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Cytochrome *c* Oxidase as a Proton-Pumping Peroxidase: Reaction Cycle and Electrogenic Mechanism

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Cytochrome oxidase (COX) is considered to integrate in a single enzyme two consecutive mechanistically different redox activities—oxidase and peroxidase—that can be catalyzed elesewhere by separate hemoproteins. From the viewpoint of energy transduction, the enzyme is essentially a proton pumping *peroxidase* with a built-in auxiliary *eu-oxidase* module that activates oxygen and prepares *in situ* H_2O_2, a thermodynamically efficient but potentially hazardous electron acceptor for the proton pumping peroxidase. The *eu-oxidase* and *peroxidase* phases of the catalytic cycle may be performed by different structural states of COX. Resolution of the proton pumping peroxidase activity of COX and identification of individual charge translocation steps inherent in this reaction are discussed, as well as the specific role of the two input proton channels in proton translocation.

KEY WORDS: Cytochrome oxidase; proton pumping; peroxidase; dioxygen reduction; catalytic cycle/time-resolved electrogenesis.

INTRODUCTION

At the Oxidase Meeting in Rieti in 1993, I came up with a model of the cytochrome *c* oxidase (COX)[2] protonmotive mechanism comprised of two distinct and well-defined *oxidase* and *peroxidase* phases. These reactions are so dissimilar in redox chemistry and energy-coupling characteristics that I suggested that they are catalyzed by different conformational states of the enzyme. This paper aims to briefly outline the contribution of our group to the studies of COX within the framework of this hypothesis and to share our views on some of the unresolved questions of current interest. A number of excellent recent reviews can be addressed for an overall picture (Ferguson-Miller and Babcock, 1996; Rich and Moody, 1997; Wikstrom *et al.*, 1997a)

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[2] Abbreviations: COX, cytochrome oxidase; Cpd, Compound; RR, resonance Raman; O, R, P, and F, oxidized, reduced peroxy, and ferryl-oxo states.

EU-OXIDASE/PEROXIDASE MODEL OF CYTOCHROME OXIDASE

A simplified catalytic cycle of COX is shown in Fig. 1. In the first phase O→R→P where O_2 is reduced to bound peroxide, heme a_3 iron operates between the ferric (III) and ferrous (II) states and reactions in the binuclear center include e^- and H^+ transfer steps and oxygen binding but no chemical bond breakage. This is a typical oxidase reaction as it involves activation of molecular oxygen, and this part of the cycle has been called an *eu-oxidase* phase (Konstantinov *et al.*, 1997a) to distinguish it from the overall oxidase activity of the enzyme. The second P→F→O half of the cycle is remarkably similar to the classical hemoprotein peroxidase mechanism (cf. Orii, 1982b), where the heme iron operates between the ferric (III) and ferryl (IV) states and the iron-peroxy complex is converted in two single-electron steps back to the oxidized form. This phase has been denoted as *peroxidase* half-reaction (Konstantinov *et al.*, 1997a).

There is an interesting analogy between the $4e^-$ *eu-oxidase/peroxidase* catalytic cycle of COX and $4e^-$ reduction of O_2 to water by ascorbate where we find

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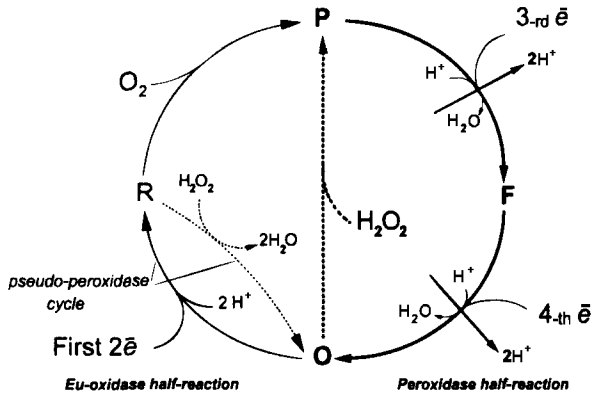

Fig. 1. The oxidase/peroxidase cycle of cytochrome *c* oxidase. The scheme considers the redox state of heme a_3. For simplicity, the oxycomplex of heme a_3 and the different intermediates at the "peroxy" level have been omitted, and the water molecules are indicated to be formed at the P→F and F→O steps, which is not necessarily the case (cf. Fig. 2 and the text). Thick lines (the right half of the cycle) depict the peroxidase cycle of COX.

ascorbate oxidase catalyzing 2-electron reduction of O_2 to H_2O_2 (Kim *et al.*, 1996) and *ascorbate peroxidase* that reduces H_2O_2 to water in two single-electron steps typical of peroxidases (Patterson and Poulos, 1995). Mention can be made of a terminal bb_3 oxidase from *Psudomonas nautica* which reduces molecular oxygen to H_2O_2 (Arnaud *et al.*, 1991; Denis *et al.*, 1989). Thus COX may indeed integrate in a single enzyme two different functions that are performed in other cases by separate hemoproteins.

TWO INTERMEDIATES AT THE FORMAL "PEROXY" LEVEL

Whereas the O, R, Oxy, and F states in the COX cycle are relatively well defined, there is currently confusion concerning the identity of the P state (see Ferguson-Miller and Babcock, 1996 and Wikstrom *et al.*, 1997a for review) showing that one P intermediate in Fig. 1 is an obvious oversimplification. Actually, the name "peroxy state" has often been used in the recent literature to refer to any intermediate resolved in the cycle after the Oxy-complex and before the 580 nm Ferryl-Oxo state.

Further pursuing the analogy with peroxidases and cytochromes P450, a minimal sequence of intermediates in the COX catalytic cycle was proposed by Konstantinov *et al.*, (1997a) as shown in Fig. 2. In this scheme, P_0 is a ferric-peroxy species with intact O–O bond homologous to Compound (Cpd) 0 of peroxidases (Baek and Van Wart, 1989; Harris and Loew, 1996a; Miller *et al.*, 1994; Rodriguez-Lopez *et al.*, 1996; Wang *et al.*, 1991) or to the doubly reduced dioxygen intermediate in cytochrome P450 (see Harris and Loew, 1996b and references therein). F_I is a ferryl-oxene species homologous to Cpd I of peroxidases that is two oxidizing equivalents above the ferric state, and F_{II} corresponds to the conventional Ferryl-Oxo state of COX (Compound F) that is one oxidizing equivalent above the ferric state and is homologous to Compound II of peroxidases. The indices 0, I, and II are meant to highlight relations of COX intermediates to the peroxidase compounds 0, I, and II. Note that F_I and F_{II} intermediates here are different from the intermediates F_I and F_{II} of Sucheta *et al.*(1997).

Recent resonance Raman (RR) experiments of Proshlyakov *et al.* (1994, 1996a,b) have resolved a new intermediate (the 804 cm^{-1} species) preceding the formation of the conventional F state in the reaction cycle (Ogura *et al.*, 1996). Although referred to sometimes as the "peroxy state," the species is actually a ferryl-oxene compound with the O–O bond broken, and it nicely fits intermediate F_I in Fig. 2.

The major question is whether the conventional intermediate P (the "607 nm" form of COX) corresponds to P_0 or F_I in Fig. 2. It is the "607 nm state" that was originally defined by Wikstrom (1981) as Compound P (Peroxy) and assigned an iron-peroxo structure (see Wikstrom *et al.*, 1997a for recent discussion). However, according to recent reports of Kitagawa's group, the 607 nm absorption band of COX is associated with the 804 cm^{-1} peak in the RR spectra and should be attributed to the ferryl-oxene intermediate (Kitagawa and Ogura, 1997). The 607 nm form is definitely at the "formal peroxy oxidation level," i.e., two-electron-deficient relative to the ferric state (Wikström, 1987; Wikström and Morgan, 1992; Verkhovsky *et al.*, 1996), but conceivably this will fit either the P_0 or F_I state in sheme II.

If the 607 nm state of COX turns out to be a ferryl-oxene intermediate F_I, the iron-peroxy form (P_0) may be suggested to have absorption characteristics similar to the oxy-complex (Kobayashi *et al.*, 1994) or ferric state (cf. Harris and Loew, 1996a) and/or not to accumulate in significant amounts as in fact is typical of peroxidases and P450. Interestingly, in the E286Q mutant of COX (*R. sphaeroides* numbering of residues is used throughout the paper) which is expected to be blocked in the catalytic cycle at the peroxy level (see below), heme a_3 is trapped in a state with increased absorption at 595 nm resembling an oxycom-

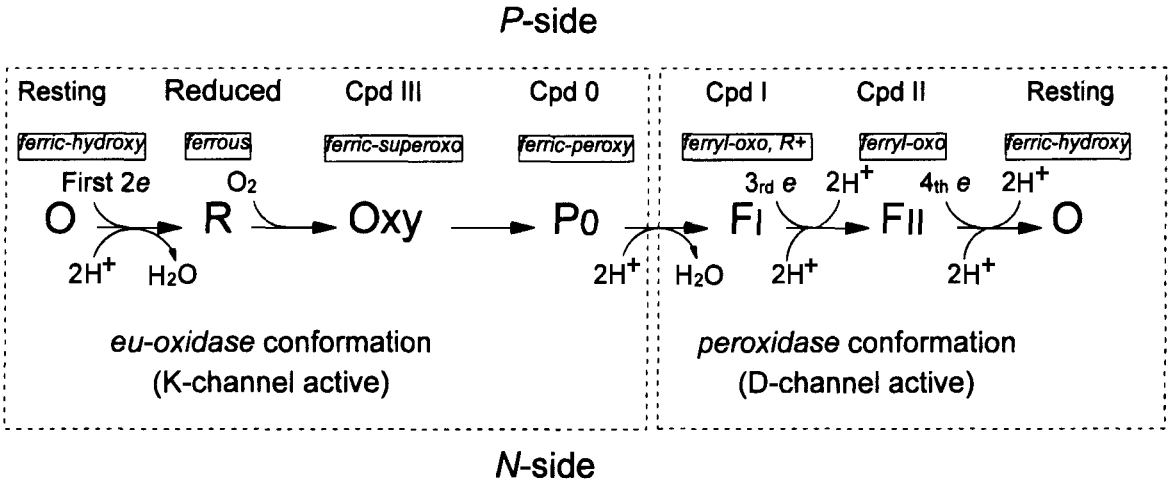

Fig. 2. Distribution of partial reaction steps between the eu-oxidase and peroxidase conformational states of COX. The intermediates in the scheme consider explicitly the state of heme a_3. Conceivably, at each of the states of a_3, there can be several intermediates, differing in the redox state of Cu_B [e.g., P_R and P_M forms of the peroxy state (Morgan *et al.*, 1996)] and of other redox centers (Sucheta *et al.*, 1997). Note that the O state of COX appears in both eu-oxidase and peroxidase boxes. The back transition of COX from peroxidase to eu-oxidase conformation (heme iron moving back to the proximal side of the heme) can be envisaged to occur at the level of the oxidized enzyme; this implies at least two different conformational states of O, which of course agrees with the notorious multiplicity of the oxidized forms of COX (Moody, 1996). As discussed in the text, the protons consumed at the $P_O \rightarrow F_I$ step may be donated by vicinal donors. Electrogenic proton uptake coupled to reprotonation of the latter (ca. 100 μs in the flow-flash experiments (Hallen and Nilsson, 1992; Sucheta *et al.*, 1997; Verkhovsky *et al.*, 1997)) can lag behind the optically resolved $P_O \rightarrow F_I$ transition and overlap with the subsequent $F_I \rightarrow F_{II}$ redox step.

plex (Konstantinov *et al.*, 1997a) [but cf. results with E286Q bo_3 oxidase (Verkhovskaya *et al.*, 1997)]. If on the other hand an iron-peroxy state of the 607 nm form is verified, and visible absorption characteristics of the 804 cm^{-1} RR intermediate are similar to those of Compound F (Morgan *et al.* 1996; Sucheta *et al.*, 1997), we may face significant confusion in earlier conclusions on the mechanism of the P→F→O phase of the cycle based on absorption spectroscopy measurements that did not discriminate between the two ferryl-oxo species. A question of special interest is whether the 804 cm^{-1} RR intermediate correlates with the poorly populated free-radical ferryl-oxo state of COX (Fabian and Palmer, 1995).

HETEROLYTIC DIOXYGEN BOND SCISSION

Scission of the O–O bond in the COX-catalyzed reaction is traditionally considered to be a reductive process requiring transfer of the 3rd electron to *dioxygen* (e.g., Blair *et al.*, 1985; Morgan *et al.*, 1994). At the same time, in many hemoproteins decay of the iron-peroxy complex originating from binding of peroxide in peroxidases and catalases or from two-electron reduction of O_2 in P450 is a rapid (ca. 10^5 s^{-1}) spontaneous process that does not require exogenous electrons but involves *proton*-assisted heterolytic cleavage of the O–O bond resulting in the formation of a Cpd I-type ferryl-oxene species and water. It is not *a priori* clear why COX should be an exception to this rule, and inclusion of heterolytic scission of the O–O bond into the COX reaction sequence ($P_O \rightarrow F_I$ step in Fig. 2) may fill in a certain gap in our understanding of the catalytic mechanism, In particular, it makes it easier to rationalize how ferryl-oxo state(s) of COX can evolve in the absence of exogenous electrons upon reaction of ferric COX with substoichiometric amounts of H_2O_2 (Fabian and Palmer, 1995) or in Compound C (Ferguson-Miller and Babcock, 1996; Kitagawa and Ogura, 1997).

Scission of the O–O bond is catalyzed in peroxidases by a His/Arg pair of residues on the distal side of the heme plane (Erman *et. al.*, 1993; Miller *et al.*, 1994; Poulos and Kraut, 1980; Rodriguez-Lopez *et al.*, 1996; Vitello *et al.*, 1993). A similar proton relay system is formed at the distal side of the heme plane in cytochromes P450 by conserved Glu (Asp) and Thr residues and hydrogen-bonded water molecules

(Harris and Loew, 1996b; Yeom *et al.*, 1995; Yeom and Sligar, 1997, and references therein). It was shown recently that in binuclear metallic complexes, the role of the second metal center in facilitating dioxygen reduction may not be electron transfer but rather acid/base catalysis of dioxygen bond scission. It is tempting to suggest that in COX, the role of the distal acid/base catalyst might be performed by Cu_B. For instance, the putative Cu_B-bound aqua-ligand (Mitchell, 1988; Fann *et al.*, 1995; Wikstrom *et al.*, 1997a) could provide the proton required for cleavage of the Fe–OOH complex.

Formation of water during heterolytic cleavage of the dioxygen bond in COX can involve vicinal proton donors not in protonic equilibrium with the aqueous phases at this step (Hallén and Nilsson, 1992; Wikstrom *et al.*, 1997a), and be a microreversible process due to the high energetic cost of ionization of the proton-donating groups in the hydrophobic environment. It is subsequent reprotonation of the local H^+ donors via the input proton channel that will make the reaction irreversible (and electrogenic). The reduced state of Cu_B could dictate an open (connected) state of the input proton channel allowing for rapid reprotonation of the proton donors in the binuclear center and promoting decay of the peroxy state.

PARTIAL REACTIONS OF COX: PEROXIDASE, PSEUDO-PEROXIDASE, CATALASE, AND SUPEROXIDE DISMUTASE ACTIVITIES

To decipher the mechanism of COX, it would be helpful to resolve the catalytic cycle into partial reactions. The $2e^-$, $2H^+$, and O_2 added to the binuclear center of COX during the eu-oxidase phase (Fig. 1) constitute a molecule of H_2O_2. Accordingly, reaction with H_2O_2 as electron acceptor may be expected to bypass the eu-oxidase phase and run through a truncated peroxidase cycle.

Earlier studies showed that H_2O_2 reacts with both ferrous and ferric forms of heme a_3 (e.g., Gorren *et al.*, 1985, 1986, 1988; Orii, 1988). Accordingly, the cytochrome *c* peroxidase activity of COX studied under anaerobic conditions to avoid oxidase reaction (Bickar *et al.*, 1982; Miki and Orii, 1986a; Orii, 1982a, 1990) proved to be heterogeneous and was dominated by H_2O_2 reduction via ferrous heme a_3, which implies a pathway quite distinct from the classical hemoprotein peroxidase mechanism (pseudo-peroxidase cycle in Fig. 1).

However, at high redox potentials of the electron donor, making reduction of heme a_3 and Cu_B unfavorable, the oxidase and pseudo-peroxidase pathways attenuate, allowing one to observe true peroxidase activity of COX proceeding via binding of peroxide with the ferric heme a_3 iron and generation of Compounds P and F (Fig. 1, thick lines) (Konstantinov *et al.*, 1997b). This enabled us to study separately the peroxidase half of the COX reaction cycle (see below).

Mention can be made of the catalase activity inherent in COX (Gorren *et al.*, 1985; Orii and Okunuki, 1963). The reaction involves one-electron oxidation of H_2O_2 to superoxide radical by Compounds P and F formed upon reaction of ferric COX with H_2O_2 (Vygodina and Konstantinov, 1988; Konstantinov *et al.*, 1992; Ksenzenko *et al.*, 1992) and was characterized in significant detail in this group in a work that still remains to be published (Peskin, Vygodina, and Konstantinov, in preparation). Recently Fabian and Palmer (1995) proposed that generation of superoxide radicals by COX is an artifact associated with oxidative destruction of heme a_3 in the enzyme. This is definitely not the case, since in our hands the radicals produced by 50 nM COX in the presence of excess peroxide can reduce almost fully 25 μM Nitro Blue Tetrazolium which implies hundreds of turnovers by the enzyme.

The ability of COX to intercept superoxide radicals was reported (Markossian *et al.*, 1978; Naqui and Chance, 1986) and confirmed in our group. However, whether this reaction is indeed superoxide dismutation (rather than, say, oxidation of superoxide) has not been established.

PROTON PUMPING IS LINKED TO THE PEROXIDASE PHASE OF THE CYCLE

The effect of membrane energization on the redox equilibrium between the O, F, and P states in mitochondria allowed Wikström (1989) to propose that each of the P→F and F→O transitions is linked to transmembrane translocations of two protons. Taking into account the $H^+/e^- = 1$ stoichiometry for the overall cycle, this indicates that all proton pumping is coupled thermodynamically to the peroxidase phase of the COX cycle and, by exclusion, that the eu-oxidase phase does not pump at all.

To test this proposal it would be best to measure directly proton or charge translocation coupled to the peroxidase partial reaction. Miki and Orii (1986a,b) described electrogenic proton pumping by liposome-

reconstituted COX coupled to oxidation of cytochrome *c* with H_2O_2 as electron acceptor. The observed H^+/e^- stoichiometry was 0.9, but since CO almost fully inhibited H^+ translocation but only about half of electron transfer, the authors considered the possibility that one of the multiple reaction pathways under their conditions might be coupled to translocation of as many as 2 protons per electron (Miki and Orii, 1986b). This pathway was assigned to the H_2O_2 reaction with ferrous heme a_3 (the pseudo-peroxidase cycle in Fig. 1), which implied that the genuine peroxidase pathway (O→P→F→O) does not pump at all.

Recently, steady-state peroxidase activity of bovine heart COX incorporated in phospholipid vesicles has been shown to be coupled to transmembrane translocation of two protons per electron under conditions eliminating contribution from the pseudo-peroxidase pathway (Vygodina *et al.*, 1994, 1997a). Collaboration with the laboratory of R. Gennis allowed us to confirm this finding with the K362M mutant of COX from *R. sphaeroides,* in which the eu-oxidase half of the catalytic cycle is knocked out by the mutation leaving the proton-pumping peroxidase phase intact (Vygodina *et al.*, 1996, 1997b). These data directly show that it is the peroxidase half of the reaction cycle that is proton-pumping.

Dynamic evidence (Wikstrom *et al.*, 1997a) for most of the proton pumping to take place in the P→F→O phase of the cycle is provided also by time-resolved measurements of charge translocation by COX during oxidation of the reduced COX by O_2 (Verkhovsky *et al.*, 1997) and in the isolated F→O and P→F transitions (Zaslavsky *et al.*, 1993; Siletsky *et al.*, 1997).

RESOLUTION OF INDIVIDUAL CHARGE TRANSFER STEPS WITHIN THE P→F AND F→O TRANSITION

The proton-pumping peroxidase half-reaction of COX is comprised of two single-electron steps (P→F and F→O) contributing about equally to charge translocation by the enzyme (Wikström, 1989; Verkhovsky *et al.*, 1997). In its turn, each of these transitions includes multiple vectorial electron and proton transfer steps that need to be resolved and identified.

Time-resolved measurements of charge translocation by COX using rapid photoinjection of a single electron into Cu_A from Ru(II)-tris-bipyridyl (RuBpy) (Zaslavsky *et al.*, 1993) or RuBpy-modified cytochrome *c* (Zaslavsky *et al.*, 1995) reveal three major electrogenic processes associated with the F→O conversion of beef heart COX as summarized in Fig. 3. Similar results have been obtained recently with aa_3 oxidase from *R. sphaeroides,* except that all three phases proved to be 2–3-fold faster in agreement with the ca. 3-fold faster turnover of the bacterial enzyme (Konstantinov *et al.*, 1997a).

The rapid phase corresponds undoubtedly to vectorial electron transfer from Cu_A to heme *a* postulated by Mitchell more than 30 years ago (Mitchell, 1966). The electron transfer from CU_A to heme *a* is physically associated with translocation of 1 charge across 1/2 of the dielectric barrier as predicted by equilibrium studies (Hinkle and Mitchell, 1970; Rich *et al.*, 1988) and verified by the 3D structure (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995, 1996). Therefore the amplitude of this well-separated phase allows us to calibrate the slower charge transfer steps in the photoelectric response.

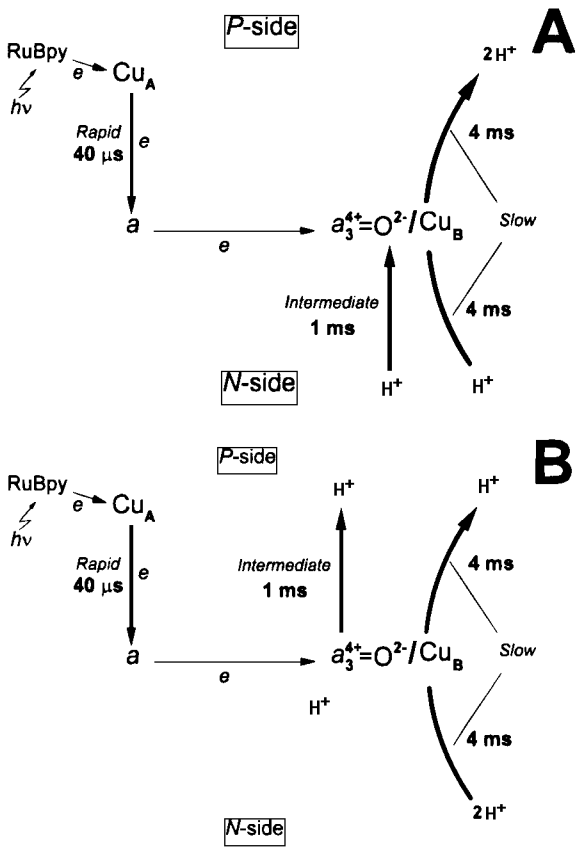

Fig. 3. Time-resolved electrogenic steps in the F→O transition of COX.

The intermediate phase (1.2 ms) equals in size the rapid one and has been assigned to the electrogenic transfer of a proton across 1/2 of the membrane (Zaslavsky *et al.*, 1993). The phase matches reasonably well the *complete* oxidation of the photoreduced heme *a* and simultaneous Fe^{4+}-to-Fe^{3+} conversion of heme a_3 at 445 and 436/412 nm, respectively (Siletsky *et al.*, in preparation). As shown in Fig. 3, the 1.2 ms electrogenic transient can be interpreted as (A) uptake of a "chemical" proton required for conversion of $Fe^{4+}=O^{2-}$ to Fe^{3+}–OH or (B) release of a pre-loaded H^+ to the P-side from a "proton trap" (Rich, 1995) (Fig. 2B). Proton uptake may seem a more natural explanation (Zaslavsky *et al.*, 1993). However, there is evidence for ca. 1 ms proton release to the P-phase coupled to the F→O transition (Nilsson *et al.*, 1990; Oliveberg *et al.*, 1991). Also the rate constant of the intermediate electrogenic phase does not depend on pH in the range 7–9.5 (Siletsky *et al.*, in preparation), which may be more consistent with the release of proton than with the uptake. Obviously, direct time-resolved measurements of proton uptake/release in the photoinduced F→O transition are necessary to discriminate between the alternatives.

The amplitude of the slow phase (×3 that of the rapid phase) corresponds to translocation across the membrane of 1.5 charges per electron. This can be rationalized as an uptake of 1 H^+ from the N phase and release of $2H^+$ to the P-phase (Fig. 3A) or vise versa (Fig. 3B). The phase decelerates upon alkalinization with apparent $pK\sim$ 8.5 (Siletsky *et al.*, in preparation) and thus can be limited by proton uptake. This principal electrogenic event with $\tau\sim$ 4–5 ms at pH 8 (which fits TN_{max} of the enzyme at this pH) lags behind the concerted oxidation of heme *a* and reduction of heme a_3 under these conditions ($\tau\sim$1.6 ms, Siletsky *et al.*, in preparation). Therefore, the major steps of proton translocation in the F→O transition may not be directly coupled to (i.e., are not simultaneous with) the reduction of the heme a_3-bound oxene, but are rather driven by protein relaxation *following* conversion of heme a_3 from the ferryl-oxo to the ferric state (e.g., by metal ligand rearrangement in the binuclear center (cf. Iwata *et al.*, 1995; Morgan *et al.*, 1994; Rich, 1995).

Analogous studies of the P→F transition following single-electron photoreduction of the P state generated by addition of low peroxide concentrations or aerobic bubbling of CO have been done recently (Siletsky *et al.*, 1997). As in the case of the F→O transition, the experiments resolve rapid, intermediate, and slow electrogenic phases (50, 0.7–0.9, and 2.5-3.5 ms), which may indicate that electrogenic mechanisms and hence the chemistry of the P→F and F→O proton pumping steps, are essentially similar. This is easier to rationalize assuming that the P→F and F→O transitions in our experiments correspond to the $F_I \rightarrow F_{II}$ and $F_{II} \rightarrow O$ steps in Fig. 2. A much faster charge translocation phase (0.2 ms) coupled to the $P(P_R) \rightarrow F$ transition during reoxidation of the fully reduced enzyme by oxygen (Verkhovsky *et al.*, 1997) is difficult to interpret in detail at this time since it has not yet been resolved into individual components.

Overall electrogenic proton movement in the F→O transition (and, according to our preliminary data, in the P→F transition as well), corresponds to transmembrane transfer of 2 electrical charges (Fig. 3) rather than of 2.5 as expected for a transmembrane pumping of 2 protons plus an uptake of 1 "chemical" proton across 1/2 of the membrane (e.g., Iwata *et al.*, 1995). This shortfall if not due to underestimation of the protonic phase contribution (Zaslavsky *et al.*, 1993), may indicate that some electrogenic proton transfer steps are left aboard the reaction span monitored in our experiments, e.g., that under the conditions of our experiments there is no uptake of "chemical" proton in the F→O transition (Wikstrom *et al.*, 1997b). Electrogenic uptake of proton(s) *following* the F→O step, i.e., coupled to conversion between several oxidized states of COX, has been considered earlier (Vygodina and Konstantinov, 1989; Babcock and Wikström, 1992). However, such a step is expected to be seen in our experiments as part of the slow electrogenic phase linked to the F→O transition, unless it is too slow to be part of the catalytic cycle.

Another possible solution is considered in Fig. 2. The scheme assumes electrogenic uptake of two protons coupled to heterolytic cleavage of the O–O bond, whereas the net result of each of the $F_I \rightarrow F_{II}$ and $F_{II} \rightarrow O$ transitions is transmembrane translocation of 2 protons (plus electrogenic Cu_A-to-heme *a* electron transfer) (cf. Fig. 3). H_2O_2 reaction with ferric COX does not result in proton release to the medium (Vygodina and Konstantinov, 1987; Konstantinov *et al.*, 1992). Therefore the two H^+ consumed at the $P_0 \rightarrow F_I$ step are likely to be delivered to the binuclear center by H_2O_2 itself and are not expected to contribute to electrogenesis in the steady-state peroxidase activity measurements by Vygodina *et al.* (1997a, 1997b), or in the time-resolved F→O and P→F assays (Zaslavsky *et al.*, 1993; Siletsky *et al.*, 1997; Konstantinov *et al.*, 1997a).

MOLECULAR ASPECTS OF REDOX-LINKED PROTON TRANSLOCATION

Redox-Linked Ionizable Groups and Proton Channels

The essential elements of COX as a proton pumping machine are the so-called *redox-linked ionizable groups* and *proton channels* (see Wikström *et al.*, 1981; Mitchell, 1988; Rich, 1996).

There are few protonatable residues around the redox core of COX. The most obvious candidates for the role of redox-linked ionizable groups are ligands of heme iron and Cu_B, heme propionates, and the highly conserved residues K362 and E286. Full reduction of COX is linked to binding of up to 3 protons (Hallén and Nilsson, 1992; Mitchell and Rich, 1994; Capitanio *et al.*, 1997) of which two are strongly coupled to oxidoreduction of the binuclear center (Mitchell and Rich, 1994; Capitanio *et al.*, 1997). The Em/pH-dependence of heme *a* in the unliganded membrane-bound COX (Wikstrom *et al.*, 1981; Artzatbanov *et al.*, 1983) and in the KCN-inhibited enzyme Artzatbanov *et al.*, 1978; Moody and Rich, 1990) indicates that the oxidoreduction of this redox center is weakly coupled to at least two ionizable groups that may interact with opposing aqueous phases (Artzatbanov *et al.*, 1978; Mitchell and Mitchell, 1988). Conceivably, attribution of the redox-linked proton uptake/release to specific redox groups is simplistic as there are probably multiple interactions of each of the redox centers with each of the ionizable groups and vice versa (see Kannt *et al.*, this volume). For instance, oxidoreduction of heme a_3 can be linked to protonation/deprotonation of Cu_B ligand(s) (Morgan *et al.*, 1994), and one of the heme *a*-linked ionizable groups (Artzatbanov *et al.*, 1978, 1983) can feel the redox state of Cu_B (Moody and Rich, 1990).

Collaborative studies with the laboratory of R. Gennis in Urbana are in progress to identify the redox-linked groups of COX. Amino acid replacements K362M and E286Q greatly suppress redox-dependent proton uptake by cyanide-inhibited *R. sphaeroides* COX at pH 8; however, the pH-dependence of the effect indicates that these residues may affect p*K* of the actual redox-linked acceptors rather than themselves being involved in the redox-linked proton binding (Siletsky, Tomson, Pecoraro, Gennis, and Konstantinov, in preparation).

As the redox-linked groups are buried deep inside subunit I, proton-conducting pathways are required to connect them with the P- and N-aqueous phases. Soon after proton pumping by COX was discovered by Wikström (1977), an operative model was proposed in which COX was postulated to have an output and two input proton channels, one loading the pumped protons at the stage of heme *a* reduction and the other involved in the uptake of protons to be consumed in water formation Konstantinov, 1977; Artzatbanov *et al.*, 1978). It is remarkable that the recently resolved three-dimensional structure of COX (Iwata *et al.*, 1995, Tsukihara *et al.*, 1995, 1996) does indeed reveal three structural domains in subunit I of the enzyme called "pores" (Tsukihara *et al.*, 1996) that look like a potential output channel (pore B) and two input proton wells (pores A and C). The two input channels were denoted operatively (Konstantinov *et al.*, 1997a) as the K-channel (pore C) for a conserved lysine residue in the middle of it (K362 in *R. sphaeroides*) and the D-channel (pore A) for a concerved aspartate in the II–III helice loop (D132 in *R. sphaeroides*) gating the channel at the N-side of the membrane.

What are the Two Input Proton Channels for?

The distinction between the four "chemical" and four "pumped" protons taken up from the N-aqueous phase has been a paradigm since the discovery of proton pumping by COX (Wikström, 1977). Morgan *et al.* (1994) and Rich (1995) considered strictly separate pathways for delivery of the two types of protons from the N-phase (but see Wikström *et al.*, this volume). Accordingly, Iwata *et al.* (1995) proposed that the K-channel conducts the "chemical" and the D-channel the "pumped" protons, which is incidentally similar to the role of the two input channels postulated earlier (Artzatbanov *et al.*, 1978; Konstantinov *et al.*, 1986). An alternative aproach is to associate the channels with different partial steps of the catalytic cycle, rather than with different kinds of protons. Within the framework of the eu-oxidase/peroxidase model it was proposed (Konstantinov *et. al.*, 1997a) that the K-channel is operational during the eu-oxidase phase of the cycle, whereas the peroxidase half-reaction is serviced by the D-channel (Fig. 2).

The effects of amino acid replacements in the K- and D-channels do not support the concept of separate pathways for "chemical" and "pumped" protons. In particular, mutations in the residues T359 and K362 within the K-channel, while inhibiting severely COX turnover, do not affect the F→O step of the catalytic

cycle (Konstantinov *et al.*, 1997a), although this step is considered to involve translocation of both chemical and pumped protons. Also, oxidation of the fully reduced K362M mutant of bo_3 quinol-oxidase by O_2 is essentially normal (Svensson *et al.*, 1995). Moreover, the "dead" mutant K362M reveals normal steady-state peroxidase activity fully coupled to proton pumping (Vygodina *et al.*, 1996, 1997b). Apparently, all protons necessary for the P→F→O phase can be delivered by the D-channel, which is difficult to reconcile with the hypothesis of Iwata *et al.* (1995). In contrast, mutations in the D-channel block electrogenic proton transfer steps in the isolated F→O transition (Konstantinov *et al.*, 1997a) and suppress the peroxidase activity of COX (Vygodina *et al.*, 1996, 1997b), but do not inhibit the O→R step (Vygodina *et al.*, 1997b) or the R→P transition (Adelroth *et al.*, 1997; Verkhovskaya *et al.*, 1997). The emerging picture agrees with the association of the K-channel with the eu-oxidase phase of the COX cycle and of the D-channel with the peroxidase half-reaction (Fig. 2).

Eu-Oxidase and Peroxidase Conformations of Cytochrome Oxidase

"Switching the channels" would accord with the proposal that the eu-oxidase and peroxidase reactions are catalyzed by different conformational states of COX (Fig. 2). Thus far, the 3D models of the oxidized and reduced states of COX do not reveal significant structural changes but notably both states correspond to the same putative eu-oxidase conformation of COX. Interestingly, the K proton pathway in the oxidized COX can be traced easily all the way through from the N-phase to a His ligand of Cu_B (Iwata *et al.*, 1995; Tsukihara *et al.*, 1996), whereas the integral D-channel pathway across the residue E286 and into the binuclear center is much less clear (Tsukihara *et al.*, 1996) although potentially discernible (Iwata *et al.*, 1995; Riistama *et al.*, 1997). Supposedly, the crystal structure of the oxidized enzyme may represent the conformational state of COX where the D-channel is not fully connected. As suggested by Konstantinov *et al.* (1997a), it may be that the formation of the strongly pulling oxene ligand at heme a_3 upon the splitting of the O–O bond ($P_0 \rightarrow F_I$ step in Fig. 2) switches the enzyme from the eu-oxidase to the peroxidase conformation. Displacement of heme iron from the proximal to the distal side of the heme plane by 0.3Å is observed upon transition from the ferric state to Cpd I in cytochrome *c* peroxidase (Fülöp *et al.*, 1994) and catalase (Gouet *et al.*, 1996).

Where in the cycle the two molecules of water are formed and how the the uptake of $8H^+$ from the N-phase divides between the K and D channels needs to be experimentally established. As pointed out by Wikstrom *et al.* (1997b), pH and energization of membrane can affect the protonation state of the intermediates and displace the steps of proton uptake and release within the cycle. In a simple provisional model (Fig. 2), the D-channel delivers the last four protons at the $F_I \rightarrow F_II \rightarrow O$ peroxidase phase of the cycle. The K-channel is proposed to conduct the protons at the O→R transition (cf. Verkhovsky *et al.*, 1995), the only step found so far to be blocked in the K362M mutant (Hosler *et al.*, 1996; Vygodina *et al.*, 1997b), but it may also deliver the second pair of protons coupled to heterolytic cleavage of the O–O bond.

Incidentally, if water is formed at the O→R and $P_0 \rightarrow F_I$ steps as depicted in Fig. 2, and proton movement in each of the $F_I \rightarrow F_II$ and $F_II \rightarrow O$ steps is formally confined to transmembrane translocation of two protons (although it can be mechanistically heterogeneous, cf. Fig. 3, the K-channel may be re-viewed as the "chemical" channel and the D-channel as the "pumping" one, though in a different sense than implied by Iwata *et al.* (1995).

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REFERENCES

Adelroth, P., Ek, M. S., Mitchell, D. M., Gennis, R. B., and Brzezinski, P. (1997). *Biochemistry*, in press.

Arnaud, S., Malatesta, F., Guigliarelli, B., Gayda, J.-P., Bertrand, P., Miraglio, R., and Denis, M. (1991). *Eur. J. Bi ochem.* **198**, 349–356.

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

Arzatbanov, V. Y., Grigoriev, V. A., and Konstantinov, A. A. (1983). *Biochemistry (Moscow)* **48**, 48–53.

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

Baek, H. K., and Van Wart, H. E. (1989). *Biochemistry* **28**, 5714–5719.

Bickar, D., Bonaventura, J., and Bonaventura, C. (1982). *Biochemistry* **21**, 2661–2666.
Blair, D. F., Witt, S. N., and Chan, S. I. (1985). *J. Am. Chem. Soc.* **107**, 7389–7399.
Capitanio, N., Vygodina, T. V., Capitanio, G., Konstantinov, A. A., Nicholls, P., and Papa, S. (1997). *Biochim. Biophys. Acta* **1318**, 255–265.
Denis, M., Arnaud, S., and Malatesta, F. (1989). *FEBS. Lett.* **247**, 475–479.
Erman, J. E., Vitello, L. B., Miller, M. A., Shaw, A., Brown, K. A., and Kraut, J. (1993). *Biochemistry* **32**, 9788–9806.
Fabian, M., and Palmer, G. (1995). *Biochemistry* **34**, 13802–13810.
Fann, Y. C., Ahmed, I., Blackburn, N. J., Boswell, J. S., Verkhovskaya, M. L., Hoffman, B. M., and Wikström, M. (1995). *Biochemistry* **34**, 10245–10255.
Ferguson-Miller, S., and Babcock, G. T. (1996). *Chem. Rev.* **7**, 2889–2907.
Fülöp, V., Phizackerley, R. P., Soltis, S. M., Clifton, I. J., Wakatsuki, S., Erman, J., Hajdu, J., and Edwards, S. L. (1994). *Structure* **2**, 201–208.
Gorren, A. C. F., Dekker, H., and Wever, R. (1985). *Biochim. Biophys. Acta* **809**, 90–96.
Gorren, A. C. F., Dekker, H., and Wever, R. (1986). *Biochim. Biophys. Acta* **852**, 81–92.
Gorren, A. C. F., Dekker, H., Vlegels, L., and Wever, R. (1988). *Biochim. Biophys. Acta* **932**, 277–286.
Gouet, P., Jouve, H.-M., Williams, P. A., Andersson, I., Andreoletti, P., Nussaume, L., and Hajdu, J. (1996). *Nature Struct. Biol.* **3**, 951–956.
Hallén, S., and Nilsson, T. (1992). *Biochemistry* **31**, 11853–11859.
Harris, D. L., and Loew, G. H. (1996a). *J. Am. Chem. Soc.* **118**, 10588–10594.
Harris, D. L., and Loew, G. H. (1996b). *J. Am. Chem. Soc.* **118**, 6377–6387.
Hinkle, P., and Mitchell, P. (1970). *Bioenergetics* **1**, 45–60.
Hosler, J. P., Shapleigh, J. P., Kim, Y., Pressler, M., Georgiou, C., Babcock, G. T., Alben, J. O., Ferguson-Miller, S., and Gennis, R. B. (1996). *Biochemistry* **35**, 10776–10783.
Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995). *Nature* **376**, 660–669.
Kim, Y. R., Yu, S. W., Lee, S. R., Hwang, Y. Y., and Kang, S. O. (1996). *J. Biol. Chem.* **271**, 3105–3111.
Kitagawa, T., and Ogura, T. (1997). In *Progress in Inorganic Chemistry* (Karlin, K. D., ed.), Wiley, New York, pp. 431–479.
Kobayashi, K., Iwamoto, T., and Honda, K. (1994). *Biochem. Biophys. Res. Commun.* **201**, 1348–1355.
Konstantinov, A. A. (1977). *Dokl. Akad. Nauk SSSR* **237**, 713–716.
Konstantinov, A. A., Vygodina, T. V., and Andreev, I. M. (1986). *FEBS Lett.* **202**, 229–234.
Konstantinov, A. A., Capitanio, N., Vygodina, T. V., and Papa, S. (1992). *FEBS Lett.* **312**, 71–74.
Konstantinov, A. A., Siletskiy, S. A., Mitchell, D., Kaulen, A.D., and Gennis, R. B. (1997a). *Proc. Natl. Acad. Sci. USA* **94**, 9085–9090.
Konstantinov, A. A., Vygodina, T. V., Capitanio, N., and Papa, S. (1997b). *Biochim. Biophys. Acta,* in press.
Ksenzenko, M. Y., Berka, V., Vygodina, T. V., Ruuge, E. K., and Konstantinov, A. A. (1992). *FEBS Lett.* **297**, 63–66.
Markossian, K. A., Poghossian, A. A., Paitian, N., and Nalbandian, R. M. (1978). *Biochem. Biophys. Res. Commun.* **81**, 1336–1343.
Miki, T., and Orii, Y. (1986a). *J. Biochem.* **100**, 735–745.
Miki, T., and Orii, Y. (1986b). *J. Biol. Chem.* **261**, 3915–3918.
Miller, M. A., Shaw, A., and Kraut, J. (1994). *Struct. Biol.* **1**, 524–531.
Mitchell, P. (1966). *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation.* Glynn Research Ltd., Bodmin, UK.
Mitchell, P. (1988). *Ann. N.Y. Acad. Sci.* **550**, 185–198.
Mitchell, R., and Mitchell, P. (1988). *Biochem. Soc. Trans.* **17**, 892–894.
Mitchell, R., and Rich, P. R. (1994). *Biochim. Biophys. Acta* **1186**, 19–26.
Moody, A. J. (1996). *Biochim. Biophys. Acta* **1276**, 6–20
Moody, A. J., and Rich, P. R. (1990). *Biochim. Biophys. Acta* **1015**, 205–215.
Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1994). *J. Bioenerg. Biomembr.* **26**, 599–608.
Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1996). *Biochemistry* **35**, 12235–12240
Naqui, A., and Chance, B. (1986). *Biochem. Biophys. Res. Communs.* **136**, 433–437.
Nilsson, T., Hallén, S., and Oliveberg, M. (1990). *FEBS Lett.* **260**, 45–47.
Ogura, T., Hirota, S., Proshlyakov, D., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996). *J. Am. Chem. Soc.* **118**, 5443–5449.
Oliveberg, M., Hallén, S., and Nilsson, T. (1991). *Biochemistry* **30**, 436–440.
Orii, Y. (1982a). *J. Biol. Chem.* **257**, 9246–9248.
Orii, Y. (1982b). In *Oxygenases and Oxygen Metabolism* (Nozaki, M., *et al.*, ed.), Academic Press, New York, pp. 137–149.
Orii, Y. (1988). *Ann. N.Y. Acad. Sci.* **550**, 105–117.
Orii, Y. (1990). In *Bioenergetics* (Kim, C. H., and Ozawa, T., eds.), Plenum Press, New York, pp 171–180.
Orii, Y., and Okunuki, K. (1963). *J. Biochem.* **54**, 207–213.
Patterson, W. R., and Poulos, T. L. (1995). *Biochemistry* **34**, 4331–4341.
Poulos, T. L., and Kraut, J. (1980). *J. Biol. Chem.* **255**, 8199–8205.
Proshlyakov, D. A., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., Appelman, E. H., and Kitagawa, T. (1994). *J. Biol. Chem.* **269**, 29385–29388.
Proshlyakov, D. A., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996a). *Biochemistry* **35**, 76–82.
Proshlyakov, D. A., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996b). *Biochemistry* **35**, 8580–8586.
Rich, P. R. (1995). *Aust. J. Plant Physiol.* **22**, 479–486.
Rich, P. (1996). In *Protein Electron Transfer* (Bendall, D. S., ed.), BIOS Scientific Publishers Ltd., Oxford, pp. 217–248.
Rich, P., and Moody, A. J. (1997). In *Bioenergetics* (Graber, P., ed.), in press.
Rich, P. R., West, I. C., and Mitchell, P. (1988). *FEBS Lett.* **233**, 25–30.
Riistama, S., Puustinen, A., Hummer, G., Dyer, R. B., Woodruff, W. H., and Wikström, M. (1997). *FEBS Lett.* **414**, 275–280.
Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996). *J. Biol. Chem.* **271**, 4023–4030.
Siletsky, S. A., Kaulen, A.D., and Konstantinov, A. A. (1997). *Eur. Biophys. J.* **26**, 98.
Sucheta, A., Georgiadis, K. E., and Einarsdóttir, O. (1997). *Biochemistry* **36**, 554–565.
Svensson, M., Hallen, S., Thomas, J. W., Lemieux, L., Gennis, R. B., and Nilsson, T. (1995). *Biochemistry* **34**, 5252–5258.
Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, T., Yaono, R., and Yoshikawa, S. (1995). *Science* **269**, 1069–1074.
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.
Verkhovskaya, M. L., García-Horsman, A., Puustinen, A., Rigaud, J.-L., Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1997). *Proc. Nat. Acad. Sci. USA* **94**, 10128–10131.
Verkhovsky, M. I., Morgan, J. E., and Wikström, M. (1995) *Biochemistry* **34**, 7483–7491.
Verkhovsky, M. I., Morgan, J. E., and Wikström, M. (1996). *Proc. Natl. Acad. Sci. USA* **93**, 12235–12239.

Verkhovsky, M. I., Morgan, J. E., Verkhovskaya, M., and Wikstrom, M. (1997). *Biochim. Biophys. Acta* **1318**, 6–10.
Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J., and Kraut, J. (1993). *Biochemistry* **32**, 9807–9818.
Vygodina, T. V., and Konstantinov, A. A. (1987). *FEBS Lett.* **219**, 387–392.
Vygodina, T. V., and Konstantinov, A. A. (1988). *Ann. N. Y. Acad. Sci.* **550**, 124–138.
Vygodina, T., and Konstantinov, A. (1989). *Biochim. Biophys. Acta* **973**, 390–398.
Vygodina, T. V., Konstantinov, A. A., Capitanio, N., and Papa, S. (1994). *EBEC Short Rep.* **8**, 37.
Vygodina, T., Mitchell, D., Pecoraro, C., Gennis, R., and Konstantinov, A. (1996). *EBEC Short Rep.* **9**, 93.
Vygodina, T. V., Capitanio, N., Papa, S., and Konstantinov, A. A. (1997a). *FEBS Lett.* **412**, 405–409.
Vygodina, T. V., Pecoraro, C., Mitchell, D., Germis, R., and Konstantinov, A. A. (1997b). *Biochemistry,* submitted.
Wang, J. -S., Baek, H. K., and Van Wart, H. E. (1991). *Biochem. Biophys. Res. Commun.* **179**, 1320–1324.
Wikström, M. (1977). *Nature* **266**, 271–273.
Wikström, M. (1981). *Proc. Natl. Acad. Sci. USA* **78**, 4051–4054.
Wikström, M. (1987). *Chem. Scr.* **27B**, 53–58.
Wikström, M. (1989). *Nature* **338**, 776–778.
Wikström, M. and Morgan, J. (1992). *J. Biol. Chem.* **267**, 10266–10278
Wikström, M., Krab, K., and Saraste, M. (1981). *Cytochrome Oxidase—A Synthesis,* Academic Press, New York.
Wikström, M., Morgan, J. E., Hummer. G., Woodruff, W. H., and Verkhovsky, M. I. (1997a). *In Frontiers of Cellular Energetics* (Papa, S., Guerrieri, F., and Tager, J., eds.), Plenum Press, in press.
Wikström, M., Morgan, J. E., and Verkhovsky, M. I. (1997b). *Biochim. Biophys. Acta* **1318**, 299–306.
Yeom, H., Sligar, S., Li, H., Poulos, T. L., and Fulco, A. J. (1995).
Yeom, H., and Sligar, S. G. (1997). *Arch. Biochem. Biophys.* **337**, 209–216.
Zaslavsky, D., Kaulen, A., and Smirnova, I. A. (1993). *FEBS Lett.* **336**, 389–393.
Zaslavsky, D. L., Smirnova, I. A., Siletsky, S. A., Kaulen, A. D., Millett, F., and Konstantinov, A. A. (1995). *FEBS Lett.* **359**, 27–30.