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Ferrocyanide-peroxidase activity of cytochrome *c* oxidase

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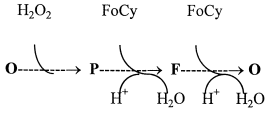
Abstract

Redox interaction of mitochondrial cytochrome *c* oxidase (COX) with ferrocyanide/ferricyanide couple is greatly accelerated by polycations, such as poly-L-lysine [Musatov et al. (1991) Biological Membranes 8, 229–234]. This has allowed us to study ferrocyanide oxidation by COX at very high redox potentials of the ferrocyanide/ferricyanide couple either following spectrophotometrically ferricyanide accumulation or measuring proton uptake associated with water formation in the reaction. At low [ferrocyanide]/[ferricyanide] ratios (E_h values around 500 mV) and ambient oxygen concentration, the ferrocyanide-oxidase activity of COX becomes negligibly small as compared to the reaction rate observed with pure ferrocyanide. Oxidation of ferrocyanide under these conditions, is greatly stimulated by H_2O_2 or ethylhydroperoxide indicating peroxidatic reaction involved. The ferrocyanide-peroxidase activity of COX is strictly polylysine-dependent and is inhibited by heme a_3 ligands such as KCN and NaN_3. Apparently the reaction involves normal electron pathway, i.e. electron donation through Cu_A and oxidation via heme a_3. The peroxidase reaction shows a pH-dependence similar to that of the cytochrome *c* oxidase activity of COX. When COX is preequilibrated with excess H_2O_2, addition of ferrocyanide shifts the initial steady-state concentrations of the Ferryl–Oxo and Peroxy compounds towards approximately 2:1 ratio of the two intermediates. It is suggested that in the peroxidase cycle



ferrocyanide donates electrons to both **P** and **F** intermediates with a comparable efficiency. Isolation of a partial redox activity of COX opens a possibility to study separately proton translocation coupled to the peroxidase half-reaction of the COX reaction cycle. © 1998 Published by Elsevier Science B.V.

Keywords: Cytochrome oxidase; Ferrocyanide; Peroxidase reaction; Hydrogen peroxide

Abbreviations: COX, cytochrome *c* oxidase; **O**, **R**, **P** and **F**: oxidized, reduced, peroxy and ferryl–oxo forms of cytochrome oxidase; FiCy, potassium ferricyanide; FoCy, potassium ferrocyanide; HEPES, 4-(2-hydroxyethyl)-1,1-piperazineethanesulphonic acid; MES, 4-morpholineethanesulphonic acid; MOPS, 4-morpholinepropanesulphonic acid; PL, poly-L-lysine, RuBpy, tris-bipyridyl complex of ruthenium (II)

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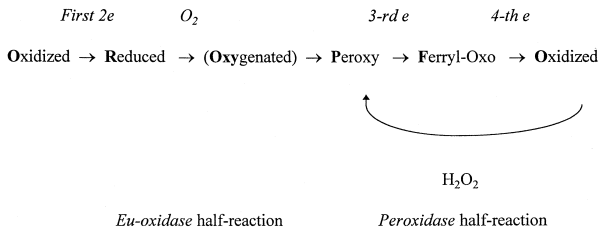
1. Introduction

Mitochondrial cytochrome *c* oxidase (COX) is a terminal respiratory chain enzyme which catalyses $4e^-$ reduction of molecular oxygen to water coupled to generation of a transmembrane difference of proton electrochemical potential, $\Delta\mu_{H+}$ [1,2]. The mechanism of redox-linked proton pumping by COX has proved to be a popular issue in molecular bioenergetic and many hypothetical ''direct'' and ''indirect'' mechanisms have been put forward [3–10].

Site-directed mutagenesis studies of bacterial oxidases closely related to the mitochondrial enzyme allowed to identify the ligands of the redox centers and to establish a number of other basic important features of the enzyme structure [11]. More recently a 3-dimensional structure of cytochrome *c* oxidase has been resolved for the bacterial [12] and mitochondrial enzyme [13,14]. This provides a solid basis for deciphering the functional mechanism by which cytochrome *c* oxidase transfers electrons and pumps protons.

Understanding the enzymatic mechanism implies resolution of the overall catalytic cycle into partial steps. A number of intermediates in the COX-catalyzed reaction have been identified (reviewed, [15–17]) as illustrated by a simplified scheme:



where the names of the intermediates (abbreviated below as **O**, **R**, **Oxy**, **P** and **F**, respectively) refer to the states of the a_3/Cu_B binuclear oxygen-reactive centre (actually, the states of heme a_3). It has to be mentioned that the structure of cytochrome oxidase compound **P** with absorption maximum at 607 nm in the difference spectrum vs. the oxidized state is not yet established and this intermediate can be either an iron–peroxo or a ferryl–oxene complex of heme a_3 (see discussion in [18,19]). In either case, **P** is at the same formal oxidation level as compound I of peroxidases, i.e. two electron deficient relative to the ferric **O** state.

The **O**-to-**P** and **P**-to-**O** halves of the COX catalytic cycle are clearly different. As emphasized by Orii [20], the **P** → **F** → **O** part associated with the transfer of the 3-rd and 4-th electrons is homologous to the sequence compound I → compound II → Ferric in the catalytic cycle of peroxidases. We denote it as peroxidase phase of the overall reaction sequence. As to the first half of the cycle (**O** → **R** → **Oxy** → **P**), it can be viewed as peroxide-yielding oxidase reaction and has been denoted as eu-oxidase to differentiate it from the overall oxidase activity of COX [21]. There is an interesting analogy between the 4-electron eu-oxidase/peroxidase catalytic cycle of COX and 4-electron reduction of O_2 to water by ascorbate where we find two separate hemoproteins: ascorbate oxidase catalyzing 2-electron reduction of O_2 to H_2O_2 [22] and ascorbate peroxidase that reduces H_2O_2 to water in two single-electron steps typical of peroxidases [23,24].

According to current thinking, it is the peroxidase half-reaction that is responsible for proton translocation across the membrane by COX [2,9,12,25]. Therefore, biochemical isolation of the peroxidase partial reaction of COX would be instrumental for analysing the mechanism of proton pumping.

Experimental resolution of the COX cycle into partial steps has been achieved in single turnover studies with the use of rapid kinetics approach (reviewed, [15–17], and see Refs. [18,26] for important recent new findings) and a number of electrogenic proton transfer steps coupled to **P** → **F** → **O** transitions have been revealed [27–30].

Alternatively, it might be possible to dissect the COX-catalysed reduction of O_2 into partial reactions by traditional enzymological approaches with the aid of suitable artificial electron donors and acceptors. This would allow for multiple turnover studies of the partial reactions under steady-state conditions.

In the catalytic cycle of COX, bound hydrogen peroxide is formed at the step of dioxygen reduction by the first two electrons and serves subsequently as the electron acceptor for the 3-rd and 4-th electrons. Therefore, investigation of COX reaction with exogenous H_2O_2 may be a promising approach to by-pass the eu-oxidase phase and separate the peroxidase part of the reaction.

In search of the partial redox activities of COX, we found earlier the oxidized enzyme to react slowly with excess H_2O_2 in a catalase or quasi-catalase cycle, in which the initially formed product with a peak at 607 nm in the difference absorption spectrum versus the oxidized enzyme (compound **P**) is reduced subsequently by two single electron transfers from two more H_2O_2 molecules, the latter oxidized to superoxide radicals [31–33]; an analogous cycle has been reported later on for myeloperoxidase [34]. This catalase activity of COX is based, in fact, on a peroxidase cycle, where the eu-oxidase part of the reaction sequence is by-passed by hydrogen peroxide addition to ferric heme a_3 and excess exogenous hydrogen peroxide serves as the source for the 3-rd and 4-th electrons. However, H_2O_2 is a very sluggish electron donor and the catalase cycle is too slow to be of practical usefulness for investigations into its energy-coupled characteristics.

Indications to COX-catalyzed peroxidation of some organic compounds can be found quite early in the literature [35]. Peroxidatic oxidation of cytochrome *c* by COX was considered more recently by a number of workers [20,36–40] and detailed studies on the rapid kinetics of COX interaction with H_2O_2 as the terminal electron acceptor have been performed in the Amsterdam group [41–44]. Moreover, Miki and Orii were able to observe membrane potential generation [37] and proton translocation [38] upon addition of H_2O_2 to an anaerobic mixture of reduced liposome-reconstituted COX with reduced cytochrome *c*, which was assigned to peroxidase activity of the enzyme.

There are two problems inherent in this type of experiments. First, it was found that H_2O_2 oxidizes the reduced COX via ferrous heme a_3 [41–43,45]. This pathway, where the heme cycles between the Fe(II) and, probably, Fe(III) states is supposed to be CO-sensitive [38], and is fully different from the classical hemoprotein peroxidase chemistry where peroxide binds to ferric heme and the latter cycles between the Fe(III) and Fe(IV) states during turnover; we would denote this peroxidation pathway as pseudo-peroxidase activity (see Section 4 and the scheme in Fig. 7). Second, it is very difficult if possible at all to fully exclude some O_2 generation from added H_2O_2 followed by consumption of the oxygen in the oxidase reaction of COX that is very much faster than the peroxidase activity under the reducing conditions of experiments in [20,36–39].

As a matter of fact, Orii [36] and Miki and Orii [38] noted heterogeneity of the H_2O_2-supported anaerobic oxidation of cytochrome *c* in their experiments, 50%–90% of the reaction being CO-sensitive. As the CO-sensitivity is diagnostic of the reaction of oxygen or hydrogen peroxide with ferrous rather than ferric heme a_3 (cf. [36]), it is likely that the pseudo-peroxidase and/or oxidase reactions dominated over the true peroxidase activity under those conditions.

In view of the importance of resolving the peroxidase activity of COX, we have considered it worthwhile to develop an approach allowing to study the activity of cytochrome oxidase with H_2O_2 as the final acceptor minimizing interference from the oxidase and pseudo-peroxidase activity of the enzyme.

Our rationale was to carry out the reaction at very high redox potential of a donor. At E_h values of ca. $+500$ mV, i.e. well above E_m values of the COX redox centres, probability of reduction of free heme a_3 or/and Cu_B by the donor pre-requisite for the oxidase and pseudo-peroxidase reactions should be very low. Moreover, the overall initial $2e^-$ step of dioxygen reduction to form bound peroxide can become thermodynamically unfavourable ($E_{m,7}$ for the O_2/H_2O_2 is about 400 mV [1,5], although it is probably higher for the binuclear centre-bound O_2/H_2O_2 couple). On the other hand, under these conditions (i) the ferric heme a_3 will react readily with exogenous H_2O_2 and (ii) the Peroxy and Ferryl–Oxo compounds formed in this reaction with E_m values of ca. $+1$ V [2,46] will provide driving force high enough to oxidize readily the high-potential donor. Therefore, peroxidase reaction will be favoured over the oxidase one both kinetically and thermodynamically.

We have chosen ferrocyanide as the high potential donor to COX. Ferrocyanide-oxidase activity of COX was studied earlier [47]. Redox potential of the ferrocyanide/ferricyanide redox couple is appropriately high (about 430 mV [48,49]) and can be easily manipulated by addition of known concentrations of ferricyanide and ferrocyanide. However, in the absence of cytochrome *c*, ferrocyanide is a very poor electron donor to COX (e.g., [50]). To overcome this difficulty we made use of previous observations that interaction of anionic reductants with the enzyme can be promoted by multivalent cations such as Ca^{2+} or

Mg^{2+} [51] or, even more efficiently, by organic polycations like polylysine [51] or clupein [47]. As discussed in [51], this is likely to imply that ferrocyanide donates electrons to COX via the physiological cytochrome *c* reactive site on the Cu_A subunit surrounded by negatively charged dicarboxylic amino acid residues [12,14,52]. Therefore, the polycation-promoted oxidation of ferrocyanide occurs in all probability by the same pathway as oxidation of cytochrome *c*, the physiological electron donor. The results presented here verify the above rationale and show that it is possible to assay ferrocyanide-peroxidase reaction of COX under aerobic conditions without significant interference from the oxidase activity.

2. Materials and methods

30% H_2O_2 ''Suprapur'' was from Merck, poly-L-lysine (5–10 kDa) from Serva, potassium ferrocyanide and potassium ferricyanide from Sigma or Fisher. Ethyl hydroperoxide was a kind gift from Dr. T. Nekipelova (Institute of Chemical Physics, Russian Acad. Sci.). Its concentration was checked by titration of optical changes of metmyoglobin converted to the ferryl state. Other chemicals were commercial products of high purity.

Hydrogen peroxide concentration was checked periodically by measuring extinction at 240 nm and using an extinction coefficient of $40\,M^{-1}\,cm^{-1}$ [53]. When kept cold (not frozen) in the dark and in the presence of 20–50 μM EDTA, the stock solutions of 5–10 mM hydrogen peroxide proved to be stable enough (less than 10% decomposition during a week).

In Moscow, COX was isolated from beef heart mitochondria essentially according to [54,55]. About 90% of the enzyme showed rapid binding of cyanide and H_2O_2. The method used in Bari included an additional purification step [56]. Enzyme concentration was calculated from the difference absorption spectra (reduced minus oxidised) using $\Delta\varepsilon_{605-630} = 27\,mM^{-1}\,cm^{-1}$ [1].

Simultaneous recordings of pH and absorbance changes were made in Bari essentially as described earlier [33,57] in a thoroughly mixed cell placed in a Johnson Foundation dual-wavelength spectrophotometer. A semi-micro glass combination electrode (Beckman Instr. Int., Geneve, No. 39030; response time < 1.5 s) was fed into a Keithley differential electrometer (model 604). The output signals were plotted on a two-channel pen recorder. The pH changes were calibrated with 1–2 μl aliquots of 10 mM HCl before and/or after each probe and ferricyanide accumulation by addition of known concentrations of ferricyanide. Other absorbance measurements were carried out in a Perkin-Elmer λ5 spectrophotomer (in Bari), or in an Aminco DW 2000 UV/VIS instrument in a dual-wavelength kinetic mode at 25°C (in Moscow); the latter applies to most of the studies on the characteristics of ferrocyanide-peroxidase reaction monitored as accumulation of ferricyanide.

Contributions of the **P** and **F** states to the difference absorption spectra of peroxide-treated COX versus the oxidized state were evaluated using approximate extinction coefficients of $5\,mM^{-1}\,cm^{-1}$ and $11\,mM^{-1}\,cm^{-1}$ for the height of the peaks at ~580 nm and 607 nm of the **F** and **P** states, respectively, measured versus a baseline connecting the points of the difference spectra at 630 nm and 510 nm. Total amount of **P** + **F** formed was estimated from the Soret region of difference spectra assuming $\Delta\varepsilon$ of $\sim 50\,mM^{-1}\,cm^{-1}$ for absorbance difference between the maximum at 433–436 nm and minimum at 412–414 nm. This is comparable to spectral characteristics of **P** and **F** assumed by other workers [46,58].

3. Results

Ferrocyanide is a purely electron donor [48,49], whereas reduction of molecular oxygen or of hydrogen peroxide to water requires uptake of 1 proton for each electron. Accordingly, reduction of O_2 or H_2O_2 by ferrocyanide should result in alkalinization.

Fig. 1 shows a typical recording of ferrocyanide oxidation by COX monitored as H^+ uptake. Addition of 200 μM ferrocyanide (FoCy) to aerobic oxidase does not result in measurable proton uptake but the reaction can be initiated subsequently by poly-L-lysine (PL). The PL-stimulated reaction slows down gradually as ferrocyanide is consumed and ferricyanide accumulates, mainly due to increasing redox potential of the donor couple. Addition of 1 mM ferricyanide (FiCy) raising E_h to ca. 500 mV almost stops the

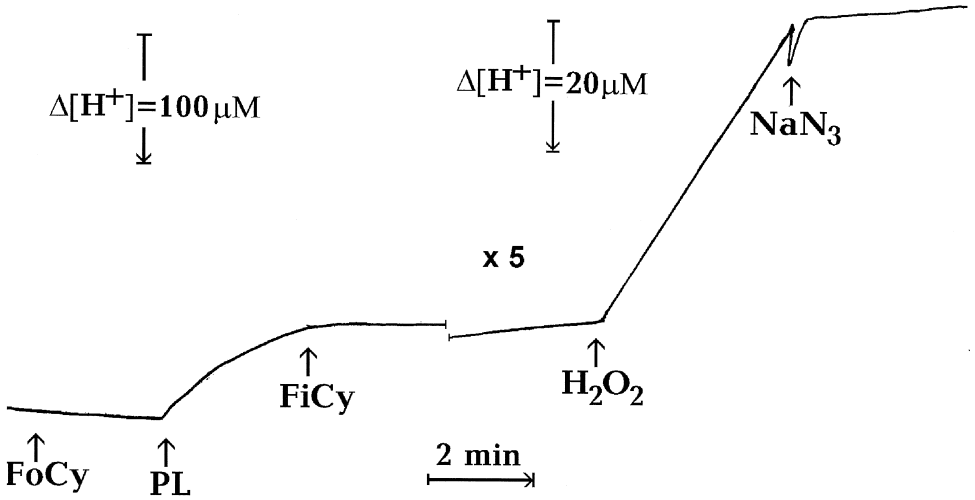


Fig. 1. Proton uptake coupled to ferrocyanide peroxidation by COX. Conditions: 0.55 μM COX in 50 mM HEPES/KOH buffer, pH = 7.5, with 0.5 mM EDTA and 0.5% Tween-80. Additions: ferrocyanide (FoCy), 0.2 mM; poly-L-lysine (PL), 25 μg/ml; ferricyanide (FiCy), 1 mM; H_2O_2, 2 mM; NaN_3, 1 mM.

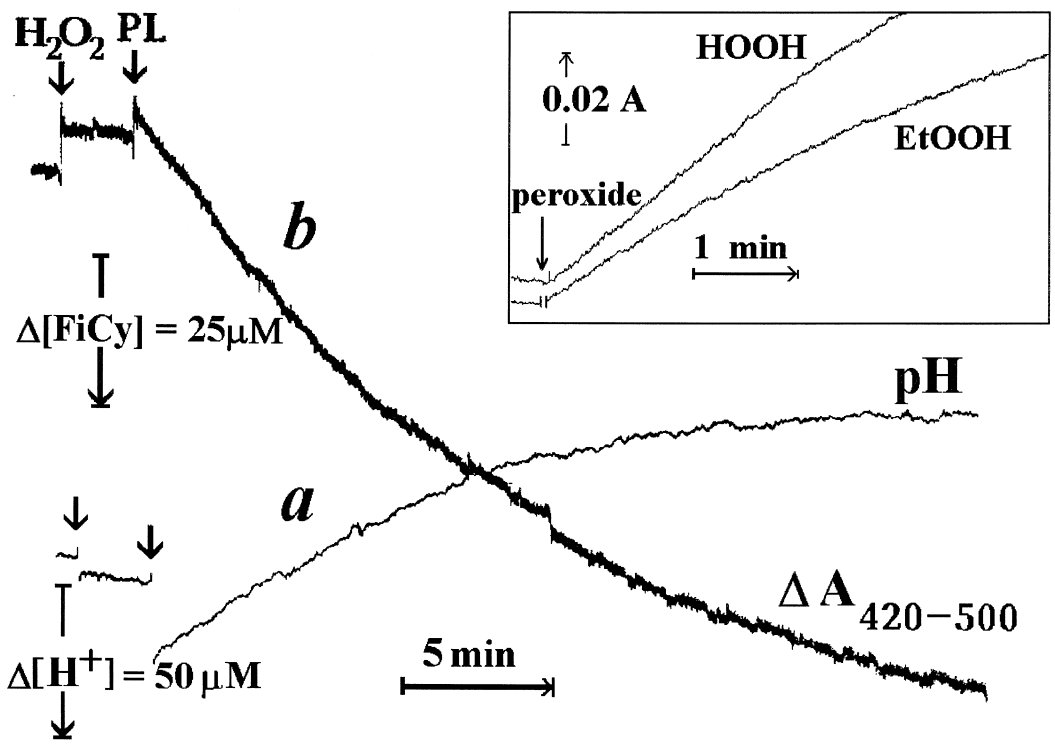


Fig. 2. Simultaneous recordings of proton uptake (a) and accumulation of ferricyanide (b) during ferrocyanide peroxidation by COX. Basic conditions, as in Fig. 1 with 0.1 mM FoCy and 1 mM FiCy; COX concentration, 0.7 μM. Additions: H_2O_2, 2 mM, PL, 20 μg/ml. Inset: COX concentration, 2 μM; ferrocyanide peroxidation is initiated by addition of 2 mM of H_2O_2 or 3 mM ethyl hydroperoxide in the presence of 40 μg/ml of PL.

reaction. Under these conditions oxidation of FoCy can be greatly stimulated by addition of H_2O_2. This H_2O_2-induced reaction is inhibited completely by azide (Fig. 1) as well as by cyanide or catalase (not shown). A number of appropriate controls have been made, including changes in the order of additions and simultaneous recordings of O_2 and proton uptake which show that at high redox potentials (FoCy/FiCy ≤ 0.1) the PL-stimulated FoCy-oxidase activity falls off whereas the FoCy-peroxidase activity persists. No COX-catalysed FoCy-peroxidase activity can be observed at these concentrations of ferrocyanide and ferricyanide in the absence of polylysine.

The peroxide-dependent proton uptake catalysed by COX could also be observed with ferrocene as the high potential electron donor (not shown). Notably, polylysine was not required in this case to elicit the activity; that is expected since ferrocene is not anionic.

Another option to measure the ferrocyanide-oxidase and peroxidase activities of COX is to follow spectrophotometrically increase in absorbance at 420 nm reporting accumulation of FiCy. Fig. 2 shows simultaneous recordings of FiCy accumulation and proton uptake linked to FoCy peroxidation by COX. The reaction can be initiated by PL (Fig. 2), H_2O_2 (Fig. 2, inset) or ferrocyanide (not shown) as the final addition. The absorbance and pH traces match each other kinetically and demonstrate $\Delta[H^+]/\Delta$[FiCy] molar stoichiometry close to 1. Peroxidation of ferrocyanide can also be observed with alkyl-substituted peroxides, such as ethyl hydroperoxide (Fig. 2, inset).

In the subsequent experiments, characteristics of the ferrocyanide peroxidase activity of COX have been explored in more detail. If not indicated otherwise, the reaction was monitored with 100 μM ferrocyanide in the presence of 1 mM ferricyanide at pH 7.5. These conditions are referred below as ''standard''.

The rate of H_2O_2-dependent oxidation of ferrocyanide increases with COX concentration in the range 0.1–4 μM. At H_2O_2 concentration of 2 mM and standard conditions with 40 μg/ml of PL, the dependence of the reaction rate on concentration of the enzyme is close to a straight line with a slope of 0.2 s^{-1} except for a small non-linear region at [COX] < 0.3 μM (data not shown).

The stimulating effect of polylysine on the ferrocyanide-peroxidase reaction is characterized more fully by the data given in Fig. 3. At COX concentrations of 0.5–1 μM and pH below 8.5, the ferrocyanide-peroxidase rate saturates at about 20 μg/ml of PL that corresponds to a slight molar excess of the polycation over COX. This concentration dependence is similar to that previously observed for stimulation of the ferrocyanide-oxidase activity of COX by PL [51].

In the presence of excess of PL, the ferrocyanide-peroxidase reaction shows saturation behaviour with respect to H_2O_2 concentration (Fig. 4). Under the standard conditions (0.1 mM FoCy, 1 mM FiCy, pH 7.5), apparent K_m for H_2O_2 is about 0.5 mM (curve a). At 500 μM FoCy, about 1.2 mM H_2O_2 is required for half-saturation of the reaction rate and the maximal rate increases from 0.4 s^{-1} to ca 1.5 s^{-1} (curve b).

It has to be emphasized that at a constant concentration of FiCy, the ferrocyanide-peroxidase reaction rate first increases with increased concentration of FoCy but above ca. 0.5 mM of the latter tends to go down (not shown). The same effect was described earlier with respect to ferrocyanide oxidase activity of COX [51] and may originate in the inhibition of the enzyme by cyanide released by ferrocyanide [47,59].

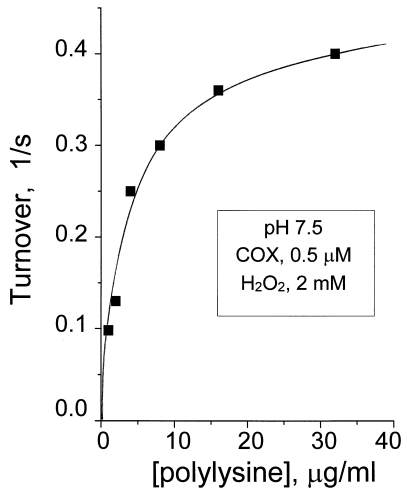


Fig. 3. Stimulation of the ferrocyanide-peroxidase activity of COX by poly-L-lysine. 0.5 μM COX in 50 mM HEPES/KOH buffer, 7.5, containing 0.5 mM EDTA and 0.5% Tween-80. FoCy, 0.1 mM, FiCy, 1 mM, H_2O_2, 2 mM.

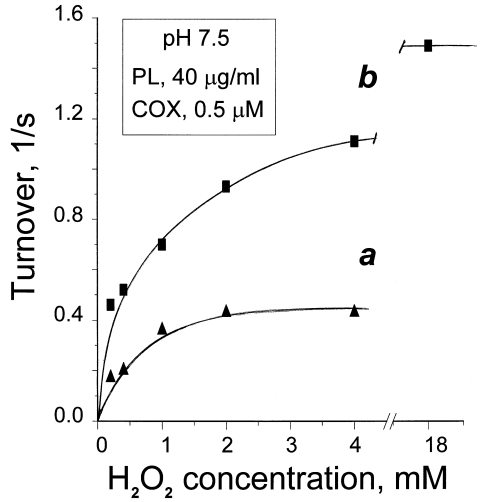


Fig. 4. Effect of H_2O_2 concentration on the ferrocyanide peroxidation by COX. 0.5 μM COX in 50 mM HEPES/KOH buffer, pH 7.5, containing 0.5 mM EDTA, 0.5% Tween-80 and also 40 μg/ml of PL and 1 mM FiCy. Concentration of ferrocyanide is (a) 0.1 mM (FoCy/FiCy = 0.1), or (b) 0.5 mM (FoCy/FiCy = 0.5).

The ferrocyanide-peroxidase activity is highly pH-dependent. The reaction shows a plateau level at pH 6–7 and decelerates with alkalinization (Fig. 5). Above pH ~ 8.5, measurements become less reliable since the stimulating effect of PL on the reaction of ferrocyanide with COX decreases (perhaps, the polycation begins to deprotonate and becomes less charged). Besides, COX-independent reduction of ferricyanide by H_2O_2 at high pH becomes significant enough to interfere with the measurements (in the reaction of one-electron oxidation of H_2O_2 to superoxide anion by ferricyanide, hydrogen peroxide becomes a stronger reductant with alkalinization by about 120 mV per pH unit, whereas E_m of ferricyanide remains pH-independent [48,49]). Notably, the pH-profile of the peroxidase reaction is rather similar to the pH-dependence of the cytochrome *c* oxidase activity of the enzyme (e.g., see [60]) as well as to the pH-dependence of the superoxide-generating catalase activity of COX [32]. As the rate of peroxide binding with ferric heme a_3 in bovine heart COX is pH-independent (Vygodina, unpublished), the pH profile in Fig. 5 may indicate that it is reductive cleavage of peroxide and/or reduction of the heme-bound oxene that requires protonation of a group with pK ~ 8.3. Notably, a group with pK 8.2 involved in proton uptake has been reported to control the rates of **P** → **F** and **F** → **O** transitions in COX in the single-turnover rapid oxidation experiments [61,62].

When excess of H_2O_2 is added to ferric cytochrome *c* oxidase, a steady state is established in which **P** and **F** states of COX are present at different proportion depending on pH, H_2O_2 concentration and some other conditions [31,63–66]. Each of the Peroxy and Ferryl–Oxo compounds is a sufficiently strong oxidant to serve as electron acceptor for the ferrocyanide/ferricyanide couple under these conditions (E_m values of the **P**/**F** and **F**/**O** transitions are about 1.1 and 0.9 V, respectively [46]). Therefore, it is of interest whether ferrocyanide reacts with both states or prefers one of them. To answer this question we have analyzed steady-state concentrations of **P** and **F** during the peroxidase turnover.

Curve a in Fig. 6(A) shows a difference spectrum of COX (vs. the oxidized state) preincubated with 100 μM H_2O_2 under the standard conditions of ferrocyanide-peroxidase activity measurements (0.1 mM FoCy + 1 mM FiCy, pH 7.4) but without poly-L-lysine. The spectrum shows a maximum at 580 nm with a β-band at 535 nm and a noticable shoulder at 607 nm indicating formation of the **F** state with some admixture of **P** typical of this peroxide concentration and pH value [63,65]. It can be seen that addition of polylysine that initiates electron donation from ferrocyanide to COX changes markedly the difference

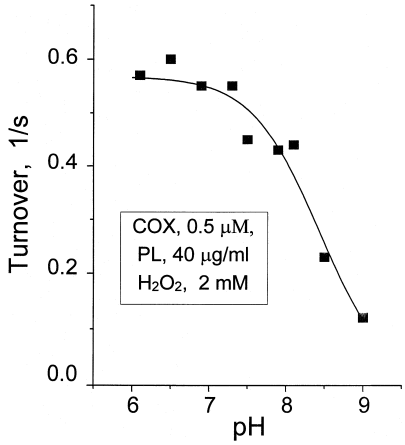


Fig. 5. pH-dependence of COX-catalysed peroxidation of ferrocyanide. Conditions, as in Fig. 3 (with 40 μg/ml of PL) except that different pH buffers were used (MES, MOPS, HEPES or TRIS) depending on pH.

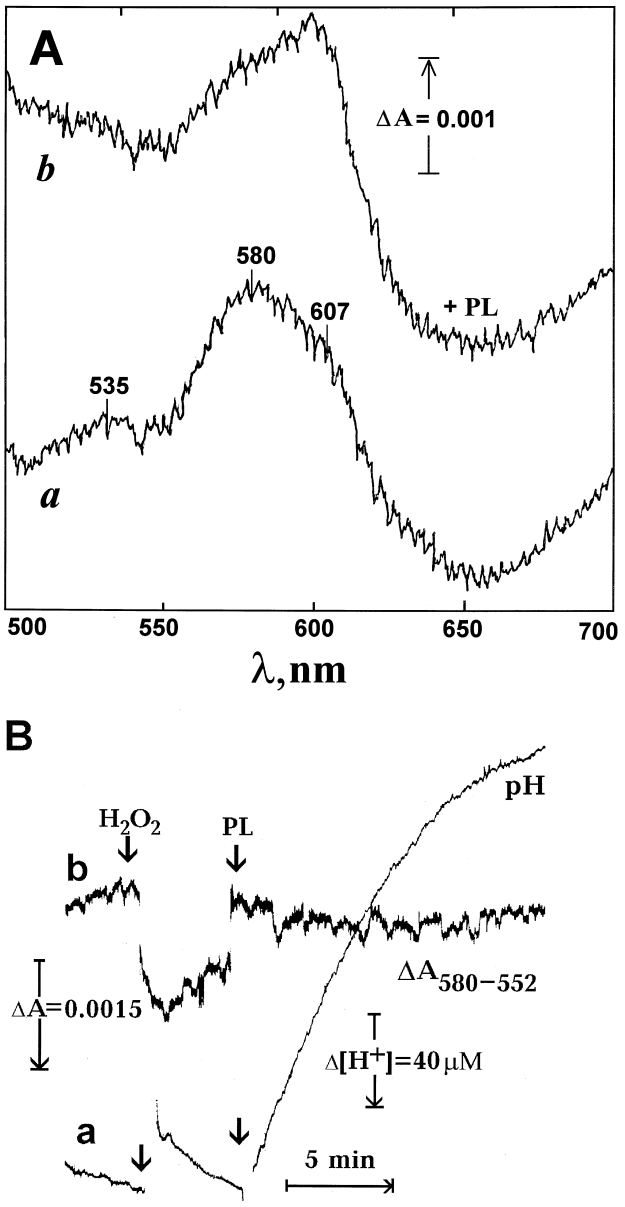


Fig. 6. Effect of ferrocyanide peroxidation on the steady-state concentrations of compounds **P** and **F** in H_2O_2-treated cytochrome oxidase. (A) Difference spectra. The sample and reference cells contain 0.6 μM COX in 50 mM HEPES/KOH buffer, pH 7.5, with 0.5 mM EDTA, 0.5% Tween-80, 0.1 mM FoCy and 1 mM FiCy. (a) 0.1 mM H_2O_2 added to the sample; (b) the same after addition of 20 μg/ml of PL. (B) Simultaneous recordings of (a) proton uptake associated with ferrocyanide oxidation and (b) absorbance changes at 580 minus 552 nm reporting concentration of compound **F**. Conditions as in Fig. 6(A). Additions: H_2O_2, 0.1 mM, PL, 20 μg/ml.

spectrum increasing steady-state concentration of **P** and decreasing that of compound **F** to a final **P**/**F** ratio of ca. 1:2. In the absence of the ferrocyanide/ferricyanide couple, PL did not induce an increase in the concentration of compound **P** (data not included). In addition, some diminution of the trough at 655 nm was observed consistently indicating an increase in concentration of the free oxidized enzyme. Significant increase in **P** with a final steady-state spectrum similar to that in Fig. 6(A) is also obtained when starting from virtually pure compound **F** at 5 mM H_2O_2 at pH 8 (not shown). On the other hand, if the experiment started with COX preequilibrated at the initial ratio of **P**/**F** ~ 0.5–0.7 (100–200 μM H_2O_2 at pH 8 [63,65]), the onset of the ferrocyanide-peroxidase reaction upon addition of PL did not result in significant change of this ratio, but rather increased concentration of the free ferric form and concomitantly decreased the total (**P** + **F**) concentration (data not shown).

Effect of ferrocyanide peroxidation on the steady-state ratio of compounds **P** and **F** in COX reacting with hydrogen peroxide is further illustrated by the kinetics recordings in Fig. 6(B), where concentration of the Ferryl–Oxo state (followed spectrophotometrically at 580 nm) and peroxidase reaction (measured potentiometrically as proton uptake) have been monitored simultaneously. Note, that downward deflection of the absorbance trace in the figure corresponds to increment of $\Delta A_{580-552}$.

Addition of H_2O_2 to the enzyme preincubated with the [FoCy]/[FiCy] = 1:10 redox buffer in the absence of poly-L-lysine results in increased absorbance at 580 nm indicating generation of compound **F** (trace b) but no proton uptake is initiated at this stage (trace a). Subsequent addition of PL initiates proton uptake linked to peroxidation of ferrocyanide (trace a) and, simultaneously, there is a drop in absorbance at 580 nm (trace b) indicating decrease in steady-state concentration of compound **F**. As FoCy peroxidation slows down due to exhaustion of ferrocyanide, there is a slow increase in $\Delta A_{580-552}$ (note a linear instrumental drift in the absorbance trace during the entire recording period in the direction of $A_{580-582}$ decrease that has to be subtracted from the experimental trace). Analogous measurements but following absorbance at 607 nm instead of 580 nm show increased steady-state concentration of

compound **P** concomitant with the onset of FoCy peroxidase reaction (data not included).

4. Discussion

There were few investigations into the peroxidase function of cytochrome *c* oxidase [20,36–44] and these studies have mainly focused on the peroxidation of cytochrome *c* assayed under anaerobic conditions to avoid contribution from oxidation of COX by molecular oxygen. As described in the Section 1, those studies are likely to be concerned mainly with the pseudo-peroxidase peroxidatic pathway which involves hydrogen peroxide interaction with ferrous heme a_3. Our data show that true peroxidase function of COX can be revealed under aerobic conditions.

Fig. 7 gives a simplified scheme of redox events taking place during peroxidase reaction of COX and shows location of the peroxidase and pseudo-peroxidase activities within the overall catalytic cycle. Normally the **P** state is generated in the eu-oxidase half-reaction of the catalytic cycle. However, the oxidized COX can react with exogenous peroxide and compound **P** is formed with $k_1 \sim 10^3\,M^{-1}\,s^{-1}$ [64] and aparent K_d of 2–3 μM [63,67]. Compound **P** can be further converted to **F**, and **F** to **O** in two consecutive single-electron reduction steps 2 and 3 corresponding to addition of the 3-rd and 4-th electrons in the overall catalytic cycle. These electrons can be donated by ferrocyanide (ferro) or cytochrome *c*. The **P** → **F** and **F** → **O** steps can also proceed with the excess H_2O_2 being itself a single-electron donor, superoxide radicals formed [31,32]. However, these reactions are very slow at peroxide concentration of several mM ($k_2^{H2O2} \sim 50\,M^{-1}\,s^{-1}$ [64], $k_3^{H2O2} \sim 3\,M^{-1}\,s^{-1}$ [33]), significantly slower than peroxidase turnover of COX with the ferrocyanide/ferricyanide couple under the conditions of our experiments, and can be neglected in this work.

The **O** → **P** → **F** → **O** reaction sequence with the heme iron operating between the ferric and ferryl states is analogous to the reaction cycle of heme peroxidases and is referred as peroxidase activity of the enzyme. However, hydrogen peroxide is able to react not only with the oxidized heme a_3, but also with the ferrous heme a_3 in the **R** state of the binuclear center [41–44]. This latter reaction pathway

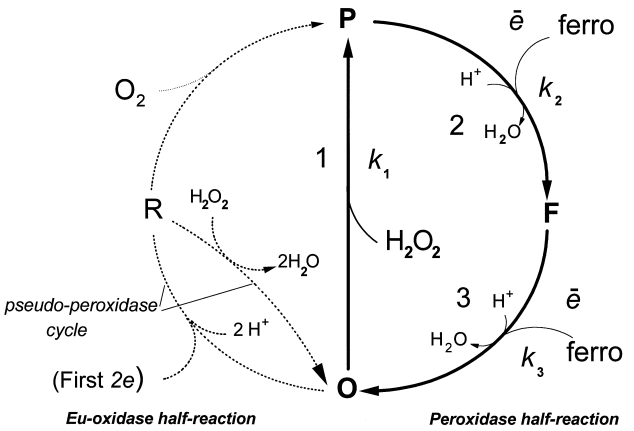


Fig. 7. Ferrocyanide-peroxidase redox cycle of COX. The peroxidase activity of COX (bold arrows) is shown as partial reaction of the overall oxidase catalytic cycle of the enzyme. The oxy-complex state is omitted from the scheme for simplicity and also since its generation in the absence of CO has not been demonstrated. The eu-oxidase half of the COX cycle (**O** → **R** → **P**) as well as the pseudo-peroxidase cycle (**O** ↔ **R**) are not operative under the conditions of our ferrocyanide-peroxidase activity measurements due to very low population of the **R** state at high redox potential of the ferrocyanide/ferricyanide couple and are depicted by dotted arrows. Generally, **P** and **F** can accept electrons during the peroxidase assay not only from ferrocyanide, but also from excess H_2O_2, the latter oxidized to superoxide anion [32,33]. However, under the conditions of present experiments, the effective rate constants of steps 2 and 3 with H_2O_2 as electron donor (k_2^{H2O2}) and (k_3^{H2O2}) are much slower than with the ferrocyanide/ferricyanide couple (k_2^{Ferro}) and (k_3^{Ferro}). Therefore, the superoxide-generating oxidation of H_2O_2 by **P** and **F** has not been included in the scheme and only reductions by ferrocyanide are indicated. Comparable steady-state concentrations of **P** and **F** during the ferrocyanide-peroxidase reaction indicate that k_2 and k_3 are not that much different, i.e. that ferrocyanide reacts with both **P** and **F** states at comparable rates.

denoted here as pseudo-peroxidase cycle (Fig. 7), was likely to predominate in the earlier anaerobic cytochrome *c*-peroxidase assays [20,36–38,40].

While studying the ferrocyanide-peroxidase activity of COX in this work we did not aim to come up with a detailed kinetics analysis of this bi-substrate reaction but rather to provide some practical guidelines as how to handle it.

4.1. Comparison with earlier studies

Earlier attempts to measure the peroxidatic function of COX without removing oxygen were counteracted by interference of the oxidase reaction. For instance, Orii [20,36,39] reported a small increase of

the rate of ferrocytochrome *c* oxidation by COX under aerobic conditions upon addition of 0.23 mM H_2O_2 (from 0.0047–0.0063 s^{-1} at 3.8 nM COX); however, as noted by the author, even this modest effect was due by 2/3 (0.0009 s^{-1}) to direct reaction of ferrocytochrome *c* with the peroxide. Thus, at equimolar concentrations of oxygen and peroxide, at least 90% of electron transfer through COX goes to molecular oxygen [39]. Our work establishes the conditions allowing to minimize contribution of the oxidase reaction so that oxidation of ferrocyanide by H_2O_2 in the presence of atmospheric oxygen is more than 90% due to the peroxidase activity of COX. This greatly facilitates investigations of this partial redox activity of the enzyme.

The rates for the ferrocyanide-peroxidase activity observed here under ''standard'' conditions (0.1 mM ferrocyanide in the presence of 1 mM ferricyanide) were typically about 0.5 s^{-1} at saturating peroxide concentration and neutral pH. The turnover increased to ca. 1.5 s^{-1} at ferrocyanide/ferricyanide ratio of 2 (Fig. 4). This is in fairly good agreement with the observed turnover number of 0.2–0.5 s^{-1} (e.g., Fig. 2 in Ref. [36]) and V_{max} of 1.9 s^{-1} reported for anaerobic cytochrome *c* peroxidase activity of solubilized COX by Orii and Miki in [38]. Higher values (ca. 10 s^{-1}) have been reported by Lodder et al. [44] for COX-catalyzed cytochrome *c* peroxidation at saturating concentrations of hydrogen peroxide (> 10 mM) under pre-steady state conditions and by Orii group for V_{max} of the reaction under steady-state turnover in proteoliposomes [37,38]. It seems that in the peroxidase assay, speaking in practical terms, submillimolar ferrocyanide in the presence of polylysine is not that much inferior to the natural electron donor cytochrome *c* at usual (several micromolar) concentrations of the latter. An important advantage of ferrocyanide is that at pH < 8.5 it does not react noticably with H_2O_2 whereas cytochrome *c* preparations usually reveal quite a significant reactivity towards peroxide (e.g., [20]).

Ferrocene has been found to be another potentially useful electron donor to COX in the peroxidase assay. However, this compound is less convenient since partition between water and hydrophobic mycelle phase is very much different for ferrocene and its conjugated oxidized form (ferricinium), which complicates control over redox potential of the ferrocene/ferricinium donor couple. Also, ferricinium is not readily available commercially. Experiments are in progress to test other artificial electron donors, like high-potential quinols, in the peroxidase assay.

4.2. Is the ferrocyanide-peroxidase activity relevant to the normal function of COX?

An obvious and important question is whether the peroxidase activity of COX is related at all to the normal catalytic mechanism of the enzyme. Alternatively, it might occur by some entirely artificial pathway. We believe that the electron transfer routes for the oxidase and peroxidase reactions of COX are much the same and the peroxidase reaction is physiologically relevant to the normal catalytic mechanism. The basic arguments are as following.

4.2.1. The electrons enter the enzyme via Cu_A and leave it via heme a_3

Although it has not been established yet with full certainty what is the redox centre of COX that reacts with the ferrocyanide/ferricyanide couple, there are good reasons to believe that the interaction takes place via Cu_A. First, Cu_A is the only redox-active metal ion in COX located at the periphery of the protein close to the solvent-exposed surface of the enzyme while the two hemes and Cu_B are buried in the protein [12–14] and are not likely to be directly accessible to hydrophilic ferrocyanide. Second, the polycation dependence of ferrocyanide oxidation in the oxidase [47,51] and peroxidase reactions (this work) corroborates the suggestion that FoCy donates electrons via the physiological negatively charged entry site on the Cu_A subunit of the enzyme [52]. We have shown earlier that heme *a* reduction by the negatively charged ferrocyanide and ascorbate is greatly accelerated by various polycations including polylysine whereas the latter did not affect interaction with neutral organic reductants and inhibited reaction with positively charged donors like hexammineruthenium [51].

As to the exit site, the detailed studies of the Amsterdam group [42–44] leave little doubt that peroxide accepts electrons via heme a_3. Accordingly, the peroxidase activity in our studies is blocked by the classical inhibitors of the oxidase reaction like cyanide and azide.

An interesting possibility is that the intraenzyme

routes for the four electrons in the cytochrome *c* oxidase reaction can be different. For instance in the compounds **P** and **F**, the electron from Cu_A could go directly to the oxygen intermediates of heme a_3 bypassing heme *a* [37,68] (an opposite possibility, i.e. heme *a* by-passed by the first two electrons, has been discussed in Refs. [69,70]). Decoupled electron transfer pathway from Cu_A directly to the binuclear centre has been considered by Capitanio et al. [71]. In this sense, peroxidase reaction could be different from the overall catalytic cycle. However, recent time-resolved studies on photochemical reduction of compounds **P** and **F** by RuBpy or RuBpy-modified cytochrome *c* [27–29,72] show that heme *a* is reduced in these compounds by Cu_A at the same rate and yield as in the ferric enzyme and about 20-fold faster than the heme a_3-bound oxygen intermediates.

4.2.2. Ferrocyanide peroxidation is energy-coupled

The data of Vygodina et al. [73], show that the ferrocyanide-peroxidase activity of cytochrome oxidase reconstituted in proteoliposomes gives rise to generation of membrane potential and is linked to proton pumping with the H^+/e^- ratio of 2 exceeding that of the oxidase reaction two-fold (cf. results of the Orii group [37,38]).

In addition, it has been found recently [74,75] that amino acid replacements D132N and E286Q in the COX input proton channel involved in proton pumping [12,21,76] inhibit peroxidase activity of COX, whereas K362M mutation in the second channel (probably involved in proton loading during the eu-oxidase part of the reaction [21,77]), while fully eliminating the cytochrome oxidase activity of the enzyme, does not affect significantly its peroxidase activity with either ferrocyanide or ferrocytochrome *c* as electron donor [74,75].

It is also noteworthy, that despite the different absolute rates, the peroxidase activity of COX shows pH-dependence rather similar to that of the cytochrome *c* oxidase reaction. Also the temperature dependences (activation energies) of cytochrome oxidase oxidation by hydrogen peroxide and dioxygen are very similar [41].

All the above arguments are in favour of the peroxidase reaction of COX being a true partial reaction of the normal catalytic cycle intimately involved in energy-transduction by the enzyme.

As found recently, there can be two **P** states of COX similar in absorption characteristics of heme a_3 but differing in the redox state of Cu_B and denoted as $\mathbf{P}_R$ (Cu_B reduced) and $\mathbf{P}_M$ (Cu_B oxidized) [18]. $\mathbf{P}_R$ and $\mathbf{P}_M$ appear as intermediates during oxidation by dioxygen of the fully reduced COX or of the 2-electron reduced enzyme, respectively [18,26]. It is likely that the **P** state in our peroxidase cycle corresponds to $\mathbf{P}_M$, although there is no direct evidence for the oxidized state of Cu_B in the peroxide-treated enzyme. Accordingly, the $\mathbf{P} \rightarrow \mathbf{F}$ step may be somewhat different in the peroxidase cycle and single-turnover oxidation of the fully reduced enzyme, although in both cases it appears to be coupled to proton pumping [29,30,78]. Which of the two **P** states is more relevant to cytochrome oxidase turnover under physiological conditions remains to be established.

4.3. Why the ferrocyanide-peroxidase reaction is so slow?

The peroxidase activity of COX as measured here (ca. $0.5\,s^{-1}$) as well as in the earlier works [20,36–44] is by about 3 orders of magnitude lower than the maximal turnover rate of the enzyme in the cytochrome *c* oxidase assay. Why is it that slow?

First, H_2O_2 binding with the ferric heme a_3 is sluggish ($k_{on} \sim 10^3\,M^{-1}\,s^{-1}$ [43,64] as compared to $10^8\,M^{-1}\,s^{-1}$ typical of oxygen binding to the reduced heme a_3 [17]). For instance, at 0.2 mM H_2O_2 the steady-state rate as limited by peroxide binding would be $0.4\,s^{-1}$ that is only about 20-fold faster than the actual ferrocyanide-peroxidase turnover number observed at this peroxide concentration and [ferrocyanide]/[ferricyanide] ratio of 0.1.

Second, population of the reduced heme *a* is quite low at high E_h of the ferrocyanide/ferricyanide couple (too low to measure; estimated, ca. 0.1% at $E_h = 500$ mV); if extrapolated to full reduction of heme *a* assuming linear dependence of the reaction on the steady-state concentration of heme *a*, the rate will reach $500\,s^{-1}$ which is about V_{max} of the enzyme. Conceivably, such an extrapolation is not fully valid but it is likely to give some reasonable rough estimate.

Earlier, Gorren et al. [41,43] found that the intramolecular electron transfer is slower in COX with

H_2O_2 as electron acceptor (15–25 s^{-1} as opposed to ca. 700 s^{-1} in the reaction of COX with O_2). They proposed that either H_2O_2 inhibited internal electron transfer in COX or that oxygen intermediates formed in the reaction of COX with dioxygen greatly speed up internal electron transfer in COX and that these intermediates are not formed in the reaction with H_2O_2. The latter conclusion is very likely to be true with respect to the pseudo-peroxidase pathway, where the reduced binuclear centre Fe(II)/Cu(I) can reduce H_2O_2 to $2H_2O$ and heme a_3 iron returns back to the ferric form without entering the higher oxidation state route. However, generation of **P** and **F** intermediates upon peroxide reaction with ferric COX has been amply confirmed [31,63–66,79,80]. Therefore, the internal electron transfer rates in the true peroxidase activity should not be lower in the peroxidase assay as compared to the overall oxidase cycle as indeed shown for the individual **P**-to-**F** and **F**-to-**O** transitions of COX with the use of RuBpy as photoactive electron donor [27,29,72].

*4.4. Ferrocyanide-peroxidase reaction of COX implies reaction of ferrocyanide with both **P** and **F***

If we consider all the 3 steps in the peroxidase cycle shown in Fig. 7 as irreversible which is a plausible assumption, the steady-state ratio of **P**, **F** and **O** in the presence of H_2O_2 and ferrocyanide/ferricyanide couple (+polylysine) will be determined essentially by the ratio of the effective rate constants of (1) H_2O_2 reaction with **O** (k_1^{H2O2}), (2) electron donation by ferrocyanide/ferricyanide couple to **P** (k_2^{Ferro}) and (3) electron donation by ferrocyanide/ferricyanide couple to **F** (k_3^{Ferro}), respectively. Under steady-state conditions, $v_2 = k_2$ [ferro][**P**] $= v_3 = k_3$ [ferro][**F**] and, accordingly, k_2 [**P**] $= k_3$ [**F**] and [**P**]/[**F**] $= k_3/k_2$. Our data show that under most conditions, the [**P**]/[**F**] ratio during the steady-state peroxidation of ferrocyanide is not that far from 1 (ca. 0.5–0.7). Hence, it is likely that the ferrocyanide/ferricyanide couple reacts with both **P** and **F** states with comparable rate constants. A somewhat faster oxidation of ferrocyanide by **P** as implied by the ~2-fold higher steady-state concentration of **F** is qualitatively consistent with the higher E_m value of compound **P** as compared to compound **F** [46].

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