The Interaction of Cytochrome Oxidase with Hydrogen Peroxide: The Relationship of Compounds P and F†

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ABSTRACT: Upon reaction of cytochrome oxidase with hydrogen peroxide, the spectral changes are complete, with slightly less than 1 equiv of hydrogen peroxide per cytochrome oxidase. At pH 8 the product is a mixture of the P and F forms, while at pH 6 the product is exclusively the F form. These data are inconsistent with current interpretations of the structure of compounds P and F. Two stable radical species are detected by EPR; the relative amounts of these species are pH dependent. The MCD spectra of pure P and F are reported. It is suggested that compound F is a hydrogen peroxide adduct of cytochrome oxidase with cytochrome a_3 in the low-spin state and that compound P is an oxyferryl state of cytochrome a_3 in support of the recent Raman data of Proshlyakov et al. [(1994) *J. Biol. Chem. 269*, 29385−29388]. We also suggest that copper B is in the trivalent state in compound P.

In 1981 Wikström described two spectral forms of cytochrome oxidase which were observed in mitochondria following energization with ATP (Wikström, 1981). The first form is characterized by a sharp absorption maximum at 607 nm in a difference spectrum derived by comparison to oxidized oxidase. In time, this first species was converted to a second characterized by an absorption maximum at about 580 nm. These intermediates were proposed to be the peroxy (P) and ferryl (F) intermediates of the oxidase catalytic cycle (Wikström & Morgan, 1992).

Similar spectral changes are observed after addition of hydrogen peroxide to oxidized cytochrome oxidase (Bickar *et al.*, 1982). At low concentration of hydrogen peroxide (typically micromolar) the principal species has an absorption maximum at 606−607 nm (Vygodina & Konstantinov, 1989; Weng & Baker, 1991; Wrigglesworth, 1984). At higher concentrations of peroxide (mM) the dominant species has a maximum at about 580 nm (Vygodina & Konstantinov, 1988; Weng & Baker, 1991; Wrigglesworth, 1984). In both species the Soret band is red shifted, with a maximum at 428 nm when prepared from the fast form of cytochrome oxidase (Weng & Baker, 1991). The same spectral changes were also observed after interaction of peroxide with reoxidized (pulsed) oxidase (Gorren *et al.*, 1986; Kumar *et al.*, 1984a,b, 1988; Orii, 1988; Witt & Chan, 1987). The precise wavelengths of these species appear to vary slightly with experimental conditions; for brevity, we will refer to them using the notation CcO-607 and CcO-580, though the exact wavelengths can deviate from these values, especially at acidic pH.[1]

There are several interpretations of the CcO-607 anc CcO-580 species. It was suggested that (i) the CcO-607 and CcO-580 compounds are indeed the P and F intermediates of Wikström (1981) (Konstantinov *et al.*, 1992; Ksenzenko *et al.*, 1992; Kumar *et al.*, 1988; Orii, 1988; Vygodina & Konstantinov, 1988; Vygodina et al., 1993; Witt & Chan, 1987); (ii) the CcO-607 and CcO-580 forms represent two different binding sites for hydrogen peroxide present in oxidized oxidase (Vygodina & Konstantinov, 1987); (iii) the CcO-607 form is F form while the CcO-580 species represents oxidized oxidase with cytochrome a_3 in a low-spin state (Wrigglesworth *et al.*, 1988); and (iv) the CcO-607 and CcO-580 species are both forms of F but with a free radical center in the CcO-607 species (Weng & Baker, 1991).

The conversion of P to F in the cytochrome oxidase catalytic cycle is accomplished by the one-electron reduction of P. A similar reaction has been proposed for the transition of CcO-607 (P) to CcO-580 (F) form prepared by hydrogen peroxide. In this case, the reductant is believed to be a second equivalent of H_2O_2 (Konstantinov *et al.*, 1992; Ksenzenko *et al.*, 1992; Vygodina & Konstantinov, 1988; Vygodina *et al.*, 1993; Witt & Chan, 1987).

In this study we have examined the interaction of oxidized oxidase with hydrogen peroxide at several pH values ranging from 6.1 to 8.0 and find that 1 equiv of hydrogen peroxide is sufficient to produce both CcO-607 and CcO-580. Furthermore, formation of CcO-580, but not CcO-607, is accompanied with the production of "radical-like" EPR signals at $g = 2$.

MATERIALS AND METHODS

Dodecyl β-D-maltoside (DM) and 30% hydrogen peroxide were purchased from Aldrich. Tris(hydroxymethyl)aminomethane (Tris), *N*-(2-acetoamido)iminodiacetic acid (ADA), xanthine, superoxide dismutase, and nitroblue tetrazolium were from Sigma. Xanthine oxidase was prepared in this laboratory by J. Howarth using the method of Massey *et al.*

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[1] Abbreviations: CcO, cytochrome oxidase; CcO-607 and CcO-580, the 607 nm and 580 nm absorbing species produced by hydrogen peroxide; DM, dodecyl maltoside; Tris, tris(hydroxymethyl)aminomethane; ADA, *N*-(2-acetamide)iminodiacetic acid; NBT, nitroblue tetrazolium; DPPH, diphenylpicrylhydrazyl.

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(1969). D_2O_2 was prepared by making up a stock solution of H_2O_2 in D_2O and incubating for 5 h at 4 °C.

The fast form of cytochrome oxidase (Palmer *et al.*, 1988) was prepared by a modification of the method of Hartzell and Beinert (1974) developed in this laboratory (Baker *et al.*, 1987). To remove residual ammonium sulfate from the preparation, the enzyme was diluted several times into 100 mM Tris−sulfate buffer, pH 8, containing 0.1% DM, followed by concentration using Amicon Centriflo ultrafiltration cones. This procedure yields enzyme with an absorption maximum in the Soret band at 423 nm at pH 8. Enzyme concentration (aa_3) was determined at pH 8 from the absorption spectrum of oxidized oxidase by using an extinction coefficient 158 mM^{-1} cm^{-1}.

P (CcO-607) was prepared using carbon monoxide by the following procedure: Cytochrome oxidase (3-100 μM) was dissolved in buffer (100 mM Tris, pH 8, 0.1% DM) and bubbled with CO for 30 s with no precautions to exclude air. The conversion of enzyme to P was followed using the absorption increase at 607 nm, which was complete within 3 min. The concentration of P was calculated from the difference spectrum (P-oxidized enzyme) using $\Delta A_{607-630}$ = 11 mM^{-1} cm^{-1} (Wikström & Morgan, 1992). Typically, about 80% of the enzyme is converted to P by this procedure; the maximum yield was 92%.

CcO-580 was obtained by treatment of cytochrome oxidase with hydrogen peroxide under a variety of conditions (see Results): its concentration was also determined by difference spectroscopy using $\Delta A_{580-630}$ = 5.3 mM^{-1} cm^{-1} (Wikström & Morgan, 1992). The concentration of H_2O_2 was determined from the absorbance at 240 nm using the molar absorbance of ΔA = 40 M^{-1} cm^{-1} (Bergmayer *et al.*, 1970).

Superoxide production was quantified by following the reduction of 0.5 mM nitroblue tetrazolium at 517 nm. The reaction was calibrated using 0.15 μM xanthine oxidase together with 9.6 μM xanthine, a mixture which produces 5 μM superoxide, as judged by the reduction of 50 μM cytochrome *c*.

Optical spectra were recorded in an IBM 9430 spectrophotometer or a Hewlett Packard diode array spectrophotometer. All measurements were performed at room temperature (25 °C). EPR spectra were recorded with a Varian E-6 spectrometer equipped with an Air Products low temperature Dewar and transfer line; the spectrometer was interfaced to a personal computer. All data were analyzed and graphs prepared using Igor Pro (Wavemetrics, Lake Oswego, OR).

RESULTS

The Optical Properties of Compound P Prepared Using CO. Bubbling CO through an aerobic solution of CcO at room temperature for 30 s leads rapidly to the formation of a species with an increased absorbance at 607 nm and a red shift of the Soret (Figure 1). The difference spectrum CO-treated minus oxidized enzyme (Figure 1, inset) is similar to that reported earlier (Fabian & Palmer, 1995; Nicholls & Chanady, 1981) with maxima at 438, 566, and 607 nm, a minimum at 414 nm, and zero-crossings at 426 and 628 nm. Using the coefficient for the absorbance difference, 607 nm *minus* 630 nm, previously reported by Wikström (1981), we calculate a value of 65 ± 3 mM^{-1} cm^{-1} for the absorbance difference between 438 and 414 nm.

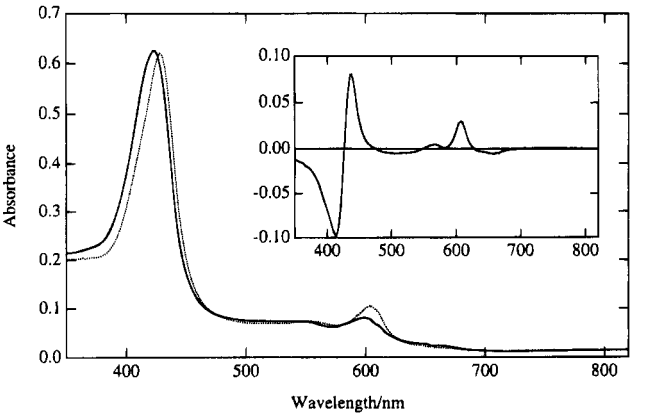


FIGURE 1: Optical spectra of cytochrome oxidase and the product of reaction with carbon monoxide under aerobic conditions. (—) Absolute spectrum of 3.9 μM cytochrome oxidase in 0.1 M Tris, pH 8.0, containing 0.1% dodecyl maltoside. (•••) After bubbling with CO for 30 s under aerobic conditions. The spectrum was recorded when the absorbance increase at 607 nm was complete. Inset: The difference spectrum (CO-treated minus control).

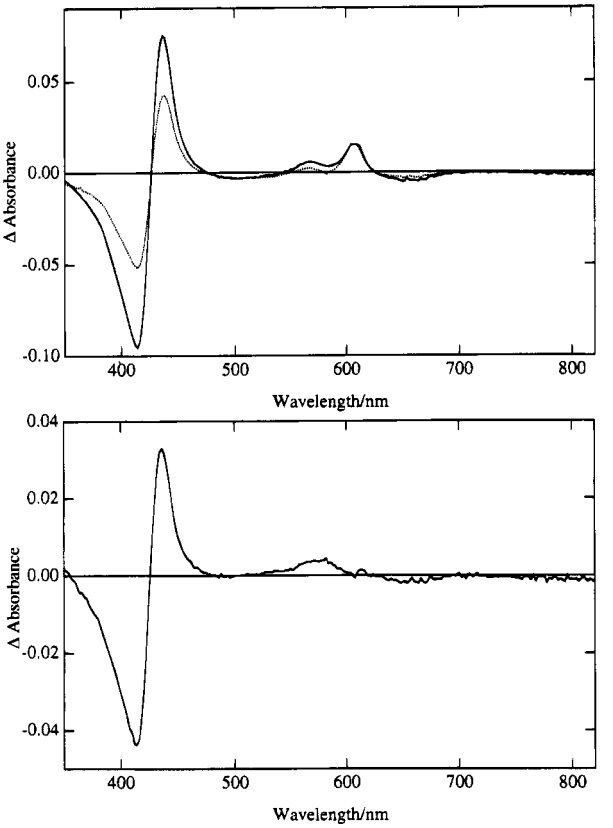


FIGURE 2: The spectral changes produced on reaction of cytochrome oxidase with H_2O_2 at pH 8.0. Upper panel: Reaction of 50 μM cytochrome oxidase with 30 μM H_2O_2. (—) The difference spectrum H_2O_2-treated minus control. (•••) Contribution to the difference spectrum of CcO−P determined from the amplitude at 607 nm, using the difference spectrum of Figure 1 as the reference. Bottom panel: Residual spectrum after subtraction of the contribution of P from the peroxide-induced difference spectrum. Conditions as in Figure 1.

Reaction of Oxidized CcO with Substoichiometric Amounts of H_2O_2. Upon reacting 50 μM oxidized CcO with 30 μM H_2O_2 at pH 8, there is an increase in absorbance at 428 nm which is complete within 3 min. The difference spectrum H_2O_2-treated minus oxidized enzyme (Figure 2, top) shows a minimum at 414 nm, a maximum at 436 nm, and two small peaks at 566 and 607 nm; the zero-crossing is at 426 nm.

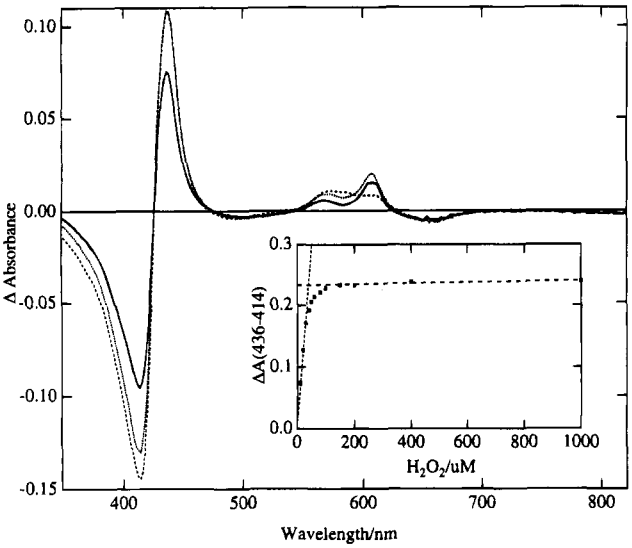


FIGURE 3: Difference spectra, H_2O_2-treated minus oxidized enzyme, for three concentrations of hydrogen peroxide at pH 8. The concentration of enzyme was 50 μM. (—) 30 μM H_2O_2; (•••) 400 μM H_2O_2; and (- - -) 10 000 μM H_2O_2. The spectra were recorded when the change in the Soret band was maximum. Inset: The change of the Soret absorbance versus peroxide concentration. (■) $A_{436-414}$. Straight lines were fitted to the initial and final data. The point of intersection was found at 40 μM H_2O_2. Other conditions as in Figure 1.

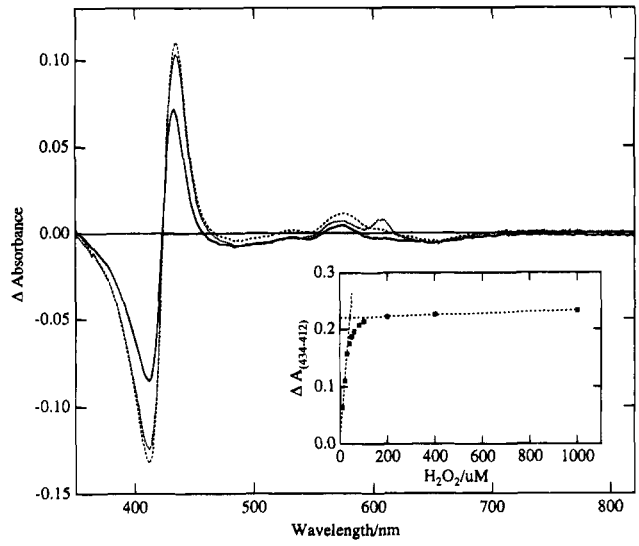


FIGURE 4: Difference spectra, H_2O_2-treated minus oxidized enzyme, for three concentrations of hydrogen peroxide, at pH 6.1. The concentration of enzyme was 50 μM. (—) 30 μM H_2O_2; (•••) 400 μM H_2O_2; and (- - -) 10 000 μM H_2O_2. The spectra were recorded when the change in the Soret band was maximum. The data have been corrected for turbidity by subtraction of a scattering standard normalized to yield zero at 350 nm in the difference spectrum. Inset: The change of the Soret absorbance versus peroxide concentration. (■) $A_{436-414}$. Straight lines were fitted to the initial and final data. The point of intersection was found at 40 μM H_2O_2. The buffer was 100 mM ADA, pH 6.1, containing 0.1% DM. Other conditions as in Figure 1.

When the difference spectrum of pure CcO-607 (Figure 1) was scaled to give the same absorbance increment at 607 nm as that produced by H_2O_2 (Figure 2, top), it became apparent that this scaled spectrum did not account for all of the absorbance changes in the Soret. The difference (Figure 2, bottom) between these two difference spectra shows a single feature at 578 nm and extrema at 413 and 436 nm, with a zero-crossing at 425 nm. This latter difference spectrum is similar to that of compound F (Vygodina & Konstantinov, 1988; Weng & Baker, 1991; Witt & Chan, 1987). A similar discrepancy between the observed absorbance changes in the Soret region and those predicted from the absorbance changes at 607 nm is obtained at molar ratios of H_2O_2:CcO as small as 0.2, with increasing concentrations of H_2O_2 leading to larger amounts of the F-like species. Repeating the experiment with concentrations of CcO as low as 3 μM and stoichiometric amounts of H_2O_2 still led to the formation of a heterogeneous product.

Using a value of 5.3 mM^{-1} cm^{-1} for the absorbance coefficient of F at 578 nm (Wikström & Morgan, 1992), we calculate a value of 67 ± 4 mM^{-1} cm^{-1} for the absorbance changes between 436−413 nm. This Soret optical coefficient is almost identical with that of CcO-607.

When separate samples of 50 μM CcO are treated with increasing concentrations of H_2O_2 in the range 10 μM−10 mM, the absorbance difference between 436 and 413 nm increases rapidly to a maximum (Figure 3, inset). Extrapolating the initial slope to intersect this limiting value leads to an intercept at 40 μM H_2O_2. Thus, the maximum absorbance changes are complete with 0.8 equiv of H_2O_2/CcO. Similar results are obtained on plotting the absorbance difference (607 minus 630 nm), which peaked at 40 μM hydrogen peroxide but began to decrease at peroxide concentrations greater than 400 μM. It should be noted that the data which are plotted represent the maximum absorbance recorded for each sample. This maximum is reached rapidly but is followed by a slow decrease in the Soret absorbance. For example, with 10 mM H_2O_2 the change in Soret absorbance is maximum immediately but then decays with a rate of 0.3 min^{-1} (see Figure 5).

Representative difference spectra from data recorded at 30, 400, and 10 000 μM H_2O_2 (Figure 3) reveal that while the absorbance changes in the Soret appear to be complete with 40 μM H_2O_2, the absorbance between 550 and 615 nm changes continuously, with a decrease in the 607 nm peak assigned to P and an increase in the 578 nm feature of F. In each case, subtraction of the difference spectrum of CcO-607 (Figure 1) scaled to the amplitude at 607 nm led to a shortfall in the difference absorbance in the Soret.

When this experiment is conducted in the pH range of 6.1−6.3, the difference spectra are slightly changed, with the minimum at 412 nm and maxima at 434, 532, 575, and 606 nm. The form of the titration is almost identical with that observed at pH 8 (Figure 4, inset); in more than 10 experiments the intercept ranged from 0.8 to 0.95 H_2O_2:CcO.

However, the behavior of the absorbance between 550 and 615 nm at pH 6.1 is different from that observed at pH 8. The spectra recorded at ratios of H_2O_2:CcO less than 0.8:1 have no obvious feature at 607 nm, and only the peak at 575 nm due to F is apparent (Figure 4). A clear peak due to the formation of CcO-607 was not detected until the ratio H_2O_2:CcO exceeded 0.8:1. Significant amounts of CcO-607 were formed at higher concentrations of H_2O_2 (e.g., 400 μM, Figure 4), while massive amounts of H_2O_2 once more led to the loss of the 607 nm peak.

In a separate experiment at pH 6.1, 100 μM CcO was reacted with 20 μM H_2O_2. The observed absorbance difference between 434 and 412 nm was 0.115; this corresponds to 18 μM of CcO-580 calculated using ΔA_m = 65 mM^{-1} cm^{-1} obtained from the experiment described in

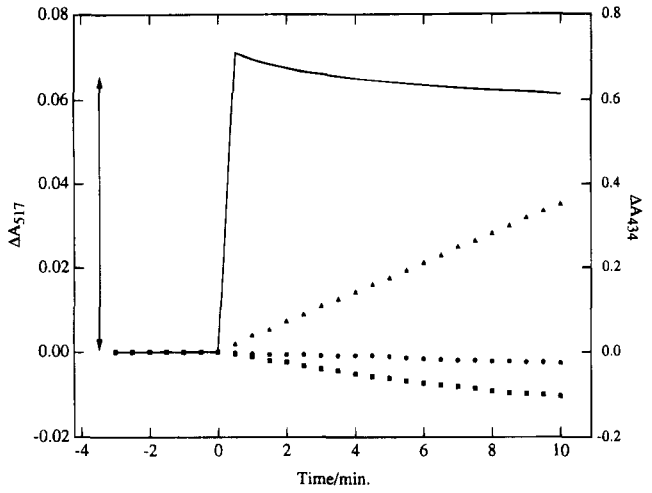


FIGURE 5: Measurement of superoxide production induced by addition of H_2O_2 to cytochrome oxidase at pH 8.0. The reaction was initiated by addition of 10 mM H_2O_2 to 5 μM cytochrome oxidase. (—) Absorbance increase and subsequent decrease at 432 nm due to formation and decay of F; (■) absorbance decrease at 517 due to F decay; (▲) absorbance increase at 517 nm in the presence of 0.5 mM nitroblue tetrazolium; (●) absorbance increase at 517 nm in the presence of 0.5 mM NBT and 100 units/mL superoxide dismutase. The vertical arrow represents the change at 517 nm produced by 5 μM superoxide prepared by adding 0.15 μM xanthine oxidase and 9.6 μM of xanthine in the presence of cytochrome oxidase, H_2O_2, and NBT.

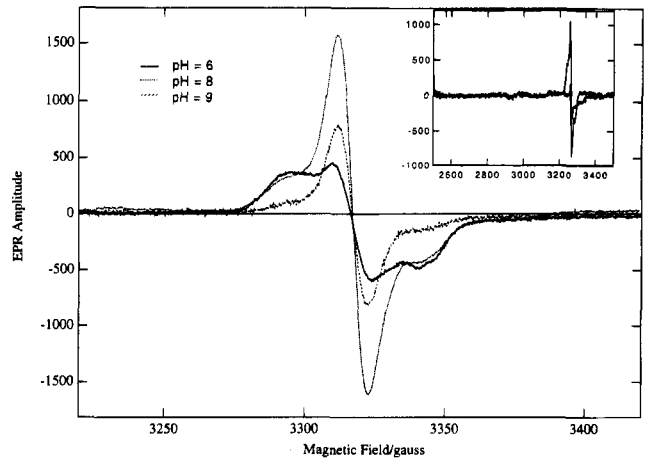


FIGURE 6: EPR spectra of free radicals produced by addition of H_2O_2 to cytochrome oxidase. 110 μM cytochrome oxidase was treated with 200 μM hydrogen peroxide at pH 6 (0.1 M ADA) and 5 mM hydrogen peroxide at both pH 8 (0.1 M Tris−sulfate) and pH 9 (0.1 M Ches). The samples were frozen immediately in liquid nitrogen. The spectra were obtained by subtraction of untreated cytochrome oxidase at the same pH from the peroxide-treated enzyme; no scaling was applied. The inset shows a wider scan of the pH 6 sample together with compound P prepared at pH 8 by the CO method; similar data were obtained at the other pH values. EPR conditions: modulation amplitude 5 G; microwave power 200 μW; temperature 12 K; microwave frequency 9.277 GHz.

Figure 2, thus providing further support for the idea that, under these conditions, the yield of F is complete with a single equivalent of peroxide.

Superoxide Is Not Produced during F Formation. One proposed mechanism for the conversion of oxidized CcO (O) to F involves 2 equiv of peroxide. The first equivalent converts O to P, which is then reduced to F by a one-electron reduction by the second equivalent of peroxide; this second equivalent is itself oxidized to superoxide (Vygodina & Konstantinov, 1988; Witt & Chan, 1987).

To establish whether or not any superoxide is formed in our experiments, we reacted 5 μM CcO with 10 mM H_2O_2 at pH 8; this led to the immediate formation of 3 μM CcO-580 as judged by the absorbance changes in the Soret (Figure 5). This absorbance subsequently decays in a very slow reaction.

Superoxide formation was followed using nitroblue tetrazolium at 517 nm; the contribution from the decay of the CcO−H_2O_2 adduct to the absorbance at this wavelength is small (Figure 5, ■). In the presence of NBT, there is an increase in absorbance with time (Figure 5, ▲), and most of this increase is eliminated by superoxide dismutase (Figure 5, ●), thus implicating superoxide formation. Using a xanthine oxidase−xanthine system adjusted to produce 5 μM superoxide, it could be calculated that the rate of superoxide production was 0.3 μM min^{-1}. Importantly, there was no instantaneous formation of superoxide immediately after addition of H_2O_2, at which time the formation of CcO-580 was complete (Figure 5).

Peroxide-Treated Cytochrome Oxidase Exhibits Free Radical EPR Spectra. As a further test for the involvement of free radical in this system, we have recorded EPR spectra of 110 μM CcO reacted with H_2O_2 at pH values of 6.3 (200 μM), 8.0 (5 mM), and 9.0 (5 mM). In each case, radical signals were detected superimposed on the principal feature of the EPR signal due to Cu_A, the intensity of which is unchanged (Figure 6, inset). The spectra of these radicals were extracted by subtracting the EPR signal of the control, enzyme untreated with H_2O_2 (Figure 6). Two signals were observed, a broad component with a peak-to-trough width of 45 G and a narrow species with a peak-to-trough width of 11 G; both were centered at $g = 2.00$, within 1 G of the DPPH standard. The relative proportion of the two signals varied with pH, with the broad signal dominating at low pH and exhibiting a constant amplitude between pH 6.3 and 8.0 and the narrow signal largest at the intermediate pH value (Figure 6). The narrow signal was partially saturated even at 12.5 μW, whereas the broad signal only began to saturate above 0.2 mW at 12 K. No hyperfine structure could be detected on either signal. When the experiment was repeated with enzyme prepared in D_2O buffer and the reaction initiated with either H_2O_2 or D_2O_2, the radical spectra were the same as described above and no collapse of the either signal was detected. Comparison of the integrated intensity of these signals with that of the Cu_A signal of the control suggested that these radicals account for about 0.07 electron per enzyme molecule. In the course of these measurements, nine enzyme preparations were used, making it most unlikely that this is some preparation-dependent artifact.

At pH 6.3, stoichiometric addition of H_2O_2 gave the same EPR spectrum as that observed using a small excess.

Incubating cytochrome oxidase with 10 mM cyanide for 10 min before reaction with 10 mM H_2O_2 for 3 min completely blocked the formation of the broad radical signal; a trace amount of a radical with a width of 11 G was observed. Bubbling CO for 30 s through a sample in which the radicals had been preformed completely eliminated both radical signals. No radical EPR signals are observed when CO is bubbled through control enzyme. Both radical signals are completely abolished upon addition of 2 equiv of reduced cytochrome *c*.

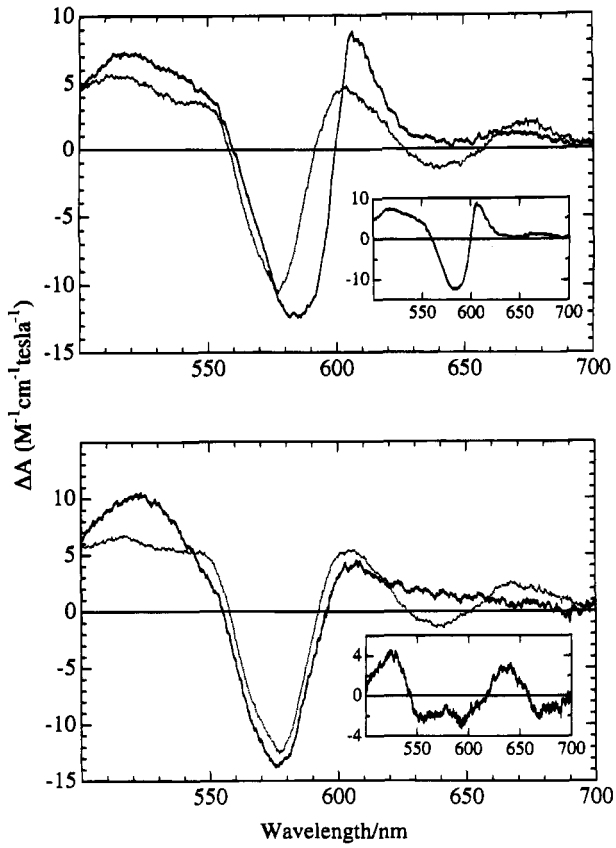


FIGURE 7: Visible MCD spectra of CcO-580 and CcO-607. Upper panel: 41 μM cytochrome oxidase was reacted with carbon monoxide in aerobic 0.1 M Tris buffer, pH 8. When the absorbance at 607 nm was maximum, the MCD spectrum was recorded. About 26 μM enzyme was converted to CcO-607. (···) Oxidized enzyme; (—) CcO-607. The inset shows the difference spectrum scaled to 100% formation of CcO-607. Lower panel: 37 μM cytochrome oxidase was reacted with 50 μM H_2O_2 in aerobic 0.1 M ADA buffer, pH 6.3. When the absorbance at 432 nm was maximum, the MCD spectrum was recorded. About 23 μM enzyme was converted to CcO-580. (···) Oxidized enzyme; (—) CcO-580. The inset shows the difference spectrum scaled to 100% formation of CcO-580.

MCD Spectra of CcO-607 and CcO-580. MCD spectra of cytochrome oxidase treated with CO (upper panel) and with a slight excess of hydrogen peroxide (lower panel) are shown in Figure 7. In both cases, the inset shows the change in MCD normalized to 100% to account for incomplete reaction as judged from the absorbance spectra. As these normalized spectra were obtained by subtraction of the untreated enzyme, they represent the change in the MCD of cytochrome a_3 upon conversion to the relevant derivative. In both cases, the spectra reveal a clear change in the 650 nm region, signifying that the high-spin state of cytochrome a_3 has been affected. Importantly, both species also show negative contributions to the spectrum between 500 and 600 nm.

The MCD spectra in the Soret region are not substantially different from those of the parent oxidase.

DISCUSSION

It is generally believed that treatment of CcO with a small excess of hydrogen peroxide at weakly basic pH leads exclusively to the formation of CcO-607. However, our data make it clear that under these conditions one always obtains a mixture of CcO-580 and CcO-607. This is true for concentrations of enzyme ranging from 3 to 110 μM, for hydrogen peroxide between 3 μM and 10 mM, and over the pH range 6.1−8.0.

This heterogeneity is most readily seen in the Soret region and appears as a discrepancy between the observed absorbance changes and those predicted from the contribution of the CcO-607 species. While the two peroxide-induced forms of CcO have clearly different spectral characteristics in the $\alpha-\beta$ region, the Soret properties are very similar, with the amplitudes of the difference spectra being identical within experimental error and the zero-crossings differing by less than 2 nm.

When experiments are conducted using high enzyme concentration, typically 50 μM, the maximum effects can be obtained with almost stoichiometric amounts of hydrogen peroxide and titrations exhibit a sharp break point. Under these conditions, we have no experiment which required more than 1 equiv of hydrogen peroxide per cytochrome oxidase to obtain the maximum spectral changes in the Soret region, and in most cases it required slightly less than 1 equiv of hydrogen peroxide. In experiments conducted at pH 6.3, the product of the reaction with stoichiometric amounts of H_2O_2 is exclusively CcO-580 and observation of CcO-607 requires levels of H_2O_2 greater than stoichiometric. At lower concentrations of enzyme hydrogen peroxide titrations are hyperbolic, reflecting the finite dissociation constant for this reaction, and the sharp break point is obscured (Vygodina & Konstaninov, 1987; Weng & Baker, 1991).

We consistently observe that the amount of CcO-580 or the CcO-580/CcO-607 mixture formed is 80−95% of theoretical, with the lower value being more common. Part of the reason for this shortfall is the presence of a small amount of slow enzyme in the preparations employed. Typically, this varies from 5% to 10% of the total enzyme. However, it is clear that the presence of some slow form is not a sufficient explanation for the discrepancy. For example, one sample of enzyme which exhibited >98% of the rapid form as judged by cyanide kinetics (and *ca.* 93% from the amplitude of the absorbance changes) gave an intercept of 0.8−0.85 in the peroxide titration. It is thus apparent that our preparation contains a small amount of one or more additional species which fail to react with hydrogen peroxide. We recently reported that the as-isolated enzyme contains small and variable amounts of P, typically 8% (Fabian & Palmer, 1995). The presence of this species together with the sluggish reaction of hydrogen peroxide with slow enzyme (Palmer *et al.*, 1988) provides one rationale for the less than unit stoichiometry that we observe.

The observation that maximum P formation is obtained with a single H_2O_2 when the concentration of CcO is 50 μM is consistent with the report that the K_d for this reaction is 5.1 μM (Weng & Baker, 1991). However, the observation that formation of the CcO-580 species is complete with a single equivalent of H_2O_2 at pH 6 contradicts all current models for this reaction.

For example, Witt and Chan (1987) suggested the following mechanism for the formation of F:

$$CcO + H_2O_2 \leftrightarrows CcO{-}H_2O_2(P)$$

$$P + H_2O_2 \leftrightarrows Fe^{IV}{=}O + O_2^- + 2H^+$$

In this mechanism the ferryl form is produced by a one-

electron oxidaton of a second equivalent of H_2O_2 with conversion of the heme iron to Fe(IV). This mechanism is clearly ruled out, both by the observed stoichiometry of one peroxide for complete reaction, and by the lack of any detectable superoxide formation at times when CcO-580 formation is complete. While our data do show, in agreement with Ksenzenko *et al.* (1992), that superoxide is produced, it is only observed during a slow secondary process which is subsequent to F formation. This slow production of superoxide may be a consequence of the destruction of heme which is believed to occur at high concentrations of H_2O_2 (Brown & Jones, 1968).

Similarly, the proposal by Weng and Baker (1991) that the 607 nm species be represented as $Fe^{IV}R\bullet{=}O$ and that it can be converted to the 580 nm species (suggested to be $Fe^{IV}{=}O$) by a second H_2O_2 cannot be supported by our observed stoichiometry nor by our observation that CcO-607 produced using carbon monoxide does not contain any radical signals. A similar assignment for CcO-607 has been advanced by Wrigglesworth *et al.* (1988); their scheme invoked partially reduced enzyme as the source of a single reducing equivalent so that reaction with 1 equiv of H_2O_2 leads to the formation of a ferryl form with a peak at 607 nm. As we initiate our reactions with oxidized enzyme, this possibility is not available in our case.

The suggestion that there are two binding sites for H_2O_2 which can be occupied simultaneously (Vygodina & Konstantinov, 1987), and that these sites represent the 607 nm and 580 nm species, would also seem to be ruled out by our data. If this were the case, it should not be possible to obtain maximum formation of both species until 2 equiv of H_2O_2 have been added.

We suggest a refinement of this suggestion of two binding sites which proposes that occupation of these sites is mutually exclusive. Maximum occupancy would then be achieved with a single equivalent of hydrogen peroxide, but the spectral data would reflect the extent to which each site is occupied. Thus, at pH 6.3 it would appear that the site responsible for the 575 nm band is exclusively occupied, but at higher pH values both sites exhibit partial occupation.

What are these two binding sites? Vygodina and Konstantinov (1987) suggested that the 607 nm species represented binding of H_2O_2 to Cu_B while the 570 nm species reflected binding of H_2O_2 to the heme iron of cytochrome a_3. There is general agreement that the binuclear center contains a variety of binding sites (Li & Palmer, 1993; Yoshikawa & Caughey, 1992), and indeed there is increasing evidence that binding of gaseous ligands to Cu_B precedes binding to cytochrome a_3, at least in the reactions of reduced enzyme (Blackmore *et al.*, 1991; Einarsdottir *et al.*, 1993). However, Vygodina and Konstantinov's (1987) assignment of optical species to ligand binding sites appears to be arbitrary, and there is no obvious reason to exclude the opposite assignments.

As both binding sites clearly affect the optical properties of cytochrome a_3, another possibility is that the two species represent alternative modes of reaction of hydrogen peroxide with the heme iron.

Recently, Proshlyakov *et al.* (1994) have reported using 607 nm excitation to observe a Raman-active mode at 803 cm^{-1} in cytochrome oxidase treated with a large excess of hydrogen peroxide for 0.4 s. This mode moved to 769 cm^{-1} when the reaction was initiated with $H_2{}^{18}O_2$, while enzyme treated with the mixed $H_2{}^{16}O{}^{18}O$ reagent simply exhibited both modes and did not show any intermediate frequencies. These data rule out the 803 cm^{-1} mode as being due to the O−O stretch of Fe−O−O and make it rather likely the 803 cm^{-1} mode in the Fe−O stretch of $Fe^{IV}{=}O$. Because the intensity of the Raman scattering decreased when 580 nm excitation was used, Proshlyakov *et al.* (1994) ascribed this mode to CcO-607.[2] We have recently observed Raman modes similar to those described by Proshlyakov *et al.* (1994) in CcO-607 prepared by the carbon monoxide method (S. Franzen, R. B. Dyer, W. H. Woodruff, M. Fabian, and G. Palmer, work in progress).

The conclusion of Proshlyakov *et al.* (1994) is supported by our MCD data. Both CcO-580 and CcO-607 exhibit finite visible MCD, with maximum differential absorbance(s) in the range 4−10 M^{-1} cm^{-1} T^{-1} (Figure 7). Both peroxidase compound I and catalase compound I have MCD of comparable intensity, but in these compounds the MCD is exclusively positive throughout almost all of the visible range (Maranon *et al.*, 1994). Thus it can be confidently asserted that neither of the species we are studying contains the porphyrin π-cation radical. Moreover, Browett and Stillman (1980) found that the MCD spectrum of catalase compound II resembles that of low-spin ferrous protoheme with resolved $\alpha-\beta$ bands; this correlation has been extended to several other peroxidases (Cheeseman *et al.*, 1991). The MCD spectrum of CcO-607 that we have obtained is strikingly like that of bis-imidazole ferrous heme *a* (Carter & Palmer, 1982), both species exhibiting positive MCD close to the absorption maximum with a more intense trough located some 500−700 cm^{-1} to higher energy. The observation that the visible MCD of $Fe^{IV}{=}O$ resembles the low-spin ferrous derivative of the parent heme is consistent with the conclusion that CcO-607 has the oxyferryl structure.

The consistency of the Raman and MCD data makes the oxyferryl assignment for CcO-607 more convincing but raises the issue of the source of the second electron required to cleave H_2O_2 in the formation of the Fe=O bond. By analogy to the peroxidases, the most obvious source(s) would be either the porphyrin ring or an amino acid radical. However, as noted above, the former is convincingly eliminated because oxidation of the porphyrin ring should lead to dramatic absorbance changes throughout the Soret and visible region and result in a very characteristic visible MCD; neither of these effects are observed.

Thus our finding that peroxide produces the free radical signals in the enzyme might be taken as evidence that an amino acid is being oxidized during this reaction. Such behavior has been observed with several heme proteins (Davies, 1991; Davies & Puppo, 1993; McArthur & Davies, 1993) and is normally believed to be due to oxidation of amino acids, notably tyrosine, promoted by the reaction of hydrogen peroxide with the heme iron.

Most of the EPR signals produced in this way exhibit EPR linewidths in the range 22−33 G (Debus *et al.*, 1988; Moan & Kaalhaus, 1975; Whitaker & Whitaker, 1990). The only spectrum of an amino acid radical we have found with an overall width comparable to the 45 G species is a 46 G wide

[2] This observation is not completely convincing because the 580 nm excited scattering would fall at 607 nm while the 607 nm derived scattering would fall at 638 nm; the extent of self-absorption is much larger in the former situation.

species produced as an intermediate in the catalytic cycle of lysine 2,3-aminomutase and which appears to arise by hydrogen abstraction at C_3 of lysine (Ballinger *et al.*, 1992); this EPR spectrum exhibited clearly resolved hyperfine structure. It should also be noted that the EPR signals of modified tyrosine such as is found in galactose oxidase (Whitaker, 1994) and the topaquinone found in monamine oxidases (Warncke *et al.*, 1994) also have linewidths in the 20−30 G range and exhibit clear hyperfine structure.

We do not know of any example of an amino acid radical with a linewidth as small as 11 G, though comparably narrow signals are observed with both flavins (Palmer *et al.*, 1971) and ubiquinones (Tsai & Palmer, 1983). Furthermore, neither signal present in cytochrome oxidase exhibited the hyperfine structure frequently present in spectra of amino acid radicals, nor could any contribution from solvent or substrate-derived deuterons be detected. However, the radical signals clearly involve the binuclear center, for they cannot be elicited in cyanide-treated enzyme.

Despite the lack of any obvious amino acid prototype, it is difficult to identify a plausible alternative source for these signals. The observed g value is essentially that of the free electron, and thus paramagnets based on metals or those which are oxygen or sulfur centered can be ruled out, for such species will exhibit g values clearly different from 2.00. However, the involvement of the binuclear center and the apparent kinetic competence (see below) of these radicals raise intriguing possibilities. Although these radicals are present at pH 6.3, the apparently low yield makes it impossible to state that the signals are not associated with CcO−P, possibly arising from reaction of Cu_B(III) with one of its ligands.

Furthermore, if an amino acid is indeed the source of this second reducing equivalent, one has difficulty explaining the absence of any signal attributable to Cu_B in the EPR spectrum of CcO-607 generated using either H_2O_2 or CO. The oxyferryl species is an $S = 1$ spin system while Cu_B(II) has $S = {}^1/_2$. With this composition the binuclear center would have an odd number of electrons, and it should be possible to detect some EPR contribution from the copper component. In the absence of exchange interaction, the copper EPR should be obvious, and even if the copper were to suffer dipolar broadening from the rapidly relaxing ferryl center, there should still be a residual anisotropic contribution from those enzyme molecules oriented so that relaxation broadening is negligible (Leigh, 1970). This should still be true if the two centers are exchange coupled, though how this coupling might be accomplished in the presence of the intervening oxene is not obvious. When the coupling constant (J) is much larger than the zero-field splitting (D) of the ferryl species (strong coupling), the resulting $S' = {}^3/_2$ manifold becomes the ground state because the electrons on Fe and Cu are in orthogonal orbitals and the exchange coupling will be ferromagnetic; in the simplest case, this yields an EPR spectrum with g values of 4, 4, and 2. However, if the coupling is weak, the exchange coupling combines with the zero-field splitting of the Fe=O center to produce three Kramers' doublets, the lowest of which has an effective spin of ${}^1/_2$ (Schultz *et al.*, 1979). Using typical g values for the ferryl and copper centers, the system will exhibit effective g values of $g_{\|} \approx 2.1$, $g_{\perp} = 2.1-4.3$, the value of $g_{\perp}$ increasing with the ratio of J/D (Rutter *et al.*, 1984). We do not detect any new EPR in this region in either CcO-607 or CcO-580, the difference EPR to low field of $g = 2$ being featureless (Figure 6, inset).

Given our failure to detect any EPR from Cu_B in CcO-607, the possibility must be considered that the source of the second electron is Cu_B itself and that the electronic formulation of the binuclear center in CcO-607 is Fe^{IV}=O...Cu_B(III). Trivalent copper is not a common series, but Margerum and his colleagues (Bossu *et al.*, 1977) have described a family of copper compounds which have this oxidation state. While these species are most readily formed using several deprotonated peptide nitrogens as donor ligands, the current description of Cu_B includes at least three histidine residues (Hosler *et al.*, 1993); three imidazolate residues may be equally effective in stabilizing the Cu(III) oxidation state, particularly in the low dielectric provided by the nonpolar transmembrane helices.

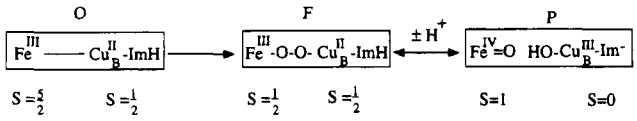
What then is CcO-580? The MCD spectra of both CcO-607 and CcO-580 show clear changes around 650 nm, a region which is a signature of high-spin cytochrome a_3 (Carter & Palmer, 1982), and the changes in the 650 MCD observed in these two derivatives imply that the high-spin character of a_3 has been modified. The basis for this loss in CcO-607 follows from the discussion above. In the case of CcO-580, an obvious possibility is that a_3 has been converted to the low-spin form. Bis(1-methylimidazole)ferric heme a has absorbance maxima at 587 and 543 nm (Carter & Palmer, 1982); these wavelengths can be compared to the values of 575 and 532 nm found in CcO-580 at pH 6.3. The similar lineshape and the typical separation of the two bands in the two compounds suggest that the two features observed in CcO-580 are the α and β components of low-spin ferric heme.[3] Similarly, the shift of the Soret peak to the red on formation of CcO-580 is consistent with a low-spin assignment though, as formation of CcO-607 also produces a red shift, this is not an unique possibility. Further support for our assignment is provided by the similarity of the absorbance spectrum of CcO-580 to that of the cyanide complex of CcO (van Buuren *et al.*, 1972; Fabian & Palmer, 1995). In this complex cytochrome a_3 is unequivocally low-spin, with the cyanide present as a bridge connecting the iron with Cu_B (Orosz *et al.*, 1994).

The possibility that the peroxide present in F bridges the two metals would appear to be discounted by the observation that both P and F can be generated by alkyl hydrogen peroxides (Vygodina *et al.*, 1993). However, before this observation is taken as definitive, one would need to be assured that these reagents were in fact free of contaminating hydrogen peroxide, for the alkyl peroxides are employed in high concentration and trace contamination with H_2O_2 would be sufficient to yield the observed result.

In any event, if CcO-607 and CcO-580 do represent competing modes of reaction of hydrogen peroxide with cytochrome oxidase, then this competition is pH dependent with an apparent pK close to 7 (Vygodina & Konstantinov,

[3] Low-spin Fe(IV), Fe(III), and Fe(II) differ by having increasing numbers of dπ electrons. Thus, as we move across the series, the porphyrin to Fe⇒π donation decreases, the pyrrole N⇒Fe bonds lengthen, and the porphyrin macrocycle is able to adopt a more planar conformation. The presumed result is a sharpening of the visible transition with decreasing metal oxidation state. In addition, removal of the vacancies in the dp orbitals eliminates some pathways for internal conversion and increases the natural (radiative) lifetime (Adar *et al.*, 1976).

Scheme 1



1988). In view of the belief that the binuclear center contains four histidine residues (Hosler *et al.*, 1993), the presence of such a p*K* value is not surprising. Thus one possibility is that a protonation state of an active center histidine is modulating the mode of interaction of hydrogen peroxide with the binuclear center.

The picture that emerges is summarized in Scheme 1. CcO-580 is a low-spin derivative of cytochrome a_3 with a bound peroxide ligand which is possibly additionally ligated to Cu_B. In this state, one of the histidine residues coordinated to Cu_B is protonated; this would be the situation at pH 6. Upon raising the pH, the histidine becomes deprotonated, the formation of Cu_B(III) is facilitated, and both metal ions are oxidized with cleavage of the O−O bond to yield CcO-607. It will of course be apparent that these two assignments are precisely the opposite of those proposed by Wikström and Morgan (1992), but there seems to be no particular reason from their reverse electron transfer data to prefer one set of assignments over the other.

Do the radical signals have any significance? Their presence in CcO-580 is particularly tantalizing in view of the report by Wilson *et al.* (1982) that similar radical signals are present during steady-state turnover of CcO at pH 7.2 in the presence of reduced cytochrome *c* and oxygen. Wilson *et al.* (1982) reported that the radical was a superposition of two species. The narrow component had a peak-to-trough width of 8 G, but the width of the broad component cannot be assessed from the published data; their spectrum most closely resembles that which we observe at pH 8. The maximum yield was 1% when cytochrome *c* was the reductant but was as large as 10% with a reducing system composed of TMPD plus ascorbate; this increased yield is not simply due to TMPD or ascorbate radicals, for the linewidths observed with the enzyme are too large. Wilson *et al.* (1982) found that the kinetic behavior of the radical-(s) paralleled that of cytochrome *a*. In their system there should be no hydrogen peroxide present, and thus formation of these radical signals cannot be ascribed as a side reaction of hydrogen peroxide.

There are at least two possible explanations for the relatively low yield of these free radical signals. The most obvious is that they arise from interaction of H_2O_2 with a minority species present in our preparation; one candidate for this species is the endogenous P (Fabian & Palmer, 1995). The second is that the low integrations are a consequence of selective line broadening arising from dipolar interactions with a nearby strongly relaxing paramagnet such as Fe(IV); in this instance, only enzyme molecules with the radical−Fe axis oriented in a narrow range of angles with respect to the magnetic field would contribute to the spectrum, the EPR absorption of the remainder being sufficiently broadened as to be undetectable (Leigh, 1970).

Of course, these explanations do not account for the almost quantitative production of P obtained by reacting enzyme with carbon monoxide in the presence of oxygen compared with the yield obtained on reaction of CcO with hydrogen peroxide. The only obvious difference between these two ways of making P is that the enzyme passes through the mixed-valence state in the CO method and that the putative bridging ligand will already have been jettisoned. The significance of this difference is not all clear.

The observation that the proportions of the two radicals induced by hydrogen peroxide vary with pH raises the possibility that the parent species are somehow involved in the proton translocating apparatus. There is evidence to suggest that the process of proton translocation is associated with the conversion of P to F and of F back to oxidized enzyme (Wikström, 1989). The extent to which these radicals are involved in this process is an obvious area for future experiments.

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