Cytochrome *c* Oxidase as a Redox-Linked Proton Pump

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Contents

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I. Introduction

Cytochrome *c* oxidase is not just an enzyme but a molecular machine, since it can use the free energy released in the exergonic catalytic reaction to pump protons against a concentration gradient across a membrane. Thus, the function of this respiratory protein complex cannot be described simply in terms of effects involved in chemical catalysis. In particular, the species transported, the proton, and the substrates of the catalytic reaction do not exchange matter, as in a chemical reaction, but free energy only. As a consequence, the catalytic site and the proton binding site do not have to be in close physical contact but can be in separate subunits of the protein complex, like the substrate and effector binding sites in an allosteric enzyme.

The reaction catalyzed by cytochrome oxidase is

$$4c^{2+} + O_2 + 4H^+ \rightarrow 4c^{3+} + 2H_2O \qquad (1)$$

where c^{2+} and c^{3+} represent cytochrome *c* in its reduced and oxidized forms, respectively. This is the terminal reaction of the so-called respiratory chain, which provides most of the free energy needed for the life processes of aerobic organisms by coupling the electron transport to the synthesis of adenosine triphosphate (ATP). Because of its central role in oxidative phosphorylation, cytochrome oxidase has a wide biological distribution. It is present in all animals and plants, in aerobic yeasts, and in some bacteria. It is an integral membrane protein, being firmly associated with the inner membrane of mitochondria, the respiratory organelle of eukaryotic organisms, or, in bacteria, the plasma membrane.

The free energy liberated in the reaction in eq 1 is temporarily stored in the form of an electrochemical potential across the membrane. This potential is then used to drive the synthesis of ATP, catalyzed by ATP

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synthase, in accordance with the chemiosmotic mechanism of Mitchell.[1] In part, the electrochemical potential is formed because cytochrome c donates electrons from the outside of the membrane, whereas the protons consumed in water formation are taken up from the inside.[2] This can, however, result only in an H^+/e^- stoichiometry of 1, but there is good experimental evidence[3] that the actual stoichiometry is close to 2. This is achieved because the electron-transfer reaction in eq 1 is coupled to proton translocation, as first demonstrated by Wikström.[4] The coupled reaction can be written

$$4c^{2+} + O_2 + (4 + n)H_1^+ \rightarrow 4c^{3+} + 2H_2O + nH_2^+ \quad (2)$$

in which the subscripts refer to two sides of the membrane. The value of n can vary between 0 and 4 depending on conditions (section IV.C), the highest value corresponding to a total stoichiometry of $2H^+/e^-$, with one scalar and one vectorial proton.

It is the purpose of this paper to review our present knowledge concerning the mechanism of the reaction catalyzed by cytochrome oxidase and the coupling between this reaction and proton translocation. The review is primarily aimed at the general chemical reader, who is not a specialist on cytochrome oxidase or other vectorial biochemical systems. For this purpose, emphasis will be on relatively well-established knowledge, whereas issues that are at present controversial will be treated more sparingly. I have, however, recently written a review[5] dealing mainly with unsolved problems and controversial issues. A comprehensive book attempting a synthesis of the knowledge available at the time was published in 1981.[6] A number of recent reviews or symposium volumes are also available.[7]

The literature on cytochrome oxidase is overwhelming. A comprehensive review[8] published in 1969 contained no less than 584 references, but most investigations on cytochrome oxidase have been carried out in the two decades following that year. Even if my treatment will also be comprehensive, emphasis will be on recent investigations (1980–1989). Thus, reference to older literature will be mainly indirect, to earlier reviews,[7] but some particularly important old papers will be quoted. Before the main review, a few milestones in the history of cytochrome oxidase research will be briefly recapitulated.

Warburg demonstrated[9] in 1929 that the photochemical action spectrum for the dissociation of the CO complex of the reduced "Atmungsferment" is similar to the corresponding spectrum for hemoglobin. He introduced[10] the concept of the respiratory chain in 1933, when he suggested that the Atmungsferment is the terminal component of a chain of cytochromes. This view was not accepted by Keilin, who had discovered the cytochromes during the 1920s,[11] until 1938, when Keilin showed[12] that "indophenol oxidase", his name for the Atmungsferment, can oxidize cytochrome c. At first, Keilin did, however, not believe that cytochrome c oxidase, as he now named the enzyme, is a heme protein, instead emphasizing its copper content.[13] The views of Warburg and Keilin were reconciled in 1939, when Keilin discovered[14] that his cytochrome a, having an absorption line close to 600 nm, was heterogeneous and had a component, cytochrome a_3, which behaved as Warburg's Atmungsferment. The term cytochrome a has been retained for the component that does not react directly with dioxygen.

II. Structure of Cytochrome Oxidase

A. Solubilization and Purification

Much work on cytochrome oxidase has been carried out with mitochondria or submitochondrial particles, but this paper will deal mainly with studies involving isolated cytochrome oxidase. Isolation of a membrane protein requires that it is first solubilized with the aid of a surface-active agent. With cytochrome oxidase, this was first achieved by the use of cholate in pioneering investigations of Straub,[15] recently elected President of Hungary, and of Okunuki and co-workers[16] in Japan. Most methods used today are still based on this early work, but sometimes deoxycholate instead of cholate is used for the solubilization step.[17] Long exposure to bile salts is detrimental to the integrity of the protein, so that in most modern procedures these are replaced by nonionic detergents, such as Tween, in the later part of the preparations. The final purification is usually performed by fractional ammonium sulfate preciptations, but a number of chromatographic methods are also available.[6] A detailed description of three commonly used solubilization and purification methods has been given by Hartzell et al.,[18] and extensive comparisons of the properties of the various preparations have been presented.[6,18] The heme and copper contents as well as the spectral and redox properties of the oxidase complexes prepared by different methods are remarkably similar. The largest differences between preparations are found in the phospholipid contents, which vary in the range 0.01–0.3 mg/mg of protein. Preparations with low phospholipid contents generally show an activity less than maximal, but they can often be activated by the addition of phospholipids.

There is no reason to believe that the structural and functional properties of isolated cytochrome oxidase are grossly perturbed compared to the enzyme in intact mitochondria. Thus, the maximal turnover numbers are similar (200–300 s^{-1}).[18] In addition, the isolated oxidase, which has been reconstituted into phospholipid vesicles, has been shown to pump protons.[19] The isolated cytochrome oxidase does, however, show several signs of heterogeneity,[20] which can cause great difficulties in the detailed interpretation of some spectroscopic and kinetic data. For example, most preparations contain a fraction of the enzyme that reacts very slowly with cyanide and displays a $g12$ signal in the EPR spectrum (section III.B),[21] whereas such a fraction is not found in mitochondria or submitochondrial particles. Recently two groups have described[21,22] purification procedures yielding homogeneous oxidase lacking the anomalous fraction.

B. Subunits

Mammalian and yeast cytochrome oxidases contain 12 or 13 different polypeptides,[23–25] the three heaviest of which, called I–III, are coded for by mitochondrial DNA. Bacterial oxidases of the aa_3 type, on the other hand, have at most three subunits.[26] The enzyme from *Paracoccus denitrificans*, for example, contains two subunits only as isolated,[27] even if determination of the

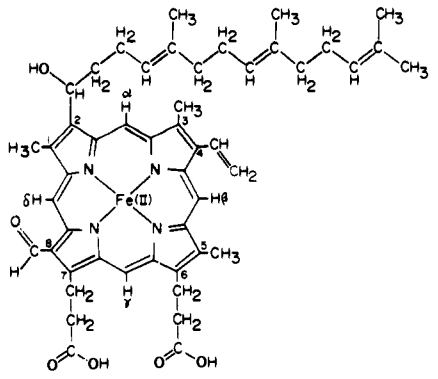

Figure 1. Chemical structure of heme A.

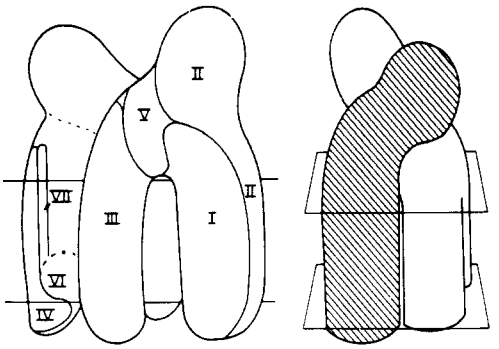

Figure 2. Membrane topography of cytochrome oxidase and arrangements of subunits in the dimer. Reprinted from ref 37; copyright 1983 Elsevier Science Publishers BV.

nucleotide sequence of part of the mitochondrial genome revealed the presence of a gene also for subunit III.[28] The two-subunit protein is, however, functional both in transferring electrons from cytochrome c to O_2 and in pumping protons. This suggests that subunits I and II constitute the functional core of the oxidase, containing all the redox centers, and that subunit III and the nuclear-coded polypeptides have other, hitherto poorly defined, functions, possibly in regulation of the enzymatic activity and the assembly of the complex.[24,25]

The amino acid sequences of all the subunits of beef heart cytochrome oxidase have been determined, by sequencing of DNA in the case of the mitochondrially synthesized polypeptides[29] and by sequencing the proteins for the nuclear-coded subunits.[23] According to Buse et al.,[23] there are 12 different polypeptides all present in a 1:1 stoichiometry with the exception of the lightest subunit, which has a stoichiometry of 2. From this they calculate an exact M_r of 202 787, which is close to the value for the monomer determined from hydrodynamic properties.[7]

Sequence information is now available for cytochrome oxidase from a large number of prokaryotic and eukaryotic species,[7,30] but a detailed discussion of this is outside the scope of the present review. It should be mentioned, however, that the number of strictly conserved amino acid residues in subunits I and II puts some restrictions on the ligand structure of the metal centers, and this will be considered in section III.D.

C. Metal Components

It has long been established that the functional unit of cytochrome oxidase contains four redox-active metal centers. Two of these, cytochromes *a* and a_3, contain heme A (Figure 1)[31] coordinated in different ways in subunit I.[30] The heme group is not covalently linked to the protein. Its typical features are the carbonyl group at position 8 and the long isoprenoid chain in position 2 of the porphyrin ring.

The other two redox-active metals are two copper ions, designated Cu_A and Cu_B, also bound in different ways to the protein. Cu_A is located in subunit II,[5,30] whereas Cu_B is most likely found in subunit I, together with the two cytochromes.[30]

Recently it has been found that beef heart cytochrome oxidase, in addition to the four redox centers, contains one strongly bound zinc and one magnesium ion.[32] The functions of these ions, if any, are not known, and they will therefore not be further considered in this review. It has also been shown[33-35] that the number of copper ions in many oxidase preparations is 3, not 2,[36] but there is evidence that the third copper is nonfunctional.

The immediate ligand environments of the redox centers are known in some detail. This knowledge is, however, largely derived from a variety of spectroscopic information, and it will consequently be summarized after the discussion of the spectroscopic properties in section III.

D. Membrane Topography and Interaction with Phospholipids

Structural knowledge about cytochrome oxidase is very limited above the level of primary structure, since it has so far proved impossible to grow crystals of a high enough quality for high-resolution X-ray studies. It has, however, been possible to prepare two-dimensional crystals suitable for electron microscopy and image reconstruction.[37] From such studies the overall shape in three dimensions, together with the position in the lipid bilayer, has been inferred (Figure 2). Cytochrome oxidase is clearly a transmembrane protein that is inserted asymmetrically through the membrane. The overall shape is like a Y, with the stalk extending about 50 Å above the membrane plane on the cytoplasmic side and the arms crossing the lipid bilayer and extending about 10 Å on the side of the mitochondrial matrix. The distance between the centers of the two arms has been estimated to be about 40 Å.

Figure 2 also suggests the arrangements of the major polypeptides, as deduced from chemical-labeling and cross-linking studies.[37] It can be seen that both subunits I and II are transmembrane components. A large part of subunit II is, however, outside the membrane on the cytosolic side, and Cu_A is probably located in this region.[30,37] The other redox centers, on the other hand, are in the portion of subunit I embedded in the membrane, even if cytochrome *a* is close to the cytosolic surface.[30]

Cytochrome oxidase in the two-dimensional crystals is present as a dimer of the functional unit (Figure 2). It is known that the monomeric enzyme is fully competent to carry out catalytic cytochrome c oxidation,[38] but there has been considerable discussion concerning the question whether it can couple this reaction to the translocation of protons.[39,40] A reciprocating-site model for the proton pump has been proposed,[6] and this requires the presence of a dimer. The experimental evidence on this point is, however, ambiguous,[39] and no clear answer can be given at present.

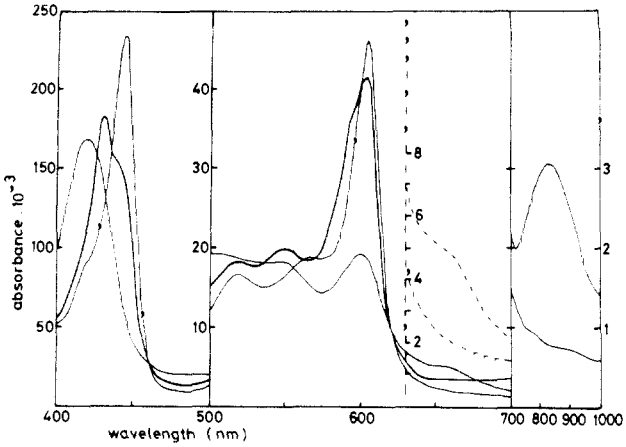

Figure 3. Optical absorption spectra of oxidized, reduced, and reduced CO complex of cytochrome oxidase. The spectrum with the highest absorbance at 445 and 605 nm refers to the reduced enzyme, whereas the absorption band in the near-infrared stems from the oxidized oxidase.

A lipid (or detergent) environment is necessary to prevent aggregation of cytochrome oxidase, but it does not otherwise seem to be a requirement for catalytic activity.[6,41] It has, however, already been mentioned (section II.A) that the addition of phospholipids increases the turnover rate, and cardiolipin is particularly effective in this regard.[41] Recent evidence[41] suggests that that cardiolipin increases the rate of a conformational transition, which is part of the proton-translocation machinery (cf. section V.A.2.).

III. Spectroscopic and Magnetic Properties

A. Optical Absorption, Resonance Raman, and MCD Spectra

Figure 3 gives the optical spectra of oxidized and reduced cytochrome oxidase as well as of the CO complex of the reduced enzyme. The spectrum is entirely attributable to heme transitions in cytochromes *a* and a_3, except that the weak band at 655 nm has been related to an interaction of oxidized cytochrome a_3 with Cu_B[42] and that the absorption band in the near-infrared is largely due to oxidized Cu_A.[43]

Vanneste[44] has attempted to determine the (reduced − oxidized) difference spectra of the individual heme components by utilizing the stabilization of oxidized cytochrome a_3 by CN^- and of reduced cytochrome a_3 by CO. This procedure presupposes that there are no spectral interactions between the hemes, which is not quite true.[45] To a first approximation it can, however, be concluded that the two cytochromes contribute about equally to the difference spectrum at 445 nm, whereas cytochrome *a* is responsible for nearly 80% of the absorbance change at 605 nm. These relative contributions are also apparent from the spectrum of the CO compound (Figure 3), since in this the absorption of reduced cytochrome a_3 is shifted downward at both wavelengths, whereas the absorption of cytochrome *a* is largely unperturbed.

Resonance Raman spectra show that, in oxidized cytochrome oxidase, cytochrome *a* is in the low-spin and cytochrome a_3 in the high-spin state.[46] Comparisons with model compounds suggest that cytochrome *a* has two imidazole groups as axial ligands. The same conclusion is reached from magnetic circular dichroism (MCD) spectra.[47] These also show that there are charge-transfer bands associated with Cu_A not only in the near-infrared but also around 500 nm.

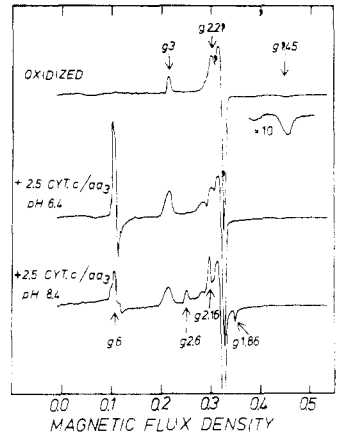

Figure 4. EPR spectra of oxidized (top) and partially reduced cytochrome oxidase at pH 6.4 (middle) or 8.4 (bottom).

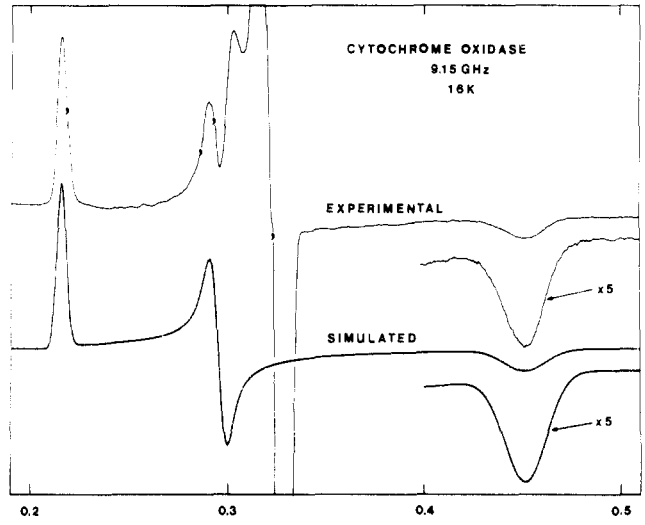

Figure 5. Experimental and simulated EPR spectrum of cytochrome oxidase, showing the low-spin signal in detail. Reprinted from ref 48; copyright 1976 Elsevier Science Publishers BV.

B. EPR Properties

The electron paramagnetic resonance (EPR) spectrum at 9 GHz of oxidized cytochrome oxidase is dominated by contributions from a low-spin Fe^{3+} with lines at *g* 3.0, 2.2, and 1.5 and a narrow signal at *g*2 ascribed to Cu_A^{2+} (Figures 4 and 5). The heme signal can be satisfactorily simulated on the assumption that it is due to a magnetically isolated low-spin Fe^{3+} (Figure 5),[48] and it thus originates from cytochrome *a*. The EPR signal of Cu_A^{2+} is unique, with very low *g* values, more like that of a free radical.

Cytochrome a_3 and Cu_B are not detectable by EPR in the oxidized enzyme. This is caused by an antiferromagnetic coupling between these two ions, as first suggested by Van Gelder and Beinert.[49] This is evidenced by the appearance of a high-spin Fe^{3+} signal at *g*6 on partial reduction (Figure 4); this arises from molecules in which cytochrome a_3 is oxidized but Cu_B reduced. At high pH, the *g*6 signal decreases at the same time as a new low-spin Fe^{3+} signal with lines at *g* 2.6, 2.16, and 1.86 appears (Figure 4). This is nearly identical with the signal displayed by metmyoglobin hydroxide and can consequently be ascribed Fe^{3+}–OH^-

in cytochrome a_3.[50] Histidine has been shown to be the fifth, axial ligand to cytochrome a_3 by elegant EPR experiments with the NO derivatives of yeast cytochrome oxidase in which [^{15}N]histidine had been incorporated.[51] Under some conditions, an EPR signal from Cu_B^{2+} can be observed,[52]

Magnetic susceptibility measurements both at low temperatures[53] and at room temperature[54] (with an NMR technique) are consistent with the presence of two magnetically isolated spin $S = {}^1/_2$ centers and a spin-coupled $S = 2$ center. Since the bimetallic cytochrome a_3–Cu_B site is fully coupled even at room temperature, it must be concluded that the exchange integral $-J$ is greater than 200 cm^{-1}.

C. ENDOR, Mössbauer, and EXAFS Studies

Electron nuclear double resonance (ENDOR) measurements on cytochrome oxidase from yeast, in which isotopically labeled amino acids has been incorporated, have been very helpful in identifying the ligands in the various metal sites. Thus, the hyperfine lines from [^{15}N]histidine unambiguously identify this amino acid as an axial ligand to the heme of cytochrome a, and comparisons with model compounds support a bis(imidazole) coordination.[55] Similarly, Cu_A has been shown to have at least one histidine and one cysteine as ligands.[56] ENDOR spectra of the enzyme form displaying an EPR signal from Cu_B^{2+} suggest that this ion is coordinated in a similar manner as type 3 Cu^{2+} in laccase, with three imidazole nitrogens and H_2O or OH^- as ligands.[52,57]

Cytochrome oxidase from bacteria or yeast can be prepared enriched in ^{57}Fe. Mössbauer studies on such samples have shown that cytochrome a_3 is in the high-spin state in both the oxidized and reduced forms.[58,59] The spectrum of reduced cytochrome a_3 is very similar to that of myoglobin, suggesting that one axial coordination site is unoccupied and free to bind O_2.

All published extended X-ray absorption fine structure (EXAFS) or other X-ray absorption studies on cytochrome oxidase have recently been reviewed.[60] The technique cannot distinguish between N and O or S and Cl. It is also severely limited by the presence of more than one ion of a kind, e.g., Cu_A and Cu_B, since it only yields the average site structure for these ions. For oxidized cytochrome oxidase, it has been concluded that the two copper sites have 6 ± 1 (N, O) at 1.99 ± 0.03 Å and 2 ± 1 (S, Cl) at 2.28 ± 0.02 Å. Measurements on samples in which Cu_A had been removed exhibited one Cu–(S, Cl) interaction at the same distance as in the native enzyme, consistent with at least one cysteine S as ligand to Cu_A.

Recent work[60] suggests that Cl^- is a bridging ligand between cytochrome a_3 and Cu_B in resting oxidase. The Fe–Cu distance in this site from EXAFS is about 3 Å. X-ray absorption studies have also confirmed the presence of zinc in the enzyme and suggest that it is coordinated to three or four sulfur atoms.

D. Structure of the Redox Centers

The spectroscopic properties just reviewed allow the assignment of probable ligand structures to the four redox centers. Coupled with knowledge about conserved amino acids, it is also possible to conclude in which subunits the centers are located. Thus, subunit

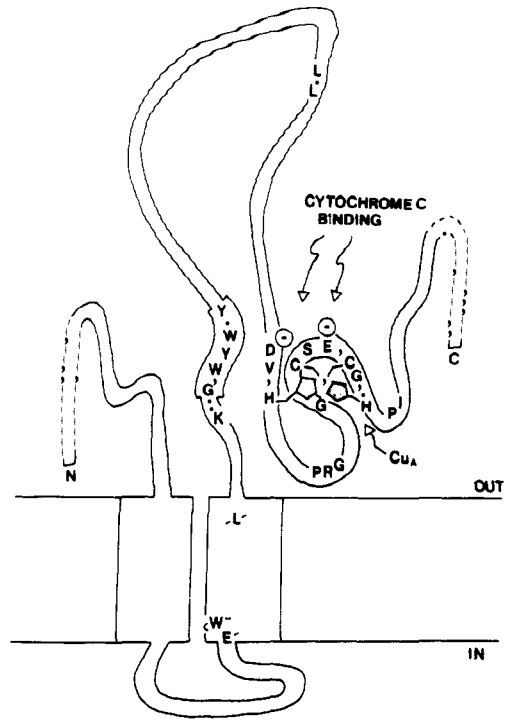

Figure 6. Schematic model of subunit II of cytochrome oxidase. Two hydrophobic helices anchor the subunit to the membrane. The Cu_A binding site is indicated in a location outside the membrane close to the COOH-terminal end of the peptide. Invariant amino acid residues are shown. Reprinted from ref 30; copyright 1987 Oxford University Press.

II is the only one that contains conserved cysteines, and it undoubtedly provides the binding site for Cu_A. This subunit has only two conserved histidines, which probably both are ligands to Cu_A, so that all other metal centers must be in subunit I.

Subunit II is presumably anchored to the membrane by two transmembrane helices, with the binding site for Cu_A located outside the membrane on the cytosol side in the COOH-terminal part of the peptide chain (Figure 6).[30,37] In this region, it shows sequence homologies with azurin, whose crystal structure is known. This has allowed Holm et al.[30] to construct a three-dimensional model of the Cu_A site, in which the metal ion is coordinated to two cysteine and two histidine residues. The site differs from azurin, which has only one ligand cysteine, which could explain why the EPR spectrum of Cu_A^{2+} is unique; the presence of two cysteines results in an extremely high degree of electron delocalization away from sulfur onto copper, giving the EPR spectrum a free-radical character.[61] It has, however, been reported[62] that a bacterial oxidase contains a single cysteine residue but gives a typical Cu_A^{2+} EPR signal. In addition, one of the ligand cysteines (Cys-200 in the bovine enzyme) appears not to be conserved in wheat, but in this case the EPR spectrum is not known.

Subunit I contains nine conserved histidine residues, which is enough to provide the six ligands implicated by spectroscopic data: two for cytochrome a, one for cytochrome a_3, and three for Cu_B. Holm et al.[30] have constructed a model for these three sites as well. In this, cytochrome a is coordinated to two histidine residues close to the membrane surface in separate transmembrane helices. Cytochrome a_3–Cu_B is suggested to be located between the same helices, with a third helix providing two of the Cu_B ligands. This places the two

heme groups very close to each other (14 Å center to center), which would seem to be at variance with the relatively slow electron transfer from cytochrome *a* to the bimetallic site (section V.C.). In addition, electron spin relaxation indicates that the distance is about 20 Å.[63] For this reason, I have suggested an alternative model, which retains the structure of cytochrome *a* but has cytochrome a_3 bound to one of the transmembrane helices on the opposite side from cytochrome *a*.[64]

IV. Redox Properties

A. Reduction Potentials

1. Redox Titrations

Two types of redox titrations have been carried out with cytochrome oxidase. The first one is potentiometric titrations in which the redox state is monitored as a function of the applied potential. It has long been known[6,7] that if the degree of reduction is measured optically, almost identical Nernst plots are obtained at 605 and 445 nm. The plots are sigmoidal in shape and correspond to two midpoint potentials of 370 and 230 mV. Originally it was thought that these values represent the individual potentials of the two cytochromes. It was then, however, difficult to explain that the same curves were obtained at the two wavelengths, in view of the large differences in the relative spectral contributions of cytochromes *a* and a_3 (section III,A). In a review[65] written in 1973, I pointed out that the results could be interpreted in terms of a negative potential interaction between the two cytochromes, so that the potential of one center depends on the redox state of the other. In this way both cytochrome *a* and a_3 would contribute to the high- as well as to the low-potential part of the Nernst curve. This concept is also incorporated in the so-called neoclassical model, which was introduced by Nicholls[66] and further elaborated by Wikström et al.[67] A shortcoming in this model is, however, that it only considers heme–heme interactions and ignores the two other redox sites.

The second type of redox titrations is anaerobic reductive titrations with a reductant having a lower potential than any of the electron acceptors in the enzyme. In this type of titration, the center with the highest potential will be reduced first. Titrations monitored by MCD or EPR are particularly informative, since with these techniques the reduction of individual redox sites can be followed. For example, in EPR titrations,[68] there is very little change in the *g*2 signal with the first electron equivalent added, whereas there is a substantial decrease in the *g*3 signal. This demonstrates that the reduction potential of cytochrome *a* is higher than that of Cu_A. A striking result is the finding that cytochrome *a* is not fully reduced until four electron equivalents have been added. This is consistent with a negative potential interaction, since it means that cytochrome *a* has both the highest and the lowest potential. Such an interpretation is supported by the behavior of the *g*6 signal from oxidized cytochrome a_3 in molecules with Cu_B reduced. This signal starts to appear in the very beginning of the titration, is maximal when two electron equivalents have been added, but does not disappear completely until the fourth equivalent has been added. Thus, cytochrome a_3 also has the highest as well as the lowest potential, in agreement with the negative-interaction model.

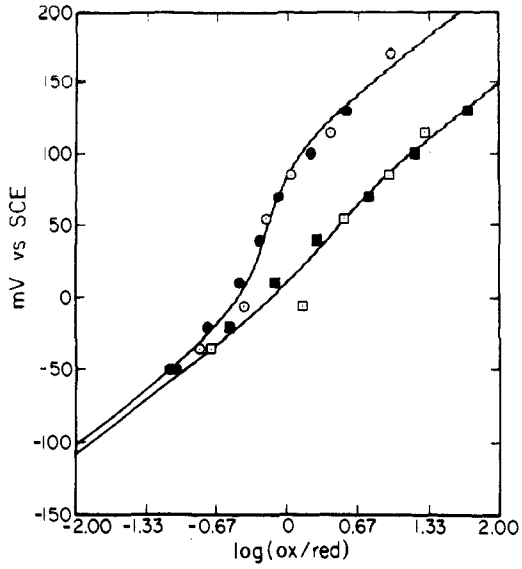

Figure 7. Nernst plots for cytochrome *a* (circles) and cytochrome a_3 (squares) in a spectroelectrochemical titration of cytochrome oxidase. Reprinted from ref 72; copyright 1986 The American Society for Biochemistry & Molecular Biology.

2. Interactions

In my 1973 review,[65] I pointed out that a completely general model for a protein with four electron-acceptor sites must include $2^4 - 2$ or 14 midpoint potentials and an equal number of extinction coefficients. If only pairwise interactions are considered, then 10 parameters are sufficient. Analyses[65,69] based on such a model showed that interactions must be included, but then a unique solution could not be found to describe the data available at the time.

In recent years, the problem of site–site interactions has been reinvestigated by Ellis, Blair, and colleagues in studies employing modern thin-layer spectroelectrochemical methods.[70-72] First, it was demonstrated that there are spectral interactions between the hemes, but these are so weak (section III.A)[45] that they can be ignored in the analysis of the spectroelectrochemistry. The redox behavior was then determined for the native and carbon monoxide inhibited enzyme as a function of pH and temperature. In the analysis of the data in the form of Nernst plots, the individual contributions of the two cytochromes were deconvoluted by measurements at two wavelengths (605 and 443 nm), as illustrated in Figure 7. A nonlinear curve-fitting program allowed the estimation of the interaction potential at each site independent of the other sites. In this way, it was shown that cytochrome *a* displays a negative interaction with all three of the other redox sites and that there is also such an interaction between cytochrome a_3 and Cu_B. Another important finding of this study is that the reduction potential of cytochrome *a* is only moderately dependent on pH, because this has implications for the function of cytochrome oxidase as a proton pump (section VI.A).

B. Effect of Ligands

Much attention has been paid to the effect of ligands to cytochrome a_3 on the distribution of electrons among

the four redox centers of cytochrome oxidase.[7] In particular, the effects of CO, which stabilizes reduced cytochrome a_3, and of N_3^- and CN^-, which bind to the oxidized center, have been investigated. Initially the results were interpreted without any consideration of potential interactions, but a thorough reevaluation within the framework of the neoclassical model has been presented by Wikström et al.[6] Their analysis showed that the experimental results in the presence of inhibitors could be fairly well described in terms of the model, except in the case of cyanide where a negative interaction between cytochrome *a* and Cu_A had to be introduced. Such an interaction was later demonstrated directly by Ellis et al.[70] with the CO-inhibited oxidase; because of the increased precision in their measurements, they could detect a small deviation from simple Nernstian behavior, which had not been observed in earlier studies.

The cytochrome *a* potential in the CO compound is 274 mV, which is intermediate between the high and low potentials in the uncomplexed enzyme.[70] The Cu_A potential is 285 mV and the interaction potential (with cytochrome *a*) 40 mV. In the presence of azide, the cytochrome *a* potential has been estimated to be 360 mV, which agrees with the conclusion in the interacting model that the potential should be high when the other centers are oxidized.[6] It is, therefore, surprising that the cyanide compound gives a much lower potential for cytochrome *a*.[6] However, the data are anomalous, as the slope in the Nernst plot gives $n = 0.5$. This is expected in the middle of the titration in the case of negative interaction, since the Nernst equation is formally equivalent to the Hill equation used to diagnose cooperativity in ligand binding.[65] A possible explanation is that Cu_B can be reduced in the cyanide but not in the azide compound, so that the negative interaction with this site manifests itself in the former case.

V. Kinetic Properties and Catalytic Mechanism

A. Steady-State Kinetics

1. Basic Features

Smith and Conrad reported[73] in 1956 that when the oxidation of cytochrome *c* by O_2, catalyzed by cytochrome oxidase, is followed spectrophotometrically, the time course is exponential, and not zero-order, even at very high concentrations of cytochrome *c*. They furthermore found that the apparent first-order rate constant decreased with increasing total concentration of cytochrome *c*. The initial velocity, calculated by multiplying the rate constant by the initial concentration of ferrocytochrome *c*, varies with substrate concentration according to the Michaelis–Menten equation (but cf. section V.A.2). Minnaert[74] later confirmed this basic kinetic behavior and showed that the rate equation has the general form

$$\frac{v_0}{[E]} = \frac{k_{cat}[S]}{K_m + ([S] + [P])} \quad (3)$$

where v_0 is the initial velocity, E is cytochrome oxidase, S and P represent ferri- and ferrocytochrome *c*, respectively, and k_{cat} and K_m are constants. He formulated five mechanisms consistent with this kinetic equation, and his analysis favored his mechanism IV:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2[O_2]} EP \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + P \quad (4)$$

The essential feature here is that S and P interact with E with the same rate constants, and hence identical binding constants, so that P acts as a competitive inhibitor with respect to S. A later extensive analysis of the steady-state behavior of cytochrome oxidase by Myers and Palmer[75] showed that such an equivalence of symmetrically related constants is a comprehensive requirement for any viable mechanism of this enzyme.

Minnaert's mechanism IV does, of course, represent a gross oversimplification beyond the basic requirement just discussed. In a complete catalytic cycle, four molecules of S are converted to P and there are four different forms of E responsible for these consecutive conversions. In addition, S and O_2 react at different sites on E, so that several intramolecular electron-transfer steps must be included in the mechanism. It has been shown, however, that a more realistic catalytic cycle, as long as it is sequential in nature, yields a steady-state rate equation that reduces to eq 3.[76] Branched mechanisms, on the other hand, can result in kinetic equations containing quadratic terms in [S] (cf. section V.A.2).[75]

2. Nonhyperbolic Kinetics

It has long been known that if a very wide range of cytochrome *c* concentration is used, particularly at low ionic strength, then the kinetics of cytochrome oxidase no longer obeys eq 3.[77-79] Instead, the kinetic equation is nonhyperbolic but can be written as the sum of two hyperbolic, or Michaelis–Menten, terms. Originally this was taken as evidence for the existence of two active sites with different affinities for the substrate,[77] but more recently three other possible explanations have been actively discussed.

The *regulatory site mechanism*[60,81] assumes a single catalytic site and an adjacent regulatory site where cytochrome *c* binds without transferring electrons. Cytochrome *c* bound at the regulatory site repels cytochrome *c* at the active site, thereby decreasing the affinity for both substrate and product. This mechanism can explain the nonhyperbolic kinetics, provided that product dissociation is rate-limiting. The *negative cooperativity mechanism*[82,83] presumes that the enzyme functions as a dimer in which there is electrostatic or steric repulsion between cytochrome *c* molecules bound in the two active sites. Finally, in the *conformational transition mechanism*[84] the enzyme oscillates between two conformations with different affinities for cytochrome *c*. The two conformations are here assumed to regulate (gate) the electron flow in the function of the oxidase as a proton pump (section VI.A).

During the last year there have been at least three studies aimed specifically at differentiating between these three hypotheses.[41,85,86] In one of them,[85] the kinetics as well as the binding of cytochrome *c* were measured with monomeric, dimeric, and oligomeric oxidase. It was found that the binding was always linear, whereas the kinetics was biphasic, independent of the aggregation state of the enzyme. This eliminates the negative cooperativity mechanism. Furthermore, no correlation was found between the Michaelis constants and the binding constants, when these parameters were measured under different conditions or with

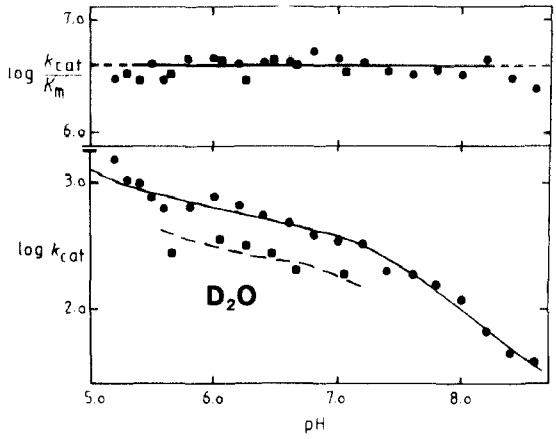

Figure 8. Effect of pH and D_2O on k_{cat} and k_{cat}/K_m for cytochrome oxidase in phospholipid vesicles at 25 °C. Experimental points: ●, in H_2O; ■, in D_2O. The solid line in the plot of log k_{cat} is simulated with eq 5 and the following parameters: k_1 = 250 s^{-1}, k_2 = 580 s^{-1}, k_3 = 4500 s^{-1}; pK_1 = 7.8, pK_2 = 6.8, pK_3 = 4.5. Reprinted from ref 88; copyright 1984 The Royal Swedish Academy of Sciences.

oxidase from different species. This is strong evidence against the regulatory site mechanism, which predicts such a correlation. Later, an even stronger contradiction to this prediction was provided by the use of a number of mutants of cytochrome *c*, which greatly extended the range of the experimental parameters.[86] The lack of correlation between the binding constants and the Michaelis constants shows that product dissociation cannot be rate-limiting, as required in the regulatory site mechanism. Actually this conclusion also follows from eq 3, since this can only be obtained from the mechanism in eq 4, if there is rapid equilibrium between E, S, and P. Finally, the demonstration[41] that nonhyperbolic kinetics is obtained in the complete absence of negative phospholipids also excludes the regulatory site mechanism, since such lipids were assumed to provide the regulatory site. Thus, the conformational transition mechanism appears to be the only viable explanation to the nonhyperbolic kinetics.

3. pH and Ionic Strength Dependence

The steady-state kinetics of cytochrome oxidase is strongly dependent on both pH and ionic strength. At high ionic strength, where eq 3 applies, k_{cat} increases continuously with decreasing pH in the experimentally available range (8.6–4.6), whereas k_{cat}/K_m remains essentially constant.[87-89] Experimental results with the enzyme in uncoupled phospholipid vesicles are given as an example in Figure 8.[88] The pH dependence of k_{cat} can be described by the following equation:[87,88]

$$k_{cat} = \frac{k_1[H^+]/K_1 + k_2[H^+]^2/K_1K_2 + k_3[H^+]^3/K_1K_2K_3}{1 + [H^+]/K_1 + [H^+]^2/K_1K_2 + [H^+]^3/K_1K_2K_3} \quad (5)$$

In eq 5, k_1, k_2, and k_3 represent k_{cat} for singly, doubly, and triply protonated oxidase, respectively, whereas K_1, K_2, and K_3 are the corresponding acid dissociation constants. This equation can be used to simulate the experimental results with the parameters given in the caption to Figure 8. Thus, the protonation of three groups with apparent pK_a values of 7.8, 6.8, and 4.5, respectively, increases the catalytic activity of cytochrome oxidase. It should be emphasized that the pK_a values are apparent and have no simple physical meaning in the case of such a complex mechanism as that of cytochrome oxidase, in which four different enzyme forms are involved in four consecutive substrate–product conversions. The results do show, however, that the rate of the slowest step(s) in the overall reaction is increased by the binding of up to three protons.

There is evidence that the rate-limiting step in the catalytic cycle of cytochrome oxidase is the internal electron transfer from cytochrome *a* and Cu_A to the bimetallic site.[76,90,91] These steps have, however, rate constants larger than k_{cat} in some states of the enzyme, as discussed in sections V.B and C. Therefore, I have argued that the internal electron transfer is limited by a slow, pH-dependent conformational change.[92] It is then tempting to consider that the pH dependence of the steady-state kinetics is directly related to the function of cytochrome oxidase as a proton pump (section VI). This idea is supported by the observed solvent isotope effect on k_{cat} (Figure 8), since this indicates that the rate-limiting step involves a proton transfer.

The parameter k_{cat}/K_m, which has the dimensions of a second-order rate constant, is not affected by pH (Figure 8). This may seem surprising, since it is generally considered that electrostatic interactions play an important role in the reaction between cytochrome *c* and the oxidase. That this is really true is evidenced by the fact that an increase in the ionic strength decreases k_{cat}/K_m but has little effect on k_{cat}.[87,88] The ionic strength dependence is, however, the same when the oxidase has a positive total charge (pH 6.2) or a negative one (pH 8).[93] From this it has been concluded that the electrostatic interaction between cytochrome *c* and the oxidase involves mainly the local interaction domains on the two proteins rather than total monopoles or dipoles. The lack of a pH dependence of k_{cat}/K_m would then indicate that the charged groups in these domains do not titrate in the pH range used.

It may be noted that the value of k_{cat}/K_m (1.1×10^6 M^{-1} s^{-1}) is very close to the second-order rate constant for the reaction between cytochrome *c* and cytochrome oxidase (2.0×10^6 M^{-1} s^{-1}).[94] This has the consequence that, at low concentrations of cytochrome *c*, the reaction is essentially diffusion-controlled. Thus, cytochrome oxidase satisfies one of the criteria for a "perfectly evolved enzyme", as defined by Albery and Knowles.[95]

B. Reaction with Cytochrome *c*

The anaerobic reaction between reduced cytochrome *c* and oxidized cytochrome oxidase was first investigated by Gibson et al.[96] in 1965 and has subsequently been further explored by a large number of investigators.[6,7] It is not known whether cytochrome *a* or Cu_A is the primary electron acceptor, because these two centers are reduced synchronously on the stopped-flow time scale.[97] This indicates that they are in rapid redox equilibrium, and this has recently been demonstrated directly by two different approaches.[98,99] In both studies, rate constants of the order of 10^4 s^{-1} in both directions were observed. An objection to the first set of experiments is that they were carried out with the three-electron reduced enzyme. Thus, the oxidase may have been in a different conformtional state compared to the oxidized enzyme, since in a redox-linked proton

pump there must be reduction-induced conformation changes (section VI.A). The same rate constants were, however, observed in experiments starting from the fully oxidized enzyme, which was very rapidly reduced by a free radical produced by pulse radiolysis.[99] In these experiments, Cu_A was reduced before cytochrome *a*, but the strongly reducing radical could well react with a different center compared to cytochrome *c*. In terms of mechanism, the question of the primary acceptor of electrons from cytochrome *c* is a purely academic one, however, because the redox equilibrium between cytochrome *a* and Cu_A is much more rapid than the rate of input of electrons from cytochrome *c* at any reasonable concentration.

The rapid redox equilibrium between cytochrome *a* and Cu_A cannot pertain under all conditions, because rapid Cu_A (1.4×10^4 s^{-1}) and slow cytochrome *a* (400 s^{-1}) oxidation has been observed anaerobically in the two-electron reduced enzyme.[100,101] In addition, when the fully reduced enzyme is reoxidized with O_2, Cu_A is oxidized with a rate of 7500 s^{-1}, whereas the rate for cytochrome *a* oxidation is 750 s^{-1}.[102]

From the two primary acceptors, the electrons are transferred intramolecularly to the bimetallic cytochrome a_3–Cu_B site, which is the dioxygen reducing center. Gibson et al.[96] followed this electron transfer by measuring the onset of photosensitivity, which is a property of the CO compound when the site is reduced. Surprisingly, they found the rate to be too low for this step to be part of the catalytic cycle.

A resolution to this dilemma was provided by Antonini and co-workers,[103,104] who showed that the electron-transfer rate is much higher in the pulsed oxidase, produced by reoxidizing the fully reduced enzyme with a pulse of oxygen. There is evidence that, in the pulsed enzyme, the intramolecular electron transfer, or perhaps a conformation change associated with it, provides the main limit on k_{cat}, as already briefly discussed in section V.A.3.[90,91]

C. Reduction of Dioxygen

One of the most intriguing mechanistic problems with cytochrome oxidase is the question how this enzyme can couple one-electron transfer from cytochrome *c* to the four-electron reduction of dioxygen. The key structural feature of the enzyme is undoubtedly the presence of a bimetallic dioxygen-reducing center, since this is found also in the few other oxidases which can reduce dioxygen all the way to two molecules of water.[105] In such a center, peroxide can be rapidly formed as one of the first intermediates by a concerted two-electron transfer, thereby bypassing the thermodynamically unfavorable one-electron reduction of dioxygen to superoxide.

Early flow–flash experiments, in which the reaction of the fully reduced enzyme with dioxygen was initiated by photolysis of the CO compound, were interpreted in terms of a sequential mechanism.[102] In this, dioxygen binding and cytochrome a_3 oxidation were suggested to be synchronous. This step was then followed by sequential oxidation of Cu_A and cytochrome *a*. Further evidence for such a mechanism was obtained from combined optical and EPR kinetic measurements,[106] based on the low-temperature technique of Chance et al.[107] Three successive intermediates were observed.

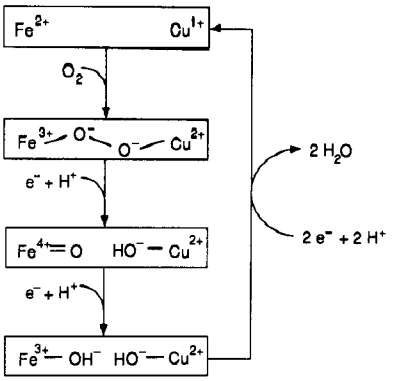

Figure 9. Mechanism of dioxygen reduction at the cytochrome a_3–Cu_B site.

The first one did not involve any electron transfer from cytochrome *a* or Cu_A, whereas the next two were formed by two successive one-electron transfers. A quantitative account of the results required, however, the introduction of branch at the three-electron stage of dioxygen reduction. Recent flow–flash results could also only be accounted for in terms of a branched mechanism.[108] These branches may occur only when the reaction is started from the fully reduced enzyme, which is probably an artificial state, not formed during normal turnover.

A reaction cycle for the reduction of dioxygen at the bimetallic site is presented in Figure 9. The intermediate at the three-electron reduced stage has been shown to give an EPR signal from Cu_B^{2+} with unusual relaxation properties, which is consistent with the structure involving a ferryl ion.[109,110] Later low-temperature work has indicated that this species is formed from the peroxide via an additional intermediate in which cytochrome a_3 is present as Fe^{2+}.[111] An identical reaction cycle has been proposed by Wikström, on the basis of experiments with partial reversal of the reaction in mitochondria at a high electrochemical potential gradient.[112,113]

Recently, the pH and temperature dependencies of the rates of the kinetic phases observed by flow–flash have been determined.[114] The rates of the two slowest phases decrease above pH 8, as expected because of the uptake of protons in these steps. Even at the highest pH (9.0), the rates are much higher than k_{cat}, however, demonstrating that the pH dependence of this parameter does not involve any of the dioxygen-reducing steps. Application of Marcus theory[115] to the temperature dependence data indicates that, in the rapid one-electron transfer from Cu_A to the peroxo intermediate, the reorganization energy is largely balanced by the driving force, which is about 62 kJ mol^{-1} for this step.[113]

VI. Proton Pump

A. General Principles of Redox-Linked Proton Pumps

DeVault in 1971 presented one of the earliest analyses of the principles that must govern the operation of any redox-linked proton pump.[116] Such a pump is driven by an exergonic electron-transfer reaction between a donor (D) and an acceptor (A):

$$D(e^-) + A \rightarrow D + A(e^-) \quad (6)$$

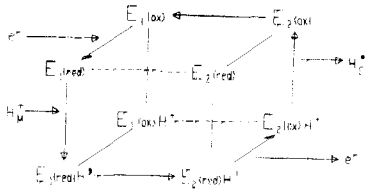

Figure 10. Cubic scheme for a redox-linked proton pump. For explanations, see the text.

One important principle pointed out by DeVault is that the donor should react with a discrete input state of the transducer, here designated E_1, whereas the acceptor should react with the output state, E_2, only. Thus, the reaction in eq 6 cannot be completed unless the transducer undergoes a transition from E_1 to E_2.

The requirement of separate input and output states, called specificity of the first kind by Krab and Wikström,[117] is part of the cubic scheme of Wikström and co-workers.[6,117-119] This scheme, which is illustrated in Figure 10, is actually quite general and can easily be modified to describe any biological energy transducer, e.g., ATP-driven ion pumps. Figure 10 shows that E_1 and E_2 are also the input and output states, respectively, for H^+. In other words, in E_1 the proton-translocating group can only equilibrate with H^+ on side 1 of the membrane, and in E_2 it can only equilibrate on side 2. By joining the input–output states for e^- and H^+, the model comprises eight states. This simplification is not necessarily valid, however, and a more general scheme should obviously include 16 states.

Specificity of the first kind is not sufficient for the transducer in Figure 10 to pump protons. The input–output transitions must also have a high degree of specificity, also called specificity of the second kind.[117] Thus, the rate constants for the transitions along the horizontal arrows in Figure 10 must be much higher than those for the transitions along the dashed lines. After all, if the transition from E_1(red) to E_2(red) were rapid, the transducer would catalyze electron transfer from D to A (eq 6) without any proton translocation. Such a situation is called "slip" and will be further considered in section VI.C. There could also be a slip with respect to H^+, if E_2(ox)H^+ could return to E_1(ox)H^+. Such a slip would be equivalent to a proton "leak" (section VI.C).

Specificity of the first kind is sometimes called "gating".[120] In my opinion, this term is unfortunate, since a gate can only be closed or open. In the open state, it allows access to both sides of the membrane. Such a situation must never be allowed in an ion pump, however, because in an open gate the flow will always be with the concentration gradient (passive transport), not against it (active transport). Thus, "alternating access"[121] is a better alternative term for specificity of the first kind.

One often sees the statement that a thermodynamic linkage between the redox and protolysis reactions is a requirement for a redox-linked proton pump. This is obviously not true, since thermodynamics is independent of pathway. The only thermodynamic requirement is that the driving force ($-\Delta G°$, where $\Delta G°$ is the standard free energy change) for the reaction in eq 6 is larger than the electrochemical potential against which the pump has to work. A thermodynamic linkage, often called a membrane Bohr effect,[122] would provide a definite kinetic advantage, however, because the states in which the transducer should undergo rapid transitions, namely E_1(red)H^+ and E_2(ox) (Figure 10), would be highly populated.

Interactions are reciprocal, so that if the pK_a of the proton-translocating group is increased on reduction of the pump redox site, then the reduction potential of the redox site must increase on protonation of the transducer. It has, however, already been mentioned (section IV.A.2) that the pH dependence of the reduction potentials of cytochrome *a* and Cu_A, the most likely candidates as pump elements (section VI.D), is small. For this reason, I formulated a few years ago a transition-state mechanism.[123] In this, it was assumed that the proton-translocating group has a constant, low pK_a in both E_1 and E_2. Even if protonation is an uphill reaction in this case, the system will come to equilibrium by picking up a proton on one side of the membrane and leaving it off on the other, because the $E_1 \rightarrow E_2$ transition in the reduced transducer is assumed to be catalyzed by H^+, whereas the return to E_1 in the oxidized transducer is assumed to be inhibited by H^+. In other words, binding of H^+ stabilizes the transition state for the input–output transition. A consequence of such a mechanism is, of course, that the pump would operate at a rate that is only a small fraction of the potential rate at a low (unphysiological) pH. This would perhaps appear to make the transition-state mechanism unattractive, but we have earlier seen that cytochrome oxidase does, in fact, behave exactly in this way (Figure 8).

The cubic scheme (Figure 10) does not say anything about the mechanism of coupling between the redox and protonation reactions. Thus, it could be *direct*, in which case the redox center also provides the proton-translocating group, but it could just as well be *indirect*, in which case the two sites are topologically remote, like the substrate and effector binding sites in an allosteric enzyme. There is some confusion in the literature on this point. Mitchell, for example, appears to think that the term redox-linked proton pump always implies indirect coupling.[124] His own redox-loop concept[1] does, of course, provide a direct mechanism, but a direct coupling may operate also in proton pumps.[117,125]

B. Stoichiometry and Thermodynamics

There have been considerable disagreements in the literature on the value of n in eq 2. Expressed as the H^+/e^- stoichiometry (i.e., $n/4$), values of 0, 1, or 2 have been maintained by various investigators.[118,119] Thermodynamics can, of course, set an upper limit, since the electrochemical potential ($\Delta\mu_{H^+}$) cannot exceed the driving force ($-\Delta G°$) of the reaction of eq 2. Thus, the following equation should give the maximum value (m) of the H^+/e^- stoichiometry of the proton pump

$$-\Delta G°/F = \Delta E° = (m + 1)\frac{\Delta\mu_{H^+}}{F} \qquad (7)$$

where $\Delta E°$ represents the difference in standard reduction potential between O_2 and cytochrome *c* and F is the Faraday constant; the digit 1 refers to the scalar proton (eq 2).

In mitochondria in state 4, i.e., a state where $\Delta\mu_{H^+}$ is maximal and there is no net synthesis of ATP or electron flow, $\Delta\mu_{H^+}$ has been estimated to be slightly more

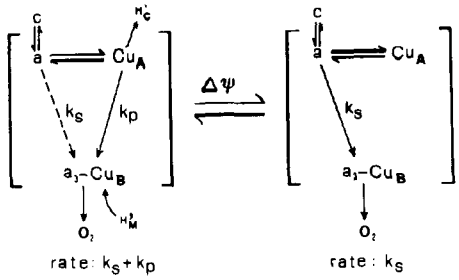

Figure 11. Branched mechanism for electron transfer and proton pumping in cytochrome oxidase. The pumping and slipping conformations are in equilibrium linked by the membrane potential ($\Delta\psi$). For further explanations, see the text. Reprinted from ref 131; copyright 1988 New York Academy of Sciences.

than 0.2 V.[126] Since $\Delta E°$ is about 0.5 V, it would appear that m cannot be much larger than 1 and that a stoichiometry as high as 2 is absolutely impossible on thermodynamic grounds.

An objection to the type of analysis just given is that the stoichiometries are, for obvious reasons, not determined experimentally in state 4, and they could well be higher at low values of $\Delta\mu_{H^+}$. A recent experimental study of the thermodynamics of the proton pump has shown that $m = 2$ becomes possible, if $\Delta\mu_{H^+}$ is lower than 0.15 V,[127] in agreement with simple deliberations based on eq 7. It has, however, also been demonstrated that the stoichiometry is constant in the $\Delta\mu_{H^+}$ range of 0.1–0.2 V.[128] Thus, it is unlikely that the H^+/e^- stoichiometry is ever greater than 1, and it may be even smaller under some conditions, if there are slips in the pump.

It should be noted that the stoichiometry discussed so far is the average stoichiometry for the four electrons transferred in the reaction of eq 2. Wikström[129] has recently reported that actually only the last two of these are coupled to proton translocation. If this is true, then the H^+/e^- stoichiometry of the steps that mechanistically do the pumping is 2. It should be noted that this causes no great thermodynamic problem, since only a small part of the free energy available from the reaction in eq 2 is released in the first two electron transfers, the reduction potential of cytochrome c and the average potential of cytochrome oxidase being not very different. It has, in fact, been estimated that the driving force for electron transfer from Cu_A to the peroxy and oxyferryl intermediates (Figure 9) is about 0.6 V.[113] Thus, according to eq 7, $m = 2$ is possible even at $\Delta\mu_{H^+}$ close to 0.2 V.

C. Slips and Leaks

The reaction in eq 2, unlike that in eq 1, does not have an equilibrium far to the right. Thus, as the electrochemical potential is built, the electron transfer from cytochrome c to O_2 will slow (respiratory control). The slow reaction that still proceeds in state 4 respiration has generally been ascribed to a passive leak of protons back across the lipid bilayer. Brunori et al.[130] have, however, provided kinetic evidence that this cannot be the whole explanation. Instead, they postulated that the electrochemical potential shifts an equilibrium from a pumping conformation (P) to a slipping state (S), as illustrated in Figure 11.[131] According to this hypothesis, pumping is associated with the internal electron transfer from Cu_A to the bimetallic site but there is also a slower pathway for the internal electron transfer from cytochrome a; this does not pump protons. Such parallel pathways have been demonstrated in experiments in which Cu_A has been made redox-inactive by chemical modification.[132] In P, both pathways are operative, and since k_p is larger than k_s, most of the internal electron transfers will lead to proton translocation. In S, on the other hand, only the slipping pathway is functioning.

There are also other indications that the pump may slip under some conditions. In section V.A.2, I presented evidence that the origin of the nonhyperbolic steady-state kinetics is the existence of two conformations that accept electrons from cytochrome c at different rates. In view of the principles discussed in section VI.A, it is very probable that these conformations correspond to the separate input and output states for electrons. The different electron transfer rates in the two conformations would in part be regulated by the changes in potential brought about by the redox interactions (section IV.A.2). Thus, in the input state, cytochrome a has a high potential, whereas the potential of cytochrome a_3 is low. In this way, the reduction of cytochrome a by cytochrome c is rapid but the internal electron transfer is slow. In the output state, on the other hand, the potential of cytochrome a is low but that of cytochrome a_3 high. Consequently, cytochrome a reduction is slow, and internal electron transfer is rapid in this state. This analysis makes it natural to suggest that the S state of Brunori et al. (Figure 11)[130] corresponds to the input state, whereas P is the output state of the proton pump.

The pump may also slip with respect to protons, which would take place if the protonated enzyme in the output state can return to the input state without prior release of the proton. That this actually occurs is evidenced by the finding that the respiratory control ratio, i.e., the ratio in respiration rate in the coupled (state 4) and uncoupled (state 3) states, decreases as the inside pH becomes close to or lower than the pK_a of the proton-translocating group.[133,134]

The occurrence of reaction slips, also called intrinsic uncoupling,[133,135] may seem surprising, since it means that the transducer loses free energy as heat. Theoretical analyses show, however, that slips may be a consequence of difficulties in achieving the stringent control of electron-transfer rates that is required to avoid them.[120,135] Furthermore, slips may become significant only under conditions that are unphysiological, for example, very high concentrations of cytochrome c,[84] or very low pH values,[133] Moderate slips may, in fact, be advantageous, since they allow the pump to operate with a reasonable efficiency over a relatively wide range of electrochemical potential or electron-transfer rate.[120,136] Slips of the type illustrated in Figure 11 at high electrochemical potentials are favorable in the case of cytochrome oxidase, as the scalar reaction will still continue with a concomitant consumption of protons from the inside.[2]

D. Molecular Mechanism

Many hypothetical mechanisms for the proton pump in cytochrome oxidase have been formulated in the literature, as summarized by Krab and Wikström.[117] Most of them involve cytochrome a or Cu_A as the pump element and one of the metal ligands as the proton-

translocating group. There is, however, very little experimental support for any of these mechanisms, and I will not discuss them here. In my opinion such direct mechanisms are, in fact, very unlikely, particularly in view of the demonstration by Wikström[129] that only the two final of the four electron transfers from cytochrome *c* to O_2 pump protons, with a H^+/e^- stoichiometry of 2. Instead, I will in this section briefly consider how some of the established knowledge of the structural, redox, and kinetic properties of the oxidase, summarized in earlier sections, may be related to the proton pump.

My suggestion is that the key reaction in the proton pump is the internal electron transfer from Cu_A to the cytochrome a_3–Cu_B site. This is slow in the input state E_1, which corresponds to S in Figure 11, but rapid in the output state E_2 or P. The rate-limiting step in the overall reaction cycle is the transition from E_1 to E_2. This transition is catalyzed by the binding of two protons:

$$E_1 + 2H^+ \rightleftharpoons E_1(H^+)_2 \rightleftharpoons E_2(H^+)_2 \qquad (8)$$

If these suggestions are correct, then the pH dependence of the steady-state kinetics should be directly related to the reaction in eq 8. Simulations of results from transient kinetics show that the pK_a of one of the proton-binding groups is 6.4,[92,94] which agrees with the pH dependence of the intrinsic uncoupling.[133] Thus, the reaction is slow at pH 7.4, since 10% only of the enzyme is in the form $E_1(H^+)_2$. This means that the pump operates according to the transition-state mechanism (section VI.A).[123] It can be shown that a low pK_a, even if it results in a decreased electron-transfer rate, has an advantage because it results in high efficiency over a wide range of rates.[120,136]

Experiments of the type summarized in section V.C have shown that electron transfer from Cu_A to the peroxo intermediate in the bimetallic site, which is one of the proton-pumping steps,[129] occurs with a rate of about 10^4 s^{-1}, which is then the rate in the electron output state E_2 (or P). It can be demonstrated that the slip rate must in such a case not be greater than 0.1 s^{-1}, if the efficiency should not fall below 90% of the maximum efficiency at a $\Delta\mu_{H^+}$ of 0.2 V.[136] Thus, the rate constant for internal electron transfer in E_1, which corresponds to k_s in Figure 11, must be at least a factor 10^5 lower compared to the rate in E_2. To discuss how such a stringent control of the electron-transfer rates can be achieved, we must first consider the main factors determining the rate of internal electron transfer between two redox centers some distance apart in a protein.

According to Marcus theory,[115] the main factors regulating the rate of intramolecular electron transfer are (i) the driving force, i.e., $-\Delta G°$ for the reaction; (ii) the reorganization energy (λ) related to different nuclear positions in the oxidized and reduced centers; (iii) the distance between the two centers; and (iv) the intervening medium. We know a good deal about the relative importance of these factors for electron transfer in proteins thanks to model studies with proteins of known structure, particularly by Gray and co-workers.[137] Together with Gray, I have applied this knowledge to redox-driven proton pumps,[138] and our analysis suggests that the main factor controlling the electron-transfer rate in such pumps is λ.

I have already suggested (section VI.C) that the observed redox interactions in cytochrome oxidase are related to the need to control the electron-transfer rates in the various states of the pump. The interaction energies are at most a few tenths of a volt, however, which at a distance of 15 Å with λ = 0.5 V could only change the rate by a factor of 10.[137] Combined with a change in λ of 1.0 V, the factor would, on the other hand, be 10^5, as required in cytochrome oxidase.

How such a change in λ is achieved structurally we can only speculate about. Together with Brzezinski, I have suggested that reduction of cytochrome *a* and Cu_A triggers a conformational change, which in an allosteric fashion is transmitted to the bimetallic site, changing its structure in such a way as to lower λ.[101] That there is a change in the structure of the oxidized cytochrome a_3–Cu_B site is evidenced by an altered cyanide reactivity on reduction of the primary acceptors.[139] The structural change is probably transmitted from the primary electron acceptors to the bimetallic site via transmembrane helices, as suggested by Williams.[140] The effect of pressure on the reduction level of cytochrome *a* in the aerobic steady state indicates that this component contracts on reduction.[141] It could then, via the helix, exercize a pull on the bimetallic site.

Despite the fact that cytochrome oxidase undoubtedly is the best characterized of the redox-linked proton pumps operating in respiration and photosynthesis, our knowledge about its mechanism on a molecular level is obviously very rudimentary. The scheme for the coupling of the catalytic electron-transfer reaction to the proton translocation, which I have outlined here, manages to incorporate a significant number of established redox and kinetic properties, but it is still largely hypothetical. Unfortunately, it is probably impossible to draw a more concrete picture without a high-resolution X-ray structure. Our hope thus rests on progress in the methodology for the determination of the structure of integral membrane proteins. The brilliant achievements made in the study of a photosynthetic reaction center[142] suggest that our hope may not be entirely in vain.

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VIII. Literature Citations

(1) Mitchell, P. *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*; Glynn Research: Bodmin, 1966.
(2) Wikström, M. *FEBS Lett.* **1988**, *231*, 247–252.
(3) Wikström, M.; Saraste, M. *Bioenergetics*; Elsevier: Amsterdam, 1984; pp 49–94.
(4) Wikström, M. K. F. *Nature* **1977**, *266*, 271–273.
(5) Malmström, B. G. *Arch. Biochem. Biophys.* **1990**, *280*, 233–241.
(6) Wikström, M.; Krab, K.; Saraste, M. *Cytochrome Oxidase - A Synthesis*; Academic Press: London, 1981.
(7) Wikström, M.; Saraste, M.; Penttilä, T. *Enzymes of Biological Membranes*; Plenum Press: New York, 1985; pp 111–148. Brunori, M.; Antonini, G.; Malatesta, F.; Sarti, P.; Wilson, M. T. *Adv. Inorg. Biochem.* **1988**, *7*, 93–153. Papa, S.; Chance, B.; Ernster, L. *Cytochrome Systems*; Plenum Press: New York, 1987. Vänngård, T. *Biophysical Chem-*

istry of Dioxygen Reactions in Respiration and Photosynthesis; Cambridge University Press: Cambridge, 1988. Brunori, M.; Chance, B, *Cytochrome Oxidase*; New York Academy of Sciences; New York, 1988.
(8) Lemberg, M. R. *Physiol. Rev.* **1969**, *49*, 48–21.
(9) Warburg, O.; Negelein, E. *Biochem. Z.* **1929**, *214*, 64–100.
(10) Warburg, O.; Negelein, E.; Haas, E. *Biochem. Z.* **1933**, *266*, 1–8.
(11) Keilin, D. *Proc. R. Soc. London* **1925**, *B98*, 312–339.
(12) Keilin, D.; Hartree, E. F. *Proc. R. Soc. London* **1938**, *B125*, 171–186.
(13) Keilin, D. *The History of Cell Respiration and Cytochrome*; Cambridge University Press: Cambridge, 1966; pp 223–224.
(14) Keilin, D.; Hartree, E. F. *Proc. R. Soc. London* **1939**, *B127*, 169–191.
(15) Straub, F. B. *Z. Physiol. Chem.* **1941**, *268*, 227.
(16) Yakaushiji, E.; Okunuki, K. *Proc. Imp. Acad. Jpn.* **1940**, *17*, 38.
(17) Fowler, L. R.; Richardson, S. H.; Hatefi, Y. *Biochim. Biophys. Acta* **1962**, *64*, 170–173.
(18) Hartzell, C. R.; Beinert, H.; Van Gelder, B. F.; King, T. E. *Methods Enzymol.* **1978**, *53*, 54–66.
(19) Casey, R. P.; Azzi, A. *FEBS Lett.* **1983**, *154*, 237–242.
(20) Hartzell, C. R.; Beinert, H.; Babcock, G. T.; Chan, S. I.; Palmer, G.; Scott, R. A. *FEBS Lett.* **1988**, *236*, 1–4.
(21) Baker, G. M.; Noguchi, M.; Palmer, G. *J. Biol. Chem.* **1987**, *262*, 595–604.
(22) Brandt, U.; Schägger, H.; von Jagow, G. *Eur. J. Biochem.* **1989**, *182*, 705–711.
(23) Buse, G.; Meinecke, L.; Bruch, B. *J. Inorg. Biochem.* **1985**, *23*, 149–153.
(24) Kadenbach, B.; Kuhn-Nentwig, L.; Buge, U. *Curr. Top. Bioenerg.* **1987**, *15*, 114–162.
(25) Capaldi, R. A.; Takamiya, S.; Zhang, Y. Z.; Gonzalez-Halphen, D.; Yanamura, W. *Curr. Top. Bioenerg.* **1987**, *15*, 91–113.
(26) Ludwig, B. *FEMS Microbiol. Rev.* **1987**, *46*, 41–56.
(27) Ludwig, B.; Schatz, G. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 196–200.
(28) Raitio, M.; Jalli, T.; Saraste, M. *EMBO J.* **1987**, *6*, 2825–2833.
(29) Anderson, S.; De Bruijn, M. H. L.; Coulson, A. R.; Eperon, I. C.; Sanger, F.; Young, I. G. *J. Mol. Biol.* **1982**, *156*, 683–717.
(30) Holm, L.; Saraste, M.; Wikström, M. *EMBO J.* **1987**, *6*, 2819–2823.
(31) Caughey, W. S.; Smythe, G. A.; O'Keefe, D. H.; Maskasky, J. E.; Smith, M. L. *J. Biol. Chem.* **1975**, *250*, 7602–7622.
(32) Einarsdóttir, O.; Caughey, W. S. *Biochem. Biophys. Res. Commun.* **1985**, *129*, 840–847.
(33) Bombelka, E.; Richter, F.-W.; Stroh, A.; Kadenbach, B. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 1007–1014.
(34) Steffens, G. C. M.; Biewald, R.; Buse, G. *Eur. J. Biochem.* **1987**, *164*, 295–300.
(35) Öblad, M.; Selin, E.; Malmström, B.; Strid, S.; Aasa, R.; Malmström, B. G. *Biochim. Biophys. Acta* **1989**, *975*, 267–270.
(36) Beinert, H. *The Biochemistry of Copper*; Academic Press: New York, 1966; pp 213–234.
(37) Capaldi, R. A.; Malatesta, F.; Darley-Usmar, V. M. *Biochim. Biophys. Acta* **1983**, *726*, 135–148.
(38) Hakvoort, T. B. M.; Moolenaar, K.; Lankvelt, A. H. M.; Sinjorgo, K. M. C.; Dekker, H. L.; Muijsers, A. O. *Biochim. Biophys. Acta* **1987**, *894*, 347–354.
(39) Bickar, D.; Lehninger, A.; Brunori, M.; Bonaventura, J.; Bonaventura, C. *J. Inorg. Biochem.* **1985**, *23*, 365–372.
(40) Antonini, G.; Brunori, M.; Malatesta, P. S.; Wilson, M. T. *J. Biol. Chem.* **1987**, *262*, 10077–10079.
(41) Marsh, D.; Powell, G. L. *Bioelectrochem. Bioenerg.* **1988**, *254*, 73–82.
(42) Beinert, H.; Hansen, R. E.; Hartzell, C. R. *Biochim. Biophys. Acta* **1976**, *423*, 339–355.
(43) Beinert, H.; Shaw, R. W.; Hansen, R. E.; Hartzell, C. R. *Biochim. Biophys. Acta* **1980**, *591*, 458–470.
(44) Vanneste, W. H. *Biochemistry* **1966**, *5*, 838–848.
(45) Blair, D. F.; Bocian, D. F.; Babcock, G. T.; Chan, S. I. *Biochemistry* **1982**, *21*, 6928–6935.
(46) Babcock, G. T.; Callahan, P. M.; Ondrias, M. R.; Salmeen, I. *Biochemistry* **1981**, *20*, 959–966.
(47) Eglinton, D. G.; Hill, B. C.; Greenwood, C.; Thomson, A. J. *J. Inorg. Biochem.* **1984**, *21*, 1–8.
(48) Aasa, R.; Albracht, S. P. J.; Falk, K.-E.; Lanne, B.; Vänngård, T. *Biochim. Biophys. Acta* **1976**, *422*, 260–272.
(49) Van Gelder, B. F.; Beinert, H. *Biochim. Biophys. Acta* **1969**, *189*, 1–24.
(50) Lanne, B.; Malmström, B. G.; Vänngård, T. *Biochim. Biophys. Acta* **1979**, *545*, 205–214.
(51) Stevens, T. H.; Chan, S. I. *J. Biol. Chem.* **1981**, *256*, 1069–1071.
(52) Reinhammar, B.; Malkin, R.; Jensen, P.; Karlsson, B.; Andrésson, L.-E.; Aasa, R.; Vänngård, T.; Malmström, B. G. *J. Biol. Chem.* **1980**, *255*, 5000–5003.
(53) Tweedle, M. F.; Wilson, L. J.; García-Iniguez, L.; Babcock, G. T.; Palmer, G. *J. Biol. Chem.* **1978**, *253*, 8065–8071.
(54) Falk, K.-E.; Vänngård, T.; Ängström, J. *FEBS Lett.* **1977**, *75*, 23–27,
(55) Martin, C. T.; Scholes, C. P.; Chan, S. I. *J. Biol. Chem.* **1985**, *260*, 2857–2861.
(56) Stevens, T. H.; Martin, C. T.; Wang, H.; Brudvig, G. W.; Scholes, C. P.; Chan, S. I. *J. Biol. Chem.* **1982**, *257*, 12106–12113.
(57) Cline, J.; Reinhammar, B.; Jensen, P.; Venters, R.; Hoffman, B. M. *J. Biol. Chem.* **1983**, *258*, 5124–5128.
(58) Kent, T. A.; Münck, E.; Dunham, W. R.; Filter, W. F.; Findling, K. L.; Yoshida, T.; Fee, J. A. *J. Biol. Chem.* **1982**, *257*, 12489–12492.
(59) Wang, H.; Sauke, T.; Debrunner, P. G.; Chan, S. I. *J. Biol. Chem.* **1988**, *263*, 15260–15263.
(60) Scott, R. A. *Annu. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 137–158.
(61) Martin, C. T.; Scholes, C. P.; Chan, S. I. *J. Biol. Chem.* **1988**, *263*, 8420–8429.
(62) Zimmerman, B. H.; Nitsche, C. I.; Fee, J. A.; Rusnak, F.; Münck, E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5779–5783.
(63) Brudvig, G. W.; Blair, D. F.; Chan, S. I. *J. Biol. Chem.* **1984**, *259*, 11001–11009.
(64) Malmström, B. G. *FEBS Lett.* **1989**, *250*, 9–21.
(65) Malmström, B. G. *Q. Rev. Biophys.* **1973**, *6*, 389–431.
(66) Nicholls, P. *Dynamics of Energy-Transducing Membranes*; Elsevier: Amsterdam, 1974; pp 39–50.
(67) Wikström, M. K. F.; Harmon, H. J.; Ingledew, W. J.; Chance, B. *FEBS Lett.* **1976**, *65*, 259–277.
(68) Babcock, G. T.; Vickery, L. E.; Palmer, G. *J. Biol. Chem.* **1978**, *253*, 2400–2411.
(69) Lanne, B.; Vänngård, T. *Biochim. Biophys. Acta* **1978**, *501*, 449–457.
(70) Ellis, W. R., Jr.; Wang, H.; Blair, D. F.; Gray, H. B.; Chan, S. I. *Biochemistry* **1986**, *25*, 161–167.
(71) Wang, H.; Blair, D. F.; Ellis, W. R., Jr.; Gray, H. B.; Chan, S. I. *Biochemistry* **1986**, *25*, 167–171.
(72) Blair, D. F.; Ellis, W. R., Jr.; Wang, H.; Gray, H. B.; Chan, S. I. *J. Biol. Chem.* **1986**, *261*, 11524–11537.
(73) Smith, L.; Conrad, H. *Arch. Biochem. Biophys.* **1956**, *63*, 403–413.
(74) Minnaert, K. *Biochim. Biophys. Acta* **1961**, *50*, 23–34.
(75) Myers, D.; Palmer, G. *Cytochrome Oxidase*; New York Academy of Sciences: New York, 1988; pp 85–97.
(76) Malmström, B. G.; Andréasson, L.-E. *J. Inorg. Biochem.* **1985**, *23*, 233–242.
(77) Nicholls, P. *Oxidases and Related Redox Systems*; Wiley: New York, 1965; pp 764–777.
(78) Errede, B.; Haight, G. P., Jr.; Kamen, M. D. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 113–117.
(79) Ferguson-Miller, S.; Brautigan, D. L.; Margoliash, E. *J. Biol. Chem.* **1976**, *251*, 1104–1115.
(80) Speck, S. H.; Dye, D.; Margoliash, E. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 347–351.
(81) Sinjorgo, K. M. C.; Meijling, J. H.; Muijsers, A. O. *Biochim. Biophys. Acta* **1984**, *767*, 48–56.
(82) Capaldi, R. A.; Darley-Usmar, V.; Fuller, S.; Millet, F. *FEBS Lett.* **1982**, *138*, 1–7.
(83) Nalecz, K. A.; Bolli, R.; Azzi, A. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 822–828.
(84) Brzezinski, P.; Malmström, B. G. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4282–4286.
(85) Michel, B.; Bosshard, H. R. *Biochemistry* **1989**, *28*, 244–252.
(86) Michel, B.; Mauk, A. G.; Bosshard, H. R. *FEBS Lett.* **1989**, *243*, 149–152.
(87) Wilms, J.; Van Rijn, J. L. M. L.; Van Gelder, B. F. *Biochim. Biophys. Acta* **1980**, *593*, 17–23.
(88) Thörnström, P.-E.; Soussi, B.; Arvidsson, L.; Malmström, B. G. *Chem. Scr.* **1984**, *24*, 230–235.
(89) Gregory, L. C.; Ferguson-Miller, S. *Biochemistry* **1988**, *27*, 6307–6314.
(90) Brzezinski, P.; Thörnström, P.-E.; Malmström, B. G. *FEBS Lett.* **1986**, *194*, 1–5.
(91) Sarti, P.; Antonini, G.; Malatesta, F.; Vallone, B.; Brunori, M. *Cytochrome Oxidase*; New York Academy of Sciences: New York, 1988; pp 161–166.
(92) Malmström, B. G. *Chem. Scr.* **1987**, *27B*, 67–72.
(93) Sinjorgo, K. M. C.; Steinebach, O. M.; Dekker, H. L.; Muijsers, A. O. *Biochim. Biophys. Acta* **1986**, *850*, 108–115.
(94) Thörnström, P.-E.; Brzezinski, P.; Fredriksson, P.-O.; Malmström, B. G. *Biochemistry* **1988**, *27*, 5441–5447.
(95) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631–5640.
(96) Gibson, Q. H.; Greenwood, C.; Wharton, D. C.; Palmer, G. *J. Biol. Chem.* **1965**, *240*, 888–894.
(97) Antalis, T. M.; Palmer, G. *J. Biol. Chem.* **1982**, *257*, 6194–6206.
(98) Morgan, J. E.; Li, P. M.; Jang, D.-J.; El-Sayed, M. A.; Chan, S. I. *Biochemistry* **1989**, *28*, 6975–6983.

(99) Kobayashi, K.; Une, H.; Hayashi, K. *J. Biol. Chem.* **1989**, *264*, 7976–7980.
(100) Boelens, R.; Wever, R.; Van Gelder, B. F. *Biochim. Biophys. Acta* **1982**, *682*, 264–272.
(101) Brzezinski, P.; Malmström, B. G. *Biochim. Biophys. Acta* **1987**, *894*, 29–38.
(102) Greenwood, C.; Gibson, Q. H. *J. Biol. Chem.* **1967**, *242*, 1782–1787.
(103) Antonini, E.; Brunori, M.; Colosimo, A.; Greenwood, C.; Wilson, M. T. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3128–3132.
(104) Brunori, M.; Colosimo, A.; Rainoni, G.; Wilson, M. T.; Antonini, E. *J. Biol. Chem.* **1979**, *254*, 10769–10775.
(105) Malmström, B. G. *Annu. Rev. Biochem.* **1982**, *51*, 21–59.
(106) Clore, G. M.; Andrēsson, L.-E.; Karlsson, B.; Aasa, R.; Malmström, B. G. *Biochem. J.* **1980**, *185*, 139–154.
(107) Chance, B.; Saronio, C.; Leigh, J. S. *J. Biol. Chem.* **1975**, *250*, 9226–9237.
(108) Hill, B. C.; Greenwood, C. *Biochem. J.* **1984**, *218*, 913–921.
(109) Karlsson, B.; Aasa, R.; Vänngård, T.; Malmström, B. G. *FEBS Lett.* **1981**, *131*, 196–188.
(110) Hansson, Ö.; Karlsson, B.; Aasa, R.; Vänngård, T.; Malmström, B. G. *EMBO J.* **1982**, *1*, 1295–1297.
(111) Blair, D. F.; Witt, S. N.; Chan, S. I. *J. Am. Chem. Soc.* **1985**, *107*, 7389–7399.
(112) Wikström, M. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4051–4054.
(113) Wikström, M. *Chem. Scr.* **1988**, *28A*, 71–74.
(114) Oliveberg, M.; Brzezinski, P.; Malmström, B. G. *Biochim. Biophys. Acta* **1989**, *977*, 322–328.
(115) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322.
(116) DeVault, D. *Biochim. Biophys. Acta* **1971**, *225*, 193–199.
(117) Krab, K.; Wikström, M. *Biochim. Biophys. Acta* **1987**, *895*, 25–39.
(118) Wikström, M.; Krab, K. *Biochim. Biophys. Acta* **1979**, *549*, 177–222.
(119) Wikström, M.; Krab, K.; Saraste, M. *Annu. Rev. Biochem.* **1981**, *50*, 623–655.
(120) Blair, D. F.; Gelles, J.; Chan, S. I. *Biophys. J.* **1986**, *50*, 713–733.
(121) Tanford, C. *Annu. Rev. Biochem.* **1983**, *52*, 379–409.
(122) Papa, S. *Biochim. Biophys. Acta* **1976**, *456*, 39–84.
(123) Malmström, B. G. *Biochim. Biophys. Acta* **1985**, *811*, 1–12.
(124) Mitchell, P. *FEBS Lett.* **1987**, *222*, 235–245.
(125) Malmström, B. G. *FEBS Lett.* **1988**, *231*, 268–269.
(126) Nicholls, D. G. *Eur. J. Biochem.* **1974**, *50*, 305–315.
(127) Murphy, M. P.; Brown, G. C.; Brand, M. D. *FEBS Lett.* **1985**, *187*, 16–20.
(128) Brown, G. C. *J. Biol. Chem.* **1989**, *264*, 14704–14709.
(129) Wikström, M. *Nature* **1989**, *338*, 6776–6778.
(130) Brunori, M.; Sarti, P.; Colosimo, A.; Antonini, G.; Malatesta, F.; Jones, M. G.; Wilson, M. T. *EMBO J.* **1985**, *4*, 2365–2368.
(131) Malatesta, F.; Antonini, G.; Sarti, P.; Vallone, B.; Brunori, M. *Cytochrome Oxidase*; New York Academy of Sciences: New York, 1988; pp 269–276.
(132) Gelles, J.; Chan, S. I. *Biochemistry* **1985**, *24*, 3963–3972.
(133) Maison-Peteri, B.; Malmström, B. G. *Biochemistry* **1989**, *28*, 3156–3160.
(134) Papa, S.; Capitanio, N.; Steverding, D. *Cytochrome Oxidase*; New York Academy of Sciences: New York, 1988; pp 238–253.
(135) Pietrobon, D.; Zoratti, M.; Azzone, G. F.; Caplan, S. R. *Biochemistry* **1986**, *25*, 767–775.
(136) Brzezinski, P. Ph.D. Thesis, Göteborg University, 1989.
(137) Lieber, C. M.; Karas, J. L.; Mayo, S. L.; Albin, M.; Gray, H. B. *Design of Enzymes and Enzyme Models*; Robert A. Welch Foundation: Houston, 1987; pp 9–33.
(138) Gray, H. B.; Malmström, B. G. *Biochemistry* **1989**, *28*, 7499–7505.
(139) Jones, M. G.; Bickar, D.; Wilson, M. T.; Brunori, M.; Colosimo, A.; Sarti, P. *Biochem. J.* **1984**, *220*, 57–66.
(140) Williams, R. J. P. *FEBS Lett.* **1987**, *226*, 1–7.
(141) Kornblatt, J. A.; Hui Bon Hoa, G.; Heremans, K. *Biochemistry* **1988**, *27*, 5122–5128.
(142) Deisenhofer, J.; Epp, O.; Miki, K.; Huber, R.; Michel, H. *Nature* **1985**, *318*, 618–624.