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The origin of the low-spin character of the resting state of cytochrome $P450_{cam}$ investigated by means of active site analogues

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Crown-capped iron(S$^-$) porphyrins 1·H_2O and 2·H_2O and their corresponding Ba^{2+} complexes have been prepared as active site analogues of the resting state of cytochrome $P450_{cam}$. cw-EPR studies and electronic structure calculations at the density functional theory (DFT) level of model systems suggest a functional role of the water cluster of $P450_{cam}$.

Cytochrome P450s are naturally abundant heme-thiolate proteins. These enzymes are extremely important to mammals because of their central role in the metabolism of drugs and the synthesis of hormones.[1] Current knowledge regarding the various catalytic reactions of heme-thiolate proteins rests to a large extent on studies of cytochrome $P450_{cam}$ (CYP101), a cytosolic protein which was isolated from *Pseudomonas putida* and has been overexpressed in other bacteria.

The crystal structure of the resting state of cytochrome $P450_{cam}$ revealed that the substrate binding pocket is occupied by a cluster of six hydrogen bonded water molecules, one of which is coordinating to iron as sixth ligand.[2] Surprisingly, the resting state is predominantly low-spin and changes to a high-spin system when substrate camphor binds and the water cluster is removed completely. Simultaneously, the redox potential shifts cathodically from −300 to −170 mV so that the subsequent reduction of Fe^{III} to Fe^{II} by the redox protein putidaredoxin (−196 mV) becomes thermodynamically favorable. Thus spin state changes are significant in regulating the catalytic cycle of P450s and furthermore are assumed to trigger the mode/reactivity of oxygen insertion by $O{=}Fe^{IV}(porph^{+\cdot})(CysS^-)$.[3]

Contradictory arguments have been published to explain the origin of the low-spin resting state of $P450_{cam}$. On the one hand, Poulos *et al.*[4] suggested that polarization of the coordinated water molecule through the hydrogen bonding network in the water cluster could result in a distinctive hydroxide character (strong ligand), thus leading to a low-spin complex. On the other hand, by comparing simulated and experimental ^{17}O ESEEM and pulsed ENDOR/four-pulse ESEEM spectra of the resting state of $P450_{cam}$, Goldfarb *et al.*[5] concluded that the distal ligand is a water molecule rather than a hydroxide ligand. Semiempirical calculations by Loew and Harris[6a] suggested a significant contribution of the electrostatic field of the surrounding protein stabilizing the low-spin resting state. It was proposed by the same authors that a point charge above the porphyrin plane would simulate the electrostatic field of the protein and push the spin equilibrium towards low-spin.[6b]

Using various EPR techniques we have recently shown that active site analogues H_2O–Fe(III)(porph)(ArS^-) are definitely high-spin and only change to the low-spin state if the coordinating water is replaced by 1,2-Me_2imidazole.[7] These results clearly indicated that the coordination of water to Fe(III) of the heme thiolate cofactor is insufficient to stabilize the low-spin state of the resting state of $P450_{cam}$. Accordingly, we designed the active site analogues **1** and **2** to distinguish experimentally between two effects: (i) hydroxide character of the coordinating water and (ii) contribution of the protein's electrostatic field. In this context some structural features of **1** and **2** are worth noting, such as the sterically congested S^- ligand coordinating to Fe(III) and a crown ether capping the distal face of the porphyrin. 1,10-Diaza-18-crown-6 is known to bind divalent cations[8] such as Ba^{2+} and would allow investigation of the electrostatic field effect. Moreover, the oxygens of the crown ether can act as H-bond acceptors[9] to water coordinating to iron if placed at a proper distance. In contrast to **2**, hydrogen bonding is possible in **1** and hence in the latter the diazacrown cap should mimic a possibly polarized water cluster in $P450_{cam}$ and a HO^- like character of the coordinating water molecule, respectively.

We report here the synthesis and characterization of **1** and **2**, their corresponding water and Ba^{2+} adducts and DFT calculations of relative stabilization energies.

As depicted in Scheme 1, the synthesis of **1** and **2** commenced with the classical porphyrin condensation[10] of dipyrromethane **3**[11] and benzaldehyde **4**.[12] Subsequent oxidation of the resulting porphyrinogens followed by deprotection furnished a 6 : 4 mixture of two atropisomers, αα-**5** and αβ-**5**, respectively, which were separated by flash chromatography after acetylation of the amino groups. Then methyl ethers were cleaved to give porphyrin αα-**6**, which was ready for the coupling with protected sulfur bridge **7** under high-dilution conditions to yield **8**.[13] After distal deprotection we took advantage of the protocols of Collman *et al.*[14] and Guilard *et al.*[9] to obtain protected crown-capped porphyrins **9** and **10**.[15] Successful capping was obvious from the high field shift of the methylene groups of the crown ether as observed in ^1H NMR. Cleavage of the sulfur protecting group and insertion of iron completed the synthesis of **1** and **2** which showed characteristic UV–vis (λ_{max} (toluene) = 404 and 406 nm, respectively) and ESI-MS spectra (*m*/*z* 1469 $[M + Na]^+$ and *m*/*z* 1497 $[M + Na]^+$, respectively).

In dry toluene both **1** and **2** showed cw-EPR spectra characteristic of pure high-spin iron porphyrins with a strong rhombic contribution from the ligand field (Fig. 1a and 1b, top trace). The EPR spectrum of **2** in water-saturated toluene (Fig. 1a, bottom trace) was identical with the spectrum in dry toluene. In contrast, when **1** was measured in water-saturated toluene low-spin signals in the EPR spectrum were observed (Fig. 1b, bottom trace). These *g*-values are very similar to those obtained for the low-spin resting state of $P450_{cam}$ (g = 2.45, 2.26, 1.91).[16] In addition, we have already shown that the same low-spin signals (g = 2.45, 2.23, 1.91) can be obtained quantitatively, if an enzyme model with an open distal face is treated with an excess of 1,2-Me_2Im, a strong ligand known to yield a pure low-spin complex.[7b]

The results suggest that water binds to the central metal of **1** and forms two hydrogen bonds with crown ether oxygens (Scheme 1). The incomplete conversion of the high-spin state of **1** to its low-spin state can be explained by the small excess of water present in toluene (~3–4 equivalents).

To probe the influence of charges (electrostatic field effects) on the spin state of iron(III) the complexes **1**·H_2O and **2**·H_2O were treated with an excess of $Ba(ClO_4)_2$. Binding of Ba^{2+}, as evident from ESI-MS spectra, however, did not change the cw-EPR spectra of the compounds. In fact **2**·H_2O–Ba^{2+} remains high-spin and **1**·H_2O–Ba^{2+} shows the same mixture of high- and low-spin state as **1**·H_2O. Since adducts **1**·H_2O and **2**·H_2O, due to their structural differences, are suitable to separate the polariza-

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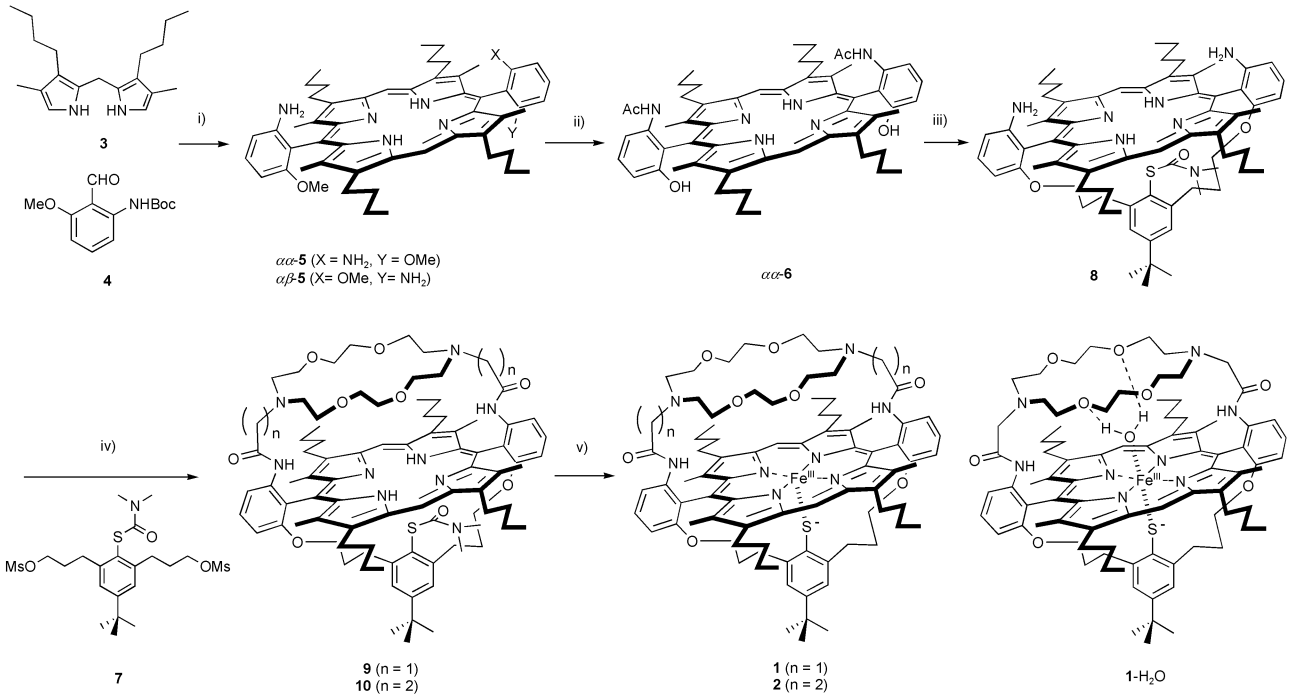

Scheme 1 *Reagents and conditions*: (i) (1) cat. PTSA, MeCN, rt, (2) DDQ, MeCN–THF, rt, (3) TFA, CH_2Cl_2, rt, 38% (3 steps); (ii) (1) AcCl, DMAP, pyridine, CH_2Cl_2, rt, (2) flash chromatography, (3) $AlCl_3$, EtSH–CH_2Cl_2 3 : 2, rt, 36% (3 steps); (iii) (1) Cs_2CO_3, DMF, 60 °C, **7**, high-dilution, (2) 6 M HCl, MeOH, reflux, 77% (2 steps); (iv) for n = 1 (1) chloroacetyl chloride, $CHCl_3$, rt, (2) 1,10-diaza-18-crown-6, EtOH, reflux, 44% (2 steps); for n = 2 (1) acryloyl chloride, Et_3N, CH_2Cl_2, rt, (2) 1,10-diaza-18-crown-6, MeOH–CH_2Cl_2, 80 °C, 46% (2 steps); (v) (1) KOMe, dioxane, reflux, (2) $FeBr_2$, 2,6-lutidine, toluene, reflux, 44% for n = 1 (2 steps), 49% for n = 2 (2 steps).

tion of water from distant charge effects we conclude that they are of no significance, stabilizing the low-spin state of Fe(III) in heme thiolate proteins. In contrast, our results clearly demonstrate that a water molecule which is hydrogen bonded *and* coordinating to iron(III), as in **1**·H_2O, stabilizes the low-spin over the high-spin state.

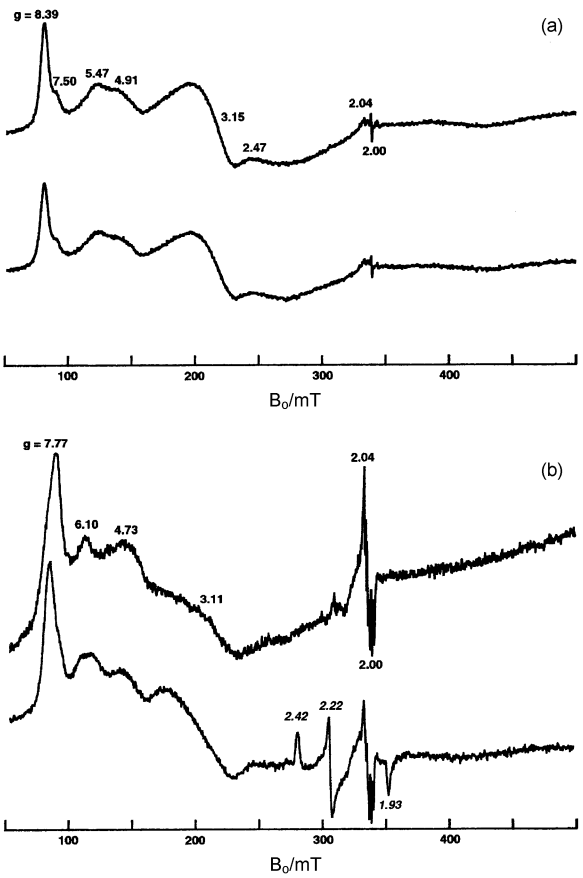

Fig. 1 (a) cw-EPR spectra of **2** in dry toluene (top) and water-saturated toluene (bottom). (b) cw-EPR spectra of **1** in dry toluene (top) and water-saturated toluene (bottom). Conditions: ν = 9.46 GHz, P = 20 mW, T = 100 K, mod. freq. 100 kHz, mod. ampl. 0.52 mT.

To corroborate the experimental results *ab initio* calculations[17] were carried out for **11** and **12** (Fig. 2). In **11** the optimized structure of the low-spin state is favored by only 2.5 kcal mol^{-1} over the high-spin state. This amounts *e.g.* to a change of only 0.1 Å in the *d*(Fe–porphyrin plane) distance which is a typical amplitude.[18] For H-bonding of the water molecule to ether oxygens as in **12** (Fig. 2) the low-spin state is unambiguously lower in energy by 7.2 kcal mol^{-1} compared to the high-spin state. Further, H-bonding to ether oxygens results in a significant change of the negative nuclear charge on the oxygen atom of the coordinated water molecule in the low-spin case (−0.86 for **11** to −0.91 for **12**) and in the high-spin case (−0.89 for **11** to −0.97 for **12**). One possibility to validate the calculations is to compare the optimized structures with results from X-ray experiments. In fact, structural parameters r_{FeS}, r_{FeO}, r_{FeNp} and *d*(Fe–porphyrin plane) obtained for **11** and **12** all agree to within 0.1 Å with experimental values for the $P450_{cam}$ resting state.[2]

Finally it is important to note that the cw-EPR experiments reported here are not designed to prove a spin equilibrium which has been shown to exist for the resting state of $P450_{cam}$. From temperature dependent UV–titration experiments Sligar[21] calculated that the low-spin state of the resting state of $P450_{cam}$ is favored by a small ΔG = 1.44 kcal mol^{-1}. In view of the relatively large ΔG = 7.2 kcal mol^{-1} in favor of the low-spin state of **12** and assuming the same order of magnitude for **1**·H_2O, it is reasonable to conclude that the cw-EPR of **1**·H_2O (Fig. 1b) does not display an equilibrium situation. Treating **1** with a small excess of water in toluene[22] we rather observed a mixture of **1** (high-spin) and **1**·H_2O (low-spin). Since under the same conditions the low-spin form is absent in the cw-EPR of **2**·H_2O it follows that H-bonding to ether oxygens is essential to stabilize the low-spin state of $H_2O\cdots Fe(III)\cdots S^-$.

In summary, experiments and calculations support the proposal that polarization in the water cluster plays an important role in the observed low-spin resting state of $P450_{cam}$.

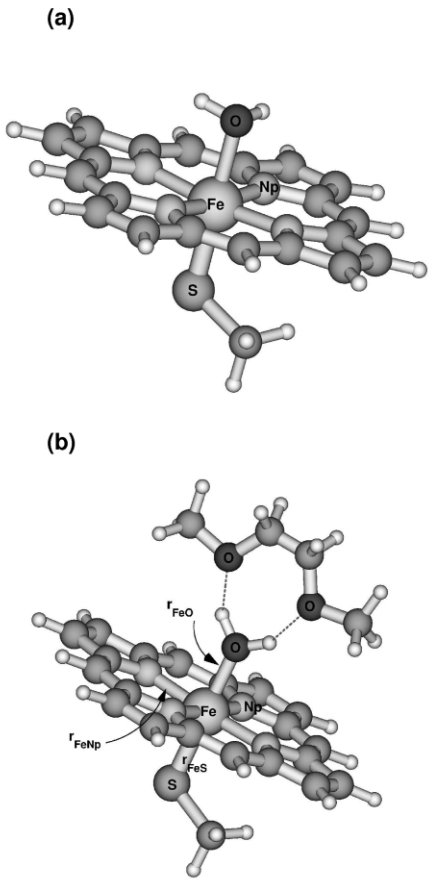

Fig. 2 (a) Heme–water complex **11** and (b) heme–water–ether complex **12** used in the calculation. The latter consists of the water molecule, the heme group with the central Fe atom, the proximal CH_3S^- ligand bound to the iron atom, and the model ether $CH_3OCH_2CH_2OCH_3$.

Accordingly, the water coordinating to iron has a functional role[23] not only in adjusting E_o to −300 mV but also in fine-tuning the spin state of the cofactor.

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