Identification of a Porphyrin π Cation Radical in Ascorbate Peroxidase Compound I[†]

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ABSTRACT: Electron paramagnetic resonance (EPR) spectroscopy has been used to analyze the ascorbate peroxidase Fe^{3+} resting state and to compare the reaction product between the enzyme and H_2O_2, compound I, with that of cytochrome *c* peroxidase. Because ascorbate peroxidase has a Trp residue in the proximal heme pocket at the same location as the Trp191 compound I free radical in cytochrome *c* peroxidase [Patterson, W. R., & Poulos, T. L. (1995) *Biochemistry 34*, 4331–4341], it was anticipated that ascorbate peroxidase compound I might also contain a Trp-centered radical. However, the ascorbate peroxidase compound I EPR spectrum is totally different from that of cytochrome *c* peroxidase. Immediately after the addition of H_2O_2, the 7.5 K EPR spectrum of ascorbate peroxidase compound I exhibits an axial resonance extending from $g_\perp = 3.27$ to $g_\parallel \sim 2$ that disappears within 30 s, presumably due to endogenous reduction of compound I. In contrast, cytochrome *c* peroxidase compound I exhibits a long-lived $g \sim 2$ signal associated with the Trp191 cation free-radical [Houseman, A. L. P., et al. (1993) *Biochemistry 32*, 4430–4443]. Recently, the 2 K EPR spectrum of a catalase compound I was found to exhibit a broad signal extending from $g_\perp = 3.45$ to $g_\parallel \sim 2$ and was interpreted as a porphyrin π cation radical [Benecky, M. J., et al. (1993) *Biochemistry 32*, 11929–11933]. On the basis of these comparisons, we conclude that ascorbate peroxidase forms an unstable compound I porphyrin π cation radical, even though it has a Trp residue positioned precisely where the Trp191 radical is located in cytochrome *c* peroxidase.

Peroxidases are heme enzymes that react with H_2O_2 to form a powerful enzymatic oxidizing agent known as compound I. In compound I, the heme iron has been oxidized from Fe^{III} to Fe^{IV}=O and the porphyrin or an amino acid side chain has been oxidized to a radical. Hence, compound I is two oxidizing equivalents above the resting state and oxidizes two substrate molecules via two consecutive, one-electron transfer reactions.

Cytochrome *c* peroxidase (CCP)[1] has long been the paradigm peroxidase for structure–function studies designed to elucidate the essential protein interactions that influence the heme moiety and, thus, the catalytic cycle of peroxidases. However, CCP is distinguished from other well-characterized peroxidases due to its preference for the protein cytochrome *c* as substrate and the presence of a compound I amino acid cation radical located on Trp191 (Sivaraja et al., 1989). Other well-studied peroxidases utilize small organic and inorganic molecules as substrate and, presumably as result of having

Phe or an aliphatic side chain at the position occupied by the Trp radical in CCP, form compound I porphyrin π cation radicals.

Recently, the cDNA sequence of pea cytosolic ascorbate peroxidase was reported (Mittler & Zilinskas, 1991) revealing that the amino acid alignments of ascorbate peroxidase and CCP are 33% identical, including Trp at sequence positions analogous to CCP Trp51 and Trp191. The APX cDNA has been cloned into an *Escherichia coli* expression system, the recombinant protein has been expressed, purified, and characterized (Patterson & Poulos, 1994), and the crystal structure has been determined (Patterson & Poulos, 1995). The crystal structure of APX shows that Trp179 is in a conformation, relative to the heme plane and proximal His ligand, identical to that of CCP Trp191 (Patterson & Poulos, 1995). APX is, then, the first example of a peroxidase that utilizes a small, organic molecule like ascorbate as a substrate, yet contains a Trp residue analogous to CCP Trp191. Preliminary UV–vis spectrophotometry indicated that the reaction between APX and H_2O_2 may form a porphyrin π cation radical that rapidly converts to a spectrum characteristic of compound II, with an oxyferryl Fe^{IV}=O center and no porphyrin radical (Patterson & Poulos, 1995). To more accurately describe the nature of the APX compound I radical, we have compared the EPR properties of APX compound I with those of other well-characterized systems (Benecky et al., 1993; Houseman et al., 1993).

EXPERIMENTAL PROCEDURES

CCP and APX were expressed and purified according to published procedures [Darwish et al. (1991) and Patterson

and Poulos (1994), respectively]. Enzyme concentrations were determined from heme molar extinction coefficients, $\epsilon_{408} = 97$ mM^{-1} cm^{-1} for CCP and $\epsilon_{403} = 114$ mM^{-1} cm^{-1} for APX, determined from pyridine hemochromogen assays (Paul et al., 1953). APX in 50 mM potassium phosphate (pH 7) and CCP in 100 mM potassium phosphate (pH 6.5) were concentrated for EPR analysis by ultrafiltration (Amicon YM-30 and YM-50 membranes). H_2O_2 was obtained as a 30% (w/v) solution (Sigma) and standardized by titration with potassium permanganate (Fowler & Bright, 1935).

The resting state EPR samples were prepared with and without 50% glycerol in final volumes of 300 μL and frozen in EPR tubes to 77 K. For the glycerol sample, the protein was concentrated 2-fold and subsequently diluted 1:2 upon the addition of 100% glycerol to achieve the appropriate enzyme concentration in 50% glycerol. The APX I and CCP I samples (Figure 2) were prepared by adding 350 μL of 0.44 mM H_2O_2 to 350 μL of 0.35 mM APX or CCP, which provided a 1.25 molar excess of H_2O_2. Aliquots were taken at the indicated time points into EPR tubes and immediately frozen to 77 K. The APX I sample analyzed in Figure 3 was prepared by rapidly adding 100 μL of 0.60 mM H_2O_2 to 250 μL of 0.25 mM APX (a 1.1 molar excess of H_2O_2) and frozen to 77 K in less than 5 s.

EPR spectra were collected on a Bruker ESP300 spectrometer equipped with an Air Products LTR3 liquid helium cryostat at 7.5 K. Instrument settings are given in the figure legends.

RESULTS

Figure 1 compares the 7.5 K EPR derivative spectra of APX in the resting ferric state with and without 50% glycerol. Glycerol was added as a precautionary measure on the basis of previous CCP EPR at 10 K results, indicating that 50–60% glycerol will inhibit freezing-induced conversion to a ferric low-spin conformation (Yonetani & Anni, 1987). The ferric state of APX exhibits signals (Figure 1A) at $g = 6.00$ and $g = 5.24$ that are similar to those of CCP (Yonetani & Anni, 1987). Additional features at $g = 2.68, 2.21$, and 1.78 clearly indicate that the Figure 1A sample contains some low-spin, $S = 1/2$ species analogous to that observed in CCP in the absence of glycerol (Yonetani & Anni, 1987). Figure 1B is the EPR derivative spectrum of a separate sample containing 50% glycerol. Under instrument conditions identical to those in Figure 1A, glycerol reduced, but did not abolish, the intensities of the low-spin signals relative to the high-spin $g \sim 6$ and $g \sim 2$ signals.

Figure 2 compares the 7.5 K EPR derivative spectra of CCP and APX after the addition of 1.25 molar equivalents of H_2O_2. Parts B–D of Figure 2 were placed on the same relative intensity scale as Figure 2A. Figure 2A,B shows the stable signal ($g_{\parallel}^{eff} = 2.04$, $g_{\perp}^{eff} = 2.01$) associated with the Trp191 radical in CCP I at 30 and 300 s after the addition of H_2O_2. In Figure 2B, the Trp radical signal at $g \sim 2$ decreases slightly over 300 s, with the appearance of a small, but detectable, ferric signal at $g = 6.00$. The APX I spectra (Figure 2C,D) show that, compared to the CCP I free radical signal, APX I is essentially EPR silent at 40 and 300 s after the addition of H_2O_2. To better observe the trace signals in these samples, the relative intensity scales of Figure 2C,D were reduced by $\sim$100-fold and are presented in Figure 2E,F. The 40 s APX spectrum in Figure 2E shows a small $g =$
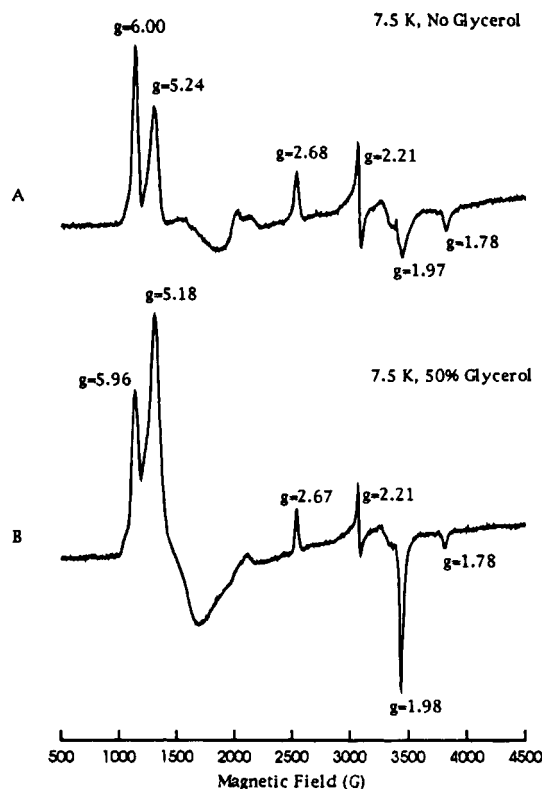

FIGURE 1: 7.5 K derivative EPR spectra of resting state APX: (A) 0.24 mM APX in 50 mM potassium phosphate, pH 7; microwave frequency, 9.52 GHz; microwave power, 2.0 mW; modulation amplitude, 4.63 G; modulation frequency, 100 kHz; field sweep rate, 11.9 G/s; time constant, 0.66 s; gain, 3.2×10^5. (B) Conditions were the same as in (A), except that the enzyme concentration was 0.25 mM in 25 mM potassium phosphate (pH 7) and 50% glycerol.

6.00 signal and a broad axial signal beginning at $g_{\perp} \sim 3.3$ and extending to $g_{\parallel} \sim 2$. The 300 s APX spectrum in Figure 2F shows a slight increase in the $g_{\perp} = 6.00$ signal due to high-spin, ferric heme and a reduction of the axial $g_{\perp} \sim 3.3$ to $g_{\parallel} \sim 2$ signal, relative to Figure 2E.

To better characterize the APX signals present upon reaction with H_2O_2, we performed a quick-freeze experiment, as described in the Experimental Procedures. During the 5 s time period allowed for addition/mixing of 1.1 molar equivalents of H_2O_2 to APX, a color change was immediately observed from the red resting state to a distinct green color, which rapidly changed to a darker red/brown. Figure 3 is the 7.5 K EPR spectrum obtained 5 s after the addition of H_2O_2. Two small, residual signals at $g_x = 6.00$ and $g_y = 5.24$ were observed, in addition to a much larger axial signal at $g_{\perp} = 3.27$ to $g_{\parallel} = 1.99$ that was first observed in the 40 s spectrum in Figure 2E. As will be discussed, we propose that the broad resonance observed in Figure 3 is associated with a porphyrin π cation radical.

DISCUSSION

The ferric resting state spectra presented in Figure 1 showed some effects of added glycerol on the axial, high-spin signals near $g \sim 6$, as well as on the low-spin signals at $g_x = 2.68$, $g_y = 2.21$, and $g_z = 1.78$. These changes may be interpreted as a glycerol-induced decrease in the amount of low-spin species relative to the high-spin state. This result is similar to the known effect of glycerol on CCP in preventing the formation of a low-spin species during freezing (Yonetani & Anni, 1987). However, in the case of
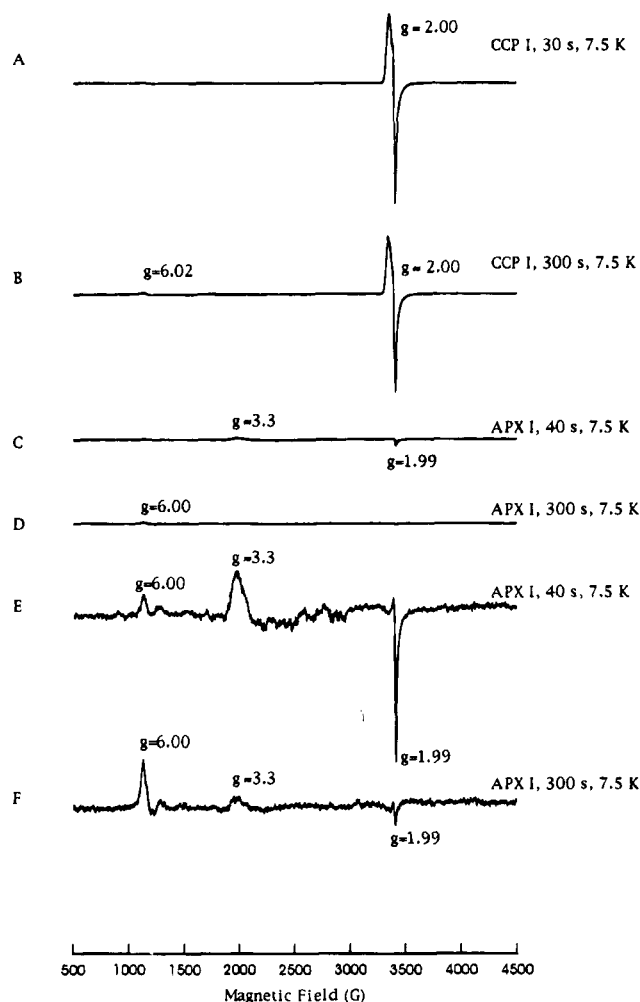
FIGURE 2: 7.5 K derivative EPR spectra of compound I states of CCP and APX; (A) 0.18 mM CCP in 100 mM potassium phosphate (pH 6.5) and frozen 30 s after the addition of 1.25 molar equivalents of H_2O_2; microwave frequency, 9.52 GHz; microwave power, 50 mW; modulation amplitude, 4.63 G; modulation frequency, 100 kHz; field sweep rate, 47.6 G/s; time constant, 0.082 s; gain, 2.0 × 10^3. (B) Conditions were the same as in (A), except that the sample was frozen 300 s after the addition of H_2O_2. (C) 0.18 mM APX in 50 mM potassium phosphate (pH 7), frozen 40 s after the addition of 1.25 molar equivalents of H_2O_2. Instrument settings for (C) and (D) were the same as in (A). (D) Conditions were the same as in (C), except that the APX I sample was frozen 300 s after the addition of H_2O_2. (E) and (F) are the same spectra as in (C) and (D), respectively, but the intensity scale has been reduced by ~100-fold relative to the spectra in (A−D).

APX, 50% glycerol did not completely prevent the formation of the low-spin species. The nature of this low-spin state for CCP is not completely understood, but has been suggested to arise from strong axial coordination of the distal water or the distal His52 due to conformational changes induced by freezing (Yonetani & Anni, 1987; Smulevich et al., 1989). The residual low-spin species observed for APX in the presence of 50% glycerol thus may represent the inability of the glassing agent to completely prevent such a conformational change for APX, or it may indicate a true coexistence of high- and low-spin states for the resting state enzyme.

The CCP I EPR spectra at 7.5 K, presented in Figure 2A,B, display the well-documented features of the Trp191 cation free-radical. Our spectra show the characteristic $g \sim 2$ signal that is commonly interpreted as having effective axial
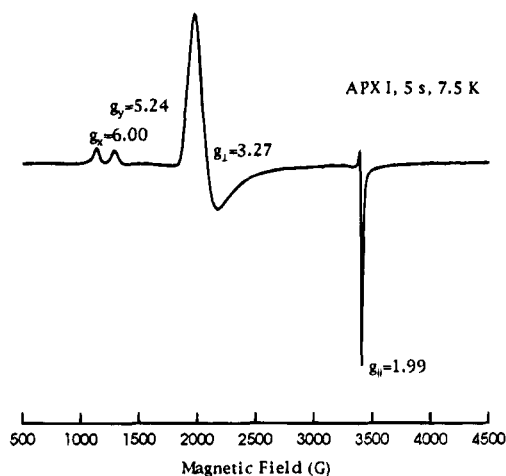

FIGURE 3: 7.5 K derivative EPR spectrum of compound I APX: 0.16 mM APX in 50 mM potassium phosphate (pH 7) and 1.1 molar equivalents of H_2O_2, frozen 5 s after mixing; microwave frequency, 9.52 GHz; microwave power, 20.0 mW; modulation amplitude, 10.4 G; modulation frequency, 100 kHz; field sweep rate, 23.8 G/s; time constant, 0.328 s; gain, 2.5 × 10^4; background corrected. The instrumental conditions were varied from those in Figure 2 to optimize the APX I signal.

symmetry with $g_{\parallel}^{eff} = 2.04$ and $g_{\perp}^{eff} = 2.01$ (Houseman et al., 1993). However, it has been argued that such a signal with $g_{\parallel} > g_{\perp}$ cannot arise from a simple model for an $S = 1/2$ radical that is exchanged coupled to the $S = 1$ ferryl heme center. A model involving a distribution of conformations in which individual values of the exchange coupling parameter, J, range from slightly less than zero to slightly greater than zero (i.e., antiferromagnetic $\rightarrow$ ferromagnetic coupling) was necessary to explain the line shape of this radical (Houseman et al., 1993). For the purpose of our study, we assume that if a Trp radical were formed in APX I, then the near-identical position and interactions of the analogous Trp residues in APX and CCP would give rise to signals similar to those observed in Figure 2A,B.

Figure 2C,D demonstrates that, relative to the signal observed for the Trp radical of CCP I, the product of reacting APX with H_2O_2 after 40 and 300 s is essentially EPR silent. We conclude that the initial formation of APX I was unstable and rapidly decayed to compound II containing the oxyferryl center ($Fe^{4+}=O$) and no radical. At high gain, the data in Figure 2E,F support this conclusion. Figure 2E, 40 s after the addition of H_2O_2 to APX, shows a barely visible $g = 6$ signal, indicative of resting state Fe^{3+}, and a broader axial resonance at $g_{\perp} = 3.3$ and $g_{\parallel} = 2$. At 300 s after the addition of H_2O_2 to APX, Figure 2F shows that the $g = 6$ signal increased only slightly while the axial signal essentially decayed. These data suggest that an EPR active state is formed initially upon reaction of APX with H_2O_2, but largely decays within 30 s to a longer lived EPR silent state (i.e., compound II) that persists even at 300 s.

The EPR spectrum of the unstable species was more efficiently trapped in the quick-freeze experiment (Figure 3). The residual low-field resonances at $g_x = 6.00$ and $g_y = 5.24$ observed in this sample were most likely due to incomplete reaction of the enzyme with H_2O_2 under rapid-mixing conditions. Interestingly, the color change from red to green to brown that was observed during the quick-freeze experiment is qualitatively significant and supports the conclusion that APX proceeds from the resting state to a

green compound I intermediate (i.e., porphyrin π cation radical), which is rapidly reduced to compound II. The g values of the broad axial resonance observed from $g_\perp = 3.27$ to $g_\parallel = 1.99$ are remarkably similar to the EPR spectra of compound I from *Micrococcus lysodeikticus* catalase (cat I) (Benecky et al., 1993). Reaction of *M. lysodeikticus* catalase with peracetic acid resulted in a species with an axial EPR spectrum ($g_\perp = 3.32$) that was assigned to a porphyrin π cation radical on the basis of proton couplings observed by ENDOR (Benecky et al., 1993). The signal observed for APX differs slightly from that for cat I, in that the APX I signal was readily observed by absorption derivative mode EPR at 7.5 K while the cat I signal at 2 K was seen as an adiabatic, rapid-passage signal. The different temperatures employed for the two measurements may be primarily responsible for this difference as the strongly saturated signal at 2 K for cat I would favor the attainment of rapid-passage conditions. At 7 K, we observe that the APX I signal becomes saturated only above 20 mW, so that, under the conditions of Figure 3, normal absorption derivative EPR would be expected. By close analogy to the interpretation of the cat I EPR signal (Benecky et al., 1993), the value of $g_\perp = 3.27$ observed for APX I is determined by the exchange interaction with the $S = 1$ ferryl heme such that

$$g_\perp \sim g_\parallel + 2g_\perp^{Fe}(J/D)$$

where $g_\perp^{Fe}$ for ferryl heme is approximately 2.25 (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 ferryl heme center, a value for $g_\perp$ that is greater than $g_\parallel \sim 2$ results from ferromagnetic coupling ($J > 0$). For APX I, the radical appears to be ferromagnetically coupled to the heme with $J/D = 0.28$. These values compare remarkably well with those of the porphyrin π cation radical of cat I, where $J/D = 0.4$ (Benecky et al., 1993). On the basis of the distinct difference observed between the CCP I and APX I EPR spectra and the close association of the broad $g_\perp = 3.27$ to $g_\parallel \sim 2$ resonance between *M. lysodeikticus* cat I and APX I, we propose that APX also exhibits a porphyrin π cation radical in compound I.

The results presented here were unexpected since the sequence alignments (Mittler & Zilinskas, 1991; Welinder, 1992) and now the crystal structure (Patterson & Poulos, 1995) show that the APX active site, including the location

and local-hydrogen bonding interactions involving the two active site Trp residues, is the same as that in CCP. Why, then, does APX not form a Trp-centered radical? The refined crystal structure of APX (Patterson & Poulos, 1995) shows that APX has a cation located about 8 Å from the proximal Trp residue. Although CCP has the same polypeptide conformation in this region, the side chain ligands in APX are replaced by nonliganding residues in CCP, so that CCP is unable to bind a cation at this site. We hypothesize that the cation is one contributing factor that raises the redox potential of the proximal Trp in APX relative to the homologous Trp in CCP, and hence, the proximal Trp in APX is more difficult to oxidize and cannot form a stable cation radical.

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