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PEROXIDASE ACTIVITY OF CYTOCHROME *C* FACILITATES THE PROTOPORPHYRINOGEN OXIDASE REACTION

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Abstract – Protoporphyrinogen oxidase (PPO) catalyzes the penultimate reaction in heme biosynthesis. The 'oxygen dependent' form of this enzyme can utilize three molecules of oxygen as electron acceptors in the reaction. In the current study, the ability of cytochrome *c* to serve as an electron acceptor for PPO was examined. Cytochrome *c* was found to enhance the catalytic rate of *Drosophila melanogaster* PPO under reduced oxygen conditions, and cytochrome *c* became reduced during PPO catalysis. Further kinetic analysis under anaerobic conditions revealed that hydrogen peroxide, a byproduct of the PPO reaction, is required for this rate enhancement to occur. This suggests that the generation of free radicals via the peroxidase activity of cytochrome *c* plays a part in this rate enhancement, rather than cytochrome *c* acting as an electron acceptor for the PPO reaction. Given the abundance of cytochrome *c* in the intermembrane space of mitochondria, the cellular location of PPO, this process may potentially impact on the synthesis of heme *in vivo* particularly in conditions of low oxygen or hypoxia.

Key words: Heme biosynthesis, protoporphyrin, tetrapyrrole, free radical; hydrogen peroxide.

INTRODUCTION

The six-electron oxidation of protoporphyrinogen to protoporphyrin is catalyzed by either an oxygen-dependent, FAD-containing enzyme found in eukaryotes and strictly aerobic bacteria (protoporphyrinogen oxidase (PPO), E.C.1.3.3.4) (32), or an oxygen-independent enzyme in facultative or anaerobic bacteria (26). These two protoporphyrinogen oxidases are distinct proteins and are unrelated. In the case of *Escherichia coli*, which possesses oxygen independent PPO activity, electrons are thought to be shuttled to the electron transport chain (24). In eukaryotic organisms, oxygen-dependent PPO has been localized to the cytosolic side of the inner mitochondrial membrane and utilizes molecular oxygen as an electron acceptor (13, 17, 7). Early work was performed on PPO purified from the native host (9, 37, 2), whereas subsequent studies have been performed on purified recombinant proteins: the human, *Bacillus subtilis*, *Myxococcus xanthus* and *Aquifex aeolicus* enzymes have been cloned, overexpressed in *Escherichia coli* and purified to homogeneity (11, 8, 10, 6, 38). All of these studies have reported the presence of a flavin cofactor.

The crystal structures of PPO from *M. xanthus* (3) and mitochondrial PPO from tobacco (27) have recently been solved. A putative reaction

Abbreviations : cyt *c*, cytochrome *c*; ***E. coli,*** *Escherichia coli;* **MOPS,** 4-morpholinepropanesulfonic acid; **PPO,** protoporphyrinogen oxidase; **tris,** tris (hydroxymethyl) aminomethane carbonate

mechanism was proposed, involving a three-step oxidation of protoporphyrinogen using FAD as the primary electron acceptor and molecular oxygen as the terminal acceptor with three moles of hydrogen peroxide produced per turnover (17). As the next enzyme in the pathway, ferrochelatase, is associated with the inner mitochondrial membrane, it has been suggested that substrate channeling between these proteins may occur (16, 7, 33). The 3.0 Å tobacco PPO structure (27) was docked onto the 2.0 Å human ferrochelatase structure (40) to generate a modeled transmembrane complex. This model suggests that a direct interaction of PPO and ferrochelatase is permitted, even when these enzymes are embedded in a lipid bilayer (27).

In humans, mutations in the PPO gene that result in decreased PPO activity can give rise to Variegate Porphyria (VP) (1, 14, 31), a dominantly inherited disorder characterized by photosensitive skin lesions and a propensity to develop neurovisceral crises (14, 25). 'South African VP' is an example of a founder effect mutation, where the R59W polymorphism accounts for a high proportion of VP cases (30, 23). However, this VP allele is not unique, as different VP polymorphisms have recently been detected in the South African population (5, 4), which contribute to over 120 VP mutations now reported worldwide (39, 28, 18-20).

Previously, it has been demonstrated that the electron acceptors coenzyme Q_0, coenzyme Q_6, and coenzyme Q_{10} inhibit PPO activity when present at concentrations greater than 10 μM (17). The current study examines the possibility that cyt *c*, which is located in the same cellular compartment, might serve as an electron acceptor for PPO. The results suggest that hydrogen peroxide, a product of oxygen dependent PPO activity, mediates the stimulatory action of cytochrome *c* on PPO activity. Given the proximity of PPO and cyt *c*, we have examined the ability of cyt *c* to serve as an electron acceptor either in addition to, or in place of molecular oxygen. The data presented herein suggest that the observed stimulatory effects of cyt *c* on the PPO reaction are mediated by hydrogen peroxide. Since cytochrome *c* has previously been shown to generate free radicals via the breakdown of hydrogen peroxide (34), the elevated PPO rates observed *in vitro* are likely to be attributable to the formation of superoxide and hydroxyl radicals via the peroxidase activity of cyt *c*.

MATERIALS AND METHODS

Materials

Protoporphyrin IX was purchased from Frontier Scientific Inc. (Logan, UT, U.S.A). Tris(hydroxymethyl)aminomethane carbonate (tris), potassium chloride, sodium dihydrophosphate and sodium metal were purchased from J.T Baker. The remaining chemicals were purchased from Sigma-Aldrich unless otherwise specified. Horse heart cyt *c* was purchased from Sigma (≥99% purity by SDS-PAGE).

Cloning

The DNA for the putative protoporphyrinogen oxidases from *D. melanogaster* was amplified from genomic DNA using the polymerase chain reaction. The 5' end was designed to contain an *Nco*I restriction site as well as codons for a His_6-tag for Co^{2+}-chelate chromatography. The 3' end was extended to contain a *Hin*dIII site. This fragment was cloned into the pTrcHis expression vector (Invitrogen). The resultant construct was transformed into *E. coli* JM109 cells for protein expression.

Protein expression and purification

The *D. melanogaster* PPO was overexpressed and purified to homogeneity as previously reported for human PPO (10). Protein preparations were analyzed using UV-visible spectroscopy to confirm the presence of the noncovalently bound FAD cofactor. The protein concentration was determined using a calculated extinction coefficient based on the amino acid composition (ε_{280} values of 45.6 $mM^{-1}cm^{-1}$). The concentration of cyt *c* (Sigma) was determined using a ε_{280} of 11.7 $mM^{-1}cm^{-1}$.

PPO assay 1 – Microaerobic plate reader assay

PPO activity was monitored at 37 ^{o}C using a continuous plate reader assay *via* the detection of protoporphyrin IX fluorescence (36). Concentrations of protoporphyrin IX were determined in 2.7 N HCl using ε_{408} = 262 $mM^{-1}cm^{-1}$ (15). In assays where the oxygen concentration was diminished, all buffers were sparged with nitrogen beforehand and 25 μL of mineral oil was layered on top of each reaction mixture to prevent re-equilibration of dissolved oxygen with the air. The nonenzymatic oxidation rates of protoporphyrinogen IX were monitored concomitantly and were subsequently subtracted. Reaction conditions and concentrations of reactants are quoted in the Figure legends. All assays contained 50 mM NaH_2PO_4 pH 8.0, 0.2 % Tween-20 (v/v). Glutathione was not included as this can cause the partial reduction of cyt *c*.

PPO assay 2 – Anaerobic fluorimetric assay

Reactions were carried out in a 3mL cuvette in 50 mM NaH_2PO_4 pH 8.0, 400 nM *Drosophila* PPO. All assay constituents were sparged with argon for a minimum of 20 minutes, and were added to the cuvette under a stream of argon. The cuvette was then sealed with parafilm to prevent re-equilibration of atmospheric oxygen with the reaction mixture. Where applicable, the concentrations of reactants were as follows: 200 nM *Drosophila* PPO, 10-11.7 μM protoporphyrinogen. 10 mM cyt *c* was added to all reactions after 6 minutes under a stream of argon. A Hitachi F2500 spectrofluorimeter was calibrated with known concentrations of protoporphyrin IX using excitation and emission wavelengths of 545 nm and 630 nm, respectively.

Excitation and emission slit widths were set to 2.5 nm and 10 nm, respectively.

PPO assay 3 – measurement of the hydrogen peroxide:protoporphyrin IX ratio

This assay was performed using a Shimadzu RF-5301PC spectrofluorimeter at 25 ^{o}C. All PPO samples were in 50 mM NaH_2PO_4 pH 8.0, 0.2 % Tween-20 (v/v). Assays were performed in a 3 mL fluorescence quartz cuvette (SCC) using 200 nM PPO, 3 μM protoporphyrinogen, and ± 5 μM cyt *c*. The concentration of hydrogen peroxide was measured by quantification of its complex with homovanillic acid, which fluoresces at 480 nm (21). The reaction buffer was sparged with nitrogen for 20 minutes prior to addition of reactants. 5 mM homovanillic acid and 4 μM horseradish peroxidase were used to convert all hydrogen peroxide to this fluorescent product. Assays were started by the addition of PPO, and fluorescence emission scans (400 – 700 nm) were taken every minute using an excitation wavelength of 315 nm and a 345 nm highpass filter at the emission side of the cuvette. Slit widths were set to correspond to excitation and emission bandwidths of 3 nm and 10 nm, respectively. Fluorescence emission peaks were integrated (Datamax) and plotted against time. For the evolution of hydrogen peroxide, 405 – 525 nm peaks were integrated, and for protoporphyrin IX quantification, 609 – 658 nm peaks were used. The instrument was calibrated with known concentrations of hydrogen peroxide and protoporphyrin IX. Duplicate experiments were performed in the absence of PPO, and these data were subsequently subtracted.

RESULTS

Cytochrome c enhances PPO activity under microaerobic conditions

If PPO uses oxygen as an electron acceptor, hydrogen peroxide is produced, which could lead to the generation of free radicals. Since, cyt *c* is an electron carrier in the same subcellular compartment as PPO, it was of interest to determine if cyt *c* could behave as an electron acceptor for the PPO reaction, without the need for peroxide generation. PPO from *D. melanogaster* was purified as previously described (10). The UV/visible absorption spectrum of the pure protein revealed the presence of an FAD cofactor, and this protein migrated as a dimer on a gel filtration column (data not shown). Fig. 1A shows a *v vs.* [protoporphyrinogen] plot obtained when the assay buffer was not degassed, which yielded k_{cat} and K_{app} values of 5.0 ± 0.3 min^{-1} and 3.8 ± 0.6 μM, respectively.

Figure 1, panels B-D show a series of PPO kinetic assays where the concentration of ferric cyt *c* was varied under low oxygen conditions. Panel B demonstrates that cyt *c* enhances both the PPO-catalyzed rate and the nonenzymatic rate, although the former is augmented to a greater extent. Panel C, where the background curves have been subtracted, shows the accumulation of protoporphyrin IX due to PPO catalysis alone. The steady-state rates are fitted to straight lines using linear regression and these enzymatic rates were plotted against the concentration of cyt *c* (panel D) and the data were fitted to a single rectangular hyperbola. The data clearly demonstrates an increase in PPO catalysis in response to increasing cyt *c* concentrations. Figure 1D inset depicts data for the same experiment, except the assay buffer was not degassed as before. The addition of cyt *c* under these conditions does not provide a significant rate enhancement, but under decreased oxygen availability conditions the addition of cyt *c* is able to restore the catalytic rate of PPO to levels obtained under oxygen saturated conditions (approximately 120 $nMmin^{-1}$).

Cytochrome c becomes reduced during PPO turnover

If cyt *c* accepts electrons from PPO, one would expect the heme cofactor to become reduced during PPO turnover. Hence, the redox state of cyt *c* was monitored spectrophotometrically during PPO turnover. When cyt *c* becomes reduced, an absorption peak around 550 nm is evolved. Fig. 2 illustrates the change in absorbance during PPO catalysis. Panel A demonstrates that cyt *c* becomes reduced when PPO and protoporphyrinogen are present. Interestingly, there appears to be some background reduction of cyt *c* by protoporphyrinogen alone (panel B). In the absence of cyt *c* or substrate, there is no significant increase in absorbance at 550 nm (panels C-E). Panel F depicts the change in absorbance at 550 nm over time for all the assays in panels A-E. Trace 1 demonstrates an elevated rate of cyt *c* reduction during PPO catalysis. Trace 2 demonstrates that cyt *c* becomes reduced in the presence of protoporphyrinogen and absence of PPO. This is consistent with the nonenzymatic evolution of protoporphyrin in the presence of cyt *c*. These data clearly demonstrate that the rate of cyt *c* reduction is enhanced when protoporphyrinogen IX is converted to protoporphyrin IX, both enzymatically and spontaneously. Several attempts were made to isolate a PPO:cyt *c* complex via both immunoprecipitation, and passing protoporphyrinogen and cyt *c* down a cobalt affinity resin with PPO immobilized to it.

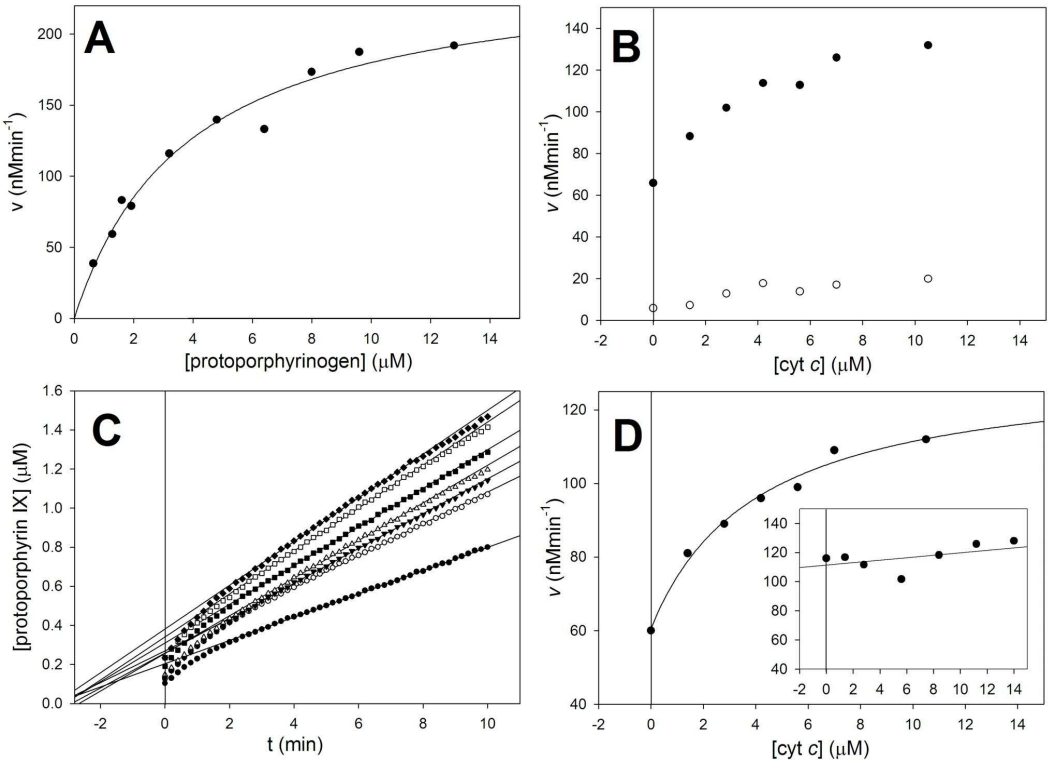


Figure 1. Cytochrome *c* enhances catalysis of *Drosophila* PPO. Reaction mixtures were at 37 °C and 50 nM PPO was present in each assay. (A) *v vs.* [protoporphyrinogen] where the assay buffer was not degassed. V_{max} = 249 ± 16 nMmin^{-1}, K_{app} = 3.8 ± 0.6 μM, k_{cat} = 5.0 ± 0.3 min^{-1} (B) *v vs.* [cytochrome *c*] in the presence (•) and absence (o) of 50 nM PPO. 3 μM protoporphyrinogen was present throughout, and the assay buffer was degassed beforehand (C) The enzyme-catalyzed evolution of protoporphyrin at a fixed concentration of protoporphyrinogen (3 μM), and at various concentrations of cyt *c*. Background data (no PPO present) were subtracted from those obtained in the presence of PPO, and the data were fitted using linear regression between 3 and 8 minutes to estimate the steady-state rates. The concentrations of cyt *c* were: 0 μM (•); 1.4 μM (o); 2.8 μM ▼; 4.2 μM (▽); 5.6 μM (■); 7.0 μM (□); 10.5 μM (◆). (D) The calculated rates from panel C were plotted against the concentration of cytochrome *c*. The assay buffer was degassed in these assays. The inset shows data for the same experiment except the buffer was not degassed.

Unfortunately, no complex could be isolated, which suggests that any putative PPO:cyt *c* complex is short lived, and direct reduction of the heme cofactor of cyt *c* by the FAD of PPO seems unlikely. Furthermore, FAD cofactor of *N. tobaccum* PPO, a close homologue of *D. melanogaster* PPO, is embedded in a deep cleft (27), adding weight to the theory that cyt *c* is not reduced directly by PPO.

Anaerobic assays reveal that hydrogen peroxide is required for the stimulatory effects of cyt c

In the absence of oxygen, cyt *c* appears to diminish the PPO catalytic rate (Table 1). However, when 100 μM hydrogen peroxide is added in the presence of PPO and absence of oxygen, the stimulatory effects of cyt *c* are restored (Table 1). This suggests that the previously characterized peroxidase activity of cyt *c* (34) is in some way enhancing the PPO reaction. One possibility is that superoxide and hydroxyl radicals produced during cyt *c* peroxidation reaction are reacting with protoporphyrinogen or intermediates in the reaction. This is summarized in Scheme 1.

Scheme 1

1) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \cdot O_2^- + 2H^+$
2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$
3) $Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2$
4) $\cdot O_2$- + porphyrinogen intermediate + H^+ $\rightarrow H_2O$ + protoporphyrin IX
5) $\cdot OH$ + porphyrinogen intermediate $\rightarrow H_2O$ + protoporphyrin IX

This could explain why the absence of oxygen, and resultant absence of hydrogen peroxide, diminished the stimulatory effects of cyt *c* on PPO catalysis (Fig. 1D).

To test if superoxide does indeed augment the PPO reaction, PPO was assayed in the presence of 10 μM cyt *c* and 200 μgmL^{-1} superoxide dismutase (SOD). The addition of SOD abolished the stimulatory effects of cyt *c* on PPO activity (data not shown), suggesting that superoxide radicals are involved in the cyt *c*-mediated stimulation of PPO activity.

Cyt c diminishes the evolution of hydrogen peroxide by PPO

If the peroxidase activity of cyt *c* is responsible for augmented PPO activity, one would expect the addition of cyt *c* to diminish the evolution of hydrogen peroxide by PPO. Hydrogen peroxide concentrations were measured simultaneously with protoporphyrin production (Fig. 3). Fig. 3A shows the accumulation of these products in the presence of 5 μM cyt *c*. The catalytic rates were estimated using linear regression and the ratio of [H_2O_2]: [protoporphyrin] was found to be 2.6. The same experiment was performed in the absence of cyt *c* (panel B) and the ratio was 3.2. This demonstrates lower hydrogen peroxide levels when cyt *c* is present, which is consistent with the breakdown of hydrogen peroxide by cyt *c*.

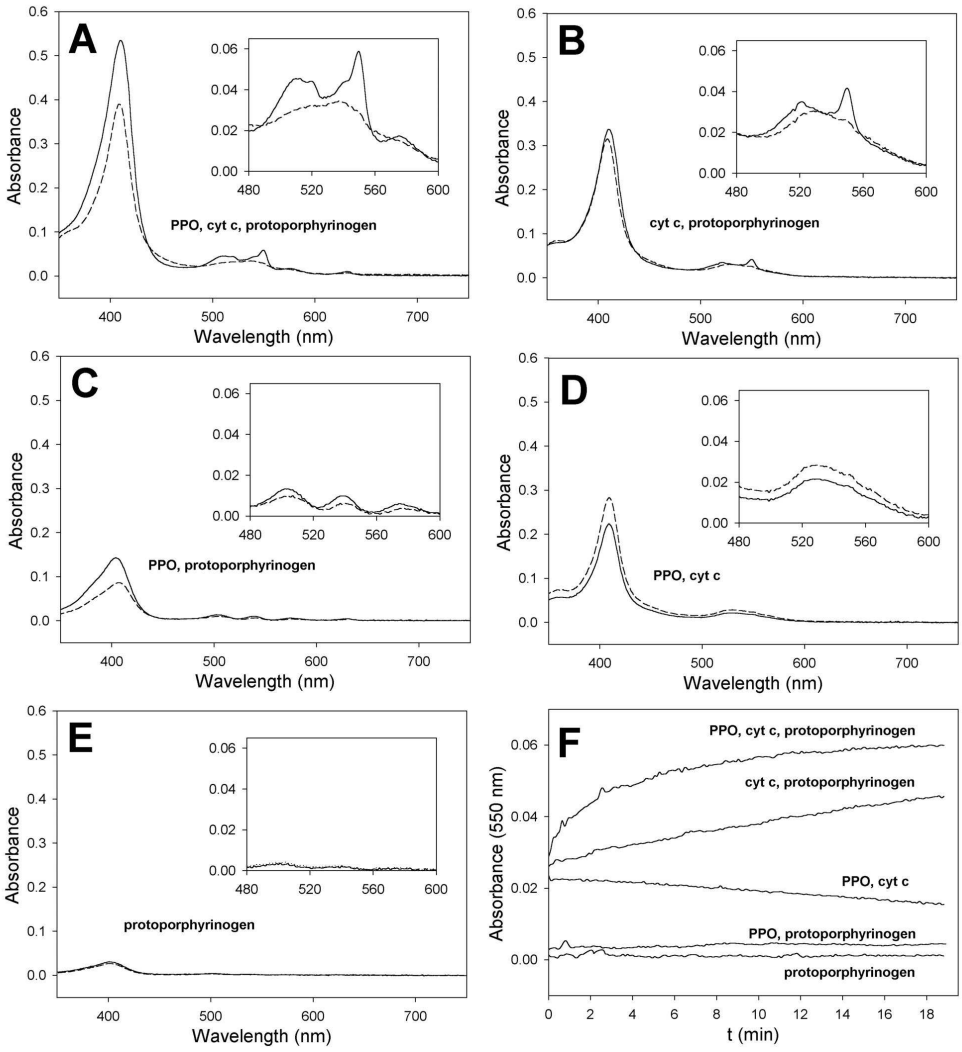


Figure 2. Cytochrome *c* is reduced during PPO catalysis. PPO assays were set up in a 1 mL quartz cuvette and the reactions were started by the addition of PPO. Where present, the concentrations of reactants were: 0.5 μM PPO, 5 μM cyt *c*, 0.6 μM protoporphyrinogen. The reaction buffer was sparged with nitrogen for 20 minutes prior to addition of reactants. Absorbance changes were measured every 30 s using a Cary-50 spectrophotometer. For clarity, only those spectra taken at t = 0 (*dashed lines*) and t = 18 min (*solid lines*) are shown. The insets in panels A-E are included to highlight the change in cyt *c* absorbance around 550 nm. Panel F depicts the change in absorbance at 550 nm over time, for all the assays in panels A-E. Assay components are listed in bold in the main panels.

Table 1. Hydrogen peroxide enhances the stimulatory effects of ferric cyt *c* under anaerobic conditions. Under these conditions, very little hydrogen peroxide will be present, the conjugate product of oxygen in the PPO reaction. PPO assays were conducted under anaerobic conditions following the sparging of all assay constituents with argon for a minimum of 20 minutes. Where applicable, the concentrations of reactants are as follows: 200 nM *Drosophila* PPO, 10 μM protoporphyrinogen. 10 μM cyt *c* was added to all reactions after 6 minutes under a stream of argon. Reaction rates were calculated using linear regression.

Assay conditions	PPO activity –cyt *c* ($\mu Mmin^{-1}$)	PPO activity +10 μM cyt *c* ($\mu Mmin^{-1}$)
+PPO, +protoporphyrinogen IX	0.097	0.059
+PPO, +protoporphyrinogen IX, +H_2O_2	0.099	0.205
-PPO, +protoporphyrinogen IX	0.00	0.030

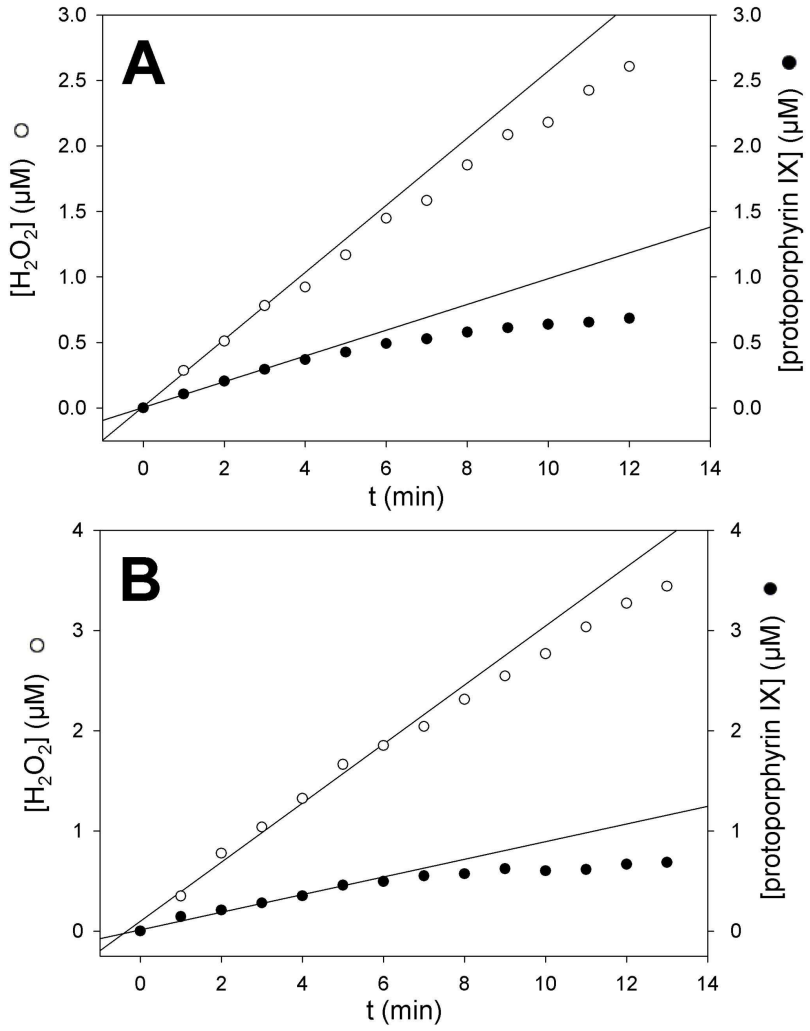


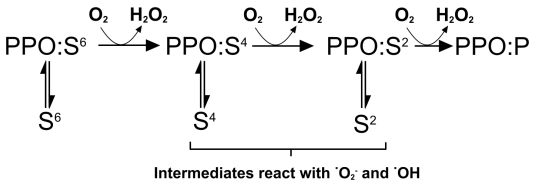
Figure 3. The effect of cyt *c* on the evolution of hydrogen peroxide (o) and protoporphyrin IX (•) during PPO catalysis. 200 nM PPO was assayed for hydrogen peroxide and protoporphyrin production in the presence (panel A) and absence (panel B) of 5 μM cyt *c*. 3 μM protoporphyrinogen was present in each assay. The ratio of [H_2O_2]:[protoporphyrin] is 2.6 and 3.2 in the presence and absence of cyt *c*, respectively.

DISCUSSION

The kinetic parameters for *D. melanogaster* PPO (Fig. 1A) are similar to those previously obtained for *Homo sapiens* PPO (k_{cat} = 10.5 min^{-1}, K_{app} = 1.7 μM (10)) and *Myxococcus xanthus* PPO (k_{cat} = 5.2 min^{-1}, K_{app} = 1.6 μM (8)). The current work describes the first *in vitro* evidence that PPO activity is enhanced by ferric cyt *c*. The observation that this stimulatory effect is only seen at low [O_2] (Fig 1D), and that cyt *c* becomes reduced during PPO turnover (Fig. 2), might suggest that oxygen and cyt *c* perform the same function and are both reduced directly by PPO. However, if this were the case, one might expect the presence of ferric cyt *c* to be sufficient for PPO activity to proceed under anaerobic conditions, which is not the case (Table 1). Furthermore, several attempts were made to isolate a PPO:cyt *c* complex using affinity chromatography and immunoprecipitation, which were largely unsuccessful, indicating that the association between these proteins is either nonexistent or transient. Hence, other avenues were explored, and the generation of radicals by cyt *c* peroxidase activity became the working hypothesis.

The stimulatory effects of cyt *c* appear to be mediated by hydrogen peroxide (Table 1). Given that hydrogen peroxide levels are similar during both aerobic and near-anaerobic growth (12), and cyt *c* concentrations in the intermembrane space are approaching mM levels (0.7 mM) (22), it seems feasible that the peroxidase activity of cyt *c* could have a profound influence on PPO catalysis *in vivo*. Furthermore, since the K_m for oxygen of PPO is around 125 μM (17), and mitochondrial oxygen concentrations are more commonly around 30 μM (35), and can plummet much lower during hypoxia, the existence of an alternative mechanism for protoporphyrinogen oxidation has obvious benefits. Even if the protoporphyrinogen concentration was saturating, a K_m of 125 μM would yield only 19% of maximal PPO activity at 30 μM oxygen. Hence, the involvement of cyt *c* in PPO catalysis could provide an important mechanism to avoid the accumulation of protoporphyrinogen under normoxic conditions, and could be essential for the synthesis of heme during hypoxia.

The hydrogen peroxide hypothesis does raise an important question: why are the stimulatory effects of cyt *c* not as profound at high concentrations of oxygen (where more hydrogen peroxide would be present as a result of PPO turnover)? To provide an explanation, one must consider the previously proposed porphyrinogen dihydro- and tetrahydro-intermediates (29, 27) that are likely to form during the PPO reaction. When the oxygen concentration is high, it is conceivable that these intermediates may have fewer opportunities to dissociate from PPO when oxygen is saturating. Although Koch *et al*. (27) propose a model where intermediates do not dissociate from PPO, there is no kinetic evidence to verify this. If an equilibrium exists between free and PPO-bound intermediates, it seems likely that the free intermediates would be susceptible to oxidation by hydroxyl and superoxide radicals. This provides a possible explanation why the peroxidase activity of cyt *c* does not significantly affect PPO activity at high oxygen levels. It is important to remember that under saturating oxygen concentrations, cyt *c* does significantly increase the nonenzymatic oxidation of protoporphyrinogen in the absence of PPO, and it is just the PPO-catalyzed rate that remains unaffected. Under these conditions, there will be a high concentration of porphyrinogen intermediates free in solution, so the increased rate of protoporphyrin evolution could be explained by the accelerated oxidation of these intermediates by cyt *c*-generated radicals. Similarly, if these intermediates are dissociated from PPO under low oxygen conditions, the presence of cyt *c*-derived radicals shown in Scheme 1 could speed up the rate of protoporphyrinogen oxidation. This is illustrated in Scheme 2.



Scheme 2

Where S^6 is protoporphyrinogen IX substrate, S^4 is a tetrahydro intermediate, S^2 is a dihydro-intermediate, and P is protoporphyrin IX. The presence of superoxide dismutase abolishes the stimulatory effects of cyt *c* on PPO activity, which adds weight to the involvement of radicals in the cyt *c*-mediated rate enhancements observed in Fig. 1. However, it remains unclear as to which protoporphyrin IX precursor is targeted by superoxide.

In the presence of oxygen, PPO has been shown to produce 3 moles of hydrogen peroxide per mole of protoporphyrin IX (17), which is comparable to the data in Fig. 3. The presence of cyt *c* does appear to diminish the concentration of hydrogen peroxide, which is consistent with the hypothesis that peroxidase activity is responsible for the stimulatory effects of cyt *c* on PPO activity.

The current work is consistent with a novel reaction mechanism for PPO involving a cyt *c*-mediated peroxidation reaction, a plausible model given weight by the *in vivo* concentrations of cyt *c*, oxygen, and hydrogen peroxide. Given the importance of this enzyme in Variegate Porphyria and as a herbicide target in plants, it will be of interest to focus on the influence of cyt *c* and hydrogen peroxide on PPO catalysis in these systems. Furthermore, radical generation via peroxidase activity may provide a means of protoporphyrinogen oxidation in prokaryotes that do not possess an oxygen-dependent PPO enzyme.

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