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Mechanistic and Phenomenological Features of Proton Pumps in the Respiratory Chain of Mitochondria

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Various direct, indirect (kinetic and thermodynamic), and combined mechanisms have been proposed to explain the conversion of redox energy into a transmembrane protonmotive force (Δp) by enzymatic complexes of respiratory chains. The conceptual evolution of these models is examined. The characteristics of thermodynamic coupling between redox transitions of electron carriers and scalar proton transfer in cytochrome *c* oxidase and its possible involvement in proton pumping is discussed. Other aspects dealt with in this paper are: (i) variability of $\leftarrow H^+/e^-$ stoichiometries, in cytochrome *c* oxidase and cytochrome *c* reductase and its mechanistic implications; (ii) possible models by which the reduction of dioxygen to water at the binuclear heme-copper center of protonmotive oxidases can be directly involved in proton pumping. Finally a unifying concept for proton pumping by the redox complexes of respiratory chain is presented.

KEY WORDS: Redox proton pumps; redox Bohr effect; respiratory chains; cytochrome *c* oxidase; cytochrome *c* reductase; NADH ubiquinone oxidoreductase.

EVOLUTION OF CONCEPTS

Each of the three enzymatic complexes making up the respiratory chain of mitochondria converts redox energy into a transmembrane protonmotive force (Δp) (Mitchell, 1966, 1987a; Papa, 1976; Boyer *et al.*, 1977; Wikström and Saraste, 1984; Malmström, 1989; Rich, 1991). The cytochrome system, in particular, was originally conceived by Mitchell (1966) as consisting of an outward directed hydrogen-conducting quinone arm connected, in series, to an inward electron-carrying arm of metal centers of bc_1 and aa_3 cytochrome complexes (redox loop), resulting in the effective translocation of $2H^+$ from the N to the P side of the membrane per mole of quinol oxidized by oxygen direct mechanism. Papa *et al.* (1975) found, however, that the $\leftarrow H^+/2e^-$ stoichiometry for electron flow in the bc_1 complex of mitochondria from ubiquinol to cytochrome *c* is 4 and not 2 (see also Lawford and Garland, 1973). This was followed by the report of Wikström (1977) and Wikström and Krab (1979) that further electron flow from cytochrome *c* to oxygen resulted in additional proton release ($\leftarrow 2H^+/2e^-$). Such a conclusion, after substantial opposition (Mitchell and Moyle, 1983; Papa and Lorusso, 1984), came to be generally accepted (West *et al.*, 1986; Papa *et al.*, 1987). To explain these $\leftarrow H^+/2e^-$ stoichiometries in terms of direct mechanism. Mitchell introduced the protonmotive quinone cycle for the bc_1 complex (Mitchell, 1976) and the oxygen cycle for cytochrome *c* oxidase (Mitchell *et al.*, 1985), subsequently replaced by the Cu loop (Mitchell, 1987b) (Fig. 1a).

Based on the principle of cooperative thermodynamic linkage in allosteric proteins (Bohr effects) (Wyman, 1968) and the finding that linkage between electron transfer at the metal and protolytic events does occur in cytochromes (redox Bohr effects) (Dutton and Wilson, 1974; Papa, 1976; Papa *et al.*, 1979), Papa *et al.* (1973) proposed an indirect cooperative model for proton pumping by cytochromes

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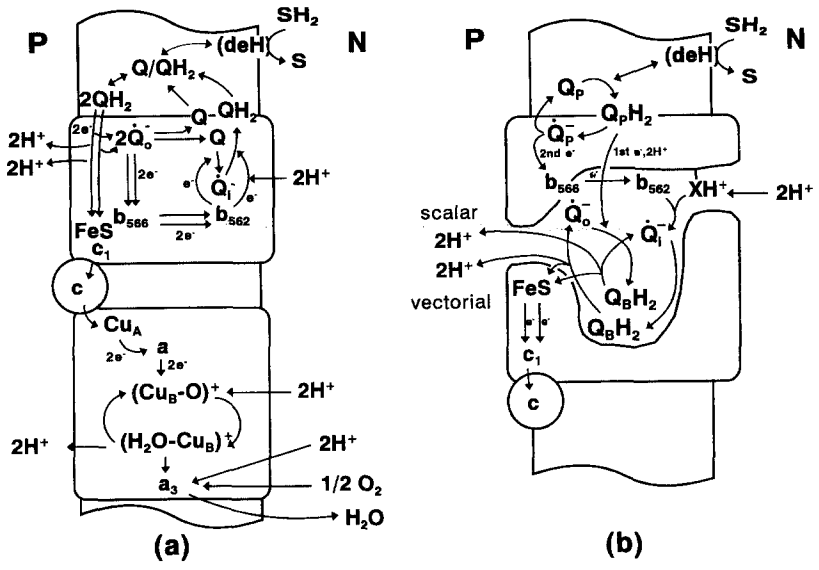

Fig. 1. Models for redox and protonmotive activity of the cytochrome system of mitochondria. a: Ubiquinone cycle for the bc_1 complex and Cu_B loop for cytochrome *c* oxidase complex. In the Cu_B loop proton translocation from the N to the P side is mediated by reorientation around Cu_B of OH^- (or O^-) and H_2O (Mitchell, 1987b). b: Q-gated H^+ pump for the bc_1 complex. The ubiquinone cycle is based on cycling of one of the two electrons donated by ubiquinol of the pool to the bc_1 complex and might involve exchange of protein-bound quinone with the pool (Mitchell, 1976, 1987a). The Q-gated pump envisages a linear split pathway for electron transfer mediated by nonexchangeable protein-stabilized quinol/semiquinone, which provides pumping of $2H^+/e^-$ (Papa *et al.*, 1983b, 1989).

(vectorial Bohr mechanism) (see also Von Jagow and Sebald, 1980). The cooperative mechanism, applied to the bc_1 complex, was later developed into a combined model in which redox Bohr effects were conceived to operate in series with protonmotive redox catalysis by the protein-bound quinol/semiquinone couple (Q-gated proton pump) (Papa *et al.*, 1983b, 1989) (Fig. 1b).

A commonly used, minimal model for redox proton pump is the eight-state "cubic" formalism developed by Wikström *et al.* (1981). This describes the essential steps of the pump and can be generally applied to both direct and indirect coupling mechanisms. The cubic model emphasizes the role of reorientation of the electron and proton transfer centers [alternating access of the centers to their reactants (cf. Malmström, 1985)] and the feasibility of kinetic linkage (cf. Malmström, 1985; Blair *et al.*, 1986).

The concept of indirect kinetic linkage, i.e., high kinetic preference of coupled protonmotive electron-transfer reaction over decoupled electron transfer (slips), is clearly described in the transition-state mechanism developed by Blair *et al.* (1986) and Malmström (1985, 1989). In this mechanism, the transition-state of an electron-transfer step is proposed to be stabilized by protonation, thus promoting, through reorientation of the redox and protolytic center(s), electron transfer from a donor to an acceptor and proton translocation from the input (N) to the output (P) sides (Malmström, 1985, 1989). The pump can exhibit variable slipping with regard to electrons or protons (Wikström *et al.*, 1981; Blair *et al.*, 1986; Malmström, 1989). A drawback of the transition state mechanism may be represented by low population of the protonated "transition state" in the coupled state in which the N phase experiences an alkaline pH. This difficulty can be minimized by redox Bohr effects which would provide kinetic advantage by promoting protonation of the transition state when, in its reduced state, it is accessible to protons from the N phase (Malmström, 1985; Blair *et al.*, 1986).

REDOX BOHR EFFECTS

Redox Bohr effects result in pH dependence of the midpoint redox potential of the electron carriers

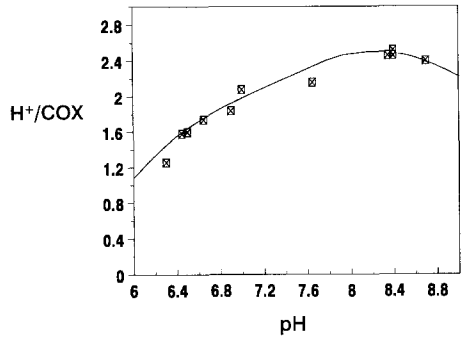

Fig. 2. pH dependence of the redox-Bohr effect (H^+/COX coupling number) in soluble cytochrome *c* oxidase. Purified cytochrome *c* oxidase (2 μM) suspension supplemented with 2 μM cytochrome *c* and a small amount of rat liver mitochondria (0.5 μM aa_3) was made anaerobic by excess succinate in the presence of CCCP. After addition of malonate, oxygenation was brought about by repetitive pulses of oxygenated medium (5 μM dioxygen). Oxidoreduction of hemes $a + a_3$ and Cu_A was monitored spectrophotometrically, and the scalar proton uptake and release, associated with the full reduction and oxidation of the oxidase redox centers, respectively, was measured by pH electrode (T. Vygodina, N. Capitanio, G. Capitanio, A. Konstantinov, and S. Papa, unpublished observations).

involved (Urban and Klingenberg, 1969; Wilson *et al.*, 1972; Dutton and Wilson, 1974; Van Gelder *et al.*, 1977; Blair *et al.*, 1985) and in scalar-proton transfer associated to their oxido-reduction (Papa *et al.*, 1979, 1986).

In the cytochrome *c* oxidase isolated from beef-heart mitochondria the number of scalar H^+/COX taken up upon full reduction of hemes a, a_3 Cu_A, and Cu_B, and released per mole of soluble oxidase upon oxidation of these centers, increases with pH from 1 at acidic to 2.5 at alkaline pH (Fig. 2) (cf. Hallen and Nilsson, 1992; Mitchell and Rich, 1994). In the cyanide-liganded oxidase with heme a_3 blocked in the oxidized state, the scalar H^+/COX transfer associated to oxido-reduction of heme *a*, Cu_A and Cu_B (Wrigglesworth *et al.*, 1988; Moody and Rich, 1990), when plotted as a function of pH, fits a bell-shaped curve resulting from two hypothetical redox-linked protolytic groups (Capitanio *et al.*, 1990). Since the E_m of Cu_A is pH independent (Erecinska *et al.*, 1971) the p*K* shifts of the two protolytic groups have to be linked to oxido-reduction of heme *a* (Artzatbanov *et al.*, 1978) and/or Cu_B whose E_m in the cyanide-liganded oxidase are apparently pH dependent (Moody and Rich, 1990). In the CO-liganded oxidase, where heme a_3 and Cu_B are held in the reduced state, the E_m of heme *a* is, practically, pH independent (Ellis *et al.*, 1986).

Redox Bohr effects in the unliganded oxidase derive from three or more protolytic groups undergoing p*K* shift upon oxido-reduction of the oxidase. Two are associated to heme *a* (and possibly CuB) (Capitanio *et al.*, 1990; Mitchell and Rich, 1994), and the other protolytic group(s) might be associated with heme a_3. One group could be represented by H_2O_2 ligated to the heme-iron (Rich, 1991; Konstantinov *et al.*, 1992), the others by protolytic residues in the protein. Genetic analysis of bacterial a, a_3 cytochrome *c* oxidase has shown that there are in subunit I, in the proximity of heme *a* and the binuclear heme a_3–Cu_B center, a number of conserved protolytic residues whose replacement by site-directed mutagenesis affects spectral and functional characteristics of the redox centers (Hosler *et al.*, 1993, 1994; Thomas *et al.*, 1993). These include tyrosine, threonine, and lysine residues whose p*K*s are in the range of those apparently involved in redox Bohr effects.

VARIABILITY OF THE H^+/e^- STOICHIOMETRY OF PROTON PUMPS

Direct models of redox proton pumps predict fixed and integral $\leftarrow H^+/e^-$ stoichiometries when electrons flow through a coupling site (Mitchell, 1966, 1987a). The stoichiometry could, however, decrease below the predicted mechanistic values if a fraction of the electrons flow through a parallel decoupled redox pathway. Indirect models can produce, in principle, variable stoichiometries due to electron and/or proton slips (Wikström *et al.*, 1981; Blair *et al.*, 1986; Malmström, 1990).

The issue has been extensively addressed by examining the steady-state relationship between respiratory rate and Δp in mitochondria and other membranes. The observed nonlinearity of this relationship (Nicholls, 1974; Pietrobon *et al.*, 1983; Murphy, 1989; Brown and Brand, 1986) is taken by some authors as evidence of slip in proton pumps (Pietrobon *et al.*, 1983; Luvisetto *et al.*, 1991; see also Murphy and Brand, 1988). Others, however, argue against this interpretation and consider the nonlinearity to be due to nonohmic increase of membrane proton conductance at high Δp (leak) (Brown and Brand, 1986; Brown, 1989). The $\leftarrow H^+/e^-$ stoichiometry of pumps in the respiratory chain is measured directly with two methods: the pulse method based on determination of the extent of H^+ release associated to consumption of a known amount of oxygen, or a reductant added to the system (Mitchell *et al.*, 1979),

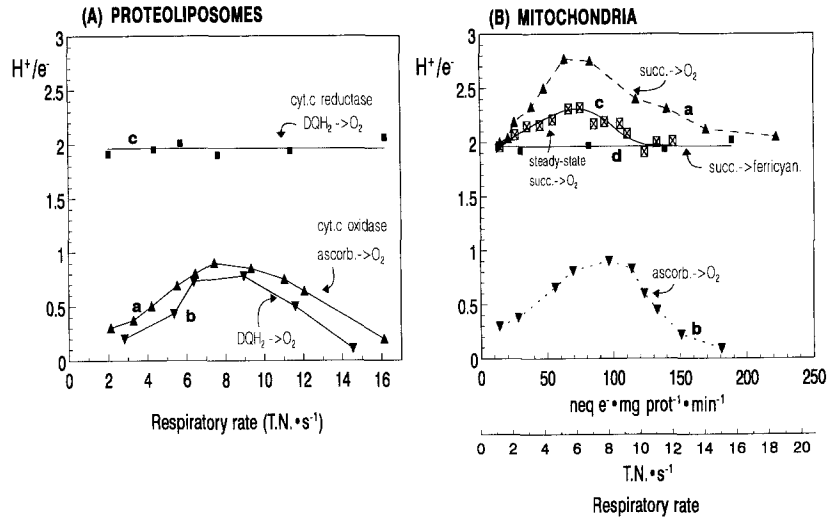

Fig. 3. Dependence of the $\leftarrow H^+/e^-$ ratio on the rate of electron flow in reconstituted cytochrome *c* oxidase and cytochrome *c* reductase and in intact mitochondria. Panel A: (a) Cytochrome *c* oxidase liposomes were supplemented with cytochrome *c* and ascorbate plus *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD). The respiratory rate was varied changing the concentration of TMPD. (b) The oxidase vesicles were supplemented with cytochrome *c*, duroquinol, and a trace of soluble cytochrome *c* reductase whose concentration was changed to vary the overall respiratory rate. (c) Cytochrome *c* reductase vesicles were supplemented with ferricytochrome *c*, a trace of soluble cytochrome *c* oxidase, and duroquinol. The respiratory rate was varied by titrating with antimycin A. In all the experiments respiration was initiated by the addition of ferricytochrome *c*, and the $\leftarrow H^+/e^-$ ratios were obtained from the initial rates of electron flow and proton translocation measured spectrophotometrically. For other details see Capitanio *et al.* (1991). Panel B: (a) Rat-liver mitochondria were supplemented with succinate; the respiratory rate was varied with the addition of malonate. (b) Mitochondria were supplemented with ascorbate and TMPD. The $\leftarrow H^+/e^-$ ratio was measured from initial rates elicited by the addition of the substrate. (c) Mitochondria were supplemented with succinate and the $\leftarrow H^+/e^-$ ratio was obtained from the rates of respiration and proton translocation measured at the aerobic steady-state. (d) KCN-Inhibited mitochondria were supplemented with ferricyanide, and the $\leftarrow H^+/e^-$ ratio was obtained from the initial rates of electron flow and proton release elicited by the addition of succinate. For other details see Papa *et al.* (1991). The points of all the curves represent the mean of six or more experiments.

and the rate method in which the stoichiometry is obtained from measurement of initial (Reynafarje *et al.*, 1979) or steady-state rates (Papa *et al.*, 1973; Murphy and Brand, 1988) of H^+ translocation and e^- flow. The prevailing conditions used in the pulse method are such that it is difficult to adjust the rate of e^- flow and the ensuing Δp. These parameters can, on the other hand, be more easily adjusted in the rate method, and recently Papa *et al.* (Papa *et al.*, 1991; Capitanio *et al.*, 1991; Cocco *et al.*, 1992), using this method, have shown that the intrinsic $\leftarrow H^+/e^-$ stoichiometry of the cytochrome system of mitochondria varies under the influence of kinetic and thermodynamic factors. A systematic study of the protonmotive activity of cytochrome *c* oxidase and cytochrome *c* reductase in intact mitochondria and in the purified enzymes reconstituted in phospholipid vesicles was carried out by these authors. Measurements were carried out in the presence of valinomycin so that K^+ migration prevented build-up of a membrane potential. The $\leftarrow H^+/e^-$ stoichiometry in cytochrome *c* oxidase vesicles measured from initial rates at level flow, i.e., under conditions of negligible Δp, first increases with the rate of electron flow to about one, then decreases again upon further enhancement of the rate (Fig. 3) (Capitanio *et al.*, 1991). In cytochrome *c* reductase, reconstituted in vesicles and in mitochondria, the $\leftarrow H^+/e^-$ stoichiometry is, at level flow, two, independently of the actual rate of e^- flow (Fig. 3) (Papa *et al.*, 1991; Capitanio *et al.*, 1991; Cocco *et al.*, 1992).

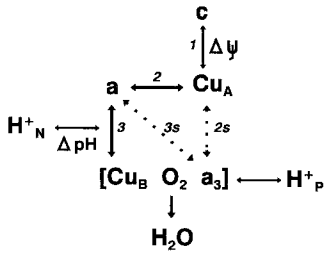

Fig. 4. Sequence of electron transfer steps in cytochrome *c* oxidase. The proton-coupled electron transfer pathway is shown by continuous arrows, and the decoupled electron transfer pathway (slips) by dotted arrows.

The rate dependence of the $\leftarrow H^+/e^-$ stoichiometry in the oxidase (see also Proteau *et al.*, 1983) can result from slips in e^- transfer. It is thought that, to be coupled to proton pumping, e^- have to follow the sequence cytc $\rightarrow Cu_A \rightarrow$ heme $a \rightarrow Cu_B \rightarrow$ heme a_3 (Fig. 4) (Babcock and Wikström, 1992; see, however, Brown *et al.*, 1994). Direct e^- slip from Cu_A to Cu_B, bypassing heme *a*, or from heme *a* to heme a_3, bypassing Cu_B (Babcock and Wikström, 1992), will result in decoupling of proton pumping (Fig. 4). Electron slip could depend on the redox pressure imposed on the enzyme, negative cooperativity between the high and low potential redox centers (Nicholls and Petersen, 1974; Wikström *et al.*, 1981; Mitchell *et al.*, 1992), and the relative adjustment of the kinetics of the various redox steps. The low efficiency of proton pumping observed in oxygen pulses of the fully reduced enzyme (Papa *et al.*, 1987; Oliveberg *et al.*, 1991), as compared to that obtained in reductant pulses of the oxidized enzyme (Wikström and Krab, 1979; Papa *et al.*, 1987a), could provide an example of how the activity of the pump can be affected by these and other factors (Papa, 1988).

The rate independence of the $\leftarrow H^+/e^-$ stoichiometry in cytochrome *c* reductase vesicles would suggest, that in this system, H^+ transfer reactions are considerably slower than redox events, thus making negligible the effect of eventual rate-dependent e^- slips on proton pumping.

Measurements in intact mitochondria (Fig. 3) show that under level flow conditions the $\leftarrow H^+/e^-$ ratio for succinate respiration varies from minima of two at extreme high (cf. Papa *et al.*, 1980a, 1980b, 1983a) and low respiratory rates (cf. Lorusso *et al.*, 1979) to about three at intermediate rates, with a rate dependence attributable to that exhibited by the $\leftarrow H^+/e^-$ ratio for cytochrome *c* oxidase (Fig. 3) (Papa *et al.*, 1991; Capitanio *et al.*, 1991). It can be noted that the range of electron transfer rates in which the $\leftarrow H^+/e^-$ stoichiometry changes corresponds to the physiological range of respiratory rates of mitochondria in tissues (Fitzgerald, 1976) and amounts to not more than 10% of the turnover of cytochrome *c* oxidase (see Fig. 3).

Under steady-state conditions the $\leftarrow H^+/e^-$ ratio for succinate respiration still exhibits some rate dependence. At steady state, the highest ratios attainable at intermediate flow rates are, however, significantly lower than those observed for the same rates at level flow, this providing evidence that, besides the flow rate, also Δp, in fact its ΔpH component ($\Delta\psi$ was collapsed by valinomycin), affects the H^+/e^- stoichiometry (cf. Murphy and Brand, 1988). This is confirmed by other experiments showing that ΔpH, but not $\Delta\psi$, depresses the efficiency of proton pumping (Cocco *et al.*, 1992; see also Murphy, 1989).

By specifically depressing proton-coupled electron flow, Δp can enhance contribution of decoupled e^- slips. Furthermore, alkalinization, at steady state, of the N phase can result in proton slip due to loss of protonation asymmetry of the critical proton-translocating center in the pump in the input state (Capitanio *et al.*, 1990; Cocco *et al.*, 1992). This protonation step might have a limited kinetic capacity (Hallen, 1993). Consistent with this view is the finding that the H^+/e^- ratio for succinate respiration at steady state in "inside out" submitochondrial vesicles, which expose the input state of the pump to the outer space, approaches the value of three at slightly acidic pH and in the presence of NaSCN to collapse Δp (Papa *et al.*, 1973).

An analogous situation seems to be met in the light-driven pump of bacteriorhodopsin where the input proton channel is the principal electrical barrier to movement of protons across the membrane (Henderson *et al.*, 1990). There are observations indicating that also this pump might "slip" at high Δp (Westerhoff and Dancshazy, 1984).

It is interesting to note that the rate dependence of the $\leftarrow H^+/e^-$ stoichiometry observed for cytochrome *c* oxidase at level flow diminished at the steady state. Under the latter conditions the critical factor for proton pumping seems to be represented for both the oxidase and the reductase by transmembrane ΔpH which imposes a limitation to protonation of the pump in the input state. It is, however, possible that under actively phosphorylating conditions (state 3), proton-coupled uptake of phosphate and respiratory substrates and proton-influx for ATP synthesis

Table I. Proposed Molecular Mechanisms for Redox-Driven Proton Pumping by Cytochrome *c* Oxidase

(A) Metal-ligand exchanges

(i) Redox-linked exchange of protolytic residues at Cu_A (Chan and Li, 1990), Cu_B (Larsen *et al.*, 1992) or heme iron (Wiström and Krab, 1978)

(ii) Redox-linked reorientation of OH^- and H_2O ligands (Mitchell, 1987b, 1988; Rich, 1991) or O_2, and H_2O_2 (see text) at Cu_B

(iii) O_2-Triggered protolytic ligand exchange between Cu_B and heme a_3 (Woodruff, 1993).

(iv) Redox-linked exchange of hystidine and tyrosine at the fifth coordination position of Fe a_3 (Rousseau *et al.*, 1993).

(B) Redox-linked p*K* shifts

(i) Redox-linked modification of a hydrogen bond between the formyl oxygen of heme *a* and a protolytic group in the protein (De Paula *et al.*, 1990; but see Babcock and Wikström, 1992)

(ii) Redox-linked conformational change in the protein with p*K* shifts in metal ligands combined with p*K* shifts in communicating protolytic residues (see text).

contribute to prevent establishment of a large ΔpH, and possibly to enhance H^+ concentration at the N input side of pumps, so that proton translocation by the cytochrome system can approach maximal efficiency.

The $\leftarrow H^+/e^-$ stoichiometry in vesicle-reconstituted cytochrome *c* oxidase seems also to be affected by the pH of the external medium, increasing in the pH range 6.0 to 7.0–7.5 (Papa *et al.*, 1987). Low H^+/e^- ratios at acidic pH could result from proton slip caused by depression of deprotonation of the pump in the output state at the P side (Maison Peteri and Malmström, 1989). A pH dependence of the $\leftarrow H^+/e^-$ ratio similar to that observed in cytochrome *c* oxidase vesicles has also been observed in the cytochrome *bo* quinol oxidase (Verkhovskaya *et al.*, 1992).

THE CATALYTIC CYCLE IN HEME-COPPER OXIDASES AND PROTON TRANSLOCATION

It is generally agreed that the Cu_B–heme a_3 (heme *o*) binuclear center, constantly associated with the largest subunit I in the members of the superfamily of cytochrome *c* and quinol oxidases (Hosler *et al.*, 1993), is directly involved in the coupling between dioxygen reduction to H_2O and proton pumping (Mitchell, 1988; Malmström, 1990, Babcock and Wikström, 1992; Rousseau *et al.*, 1993; Woodruff, 1993). This is, however, not meant to exclude a role of the low-potential centers (Chan and Li, 1990), in particular of heme *a* (Babcock and Wikström, 1992).

The key reactions in the four-electron reduction of dioxygen to H_2O (Malmström, 1990; Babcock and Wikström, 1992) are: (1) two-electron reduction of Cu_B and heme a_3; (2) oxygen binding, and (3) its reduction to heme a_3-bound peroxide (P); (4) delivery of the 3rd electron with formation of the first H_2O molecule and the oxy-ferryl derivative of heme a_3 (F); (5) delivery of the 4th electron with formation of the second H_2O molecule and reconversion of the binuclear center to the fully oxidized state (O). The first two protons consumed in the reduction of dioxygen to H_2O are apparently taken up by the enzyme upon reduction of heme a_3 and Cu_B (Mitchell *et al.*, 1992; Kostantinov *et al.*, 1992; see also Guerrieri *et al.*, 1981); the 3rd and 4th protons are taken up separately in reaction (4) and (5) respectively; according to Babcock and Wikström (1992), of the four protons, two are taken up in reaction (4) and two in reaction (5). Wikström (1989) has proposed that the four electrons consumed in the reduction of dioxygen are not all equivalent for proton pumping as only the transfer of the 3rd [reaction (4)] and 4th electron [reaction (5)] would be associated to pumping of 2 protons per electron.

As regards the molecular mechanism by which proton pumping would be associated to electron transfer, various models have been proposed. These can be grouped in two types: exchange of protolytic ligands at the metal centers (A) and redox-linked p*K* shifts of protolytic groups in the enzyme (B). Specific examples of both types, which have recently appeared in the literature, are summarized in Table I.

In Fig. 5, two of the possible models by which the oxygen chemistry at the binuclear center could be directly involved in the protonmotive activity of heme copper oxidases are presented (cf. Mitchell, 1987b, 1988; Rich, 1991). Both models (a) and (b) incorporate the various oxygen-intermediate derivative of heme a_3 resolved by resonance Raman spectroscopy (Varotsis *et al.*, 1993). Model (a) is a minimal scheme assuming anisotropic protonation from the N phase and deprotonation to the P phase of the peroxy (P) and oxy-ferryl (F) derivative of heme a_3, protonmotive steps resulting in the translocation of $2H^+$ from the N to the P phase per e^- transferred in the

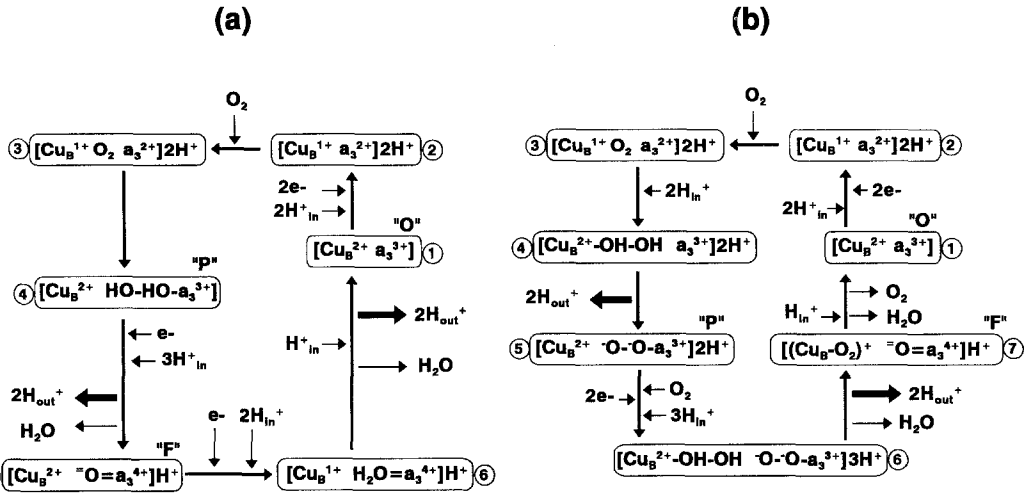

Fig. 5. Tentative models for protonmotive dioxygen reduction in heme-copper oxidases. Model (a) is based on anisotropic protonation of the "P" and "F" intermediates of heme a_3; model (b) is based on initial production of hydrogen peroxide on Cu_B and its anisotropic (de)protonation. The protons shown in the models, in addition to those directly involved in the oxygen reduction chemistry, are Bohr protons transferred by protolytic amino-acid residues. For details see text.

conversion of "P" to "F" and "F" to "O." Model (b) introduces the proposal that a central process in proton pumping is the production of hydrogen peroxide on Cu_B. It is conceived that the first two electrons, which reduce Cu_B and heme a_3, are transferred to dioxygen bound to the binuclear center with formation of Cu^{2+}-peroxide protonated from the N phase (cf. Rich, 1991). This step is followed by exchange of H_2O_2 from Cu_B^{2+} to a_3^{3+} (via a bridged intermediate?) where it finds an environment favoring its deprotonation in the P phase, with pumping of the first two protons.

The 3rd and 4th electrons, accompanied by binding of a second O_2 and 3 N protons, produce a second Cu_B^{2+}–H_2O_2. One electron of Cu_B^{2+}–H_2O_2 is then donated to ^{-}O–^{-}O–a_3 with formation of compound F, the first H_2O molecule, and pumping in the P phase of the other two protons. The remaining one-electron species $(Cu_B–O_2)^+$ transfers the 4th electron to the F compound with formation, with the uptake of a N proton (cf. Vygodina and Konstantinov, 1988; Konstantinov *et al.*, 1992), of the second H_2O and reconversion of the binuclear center to the fully oxidized state.

In this context it can be recalled that Vygodina and Konstantinov (1987) have proposed that H_2O_2 dismutation at the Cu_B–heme a_3 center could be involved in electrogenic proton translocation.

Enzyme-bound Cu^{2+}-peroxide complex is considered as an intermediate in copper monooxygenases (Klinman and Brenner, 1988). No direct evidence seems, however, to have been reported so far for its formation in heme-copper oxidases (see, however, Vygodina and Konstantinov, 1987, 1989). Its detection might have escaped since Cu_B absorbance is negligible compared to heme a_3.

Evidence has been presented suggesting that Cu_B is the site of the initial ligation step in the physiological function of heme-copper oxidases and that this ligation step may be involved in the link between the redox and proton pumping functions (Woodruff, 1993; Lemon *et al.*, 1993). The present model (b) makes use of the concept, introduced by Mitchell, of ligation and redox-linked reorientation of oxygen species on Cu_B (Mitchell, 1987b; see also Rich, 1991). In Mitchell's model the ligands to Cu_B are OH^- (or O^-) and H_2O; in the present model, O_2 and H_2O_2. H_2O_2 could correspond to the hypothetical ligand proposed by Woodroof (1993) to shuttle from Cu_B in the protonated state to heme a_3 where it is deprotonated. Our proposal that the ligand in question is oxygen peroxide itself eliminates the difficulty to understand how the exchange of a hypothetical endogenous ligand will be able to couple to redox events when the oxo-ligands are bound to heme a_3 (Rousseau *et al.*, 1993).

Bohr effects, apparently shared by Cu_B and heme *a* (Moody and Rich, 1990), could represent a cooperative device by which heme *a* participates in the pump by coupling electron delivery to Cu_B with proton translocation from the N phase to the Cu_B $-O^{-}-O^{-}$ compound.

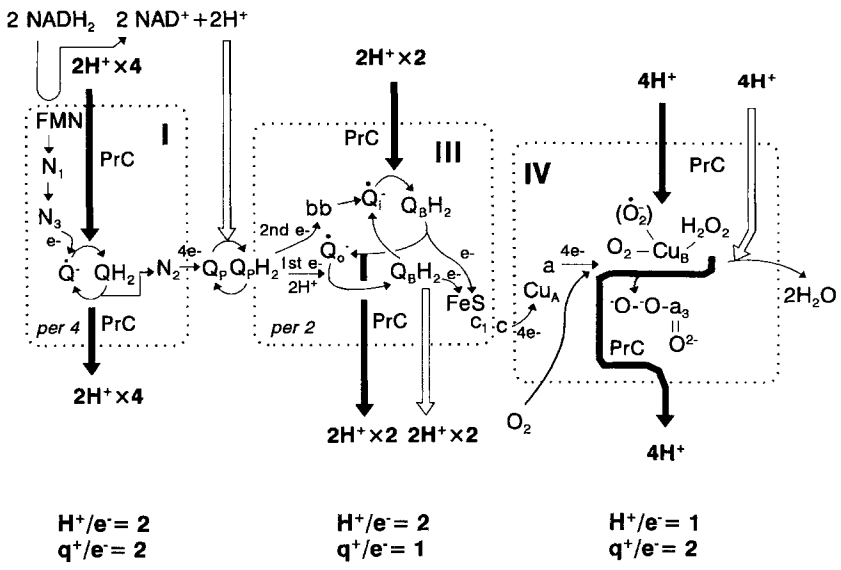

Fig. 6. General scheme for proton pumping at the three coupling sites of the mitochondrial respiratory chain in complex I, III, and IV, respectively. The maximal $\leftarrow H^+/e^-$ and $\leftarrow q^+/e^-$ ratios attainable for the three sites are given at the bottom of the scheme. Proton pumping in complex I is based on the information reviewed in Weiss *et al.* (1991) and translocation of $2H^+/e^-$ by protein-bound $\dot{Q}^-/QH_2$. For proton translocation in complex III the Q-gated proton pump (Papa *et al.*, 1983b, 1989) is shown. For complex IV a simplified version of the model of Fig. 5 is presented. PrC stands for proton-conducting pathway in proteins.

It can be noted that in the model of Fig. 5, if electrons delivered by the low-potential centers by pass Cu_B, and H_2O_2 is formed and cleaved directly on heme a_3, decoupling of proton pumping could occur. Furthermore, peroxidative oxidation of ferrocytochrome *c* could result in the release of $2H^+/e^-$ (cf. Miki and Orii, 1986): one H^+ would be scalar and associated to reaction of H_2O_2 with the binuclear center; the second would be vectorial and associated to oxidation of ferrocytochrome *c*.

TOWARDS A UNIFYING CONCEPT FOR PROTON PUMPING IN THE RESPIRATORY CHAIN

The maximal $\leftarrow H^+/e^-$ stoichiometry and $\leftarrow q^+/e^-$ stoichiometries observed for complex I (Weiss *et al.*, 1991), III (Papa and Lorusso, 1984), and IV (Wikström and Saraste, 1984) of the mitochondrial respiratory chain are 2 and 2, 2 and 1, and 1 and 2 respectively (see Fig. 6). A detailed tentative mechanism for proton pumping in the oxidase (complex IV) has just been described in the previous section. For the pumping activity of complex III two models are available: the Q-cycle (Mitchell, 1976) and the Q-gated proton pump Papa *et al.* 1983b, 1989). The former mechanism has, so far, met with general favor (Lenaz *et al.*, 1990). The relative merits of the two models is discussed in Papa *et al.* 1989. For the Q cycle the reader can refer to the many reviews written on the issue (Lenaz *et al.*, 1990). As regards the Q-gated proton pump, it can be noted that the two electrons transferred from ubiquinol to cytochrome *c* are not equivalent for proton pumping (Papa *et al.*, 1989). The transfer of the first electron of the quinol of the pool to cytochrome *c* is associated with release in the P phase of two scalar protons, and the transfer of the second electron results in vectorial transport of $2H^+$ and 2 q^+ from the N to the P phase (Papa *et al.*, 1989), as it is proposed to occur in the transfer of the 3rd electron from Cu_B^{2+}–H_2O_2 to a_3–O^-–O^- to form the F compound in the oxidase.

The situation in NADH-ubiquinone oxidoreductase (complex I) might appear to be more complicated due to the presence in this complex of different organic and metal redox centers (Weiss *et al.*, 1991). Of the $\leftarrow 2H^+/e^-$ pumped by the complex, one is considered to be associated to redox cycling of the intrinsic quinone molecule, apparently located

between two-electron carrying Fe–S centers (N_3 and N_2); the second is attributed to an as yet unidentified redox process on the substrate side of N_3 (Weiss *et al.*, 1991). Recently, Kotylar *et al.* (1990) have presented evidence showing the existence in the complex of a protein-stabilized, rotenone-sensitive, $g = 2.00$ ubisemiquinone similar to the antimycin-sensitive ubisemiquinone of complex III (Ohnishi and Trumpower, 1980), the central element in the Q-gated pump (Papa *et al.*, 1983b, 1989; Lorusso *et al.*, 1989).

We would like to propose that, as in complex III, also in complex I electron transfer by the protein-stabilized ubisemiquinone/quinol couple from the N_3 to N_2 FeS centers results in vectorial transport across the osmotic barrier of the membrane from the N to the P phase of 2 protons per electron.

This similarity that emerges here for the three complexes seems to allow the conclusion that in each of the complexes the central element in redox-linked proton pumping is provided by protonmotive redox catalysis at the primary reaction centers: $U\dot{Q}^-/UQH_2$ in complex I and III and $O_2/(\dot{O}_2^-)/H_2O_2$ in complex IV. Transmembrane proton pumping will result in each of the three complexes from the combination of the protonmotive redox events at the catalytic centers and cooperative proton-input pathways in the protein from the N phase to the catalytic center and proton-output pathways from the center to the P phase, much as in the case of bacteriorhodopsin. The advanced structural and physicochemical knowledge available for this light-driven pump has made it possible to identify the critical aspartic residue involved in deprotonation of the Schiff base of the retinal to the external side and the aspartic residue responsible for refilling the proton fault at the active site from the cytosolic side (Henderson *et al.*, 1990). Information has also been obtained on other residues in the input and output proton conduction pathways (Henderson *et al.*, 1990).

We believe that the rapid progress being made in the knowledge of the structural and functional features of redox proton pumps, contributed nowadays by site-directed mutagenesis and sophisticated spectroscopic analysis, will allow a better understanding of the molecular structure and mechanism of redox proton pumps in prokaryotic and eukaryotic cells.

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NOTE ADDED IN PROOF

After this manuscript was submitted a paper has appeared (Larsen, R. W. (1994) FEBS Lett. 352, 365–368) with data indicating initial binding of H_2O_2 to Cu_B preceding its binding to heme a_3.

REFERENCES

Artzatbanov, V. Y., Konstantinov, A. A., and Skulachev, V. P. (1978). *FEBS Lett.* **87,** 180–185.

Babcock, G. T., and Wikström, M. K. F. (1992). *Nature (London)* **356,** 301–309.

Blair, D. F., Ellis, W. R., Wang, H., Gray, H. B., and Chan, S. I. (1985). *J. Biol. Chem.* **261,** 11524–11537.

Blair, D. F., Gelles, J., and Chan, S. I. (1986). *Biophys. J.* **50,** 713–733.

Boyer, P. D., Chance, B., Ernster, L., Mitchell, P., Racker, E., and Slater, E. C. (1977). *Annu. Rev. Biochem.* **46,** 955–1026.

Brown, G. C. (1989). *J. Biol. Chem.* **264,** 14704–14709.

Brown, G. C., and Brand, M. D. (1986). *Biochem. J.* **234,** 75–81.

Brown, S., Rumbley, J.N., Moody, A.J., Thomas, J.W., Gennis, R.B., and Rich, P.R. (1994). *Biochim. Biophys. Acta* **1183,** 521–532.

Capitanio, N., De Nitto, E., Villani, G., Capitanio, G., and Papa, S. (1990). *Biochemistry* **29,** 2939–2945.

Capitanio, N., Capitanio, G., De Nitto, E., Villani, G., and Papa, S. (1991). *FEBS Lett.* **288,** 179–182.

Chan, S. I., and Li, P. M. (1990). *Biochemistry* **29,** 1–12.

Cocco, T., Lorusso, M., Di Paola, M., Minuto, M., and Papa, S. (1992). *Eur. J. Biochem.* **209,** 475–481.

De Paula, J. C., Peiffer, W. E., Ingle, R. T., Centeno, J. A., Ferguson-Miller, S., and Babcock, G. T. (1990). *Biochemistry* **29,** 8702–8706.

Dutton, P. L., and Wilson, D. F. (1974). *Biochim. Biophys. Acta* **346,** 165–212.

Ellis, W. R., Wang, H., Blair, D. F., Gray, H. B., and Chan, S. I. (1986). *Biochemistry* **25,** 161–167.

Erecinska, M., Chance, B., and Wilson, D. F. (1971). *FEBS. Lett.* **16,** 284–286.

Fitzgerald, L. D. (1976). *Biological Handbooks, Cell Biology* (Altman, P. L., and Dittmer Katz, D., eds.), Fed. Am. Soc. Expt. Biology, Bethseda, Maryland, Vol. 1, pp. 72–89.

Guerrieri, F., Maida, I., and Papa, S. (1981). *FEBS Lett.* **125,** 261–265.

Hallen, S. (1993). Phd Thesis, Bibliotekets Reproservice, Göteberg.

Hallen, S., and Nilsson, T. (1992). *Biochemistry* **31,** 11853–11859.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., and Downing, K. H. (1990). *J. Mol. Biol.* **213,** 899–929.

Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgia, C., Fetter, J., Shapleigh, J., Tecklenburg, M. M. J., Babcock, G. T., and Gennis, R. B. (1993). *J. Bioenerg. Biomembr.* **25,** 121–136.

Hosler, J. P., Shapleigh, J. P., Tecklenburg, M. H. J., Thomas, J. W., Kim, Y., Espe, M., Fetter, J., Babcock, G. T., Alben, J. O., Gennis, R. B., and Ferguson-Miller, S. (1994). *Biochemistry* **33,** 1194–1201.

Klinman, J. P., and Brenner, M. (1988). In *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., and Morrison, M. eds.), Alan Liss, New York, pp. 227–246.
Konstantinov, A. A., Capitanio, N., Vygodina, T. V., and Papa, S. (1992). *FEBS Lett.* **264,** 71–74.
Kotylar, A. B., Sled, V. D., Moroz, I. A., and Vinogradov, A. D. (1990). *FEBS Lett.* **264,** 17–20.
Larsen, R. W., Pam, L. P., Musser, S. M., Li, Z., and Chan, S. I. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 723–727.
Lawford, H. G., and Garland, P. B. (1973) *Biochem. J.* **136,** 711–720.
Lemon, D. D., Calhaun, M. W., Gennis, R. B., and Woodruff, W. H. (1993). *Biochemistry* **32,** 11953–11956.
Lenaz, G., Barnabei, O., Rabbi, A., and Battino, M., eds. (1990). *Highlights in Ubiquinone Research,* Taylor and Francis, London.
Lorusso, M., Capuano, F., Boffoli, D., Stefanelli, R., and Papa, S. (1979). *Biochem. J.* **182,** 133–147.
Lorusso, M., Cocco, T., Boffoli, D., Gatti, D., Meinhardt, S., Ohnishi, T., and Papa, S. (1989). *Eur. J. Biochem.* **179,** 535–540.
Luvisetto, S., Conti, E., Buso, M. and Azzone, G. F. (1991). *J. Biol. Chem.* **266,** 1034–1042.
Maison Peteri, B., and Malmström, B. G. (1989). *Biochemistry* **28,** 3156–3160.
Malmström, B. G. (1985). *Biochim. Biophys. Acta* **811,** 1–12.
Malmström, B. G. (1989). *FEBS Lett.* **250,** 9–21.
Malmström, B. G. (1990). *Chem. Rev.* **90,** 1247–1260.
Miki, T., and Orii, Y. (1986). *J. Biol. Chem.* **261,** 3915–3918.
Mitchell, P. (1966). *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation,* Glynn, Research Ltd., Bodmin, U.K.
Mitchell, P. (1976). *J. Theor. Biol.* **62,** 327–367.
Mitchell, P. (1987a). In *Advances in Membrane Biochemistry and Bioenergetics* (Kim, C. H., Tedeschi, H., Diwan, J. J., and Salerno, J. C., eds.), Plenum, New York and London, pp. 25–52.
Mitchell, P. (1987b). *FEBS Lett.* **222,** 235–245.
Mitchell, P. (1988). In *Cytochrome Oxidase. Structure Function, and Physiopathology* (Brunori, M., and Chance, B., eds.), *Ann. N. Y. Acad. Sci.* **550,** 185–198.
Mitchell, P., and Moyle, J. (1983). *FEBS Lett.* **151,** 167–178.
Mitchell, P., Moyle, J., and Mitchell, R. (1979). *Methods Enzymol.* **55,** 627–640.
Mitchell, P., Mitchell, R., Moody, A. J., West, I. C., Baum, H., and Wrigglesworth, J. M. (1985). *FEBS Lett.* **188,** 1–7.
Mitchell, R., and Rich, P. R. (1994). *Biochim. Biophys. Acta,* **186,** 19–26.
Mitchell, R., Mitchell, P., and Rich. P. R. (1992). *Biochim. Biophys. Acta* **1101,** 188–191.
Moody, A. J., and Rich, P. R. (1990). *Biochim. Biophys. Acta* **1015,** 205–215.
Murphy, M. P. (1989). *Biochim. Biophys. Acta* **977,** 123–141.
Murphy, M. P., and Brand, M. D. (1988) *Eur. J. Biochem.* **173,** 645–651.
Nicholls, D. G. (1974). *Eur. J. Biochem.* **50,** 305–315.
Nicholls, P., and Petersen, L. C. (1974). *Biochem. Biophys. Acta* **357,** 462–467.
Ohnishi, T., and Trumpower, B. L. (1980). *J. Biol. Chem.* **255,** 3278–3284.
Oliveberg, M., Hallen, S., and Nilsson, T. (1991). *Biochemistry* **30,** 436–440.
Papa, S. (1976). *Biochim. Biophys. Acta* **456,** 39–84.
Papa, S. (1988). In *Oxidases and Related Redox Systems.* (Mason, H. S., ed.), Elsevier Amsterdam, pp. 49–94.
Papa, S., and Lorusso, M. (1984). In *Biomembranes* (Burton, R. M., and Guerra, F. C., eds.), Plenum, London, pp. 257–290.
Papa, S., Guerrieri, F., Lorusso, M., and Simone, S. (1973). *Biochimie* **55,** 703–716.
Papa, S., Lorusso, M., and Guerrieri, F. (1975). *Biochim. Biophys. Acta* **387,** 425–440.
Papa, S., Guerrieri, F., and Izzo, G. (1979). *FEBS Lett.* **105,** 213–216.
Papa, S., Capuano, F., Markert, M., and Altamura, N. (1980a). *FEBS Lett.* **177,** 243–248.
Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., Boffoli, D., Capuano, F., Capitanio, N., and Altamura, N. (1980b). *Biochem. J.* **192,** 203–218.
Papa, S., Guerrieri, F., Izzo, G., and Boffoli, D. (1983a). *FEBS Lett.* **157,** 15–20.
Papa, S., Lorusso, M., Boffoli, D., and Bellomo, E. (1983b). *Eur. J. Biochem.* **137,** 405–412.
Papa, S., Guerrieri, F., and Izzo, G. (1986). *Methods Enzymol.* **126,** 331–343.
Papa, S., Capitanio, N., and De Nitto, E. (1987). *Eur. J. Biochem.* **164,** 507–516.
Papa, S., Lorusso, M., Cocco, T., Boffoli, D., and Lombardo, M. (1989). In *Highlights in Ubiquinone Research* (Lenz, G., Barnabei, O., Rabbi, A., and Battino, M., eds.), Taylor and Francis, London, pp. 122–135.
Papa, S., Capitanio, N., Capitanio, G., De Nitto, E., and Minuto, M. (1991). *FEBS Lett.* **288,** 183–186.
Pietrobon, D., Zoratti, M., and Azzone, G. F. (1983). *Biochim. Biophys. Acta* **723,** 317–321.
Proteau, G., Wrigglesworth, J. M., and Nicholls, P. (1983). *Biochem. J.* **210,** 199–205.
Reynafarje, B., Brand, M. D., Alexandre, R., and Lehninger, A. L. (1979). *Methods Enzymol.* **55,** 640–656.
Rich, P. R. (1991). *Biosci. Rep.* **11,** 539–568.
Rousseau, D. L., Ching, Y. C., and Wang, J. (1993). *J. Bioenerg. Biomembr.* **25,** 165–176.
Thomas, J. W., Lemieux, L. J., Alben, J. O., and Gennis, R. B. (1993). *Biochemistry* **32,** 11173–11180.
Urban, P. F., and Klingenberg, M. (1969). *Eur. J. Biochem.* 519–525.
Van Gelder, B. F., Van Rijin, J. L. M. L., Schilder, G. J. A., and Wilms, J. (1977). In *Structure and Function of Energy- Transducing Membranes* (Van Dam, K., and Van Gelder, B. F., eds.), Elsevier North-Holland Biomedical Press, Amsterdam, pp. 61–68.
Varotsis, C., Zhang, Y., Appelman, E.H., and Babcock, G.T. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 237–241.
Verkhovskaya, M., Verkhovsky, M., and Wikström, M. K. F. (1992). *J. Biol. Chem.* **267,** 14559–14562.
Von Jagow, G., and Sebald, W. (1980). *Annu. Rev. Biochem.* **49,** 281–314.
Vygodina, T., and Konstantinov, A. A. (1987). *FEBS Lett.* **219,** 387–392.
Vygodyna, T., and Konstantinov, A. A. (1988). *Ann. N. Y. Acad. Sci.* **550,** 124–138.
Vygodina, T., and Konstantinov, A. A. (1989). *Biochim. Biophys. Acta* **937,** 390–398.
Weiss, H., Friedrich, T., Hofhaus, G., and Preis, D. (1991). *Eur. J. Biochem.* **197,** 563–576.
West, I. C., Mitchell, R., Moody, A. J., and Mitchell, P. (1986). *Biochem. J.***236,** 15–21.
Westerhoff, H. V., and Dancshazy, Zs. (1984). *Trends Biochem. Sci.* **9,** 112–117.
Wikström, M. K. F. (1977). *Nature (London)* **266,** 271–273.
Wikström, M. K. F. (1989). *Nature (London)* **338,** 776–778.
Wikström, M. K. F., and Krab, K. (1978). In *Energy Conservation in Biological Membranes* (Schäfer, G., and Klingenberg, M., eds.), Springer-Verlag, Berlin, pp. 128–139.
Wikström, M.K.F., and Krab, K. (1979). *Biochim. Biophys. Acta* **549,** 177–222.
Wikström, M. K. F., Krab, K., and Saraste, M. (1981). *Cytochrome oxidase. A synthesis,* Academic Press New York and London.
Wikström, M. K. F., and Saraste, M. (1984). In *Bioenergetics* (Ernster, L., ed.), Elsevier, Amsterdam, pp. 49–94.
Wilson, D. F., Lindsay, J. G., and Brocklehurst, E. S. (1972). *Biochim. Biophys. Acta* **256,** 277–286.
Woodruff, W. H. (1993). *J. Bioenerg. Biomembr.* **25,** 177–188.
Wrigglesworth, J. M., Elsden, J., Chapman, A., Van der Water, N., and Grahm, M. F. (1988) *Biochim. Biophys. Acta* **936,** 452–464.
Wyman, J. (1968). *Q. Rev. Biophys.* **1,** 35–81.