Heme Spin States and Peroxide-induced Radical Species in Prostaglandin H Synthase*

Richard J. Kulmaez†
From the Department of Biological Chemistry, University of Illinois at Chicago, Chicago, Illinois 60680
Ah-Lim Tsai and Graham Palmer
From the Department of Biochemistry, Rice University, Houston, Texas 77251

We have examined the optical, magnetic circular dichroism, and electron paramagnetic resonance (EPR) spectra of pure ovine prostaglandin H synthase in its resting (ferric) and ferrous states and after addition of hydrogen peroxide or 15-hydroperoxyeicosatetraenoic acid. In resting synthase, the distribution of heme between high- and low-spin forms was temperature-dependent: 20% of the heme was low-spin at room temperature whereas 50% was low-spin at 12 K. Two histidine residues were coordinated to the heme iron in the low-spin species. Anaerobic reduction of the synthase with dithionite produced a high-spin ferrous species that had no EPR signals. Upon reaction with the resting synthase, both hydroperoxides quickly generated intense (20-40% of the synthase heme) and complex EPR signals around \( g = 2 \) that were accompanied by corresponding decreases in the intensity of the signals from ferric heme at \( g = 3 \) and \( g = 6 \). The signal generated by HOOH had a doublet at \( g = 2.003 \), split by 22 G, superimposed on a broad component with a peak at \( g = 2.085 \) and a trough at \( g = 1.95 \). The lipid hydroperoxide generated a singlet at \( g = 2.003 \), with a linewidth of 25 G, superimposed on a broad background with a peak at \( g = 2.005 \) and a trough around \( g = 1.9 \). These EPR signals induced by hydroperoxide may reflect synthase heme in the ferryl state complexed with a free radical derived from hydroperoxide or fragments of hydroperoxide.

The heme-enzyme prostaglandin H (PGH)\(^1\) synthase exhibits two catalytic activities: a cyclooxygenase activity that converts arachidonic acid to prostaglandin \( G_\text{2} \) with incorporation of two molecules of oxygen, and a peroxidase activity that can reduce a wide variety of hydroperoxides to the corresponding alcohol (1, 2). Both activities require 1 mol of the heme prosthetic group/mol of synthase dimer (3).

Free radicals have been implicated in both of the reactions of the synthase, functioning as substrates for the peroxidase activity and as obligatory initiators of the cyclooxygenase reaction (11). Recent evidence (12, 13) has lent credence to an earlier hypothesis (14) that the process of initiating the cyclooxygenase reaction begins with intermediates of the peroxidase reaction. This emerging realization of the importance of the peroxidase intermediates suggested that a detailed spectroscopic examination of the interaction of hydroperoxides with pure PGH synthase might be useful. In the present study we have examined the interaction of pure ovine PGH synthase with hydrogen peroxide and 15-HPETE. As part of this study we have determined the EPR and MCD characteristics of the enzyme in several pertinent oxidation and spin states. The results of this examination have provided important fundamental information about the heme environment and spin state and have shown that the interaction of the synthase with hydroperoxides generates an intense paramagnetic species associated with the synthase heme. The insights obtained into the identity of this radical species should be valuable in establishing its relationship to the peroxidase and cyclooxygenase reactions catalyzed by the synthase.

**MATERIALS AND METHODS**

Heme, glutathione, and glutathione peroxidase were obtained from Sigma and arachidonic acid was from NuChek Preps Inc., Elysian, MN. Hydrogen peroxide (30%) was purchased from Fisher. Stock solutions were prepared daily and standardized spectrophotometrically at 240 nm (15). 15-HPETE was prepared by the method of Graff (16) and assayed by thin-layer chromatography (16) or iodometrically (17).

The apoprotein of PGH synthase was purified to homogeneity from ram seminal vesicles (18). The holoenzyme was reconstituted by addition to the apoprotein of an excess of hematin (2 eq/synthase dimer) in the absence of peroxidase cosubstrate, followed by incubation at room temperature for 30 min. The excess heme was then removed by passage of the incubation mixture over a small column of DEAE-cellulose (3).

Protein was assayed by the method of Peterson (19). The heme content of the holoenzyme was determined using the reduced pyridine hemochrome procedure (20). For this assay, a portion of the enzyme catalyzed by the synthase. In the case of the peroxidase reaction, addition of hydroperoxides to micromolar preparations of the synthase resulted in the formation of a free radical (4, 5) that appeared to be hemeprotein-associated (6). This hydroperoxide-dependent free radical seemed to be involved in the oxidation of cosubstrates during the peroxidase reaction (7). For its part, the cyclooxygenase reaction has long been presumed to proceed via a radical mechanism (4, 8) and, indeed, arachidonate-derived free radicals consistent with such a mechanism have been trapped during the cyclooxygenase reaction (9, 10).

Hydroperoxides have roles in both reactions of the synthase, functioning as substrates for the peroxidase activity and as obligatory initiators of the cyclooxygenase reaction (11). Recent evidence (12, 13) has lent credence to an earlier hypothesis (14) that the process of initiating the cyclooxygenase reaction begins with intermediates of the peroxidase reaction. This emerging realization of the importance of the peroxidase intermediates suggested that a detailed spectroscopic examination of the interaction of hydroperoxides with pure PGH synthase might be useful. In the present study we have examined the interaction of pure ovine PGH synthase with hydrogen peroxide and 15-HPETE. As part of this study we have determined the EPR and MCD characteristics of the enzyme in several pertinent oxidation and spin states. The results of this examination have provided important fundamental information about the heme environment and spin state and have shown that the interaction of the synthase with hydroperoxides generates an intense paramagnetic species associated with the synthase heme. The insights obtained into the identity of this radical species should be valuable in establishing its relationship to the peroxidase and cyclooxygenase reactions catalyzed by the synthase.

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† To whom correspondence should be addressed Dept. of Biological Chemistry, University of Illinois at Chicago, 1853 W. Polk St., Chicago, IL 60680.

1 The abbreviations used are: PGH, prostaglandin H; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid.
ence of 24.5 mM cm (20).

The formation of a precipitate. A few grains of dithionite were then added to the cuvette before addition of 100 µl of pyridine; this sequence avoided the formation of a precipitate. A few grains of dithionite were then added and the heme content was calculated from the difference in absorbance at 556 and 544 nm, using an extinction coefficient difference of 1.77 × 104 M⁻¹ cm⁻¹ (20).

MCD spectra were obtained on a Jasco J-500C spectropolarimeter equipped with a 1.3 tesla electromagnet and a Jasco DF-500 data system for instrument control, data accumulation, and processing. EPR spectra were recorded with a Varian E-6 spectrometer equipped with an Air Products flexible transfer line for low temperature operation. Microwave frequencies were measured with a Hewlett-Packard 5342A  microwave frequency counter. Both instruments were interfaced to the laboratory data system for data storage, manipulation, and display.

The temperature dependence of the MCD spectrum of PGH synthase was determined by cooling the cuvette of 36-mm path length. A glass Dewar flask with unsealed walls was fitted between the polecaps of the electromagnet, and the sample in the Dewar flask was cooled using cold nitrogen gas obtained by boiling liquid nitrogen.

The temperature of the sample was monitored with a platinum resistance thermometer mounted on the well of the cuvette. Path length changes due to solvent contraction upon cooling were monitored by recording the absorption spectrum of the sample at each temperature using the "high-tension" mode of the spectropolarimeter.

Stopped-flow experiments were performed at 22 °C in a Gibson-Durrum apparatus using a 2-cm path length observation cell. Kinetic data were collected at appropriate wavelengths using an OLIS model 3290 data system. The dead time of the apparatus was 3 ms. The time interval between data points was set at 2 or 5 ms to monitor rapid absorbance changes and increased when slower changes were to be followed. Each kinetic trace was the average of at least two replicates.

Transient changes in the EPR spectrum produced by addition of H2O2 were followed using the rapid-mixing apparatus described by Ballou and Palmer (21). Because the optical changes observed in the stopped-flow experiments persisted for more than a second, the reaction between enzyme and substrate was quenched by squirting the reacting mixture into an ice-cold EPR tube that was immediately plunged into an acetone/dry ice mixture. When 15-HPETE was used as substrate, it was added to the enzyme in a small volume of ethanol and manual mixing was used.

Quantitation of the EPR radical signal was performed in the usual way using a copper standard. The low-spin EPR signal was quantitated by the method of Aasa and Vanngard (22) using the g = 3 feature of cytochrome a as the reference species.

Cyclooxygenase activity was measured at 30 °C using an oxygen electrode (23). The reaction mixture (3.0 ml) contained 0.1 M potassium phosphate (pH 7.2), 1 mM phenol, and 50 µM arachidonic acid. The specific activity of the synthase holoenzyme preparations used in these studies ranged between 80 and 150 nmol of O2/min/mg of protein.

The peroxidase activity of the synthase was assayed with H2O2 as substrate (13). At glycerol concentrations between 0 and 70% the activity was inversely related to the viscosity of the medium. No inhibitory effect of the glycerol per se was apparent in this concentration range.

RESULTS AND DISCUSSION

EPR and MCD Properties of the Resting Enzyme—The MCD spectrum of pure PGH synthase in the Soret region is shown in Fig. 1 (left). The enzyme exhibits a derivative-like spectrum centered at 412 nm with a peak at 408 and a trough at 425 nm. These wavelengths are to be compared with the absorption maximum for this form of the enzyme which is found at 411 nm. The observed MCD lineshape is similar to those exhibited by authentic low-spin heme centers (24). Were the enzyme to be fully low-spin, however, we would expect the peak-to-trough amplitude of the MCD to exceed 100 (M cm-tesla) (24). Therefore, it must be concluded that a substantial proportion of the enzyme is present in the high-spin state. This interpretation is supported by several additional pieces of data.

First, upon conversion of the heme center to the completely low-spin cyanide derivative there is a dramatic increase in the intensity of the MCD (Fig. 1) to a value comparable to that observed with other heme-cyanide derivatives (24). The zero-crossing shifts to 425 nm which is the same wavelength as the maximum in the absorption spectrum of the synthase-cyanide complex (Fig. 2). The general shape of the MCD spectral envelope is not changed appreciably by treatment with cyanide, supporting the conclusion that the MCD spectrum observed with the resting enzyme reflects the low-spin component present in the untreated synthase.

Second, addition of fluoride, a weak-field ligand, to the enzyme reduced the intensity of the MCD spectrum without any major changes in the overall spectral shape (Fig. 1). As this reagent is expected to convert all of the heme to the high-spin form, this effect is consistent with the presence of some low-spin heme in the enzyme sample.

Although there does not appear to be a definitive relationship between the intensity of the Soret MCD and the amount of low-spin species present in a heme protein (24, 25), there does appear to be a semiquantitative correlation (24), thus, from the relative sizes of the Soret MCD peak-to-trough of the untreated enzyme and its cyanide derivative we can estimate that some 20% of the enzyme is in the low-spin form.

An obvious corollary to this conclusion is that the balance of the enzyme is high-spin. High-spin heme does not have unique MCD in the Soret region but does exhibit a diagnostic trough associated with the characteristic charge transfer band present in the near-infrared. The presence of such a charge transfer band is demonstrated in Fig. 1 (right) where a weak negative feature is clearly present at about 640 nm. This feature is shifted slightly upon formation of the high-spin enzyme-fluoride complex and is eliminated upon addition of cyanide which converts the heme to the low-spin form (Fig. 1, right). This charge transfer band can also be seen at 635 nm in the absorbance spectrum of the resulting synthase but not in that of the cyanide derivative (Fig. 2).

Evidence documenting that the Soret MCD indeed arises from a paramagnetic species is presented in Fig. 3 which shows that the spectral features that are observed at room temperature increase in intensity as the temperature is lowered. The intensity of the spectrum at 167 K is about three times that observed at room temperature and, to the rather limited accuracy of the data, the intensity of the spectrum appears to be a linear function of reciprocal temperature at both wavelengths tested (Fig. 3, inset). However, it would appear that there is also a temperature-dependent change in spin state with the low-spin form being favored as the temperature is lowered. Thus, the amplitude of the MCD increases more rapidly than is predicted by the Curie law, which requires that the intensity change by less than a factor of 2 over the temperature range studied.

Furthermore, the intensity does not extrapolate to zero at infinite temperature, as would be the case for a well-behaved paramagnet. (24, 25).

The heterogeneity in the spin state of the heme of PGH synthase deduced from the room temperature MCD spectra is also apparent in the EPR spectrum recorded at 12 K, where clear evidence for both high- and low-spin EPR species is apparent (Fig. 4). The prominent features of the spectrum present at about 1000 G are due to high-spin heme. At least two forms of high-spin heme are apparent in the data, an intense component of rhombic symmetry responsible for the peak at g = 6.6 and the derivative feature at g = 5.3 together with a much weaker component with apparent axial symmetry...
FIG. 1. MCD spectra of ferric PGH synthase (---) and its cyanide (--) and fluoride (----) derivatives in the Soret (left) and visible (right) regions. The data were recorded at 25°C in 50 mM potassium phosphate, pH 7.2, containing 30% glycerol. The synthase concentration was 11 μM subunit with 5.8 μM bound heme. The ligands were present at 0.1 M.

which contributes the feature at \( g = 6.0 \). The feature at \( g = 6 \) was more intense (relative to the \( g = 6.6 \) and \( g = 5.3 \) features) in a synthase preparation with lower specific activity (not shown). The trough at \( g = 2.0 \) is presumably the parallel component of the rhombic species; it shows a slight asymmetry possibly due to the parallel contribution from the axial high-spin species. Heterogeneity in the high-spin EPR has previously been noted in data obtained at 90 K by Ruf et al. (26), who attributed the rhombic species to specifically bound heme and the minor species with a \( g \)-value of about 6.3 to adventitious heme.

The second important feature in the spectrum is the symmetric peak at \( g = 2.98 \). This is the \( g_z \) component of the low spin species. It is presumably associated with the derivative feature present at 2.18 (\( g_x \)) but the third feature, a trough due to \( g_y \), which should be present at \( g = 1.56 \), could not be found. This feature is often very broad and difficult to detect except in very concentrated solutions and at high instrumental sensitivity.

Integration of the peak at \( g = 2.98 \) and comparison with the same feature of a low-spin heme standard using the method of Aasa and Vanggard (22) indicated that about 48% of the heme present in the enzyme is low-spin at this temperature. It follows that 52% of the heme contributes to the high-spin signal.

Blumberg and Peisach (27) have introduced a technique for identifying the axial ligands to low-spin hemes. This is accomplished by calculation of the axial and rhombic ligand field parameters that can be obtained from the three \( g \)-values. Using the improper coordinate system emphasized by Blumberg and Peisach together with the observed \( g \)-values, these parameters were calculated to be 3.3 and 3.0, respectively. With these values an entry can be made in a Blumberg-Peisach diagram (Fig. 5) which correlates the axial ligand field with the rhombicity, the ratio of rhombic to axial fields (see Ref. 28 for a full description of this procedure). The entry for the synthase falls well within the domain (B) characteristic.

The calculations can also be performed using a proper-axis system. The values for the axial and rhombic terms in a proper system are 3.9 and 1.8, which again are typical of normal bis-imidazole coordination. The ratio of these quantities (0.46), the rhombicity, is much larger than that observed with strained hemes (<0.2) implying that the planes of the two imidazole ligands are essentially parallel to one another.

These quantities are expressed in units of the spin-orbit coupling constant. The values are not especially sensitive to changes in the value of \( g \), and thus an incorrect assignment in \( g \), does not affect the conclusions drawn.
FIG. 3. Temperature dependence of the Soret MCD spectrum of PGH synthase (30 μM heme). The figure shows spectra obtained at 304 and 167 K and the inset shows the dependence of the MCD intensity at 408 (□) and 423 (□) nm. The normalized MCD intensity is the ratio of the absolute MCD amplitude obtained at each temperature relative to that obtained at 167 K at 423 nm. The open symbols represent the data obtained while cooling the sample and the solid symbols are the data points obtained upon warming.

Consequently, it can be asserted that the low-spin species present in resting PGH synthase has 2 histidine residues coordinated to the heme iron.

The MCD spectrum of PGH synthase after reduction with dithionite (Fig. 6) is typical of high-spin ferrous hemes such as deoxymyoglobin and ferrous horseradish peroxidase (24, 29). There is a weak, broad trough at short wavelengths and a narrow and intense peak at longer wavelength with the zero-crossing occurring at the maximum in the absorption spectrum. Between 450 and 700 nm, the MCD is very weak and featureless (Fig. 6, left and right); this establishes that the fraction of enzyme that was low-spin in the ferric state has been converted to the high-spin state, for low-spin ferrous bis-imidazole compounds exhibit a very characteristic intense and narrow MCD A-term in the α-band. No evidence for this A-term was found in the data.

Formation of the low-spin cyanide derivative (Fig. 6) eliminates the high-spin ferrous MCD spectrum5 in the Soret region and leads to the appearance of a typical MCD A-term with a crossover at 562 nm corresponding to the α-band maximum in the visible spectrum (Fig. 2).

As expected, no EPR signals were observed in the reduced enzyme.

5The remaining small peak at 434 nm could reflect a small proportion of unreacted high-spin material, although the concentration of cyanide used was well above the Kd for the heme-cyanide complex in the resting synthase (0.2 mM) (18) and the mixture was incubated for 90 min before the spectrum was recorded.
FIG. 5. Plot of the axial ligand field (in units of the spin orbit coupling constant) versus rhombicity for a variety of low-spin hemoproteins using data summarized in Refs. 27 and 36. The entry for PGH synthase (PGHS) was determined taking \( g_x \) to be 2.98 and \( g_y \) to be 2.19 and assuming that the sum of the squares of the three \( g \)-values is 16 (35). The improper coordinate system of Blumberg and Peisach (27) was adopted, and the abbreviations are those used in Ref. 35.

FIG. 6. The MCD spectrum of dithionite-reduced PGH synthase (dotted line) and its cyanide derivative (solid line) in the Soret (left) and visible (right) regions. Conditions were as described in the legend to Fig. 1.

Reactions of PGH Synthase with Hydroperoxide: Spectroscopic Changes—Reaction of PGH synthase (12 \( \mu \)M heme) with 2 eq of hydrogen peroxide led to rapid changes in the optical spectrum in the Soret region (Fig. 7, left) which were complete within 500 ms; the data could be fit with a single exponential with an apparent rate constant of about 20 s\(^{-1}\). Higher concentrations of peroxide result in more rapid rates. For example, with 100 eq of peroxide the rate constant was about 50 s\(^{-1}\). Subsequently there was a much slower reaction which resulted in absorbance changes of opposite sign. This slow reaction appeared to consist of several phases with about half of the reaction occurring in about 5 s and the remainder requiring a minute for completion.

By repeating the experiment at a number of wavelengths it was possible to extract the difference spectrum for the optical changes at several reaction times (Fig. 7, right). During the first 500 ms the difference spectrum exhibited a minimum at 414 nm, a zero-crossing at about 420 nm and a maximum at...
430 nm; the peak and trough of the difference spectra were of equal amplitude. During the subsequent slow reaction there was almost no change in amplitude at the trough at 414 nm but the peak at 430 nm decreased sharply and shifted slightly to 433 nm; the zero-crossing shifted to 427 nm.

At low molar ratios of H₂O₂ to synthase the absorbance spectrum eventually reverted to that of the original enzyme. With higher levels however, the optical properties of the final product did not return to that of the original enzyme solution and significant bleaching of the chromophore between 405–420 nm was observed (data not shown).

These absorbance changes appear to be similar to those obtained by Lambeir et al. (30) upon mixing the synthase with an organic peroxide for 135 ms. However, in this latter work, the peak at 430 nm was only about half as large as the trough present at 408 nm; the zero-crossing was present at 420 nm. Furthermore, in the case of the reaction with organic hydroperoxide (30), the optical changes at 410 nm were complete by 10 ms, in which time the absorbance at 420 nm also decreased; the absorbance at 420 nm subsequently increased at a rate similar to the increase in absorbance at 430 nm. In our experiments with hydrogen peroxide (a much slower-reaction hydroperoxide) (13), the absorbance changes that occurred during the first 2 s could be described by a single exponential, implying the existence of only a single rate-determining reaction, and the magnitude of the absorbance changes at 430 nm was somewhat less than was found by Lambeir et al. This difference in magnitude suggests that a smaller proportion of the enzyme accumulated as the 430 nm absorbing species when hydrogen peroxide was used as substrate. The apparent discrepancies between the present results and those of Lambeir et al. may arise from the very much smaller rate of reaction of the synthase with H₂O₂ than with organic hydroperoxide (a value of 9 × 10⁶ versus 7 × 10⁷ M⁻¹ s⁻¹ (13). In the case of the slower-reaction H₂O₂, the rate of conversion of a Compound I to the next intermediate may be as fast as its formation, and little would accumulate.

Although the absorbance changes we observed are similar to those found upon formation of compound ES of cytochrome c peroxidase and are clearly different from those found upon formation of horseradish peroxidase Compound I, the spectral changes seen with the synthase are smaller than those observed when cytochrome c peroxidase was reacted with hydrogen peroxide (31).

The intermediate produced upon reaction of PGH synthase (30 μM heme) with 5 eq of hydrogen peroxide was trapped by freezing in acetone/dry ice within about 1 s after mixing and examined by EPR at 12 K. Several striking differences between the EPR spectrum of the sample after reaction and that of the resting enzyme were apparent (Fig. 8, A and B). First, there was a substantial reduction in the amplitude of both the high-spin and low-spin EPR resonances, with the sizes of the signals at g = 6.6 and g = 2.98 reduced to about one-half of those observed with the resting enzyme (Fig. 8, A versus B). Second, a new EPR signal appeared in the g = 2
Fig. 9. Expanded EPR spectra of the product of reaction of PGH synthase with peroxides. Upper trace, after reaction with hydrogen peroxide. This is the same sample as in Fig. 8B. Lower trace, synthase (86 μM heme) was treated with 2 eq of 15-HPETE for 2 s. Both spectra were recorded with 10-G modulation and 4-milliwatt power and are consequently somewhat saturated. Spectra obtained at much smaller modulation amplitudes did not show any splitting of the $g = 2$ component of the 15-HPETE-treated sample. The microwave frequency was 9.2129 GHz. Left inset, saturation behavior of the broad (C) and narrow (O) components of the lower spectrum. Right inset, saturation behavior of the broad (C) and narrow (O) components of the upper spectrum.

Region. Similar changes in the EPR spectrum were observed when much more dilute solutions of the synthase (1.3 μM subunit) were reacted with hydrogen peroxide (data not shown). Upon further incubation after addition of substrate, the heme EPR signals slowly returned to their original sizes (Fig. 8, C and D); about 25 min at room temperature was required for the original EPR spectrum to be restored. The apparent persistence of the $g = 2$ signal seen in Fig. 8 compared to the peroxidase activity of the synthase (Fig. 1 in Ref. 13) may be a consequence of the very different reaction conditions in the two studies. Interestingly, the intensity of the feature at $g = 6$, which has been ascribed to nonfunctional heme (26), remained relatively unchanged after addition of hydroperoxide (Fig. 8, A versus B), suggesting that it was not involved in formation of the radical species.

Examination of the $g = 2$ region (Fig. 9, upper trace) reveals that the hydrogen peroxide-induced signal appears to consist of two components: a narrow doublet at $g = 2.003$, which is split by about 22 G and 4-milliwatt power and has an overall peak-to-trough width of 40 G, together with a much broader component with contributions from a peak at $g = 2.085$ and a trough at $g = 1.95$. The overall shape of the signal resembles more closely the EPR spectrum of the ES complex of cytochrome c peroxidase (32) than that of horseradish peroxidase Compound I (33). The EPR characteristics of the narrow component do not resemble those of common oxygen-containing radicals, but do have some features in common with the radical signal obtained when crude microsomal PGH synthase was treated with arachidonic acid (see Tables I and II in Ref. 6).

Qualitatively similar EPR spectra were obtained when the EPR experiment was repeated using 15-HPETE as substrate (Fig. 9, lower trace). The high- and low-spin EPR signals were reduced in intensity by about 50% while a new resonance appeared at $g = 2$. This new resonance consists of a narrow unresolved singlet at $g = 2.003$, having a linewidth of 25 G and superimposed upon a broad background. In this instance the radical signal appears to be very similar to that reported earlier for a mixture of the microsomal enzyme and arachidonate (6). The differences in radical lineshape observed with the two substrates suggest that the hydroperoxides, or fragments from them, are intimately associated with the radical species. In the case of $H_2O_2$ the observed splitting would be ascribed to the single hydrogen nearby, whereas in the case of the lipid hydroperoxide the interaction of a carbon atom should significantly reduce the hyperfine coupling to the nearest hydrogen atom, and any residual splitting would be lost in the overall linewidth of the EPR signal. The splitting of 22 G observed with $H_2O_2$ is significantly larger than has been observed with authentic $HOO^*$ (Table II in Ref. 6), and the observed $g$-value is clearly smaller than is expected for a species which has a significant amount of unpaired electron localize localized on oxygen, so it is most unlikely that the EPR signals arise from a simple hydroperoxy radical.

Integration of the $g = 2$ signals was complicated by the difficulty in making a correction for the signals originally present at $g = 2$ from the heme center. By assuming that the intensity of the signals at $g = 2$ were reduced in direct proportion to the heme signals at $g = 3$ and 6, we estimated that the new signals at $g = 2$ might account for as much as 0.4 electrons (i.e., 40% of heme), when hydrogen peroxide was the substrate, and 0.3 electrons, when 15-HPETE was the substrate. The corresponding lower limits, calculated with the assumption that the intensity of the original signal present at $g = 2$ was unchanged, were estimated to be 0.3 and 0.2 electrons, respectively. It should be emphasized that most of this intensity is contributed by the broad component, with the narrow species contributing no more than a few percent to the EPR integral.

The power saturation characteristics of the two EPR species reveal that the signals induced by hydrogen peroxide relax much more effectively than those studied in the presence of the 15-HPETE (Fig. 9, inset). The $P_s$ of the narrow and broad signals are 0.25 and 1 milliwatt, respectively, in the former case and 0.05 milliwatt for both species in the latter case.

Egan et al. (34) have previously reported that no EPR signal was generated when purified synthase was incubated with hydroperoxide. Their description of reaction conditions was not detailed enough for comparison with our results and so the reason for their inability to observe this strong signal is not clear.

In view of its high yield and rapid formation it seems reasonable to believe that this new paramagnet represents a catalytically important species. Several possibilities exist: in each case an organic radical is adjacent to iron, but, provided the iron atom is paramagnetic, any oxidation state between II and IV, is possible. Attempts to demonstrate that the heme was in the ferrous oxidation states (using freeze-trapping MCD experiments) failed and so, drawing mainly on analogies to other peroxidase systems, it seems most likely that a species containing Fe(IV)-R is involved and that the EPR signal arises from a weak exchange interaction between a zero-field split state of the heme and the radical center, as has been proposed for horseradish peroxidase (33).

The further characterization of the radical species will unquestionably be important in establishing the mechanism of action of this important enzyme.

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