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Reactivity and Redox Potential of Heme-Thiolate Proteins – Results from Enzymes and Enzyme Models

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Abstract: The redox potential is a characteristic parameter of various intermediates of the catalytic cycle of heme-thiolate proteins (cytochromes P450, NO-synthase, chloroperoxidase) which significantly influences catalytic turnover. E_0 values of these proteins are surprisingly positive compared to synthetic active site analogues that have an arylthiolate or an alkylthiolate coordinating to the iron. This report examines factors underlying this phenomenon and describes the design of enzyme mimics having redox potentials close to those of heme-thiolate proteins

Keywords: Cytochrome P450 · Enzymes · Enzyme models · Iron porphyrins · Iron–sulfonate coordination · Iron–thiolate coordination · Redox potential

Introduction

Heme-thiolate proteins comprise the enzymes cytochrome P450 [1], NO-synthase [2], and chloroperoxidase (CPO). The two first proteins are extremely important to mammals due to their central role in the metabolism of drugs/synthesis of hormones and the production of •NO as a signal messenger, respectively. In contrast CPO has attracted attention mainly because of its diverse reactivity profile [3] and its peculiar reaction mechansim [4–6].

Current knowledge regarding the various catalytic reactions of heme-thiolate proteins [7][8] rests to a large extent on studies of cytochrome $P450_{cam}$ [9]. This cytosolic protein can be easily purified from *Pseudomonas putida* and has been overexpressed in other bacteria [10]. X-ray structures of various forms of $P450_{cam}$ [11–16] have been obtained, providing touchstones for understanding the topology at the active site. The enzyme contains an iron(III) protoporphyrin(IX) complex **1** bound to the protein *via* its two propionate site chains and a thiolate ligand from Cys357 coordinating to the iron from the proximal site (Scheme 1). Since its early discovery, the significance of the thiolate ligand for the chemical reactivity of the system [17], and its influence on the enzyme's UV and EPR spectral properties and electrochemistry have been a matter of debate and have initiated intense investigations [18].

The catalytic cycle of $P450_{cam}$ (Scheme 1) [18], and hence that of heme-thiolate proteins more generally, has been established by taking into account the X-ray structures and Laue snapshots [19] of certain intermediates, numerous mechanistic studies on various P450s and chemical models thereof [18]. Accordingly, the sequence of events in the catalytic cycle of the heme-thiolate proteins is certain up to intermediate **2**.

Subsequent steps, though lacking solid characterization, have been deduced from studies using site-specific mutagenesis, by comparing spectroscopic parameters of enzymes and model compounds, and by measuring isotope effects. The formation of a high-valent iron(IV) oxo porphyrin radical cation intermediate **3** by protonation of the peroxo complex **2** and subsequent O–O bond cleavage is in agreement with data from different sources. By analogy to model studies [18] this intermediate is believed to have electronic structure **3** rather than **4**. For the past two decades, the mechanism of P450-catalyzed hydroxylations has been described as a two-step reaction of **3** with the substrate: hydrogen abstraction by the iron-oxo of **3** gives a substrate alkyl radical **6** that is immediately trapped by HO• from the iron, yielding the hydroxylated substrate **7** and the water-free form of the enzyme in its resting state **1** [18]. Recently, however, several aspects of the catalytic cycle have been questioned, including: i) the origin of the low-spin state of **5** [20], the resting state of $P450_{cam}$ enzymes [20], ii) the electronic nature of compound **3/4** [18][21], and iii) the two-step oxygen-rebound mechanism [22][23]. In the present account we focus on the significance of the redox potential of P450 intermediates and corresponding synthetic active site analogues.

Results and Discussion

Three steps of the reaction sequence shown in Scheme 1 are obviously redox sensitive: i) reduction of iron(III) **9** → iron(II) **10**, ii) addition of an electron to the terminal oxygen bound to iron (**11** →

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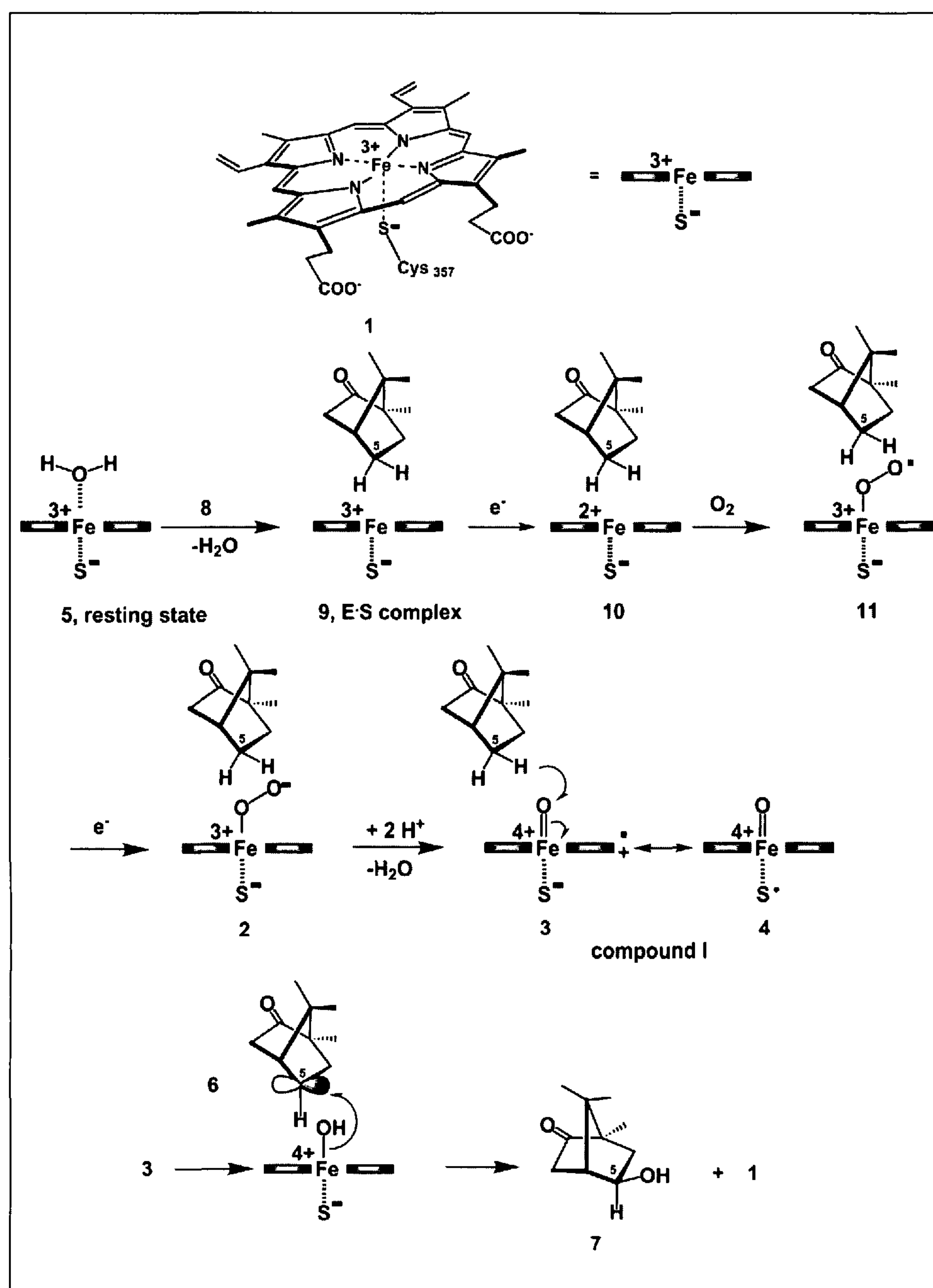


Scheme 1. Catalytic cycle of cytochrome $P450_{cam}$

2), and the interaction of **3/4** with the substrate.

The redox potential of **3/4** is not known but, since it is a strong oxidant, it can abstract H• from substrates (Scheme 1), as well as accept electrons from suitable substrates such as amines [18]. The transformation of **11** → **2** is required to initiate oxygen cleavage, and it is known in certain cases that the donation of an electron to **11** is rate limiting, leading to a small, not fully expressed k_H/k_D isotope effect or H• removal [18], for example in the hydoxylation of camphor (Scheme 1).

The six-coordinate resting state of $P450_{cam}$ (**5**) is an important intermediate in the catalytic cycle. Due to the presence of the thiolate ligand its redox potential is very negative (E_0= –290 mV) [24], precluding reduction by NADPH *via* putidaredoxin [25][26]. Thus, **5** is essentially inert with respect to catalysis and is only activated upon removal of the coordinating water and subsequent binding of the substrate camphor **8** to yield the high-spin iron(III) complex **9** [26–28] (E_0= –170 mV) [23] which can be subsequently reduced to **10**. This fine-tuning of the redox potential of **5** to –290 mV [24], one of the most elegant examples of gating biological electron transfer, is accomplished by two factors. First, the iron(III) adopts a low-spin state, and second, the thiolate ligand provided by Cys357 is hydrogen bonded to two amino acids from the protein [27].The latter aspect, not immediately recognized in the first X-ray structures of $P450_{cam}$, was predicted from the E_0 values of the synthetic active site analogues **12** [28] and **13** [29] (Fig.). Both display rather negative redox potentials (–607 and –714 mV), indicating a clear correlation between E_0 and the effective charge at S^-.

Several possibilities exist for generating catalytic enzyme models having E_0 values close to $P450_{cam}$ (E_0= –290 mV [24]) or CPO (E_0= –140 mV [30]). The synthetically most convenient approach involves the preparation of electron-deficient porphyrins like **14** and **15** (Fig.). Nitro-substituted iron porphyrins, derived from parent compound **15**, provide access to enzyme models with E_0 values

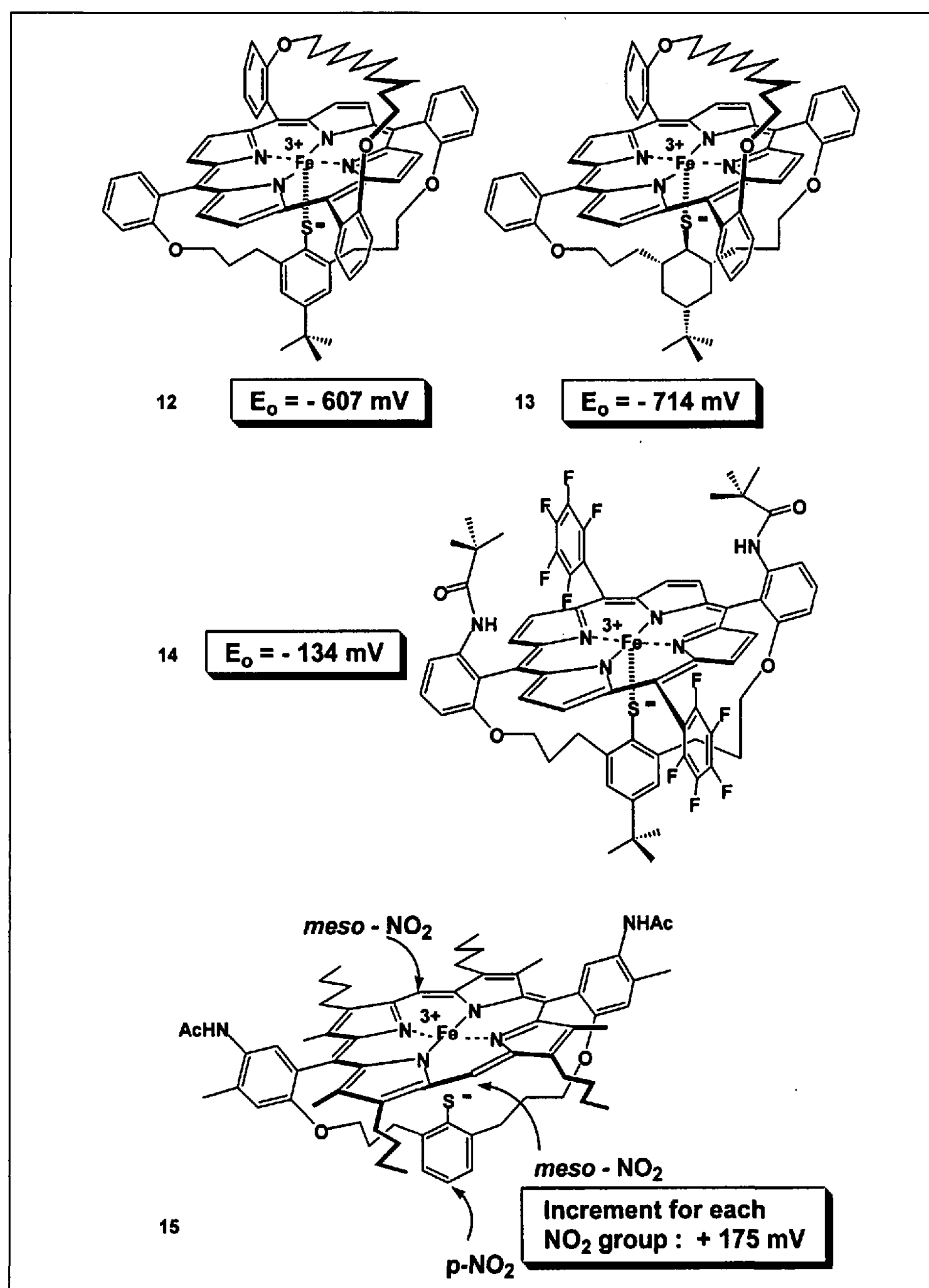
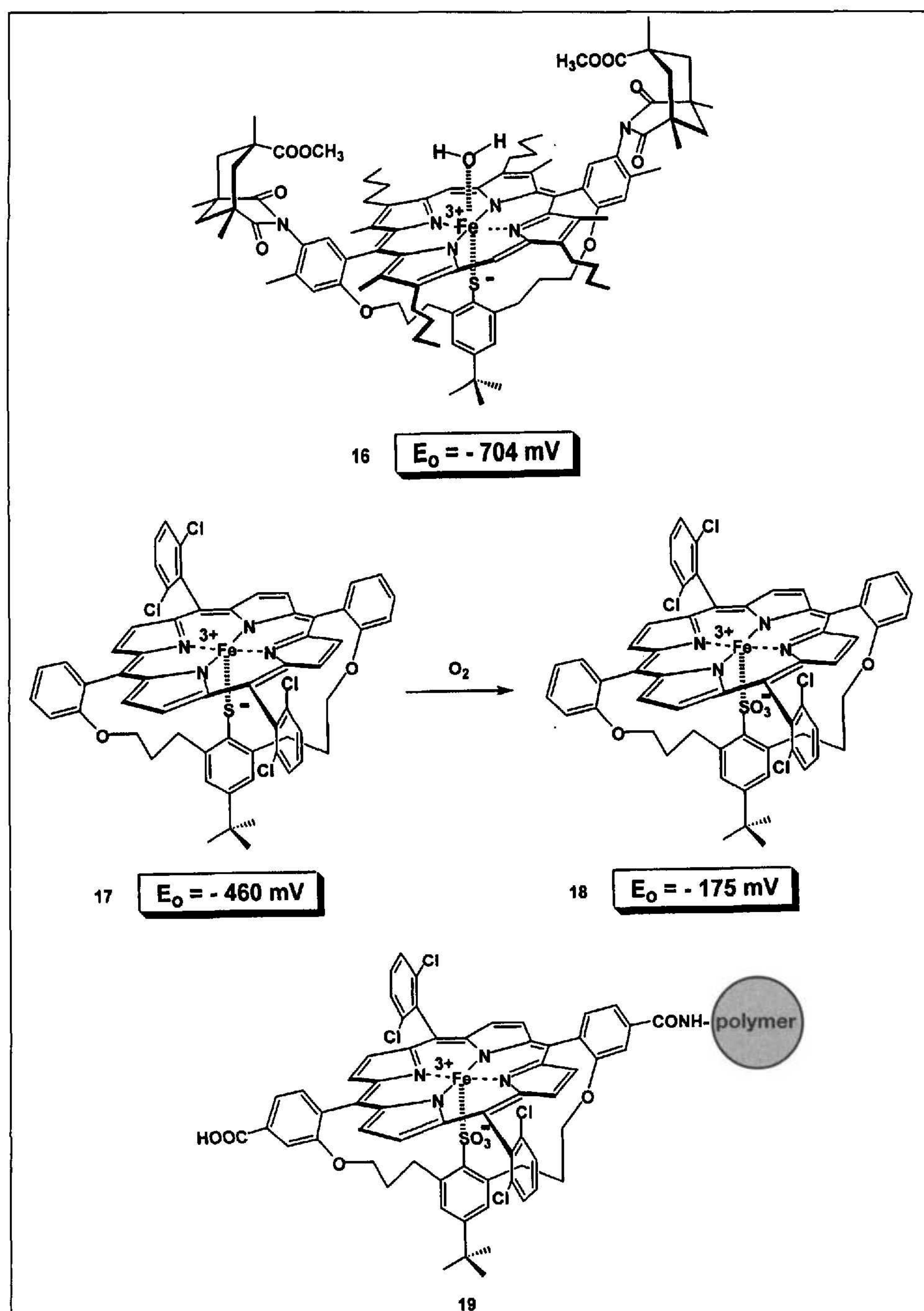


Fig. Redox potentials (E_0) of various synthetic enzyme models of heme-thiolate proteins. E_0 obtained from cyclic voltammograms

between –500 and –200 mV depending on the degree of nitro substitution. However, these complexes are rather difficult to prepare [31] by direct nitration of the porphyrin. In contrast, model compound **14**, containing two pentafluorophenyl substituents as electron-withdrawing groups in the meso-position of the porphyrin plane, is easily accessible and exhibits a redox potential (E_0= –134 mV) comparable to that of CPO [32].

From these model studies we can conclude that E_0 is largely dependent on the electron-donating character of the proximal S^- ligand (*e.g.* **15**, with a *para*-NO_2 substituted thiophenolate ligand) and the structure of the porphyrin and can be further modulated by substituents protecting the distal site of the porphyrin. It is interesting to note that the ΔE_0 between **12** and **16** [33] is about 100 mV suggesting that E_0 depends on the polarity of the distal site. Remarkable anodic shifts are observed only if the electron density at S^- is reduced, as in **15**, or electron donation from the porphyrin ligand is reduced, as in **14** and **15**. If these arguments are also valid for $P450_{cam}$, then the main contribution to the ΔE_0 of +400 mV between the enzyme's resting state and the model compound can be attributed to H-bonding to the thiolate of cysteine 357. Smaller effects may result from the protein environment of the distal substrate pocket. The catalytically important 120 mV anodic shift **7** → **9**, however, is associated with the change from a low-spin to a high-spin state. Though analogues have been prepared (E_0 = –350 mV) that mimic the unique $S^-_{cys}\cdots H\text{–}N$ bonding of $P450_{cam}$, these models are unsuitable for catalytic reactions [34][35].

A third possiblity for varying the redox potential of heme-thiolate proteins involves a very convenient modification of the thiolate ligand, *i.e.* oxidation to the sulfonate SO_3^- (Scheme 2). In agreement with our expectations, by distributing the single negative charge of S^- (**17**) over a much larger space volume in the SO_3^- group, a very significant anodic shift of 285 mV to E_0 = –175 mV (see 18) can be achieved which, within experimental error, coresponds exactly to the value of **9**,



Scheme 2. E_0 of enzyme mimics of cytochrome P450

the high-spin E·S complex of cytochrome $P450_{cam}$.

Interestingly these unusual modifications of the heme-thiolate model system produced good catalysts for epoxidations both in homogenous (**17**-PhIO, **18**-PhIO) and heterogenous fashion (**19**-PhIO) [36].

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