

Proton pumping by cytochrome *c* oxidase is coupled to peroxidase half of its catalytic cycle

T.V. Vygodina^a, N. Capitanio^b, S. Papa^b, A.A. Konstantinov^{a,*}

^a*A.N. Belozerskiy Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia*

^b*Institute of Medical Chemistry and Biochemistry, University of Bari, Bari, Italy*

Received 5 May 1997

Abstract The four-electron reaction cycle of cytochrome oxidase is comprised of an *eu-oxidase* phase in which the enzyme receives the first two electrons and reduces oxygen to bound peroxide and a *peroxidase* phase in which the peroxy state formed in the *eu-oxidase* half of the cycle is reduced by the 3rd and 4th electrons to the ferryl-oxo state and oxidized form, respectively. Here we show that the ferrocyanide-peroxidase activity of cytochrome *c* oxidase incorporated in phospholipid vesicles is coupled to proton pumping. The H^+/e^- ratio for the ferrocyanide-peroxidase partial reaction is twice higher than for the overall ferrocyanide-oxidase activity and is close to 2. These results show that proton pumping by COX is confined to the *peroxidase* part of the enzyme catalytic cycle (transfer of the 3rd and 4th electron) whereas the *eu-oxidase* part (transfer of the first two electrons) may not be proton pumping.

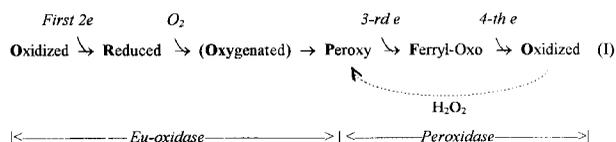
© 1997 Federation of European Biochemical Societies.

Key words: Cytochrome oxidase; Proton pumping; Peroxidase; Proteoliposome; Oxygen intermediate; Membrane potential

1. Introduction

Cytochrome *c* oxidase (COX) catalyses 4-electron reduction of dioxygen to water by cytochrome *c* and pumps protons electrogenically across the membrane at the expense of this highly exergonic reaction [1,2]. The mechanism of the redox-linked proton pumping is an intriguing issue and one of the first questions to be concerned is which of the numerous partial redox steps of the catalytic cycle are directly linked to translocation of protons.

The COX reaction sequence includes a number of intermediates [3,4] as described by a simplified scheme



in which intermediate states of heme a_3 in the heme a_3/Cu_B binuclear oxygen-reducing centre are indicated. We use below traditional abbreviations **O**, **R**, **Oxy**, **P** and **F** to denote these intermediates.

The reaction sequence in the scheme is comprised of two

distinct parts. The **O** → **R** → **Oxy** → **P** half (transfer of the first 2 electrons) is a typical oxidase reaction (peroxide-yielding), heme a_3 cycling between the ferric and ferrous states. We denote it as *eu-oxidase* part of the COX catalytic cycle. The **P** → **F** → **O** sequence (transfer of the 3rd and 4th electrons) is analogous to the catalytic cycle of heme-containing peroxidases where the iron-porphyrin group cycles between the ferric and ferryl state. We denote it as *peroxidase* part.

Wikstrom showed that effect of protonmotive force on the equilibrium ratio of intermediates **P**, **F** and **O** in liver mitochondria is consistent with each of the **P** → **F** and **F** → **O** transitions being coupled to translocation of 2 protons [4]. Taking into consideration the net H^+/e^- ratio of 1 for the overall COX reaction [1], this indicated that transfer of the first two electrons may not be coupled to translocation of protons, and all proton pumping is associated with transfer of the 3rd and 4th electrons. However, conclusions based on interpretation of partial equilibria between *some* of the intermediates within the entire catalytic cycle can only be provisional. It would be useful to resolve the COX catalytic cycle into partial reactions and to measure directly proton and charge translocation coupled to the isolated partial reactions under single-turnover and/or steady-state conditions.

The **P**-state of COX can be generated by reacting the ferric enzyme with hydrogen peroxide (see scheme) [5–8]. This allows to truncate the COX reaction cycle bypassing the *eu-oxidase* part and to isolate biochemically the *peroxidase* half-reaction. It was shown earlier [9–13] that under anaerobic conditions, COX catalysed slow oxidation of cytochrome *c* upon addition of hydrogen peroxide. Under aerobic conditions, H_2O_2 could not compete with oxygen to any significant extent and contribution of peroxidatic oxidation of cytochrome *c* was barely visible [14]. Similar conclusions were arrived at in the pre-steady state studies of the Amsterdam group [15–17].

There are two problems inherent in the anaerobic cytochrome *c* peroxidation experiments. First, despite all the precautions [11], it is extremely difficult if possible at all to ensure that minute amounts of oxygen are not formed from hydrogen peroxide in the presence of COX and cytochrome *c*. The rate constant of O_2 reaction with the reduced heme a_3 is about 10^5 -fold higher than that of hydrogen peroxide binding. Therefore, generation of just nanomolar concentrations of oxygen from ca. 10^{-3} M peroxide used in [10–13] could result in the reaction going by the oxidase rather than peroxidase pathway. Second, under reducing conditions hydrogen peroxide reacts with COX via the CO-sensitive pathway in which H_2O_2 interacts with ferrous state of heme a_3 [11,13] (the peroxide-dependent **O** → **R** → **O** cycle in Fig. 2). This pathway implying two-electron reduction of H_2O_2 by the binuclear centre is very different from the classical peroxidase mecha-

*Corresponding author. Fax: (7) (095) 939 03 38.

E-mail: konst@libro.genebee.msu.su

Abbreviations: COX, cytochrome *c* oxidase; COV, cytochrome *c* oxidase vesicles; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; **O**, **R**, **P**, **F**, oxidized, reduced, peroxy and ferryl-oxo forms of cytochrome *c* oxidase

nism as the enzyme does not pass through the higher oxidation states of heme iron. Rather this peroxide shunt corresponds to the oxidase reaction where O_2 is mimicked by H_2O_2 . Therefore we would denote this partial reaction as a *pseudo-peroxidase* pathway to distinguish it from the $O \rightarrow P \rightarrow F \rightarrow O$ true peroxidase pathway analogous to the catalytic cycle of peroxidases (Fig. 2). Actually, Miki and Orii reported on the heterogeneity of anaerobic cytochrome *c* peroxidation in their experiments, and concluded that multiple reaction mechanisms coexisted in their experimental model [13].

It would be worthwhile to find conditions allowing to study true peroxidase activity of COX aerobically without interference from the oxidase and pseudo-peroxidase reactions. As described in [18,19] this can be done with ferrocyanide as electron donor at high redox potentials of the ferrocyanide/ferricyanide couple (ca. 450–500 mV) which renders reduction of ferric heme a_3 ($O \rightarrow R$ step of the catalytic cycle) thermodynamically unfavourable and eliminates efficiently the eu-oxidase and pseudo-peroxidase pathways.

In this paper we show that the ferrocyanide-peroxidase activity of COX incorporated in phospholipid vesicles is coupled to proton pumping. Moreover, proton pumping in the peroxidase reaction is twice more efficient ($H^+/e^- = 2$) than in the overall ferrocyanide-oxidase reaction ($H^+/e^- = 1$). This finding corroborates the hypothesis [20] that proton pumping by COX is specifically linked to the *peroxidase* ($P \rightarrow F \rightarrow O$) half-reaction of the catalytic cycle. These results have been reported at the 8th EBEC Meeting in Valencia in 1994 [18].

2. Materials and methods

2.1. Chemicals

Hydrogen peroxide ('Suprapur') was from Merck. Phosphatidyl choline from dried egg yolk type X-E and poly-L-lysine (MW 4600–6900) from Sigma. Potassium salts of ferrocyanide and ferricyanide ('Analar' grade) were from Fisher. Other chemicals were commercial products of high grades from conventional sources.

2.2. Preparations

COX was isolated from beef heart mitochondria according to the protocol [21] as modified in the Bari group [22] and reconstituted in phospholipid vesicles by a conventional cholate dialysis method [23]. COV are traditionally made from soya bean phospholipids (asolectin) [23]. However, the ferrocyanide-peroxidase reaction has to be investigated in the presence of polylysine that greatly stimulates redox interaction of the ferrocyanide/ferricyanide couple with COX [24]; polylysine binds with the negatively charged asolectin liposomes and agglutinates them. Therefore, we have prepared COV using neutral phospholipids (phosphatidyl choline). At ca. 10^{-3} M peroxide, the ferrocyanide-peroxidase activity is rather slow (less than 1 s^{-1} [19]) and at low turnover rates proton pumping by COV becomes difficult to follow resulting in low apparent H^+/e^- values [25]. Therefore, very high quality of proteoliposomes is required to allow for measurements of the respiratory control and translocation of protons. After some efforts, we succeeded in routine preparation of COV with a respiratory control ratio of 20–80 with ascorbate and cytochrome *c* as electron donor. The vesicles showed at least 4-fold stimulation of the ferrocyanide-peroxidase activity by valinomycin and uncoupler.

2.3. Measurements

Proton translocation and ferrocyanide oxidation were monitored simultaneously in a thoroughly stirred optical cell placed in a Johnson Foundation dual-wavelength spectrophotometer essentially as described [26,27]. pH was monitored with a rapid semi-micro glass combination electrode (Beckman No. 39030; time constant < 1.5 s). Ferrocyanide oxidation to ferricyanide was followed spectrophotometrically at 420 minus 500 nm. The pH and optical responses were

calibrated by addition of known concentrations of HCl and ferricyanide.

3. Results

Fig. 1A shows proton pumping coupled to ferrocyanide-peroxidase activity of COX incorporated in phosphatidyl choline liposomes. Addition of 100 μ M ferrocyanide to valinomycin-supplemented COV in the presence of 1 mM ferricyanide and 4 mM H_2O_2 initiates almost linear increase of absorbance at 420 nm (accumulation of ferricyanide) with the rate of 0.26 s^{-1} . No ferricyanide generation is observed in the absence of hydrogen peroxide (not shown, cf. [19]). Oxidation of ferrocyanide is accompanied by transient acidification of the medium followed by a slower phase of net proton consumption. This is a typical proton pumping trace behaviour as observed in conventional experiments with cytochrome *c* as electron donor [23,25]. The acidification is not observed in the absence of valinomycin (data not included) and is abolished by the uncoupler CCCP (Fig. 1B, trace 1). The ferrocyanide-peroxidase reaction is fully inhibited by KCN (Fig. 1B, trace 3).

The COX-catalysed peroxidatic oxidation of ferrocyanide is stimulated ~ 4 -fold (to 1.0 s^{-1}) in the presence of the uncoupler CCCP (cf. Fig. 1A and B) indicating that the reaction is coupled to membrane energization. The ratio of the steady-state rates of ferrocyanide oxidation and proton consumption linked to H_2O_2 reduction to H_2O in the uncoupled COV is close to 1 in agreement with the data on purified COX [19].

A rapid downward deflection of the pH trace has been observed upon addition of ferrocyanide. This effect is also seen in the presence of the uncoupler or KCN (Fig. 1B) and can reflect an artifactual glass electrode response and/or a minute change of pH induced by ferrocyanide addition. The essential point is that this artifactual response is reproducible and is complete within the mixing time. Therefore it can be easily corrected for and does not interfere with the kinetics of proton release.

The ratio of the rate of proton translocation (0.53 s^{-1}) to the rate of ferricyanide accumulation (0.26 s^{-1}) in Fig. 1A gives an H^+/e^- ratio of 2 when measured in a traditional way as the ratio of slopes of the initial almost linear parts of the traces (ca. 30 s); the maximal scatter observed in different experiments was 1.5–2.5. This value is twice higher than H^+/e^- of 1 observed for the cytochrome *c* oxidase reaction in most laboratories under optimal conditions (e.g. see [25,28,29]).

It can be noted that the absolute H^+/e^- values as obtained in this kind of measurements are prone to errors. This is partly due to the non-linear character of the pH-trace, so that drawing a tangent to the initial part of the curve may be a somewhat arbitrary procedure. To minimize this problem, we have repeated our measurements with COV catalysing ferrocyanide-oxidase reaction and adjusting the conditions as close as possible to those of the ferrocyanide-peroxidase assay to allow for direct comparison between the overall *oxidase* and partial *peroxidase* activities of COX.

The results are shown in Fig. 1C. The pH traces in A and C are superimposable in their initial parts from the reaction onset to the top of the curve. It can be seen that the same rate and amplitude of the transient acidification due to proton pumping is obtained at a 2-fold lower rate of ferrocyanide

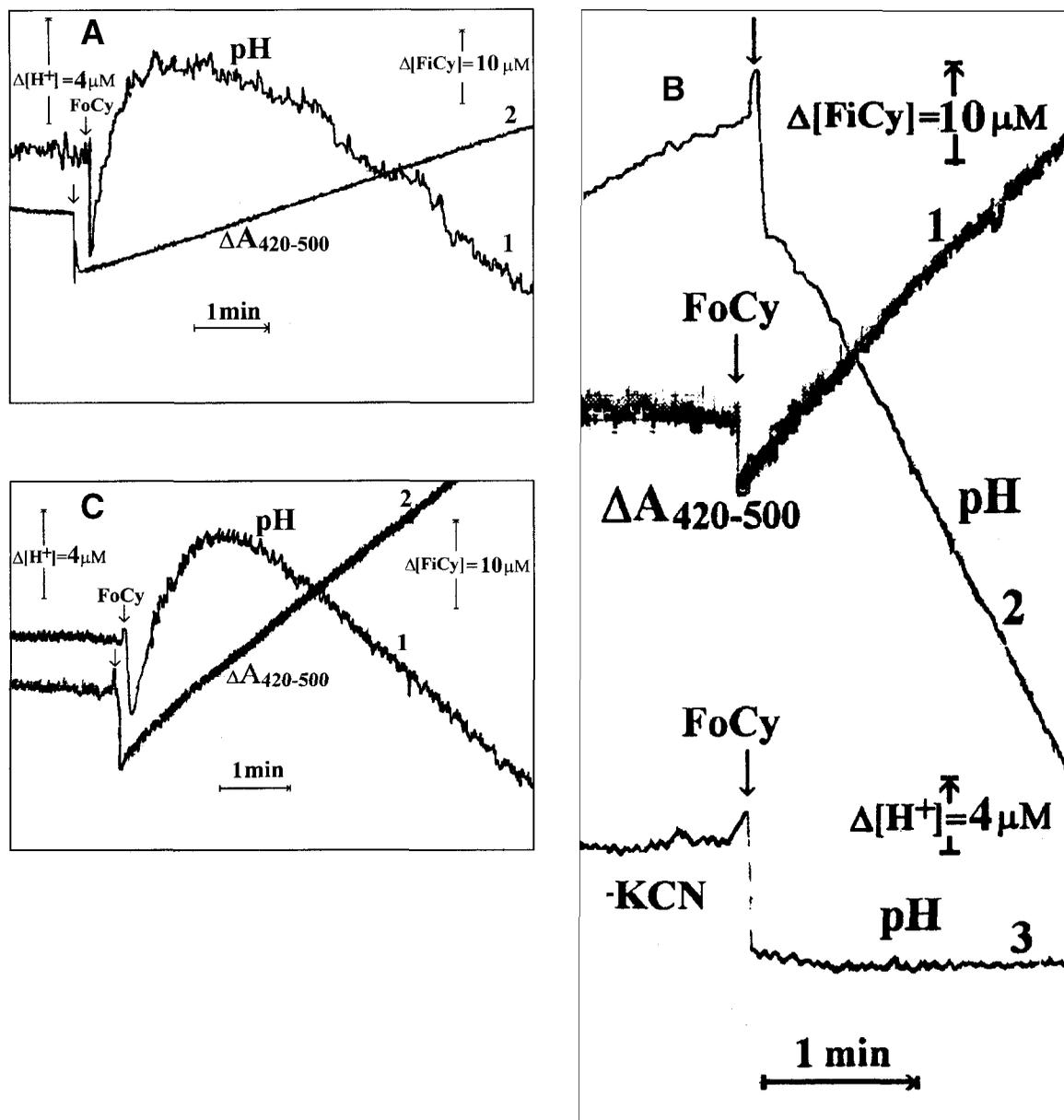


Fig. 1. Proton translocation coupled to ferrocyanide-peroxidase (panels A and B) and ferrocyanide-oxidase (panel C) activity of cytochrome *c* oxidase. Traces: 1 and 3, pH-changes monitored with a combination glass electrode. 2, accumulation of ferricyanide monitored spectrophotometrically. Note, that the pH and absorbance traces are displaced by ~ 15 s relative to each other on the chart due to position of the two pens in the dual-channel strip chart recorder. In panels A and B, the reaction mixture contained COV ($0.3 \mu\text{M}$ in COX) in 0.3 M sucrose, 0.2 mM EDTA, 50 mM KCl, $40 \mu\text{g/ml}$ of poly-L-lysine and $1 \mu\text{g/ml}$ of valinomycin. pH in the cell has been adjusted to 7. (A) The ferrocyanide-peroxidase activity (ferrocyanide: H_2O_2 -oxidoreductase) is initiated by addition of $100 \mu\text{M}$ ferrocyanide (FoCy) in the presence of 1 mM ferricyanide and 4 mM hydrogen peroxide. Under these conditions, contribution of the oxidase reaction to the rate of ferrocyanide oxidation is below detection limit. (B) Conditions, as in A but in the presence of the uncoupler ($1 \mu\text{M}$ CCCP, traces 1 and 2) or 4 mM KCN (trace 3). (C) The ferrocyanide-oxidase reaction (ferrocyanide: O_2 -oxidoreductase) is initiated by addition of $100 \mu\text{M}$ ferrocyanide to aerobic vesicles. 0.5 mM ferricyanide has also been included in the medium to adjust the rate of proton translocation to that observed in the peroxidase reaction.

oxidation in case of the peroxidase reaction (accordingly, the H^+/e^- ratio calculated for the ferrocyanide-oxidase activity of COX is $0.9\text{--}1.0$). This result clearly demonstrates that irrespective of the absolute H^+/e^- ratio values, the peroxidase reaction translocates protons twice more efficiently (speaking of the H^+/e^- ratio) than the overall oxidase cycle.

4. Discussion

The major conclusions of this work are summarized in the

scheme in Fig. 2. Our data show that the ferrocyanide-peroxidase activity of COX, although slow as limited by the rate constant of H_2O_2 binding with heme a_3 [19], is coupled to energization of membrane and translocation of protons. Moreover, the $\text{H}^+/\text{e}^- = 2$ ratio for peroxidation of ferrocyanide is twice higher than for its oxidation by dioxygen or than the H^+/e^- ratio of 0.9 reported for anaerobic peroxidation of cytochrome *c* [13]. These results, reported earlier at the 8th EBEC Conference in 1994 [18], corroborate the hypothesis of Wikstrom [4] that proton-pumping by COX is associated ex-

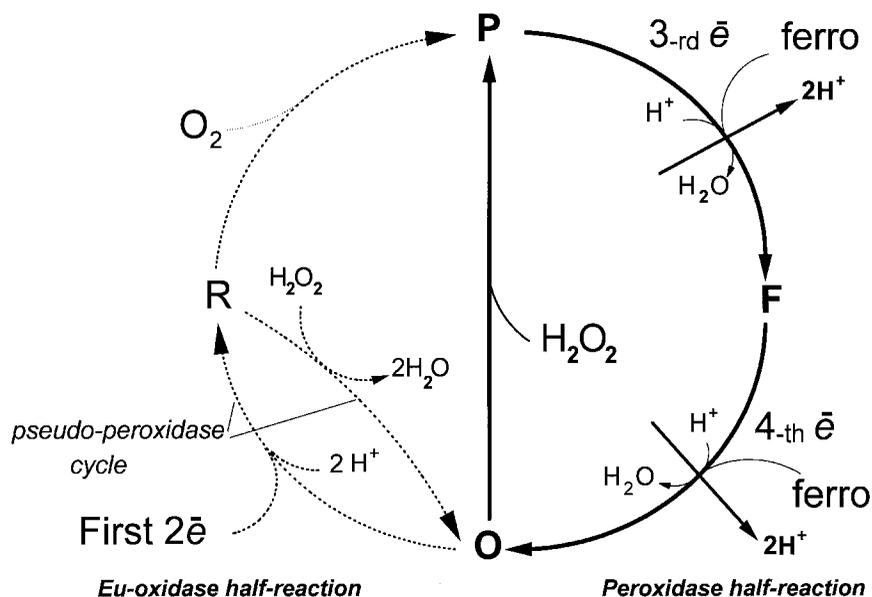


Fig. 2. Proton translocating peroxidase cycle of COX. The scheme illustrates the reactions involved in the ferrocyanide-peroxidase activity and overall catalytic cycle of COX. The proton-pumping peroxidase cycle $\text{O} \rightarrow \text{P} \rightarrow \text{F} \rightarrow \text{O}$ is depicted by bold lines. The *eu-oxidase* part of the cycle is not operational under the conditions of the ferrocyanide-peroxidase assay and is given by dashed lines. The peroxide-dependent $\text{O} \rightarrow \text{R} \rightarrow \text{O}$ cycle in the *eu-oxidase* part of the scheme corresponds to the pseudo-peroxidase activity observed by the Orii group [10–13] and, probably, by other workers [9,15–17] under the conditions of *anaerobic* cytochrome *c* peroxidase assay. It can be seen that this partial reaction is totally different from the peroxidase activity of COX along the $\text{O} \rightarrow \text{P} \rightarrow \text{F} \rightarrow \text{O}$ pathway studied in this work. Although not included in the figure, there should be several sub-states of **P** considered in the scheme (cf. [30]) so that the *protonated* form of **P** formed upon H_2O_2 reaction with the **O**-state [27,40] is not fully identical with the *unprotonated* **P** formed initially upon the $2e^-$ reduction of O_2 [2] (omitted from the scheme).

clusively with the transfer of the 3rd and 4th electrons (and see [30] for recent discussion and references).

First indications to $\text{H}^+/e^- > 1$ ratio linked to peroxidatic function of COX can be found in the works of the Orii group in 1986 [12]. Miki and Orii noticed heterogeneity of the anaerobic cytochrome *c* peroxidase activity of COX: in their experiments CO fully inhibited proton pumping but only 50% of electron transfer. The authors proposed that it is reaction of H_2O_2 with COX via *ferrous* heme a_3 (their 'mechanism I' as denoted in [13], the pseudo-peroxidase $\text{O} \rightarrow \text{R} \rightarrow \text{O}$ cycle in Fig. 2) that is CO-sensitive and drives proton translocation with $\text{H}^+/e^- \sim 2$, while the CO-insensitive pathway corresponds to peroxide reaction with the *ferric* heme a_3 ('mechanisms II or III' as mentioned in [13], $\text{O} \rightarrow \text{P} \rightarrow \text{F} \rightarrow \text{O}$ route in Fig. 2) and is not coupled to proton pumping at all [13]. In this respect, our conclusions are directly opposite to those of the Orii group. The results of this work have been obtained under highly oxidizing conditions that eliminate the $\text{O} \rightarrow \text{R}$ step of the COX catalytic cycle and make the pseudo-peroxidase $\text{O} \rightarrow \text{R} \rightarrow \text{O}$ pathway unlikely [19]. Hence it is the classical peroxidase mechanism $\text{O} \rightarrow \text{P} \rightarrow \text{F} \rightarrow \text{O}$, where peroxide binds with *ferric* heme a_3 and generates higher oxidation states of heme iron, that drives proton translocation with 2H^+ per electron efficiency.

Electrogenicity of the isolated F-to-O transition of COX in single-turnover experiments corresponds to electrogenic pumping of protons with H^+/e^- stoichiometry of 1.5 [31]. Although higher than 1, which favours qualitatively the hypothesis of Wikstrom [4], the H^+/e^- ratio of 1.5 observed in these experiments is still non-negligibly lower than $\text{H}^+/e^- = 2$ as implied by [4]. It was suggested that intermediates **P** and **F** formed in the reaction of ferric COX with H_2O_2 may not be fully physiological due to their incorrect protonation states

(M. Wikstrom, P. Rich, personal communications to A.A.K.; see [30] for more specific discussion). It is therefore noteworthy that according to the present data, the H_2O_2 -generated intermediates are likely to be physiologically competent as evidenced by the H^+/e^- ratio of 2 observed for the steady-state peroxidase reaction. It is possible that the partial reactions as studied in (sub)single turnover transitions [31] do not encompass the full energy-transducing span of the peroxidase reaction and some of the electrogenic proton transfer steps inherent in the peroxidase cycle of COX are left out (cf. [30]); obviously, these steps must be present when the enzyme runs through the entire cycle in the multiple-turnover steady-state studies.

Profound differences between the *eu-oxidase* and *peroxidase* reaction phases of COX in the redox chemistry and energy-transducing mechanisms led to a hypothesis presented by one of us (A.A.K.) at the Cytochrome Oxidase Conference in Rieti in 1992 that these half-reactions may be catalysed by two structurally different states of the enzyme (see also [32]). Evidence for the *eu-oxidase* and *peroxidase* phases of the COX cycle using different input proton channels [33,34] has been reported recently [32,35].

It is interesting to mention in this connection that enzymatic 4-electron reduction of oxygen to water by ascorbate can proceed in two discrete 2-electron steps catalysed by two separate hemoproteins: an ascorbate oxidase that reduces O_2 to H_2O_2 [36] and ascorbate peroxidase that reduces H_2O_2 to water [37,38]. An analogy to the *eu-oxidase* and *peroxidase* halves of the COX cycle is obvious except that cytochrome oxidase integrates both reactions in a single enzyme.

The possibility to run separately the proton-pumping peroxidase half of the COX cycle is consistent with the hypothesis that the *eu-oxidase* and *peroxidase* halves of COX cata-

lytic cycle are relatively independent of each other. Accordingly, recent experiments of the Moscow group in collaboration with R. Gennis's laboratory in Urbana (IL) have shown that K→M replacement of the conserved Lys-362 in one of the two input proton channels revealed in subunit I of COX [33,34] inhibits the *eu-oxidase* part of the COX reaction sequence without affecting the *peroxidase* activity of COX [39].

Acknowledgements: We would like to thank Prof. V.P. Skulachev for his interest in this work and helpful comments. This work is supported in part by the Grants of Russian Fund for Basic Research 94-04-12726a and 97-04-49765 (A.A.K.), and 97-04-49144 (T.V.V.). T.V.V. and A.A.K. participated in a Bilateral Collaboration Research Project between the Universities of Moscow and Bari and were supported by a grant from UNESCO Global Network for Molecular and Cell Biology.

References

- [1] Wikström, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase – A Synthesis, Academic Press, New York.
- [2] Babcock, G.T. and Wikström, M. (1992) Nature 356, 301–309.
- [3] Babcock, G.T. and Varotsis, C. (1993) J. Bioenerg. Biomembr. 25, 71–80.
- [4] Wikström, M. (1989) Nature 338, 776–778.
- [5] Wrigglesworth, J. (1984) Biochem. J. 217, 715–719.
- [6] Vygodina, T.V. and Konstantinov, A.A. (1988) Ann. N.Y. Acad. Sci. 550, 124–138.
- [7] Vygodina, T. and Konstantinov, A. (1989) Biochim. Biophys. Acta 973, 390–398.
- [8] Weng, L. and Baker, G.M. (1991) Biochemistry 30, 5727–5733.
- [9] Bickar, D., Bonaventura, J. and Bonaventura, C. (1982) Biochemistry 21, 2661–2666.
- [10] Orii, Y. (1982) in: Oxygenases and Oxygen Metabolism (Nozaki, M. et al., Eds.), pp. 137–149, Academic Press, New York.
- [11] Orii, Y. (1982) J. Biol. Chem. 257, 9246–9248.
- [12] Miki, T. and Orii, Y. (1986) J. Biochem. 100, 735–745.
- [13] Miki, T. and Orii, Y. (1986) J. Biol. Chem. 261, 3915–3918.
- [14] Orii, Y. (1990) in: Bioenergetics (Kim, C.H. and Ozawa, T., Eds.), pp. 171–180, Plenum Press, New York.
- [15] Gorren, A.C.F., Dekker, H. and Wever, R. (1985) Biochim. Biophys. Acta 809, 90–96.
- [16] Gorren, A.C.F., Dekker, H. and Wever, R. (1986) Biochim. Biophys. Acta 852, 81–92.
- [17] Gorren, A.C.F., Dekker, H., Vlegels, L. and Wever, R. (1988) Biochim. Biophys. Acta 932, 277–286.
- [18] Vygodina, T.V., Konstantinov, A.A., Capitanio, N. and Papa, S. (1994) EBEC Short Rep. 8, 37.
- [19] Konstantinov, A.A., Vygodina, T.V., Capitanio, N. and Papa, S. (1997) Biochim. Biophys. Acta (submitted).
- [20] Wikström, M. and Krab, K. (1979) Biochim. Biophys. Acta 549, 177–222.
- [21] Errede, B., Kamen, M.O. and Hatefi, Y. (1978) Methods Enzymol. 52, 40–47.
- [22] Planques, Y., Capitanio, N., Capitanio, G., De Nitto, E., Villani, G. and Papa, S. (1989) FEBS Lett. 258, 285–288.
- [23] Hinkle, P.C. (1979) Methods Enzymol. 55, 751–776.
- [24] Musatov, A.P., Berka, V., Ksenzenko, M.Y., Vygodina, T.V. and Konstantinov, A.A. (1991) Biolog. Membr. (Moscow) 8, 229–234.
- [25] Capitanio, N., Capitanio, G., De Nitto, E., Villani, G. and Papa, S. (1991) FEBS Lett. 288, 179–182.
- [26] Papa, S., Guerrieri, F. and Rossi-Bernardi, L. (1979) Meth. Enzymol. 55, 614–627.
- [27] Konstantinov, A.A., Capitanio, N., Vygodina, T.V. and Papa, S. (1992) FEBS Lett. 312, 71–74.
- [28] Casey, R.P. (1984) Biochim. Biophys. Acta 768, 319–347.
- [29] Antonini, G., Malatesta, F., Sarti, P. and Brunori, M. (1993) Proc. Natl. Acad. Sci. USA 90, 5949–5953.
- [30] Wikstrom, M., Morgan, J.E. and Verkhovsky, M.I. (1997) Biochim. Biophys. Acta 1318, 299–306.
- [31] Zaslavsky, D., Kaulen, A. and Smirnova, I.A. (1993) FEBS Lett. 336, 389–393.
- [32] Konstantinov, A.A., Siletskiy, S.A., Mitchell, D., Kaulen, A.D. and Gennis, R.B. (1997) Proc. Natl. Acad. Sci. USA (submitted).
- [33] Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Nature 376, 660–669.
- [34] Tsukihara, T., Aoyama, H., Yamashita, E., Takashi, T., Yamaguichi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) Science 272, 1136–1144.
- [35] Siletsky, S.A., Kaulen, A.D., Mitchell, D., Gennis, R.B. and Konstantinov, A.A. (1996) EBEC Short Rep. 9, 90.
- [36] Kim, Y.R., Yu, S.W., Lee, S.R., Hwang, Y.Y. and Kang, S.O. (1996) J. Biol. Chem. 271, 3105–3111.
- [37] Patterson, W.R., Poulos, T.L. and Goodin, D.B. (1995) Biochemistry 34, 4342–4345.
- [38] Patterson, W.R. and Poulos, T.L. (1995) Biochemistry 34, 4331–4341.
- [39] Vygodina, T., Mitchell, D., Pecoraro, C., Gennis, R. and Konstantinov, A. (1996) EBEC Short Rep. 9, 93.
- [40] Vygodina, T.V. and Konstantinov, A.A. (1987) FEBS Lett. 219, 387–392.