EPR Detection and Characterization of Lignin Peroxidase Porphyrin π-Cation Radical†

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ABSTRACT: Lignin peroxidase (LiP) from *Phanerochaete chrysosporium* catalyzes the H_2O_2 dependent one- and two-electron oxidations of substrates. The catalytic cycle involves the oxidation of ferric-LiP by H_2O_2 by two electrons to compound I,which is an oxoferryl heme and a free radical. It has been speculated that the unpaired electron is in a π delocalized porphyrin radical. However, no direct evidence for the presence of the free radical has been reported. We present electron paramagnetic resonance (EPR) detection and characterization of compound I of LiP. The LiP compound I EPR signal is different than those reported previously for compound I of horseradish peroxidase and chloroperoxidase. However, the EPR signal of compound I of LiP (axial g tensor extending from $g_\perp = 3.42$ to $g_\parallel \sim 2$) is very similar to the EPR signals of compound I of ascorbate peroxidase and catalase from *Micrococcus lysodeikticus*, in which the radical has been identified as a porphyrin π-cation radical. On the basis of the analysis of our data and comparison with the earlier published results for compounds I of other peroxidases, we interpret the LiP compound I signal by a model for exchange coupling between an $S = 1$ oxyferryl $[Fe{=}O]^{2+}$ moiety and a porphyrin π-cation radical ($S' = 1/2$) [Schulz, C. E., et al. (1979) *FEBS Lett. 103*, 102−105]. The exchange coupling is characterized by ferromagnetic rather than an antiferromagnetic interaction between the two species. The ferric-LiP EPR signal suggests that the iron in the heme is in near perfect orthogonal symmetry and provides additional evidence of the ferromagnetic interaction between the oxoferryl iron center and the porphyrin π-cation radical.

Phanerochaete chrysosporium is in a class of fungi that are the most efficient lignin degraders in nature (Buswell & Odier, 1987; Kirk & Farrell, 1987; Hammel et al., 1994). The ability of the fungus to degrade lignin, a bulky, stereoirregular polymer of woody plants, depends largely on a nonspecific, extracellular enzymatic system synthesized upon nutrient limitation (Tien & Kirk, 1983). The nonspecificity of the lignin degrading system gives these fungi the ability to degrade a wide variety of environmental pollutants (Barr & Aust, 1994). The major component of the lignin-degrading system is a group of heme containing peroxidases (Tien & Kirk, 1983). The peroxidases are divided among two broad groups, one that oxidize various organic and inorganic compounds directly, called lignin peroxidases (LiP), and the other that require manganese for activity, called manganese-dependent peroxidases.

The catalytic cycle of LiP is similar to that of other peroxidases (Chance, 1952; Dunford, 1990; Tien, 1987). Ferric-LiP is oxidized by H_2O_2 by to an intermediate called compound I (LiP I). Compound I contains two oxidizing equivalents, one of which is stored in the enzyme as an oxoferryl moiety $[Fe(IV){=}O]^{2+}$ and the other as a radical (Tien, 1987). In most peroxidases and other heme-containing proteins that react with H_2O_2, the radical is a π-orbital delocalized porphyrin radical (Dunford, 1990). The only known exception to this rule is cytochrome *c* peroxidase which contains a tryptophan cation radical though it is thought that the tryptophan cation radical entails the formation of a porphyrin π-cation radical (Sivaraja et al., 1989). This general trend has led most to propose the same for LiP. However, no EPR observations of LiP I have been reported. One earlier attempt to detect the radical was unsuccessful (Paszczynski et al., 1986). The characterization of the radical is important to our understanding of the LiP catalysis and possible structure−function relationship for the protein and the heme prosthetic group, not only for this and other peroxidases but for heme proteins in general. Therefore, we have attempted to detect and characterize the nature of the radical in LiP compound I. We have also analyzed the ferric-heme by 4 K EPR in order to better understand the active site geometery, especially the iron coordination environment in the heme. We have also attempted to correlate the sign and magnitude of the exchange parameter [for the coupling of the oxoferryl moiety ($S = 1$) and the porphyrin π-cation radical ($S' = 1/2$)] with the nature of the proximal axial imidazolate ligand.

MATERIALS AND METHODS

Materials. Hydrogen peroxide and tartaric acid were purchased from Sigma Chemical Company. Sodium succinate and succinic acid were purchased from Mallinckrodt. All chemicals were reagent grade and were used without further purification. All buffers and solutions were prepared using purified water (Barnstead NANOpure II system; specific resistance 18.0 MΩ cm^{-1}). Pure lignin peroxidase isozyme H2 (pI = 4.4) was used throughout the study and was purified as described previously (Tuisel et al., 1990).

EPR Measurements. All EPR measurements were performed on a Bruker ESP300 spectrometer equipped with a

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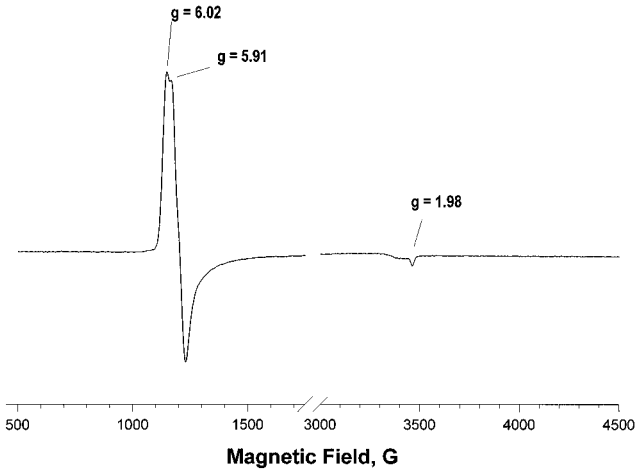

FIGURE 1: 4 K EPR spectrum of ferric-LiP. Aquisition conditions were as follows: enzyme concentration, 0.25 mM LiP in 20 mM, pH 4.5, succinate buffer; microwave frequency, 9.64 GHz; microwave power, 0.5 mW; modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 81.92 ms; scan time, 83.89 s; gain, 1×10^5.

Oxford ESR 10 helium flow cryostat and DTC-2 temperature controller. Instrument settings are given in figure legends. EPR saturation data were collected by measuring the EPR absorption derivative signal intensity as a function of microwave power at different temperatures. The saturation data were fit to the expression

$$S/P^{1/2} \propto 1/(1 + P/P_{1/2})^{1/2} \qquad \text{(a)}$$

where S is the derivative signal intensity, P is the microwave power, and $P_{1/2}$ is the half-saturation power. A nonlinear least-squares fit to the above equation yielded a $P_{1/2}$ value for each particular temperature.

RESULTS

EPR Spectrum of Ferric Lignin Peroxidase. An EPR signal of ferric-LiP at 4 K is shown in Figure 1. The signal was characteristic of a high spin iron with $g_\perp = 6$ and $g_\parallel = 1.97$, though it is evident that the EPR spectrum was a mixture of sharp axially symmetric signal at $g_\perp = 6$ and a weak rhombically distorted axially symmetric signal. A rhombically distorted high spin signal, with easily resolved x, y, and z components, was not detected. The high spin ferri-heme signal was similar to the one observed for aged C*c*P (Yonetani et al., 1987). However, unlike aged C*c*P, which exhibits a low spin ferric signal and does not react with H_2O_2, no appreciable difference could be detected for the rate of reaction of fresh or aged LiP with H_2O_2. Also, the failure to detect rhombic distortion was not accompanied by an EPR signal for a low spin ferri-heme. Thus, if the absence of rhombic distortion is due to axial binding of an internal ligand, possibly a water molecule associated with the distal histidine, the ligand field strength would be "intermediate", as it is the symmetry and the strength of the ligand field which determines the planarity and the spin state of the ferri-heme. Decreasing axial ligand field strength results in increasing tetragonal distortion ($z < x,y$) with the spin state changing from 1/2 to 5/2 to a mixture of 5/2 and 3/2 and finally with further decrease in the ligand field results in a pure 3/2 spin state. This supposition is in accord with the structural data (Poulos et al., 1993) as strong field side chains of distal residues are not within the inner sphere coordination distance of the heme iron. Furthermore, the presence of glycerol, which prevents the freezing-induced conversion of high spin to low in C*c*P, had no effect on the ferric-LiP signal (spectrum not shown) suggesting that the freezing did not induce a conformation change in the distal pocket that could have resulted in axial binding of an internal strong field ligand. Another possible explanation for lack of rhombic distortion in the ferri-heme signal is that there may be near perfect orthogonal symmetry for the iron in the heme. We prefer this supposition to the possible internal ligand binding, as is the case with C*c*P (Yonetani et al., 1987), to the sixth coordination site. This will be discussed later, and the rationale for this supposition will be clear when presented in conjunction with the analysis of the nature of the LiP compound I radical.

EPR Spectrum of Lignin Peroxidase Compound I. The EPR signal obtained subsequent to the addition of 2.0 equiv of H_2O_2 to LiP and rapid cooling is shown in Figure 2. The ferri-heme signal disappeared, and a new pattern extending from $g = 3.42$ to $g \sim 2.00$ was observed. The observation that the two signals ($g \sim 3.42$ to $g \sim 2.00$) were connected was confirmed by Fourier transform from the time to the frequency domain. Although this new signal was weak, its shape indicated that it was strongly saturated even at the low microwave power employed (53 dB), and that the 100-kHz field modulation was inducing a rapid passage condition. Concomitant with the observance of this EPR signal, the electronic absorption signal of ferric-LiP in the Soret region exhibited a decrease (inset; Figure 2A). This decrease has been taken as a measure of compound I formation. Also, bleaching of the Soret was possibly due to decrease in the energy from π to π^* transitions of the heme with a porphyrin π-cation radical. Therefore, the EPR signal was tentatively assigned to a porphyrin π-cation radical of LiP compound I resulting from the two-electron oxidation of ferric-LiP by H_2O_2.

We also investigated the stoichiometery of ferric-LiP reaction with H_2O_2 (Figure 3). The formation of LiP I was followed by measuring the EPR signal intensity of the $g \sim 2$ signal and the decrease in absorbance at 408 nm. The change in absorption at 408 nm with the addition of indicated amount of H_2O_2 is shown as plot a. The EPR signal intensity at $g \sim 2$ as a function of number of equivalents of H_2O_2 is also presented (plot b). The break in curves at the equivalent amount of H_2O_2 and the EPR signal intensity indicated that a 1:1 reaction of H_2O_2 with ferric-LiP was required for LiP I formation. Additionally, we also quantitated the concentration of species corresponding to the $g \sim 2$ signal using a Cu^{2+} reference. The integrated intensity of the $g \sim 2$ EPR signal corresponded to within 14% of the LiP concentration. Within experimental error and considering the fact that the LiP I signal was saturated even at low microwave power, the integrated spin concentration was assumed to yield one porphyrin π-cation radical per mole of LiP per equivalent of H_2O_2 added.

Spin Coupling Analysis. The presence of a broad EPR signal for LiP I is similar to HRP I (Schulz et al., 1979), CPO I (Rutter et al., 1984), *Micrococcus lysodeikticus* catalase compound I (Benecky et al., 1993), and APX I (Patterson et al., 1995). The only difference is that $g_\parallel \sim 2$ and $g_\perp < 2$ for CPO I (Rutter et al., 1984) but for others and LiP I $g_\perp > 2$. In spite of these differences, the

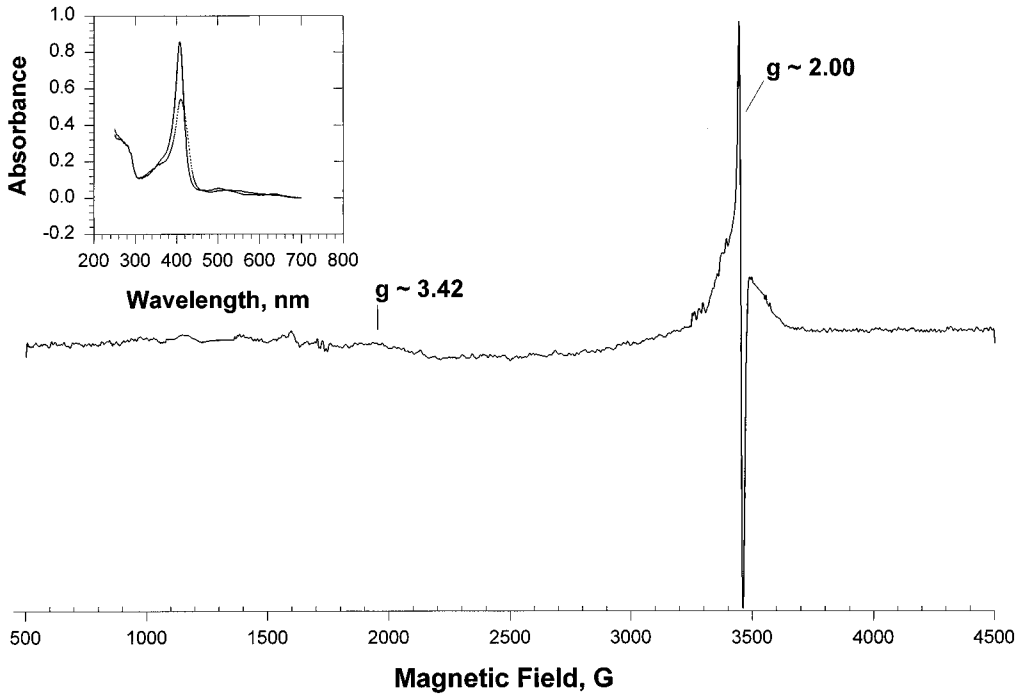

FIGURE 2: 4 K EPR spectrum of the product of H_2O_2 reaction with ferric-LiP. Aquisition conditions were as follows: reactants, 0.25 mM LiP in 20 mM, pH 4.5, succinate buffer and 0.5 mM H_2O_2; temperature, 4 K; microwave frequency, 9.64 GHz; microwave power 0.1 μW (53 dB); modulation amplitude, 5.0 G; modulation frequency, 100 kHz; time constant, 163.34 ms; scan time, 163.85 s; gain, 1×10^4. (Inset) Electronic absorption signal of ferric-LiP and its H_2O_2 reaction product. The decrease in absorbance at 409 nm is indicative of compound I formation, subsequent to a two-electron oxidation of ferric-LiP by H_2O_2.

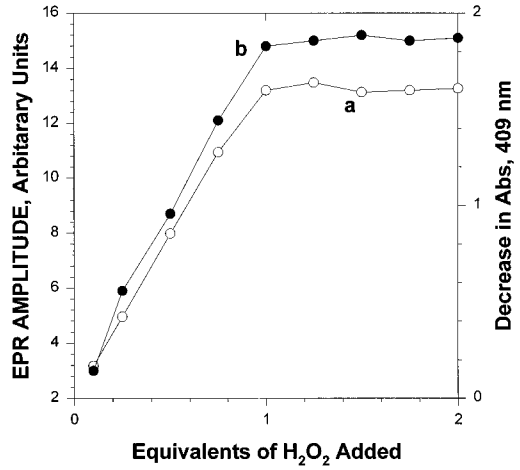

FIGURE 3: Titration of LiP with H_2O_2 followed as an absorbance change at 409 nm and the amplitude of the $g \sim 2.0$ EPR signal. The open circles represent the decrease in absorbance at 409 nm, and the closed circles represent the EPR signal intensity of the $g \sim 2$ signal using 0.25 mM LiP. Due to the high extinction coefficient of LiP (169 000 M^{-1} cm^{-1}), a small aliquot of the reaction mixture was taken and diluted to aquire the electronic absorption spectrum.

compounds I spectra of these peroxidases can be interpreted by using the spin-coupling model of Schulz et al. (1979). In this model $S = 1$ oxoferryl moiety and $S' = 1/2$ porphyrin π-cation radical are weakly exchange coupled as described by $H_{ex} = JS \cdot S'$; and this, in combination with a positive zero field splitting at the $[Fe{=}O]^{2+}$ moiety (splitting parameter, D), causes six energy levels $[(2S + 1)(2S' + 1) = (3)(2)]$ of the oxoferryl porphyrin π-cation radical system to form three Kramer's doublets. The lowest doublet is well separated in energy from the other two and gives rise to the observed EPR spectrum which can be represented in terms of the effective spin ($S_{eff} = 1/2$). The effective g values, $g_{\perp eff}$ and $g_{\parallel eff}$, are determined by the ratio J/D (Shulz et al., 1979; Rutter et al., 1984). To a first approximation, $g_{\parallel eff} \approx g_e$ and $g_{\perp eff} \approx g_e - 2g_{\perp}^{Fe}J/D$, where $g_{\perp}^{Fe}$ is associated with the isolated $[Fe{=}O]^{2+}$ moiety and is 2.25 for the oxoferryl heme (Hoffman et al., 1981) and J/D is the ratio of exchange coupling to the zero-field splitting parameter. As D is expected to be greater than zero for the oxoferryl heme center (Hoffman et al., 1981), a value for $g_{\perp}$ that is greater than $g_{\parallel}$ results from ferromagnetic coupling ($J < 0$). For LiP I the radical therefore seems to be ferromagnetically coupled to the oxoferryl heme with $|J|/D = 0.29$. These values compare remarkably well with those reported for *M. lysodeikticus* catalase I (0.40) (Benecky et al., 1993) and ascorbate peroxidase I (0.28) (Patterson et al., 1995). It is useful to note that exchange couplings characterized by this ratio cause a negligible (<3%) change in the hyperfine couplings to the protons on the porphyrin.

Further evidence that the oxoferryl moiety affects the EPR signal of LiP I comes from power saturation data. These data can also be used to determine the temperature dependence of spin−lattice relaxation time. As mentioned before, according to our model the porphyrin π-cation radical is spin coupled to the oxoferryl moiety which has a large zero field splitting parameter, D. The resulting system consists of three Kramer's doublets, the lowest of which is responsible for the observed EPR spectrum. The temperature dependence of the spin−lattice relaxation rate ($1/T$) was measured by the method of progressive saturation, and a summary of the results is shown in Figure 4. A plot of ln $P_{1/2}$ as a function of $1/T$ yielded a straight line with the slope equal to excited state energy, Δ, 33 K. For weak coupling, $|J| \ll D \sim 30$ K, the ground doublet, which gives rise to the EPR signal, is separated from the excited states by an energy $\Delta \sim D$. Since phonon-induced transitions within a doublet are forbidden in first order, spin relaxation is dominated by Orbach

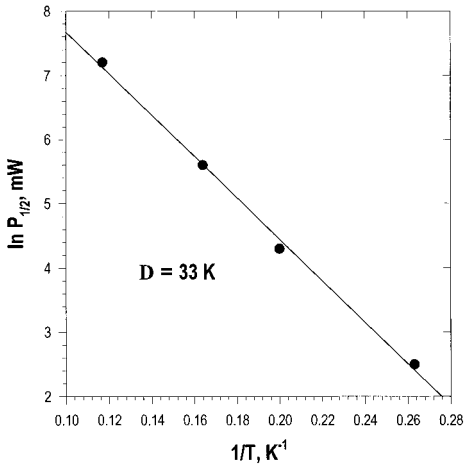

FIGURE 4: Half-saturation power of the $g \sim 2$ EPR signal of LiPI as a function of temperature. The $P_{1/2}$ values were calculated as described in Materials and Methods. All parameters were the same as those for Figure 2 except that modulation frequency equal to 1 kHz was used. The solid line is the fit of the logarithm of $P_{1/2}$ according to $S/P^{1/2} \propto 1/(1 + P/P_{1/2})^{1/2}$. The slope of the plot gives the excited state energy, $\Delta = 33$ K.

processes via excited states. Therefore, from these data the zero-field splitting can be predicted, within reasonable error, to be $\sim$33 cm^{-1}.

DISCUSSION

The results of this study show that a porphyrin π-cation radical is formed upon oxidation of ferric-LiP by H_2O_2. Although there are considerable differences between the EPR signal of LiP I, catalase I, and ascorbate peroxidase I and the analogous compounds I of HRP and CPO, all these can be analyzed in terms of spin coupling between the zero-field split oxoferryl iron ($S = 1$) and the porphyrin π-cation radical ($S' = 1/2$). An excellent discussion of the subject has been published by Benecky et al. (1993) and is essentially repeated here.

For all these compounds the spin coupling can be characterized by the sign and magnitude of exchange parameter, J, and the zero-field splitting parameter, D. The well characterized model complexes of the form $[(P)-Fe{=}O]^{2+}$ represent an example of strong ferromagnetic exchange ($J < 0$) where $|J|/D \geq 1$. Lignin peroxidase I is only the third protein bound system to exhibit ferromagnetic exchange ($J < 0$), with an intermediate strength of $|J|/D = 0.29$. Horseradish peroxidase I exhibits an extremely weak exchange, the simulation of which required that the $|J|/D$ be assigned both ferromagnetic and antiferromagnetic values (Schulz et al., 1979). CPO I, on the other hand, exhibits an appreciable antiferromagnetic exchange ($J > 0$), with $|J|/D = 1.02$ (Rutter et al., 1984). Finally, catalase I and APX I showed ferromagnetic exchange ($J < 0$) with $|J|/D = 0.40$ and 0.28, respectively. Thus, $|J|/D$ goes from being large and negative to moderately negative, through near zero to moderately positive (antiferromagnetic) in the series $[(P)-Fe{=}O]^{2+}$, catalase I, LiP I, APX I, HRP I, and CPO I. The parameter D is uniformly positive and has been found to vary over only a modest range: for example $D = 20-25$ cm^{-1} for the $[(P)Fe{=}O]^{2+}$ model compounds, $D = 22$ cm^{-1} for HRP I, $D \sim 35$ cm^{-1} for CPO I, and within reasonable error $\sim$32 cm^{-1} for LiP I. Thus, the changes in $|J|/D$ within this series of compounds I primarily result from differences in the exchange coupling, J.

The variation within this series from ferromagnetic to antiferromagnetic coupling between the $[(P)Fe{=}O]^{2+}$ and the porphyrin π-cation radical (P^+) must reflect an increasing departure from the idealized four-fold geometery provided by the coordination of iron by porphyrin. The two odd electrons in an oxoferryl, $[Fe{=}O]^{2+}$, $S = 1$, moiety reside one each in the two antibonding Fe=O molecular orbitals that have π-symmetry with respect to the diatomic axis. In a four-fold symmetric metalloporphyrin, the half-filled orbitals of the $[Fe{=}O]^{2+}$ center, and the π-molecular orbitals of the porphyrin radical are orthogonal in symmetry. In such a case, correlation effects tend to force the spins of the two magnetic subsystems to align parallel, and the result can be described in terms of a ferromagnetic exchange, $J_f < 0$. If symmetry is reduced to such a degree that the magnetic orbitals of the metal and the porphyrin are no longer orthogonal, an additional bonding interaction tends to align the subsystem spins antiparallel that can be described in terms of an antiferromagnetic contribution to the exchange, $J_{AF} > 0$. In general, the observed exchange coupling, J, is a result of the competition between the two opposing tendencies. It is reasonable to assume that the major element in this reduction of symmetry for the proteins is π-bonding between the Fe(IV) and the proximal endogenous axial ligand. For CPO I, where the axial ligand is a strongly π-bonding cysteinyl thiolate, the effective symmetry is lowered and the orbital overlap is increased to the point where antiferromagnetic coupling dominates. Catalase compound I, where the axial ligand is a phenolate (Hu & Kincaid, 1992), has a small antiferromagnetic coupling. HRP I with an imidazole ligand has balanced ferro- and antiferro-magnetic coupling, and the result is $J \sim 0$. APX I and LiP I, both also with imidazolate ligands (Patterson & Poulos, 1995; Poulos et al., 1993), have small antiferromagnetic coupling and predominately exhibit ferromagnetic exchange between the oxoferryl and porphyrin π-cation radical. This suggests that not just the kind of proximal axial ligand but its π-bonding characteristics, possibly influenced by the microenvironment, are important in determining the exchange coupling, J.

Interpretation of the variations in the values of J in terms of increasingly unsymmetric heme environment can also be correlated to the g tensors of the parent high spin ferri-heme state ($S = 5/2$). A high spin ferri-heme of four-fold effective symmetry would have an axial g-tensor of $\delta_g \propto g_y - g_x = 0$. Reduction of the symmetry through π-bonding between the ferri-heme and the axial ligand and/or by the deformations of the porphyrin will progressively increase δ_g. Consistent with the expectations, the model compounds ($[PFe{=}O]^{2+}$) are high spin with $g_x \sim g_y \sim 6$ and $\delta_g = 0$, while ferric-CPO shows a large rhombic splitting, $\delta_g = 3.10$, HRP and catalase have intermediate values and LiP has a minimal value. This suggests that the heme iron is in near perfect orthogonal symmetry in LiP and there are minimal distortions (heme puckering) due to either π-bonding with the axial ligand or the microenvironment. Therefore, LiP heme with near perfect symmetry exhibits a strong ferromagnetic exchange between the oxoferryl moiety and the porphyrin π-cation radical. It is not evident as to the effect of minimal π-bonding interation of the heme with the proximal ligand on its electronic structure. However, it is evident that the π-bonding interaction of the heme and the proximal ligand is minimal and does not affect the orthogonal symmetry of the iron. More work is needed, possibly with site-directed

mutants at the proximal ligand site, to further elucidate the nature of interaction of the heme with the proximal ligand.

In conclusion, we have shown that (i) LiP I contains a porphyrin π-cation radical that is ferromagnetically coupled to the oxoferryl moiety, (ii) the heme-iron in LiP is in near perfect orthogonal symmetry, and (iii) the proximal ligand has little or no π-bonding interaction with the heme.

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