ELSEVIER

Biochimica et Biophysica Acta 1229 (1995) 129–147

BBA Biochimica et Biophysica Acta

Review

Fast reactions of cytochrome oxidase

Ólöf Einarsdóttir *

Department of Chemistry and Biochemistry, University of California, Santa Cruz, Santa Cruz, CA 95064, USA

Received 24 October 1994; accepted 9 December 1994

Keywords: Time-resolved spectroscopy; Cytochrome oxidase; Electron transfer; Proton translocation; Carbon monoxide photodissociation

Contents

1. Introduction

Cytochrome oxidase catalyzes the reduction of dioxygen to water with electrons from ferrocytochrome *c*. This reaction serves as one of the coupling sites of the respiratory chain where the energy of the redox reaction is conserved as an electrochemical proton gradient (see Wikström et al. for review [1] for review). The mechanisms underlying the fast reactions of cytochrome oxidase, oxygen activation, electron transfer, and coupling of electron transfer to proton translocation, remain a major challenge in the field of bioenergetics [2–8]. A complete elucidation of the mechanisms of these reactions will require not only detailed knowledge of the static structure of cytochrome oxidase, but an understanding of the structural changes that occur as the protein carries out its function. Various time-resolved spectroscopic techniques can probe the temporal evolution of protein structure during turnover conditions and recent advances in these techniques have significantly expanded our knowledge of many fast biological reactions, including those of cytochrome oxidase [8–17]. The application of site-directed mutagenesis to bacterial quinol and cytochrome *c* oxidases, in combination with spectroscopic techniques, has also led to important insights into the structure and function of the heme-copper oxidases [18–27].

Abbreviations: Cu_A, copper A; Cu_B, copper B; a^{2+}, reduced cytochrome *a*; a^{3+}, oxidized cytochrome *a*; a_3^{2+}, reduced cytochrome a_3; a_3^{3+}, oxidized cytochrome a_3; TRIR, time-resolved infrared; TR^3, time-resolved resonance Raman; TRMCD, time-resolved magnetic circular dichroism; TROA, time-resolved optical absorption.

* Corresponding author. Fax: +1 (409) 4592935.

SSDI 0005-2728(94)00196-0

The reaction of cytochrome oxidase with O_2 is usually studied by the flow-flash technique developed by Gibson and Greenwood [28,29], which involves the photodissociation of CO from the reduced cytochrome a_3 in the presence of O_2. Transient optical absorption studies have provided information about the kinetics of the internal electron transfer processes [10,13,16,17,28–34], and more recently, proton uptake [35–41]. Low-temperature optical absorption and EPR trapping experiments [42–47] and time-resolved resonance Raman (TR^3) studies [11,14,48–52] have yielded useful information about the transient intermediates generated during the dioxygen reduction reaction. Other probes of cytochrome oxidase dynamics include time-resolved infrared (TRIR) [8,9,53–56] and time-resolved magnetic circular dichroism (TRMCD) [12,57,58] spectroscopies. Based on these studies, various models of the mechanism of the dioxygen reduction and the redox-linked proton translocation have emerged [2,5–8,16,17,55,59–61].

Despite considerable progress, significant questions remain. What is the exact nature of the metal-dioxygen interactions and how does O_2 activation occur? What factors control the rates of electron transfer and the uptake of scalar protons during the dioxygen reduction? How is electron transfer coupled to proton translocation? The primary focus of this review will be time-resolved spectroscopic studies of cytochrome oxidase as they relate to these questions. Experimental details will not be covered here but have recently been summarized by Woodruff et al. [8]. The emphasis will be on the enzyme isolated from bovine heart, but relevant studies on other heme-copper oxidases will be described as well.

2. Structure and function

Cytochrome oxidase is the terminal oxidase of cellular respiration, catalyzing the reduction of dioxygen to water by cytochrome *c* in all eukaryotes and many prokaryotes (see Wikström et al. and Saraste [1,62] for reviews). The enzyme plays a key role in the energy transduction in mitochondrial respiration, being responsible for over 90% of aerobic metabolism [63]. The energy derived from the reduction of dioxygen to water is conserved by the generation of a transmembrane proton gradient. This is accomplished by two means. The four protons required for the oxygen reduction are consumed from the inner (matrix) side of the membrane [64], and an additional four protons are pumped from the inside to the outside as the electrons pass through the enzyme [65]. The energy of the gradient is subsequently used by the cell to synthesize ATP [66].

The mammalian cytochrome oxidase contains at least thirteen different subunits [67] of which the three largest ones are coded by mitochondrial DNA and the remainder by nuclear DNA. The details of the structure and membrane topology of the subunits have been reviewed recently [62,68–70]. The enzyme contains two heme A chromophores (cytochromes *a* and a_3) and at least two redox active coppers (Cu_A and Cu_B) coordinated to the protein. Heme A differs from heme B in that it contains a formyl group in position 8 and a farnesyl tail of unsaturated isoprenoid groups in position 2 [71]. The function of the farnesyl chain is unknown, but it has been suggested to play a structural role in orienting the heme within the protein, act as an electron transfer wire between cytochrome a_3 and Cu_B [71], or play a role in proton translocation [12]. The bovine heart enzyme also contains one zinc and one magnesium atom [72,73], both of unknown function. EXAFS studies have suggested that the zinc is in a tetrahedral environment, coordinated mostly by sulfur ligands [74].

A third copper, Cu_x, has recently been shown to be an integral component of cytochrome oxidase from a variety of species [73,75–80]. This observation, together with spectroscopic evidence [81–83], suggests that Cu_A forms a mixed-valence dimer in the oxidized enzyme, $Cu(1.5) \cdot \cdots \cdot Cu(1.5)$, with absorbance, EPR and magnetic circular dichroism spectra similar to that of nitrous oxide reductase (N_2OR), a multicopper enzyme [81–85]. The amino acid sequence of the N_2OR C-terminus is homologous to the Cu_A-domain of subunit II of cytochrome oxidases [86], and recent phylogenetic studies indicate that cytochrome oxidases evolved from the denitrification enzymes and thus may have acquired their Cu_A binuclear center from N_2OR [87]. Subunit II of cytochrome *bo* from *E. coli* also contains the same domain but lacks Cu_A [88,89]. Recent experiments on an engineered Cu_A-like site in subunit II of cytochrome *o* from *E. coli* and on the Cu_A-binding domains of cytochrome *c* oxidase from *Paracoccus denitrificans* and the caa_3-type oxidase from *Bacillus subtilis*, support the conclusion that Cu_A is a mixed-valence binuclear copper center [89–91].

Because high-resolution X-ray studies of cytochrome oxidase are lacking, structural information on cytochrome oxidase is limited to 7 Å [92,93]. However, electron microscopic examination of two-dimensional crystals of cytochrome oxidase has provided information regarding the overall shape of the oxidase molecule and its position in the membrane [94–96]. Crosslinking and chemical labeling studies have yielded additional insight into the relative arrangements of the subunits within the enzyme [95]. Spectroscopic evidence, hydropathy plots, and recent site directed mutagenesis studies have established the locations of the metal centers and their respective ligands (see below). Distances between the redox centers have been obtained by various spectroscopic techniques [97–102].

Cytochrome oxidase belongs to a family of related enzymes of which several members are found in the cell membrane of aerobic bacteria [62,103–107]. Bacterial oxidases are significantly simpler in composition, containing three subunits with strong homology to subunits I, II and III of eukaryotes. Some of the bacterial oxidases contain

heme A as a prosthetic group and cytochrome *c* as a substrate, but in others heme A has been replaced by a different heme. In cytochrome ba_3 from *Thermus thermophilus*, the low-spin heme *a* has been replaced by a protoheme but both Cu_A and Cu_B are still present [108]. In cytochrome *bo* the heme at the binuclear site is heme O which has the same structure as heme A except for a methyl group in place of the formyl group [109,110]. In this enzyme and cytochrome aa_3-600 from *Bacillus subtilis* and cytochrome aa_3 from *Sulfolobus acidocaldarius* [111], the electron donor is ubiquinol rather than cytochrome *c* and these enzymes lack Cu_A. *Rhodobacter capsulatus* [112] and *Rhodobacter sphaeroides* [113] have recently been shown to contain an alternative *cb*-type cytochrome *c* oxidase which contains a high-spin heme B at the binuclear center. This oxidase contains Cu_B but lacks Cu_A. A similar, if not identical, enzyme in *Paracoccus* pumps protons [114], indicating that the farnesyl chain may not have a role in proton translocation as previously suggested [12]. Therefore, the common feature of the heme-copper oxidases is a binuclear center located in subunit I, containing a high-spin heme and Cu_B. Furthermore, all are able to function as redox-linked proton pumps, suggesting that the binuclear center is the site of the proton pump. This issue will be discussed in more detail in the section on Proton pumping.

3. Spectroscopic properties

Optical absorption spectra of bovine heart cytochrome c oxidase. The complex oxidation-reduction system of cytochrome *c* oxidase has been studied by various spectroscopic techniques, the most common of which is optical absorption spectroscopy [1,115]. Fig. 1 shows the spectra of oxidized, fully reduced, mixed-valence-CO and fully reduced CO-complexes of bovine heart cytochrome *c* oxidase in the Soret, visible and near-infrared regions. The 0–0 $\pi \rightarrow \pi^*$ transition leads to an intense band in the Soret region between 400–450 nm and a less intense band in the visible region, the α band, centered around 600 nm. A weaker side-band, the β band, is observed at ~ 560 nm in the visible region and is due to a 0–1 vibronic transition.

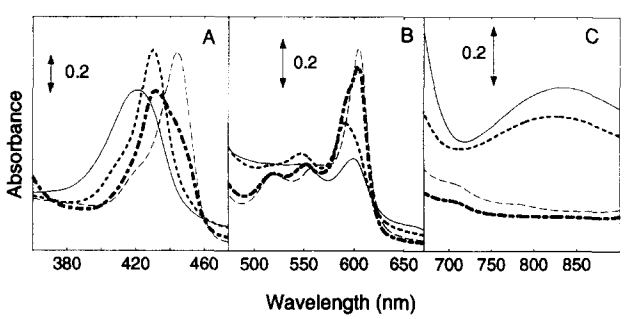


Fig. 1. Optical absorption spectra of oxidized (—), mixed-valence CO (-----), fully reduced (– – –), and fully reduced CO complex (- · - · - · -). Concentrations were : (A) 13 μM, (B) 17 μM and (C) 180 μM in cytochrome oxidase. The pathlength was 0.4 cm for the Soret region and 1 cm for the visible and the near-infrared regions. In the near-infrared region, the spectrum of the mixed-valence CO complex was up-shifted 0.133 absorbance units. Conditions: 0.1 M phosphate buffer; pH = 7.4; temperature 24° C. The concentration of CO for the CO complexes was 1 atm.

The two hemes have strongly overlapping bands, which has made assignment to individual hemes difficult. Using the photochemical action spectrum of the cytochrome a_3-CO complex and the spectra of ligand-bound derivatives (CN^- and CO), Vanneste [116] deconvoluted the reduced-minus-oxidized difference spectra of the individual hemes and their absolute spectra. The constructed spectra suggest that the two cytochromes have about equal contributions to the 445 nm band in the fully reduced enzyme whereas the absorbance of the reduced cytochrome *a* dominates (~ 80%) at 605 nm (Fig. 1). The broad band at ~ 420 nm in the oxidized enzyme is a composite of two absorption bands at ~ 414 nm and ~ 426 nm, attributed to cytochrome a_3 and cytochrome *a*, respectively [116]. The presence of these two bands has also been observed by second derivative spectroscopy [117].

The ligand-inhibition spectral procedure assumes that no spectral interactions exist between the redox centers [116] (an assumption not strictly valid [118,119]) and also ignores any possible contributions from the oxidized coppers in the Soret and visible regions. Although no optical absorption due to Cu_B has been reported, low-temperature MCD measurements have shown that Cu_A has a transition with maximum at ~ 520 nm [120–123]. We have recently obtained evidence for an optical absorption of Cu_A between 480–550 nm [119]. The visible absorption spectra of the engineered Cu_A mutant of subunit II of cytochrome *o* from *E. coli* and the Cu_A domains of cytochrome oxidase from *Paracoccus* and *Bacillus subtilis* also have an absorbance between 480–550 nm [88–91].

In addition to the $\pi \rightarrow \pi^*$ transitions, the fully oxidized enzyme shows a weak band at ~ 655 nm due to the high-spin ferric cytochrome a_3 (Fig. 1) [124,125]. This band has been postulated to be modulated by the redox state of Cu_B [124,126]. A band at 830 nm in the near-infrared region of the fully oxidized enzyme has been attributed to a charge transfer transition between Cu_A and a sulfur ligand [127,128]. We recently reported a band at 710 nm ($\epsilon = 80\ M^{-1}\ s^{-1}$) in the spectra of the fully reduced enzyme and the fully reduced CO-bound complex [129] which we attributed to low-spin cytochrome *a* (Fig. 1C). A second band at 785 nm (ϵ ~ 50 $M^{-1}\ cm^{-1}$) was observed in the ground state spectrum of the fully reduced enzyme and in the transient difference spectra following photodissociation of the fully reduced and mixed-valence CO complexes. This absorbance was attributed to the five-coordinate ferrous cytochrome a_3 [129], in agreement with previous MCD assignment of this band [130]. An analo-

gous transition has been observed in cytochrome *bo* [131]. The 785 nm band was found to have some characteristics of band III which is observed at ~ 760 nm in deoxy-hemoglobin and deoxy-myoglobin [132].

Structure of the metal centers. Cytochrome *a* is low-spin and six-coordinated in both the oxidized and reduced forms of the enzyme as demonstrated by MCD and resonance Raman spectroscopy [125,133–135]. Cytochrome a_3 is considered to be high-spin both in the resting ferric state ($S = 5/2$) and the fully reduced ferrous state ($S = 2$). Cytochrome a_3 and Cu_B are in close enough proximity, 3.0–3.8 Å [136,137], to be magnetically coupled (EPR silent) by an unknown ligand in the oxidized form(s) of the enzyme [138,139]. The identity of this ligand is unknown but sulfur [136], chloride [137] and carboxylate [140] have been proposed. Synthesis and characterization of an oxo-bridged heme-copper complex displaying a strong antiferromagnetic coupling between the two redox centers has recently been reported [141–143]. Although single turnover oxidation studies of reduced cytochrome oxidase with $^{18}O_2$ have provided strong arguments against an oxo bridge derived from dioxygen [144], an oxo bridge, possibly derived from an amino acid such as tyrosine, may be present. The coupling between cytochrome a_3 and Cu_B is broken upon reduction of the binuclear site, leaving a vacant distal site on cytochrome a_3 for small molecules such as CO, NO, CN^- and O_2 to bind.

The binding site for Cu_A is located in subunit II [88–91]. EPR and ENDOR studies have indicated that Cu_A contains at least one and perhaps two histidine ligands [145], and one cysteine [146,147]. Recent results from biochemical analysis, site-directed mutagenesis and electrospray mass spectrometry on a Cu_A-engineered mutant of subunit II of cytochrome *bo* from *E. coli* support the binuclear model of Cu_A and indicate that two cysteines, two histidines and one methionine are ligands to the two coppers [89]. Spectroscopic data suggest that cytochrome *a* is coordinated by two histidines [148] and Cu_B by three histidines [137,149,150] and that the proximal ligand to cytochrome a_3 is histidine [151]. Based on these data and sequence homology analysis [152,153], cytochrome a_3, Cu_B and cytochrome *a* are believed to be located in subunit I, which based on hydropathy analysis contains 12 (I–XII) transmembrane helices [152]. This conclusion is supported by recent site-directed mutagenesis studies of the bacterial oxidases, in which six conserved histidines in subunit I were assigned to Cu_B, cytochrome a_3 and cytochrome *a* [18–24,26]. These assignments place cytochrome a_3 between cytochrome *a* and Cu_B with cytochrome a_3 and cytochrome *a* located on opposite sides of helix X, separated by one residue [24,27]. In view of the structural homology between the cytochrome oxidases and the ubiquinol oxidases (see Saraste [62] and Trumpower and Gennis [107] for reviews), the latter should be a good model for the former. If correct, this structural arrangement may have important implications for the pathway(s) of electron transfer and control of the redox-linked proton translocation in the heme-copper oxidases [55]. Fig. 2 summarizes our current knowledge of the structure of cytochrome *c* oxidase.

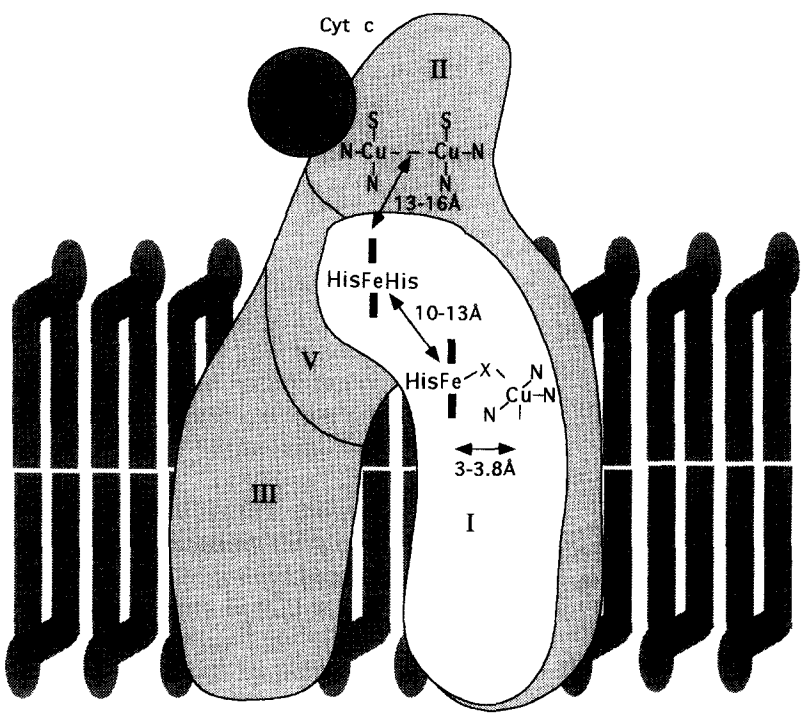
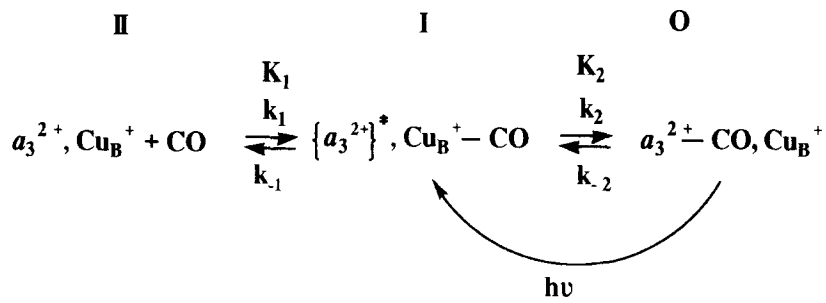


Fig. 2. Pictorial representation of the structural organization of cytochrome *c* and the cytochrome oxidase monomer in the inner mitochondrial membrane. The relative positions of some of the subunits and the redox-active metal centers are represented.



Scheme 1. The mechanism of the photodissociation and recombination dynamics of fully reduced CO-cytochrome oxidase.

4. Photodissociation and rebinding dynamics of ligand-bound cytochrome oxidase

Carbon monoxide. Most studies of fast ligand reactions of the heme-copper oxidases have focused on the photodissociation and rebinding dynamics of carbon monoxide. This is due to the high quantum yield of carbon monoxide compared to other ligands such as cyanide [154], and the fact that the CO recombination occurs on a readily observable time-scale. More importantly, the flow-flash technique developed by Gibson and Greenwood [28,29] utilizes photodissociation of CO to initiate the reaction of cytochrome oxidase with O_2. The physiological relevance of the flow-flash technique is based on the assumption that the photodissociated CO does not interfere with the dioxygen reduction reaction. Therefore, it is crucial that we understand the consequences of CO photodissociation in order to know whether the O_2 reaction observed by the flow-flash technique adequately represents the physiological O_2 reaction that occurs in the absence of CO. Secondly, the early events in the physiological function of the enzyme are O_2-ligation reactions, followed by subsequent electron transfer events. Since the CO and O_2 ligands compete for the same binding site in cytochrome oxidase, a better understanding of the CO binding dynamics is expected to give information regarding O_2 binding, the structure of the dioxygen reduction site, and the mechanism of the reduction of dioxygen to water.

Bovine heart cytochrome c oxidase. Photodissociation and recombination dynamics of the carbonmonoxy bovine heart cytochrome oxidase have been studied by FTIR at cryogenic temperatures [15,155–157] and by a combination of various time-resolved spectroscopic techniques, including time-resolved infrared (TRIR), time-resolved optical absorption (TROA), time-resolved resonance Raman (TR^3) and time-resolved magnetic circular dichroism (TRMCD) at room temperature [9,12,15,53,54,56,57,158–160]. These measurements, which span the time-scales from subpicoseconds (100 fs) to milliseconds, will be summarized below.

Infrared spectroscopy has great structural specificity and has been used effectively to monitor binding of ligands such as CO, CN^-, NO and N_3^- to hemeproteins, including cytochrome oxidase [161–165]. These studies have shown that CO binds to cytochrome a_3 at a site well isolated from the external medium [161,164]. FTIR studies of mitochondrial cytochrome oxidase preparations [155,156] and the isolated enzyme [15] have demonstrated that CO binds to Cu_B^+ under cryogenic conditions (< 180 K) following CO photodissociation from cytochrome a_3. Therefore, the infrared method has the unique advantage of being able to follow species such as Cu_B-CO, which are inaccessible to other spectroscopic probes. The FTIR method, in combination with site-directed mutagenesis studies of cytochrome aa_3 from *Rhodobacter sphaeroides* and cytochrome *bo* from *E. coli*, has recently proven very useful in determining which of the six conserved histidines in subunit I are ligands to cytochrome *a*, cytochrome a_3 and Cu_B [21–24,26,27].

In the last few years, TRIR spectroscopy has emerged

Table 1

	Rate constants		Equilibium constants
	Scheme 1		
Fully reduced	$k_1 = 5.9 \cdot 10^7\ M^{-1}\ s^{-1}$	$k_{-1} = 6.5 \cdot 10^5\ s^{-1}$	$K_1 = k_1/k_{-1} = 90\ M^{-1}$
	$k_2 = 1.1 \cdot 10^3\ s^{-1}$	$k_{-2} = 0.023\ s^{-1}$	$K_2 = k_2/k_{-2} = 4.8 \cdot 10^4$
	Scheme 2		
Mixed-valence	$k_{1f} = 8.7 \cdot 10^7\ M^{-1}\ s^{-1}$	$k_{1r} = 8.7 \cdot 10^5\ s^{-1}$	$K'_1 = k_{1f}/k_{1r} = 100\ M^{-1}$
	$k_{2f} = 1.7 \cdot 10^5\ s^{-1}$	$k_{2r} = 1.7 \cdot 10^4\ s^{-1}$	$K'_2 = k_{2f}/k_{2r} = 10$
	$k_{3f} = 1.2 \cdot 10^4\ s^{-1}$	$k_{3r} = 6.5 \cdot 10^3\ s^{-1}$	$K'_3 = k_{3f}/k_{3r} = 1.8$
	$k_{4f} = 9.5 \cdot 10^2\ s^{-1}$	$k_{4r} = 0.023\ s^{-1}$	$K'_4 = k_{4f}/k_{4r} = 4.1 \cdot 10^4$
	$k_{5f} = 49\ s^{-1}$	$k_{5r} = 113\ s^{-1}$	$K'_5 = k_{5f}/k_{5r} = 0.43$

as a powerful tool to probe the dynamics of cytochrome oxidase following photodissociation of CO bound to the fully reduced enzyme [9,12,15,53,158]. It has also recently been used to study the CO photodissociation dynamics of wild-type and mutant variants of cytochrome *bo* from *E. coli* [25]. The TRIR technique has shown that photodissociated CO binds to Cu_B^+ in bovine heart oxidase on a subpicosecond time-scale at room temperature [9,53,56], possibly by a concerted mechanism in which the Cu_B^+–CO bond is formed simultaneously with the breakage of the Fe_{a3}^{2+}–CO bond [53]. Recent femtosecond UV-Vis measurements have indicated that the photodissociation of CO from cytochrome a_3 occurs in less than 100 fs, possibly on the time-scale of one vibrational period of the Fe–CO stretch, 64 fs [54].

The picosecond to microsecond kinetics of the CO photodissociation and rebinding in fully reduced bovine heart cytochrome oxidase have been studied by UV-Vis spectroscopy at single wavelengths (445 and 610 nm) [12,15], and more recently, using multichannel detection [102,119]. The results from these studies can be summarized by the mechanism in Scheme 1. The microscopic rate constants are listed in Table 1. In the transient species generated 40 ns after CO photolysis, CO is bound to Cu_B and cytochrome a_3 is in a non-equilibrium state ($\{a_3^{2+}\}^*$ in Scheme 1) [12,15,102,119]. The absorbance spectrum of this species is red-shifted relative to the spectrum of the unliganded fully reduced enzyme at equilibrium in both the visible and Soret regions [102,119]. This transient species, in which cytochrome a_3 is high-spin as shown by TRMCD [12,57], relaxes to an intermediate with a spectrum nearly identical to the equilibrium unliganded enzyme on a microsecond time-scale (Scheme 1, intermediate II). While Brittain et al. [166] found the MCD spectra of the CO-photolyzed cytochrome oxidase trapped at 4 K and the equilibrium unliganded fully reduced complex indistinguishable, room temperature TRMCD spectra do show subtle differences between the two in both the visible and Soret regions [57]. The TR^3 spectra also display significant differences between the two forms (see below).

The nature of the structural change in cytochrome a_3 in intermediate I is not known. The results from the transient kinetics, TRIR, TRMCD and TR^3 studies were interpreted by a mechanism in which an endogenous ligand L bound to Cu_B is transferred ('shuttled') to the distal side of cytochrome a_3 upon binding of the photodissociated CO to Cu_B, with a simultaneous loss of the proximal histidine [12,57]. When CO dissociates from Cu_B^+, ligand L returns to Cu_B and the proximal histidine rebinds to the heme. The rate-limiting step for the CO recombination with cytochrome a_3 was suggested to be the dissociation of ligand L. Based on these studies, a similar behavior was suggested for other ligands, such as NO, and more importantly, O_2 [12,15]. That O_2 may also bind to Cu_B prior to binding to cytochrome a_3 has been supported by recent flow-flash studies of the reaction of the fully reduced enzyme with dioxygen [13,17,34]. The 'ligand shuttle' has been postulated to play a role in electron transfer and in the proton translocation mechanism of the enzyme [55] (see below). Light-induced structural changes in cytochrome oxidase have also been explained in terms of Woodruff's ligand shuttle model [167].

An alternative explanation for the nature of the cytochrome a_3 transient created upon CO photodissociation is that it reflects an unrelaxed proximal heme pocket geometry [159,160]. The changes in the optical and TRMCD spectra [12,15,57,102] and the shift in the resonance Raman Fe-His stretch from 222 cm^{-1} to its equilibrium position at 215 cm^{-1} on a microsecond time-scale would consequently reflect the relaxation of the proximal heme pocket to its equilibrium state [159,160]. More direct experimental evidence is needed to distinguish between the two alternatives (see Section 8).

Bacterial oxidases. Comparative transient UV-Vis and TRIR studies of the CO photodissociation and rebinding dynamics of other heme-copper oxidases, including c_1aa_3 and ba_3 from *Thermus thermophilus* and cytochrome *bo* from *E. coli* [8,25], suggest that all the oxidases examined follow the mechanism in Scheme 1, where CO in solution first binds in a pre-equilibrium with Cu_B and then transfers to cytochrome a_3 [55]. However, the stability constant of the Cu_B-CO complex (K_1 in Scheme 1) and the rate constant for transfer of CO from Cu_B to cytochrome a_3 (k_2) are different for various oxidases. For example, k_1 and k_{-1} are $2 \cdot 10^5$ $M^{-1}\,s^{-1}$ and 500 s^{-1}, respectively, for cytochrome *bo* of *E. coli* compared to $6 \cdot 10^7$ $M^{-1}\,s^{-1}$ and $7 \cdot 10^5$ s^{-1}, respectively, for the bovine heart aa_3 enzyme [8]. This indicates that the protein structure external to the binuclear site may regulate ligand entry into the site [25].

Other ligands. Whether the ligand shuttle model is applicable to other ligands, such as NO, CN^- and O_2 remains to be proven. In the NO data of Blackmore et al. [34] there is no evidence for rate limitation of NO binding to cytochrome a_3 upon photolysis of CO, indicating that at least for NO the dissociation of L is not rate limiting. The observed rate was found to be proportional to NO concentration up to 1 mM NO, giving a second-order rate constant of $\sim 1 \cdot 10^8$ $M^{-1}\,s^{-1}$. However, these results do not rule out that NO may bind to Cu_B prior to cytochrome a_3 but could simply indicate that the binding constant for the Cu_B^+-NO complex (K_1 in Scheme 1) is very small and that saturation is only achieved at much higher NO concentration (in the case of CO, saturation is observed at [CO] > 1 mM [15]). A low Cu_B-NO binding constant is supported by cryogenic measurements which have shown that the fully reduced NO complex is irreversibly photodissociated at temperatures below 5 K, presumably due to its binding to Cu_B^+ [168,169]. This temperature is considerably lower than the value of 160 K at which CO starts to recombine with cytochrome a_3^{2+} [15,156].

Studies of the photodissociation and rebinding dynam-

ics of the fully reduced cyanide complex have been hampered by the low apparent quantum yield. Following photolysis of the fully reduced CN complex, CN recombines with a bimolecular binding constant of 235 $M^{-1} s^{-1}$ [154] in agreement with a value obtained from stopped-flow studies [170]. The difference between the quantum yields of the fully reduced CO and CN complexes may due to two binding sites for CN in the fully reduced enzyme, one on cytochrome a_3 and the other on Cu_B, as previous infrared spectroscopic results have suggested [171]. Therefore, upon photolysis of CN from cytochrome a_3, CN may recombine with ferrocytochrome a_3 in a geminate reaction because it cannot bind to Cu_B. Time-resolved optical absorption studies of the photodissociation and recombination dynamics of CN on picosecond or early nanosecond time-scales should be able to determine whether this is the case.

5. Electron transfer pathways and kinetics

Numerous kinetic experiments on cytochrome oxidase have been aimed at determining which redox center – cytochrome *a* or Cu_A – is the primary acceptor of electrons from cytochrome *c* in vivo. The elucidation of the electron transfer pathway(s) from cytochrome *c* to the dioxygen site is clearly relevant to mechanisms by which these processes may be coupled to proton pumping. Two types of experiment have been used to investigate the turnover cycle of the enzyme, steady-state and transient kinetic experiments. The former will not be covered in detail here, but readers are referred to a review article by Cooper [172].

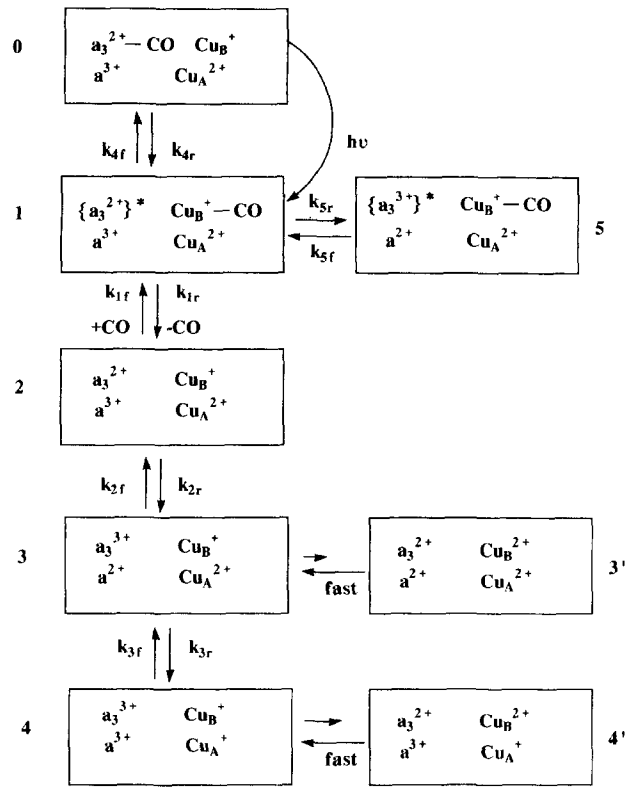
Steady-state measurements. Steady-state measurements show nonhyperbolic kinetics of the oxidation of cytochrome *c*. The biphasic kinetics were initially suggested to arise from two catalytic binding sites for cytochrome *c* [173]. More recently, Malmström and coworkers [174,175] postulated that the two phases arise from two enzyme conformations, E_1 and E_2, both of which can accept electrons, but only E_2 has the ability to pump protons. Stopped-flow measurements of the reduction of cytochrome *a* and Cu_A by cytochrome *c* also show biphasic kinetics, with the data suggesting that cytochrome *a* and Cu_A become reduced simultaneously, in two different phases [176,177], or that Cu_A may lag slightly behind [178]. However, the stopped-flow measurements of the electron input are limited by the cytochrome *c* binding rate, which has been estimated between 10^6 and $3 \cdot 10^7$ $M^{-1} s^{-1}$ [176–178]. To circumvent this rate limitation, new techniques have recently been developed to measure intra-complex electron transfer rates (see below).

While most of the early steady-state and stopped-flow experiments favored cytochrome *a* over Cu_A as the primary acceptor of electrons from cytochrome *c* in vivo (see review by Brunori et al. [68]), recent transient kinetic studies suggest the opposite. These conclusions are based on three different approaches: (1) flow-flash experiments of the reaction of the electrostatic cytochrome *c*-cytochrome *c* oxidase complex with O_2 under single turnover conditions [16,32,179], (2) transient optical absorption studies in which the apparent rates of reverse electron transfer from the binuclear center to the oxidized low-potential centers, cytochrome *a* and Cu_A, are measured following photodissociation of the mixed-valence CO complex [101,102,119,180–185], and (3) transient optical absorption measurements in which cytochrome *c* is reduced in situ by photochemically activated compounds [186–190], or alternatively, where the photoactivated system itself serves as the electron donor [191,192]. Structural data also support the idea that Cu_A is the primary acceptor of electrons from cytochrome *c* [152,193].

Flow-flash studies of the cytochrome c-cytochrome oxidase complex. Single turnover flow-flash transient optical absorption studies of the reaction of the electrostatic cytochrome *c*-cytochrome oxidase complex with oxygen indicate that the time-course for the oxidation of cytochrome *c* is very similar to that of Cu_A, in the absence of cytochrome *c*, suggesting that Cu_A is the initial electron acceptor from cytochrome *c* [16,32,179]. Hill [16] has recently modeled the sequence of the electron transfer reactions of the reduced cytochrome oxidase with oxygen in the presence and absence of bound cytochrome *c* and extracted the microscopic rate constants for each step. The forward rate constant of electron transfer between cytochrome *c* and Cu_A was estimated to be $\sim 1 \cdot 10^5$ s^{-1} [16,32]. This process was followed by electron transfer from Cu_A to cytochrome *a* with a forward rate constant of 7000 s^{-1}.

Photodissociation of the mixed-valence CO complex. Photodissociation of CO bound to the mixed valence enzyme lowers the apparent reduction potential of the binuclear site, causing a reverse flow of electrons (relative to the physiological pathway) from the binuclear center to cytochrome *a* and Cu_A [101,102,180–185]. Oliveberg and Malmström [184] observed two processes at 445 and 605 nm, with apparent rates of $2 \cdot 10^5$ s^{-1} and $1.3 \cdot 10^4$ s^{-1}, following photolysis of the bovine heart mixed-valence CO complex. The two phases were attributed to electron transfer between cytochrome a_3 and cytochrome *a* and between cytochrome *a* and Cu_A, respectively. Verkhovsky et al. [101] reached similar conclusions by following the electron transfer at selected wavelengths in the Soret region. Electron transfer between the high- and low-spin hemes has also been observed on a microsecond time-scale upon photolysis of CO from wild-type cytochrome *bo* and cytochrome oo_3 from *E. coli* and mutant variants of cytochrome *bo* [194,195]. In both cytochrome *bo* and oo_3, the same electron transfer process was also apparently observed on faster and slower time-scales [194].

We have recently used an optical multichannel spectrophotometric analyzer to monitor the intramolecular elec-



Scheme 2. A proposed mechanism for the intramolecular electron transfer and conformational events in cytochrome oxidase following photodissociation of CO bound to the mixed-valence CO-bound enzyme. From Ref. [119].

tron transfer in bovine heart cytochrome oxidase following photodissociation of the mixed-valence CO complex [102,119,185]. This method allows collection of time-resolved spectra over a wide spectral range simultaneously on time-scales of nanoseconds to seconds. The spectra were analyzed by a global exponential fitting routine combined with a singular value decomposition method. The results from this analysis are in agreement with those of Oliveberg and Malmström [184] and Verkhovsky et al. [101], but indicate that in addition to the two electron transfer steps, a conformational change at cytochrome a_3, similar to that observed in the photolyzed fully reduced CO complex [12,15,102,119], precedes the electron transfer between the two hemes. This conformational change may be a prerequisite for the subsequent heme–heme electron transfer as suggested previously by Brzezinski and Malmström [182]. Our results also indicate a millisecond process, which could reflect intramolecular electron transfer between the two hemes [119] as suggested recently by Hallén et al. [40]. These results are summarized in Scheme 2 with the respective microscopic rate constants listed in Table 1 [102,119]. From Scheme 2 and the microscopic rate constants, the time dependence of the populations of the intermediates during the photodissociation was determined [102]. These profiles indicate that ~ 12% of cytochrome a_3 becomes oxidized upon photolysis of the mixed-valence CO complex. Based on the mechanism in Scheme 2, the absorption spectra of the intermediates involved in the electron transfer dynamics of the mixed-valence enzyme were determined [102,119]. These spectra are in good agreement with calculated spectra of the intermediates in Scheme 2 (obtained from appropriate linear combinations of ground state spectra of oxidized, fully reduced, mixed-valence CO and fully reduced CO complexes) except in the spectral region between 480–550 nm. A comparison of the experimental spectrum of intermediate 3 (a_3^{3+} Cu_B^+ a^{2+} Cu_A^{2+}) and the corresponding calculated spectrum (a_3^{3+} Cu_B^{2+} a^{2+} Cu_A^+) allowed extraction of the absorbance spectrum of Cu_A^{2+} in the 480–550 nm region [119]. These results are in agreement with low-temperature MCD studies which have shown that Cu_A has an absorbance in this region [120–123]. As mentioned earlier, the visible absorption spectra of the engineered Cu_A mutant of subunit II of cytochrome *o* from *E. coli* and the Cu_A domains of cytochrome oxidase from *Paracoccus* and *Bacillus subtilis* also show an absorbance between 480–550 nm [88–91].

As pointed out by Verkhovsky et al. [101], the data for the mixed-valence CO complex do not establish whether cytochrome a_3 or Cu_B is the initial electron donor to cytochrome *a*. An equally valid alternative to Scheme 2 would involve a conformational change at cytochrome a_3, subsequent electron transfer from Cu_B to cytochrome *a*, followed by much faster electron transfer from cytochrome a_3 to Cu_B in favor of the oxidized cytochrome a_3 [101,102]. In a previous study, we suggested that the conformational change at cytochrome a_3 was followed by electron transfer from Cu_B to cytochrome *a* [185]. However, in view of recent biochemical and spectroscopic studies on mutants of the bacterial oxidases, cytochrome *bo* from *E. coli* and cytochrome aa_3 from *Rhodobacter sphaeroides*, which have indicated that cytochrome a_3 is situated between Cu_B and cytochrome *a* [24,27], it would appear more likely that cytochrome a_3 is the electron donor to cytochrome *a* as indicated in Scheme 2. This is supported by CO flash photolysis studies of a partially reduced CO complex of a His333Leu mutant of cytochrome *bo* from *E. coli* lacking Cu_B, which showed electron transfer on a microsecond time-scale, presumably between the two hemes [195].

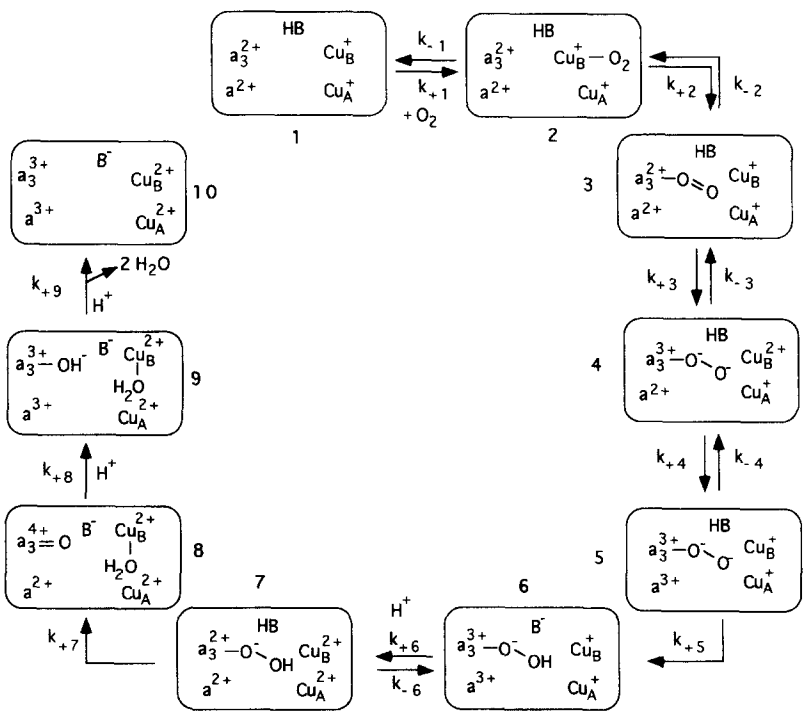
Assuming an electron path through the medium, Verkhovsky et al. [101] used the equations of Moser et al. [196,197] to calculate an edge-to-edge distance of 10–12 Å between the two hemes. Similarly, assuming a driving force of 15 mV between cytochrome *a* and Cu_A, a distance of 13–15 Å between those two redox centers was obtained [102]. These distances are in good agreement with previous spectroscopic measurements [97–100]. The short distance between the two hemes is consistent with recent site-directed mutagenesis studies of the bacterial oxidases, which indicated that cytochrome a_3 and cytochrome *a* are on opposite sides of helix X, separated by a single residue [24,27].

Photoactivatable compounds as electron donors. The intramolecular electron transfer in cytochrome oxidase has also been investigated with photoactivatable compounds as electron donors to the enzyme [191,192]. In experiments in which a 1-methylnicotinamide (MNA) radical generated by pulse radiolysis [191] or a photoactivatable tris(2,2′-bipyridyl)ruthenium (II) complex [192] were used as an electron source, Cu_A was found to be the primary electron acceptor. The apparent rate constant of $\sim 2 \cdot 10^4$ s^{-1} for the electron transfer between Cu_A and cytochrome *a* is similar to the value obtained following photodissociation of the mixed-valence CO complex.

The electron transfer from cytochrome *c* to cytochrome oxidase has been investigated by transient optical absorption spectroscopy using photogenerated semiquinone of 5-deazariboflavin [186,187] or a photosensitized uroporphyrin/NADH system [188,189] as the electron source. Most of these studies suggested a role for Cu_A in the primary input of electrons from cytochrome *c*, although the possibility of two pathways was not ruled out [186,187,189]. Nicholls and coworkers [198] pointed out that, although Cu_A is probably the physiological acceptor of electrons from cytochrome *c*, Cu_A may not be the electron acceptor for all reductants, such as TMPD and DMPT. Most recently, Pan et al. [190] investigated the electron transfer between cytochrome oxidase and several derivatives of cytochrome *c* labeled at specific lysines with tris(bipyridine)ruthenium(II). In these experiments, the cytochrome *c* is reduced in situ via excited state quenching of $Ru(II)^*$ by ferricytochrome *c*. These studies also support Cu_A as the primary electron acceptor from cytochrome *c*. The rate constants for electron transfer between cytochrome *c* and Cu_A varied from 10^3–10^5 s^{-1} for cytochrome *c* modified close to or remote from the heme crevice, respectively. The upper value is similar to that reported by Hill [16,32]. The accumulating evidence that Cu_A is the acceptor of electrons from cytochrome *c* does not seem surprising considering that Cu_A is located in subunit II [88–91], which also appears to be the binding site of cytochrome *c* [193,199]. Therefore, Cu_A is appropriately located to act as the acceptor of electrons from cytochrome *c* and donate them to cytochrome *a*.

6. The dioxygen reduction reaction

The reactions of partially or fully reduced unliganded cytochrome oxidase with dioxygen are so fast that studies by conventional stopped-flow are impractical. However, the combination of the flow-flash technique [28,29] with low-temperature optical [42,43] and EPR trapping measurements [45–47], room temperature TROA measurements [10,13,16,17,30,31,33,34,200,201] and TR^3 measurements [11,14,48–52], have allowed us to investigate the O_2 reactions in considerable detail. Experiments in which the O_2 reduction is partially reversed at a high electrochemical potential gradient have provided additional information regarding the possible nature of the transient oxygen intermediates [202,203]. More recently, the pH and D_2O dependence of proton uptake and electron transfers during the reaction between fully reduced cytochrome oxidase and oxygen were studied using the flow-flash



Scheme 3. A proposed mechanism for dioxygen activation and reduction by cytochrome oxidase (see text for details).

method [35,36]. A reaction mechanism of the dioxygen reduction at the cytochrome a_3/Cu_B site is summarized in Scheme 3 and will be discussed in detail below, together with the respective rate constants. It should be noted that in many previous experiments, the microscopic rate constants were represented by the apparent rate constants since the steps were considered unidirectional. However, recent experiments suggest that reversible steps occur at least in the early part of the reaction [16,17,33].

The first step in Scheme 3 ($1 \leftrightarrow 2$) showing O_2 binding to Cu_B^+ is supported by several observations. The recent finding that CO binds to Cu_B^+ on its route to cytochrome a_3 [12,15] suggests that O_2 may follow the same pathway. As early as 1967 Greenwood and Gibson [29] observed that the rapid phase of the flow-flash reaction of the fully reduced cytochrome oxidase with oxygen showed little wavelength dependence and saturated at high oxygen concentration, suggesting a rate-limiting step for O_2 binding to the ferrous cytochrome a_3. These observations were subsequently confirmed by others [30,31,33], most recently by elegant double-flash experiments of Blackmore et al. [34], which suggested that the intermediate might represent O_2 binding to Cu_B^+. Simulation of their data gave rate constants of $3.5 \cdot 10^8$ $M^{-1} s^{-1}$ and $5 \cdot 10^4$ s^{-1} for k_{+1} and k_{-1}, respectively. Oliveberg and Malmström [13] reported absorbance changes at 830 nm, with an apparent rate of $1 \cdot 10^8$ $M^{-1} s^{-1}$, which they attributed to O_2 binding to Cu_B^+. Recent analysis by Verkhovsky et al. [17] of the early steps in the reaction of oxygen with the fully reduced cytochrome oxidase also indicated that O_2 may bind to Cu_B^+ prior to binding to cytochrome a_3. However, the low occupancy of this intermediate precluded its observation in the optical spectra. The dissociation constant of the Cu_B-O_2 complex, $K_1 = k_{-1}/k_{+1}$, was estimated between 1.5 and 8 mM [17]. The lower value is similar to the value previously reported by Orii [33], but an order of magnitude higher than that reported by Blackmore et al. [34]. The upper limit of 8 mM is the same as the dissociation constant of the Cu_B^+-CO complex ($1/K_1$ in Table 1) [15,55,102].

The binding of O_2 to Cu_B is followed by the first-order transfer of O_2 to cytochrome a_3 (Scheme 3, $2 \leftrightarrow 3$). The presence of the cytochrome a_3^{2+}-O_2 complex has been reported by low-temperature triple trapping experiments [42,43], room temperature TROA [16,17,33,34,200] and TR^3 experiments [48,50,51,204]. Orii reported O_2 on and off rates of $9.9 \cdot 10^4$ s^{-1} and $5.4 \cdot 10^3$ s^{-1}, respectively [33]. The resulting dissociation constant, $K_2 = k_{-2}/k_{+2}$, is similar to the lower estimate of 0.04 reported by Verkhovsky et al. [17].

In the model of Babcock and Wikström [5] and Varotsis et al. [14], the formation of the cytochrome a_3-O_2 complex is followed by the production of an apparent peroxy compound (Scheme 3, $3 \leftrightarrow 4$) with electron redistribution at the binuclear center [5]. Intermediate 4, in which both cytochrome a and Cu_A are reduced, has not been observed in flow-flash-based EPR, TROA and TR^3 studies of the reaction of the fully reduced enzyme with dioxygen and whether it is a true intermediate under turnover conditions remains an open question [5]. In the model of Verkhovsky et al. [17], a peroxy or superoxy intermediate (P/S), equivalent to intermediate 4, was proposed to follow the formation of intermediate 3. The dissociation constant of this step, $K_3 = k_{-3}/k_3$, was between 3.8 and 4.8. This intermediate is purely hypothetical and its low occupancy would explain why it is not observed in the optical and resonance Raman experiments [17]. However, in the absence of electron input from the low-potential metals, cytochrome a and Cu_A, as occurs in the reaction of the mixed-valence enzyme with oxygen, a compound is formed that has a maximum at 607 nm [31,42]. A similar species is produced when hydrogen peroxide reacts with the resting or pulsed enzyme [205]. A species that is two equivalents more oxidized than the oxidized resting enzyme, with a maximum at 607 nm (peroxy or P form), is also readily observed during reversal of the O_2 reduction reaction [202,203]. It should be emphasized that the details of the structure of these compounds and that of intermediate 4 and intermediate 5 discussed below are not known, i.e., whether they are an actual peroxy species as indicated in Scheme 3 or a oxyferryl species with an oxidizing equivalent elsewhere in the molecule, either on the porphyrin or on a neighboring amino acid residue. Recent studies by Cheesman et al. [206] have provided evidence for an oxyferryl heme *o* when the fast form of cytochrome *bo* reacts with hydrogen peroxide. However, there was no evidence for a heme π-cation radical. The oxyferryl heme *o* derivative is spectroscopically identical to the product of reacting two-electron reduced cytochrome *bo* with oxygen, which for the bovine heart enzyme leads to the P form. This suggests that the P form may contain ferryl cytochrome a_3 [206].

The existence of the peroxy form is central to the question of which form of cytochrome oxidase – the mixed-valence or the fully reduced enzyme – is present under turnover conditions. This question is important because the efficiency of proton pumping is likely to depend on the nature of the oxygen reacting species [207,208]. While most of the steady-state measurements of the redox behavior of cytochrome *c*, cytochrome *a* and Cu_A in cytochrome oxidase under turnover conditions indicate that cytochrome *a* remains significantly more reduced than Cu_A [175,209,210], recent studies by Morgan and Wikström [211] suggest the opposite. Based in part on these results, Babcock and Wikström [5] postulated that the mixed-valence cytochrome oxidase is the form that reacts with O_2 under turnover conditions, as previously suggested by Clore et al. [43]. They argued that the formation of the peroxy compound and the rapid oxidation of cytochrome *a* is a short-circuit reaction unique to the fully reduced enzyme, and postulated that this premature reduction of the binuclear center corresponds to a slip in the proton pump-

ing cycle [5]. Nilsson and coworkers used similar arguments to explain why the H^+/e stoichiometry is significantly less than 1 when the proton pumping in the fully reduced cytochrome oxidase is measured by the flow-flash technique [35,212].

The results of Morgan and Wikström were recently challenged by Brunori and coworkers [207] who used multichannel transient optical absorption spectroscopy and singular value decomposition analysis to study the steady-state reduction levels of cytochrome *a* and cytochrome *c* in respiring rat heart myocytes. Under conditions in which the absorbance contribution of $TMPD^+$ was very small (the major source of possible error in previous experiments), the steady-state levels of cytochrome *c* and cytochrome *a* were found to be comparable at all times, suggesting that the mixed-valence enzyme is not the major oxygen binding species in oxidase unless electron donation to cytochrome *c* becomes rate limiting. An alternative mechanism, in which the three electron reduced cytochrome oxidase (a_3^{2+} Cu_B^+ a^{2+} Cu_A^{2+}) reacts with O_2 under turnover conditions, has also been proposed [208].

In most models the decay of the primary oxygen compound (or peroxy compound if it exists) is accompanied by the oxidation of cytochrome *a* (Scheme 3, 4 ↔ 5) [30,213]. The resulting peroxy complex (Scheme 3, intermediate 5) has not been observed in time-resolved resonance Raman measurements, but was included in the recent model of Verkhovsky et al. [17]. In this model, the forward rate of electron transfer from cytochrome *a* to cytochrome a_3 (k_{+4}) was assumed to be the same as that observed following photolysis of CO from the mixed-valence CO compound or $2.4 \cdot 10^5$ s^{-1} [101]. The much slower apparent rate of the inter-heme electron transfer in the flow-flash experiments ($\sim 3 \cdot 10^4$ s^{-1} at ~ 1 mM O_2) was attributed to the low occupancy of intermediate 4 discussed above [17]. In the model of Hill, steps 3 ↔ 4 and 4 → 5 are proposed to occur as an irreversible concerted two-electron step with a rate constant of $6 \cdot 10^4$ s^{-1} [16]. This type of mechanism also fitted the flow-flash data of the Cu_A-lacking aa_3-600 oxidase of *Bacillus subtilis* [214].

A resonance Raman stretching frequency at 358 cm^{-1} appearing on a $\sim 1 \cdot 10^4$ s^{-1} (k_{+5}) time-scale has been attributed to the subsequent formation of a three-electron reduced protonated peroxy compound (Scheme 3, 5 → 6) [14], which is consistent with the uptake of the first proton from solution on this time-scale [35,36]. In the model of Babcock and Wikström [5], this process is followed by electron transfer from Cu_B to the ferric peroxide to form a ferrous-cupric peroxide which has been reported at low temperature [46] (Scheme 3, 6 ↔ 7). Electron transfer from Cu_A to cytochrome *a* occurs on the same time-scale [10], consistent with results obtained upon photolysis of the mixed-valence CO complex [101,102,119,184]. Hill reported microscopic rate constants of $7 \cdot 10^3$ s^{-1} and $4 \cdot 10^3$ s^{-1} for k_6 and k_{-6}, respectively [16]. The oxidation of Cu_B may trigger this step by causing cytochrome *a* to go from a low-potential to a high-potential state due to anticooperative interactions between cytochrome *a* and Cu_B [13,215]. This step is followed by the cleavage of the O–O bond to form a ferryl intermediate (Scheme 3, 7 → 8). This step and subsequent steps are shown as unidirectional, where the apparent rate constants are equal to the microscopic rate constants.

The formation of the ferryl compound ($Fe^{IV} = O$) (Scheme 3, 7 → 8) has been proposed to occur with an apparent rate constant of $\sim 7 \cdot 10^3$ s^{-1} [14,30]. The step may be rate-limited by proton uptake from the medium [36]. This is supported by recent studies of cytochrome aa_3-600 quinol-oxidase from *Bacillus subtilis*, which indicate that the transfer of the third electron to the binuclear center is accompanied by a proton uptake which must occur before the O–O bond is broken [216]. A resonance mode at 786 cm^{-1} in the TR^3 spectra of the beef heart enzyme was assigned to the $Fe^{IV} = O$ intermediate [11,49,217]. The ferryl complex has been observed following one electron oxidation of the binuclear center in reversed O_2-reduction experiments [202,203]. The complex exhibits a peak at 580 nm in the optical difference spectrum (ferryl minus resting) and is identical to the oxyferryl species generated by Chan and coworkers at the three electron level of reduction [218]. Recent studies of the Cu_A-lacking cytochrome aa_3-600 quinol oxidase from *Bacillus subtilis* strongly support the involvement of discrete 'peroxy' (P) and ferryl intermediates in the catalytic cycle, providing further evidence that the dioxygen reduction cycle mechanism at the binuclear center is similar in the quinol and the cytochrome *c* oxidases [216].

Ogura et al. [52] recently showed that the 786 cm^{-1} resonance Raman band is a composite of two peaks with maxima at 785 cm^{-1} and 804 cm^{-1}, which they assigned to a hydroperoxy and a ferryl intermediate, respectively. However, recent mixed-isotope $^{16}O^{18}O$ TR^3 experiments [14] have indicated that the 785 cm^{-1} band cannot be due to a peroxy complex. Therefore, the two bands may reflect the ferryl complex and a corresponding hydrogen-bonded form.

Further reduction by one electron (presumably from cytochrome *a* via Cu_A) and uptake of a proton [36] leads to the decay of the ferryl compound and the formation of a new intermediate with a resonance Raman line at 450 cm^{-1}, assigned to a ferric hydroxide, Fe^{3+}-OH [11] (Scheme 3, 8 → 9). The rate constant for this step, k_{+8}, is ~ 800–1000 s^{-1} [10,16,36]. This phase shows a marked pH dependence and D_2O isotope effect and has been suggested to be rate-limited by proton uptake from the medium [36]. Further addition of an additional proton completes the dioxygen reduction cycle with the release of two water molecules.

As discussed above, each of several reactions – oxygen binding, electron and hydrogen transfer to oxygen, cleavage of the O–O bond, and release of two water molecules – must be carried out rapidly for an efficient O_2 reduction

to take place. Therefore, models of the active site of cytochrome oxidase must accommodate conversion of dioxygen to water along a low-energy pathway. An oxygen reduction and binding site with limited conformational flexibility and well secluded from the aqueous environment is consistent with FTIR measurements [161,163,164]. This suggests that protein residues immediately adjacent to the bound O_2 may serve as hydrogen donors. These could include the ligands to the binuclear reaction center or other residues close to the bound O_2 [36,37,164]. Flow-flash studies of pH and D_2O dependence of proton uptake and electron transfer during the reaction of fully reduced cytochrome oxidase with oxygen have indicated that proton transfer reactions are the rate limiting steps in the dioxygen reduction reaction [35,36]. These studies suggest that two protonatable groups are involved, one in close proximity to the bimetallic site and the other mediating proton transfer from the bulk medium into the dioxygen binding and reduction site [36]. Hallén et al. [40] have provided evidence for a protonatable group at the binuclear site, possibly in equilibrium with the solvent via a proton channel. Recent results by Mitchell and Rich [41] of the proton uptake by cytochrome *c* oxidase, suggested a principle of electroneutrality of redox and ligand state changes of the binuclear center, with charge compensations provided by protonation reactions. The effects of D_2O and pH on cytochrome oxidase activity also support the involvement of protons in the rate-limiting step(s) in the reduction of O_2 to water [164]. Possible proton donors include conserved serine, threonine and lysine residues in helix VIII [219], glutamic and tyrosine residues in helix VI, and certain aspartate residues in the vicinity of the binuclear center [5,6,36]. These residues could also have a role in the proton pumping function. Recent site-directed mutagenesis studies of cytochrome *bo* from *E. coli* have indicated that an aspartate residue in subunit I may play an important role in the function of the proton pump [220]. Bound water has also been suggested as a possible proton donor [221]. Based on current genetic and biophysical data, Brown et al. [6] have recently proposed a structural model for subunit I that brings several protonatable residues into the vicinity of the binuclear center, thereby providing proton channels to the aqueous phases.

7. Proton pumping

The mechanism of the redox-linked proton pump has remained elusive since the discovery of the proton translocation function of cytochrome oxidase by Wikström in 1977 [65]. These studies, which were carried out on liver mitochondria, and subsequent studies on reconstituted enzyme in phospholipid vesicles [222–224], showed that for each O_2 molecule reduced, cytochrome oxidase translocates four protons from the inside to the outside using the free energy of the electron transfer reactions as the driving force. Proton translocation may be direct, in which the ligands to one or more of the redox centers provide the protons, or indirect, in which the oxidation and reduction reactions cause conformational changes that are transmitted to proton donating groups remote from the redox centers. Both of these mechanisms require the existence of distinct states of the proton translocating element, protonated and deprotonated, as well as distinct states of the redox center(s), reduced and oxidized. The principles and basic requirements for a redox-linked proton pump have been reviewed in detail elsewhere and will not be discussed here [1,223,225]. In addition, both the direct and indirect coupling mechanisms must include an H^+/e^- pump stoichiometry of zero for the first two electrons transferred to the binuclear center, and a stoichiometry of 2 for the last two electron transfer steps, peroxy to ferryl and ferryl to the fully oxidized state [226].

Transient kinetics of proton pumping. Nilsson and coworkers [35,212] recently measured the rate of proton release during flash-induced oxidation of cytochrome oxidase reconstituted into phospholipid vesicles. The pH changes in the extravesicular medium were detected by phenol red. Proton release was found to occur synchronously with the final step in the oxidation reaction, in agreement with earlier results [226]. The rate constant of 900 s^{-1} observed for this step must be taken as a lower limit for the release of the protons from the enzyme, since the rate of proton release at the protein surface would be expected to be faster than the rate measured by uptake of protons by the indicator dye in the medium [227]. The total number of protons released during the reoxidation of the enzyme was in the range of 1 to 2 (instead of the expected value of four), and no proton release was observed in the other proton pumping step, the conversion of the peroxide to the ferryl ion intermediate [212]. The discrepancy between the measured and the expected value was attributed to a slipping reaction in the fully reduced enzyme, as proposed by other groups [5,7,35,188]. Analogous studies with the mixed-valence enzyme showed no translocated protons [35].

Postulated models. Earlier models of the redox-linked proton translocation favored either cytochrome *a* (Babcock's model) [228] or Cu_A (Chan's model) [2,229–231] as the site of the redox-linked proton pump. Proposals favoring the binuclear center as the site of energy transduction were also put forward [59,232]. The models of Mitchell and coworkers are based on a direct coupling mechanism [59,232,233] (see discussion on the definition of the terms redox loop and proton pump in Refs. [59,234]). In the latest model [59], the Cu_B center is proposed to act as the hydrogen translocator with changes in ligand geometry around the redox center providing the necessary switch between an input and an output state.

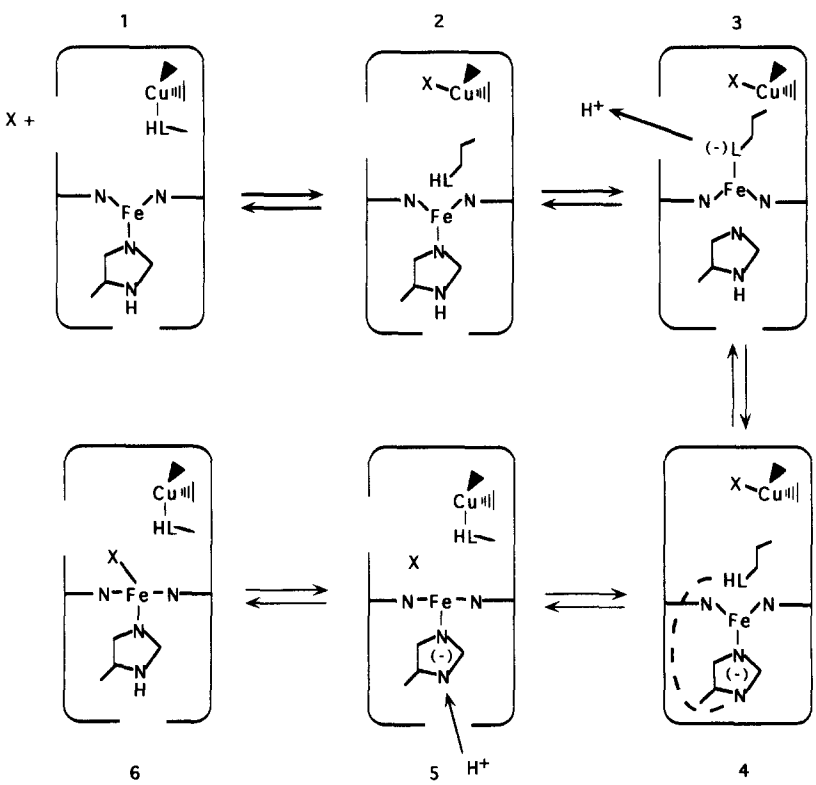
In 1989, Wikström showed that only the last two electron-transfer steps – peroxy to ferryl and ferryl to the fully oxidized enzyme – translocate protons, two protons in

each step [226]. As a result, newer models favor the binuclear center as the site of the redox-linked proton translocation. Arguments against either the formyl group of cytochrome *a* (Babcock's model) or Cu_A (Chan's model) being the site of the redox-linked proton pump are based on the observation that the quinol oxidase, cytochrome *bo* from *E. coli*, which contains neither Cu_A nor the formyl group of heme A, also pumps protons [235]. The structural homology between the two types of oxidase suggests that proton translocation occurs by the same or similar mechanism in the quinol and cytochrome *c* oxidases (however, see Musser et al. [236,237]).

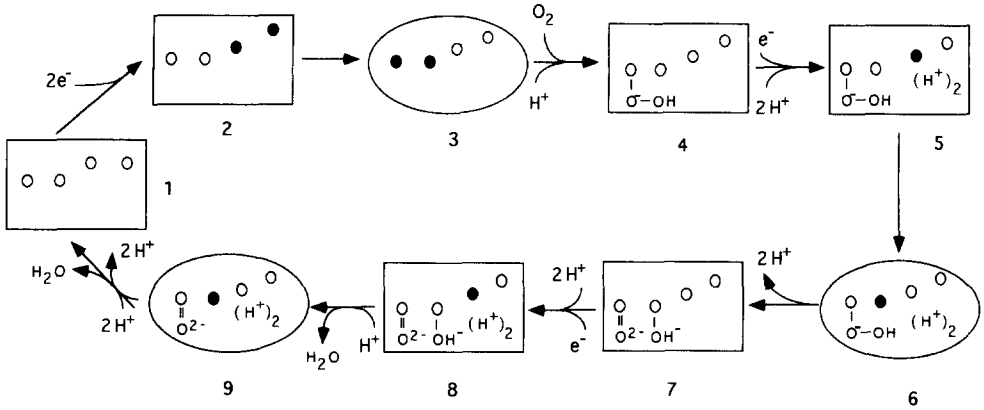
Taking into account the biphasic steady-state kinetics of cytochrome oxidase, Malmström and coworkers proposed an indirect mechanism for proton translocation involving two conformations of the enzyme, E_1 and E_2 [3,174]. They postulated that the enzyme switches between the non-proton pumping conformation, E_1, and the proton pumping conformation, E_2, only when cytochrome *a* and Cu_A are reduced, and suggested that this conformational switching is the rate-limiting step in the electron transfer to the binuclear center. The first two internal electron transfers were proposed to be uncoupled from the proton pumping with the third and the fourth electron transferred through Cu_A. A branched pathway has also been proposed by Malatesta et al. [60]. In this model, two conformations, P (pumping) and S (slipping), are characterized by different internal electron transfer pathways, through Cu_A and cytochrome *a*, respectively. The conformations are in equilibrium which is controlled by the membrane potential.

Woodruff and coworkers recently outlined a mechanism in which labile coordination chemistry at the binuclear center of cytochrome oxidase ('ligand shuttling') may play a role in the redox-linked proton translocation [8,12,55] (Scheme 4). A redox-dependent ligand switching was originally proposed by Chan and coworkers for Cu_A [229–231]. In Scheme 4 the shuttling ligand L has a labile proton. Upon binding of a ligand X, such as CO or O_2, to Cu_B ($1 \rightarrow 2$), ligand L is transferred from Cu_B to cytochrome a_3 with a loss of a proton ($2 \rightarrow 3$). Subsequently, a protein conformational change occurs which prevents proton exchange with the surroundings. The proximal histidine then reprotonates ligand L, which now has dissociated from the heme ($3 \rightarrow 4$), through a hydrogen-bonded pathway. In the next step, communication of the proton with the surroundings is reestablished and the proximal histidine is reprotonated using a different pathway ($4 \rightarrow 5$). A variation of this model was proposed by Rousseau and coworkers [61] in which the proximal ligand on cytochrome a_3 exchanges between a histidine and a tyrosine during concurrent protonation and deprotonation reactions.

Babcock and Wikström [5] recently presented a mechanism in which the reduction/oxidation cycles of Cu_B during catalysis are linked in a controlled manner to the input/output state of a proton-binding site(s), whose nature is unknown. They suggest that electron transfer from



Scheme 4. A schematic representation of proton translocation based on the ligand shuttle reactions proposed by Woodruff et al. [12]. Adapted from Ref. [8].



Scheme 5. A reaction cycle for cytochrome oxidase as a redox-linked proton pump, in which Cu_B is assumed to control the input-output transitions. The circles in the boxes represent the four redox centers, from left to right, cytochrome a_3, Cu_B, cytochrome a, and Cu_A. An open circle represents an oxidized center and a filled one a reduced center. The square boxes represent the input states and the ellipsoids the output states. Adapted from Ref. [7].

Cu_B to the heme-oxygen system, and the subsequent chemistry of the latter, provide the major driving force for proton pumping. The high potential of the peroxy and the ferryl intermediates makes them efficient acceptors of electrons from Cu_B [203]. Malmström and coworkers have also proposed a mechanism in which the reduction state of Cu_B is assumed to control the transitions between the input and output states [7,13]. This model is depicted in Scheme 5. In both models, the observed redox interactions between the metal sites [1,238] are an integral part of the pump.

8. Conclusions and future directions

Our understanding of the complex chemistry involved in the reduction of dioxygen to water by cytochrome oxidase has expanded greatly in the last decade, mostly due to fast spectroscopic devices that have allowed us to probe the details of this reaction. There now seems to be ample spectroscopic evidence to indicate that Cu_A is the primary acceptor of electrons from cytochrome *c* under turnover conditions and that cytochrome *a* is the donor of electrons to cytochrome a_3 which is in fast equilibrium with Cu_B. This pathway of electron transfer is supported by site-directed mutagenesis and biochemical and spectroscopic studies of the bacterial heme-copper oxidases [24,27].

The mode of oxygen binding in some of the transient intermediates formed when the fully reduced cytochrome oxidase reacts with dioxygen has been demonstrated by TR^3. However, the existence and nature of other intermediates, including the optical absorbance spectra and microscopic rate constants, still remain to be established. One question centers on whether O_2 binds to Cu_B^+ prior to binding to cytochrome a_3. As discussed above, there is significant indirect evidence from flow-flash studies, and more recently, double flow-flash measurements, that this is the case, but the population of this intermediate may be very low even at 1 mM oxygen concentration [17]. TROA measurements carried out at higher oxygen pressure and on submicrosecond time-scales may help establish the existence of this intermediate; however, the concentration of oxygen must not be so high that the dissociation of CO from Cu_B rate-limits the O_2 binding to Cu_B. Additionally, the O–O stretch of the Cu-O_2 intermediate might be observed by flow-flash or double flash TRIR measurements.

TROA experiments at elevated O_2 concentrations may also help establish whether a 'peroxy' intermediate(s) is formed following the binding of dioxygen to cytochrome a_3. Scheme 3 includes two such unprotonated species, intermediates 4 and 5. The former has not been observed in flow-flash TROA and TR^3 experiments, presumably due to its low occupancy. Intermediate 5 has not been observed in TR^3 but optical contribution of this intermediate has been reported [17]. It is possible that the excitation wavelength used in the TR^3 experiments is inappropriate for the observation of this intermediate. The nature of these intermediates i.e., whether they represent an actual peroxy intermediate as represented by the molecular structures for intermediates 4 and 5 in Scheme 3, rather than an oxyferryl compound with a second oxidized equivalent elsewhere in the molecule, may be resolved by TR^3 using mixed oxygen isotopes [14]. MCD and flow-flash TRMCD experiments, in combination with EPR measurements, may also help resolve this issue.

Another question concerns the oxidation state of the enzyme that reacts with dioxygen during aerobic turnover in mitochondria or in plasma membranes of bacteria. It is unclear whether flow-flash studies of the mixed-valence CO compound or the fully reduced CO complex better represent the physiological reduction of dioxygen. However, this may be moot, because even if the partially reduced enzyme represents a better model, the flow-flash experiment may not reflect the reaction under physiological conditions [239]. This is due to different electron redistribution in the CO-photolyzed mixed-valence enzyme

compared to the partially reduced enzyme in the absence of CO. In the two electron reduced enzyme in absence of CO, the electrons are distributed among all the metal centers, whereas in the CO-photolyzed initial product the electrons reside on cytochrome a_3 and Cu_B and may not have time to redistribute prior to the initial steps in the dioxygen reduction reaction. The presence of CO itself could also lead to different results than would be observed under physiological conditions because the dissociation of CO from Cu_B may rate-limit the O_2 binding to Cu_B. At typical O_2 concentrations (625 μM to 1 mM), the CO dissociation from Cu_B in the bovine heart cytochrome oxidase and the *Paracoccus* enzyme is an order of magnitude faster than the O_2 binding to Cu_B (assuming the O_2 binding has a second-order rate constant of $\sim 1 \cdot 10^8$ $M^{-1}s^{-1}$) and therefore does not pose a problem. However, this might not be the case for other heme-copper oxidases such as cytochrome *bo* of *E. coli* where the dissociation rate of CO from Cu_B is 500 s^{-1} [55]. Therefore, alternatives to the flow-flash method are needed to study the rapid reactions of cytochrome oxidase with dioxygen. One such alternative involves studying the direct reaction of the reduced unliganded enzyme with oxygen using superfast mixing methods. Another novel approach being pursued in our laboratory uses O_2 which is produced in situ by photodissociating synthetic caged dioxygen carriers (MacArthur et al., unpublished results).

The mechanism by which the redox reaction is coupled to proton translocation remains unclear. Several models have been proposed, including ligand exchange at the binuclear center, but these models are still speculative and more direct experimental evidence is needed. For instance, the observation of a cytochrome a_3–L stretching vibration using TR[3] would support the ligand shuttle concept. Alternatively, one could look for vibrations of the shuttling ligand when it is bound to cytochrome a_3 or Cu_B using FTIR at lowtemperature or TRIR at room temperature. A combination of these techniques with site-directed mutagenesis of the bacterial heme-copper oxidases and selective isotope labeling of the potential candidates for the shuttling ligand, could allow assignment of specific bands to a particular amino acid residue. Such studies have been very useful in determining the role of individual amino acids in the protonation reactions of bacteriorhodopsin [240]. They should also be able to give important insights into which amino acids residues are involved in protonation/deprotonation reactions during the dioxygen reduction and proton pumping cycles in the heme-copper oxidases. These studies assume a priori that the proton translocation mechanisms of the quinol and cytochrome *c* oxidases are the same. Based on the extensive similarities in structure and ligand binding and electron transfer properties among the two groups of oxidases, this assumption appears valid.

To understand how electron transfer is coupled to proton pumping, simultaneous time-resolved measurements of the dioxygen reduction reaction and the proton pumping are needed. In the two studies to date on this issue, the flow-flash method in combination with a pH indicator dye was used to monitor proton translocation during oxidation of the reduced enzyme reconstituted into phospholipid vesicles [35,212]. However, as mentioned above, a pH indicator dye in the aqueous bulk phase monitors proton transfer from the surface into the medium but not the appearance of protons at the ejection site of the protein. Therefore, alternative techniques that probe the kinetics of proton transfer directly at the protein/water interface, are needed. Surface-bound indicators may help establish the kinetics of proton pumping in cytochrome oxidase and allow correlation of the redox cycle to the pump cycle, as in the case of the photocycle and proton pumping cycle in bacteriorhodopsin [227].

Acknowledgements

The author's work is supported by the National Institutes of Health Grant, GM45888.

References

[1] Wikström, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase-A Synthesis, Academic Press, New York.
[2] Chan, S.I. and Li, P.M. (1990) Biochemistry 29, 1–12.
[3] Malmström, B.G. (1990) Chem. Rev. 90, 1247–1260.
[4] Wilson, M.T. and Bickar, D. (1991) J. Bioenerg. Biomembr. 23, 755–771.
[5] Babcock, G.T. and Wikström, M. (1992) Nature 356, 301–309.
[6] Brown, S., Moody, J., Mitchell, R. and Rich, P.R. (1993) FEBS Lett. 316, 216–223.
[7] Malmström, B.G. (1993) Acc. Chem. Res. 26, 332–338.
[8] Woodruff, W.H., Dyer, R.B. and Einarsdóttir, Ó. (1993) in Biological Spectroscopy, Part B, (Clark, R.J.H. and Hester, R.E., eds.), pp. 189–233, John Wiley, Chichester.
[9] Dyer, R.B., Einarsdóttir, Ó., Killough, P.M., López-Garriga, J.J. and Woodruff, W.H. (1989) J. Am. Chem. Soc. 111, 7657–7659.
[10] Oliveberg, M., Brzezinski, P. and Malmström, B.G. (1989) Biochim. Biophys. Acta 977, 322–328.
[11] Han, S., Ching, Y.-C. and Rousseau, D.L. (1990) Nature 348, 89–90.
[12] Woodruff, W.H., Einarsdóttir, Ó., Dyer, R.B., Bagley, K.A., Palmer, G., Atherton, S.J., Goldbeck, R.A., Dawes, T.D. and Kliger, D.S. (1991) Proc. Natl. Acad. Sci. USA 88, 2588–2592.
[13] Oliveberg, M. and Malmström, B.G. (1992) Biochemistry 31, 3560–3563.
[14] Varotsis, C., Zhang, Y., Appleman, E.H. and Babcock, G.T. (1993) Proc. Natl. Acad. Sci. USA 90, 237–241.
[15] Einarsdóttir, Ó., Dyer, R.B., Lemon, D.D., Killough, P.M., Hubig, S.M., Atherton, S.J., López-Garriga, J.J., Palmer, G. and Woodruff, W.H. (1993) Biochemistry 32, 12013–12024.
[16] Hill, B.C. (1994) J. Biol. Chem. 269, 2419–2425.
[17] Verkhovsky, M.I., Morgan, J.E. and Wikström, M. (1994) Biochemistry 33, 3079–3086.
[18] Lemieux, L.J., Calhoun, M.W., Thomas, J.W., Ingledew, W.J. and Gennis, R.B. (1992) J. Biol. Chem. 267, 2105–2113.
[19] Minagawa, J., Mogi, T., Gennis, R.B. and Anraku, Y. (1992) J. Biol. Chem. 267, 2096–2104.

[20] Shapleigh, J.P., Hosler, J.P., Tecklenburg, M.M.J., Kim, Y., Babcock, G.T., Gennis, R.B. and Ferguson-Miller, S. (1992) Proc. Natl. Acad. Sci. USA 89, 4786–4790.
[21] Calhoun, M.W., Thomas, J.W., Hill, J.J., Hosler, J.P., Shapleigh, J.P., Tecklenburg, M.M.J., Ferguson-Miller, S., Babcock, G.T., Alben, J.O. and Gennis, R.B. (1993) Biochemistry 32, 10905–10911.
[22] Calhoun, M.W., Lemieux, L.J., Thomas, J.W., Hill, J.J., Goswitz, V.C., Alben, J.O. and Gennis, R.B. (1993) Biochemistry 32, 13718–13724.
[23] Calhoun, M.W., Hill, J.J., Lemieux, L.J., Ingledew, W.J., Alben, J.O. and Gennis, R.B. (1993) Biochemistry 32, 11524–11529.
[24] Hosler, J.P., Ferguson-Miller, S., Calhoun, M.W., Thomas, J.W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M.M.J., Babcock, G.T. and Gennis, R.B. (1993) J. Bioenerg. Biomembr. 25, 121–136.
[25] Lemon, D.D., Calhoun, M.W., Gennis, R.B. and Woodruff, W.H. (1993) Biochemistry 32, 11953–11956.
[26] Uno, T., Mogi, T., Tsubaki, M., Nishimura, Y. and Anraku, Y. (1994) J. Biol. Chem. 269, 11912–11920.
[27] Hosler, J.P., Kim, Y.K., Shapleigh, J., Gennis, R.B., Alben, J.O., Ferguson-Miller, S. and Babcock, G.T. (1994) J. Am. Chem. Soc. 116, 5515–5516.
[28] Gibson, Q.H. and Greenwood, G. (1963) Biochem. J. 86, 541–554.
[29] Greenwood, G. and Gibson, Q.H. (1967) J. Biol. Chem. 242, 1782–1787.
[30] Hill, B.C. and Greenwood, C. (1984) Biochem. J. 218, 913–921.
[31] Hill, B.C., Greenwood, C. and Nicholls, P. (1986) Biochim. Biophys. Acta 853, 91–113.
[32] Hill, B.C. (1991) J. Biol. Chem. 266, 2219–2226.
[33] Orii, Y. (1988) Ann. NY Acad. Sci. 550, 105–117.
[34] Blackmore, R.S., Greenwood, C. and Gibson, Q.H. (1991) J. Biol. Chem. 266, 19245–19249.
[35] Oliveberg, M., Hallén, S. and Nilsson, T. (1991) Biochemistry 30, 436–440.
[36] Hallén, S. and Nilsson, T. (1992) Biochemistry 31, 11853–11859.
[37] Mitchell, R., Mitchell, P. and Rich, P.R. (1992) Biochim. Biophys. Acta 1101, 188–191.
[38] Hallén, S., Svensson, M. and Nilsson, T. (1993) FEBS Lett. 325, 299–302.
[39] Hansen, A.P., Britt, R.D., Klein, M.P., Bender, C.J. and Babcock, G.T. (1993) Biochemistry 32, 13718–13724.
[40] Hallén, S., Brzezinski, P. and Malmström, B.G. (1994) Biochemistry 33, 1467–1472.
[41] Mitchell, R. and Rich, P.R. (1994) Biochim. Biophys. Acta 1186, 19–26.
[42] Chance, B., Saronio, C. and Leigh, J.S., Jr. (1975) J. Biol. Chem. 250, 9226–9237.
[43] Clore, M.G., Andréasson, L.-E., Karlsson, B.G., Aasa, R. and Malmström, B. (1980) Biochem. J. 185, 139–154.
[44] Karlsson, B., Aasa, R., Vänngård, T. and Malmström, B.G. (1981) FEBS Lett. 131, 186–188.
[45] Hansson, Ö., Karlsson, B., Aasa, R., Vänngård, T. and Malmström, B.G. (1982) EMBO J. 1, 1295–1297.
[46] Blair, D.F., Witt, S.N. and Chan, S.I. (1985) J. Am. Chem. Soc. 107, 7389–7399.
[47] Witt, S.N. and Chan, S.I. (1987) J. Biol. Chem. 262, 1446–1448.
[48] Varotsis, C., Woodruff, W.H. and Babcock, G.T. (1989) J. Am. Chem. Soc. 111, 6439–6440.
[49] Varotsis, C. and Babcock, G.T. (1990) Biochemistry 29, 7357–7362.
[50] Han, S., Ching, Y.-C. and Rousseau, D.L. (1990) Proc. Natl. Acad. Sci. USA 87, 2491–2495.
[51] Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S. and Kitagawa, T. (1990) J. Am. Chem. Soc. 112, 5630–5631.
[52] Ogura, T., Takahashi, S., Hirota, S., Shinzawa-Itoh, K., Yoshikawa, S., Appleman, E.H. and Kitagawa, T. (1993) J. Am. Chem. Soc. 115, 8527–8536.
[53] Dyer, R.B., Petersen, K.A., Stoutland, P.O. and Woodruff, W.H. (1991) J. Am. Chem. Soc. 113, 6276–6277.
[54] Stoutland, P.O., Lambry, J.-C., Martin, J.-L. and Woodruff, W.H. (1991) J. Phys. Chem. 1991, 6406–6408.
[55] Woodruff, W.H. (1993) J. Bioenerg. Biomembr. 25, 177–188.
[56] Dyer, R.B., Peterson, K.A., Stoutland, P.O. and Woodruff, W.H. (1994) Biochemistry 33, 500–507.
[57] Goldbeck, R.A., Dawes, T.D., Einarsdóttir, Ó., Woodruff, W.H. and Kliger, D.S. (1991) Biophys. J. 60, 125–134.
[58] Goldbeck, R.A., Einarsdóttir, Ó., Dawes, T.D., O'Connor, D.B., Surerus, K.K., Fee, J.A. and Kliger, D.S. (1992) Biochemistry 31, 9376–9387.
[59] Mitchell, P. (1988) Ann. NY Acad. Sci. 550, 185–198.
[60] Malatesta, F., Antonini, G., Sarti, P., Vallone, B. and Brunori, M. (1988) Ann. N.Y. Acad. Sci. 550, 269–276.
[61] Rousseau, D.L., Ching, Y.-C. and Wang, J. (1993) J. Bioenerg. Biomembr. 25, 165–176.
[62] Saraste, M. (1990) Q. Rev. Biohys. 23, 331–366.
[63] Slater, E.C., Van Gelder, B.F. and Minnaert, K. (1965) in Oxidases and Related Redox Systems, Vol. II, pp. 667–706, John Wiley & Sons, New York.
[64] Wikström, M. (1988) FEBS Lett. 231, 247–252.
[65] Wikström, M.K.F. (1977) Nature 266, 271–273.
[66] Mitchell, P. (1966) Glynn Research, Bodmin, England,
[67] Kadenbach, B., Jarausch, J., Hartmann, R. and Merle, P. (1983) Anal. Biochem. 129, 517–521.
[68] Brunori, M., Antonini, G., Malatesta, F., Sarti, P. and Wilson, M.T. (1988) Adv. Inorg. Biochem. 7, 93–154.
[69] Capaldi, R.A. (1990) Annu. Rev. Biochem. 59, 569–596.
[70] Cooper, C.E., Nicholls, P. and Freedman, J.A. (1991) Biochem. Cell. Biol. 69, 586–607.
[71] Caughey, W.E., Smythe, G.S., O'Keeffe, D.H., Maskasky, J.E. and Smith, M.L. (1975) J. Biol. Chem. 250, 7602–7622.
[72] Einarsdóttir, Ó. and Caughey, W.S. (1984) Biochem. Biophys. Res. Commun. 124, 836–842.
[73] Einarsdóttir, Ó. and Caughey, W.S. (1985) Biochem. Biophys. Res. Commun. 129, 840–847.
[74] Naqui, A., Powers, L., Lundeen, M., Constantinescu, A. and Chance, B. (1988) J. Biol. Chem. 263, 12342–12345.
[75] Einarsdóttir, Ó. (1986) Ph.D. Thesis, Colorado State University.
[76] Bombelka, E., Richter, F.-W., Stroh, A. and Kadenbach, B. (1986) Biochem. Biophys. Res. Commun. 140, 1007–1014.
[77] Steffens, G.C.M., Biewald, R. and Buse, G. (1987) Eur. J. Biochem. 164, 295–300.
[78] Öblad, M., Selin, E., Malmström, B., Strid, L., Aasa, R. and Malmström, B.G. (1989) Biochim. Biophys. Acta 975, 267–270.
[79] Malmström, B.G. and Aasa, R. (1993) FEBS Lett. 325, 49–52.
[80] Steffens, G.C.M., Soulimane, T., Wolff, G. and Buse, G. (1993) Eur. J. Biochem. 213, 1149–1157.
[81] Kroneck, P.M.H., Anotholine, W.A., Riester, J. and Zumft, W.G. (1989) FEBS Lett. 242, 70–74.
[82] Scott, R.A., Zumft, W.G., Coyle, C.L. and Dooley, D.M. (1989) Proc. Natl. Acad. Sci. USA 86, 4082–4086.
[83] Kroneck, P.M.H., Antholine, W.E., Kastrau, D.H.W., Buse, G., Steffens, G.C.M. and Zumft, W.G. (1990) FEBS Lett. 268, 274–276.
[84] Farrar, J.A., Thomson, A.J., Cheesman, M.R., Dooley, D.M. and Zumft, W.G. (1991) FEBS Lett. 294, 11–15.
[85] Antholine, W.E., Kastrau, D.H.W., Steffens, G.C.M., Buse, G., Zumft, W.G. and Kroneck, P.M.H. (1992) Eur. J. Biochem. 209, 875–881.
[86] Zumft, W.G., Dreusch, A., Löchelt, S., Cuypers, H., Friedrich, B. and Schneider, B. (1992) Eur. J. Biochem. 208, 31–40.
[87] Saraste, M. and Castresana, J. (1994) FEBS Lett. 341, 1–4.

[88] van der Oost, J., Lappalainen, P., Musacchio, A., Warne, A., Lemieux, L., Rumbley, J., Gennis, R.B., Aasa, R., Pasher, T., Malmström, B.G. and Saraste, M. (1992) EMBO J. 11, 3209–3217.
[89] Kelly, M., Lappalainen, P., Talbo, G., Haltia, T., van der Oost, J. and Saraste, M. (1993) J. Biol. Chem. 268, 16781–16787.
[90] Lappalainen, P., Aasa, R., Malmström, B.G. and Saraste, M. (1993) J. Biol. Chem. 268, 26416–26421.
[91] Von Wachenfeldt, C., De Vries, S. and Van der Oost, J. (1994) FEBS Lett. 340, 109–113.
[92] Yoshikawa, S., Tera, T., Takahashi, Y., Tsukihara, T. and Caughey, W.S. (1988) Proc. Natl. Acad. Sci. USA 85, 1354–1358.
[93] Yoshikawa, S., Shinzawa, K., Tsukihara, T., Abe, T. and Caughey, W.S. (1991) J. Crystal Growh 110, 247–251.
[94] Fuller, S.D., Capaldi, R.A. and Henderson, R. (1979) J. Mol. Biol. 134, 305–311.
[95] Capaldi, R.A., Malatesta, F. and Darley-Usmar, V.M. (1983) Biochim. Biophys. Acta 726, 135–148.
[96] Valpuesta, J.M., Henderson, R. and Frey, T.G. (1990) J. Mol. Biol. 214, 237–251.
[97] Ohnishi, T., LoBrutto, R., Salerno, J.C., Bruckner, R.C. and Frey, T.G. (1982) J. Biol. Chem. 257, 14821–14825.
[98] Scholes, C.P., Janakiraman, R., Taylor, H. and King, T.K. (1984) Biophys. J. 45, 1027–1030.
[99] Goodman, G. and Leigh, J.S.J. (1985) Biochemistry 24, 2310–2317.
[100] Brudvig, G.W., Blair, D.F. and Chan, S.I. (1984) J. Biol. Chem. 259, 11001–11009.
[101] Verkhovsky, M.I., Morgan, J.E. and Wikström, M. (1992) Biochemistry 31, 11860–11863.
[102] Georgiadis, K.E., Jhon, N.-I. and Einarsdóttir, Ó. (1994) Biochemistry, 33, 9245–9256.
[103] Fee, J.A., Kuila, D., Mather, M.W. and Yoshida, T. (1986) Biochim. Biophys. Acta 853, 153–185.
[104] Ludwig, B. (1987) FEMS Microbiol. Rev. 46, 41–56.
[105] Chepuri, V., Lemieux, L., Au, D.C.-T. and Gennis, R.B. (1990) J. Biol. Chem. 265, 11185–11192.
[106] Castresana, J., Lübben, M., Saraste, M. and Higgins, D.G. (1994) EMBO J. 13, 2516–2525.
[107] Trumpower, B.L. and Gennis, R.B. (1994) Annu. Rev. Biochem. 63, 675–716.
[108] Zimmermann, B.H., Nitche, C.I., Fee, J.A., Rusnak, F. and Münck, E. (1988) Proc. Natl. Acad. Sci. USA 85, 5779–5783.
[109] Puustinen, A. and Wikström, M. (1991) Proc. Natl. Acad. Sci. USA 88, 6122–6126.
[110] Wu, W., Chang, C.K., Varotsis, C., Babcock, G.T., Puustinen, A. and Wikström, M. (1992) J. Am. Chem. Soc. 114, 1182–1187.
[111] Lübben, M., Kolmer, B. and Saraste, M. (1992) EMBO J. 11, 805–812.
[112] Gray, K.A., Grooms, M., Myllykallio, H., Moomaw, C., Slaughter, C. and Daldal, F. (1994) Biochemistry 33, 3120–3127.
[113] García-Horsman, J.A., Berry, E., Shapleigh, J.P., Alben, J.O. and Gennis, R.B. (1994) Biochemistry 33, 3113–3119.
[114] Raitio, M. and Wikström, M. (1994) Biochim. Biophys. Acta 1186, 100–106.
[115] Palmer, G. (1987) Pure Appl. Chem. 59, 749–758.
[116] Vanneste, W.H. (1966) Biochemistry 5, 838–848.
[117] Sherman, D., Kotake, S., Ishibe, N. and Copeland, R.A. (1991) Proc. Natl. Acad. Sci. USA 88, 4265–4269.
[118] Blair, D.F., Bocian, D.F., Babcock, G.T. and Chan, S.I. (1982) Biochemistry 21, 6928–6935.
[119] Einarsdóttir, Ó., Georgiadis, K.G. and Sucheta, A. (1995) Biochemistry 34, 496–508.
[120] Greenwood, C., Hill, B.C., Barber, D., Eglington, D.G. and Thomson, A.J. (1983) Biochem. J. 215, 303–316.
[121] Thomson, A.J., Greenwood, C., Peterson, J. and Barrett, C.P. (1986) J. Inorg. Biochem. 28, 195–205.
[122] Greenwood, C., Thomson, A.J., Barrett, C.P., Peterson, J., George, G.N., Fee, J.A. and Reichardt, J. (1988) Ann. NY Acad. Sci. 550, 47–52.
[123] Farrar, J.A., Thomson, A.J., Cheesman, M.R., Dooley, D.M. and Zumft, W.G. (1991) FEBS Lett. 294, 11–15.
[124] Beinert, H., Hansen, R.E. and Hartzell, C.R. (1976) Biochim. Biophys. Acta 423, 339–355.
[125] Carter, K. and Palmer, G. (1982) J. Biol. Chem. 257, 13507–13514.
[126] Mitchell, R., Mitchell, P. and Rich, P.R. (1991) FEBS Lett. 280, 321–324.
[127] Wharton, D.C. and Tzagoloff, A. (1964) J. Biol. Chem. 239, 2036–2041.
[128] Beinert, H., Shaw, R.W., Hansen, R.E. and Hartzell, C.R. (1980) Biochim. Biophys. Acta 591, 458–470.
[129] Einarsdóttir, Ó., Georgiadis, K.E. and Dawes, T.D. (1992) Biochem. Biophys. Res. Commun. 184, 1035–1041.
[130] Eglinton, D.G., Johnson, M.K., Thomson, A.J., Gooding, P.E. and Greenwood, C. (1980) Biochem. J. 191, 319–331.
[131] Ingledew, W.J., Bacon, M. and Rich, P.R. (1992) FEBS Lett. 305, 167–170.
[132] Eaton, W.A. and Hofrichter, J. (1981) Methods Enzymol. 76, 175–261.
[133] Babcock, G.T., Vickery, G.T. and Palmer, G. (1976) J. Biol. Chem. 251, 7901–7919.
[134] Eglinton, D.G., Hill, B.C., Greenwood, C. and Thomson, A.J. (1984) J. Inorg. Biochem. 21, 1–8.
[135] Babcock, G.T., Callahan, P.M., Ondrias, M.R. and Salmeen, I. (1981) Biochemistry 20, 959–966.
[136] Powers, L., Chance, B., Ching, Y.-C. and Angiolillo, P. (1981) Biophys. J. 34, 465–498.
[137] Scott, R.A. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 137–158.
[138] Van Gelder, B.F. and Beinert, H. (1969) Biochim. Biophys. Acta 189, 1–24.
[139] Tweedle, M.F., Wilson, L.J., Garcia-Iniquez, L., Babcock, G.T. and Palmer, G. (1978) J. Biol. Chem. 253, 8065–8071.
[140] Moody, A.J., Cooper, C.E. and Rich, P.R. (1991) Biochim. Biophys. Acta 1059, 189–207.
[141] Nanthakumar, A., Fox, S., Murthy, N.N. and Karlin, K.D. (1993) J. Am. Chem. Soc. 115, 8513–8514.
[142] Lee, S.C. and Holm, R.H. (1993) J. Am. Chem. Soc. 115, 11789–11798.
[143] Karlin, K.D., Nanthakumar, A., Fox, S., Murthy, N.N., Ravi, N., Huynh, B.H., Orosz, R.D. and Day, E.P. (1994) J. Am. Chem. Soc. 116, 4753–4763.
[144] Shaw, R.W., Rife, J.E., O'Leary, M.H. and Beinert, H. (1981) J. Biol. Chem. 256, 1105–1107.
[145] Gurbiel, R.J., Fann, Y.-C., Surerus, K.K., Werst, M.M., Musser, S.M., Doan, P.E., Chan, S.I., Fee, J.A. and Hoffmann, B.M. (1993) J. Am. Chem. Soc. 115, 10888–10894.
[146] Stevens, T.H., Martin, C.T., Wang, H., Brudvig, G.W., Scholes, C.P. and Chan, S.I. (1982) J. Biol. Chem. 257, 12106–12133.
[147] Martin, C.T., Scholes, C.P. and Chan, S.I. (1988) J. Biol. Chem. 263, 8420–8429.
[148] Martin, C.T., Scholes, C.P. and Chan, S.I. (1985) J. Biol. Chem. 260, 2857–2861.
[149] Cline, J., Reinhammar, B., Jensen, P., Venters, R. and Hoffman, B.M. (1983) J. Biol. Chem. 258, 5124–5128.
[150] Reinhammar, B., Malkin, R., Jensen, P., Karlsson, B., Andréasson, L.-E., Aasa, R., Vänngård, T. and Malmström, B.G. (1980) J. Biol. Chem. 255, 5000–5004.
[151] Stevens, T.H. and Chan, S.I. (1981) J. Biol. Chem. 256, 1069–1071.
[152] Holm, L., Saraste, M. and Wikström, M. (1987) EMBO J. 6, 2819–2823.
[153] Mueller, M., Schläpfer, B. and Azzi, A. (1988) Proc. Natl. Acad. Sci. USA 85, 6647–6651.
[154] Hill, B.C. and Marmor, S. (1991) Biochem. J. 279, 355–360.

[155] Alben, J.O., Moh, P.P., Fiamingo, F.G. and Altschuld, R.A. (1981) Proc. Natl. Acad. Sci. USA 78, 234–237.
[156] Fiamingo, F.G., Altschuld, R.A., Moh, P.P. and Alben, J.O. (1982) J. Biol. Chem. 257, 1639–1650.
[157] Einarsdóttir, Ó., Killough, P.M., Fee, J.A. and Woodruff, W.H. (1989) J. Biol. Chem. 264, 2405–2408.
[158] Stoutland, P.O., Dyer, R.B. and Woodruff, W.H. (1992) Science 257, 1913–1927.
[159] Findsen, E.W., Centeno, J., Babcock, G.T. and Ondrias, M.R. (1987) J. Am. Chem. Soc. 109, 5367–5372.
[160] Lou, B.-S., Larsen, R.W., Chan, S.I. and Ondrias, M.R. (1993) J. Am. Chem. Soc. 115, 403–407.
[161] Yoshikawa, S., Choc, M.G., O'Toole, M.C. and Caughey, W.S. (1977) J. Biol. Chem. 252, 5498–5508.
[162] Caughey, W.S. (1980) in Methods for Determining Metal Ion Environments in Proteins: Structure and Function of Metallo-proteins, (Darnall, D.W. and Wilkins, R.G., eds.), pp. 95–115, Elsevier/North-Holland, New York.
[163] Yoshikawa, S. and Caughey, W.S. (1982) J. Biol. Chem. 257, 412–420.
[164] Einarsdóttir, Ó., Choc, M.G., Weldon, S. and Caughey, W.S. (1988) J. Biol. Chem. 263, 13641–13654.
[165] Li, W. and Palmer, G. (1993) Biochemistry 32, 1833–1843.
[166] Brittain, T., Greenwood, C., Springall, J.P. and Thomson, A.J. (1992) Biochim. Biophys. Acta 703, 117–128.
[167] Hallén, S. and Brzezinski, P. (1994) Biochim. Biophys. Acta 1184, 207–218.
[168] Yoshida, S., Hori, H. and Orii, Y. (1980) J. Biochem. 88, 1623–1627.
[169] Boelens, R., Rademaker, H., Pel, R. and Wever, R. (1982) Biochim. Biophys. Acta 679, 84–94.
[170] Antonini, E., Brunori, M., Greenwood, C., Malmström, B.G. and Rotillo, G.C. (1971) Eur. J. Biochem. 23, 396–400.
[171] Yoshikawa, S. and Caughey, W.S. (1990) J. Biol. Chem. 265, 7945–7958.
[172] Cooper, C.E. (1990) Biochim. Biophys. Acta 1017, 187–203.
[173] Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1639–1650.
[174] Brzezinski, P. and Malmström, B.G. (1986) Proc. Natl. Acad. Sci. USA 83, 4282–4286.
[175] Brzezinski, P., Thörnström, P.-E. and Malmström, B.G. (1986) FEBS Lett. 194, 1–5.
[176] Antalis, T.M. and Palmer, G. (1982) J. Biol. Chem. 257, 6194–6206.
[177] Andréasson, L.E., Malmström, B.G., Strömberg, B.G. and Vänngård, T. (1982) FEBS Lett. 28, 297–301.
[178] Wilson, M.T., Greenwood, C., Brunori, M. and Antonini, E. (1975) Biochem. J. 147, 145–153.
[179] Hill, B. (1993) J. Bioenerg. Biomembr. 25, 115–120.
[180] Boelens, R. and Wever, R. (1979) Biochim. Biophys. Acta 547, 296–310.
[181] Boelens, R., Wever, R. and Van Gelder, B.F. (1982) Biochim. Biophys. Acta 682, 264–272.
[182] Brzezinski, P. and Malmström, B.G. (1987) Biochim. Biophys. Acta 894, 29–38.
[183] Morgan, J.E., Li, P.M., Jang, D.-J., El-Sayed, M.A. and Chan, S.I. (1989) Biochemistry 28, 6975–6983.
[184] Oliveberg, M. and Malmström, B.G. (1991) Biochemistry 30, 7053–7057.
[185] Einarsdóttir, Ó., Dawes, T.D. and Georgiadis, K.E. (1992) Proc. Natl. Acad. Sci. USA 89, 6934–6937.
[186] Hazzard, J.T., Rong, S. and Tollin, G. (1991) Biochemistry 30, 213–222.
[187] Pan, L.P., Hazzard, J.T., Lin, J., Tollin, G. and Chan, S.I. (1991) J. Am. Chem. Soc. 113, 5908–5910.
[188] Larsen, R.W., Winkler, J.R. and Chan, S.I. (1992) J. Phys. Chem. 96, 8023–8027.
[189] Stowell, M.H.B., Larsen, R.W., Winkler, J.R., Rees, D.C. and Chan, S.I. (1993) J. Phys. Chem. 97, 3054–3057.
[190] Pan, L.P., Hibdon, S., Liu, R.-Q., Durham, B. and Millet, F. (1993) Biochemistry 32, 8492–8498.
[191] Kobayashi, K., Une, H. and Hayashi, K. (1989) J. Biol. Chem. 264, 7976–7980.
[192] Nilsson, T. (1992) Proc. Natl. Acad. Sci. USA 89, 6497–6501.
[193] Millet, F., de Jong, C., Paulson, L. and Capaldi, R.A. (1983) Biochemistry 22, 546–552.
[194] Morgan, J.E., Verkhovsky, M.I., Puustinen, A. and Wikström, M. (1993) Biochemistry 32, 11413–11418.
[195] 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.
[196] Moser, C.C., Keske, J.M., Warncke, K., Farid, R.S. and Dutton, P.L. (1992) Nature 355, 796–802.
[197] Moser, C.C. and Dutton, P.L. (1992) Biochim. Biophys. Acta 1101, 171–176.
[198] Crinson, M. and Nicholls, P. (1992) Biochem. Cell Biol. 70, 301–308.
[199] Bisson, R., Steffens, G.C.M., Capaldi, R.A. and Buse, G. (1982) FEBS Lett. 144, 359–363.
[200] Orii, Y. (1984) J. Biol. Chem. 259, 7187–7190.
[201] Hill, B.C. and Greenwood, C. (1984) FEBS Lett. 166, 362–366.
[202] Wikström, M. (1981) Proc. Natl. Acad. Sci. USA 78, 4051–4054.
[203] Wikström, M. and Morgan, J.E. (1992) J. Biol. Chem. 267, 10266–10273.
[204] Varotsis, C., Woodruff, W.H. and Babcock, G.T. (1990) J. Am. Chem. Soc. 106, 8305–8306.
[205] Wrigglesworth, J.M. (1984) Biochem. J. 217, 715–719.
[206] Cheesman, M.R., Watmough, N.J., Gennis, R.B., Greenwood, C. and Thomson, A.J. (1994) Eur. J. Biochem. 219, 595–602.
[207] Brunori, M., Antonini, G., Malatesta, F., Sarti, P. and Wilson, M.T. (1992) FEBS Lett. 314, 191–194.
[208] Nicholls, P. (1992) Biochem. J. 288, 1070–1072.
[209] Gregory, L. and Ferguson-Miller, S. (1989) Biochemistry 28, 2655–2662.
[210] Thörnström, P.-E., Brzezinski, P., Fredriksson, P.-O. and Malmström, B.G. (1988) Biochemistry 27, 5441–5447.
[211] Morgan, J.E. and Wikström, M. (1991) Biochemistry 30, 948–958.
[212] Nilsson, T., Hallén, S. and Oliveberg, M. (1990) FEBS Lett. 260, 45–47.
[213] Han, S., Ching, Y.-C. and Rousseau, D.L. (1990) Proc. Natl. Acad. Sci. USA 87, 8408–8412.
[214] Hill, B.C. (1993) Biochem. Biophys. Res. Commun. 192, 665–670.
[215] Wikström, M. (1988) Ann. N.Y. Acad. Sci. 550, 199–206.
[216] Lauraeus, M., Morgan, J.E. and Wikström, M. (1993) Biochemistry 32, 2664–2670.
[217] Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S. and Kitagawa, T. (1990) J. Biol. Chem. 265, 14721–14723.
[218] Chan, S.I., Witt, S.N. and Blair, D.F. (1988) Chem. Scr. 28A, 51–56.
[219] Thomas, J.W., Lemieux, L.J., Alben, J.O. and Gennis, R.B. (1993) Biochemistry 32, 11173–11180.
[220] Thomas, J.W., Puustinen, A., Alben, J.O., Gennis, R.B. and Wikström, M. (1993) Biochemistry 32, 10923–10928.
[221] Kornblatt, J.A. and Kornblatt, M.J. (1992) Biochim. Biophys. Acta 1099, 182–184.
[222] Wikström, M.K.F. and Saari, H.T. (1977) Biochim. Biophys. Acta 462, 347–361.
[223] Krab, K. and Wikström, M. (1987) Biochim. Biophys. Acta 895, 25–39.
[224] Casey, R.P., Chappell, J.B. and Azzi, A. (1979) Biochem. J. 182, 149–156.
[225] Malmström, B.G. (1985) Biochim. Biophys. Acta 811, 1–12.
[226] Wikström, M. (1989) Nature 338, 776–778.
[227] Heberle, J. and Dencher, N.A. (1992) Proc. Natl. Acad. Sci. USA 89, 5996–6000.

[228] Babcock, G.T. and Callahan, P.M. (1983) Biochemistry 22, 2314–2319.
[229] Gelles, J. and Chan, S.I. (1985) Biochemistry 24, 3963–3972.
[230] Gelles, J., Blair, D.F. and Chan, S.I. (1987) Biochim. Biophys. Acta 853, 205–236.
[231] Chan, S.I. (1988) Ann. N.Y. Acad. Sci. 550, 207–222.
[232] Mitchell, P. (1987) FEBS Lett. 222, 235–245.
[233] Mitchell, P., Mitchell, R., Moody, A.J., West, I.C., Baum, H. and Wrigglesworth, J. (1985) FEBS Lett. 188, 1–7.
[234] Malmström, B.G. (1988) FEBS Lett. 231, 268–269.
[235] Puustinen, A., Finel, M., Virkki, M. and Wikström, M. (1989) FEBS Lett. 249, 163–167.
[236] Musser, S.M., Stowell, M.H.B. and Chan, S.I. (1993) FEBS Lett. 327, 131–136.
[237] Musser, S., Stowell, M.H.B. and Chan, S.I. (1993) FEBS Lett. 335, 296–298.
[238] Blair, D.F., Ellis, W.R.J., Wang, H., Gray, H.B. and Chan, S.I. (1986) J. Biol. Chem. 261, 11524–11537.
[239] Rousseau, D.L., Han, S.H., Song, S.H. and Ching, Y.C. (1992) J. Raman. Spec. 23, 551–556.
[240] Braiman, M.S., Mogi, T., Marti, T., Stern, L.J., Khorana, H.G. and Rothschild, K.J. (1988) Biochemistry 27, 8516–8520.