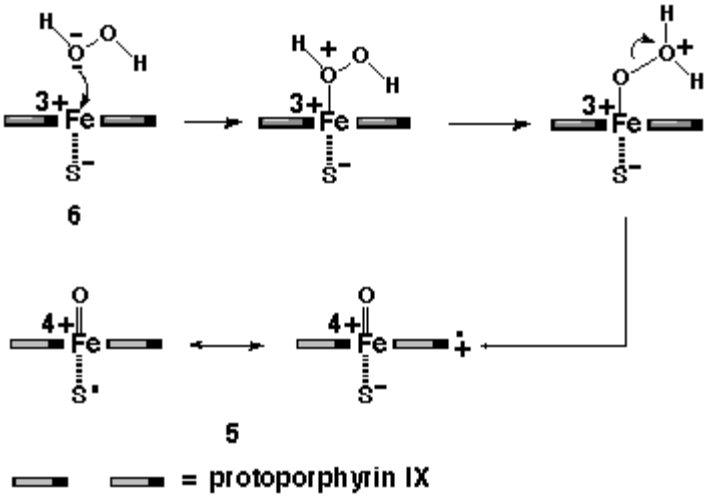
Chloroperoxidase, first isolated from the fungus *Caldariomyces fumago* [8], is the most versatile of the *heme-thiolate* proteins due to its ability to catalyze the halogenation of activated C-H bonds and reactions reminiscent of peroxidase, catalase, and cytochrome P450. Concerning chlorination chloroperoxidase (CPO) is employing H_2O_2 and Cl^- at pH 3 to react with 1,3 diketones such as **1** and **2** to yield **3** and **4**, respectively, scheme 1.

Scheme 1



It was suggested [10] that chlorination proceeds via **5**, the so called compound I, which is produced on reaction of the enzymeÕs resting state **6** with H_2O_2, scheme 2. It was also invoked [10] that **5** reacts with Cl^-, and depending on the concentration of the Cl^-, either Cl_2 or HOCl is released into solution where halogenation of the substrate occurs.

Scheme 2



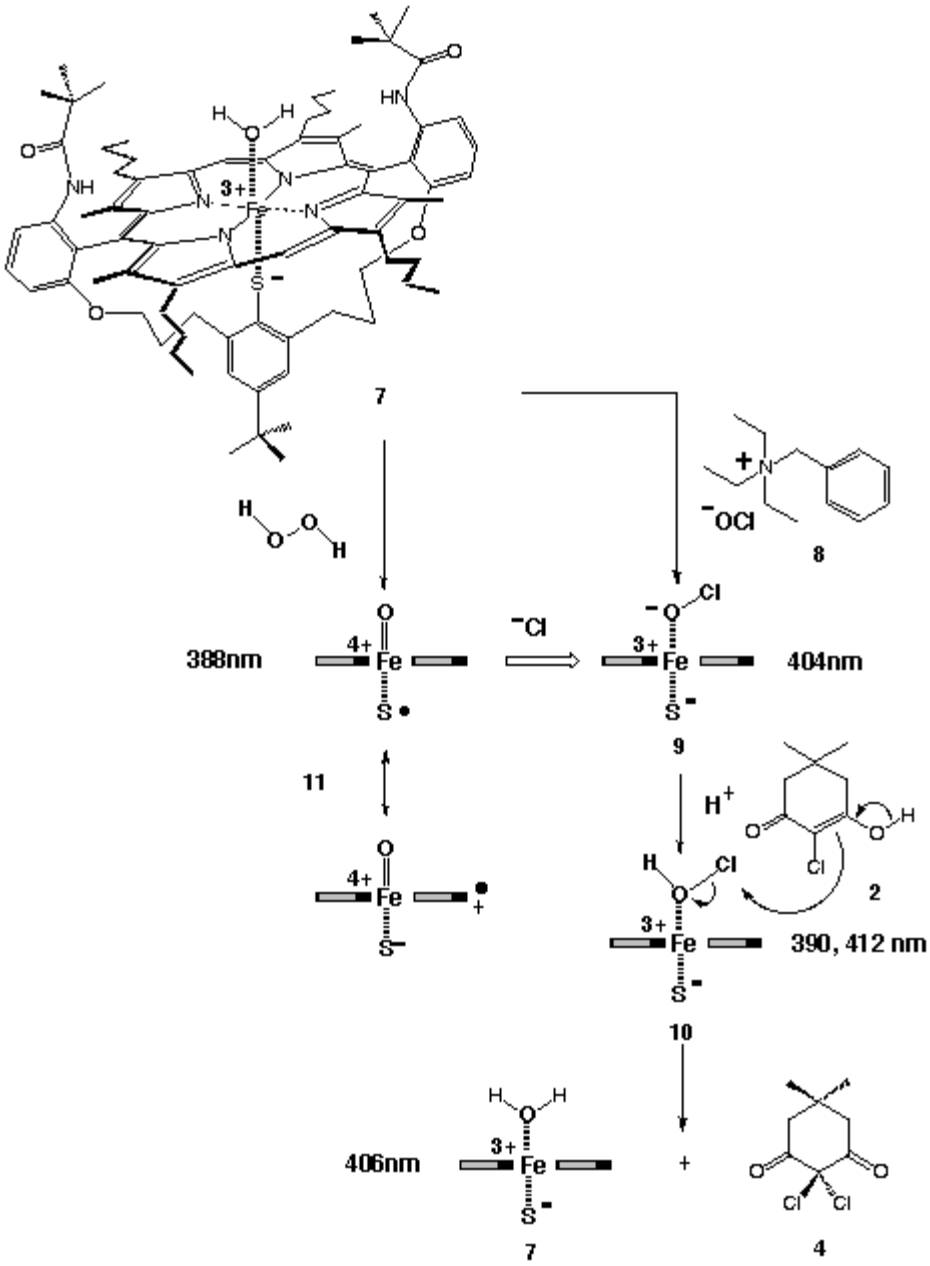
The participation of a "free halogenating species" seems to be in agreement with the observation that the halogenation of small, apolar substances often proceeds non-stereospecific [11]. In contrast, however, it has been shown e.g. that a highly substituted glycal is "halohydrated" completely stereospecific by means of CPO/KBr-H_2O_2 at pH 3 [12]. Accordingly despite of numerous investigations of the enzyme [13] the mechanism of chlorination and the identification of significant reactive intermediates remained elusive.

Our main objective was to prepare a heme-thiolate enzyme model which, we anticipated, would bind ^-OCl and even HOCl as the sixth ligand due to the presence of the coordinating S^-. We further hoped that these complexes would release Cl^+ in the presence of substrate.

The target compound **7** is a *face-protected* iron(III) diphenyl porphyrin carrying a thiophenolate ligand attached to a *bridge* tightly spanning the porphyrin plane such that the S^- is *forced* into coordination to the iron, and decomplexation from the iron is prevented for steric reasons [4]. The substrate binding site is protected against porphyrin-porphyrin interactions by means of two pivalylamido groups [14], scheme 3.

The synthesis of the porphyrin **7** [15] was pursued employing our experience with bridged porphyrins [4]. The complex **7** is a high-spin iron(III) porphyrin displaying a *Soret* band at 406 nm; in comparison the resting state of chloroperoxidase, which has no water coordinating to the iron, has a *Soret* band at 399 nm. Reduction of **7** with NaBH4 in THF yielded quantitatively the iron(II) complex (*Soret* band 418 nm) which after addition of CO displayed the expexted bathochrome shift to 440 nm.

Scheme 3



To obtain a decent concentration of ^{-}OCl in CH_2Cl_2 the benzyl triethyl ammonium salt **8** was prepared, and reacted with **7** at r.t. to obtain quantitatively the high-spin hypochlorite complex **9** ($g_{\perp}$ = 5.768 and $g_{\parallel}$ = 1.987) displaying a UVmax of 404 nm, scheme 3. This compound showed very little chlorination acticity towards monochloro dimedone **2**, however, after addition of AcOH the high-spin ($g_{\perp}$ = 5.612 and $g_{\parallel}$ = 1.955) HOCl-adduct **10** (UVmax: 390, 412 nm) was obtained which behaved reasonably well as a catalyst for the chlorination of **2**. When the proton of the HOCl ligand in **10** was replaced by a Lewis Acid turnovers up to 1500 could be obtained in chlorination reactions.

In order to mimic the ordinary enzymatic reaction **7** was treated successively with H_2O_2, Cl^-, and AcOH. The addition of H_2O_2 gave quantitatively a new complex which according to its UVmax of 388 nm is the oxo iron(IV) porphyrin **11**, equivalent to compound I (**5**), the reactive intermediate of cytochrome P450 and chloroperoxidase (UVmax: 367

nm) [16], capable of oxygen insertion. Subsequent addition of Cl^- yielded complex **9**, which on protonation furnished **10**, subsequently reacting with e.g. **2**.

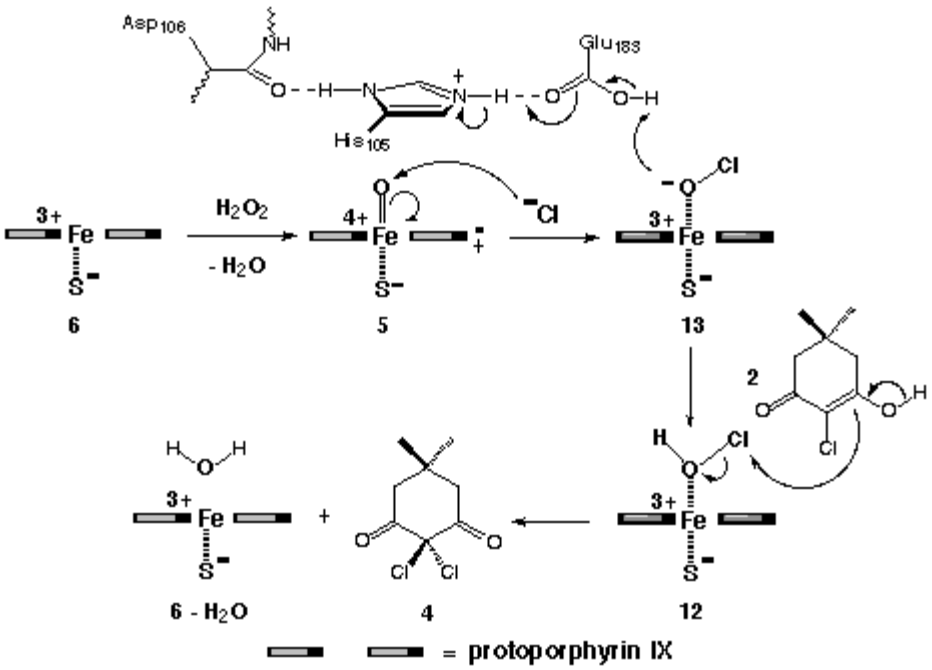
Accordingly the same intermediates can be approached in two distinct reaction pathways. These results indicate that iron(III)porphyrins with a thiolate ligand easily coordinate with -OCl and HOCl and mimic the catalytic behaviour of chloroperoxidase. Furthermore these results show that neither "free HOCl" nor $Cl^{\cdot}$ are involved in the chlorination catalyzed by CPO but imply that an iron-bound HOCl is the Cl^+ source. It is important to note, however, that intermediates, like **9** and **10**, have never been observed in the enzymatic reaction. We reasoned that these sixcoordinate complexes may have not been detected because under common reaction conditions at pH<3 intermediates corresponding to **9** and **10** are too shortlived.

Thus our first objective was to investigate commercially available chloroperoxidase from *Caldariomyces fumago* for binding of HOCl and -OCl, respecively, at different pH [17]. Screening the range from pH 3 to 9 at r.t. it became evident that only at pH 4.4 a new compound was formed. The *Soret* band at lmax = 398 nm of the resting state disappeared, and a split *Soret* band with maxima at 376 nm and 434 nm was observed similar to the UV spectrum of the synthetic enzyme model **10**. Hence we conlude that under these conditions **12**, the HOCl-adduct of CPO is produced, scheme 4. The splitting of the *Soret* band of **10**, and of **12** confirms an earlier prediction, based on MO calculations [18], that due to the presence of the proximal thiolate ligand *heme-thiolate* proteins should exhibit a split *Soret* band on addition of a sixth ligand. Until now this phenomenon has only been demonstrated for the oxygen- (**.**O-O-Fe(III)-S-) and the carbon monoxide - complex (OC-Fe(II)-S-) of cytochromes P450. For iron porphyrin model compounds, however, often only one of the two *Soret* bands is detectable.

In view of this result it seemed unlikely to obtain any information regarding the existence of a -OCl-CPO adduct using the intact enzyme. In this context we took advantage of the recently published X-ray structure of CPO which identifies the glutamate Glu183 as part of a proton relay system including the amino acids His105 and Asp106 [7]. Glu183 is located adjacent to the peroxide binding site, suggesting its active role in peroxide cleavage, scheme 2. Since it is known that the covalent modification of His105 [19], using diethyl pyrocarbonate, inactivates CPO irreversibly and completely [20] it was anticipated that the -OCl - CPO adduct would be detectable by interrupting the proton supply. At pH 6.0 His105 is the only residue of the proton shuttle system which remains protonated and hence reacts with diethyl pyrocarbonate in dry ethanol. The reaction is monitored by the increasing absorbance at l = 246 nm [19].

When CPO, containing the ethoxycarbonyl protected His105, was treated with a NaOCl solution at pH 6.0 a significant change of the UV spectrum was detected: l_{max} = 398 nm of the resting state changed to l_{max} = 406 nm which by comparison with the enzym model **9** (l_{max} = 404 nm) is interpreted in terms of formation of the -OCl-CPO adduct **13**. Given the experimental conditions, namely pH=6.0, and the pKa values for glutamate (4.27) and HOCl (7.49), we believe that HOCl first coordinates to iron and is then sufficiently acidic to protonate the adjacent Glu183, Fig. 7. It is important to note that on adjusting the acidity to pH=3.8 the absorbance of the -OCl adduct remains unchanged, and the UV maxima of the HOCl - CPO adduct are not detectable, indicating that the proton delivery is completely blocked indeed.

Scheme 4



These experiments demonstrate that both the -OCl- and the HOCl-adduct of the iron(III)protoporphyrin(IX) complex of CPO can be generated at pH=6 and pH=4.4, respectively. Both pH values are notably different from pH<3, the pH at which CPO performs the chlorination using H_2O_2 and Cl^-. It seems, however, reasonable that both intermediates are not observable at low pH under conditions when the proton delivery system is fully operative, and as we know from enzyme model studies, the final Cl+ donor is very reactive. Accordingly we propose a reaction mechanism for CPO, scheme 4, involving both intermediates consistent with our observations and supported by recent kinetic studies [21]. After the cleavage of H_2O_2, see scheme 2, the oxo iron intermediate **5** is generated. Addition of Cl- to **5** leads to the -OCl-CPO adduct **13** which is immediately protonated by the proton relay system to yield the HOCl-CPO complex **12** which is catalytically active as a Cl+ donor as we know of the equivalent synthetic enzyme model **10**.

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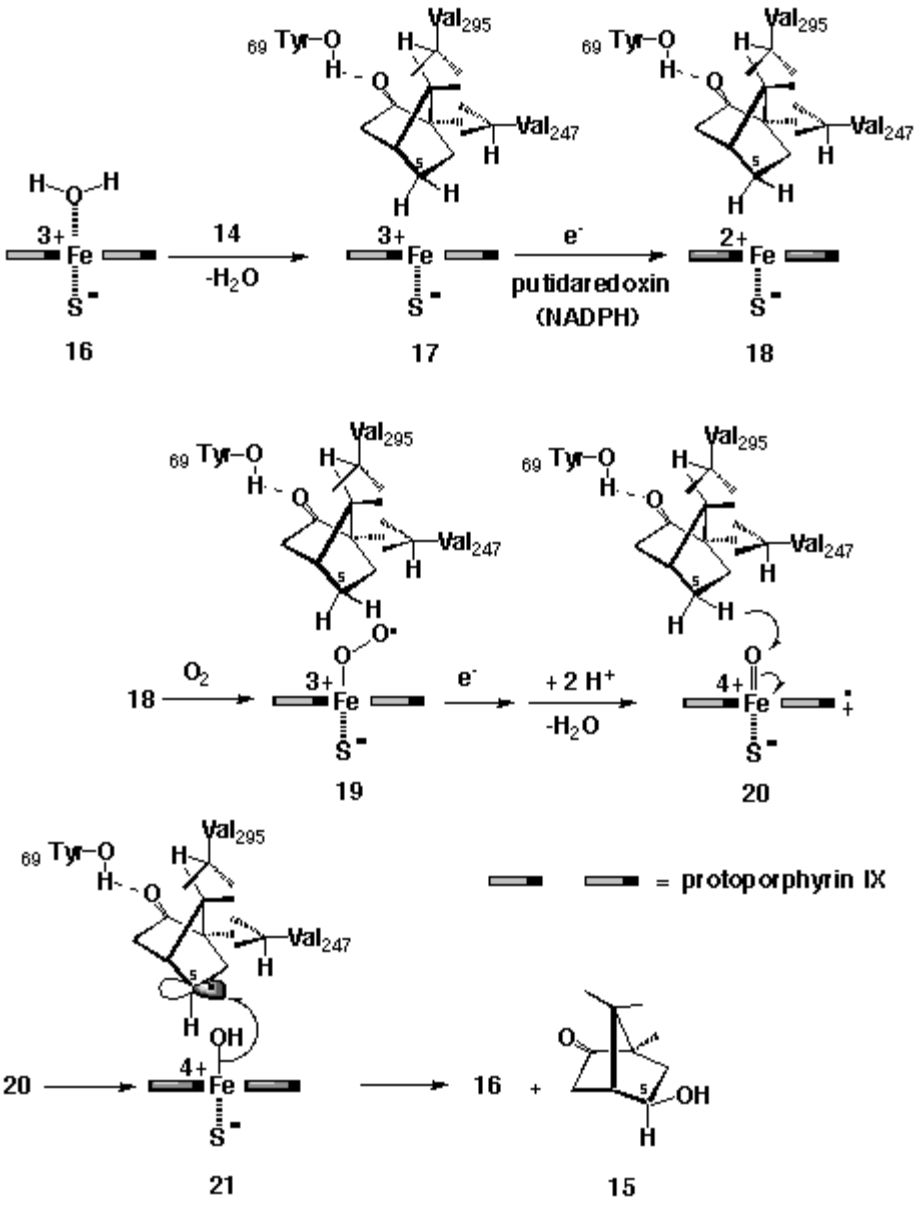
Next chapter: [The Resting State of P450cam]

[References]

The Resting State of P450$_{cam}$

Cytochrome P450$_{cam}$ from the soil bacterium *Pseudomonas putida* catalyzes the stereospecific hydroxylation of camphor **14** at the 5-*exo* position to yield 5-hydroxycamphor **15**, scheme 5. This very interesting reaction shows the unusual ability of the P450 enzymes to oxidize non-activated position. Many mechanistic studies [9] and the structure elucidation of different forms of P450$_{cam}$ [6] have contributed a great deal to our understanding of P450 action, in fact these studies have dominated the research of heme-thiolate proteins for a long time. The reaction mechanism which has been evolved from all these studies is shown in scheme 5. In the resting state **16** the substrate pocket is occupied by six water molecules of which one is coordinating to the iron. This water cluster is completely removed when camphor binds, simultaneously the spin state of the system changes from low-spin **16** to the predominantly high-spin iron(III) of the E**.**S complex **17** [22]. An anodic shift from -300mV (**16**) to -175mV (**17**) renders the latter to accept an electron NADPH via the redoxprotein putidaredoxin.

Scheme 5



The resulting iron(II)complex **18** binds oxygen. Reductive oxygen cleavage via **19** furnishes the oxo iron(IV) intermediate **20** which abstracts H**.** from the bound camphor to yield **21**. Addition of HO**.** to the *exo*-face of camphor leads to the formation of the product **15**. Regarding this complex cycle some problems remain to be solved.

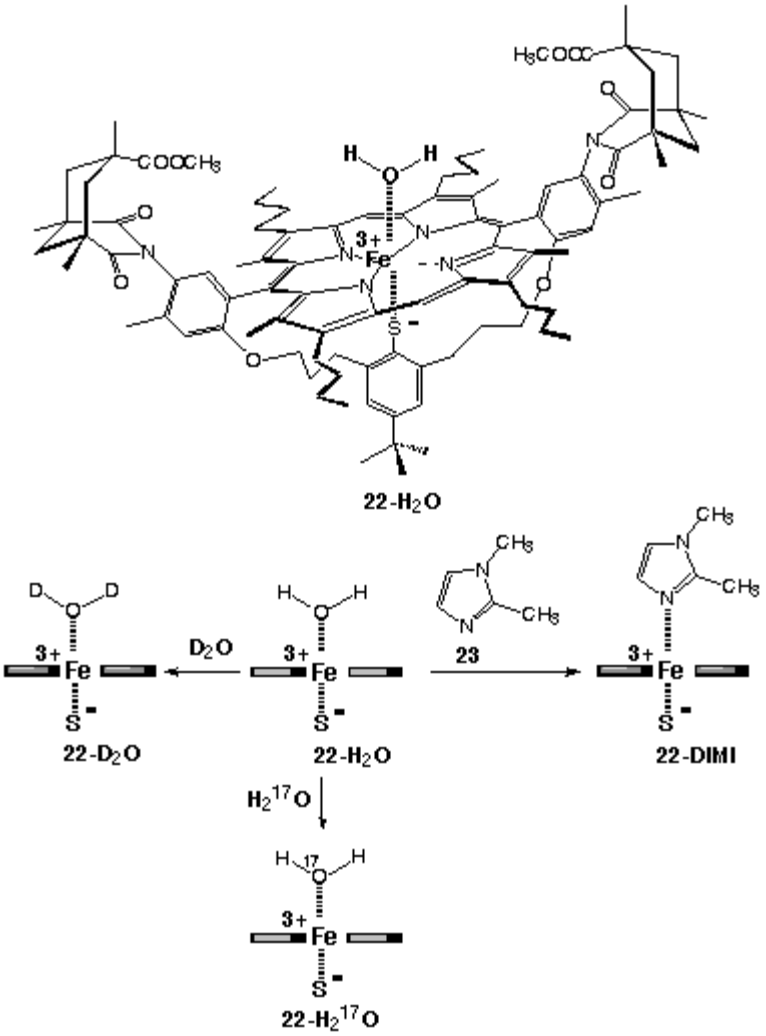
The structure of the oxygen inserting intermediate **20** has been invoked from investigating model compounds lacking the thiolate ligand [23] and the H**.** abstraction-rebound mechanism has been accepted by the P450 community *inter alia* because of results obtained from incubation of 5-*exo*- and 5-*endo*-deuterated camphor [24]. In fact both substrates gave in a different ratio deuterated and undeuterated 5-*exo*-hydroxycamphor, indicating that H**.** removal and O-insertion are separate events in the reaction sequence; this conclusion was supported by earlier radical clock experiments. Recently however both the elctronic structure of **20** [25] and the mechanistic dogma that **20** is the single possible reactive intermediate [26] to hydroxylate substrates have been questioned.

The other and equally important question, which we like to address here, concerns the low-spin character of the resting state **16**. Since the water cluster was discovered in **16**, the origin of the low-spin ground state in the resting state has been a matter of debate, as it seems unlikely that the thiolate and one of the water molecules, both weak ligands, would establish a low-spin iron(III). It was therefore suggested that HO- rather than water binds to the iron or that the hydrogen-bonded water **-** cluster induces a hydroxide-like character at the ligand water [6],[27]. From ESEEM studies using ^{17}O-enriched water bound to P450$_{cam}$, however, it was concluded that one water molecule binds non randomly to iron; the presence of a hydroxide ion as the sixth ligand was excluded [28]. Since these investigations arrived at no conclusions whatsoever concerning the origin of the low-spin character of iron(III) in the given ligand field, we decided to prepare suitable enzyme models for the resting state of P450$_{cam}$ to investigate these complexes by cw EPR, and pulse EPR and ENDOR techniques. Such studies should provide information on the possible structure and electronic nature of the first intermediate of the catalytic cycle of cytochrome P450$_{cam}$.

The P450 analogue **22-H**$_2$O was prepared from the corresponding diacid a recently synthesized iron(III)porphyrin with substrate recognition sites [29], scheme 6, and it was shown that **22** is a spectroscopical and chemical enzyme model of cytochrome P450 [30]. **22-H**$_2$O was characterized by a *Soret* band at 408nm and ESI - MS (*m/z* = 1623 [*M*+]) indicating the coordination of one molecule of water to the iron from the distal site. In the native P450$_{cam}$ the change from the hexacoordinated low-spin state **16** to the pentacoordinated high-spin state **17** can be easily monitored by cw EPR. The low-spin state **16** shows an EPR spectrum with g values 2.45, 2.26 and 1.91. The addition of the substrate camphor is accompanied by a conversion to a rhombically distorted high-spin form **17** with g=7.85, 3.97 and 1.78 [31], [32].

The cw EPR spectrum of the synthetic P450 analogue **22-H**$_2$O at 10K displays an almost axially symmetric EPR spectrum with $g_\perp$ = 5.67 and $g_\parallel$ = 2.00 which is characteristic of high-spin iron(III)porphyrins without significant rhombic contributions from the ligand field. It is established that the $g_\parallel$ extremum is along the normal of the porphyrin plane and $g_\perp$ lies in the porphyrin plane[33].

Scheme 6



Since the only indication regarding the coordination of water to iron was the ESI-MS of **22-H**2O a more complete characterization of the iron(III) ligand sphere by pulse EPR and ENDOR techniques was required. Treatment of **22-H**2O with an excess of 1,2-dimethylimidazole (DIMI) **23** induced a change to the low-spin state **22-DIMI**. It is important to note that the corresponding cw EPR spectrum shows g values (2.45, 2.23 and 1.91) almost identical to those of the native P450cam in its low-spin state **16**. This additional spectroscopical conformity between the enzyme's resting state and its model system **22** lends further evidence to the suitability of the latter as an active site analogue.

Concerning the coordination of water to FeIII, the proton hyperfine couplings of several comparable native high- and low-spin heme systems are known from ENDOR studies [34]. In the high-spin case the expected proton hyperfine coupling along gíí (a_{iso} + A(gíí)) lies around 6 MHz and is found to be predominantly dipolar in character [35]. The three-pulse ESEEM spectrum of **22-H**2O shows no evidence of such a strong proton interaction. After stirring **22-H**2O in 2-methyltetrahydrofuran (MTHF)/D2O for 12 h the corresponding deuterated complex **22-D**2O was obtained; its three-pulse ESEEM spectrum reveals an intensive deuterium signal pair centered

at the nuclear Zeeman frequency of deuterium with a splitting of 1.04 MHz.

The Fourier transformation of the ratio of the time domain signals **22-D**2O/22-H2O includes only the deuterium modulation although, in principle, residual lines at frequencies corresponding to other nuclei and their combinations should be present. The intensities of these are, however, very small and can be neglected.

From the deuterium interaction the expected proton coupling normal to the porphyrin plane can be calculated to 6.77 MHz (g_H/g_D = 6.51). A proton hyperfine coupling with exactly this value can be detected in the Davies ENDOR spectrum of **22-H**2O. The corresponding signal pair can be completely removed by exchange with D2O, in contrast all the other proton lines are not affected by the exchange reaction.

In the Davies ENDOR spectrum the deuterium coupling is not detectable since this technique is not sensitive enough at low frequencies. On the other hand, the broad proton lines can not be seen in the three-pulse ESEEM due to spectrometer deadtime. Thus Davies ENDOR and three-pulse ESEEM spectroscopy complement each other in an optimal fashion [36]. In further experiments the H_2O ligand of **22-H**$_2$O was exchanged for $H_2{}^{17}O$. The resulting complex **22-H**$_2{}^{17}$O shows no line broadening in the cw EPR, the Davies ENDOR spectrum, however, is very informative. Subtraction of the two ENDOR spectra **22-H**$_2{}^{17}$O - 22-H_2O reveals two intense ^{17}O lines centered at 1/2 A(^{17}O) with A(^{17}O) = 19.83 MHz, splitted by twice

the nuclear Zeeman frequency of ^{17}O (2n(^{17}O)= 3.98 MHz) and broadened due to the nuclear quadrupole interaction of ^{17}O (I=5/2). This coupling is remarkably strong in comparison to the very small Fe-^{17}O interaction in the low-spin state **16** where the unpaired electron is residing predominantly in the d_{yz} orbital of Fe^{III}[28].

Accordingly it is unequivocally demonstrated that **22-H**$_2$O is a hexaco-ordinated iron(III)porphyrin with a thiolate and a water molecule coordinating to the iron. In contrast to **16**, the resting state of $P450_{cam}$, the synthetic analogue **22-H**$_2$O is definitely high-spin and changes only to a low-spin system if the water ligand is exchanged for a strong ligand, such as 1,2 dimethylimidazole **23**. In view of the ESEEM studies of the enzyme $P450_{cam}$ mentioned above, [28] water rather than a stonger ligand like a hydroxide ion is coordinating to iron(III). Consequently the coordination of water to the active site's iron of cytochrome P450 is not the single determining factor to establish the low-spin character of the system. It is important to note that the results presented here provide the first experimental support for calculations reported recently [37].

INDO/ROHF and molecular dynamics simulations of cytochrome $P450_{cam}$ suggested two cooperative factors favoring the low-spin state of **16** : the presence of the water ligand decreases the energy difference between high-spin and low-spin state from 75.4 to 15.9 kJ mol^{-1}, and the electrostatic field from the protein can further stabilize the low-spin state 7.5 kJ mol^{-1} below the high-spin state.

Regarding our enzyme model a simple estimation revealed that a point charge 7Å above the porphyrin plane of **22-H**$_2$O would be suitable to establish an electric potential sufficient to decrease the energy level of the low-spin state of $P450_{cam}$ significantly below the high-spin state. Work is in progress to mimic the significance of the protein's electric field through attachment of positively charged substituents to the Kemp acids of **22-H**$_2$O [38].

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