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EVIDENCE FOR HEME π CATION RADICAL SPECIES IN COMPOUND I OF HORSERADISH PEROXIDASE AND CATALASE

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Magnetic circular dichroism spectra are reported for the compound I species of beef liver catalase (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, EC 1.11.1.6) and horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) and the π cation radical derivatives of porphyrins that have been suggested as models of the electronic configuration of the heme in the compound I species of these enzymes. Comparison of the magnetic circular dichroism spectra of the compound I species with the spectra of [Co(octaethylporphyrin)]2Br and [Co(octaethylporphyrin)]2ClO_4 indicates that in both the intermediate enzyme species the heme has been oxidized to a π cation radical. While there is a clear distinction between the magnetic circular dichroism spectra of the $^2A_{2u}$ porphyrin, [Co(III)octaethylporphyrin]2ClO_4, and the $^2A_{1u}$ porphyrin, [Co(III)-octaethylporphyrin]2Br, such specific differences are not observed in the spectra of the two enzymes. Analysis of our data suggests that the ground states in the two enzymes are far more similar than the ground states in the two model porphyrins.

Introduction

During the well known reactions of peroxidases and catalases with peroxide, an intermediate species, compound I, is formed that is two oxidizing equivalents above the native heme [1–10]. Two reaction schemes are applicable for these enzyme redox cycles [1,9,10]: the peroxidatic reaction for both peroxidases and catalases:

(1) ferric enzyme + H_2O_2 → compound I + H_2O
(2) compound I + AH_2 → compound II + AH
(3) compound II + AH_2 → ferric enzyme + AH

and the catalatic reaction observed, primarily, for catalase:

(4) ferric catalase + H_2O_2 → compound I + H_2O
(5) compound I + H_2O_2 → ferric catalase + O_2 + H_2O

Whilst the stoichiometry of these reactions has been firmly established, the exact location of the oxidizing equivalents in both compounds I and II has not been determined [1–4,11–15].

The results from magnetic susceptibility experiments [16,17], and extensive Mossbauer [18–20] and EPR [18,21–23] studies of horseradish peroxidase compound I show that the iron in the heme has an electronic configuration of Fe(IV) with an $S = 1$ spin state. The second oxidizing equivalent results in a third unpaired electron that the EPR data indicate must be located close to the paramagnetic iron [18,21]. This is quite unlike the case in cytochrome *c* peroxidase complex ES (the

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Abbreviations: MCD, magnetic circular dichroism; OEP, octaethylporphyrin.

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analogous compound I species, which is also two oxidizing equivalents above the native enzyme) where the radical is located far enough away from the Fe(IV) that an intense $g = 2.00$ signal can be seen [24]. Reduction of both catalase and horseradish peroxidase compound I species to compound II results in spectroscopic data that is now quite similar to that of cytochrome *c* peroxidase complex ES. This result implies that it is the organic free radical that is reduced, not the Fe(IV) [25–27].

Some years ago, Dolphin and coworkers suggested that certain synthetic porphyrins, when oxidized, were models of the electronic configuration in the hemes of these compound I spcies [28–32]. EPR data provided evidence for π cation radical formation following oxidation of a wide range of tetraphenyl- and octaethylporphyrins. These results also indicated that the top filled a_{2u} and a_{1u} orbitals could invert in some π cation radical complexes and that the ground state configuration of the oxidized porphyrin depended on this ordering. The optical absorption data of the resultant $^2A_{2u}$ and $^2A_{1u}$ porphyrin π cation species were quite different. From similarities in the 500–700 nm region of the absorption spectra of [Co(III)OEP]$2ClO_4$ (a^2A_{2u} ground state species) and [Co(III)OEP]$2Br(^2A_{1u})$, and the compound I species of horseradish peroxidase and catalase, it was suggested that horseradish peroxidase contains a $^2A_{2u}$ π cation radical heme, while in catalase the heme is a $^2A_{1u}$ π cation radical [29]. However, alignment of the various sets of absorption data is not at all close outside this limited 500–700 nm range.

The magnetic circular dichroism technique provides precise information on the magnetic properties of the excited states of molecules, and thus MCD data can be used to aid in the characterization of electronic configurations [33–37]. In the study described in this paper we have measured MCD spectra for each of the species described above, the two oxidized enzymes and the two cobalt octaethylporphyrin π cation radical complexes. The greater detail observed in these MCD spectra does allow a more extensive comparison between the model compound and enzyme data than was possible previously using the absorption data alone. Additionally, the MCD spectra clearly shows that although there is considerable similarity between the spectra of the two enzyme species, neither of the enzyme spectra completely match either of the [cobalt octaethylporphyrin]$^{2+}$ spectra.

Experimental

Freeze-dried horseradish peroxidase (Boehringer Mannheim; Grade I; purity number, $A_{403}/A_{280} = 3.2$) was dissolved in triply-distilled water and cooled to 0°C for all experiments. Compound I was prepared by pretreating the peroxidase by addition of 1.5 mol equiv. H_2O_2 24 h previously. A further 1.5 mol equiv. of H_2O_2 was added to the cuvette at 0°C to make the compound I used for the spectra reported here. Both the absorption spectra (Cary 219) and magnetic circular dichroism spectra (Jasco J5, with an Ithaco 319A Lock-In amplifier and an Oxford Instruments SM2 superconducting magnet) were digitized as they were recorded (Scheiring, M.J. and Stillman, M.J., unpublished data), the MCD data presented here are computer plots of the spectrum as recorded. The MCD and CD data were calibrated as previously described [38].

The absorption spectrum of the horseradish peroxidase compound I was retraced after the first scan, and this second line was identical to the first within the pen width. There was no indication of additional compound II formation in the MCD spectrum after the initial spectrum had been run. Under the MCD spectrometer settings used, the compound I CD spectrum had no significant intensity and the MCD data presented here have not had the CD spectra subtracted. Computer plots of spectra, where the CD spectrum had been substracted, were substantially noisier but there were no differences in the intensity of the bands associated with the heme spectral region.

Bovine liver catalase, a crystalline suspension in 0.01% alkyl benzyldimethyl ammonium chloride from Boehringer Mannheim, was prepared by exhaustive dialysis against triply-distilled water and finally against a $I = 0.05$ pH 7 phosphate buffer. Catalase compound I was prepared by adding a 50-fold excess of peroxoacetic acid to a solution of native catalase at 2°C [30]. Other experimental conditions were as previously reported [30]. The data presented here are retraced computer plots; the noise level of the MCD data were similar to that shown in Fig. 2.

Cobalt octaethylporphyrin was prepared according to the acetate method of metal insertion into octa-

ethylporphyrin [10] and oxidized in CH_2Cl_2 by addition of excess dilute Br_2 in CCl_4. The bromide to perchlorate ligand exchange was achieved by passing the solution over $AgClO_4$ [28,40]. The room temperature spectra of the oxidized species shown in Figs. 3 and 4 were obtained with the same equipment and in a similar fashion to the catalase compound I spectra.

Results

The absorption spectrum of horseradish peroxidase compound I, Fig. 1, clearly illustrates the low intensity and broad, poorly resolved series of bands that is characteristic of the spectra of these compound I species. We should note though that cytochrome *c* peroxidase complex ES (i.e., compound I) exhibits well resolved bands in both its absorption [27] and MCD (Laframboise, C., and Stillman, M.J., unpublished data and Ref. 42) spectra, both of which are similar to the catalase [38] and horseradish peroxidase [42,43] compound II data. The MCD spectrum of horseradish peroxidase compound I, Fig. 1, provides little evidence as to the identity of each transition. The envelope of bands between 300 and 700 nm comprises, in particular, a negative band of 660 nm, a broad, positive set of bands between 450 and 650 nm and, finally, a rather weak and distorted Soret band centered on 400 nm. In this spectrum the peak to trough line of the Soret MCD band is not a straight line, which suggests that at least two transitions are responsible for the observed line shape.

In the MCD spectra of many heme proteins the Soret band dominates the envelope of transitions [35,43,44]. While the magnitude of the absorption band is very low for compound I, approx. $50 \cdot 10^3$ l $mol^{-1} \cdot cm^{-1}$, MCD intensity for the peak to trough measurement of the distorted derivative-shaped signal observed at 400 nm of about $15 \cdot 10^4$ deg $cm^2 \cdot dmol^{-1} \cdot T^{-1}$ compares well with similar data of Nozawa et al. [44] for the native enzyme at pH 11.9, approx. $30 \cdot 10^4$, and the fluro-derivative, approx. $7 \cdot 10^4$, but is much less intense than typical low spin ferric or ferrous derivatives, where the span may be as high as the $60 \cdot 10^4$ deg $cm^2 \cdot dmol^{-1} \cdot T^{-1}$, as observed with the ferric cyano-derivative. We should note that in our earlier MCD data [42] a shoulder was

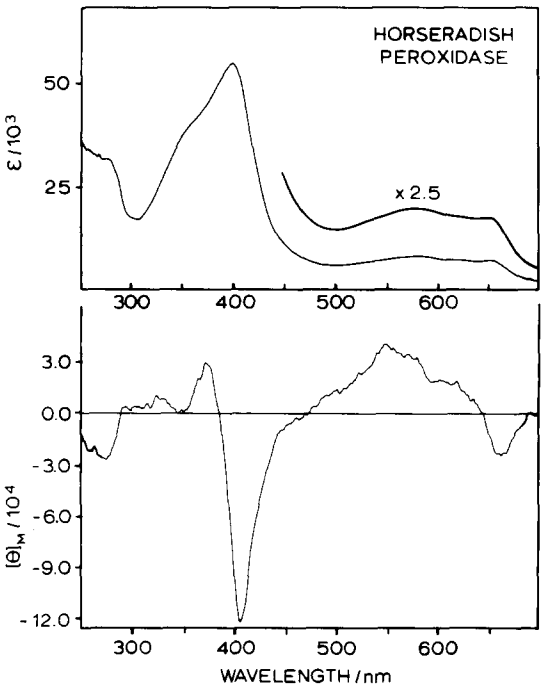

Fig. 1. The absorption and MCD spectra of $6.63 \cdot 10^{-6}$ M horseradish peroxidase compound I in triply-distilled water at 0°C.

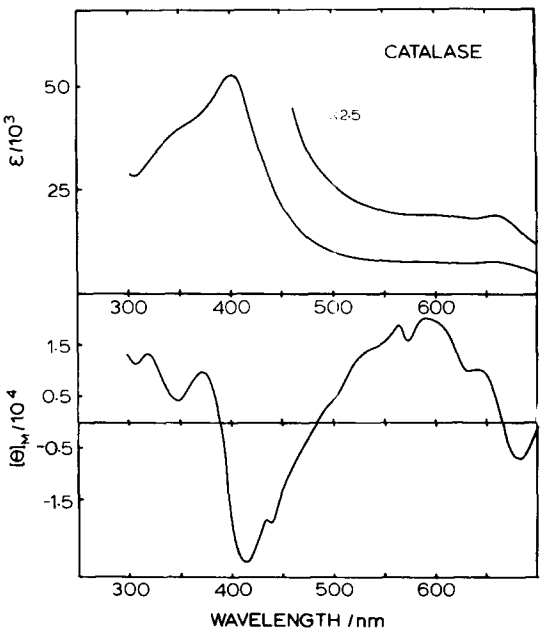

Fig. 2. The absorption and MCD spectra of $8.9 \cdot 10^{-6}$ M (per heme) beef liver catalase compound I pH 6.9, $I = 0.05$ at 2°C.

prominent to low energy of the Soret band. This shoulder, which we attribute to a compound II impurity, is absent in the spectrum of compound I shown in Fig. 1.

Fig. 2 shows the absorption and MCD spectra recorded for catalase compound I at 2°C. As for horseradish peroxidase compound I, the absorption spectrum shows little resolution apart from the band at 656 nm and the Soret band at 400 nm. The MCD spectrum is also quite similar to that found for horseradish peroxidase although the 656 nm band appears considerably better resolved in the catalase, an almost A-term shape in observed. However, with so many overlapping bands present it is possible that this derivative shape arises from the overlap of two oppositely signed B or C terms. Weak bands at 570 and 440 nm in the catalase compound I spectra arise from a compound II impurity. The bandwidths of both the absorption and MCD signals in the Soret region are slightly greater for catalase than for peroxidase and one result of this appears to be the rather steeper angle for the trace from 500 to 400 nm for the peroxidase.

Figs. 3 and 4 show absorption and MCD data for the two derivatives of $[Co(III)OEP]^{2+}$: the perchlorate derivative which has the $^2A_{2u}$ ground state, Fig. 3, and the bromide derivative which has the $^2A_{1u}$ ground state, Fig. 4 [29].

The perchlorate spectra, Fig. 3, closely resemble those of $MTPP^+$ cation radical species [41], in which the well resolved Q_{00} and Q_{vib} bands of the unoxidized porphyrins [45] have been replaced in the visible region by band envelopes which are very diffuse, having a characteristic negative band between 600 and 660 nm, and a Soret band region dominated by a narrow and intense A term centered under the most strongly absorbing band at 399 nm. It can be seen that the magnitude of the span of Soret band MCD is nearly 20-times greater than the negative band at 640 nm. This Soret band MCD intensity is close to that observed for the unoxidized Co(II)OEP, about $51 \cdot 10^4$ deg $cm^2 \cdot dmol^{-1} \cdot T^{-1}$ in both spectra [40]. By way of contrast the extinction coefficient at the Soret band for the oxidized species (approx. $70 \cdot 10^3$ $l \cdot mol^{-1} \cdot cm^{-1}$) is only one-third the magnitude of the unoxidized species. Thus, it appears that the perchlorate complex retains much of a typical

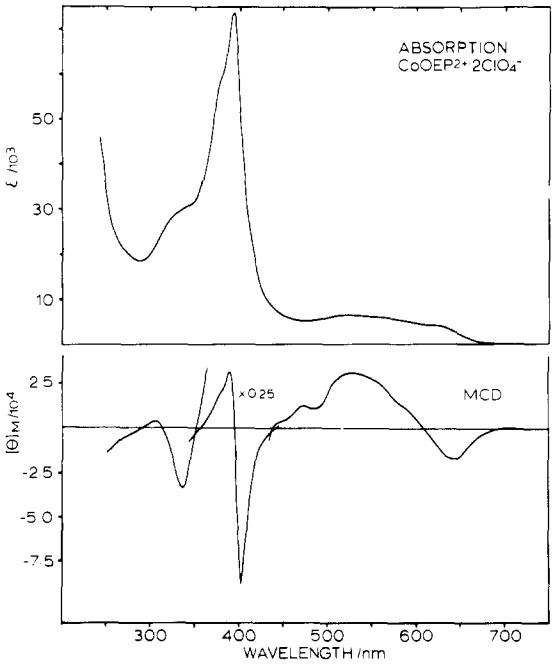

Fig. 3. The absorption and MCD spectra of $8.58 \cdot 10^{-6}$ M $[Co(III)OEP]^{2+}$ $2ClO_4^-$, diperchloro-(octaethylporphyrinato)-cobalt(III) in CH_2Cl_2 at room temperature.

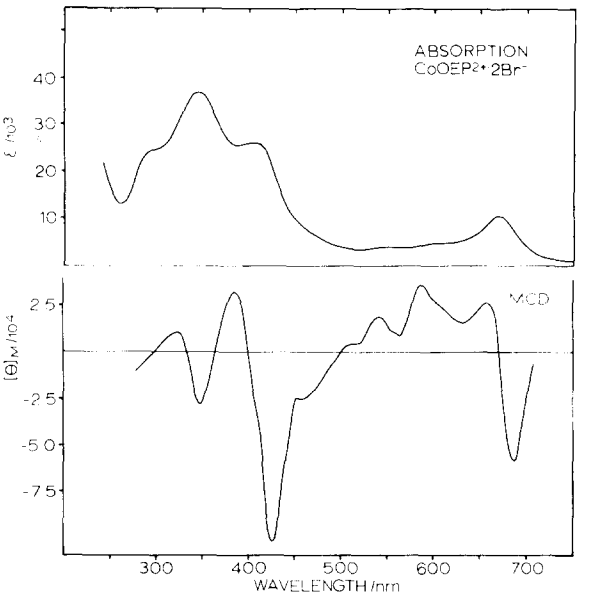

Fig. 4. The absorption and MCD spectra of $8.58 \cdot 10^{-6}$ M $[Co(III)OEP]^{2+}2Br^-$, dibromo-(octaethylporphyrinato)-cobalt(III) in CH_2Cl_2 at room temperature.

porphyrin MCD intensity. It is significant that the MCD spectrum is much better resolved than the corresponding absorption spectrum, and, additionally, the MCD data provides the intense A term marker under the Soret band as an identifier for the $^2A_{2u}$ class of oxidized ring [4].

Axial ligation by Br^-, Fig. 4, greatly reduces the Soret band MCD intensity in the [cobalt octaethylporphyrin]$^{2+}$ species. A new band is also now observed at 350 nm. There are apparently no A terms in the MCD spectrum in the Soret region. Because of the considerable reduction in intensity, the Soret band MCD intensity is now almost the same as the visible region. Under the absorption band at 670 nm we observe a reasonably well resolved derivative signal, possibly an A term or two oppositely-signed B terms. The MCD spectrum of the bromide complex can be considered to resemble that of a typical phthalocyanine rather than a porphyrin, [46,47] where the Q band intensity equals that of the Soret bands, and the resolution in the Soret region is much less distinct than in porphyrin spectra. Although this association is rather loose, it does provide support for the assignment of the ground state as $^2A_{1u}$ [29].

Discussion

It is clear when comparing the MCD spectra of catalase and horseradish peroxidase compounds I in Figs. 1 and 2, with the spectra recorded for the porphyrin π cation radical species in Figs. 3 and 4, that the hemes in both enzymes are also π cation radicals. All the major features of the enzyme data can be clearly seen in the model compound data. Comparison of the absorption spectra alone does not provide a sufficiently good alignment, especially outside the limited 500–700 nm region, for the electronic configuration of the enzyme intermediates to be determined unambiguously. However, the additional assignment criteria provided in the MCD spectra now show that the excited states in the enzymes are very similar to those found in the porphyrin π cation radical model compounds.

Because the MCD spectra of the two cobalt porphyrins are so different, it seemed likely that the MCD spectra of the enzymes would also allow identification of the specific oxidized orbital involved. This analysis would then complement the extensive treatment of the EPR and room temperature absorption data [28–31,48]. The nodal patterns of the a_{1u} and a_{2u} orbitals given by Gouterman [45] are shown in Fig. 5. In a normal porphyrin, the a_{2u} orbital is the highest occupied, while in phthalocyanines [46] and some substituted porphyrins (for example, octamethyltetrabenzpophyrin) the a_{1u} orbital lies highest. The spectral characteristics of unoxidized molecules in which the orbital ordering places a_{1u} above a_{2u} are very different compared with porphyrins with a_{2u} above a_{1u} [45,46]. However, for the enzyme data discussed here, and for [Co(III)OEP]2Br and [Mg(II)OEP]$^+$, the ordering is only thought to change following the oxidation of the π ring.

We find that the MCD spectra of the enzymes do not allow a definitive assignment of the ground state. As a result our data do not support a ground state assignment based on absorption data alone. The intense Soret A term of the $^2A_{2u}$ is absent from both sets of data. The resolved visible region A term of the $^2A_{1u}$ species seems to appear only in catalase, but even here the superposition of two B or C terms could, accidentally, give the same envelope shape. The catalase data initially looks most like the $^2A_{1u}$ spectrum, however, the visible region positive bands between 480 and 660 nm are modelled much better by the perchlorate complex, the $^2A_{2u}$ species. The lack of an A term-like envelope at 660 nm and the

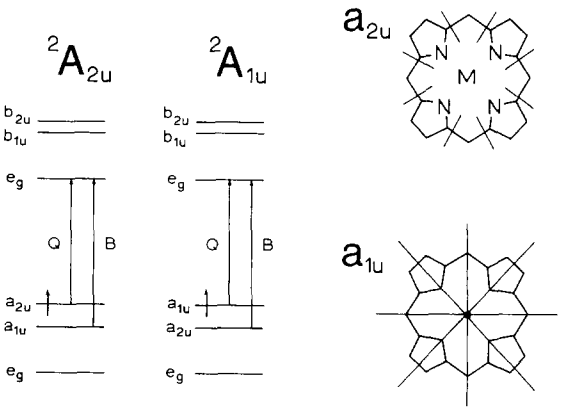

Fig. 5. Molecular orbitals, state symmetries and nodal plane patterns for the two different ground state configurations of the porphyrin π cation radical species, the $^2A_{2u}$ for the [cobalt(III)octaethylporphyrin]2ClO_4 and the $^2A_{1u}$ for the [cobalt(III)octaethylporphyrin]2Br. Data taken from Gouterman [45] and Dolphin et al. [28–32].

close resemblance of the 500–640 nm region of horseradish peroxidase compound I spectrum to the perchlorate radical spectrum seems to suggest a $^2A_{2u}$ ground state.

Unfortunately, for this analysis, the Soret region of the peroxidase spectrum is much closer in band shape to the $^2A_{1u}$ bromide spectrum than the $^2A_{2u}$ perchlorate spectrum, and there is no indication of the intensity seen in the $^2A_{2u}$ spectra (Fig. 3).

An approximate calculation of the peak to trough MCD intensities for the Soret and 650 nm bands gives about a 28 : 1 ratio for Soret-to-visible band in the $^2A_{2u}$ radicals compared with a 2 : 1 for the $^2A_{1u}$ radical, the comparable ratios for the enzymes are 7 : 1 for the peroxidase, and 6 : 1 for the catalase. The absolute values of the signals also differ considerably between each system, the horseradish peroxidase compound I spectrum is almost 5-times more intense than the catalase data. Similar intensity reductions are also observed in the MCD spectra of the ferric cyano-derivatives of these enzymes and are thought to be due to spin-state differences in metal-porphyrin interaction [43].

The paramagnetic contributions of the ferryl ion and the π cation radical to these spectra cannot be determined without temperature dependence studies. It is well known that spin-state interactions between the metal and the $a_{1u} \rightarrow e_g$ and $a_{2u} \rightarrow e_g$ transitions of both ferrous and ferric hemes result in significant and varied intensity changes in these $\pi \rightarrow \pi^*$ transitions. It would be expected that the porphyrin in these compound I species is also sensitive to the ferryl cation.

It is curious that with such completely different spectra for the model porphyrin π cation species, that the spectra of the enzymes do not reflect more clearly the molecular orbitals involved. Our conclusions from the MCD results almost suggest a blurring between the a_{1u} and a_{2u} orbitals, so that the distinction between the electron density locations shown in Fig. 5 is lost. Our result does however, support a very recent theoretical calculation by Loew and Herman [15] in which they find that the a_{1u} and a_{2u} orbitals are still very close in energy in the horseradish peroxidase oxidized heme, with a_{1u} only slightly below a_{2u}, thus the radical could easily be mixed into both orbitals. The extensive NMR study of La Mar et al. [49] offers confirmation of our view that the band at 660 nm in the optical absorption spectrum of the enzyme compound I species does not clearly indicate the ground state electronic configuration of the π cation radical.

Analysis of the reactions of H_2O_2 with either catalase or peroxidase to produce compound I suggest that an oxy-ferryl heme complex is formed [50,51]. This species may have the ferryl-oxene structure as suggested by comparison between the absorption spectra of horse erythrocyte catalase compound I and ferryl-oxene complexes prepared by reaction of FeOEP or Fe dimethylprotoporphyrin with iodosoxylene [11] and also the absorption spectra of oxidized vinylidene and carbene iron porphyrin complexes [12]. Magnetic susceptibility data indicate that these ferryl-oxene complexes have a high-spin d^4 electronic configuration. These data, together with the catalase compound I-like absorption spectrum of deutero horseradish peroxidase compound I, where there are larger paramagnetic contributions to the peripheral methyls of the heme than native horseradish peroxidase I, [13] suggest that the 670 nm band intensity may indeed be sensitive to the spin characteristics of the metal.

Much of the interest in the analysis of the electronic structure of the horseradish peroxidase and catalase compound I intermediates has been derived from the implied relationship with their functional characteristics [2,3]. However, the electronic differences, even if they are found to be more important than metal-porphyrin spin interaction changes may, in the end, yield only limited functional information. Even though the deutero horsedarish peroxidase compound I spectra [52] do not resemble the native peroxidase, its activity appears unchanged. Thus the protein appears to regulate the binding of neutral substrates which will be ionized in the heme crevice, and the mechanism of compound I reduction in either the catalytic or peroxidatic direction, as well as aiding in the stabilization of the ferryl π cation radical heme complex.

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