Journal of Bioenergetics and Biomembranes, Vol. 34, No. 4, August 2002 (© 2002)

Cytochrome *c* Oxidase: The Mechanistic Significance of Structural H^+ in Energy Transduction

Baltazar Reynafarje[1] and Jorge Ferreira[2,3]

Received April 30, 2002; accepted June 3, 2002

Changes in the bulk-phase concentration of O_2 and H^+ associated with the reduction of O_2 to water are simultaneously determined in reactions catalyzed by *fully reduced* cytochrome *c* oxidase both isolated and embedded in liposomes. Consistent with the polyphasic kinetics of electron transfer through the oxidase, the time course of O_2 consumption and H^+ translocation exhibit the following novel characteristics: (1) The *uptake of scalar protons* (H_m^+), the *ejection of vectorial protons* (H_v^+), and the *consumption* of O_2, all proceed in a kinetically polyphasic process. (2) *During the first phase of the reaction* the rates of O_2 uptake and H^+ transfer are extremely fast and compatible with the rates of electron flow through the oxidase. (3) The K_m of the oxidase for O_2 is close to 75 μM, the same for O_2 consumption and scalar H^+ uptake. The V_{max} of O_2 reduction to water in reactions catalyzed by the isolated enzyme is, at least, 0.5×10^4 s^{-1}. (4) The extent of vectorial H^+ ejection by cytochrome *c* oxidase embedded in liposomes is an exponential function dependent on both *enzyme concentration* and extent of O_2 consumption. (5) The H^+/O stoichiometry of H^+ ejection is a variable that may reach a maximum value of 4.0 only when the enzyme undergoes *net oxidation* at extremely high enzyme/O_2 molar ratios. It is postulated that the generation of useful energy at the level of cytochrome *c* oxidase depends not only on the number of molecules of O_2 reduced to water but also on the *extent and state of reduction and/or protonation of the enzyme*.

KEY WORDS: Cytochrome oxidase; H^+/O stoichiometry; structural protons; proton pumping; energy transduction.

INTRODUCTION

The classic chemiosmotic hypothesis (Mitchell, 1961) postulates that a *bidirectional translocation of H^+* across the mitochondrial inner-membrane couples the transfer of electrons with the synthesis of ATP. The proton-motive force (Δ_P), generated during the respiratory process of H^+ ejection, drives the synthesis of ATP by drawing the return of ejected H^+ to the mitochondrial matrix through the ATP synthase. A central but still contentious element of the hypothesis concerns the stoichiometric relationship that exists between all, the flow of electrons, the translocation of H^+ (in and out of the mitochondria), the consumption of O_2, and the synthesis of ATP (see Brand, 1994). The Mitchell's hypothesis postulates that, like in a simple chemical reaction, the $H^+/2e^-$, H^+/O, H^+/ATP, and ATP/O stoichiometries are constants. To this day, however, the value of these stoichiometries remains an open question. In fact, the author of the hypothesis stated, "the mechanism of energy transduction will not be elucidated until the question of the H^+/O ratio is resolved" (Mitchell *et al.*, 1986). The main reason for the delay in solving this important problem may stem from the inherent difficulties in determining *simultaneously* the rates and extents of all, O_2 consumption, electron transfer, H^+ uptake,

Key to abbreviations: Δp, electrochemical gradient of protons (proton-motive force); Fe_{a3}–Cu_B, oxygen-binding and catalytic site of cytochrome *c* oxidase; H_m^+, scalar protons originated in the external medium; H_s^+, external protons that originate in the substrate; H_v^+, vectorial protons extruded by the enzyme; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TMPD, *N*,*N*,*N*′,*N*′-tetraphenylenediamine; TN, turnover number.

[1]Department of Biological Chemistry, The Johns Hopkins University School of Medicine 725 N. Wolfe Street, Baltimore, Maryland 21205.

[2]Programa de Farmacología Molecular y Clínica, Facultad de Medicina, Universidad de Chile, Independencia 1027, Casilla 70086, Santiago 7, Chile.

[3]To whom correspondence should be addressed; e-mail: jferreir@machi.med.uchile.cl.

0145-479X/02/0800-0259/0

and H^+ extrusion. The pioneering work of Gibson and Greenwood (1963) and Greenwood and Gibson (1967) has provided the bases and the tools to study in detail the kinetics of electron transfer through the oxidase. Thus, in spite of the complexity and polyphasic nature of the electrical process (Babcock and Wikström, 1992; Hill, 1991; Hill and Greenwood, 1984), detailed mechanisms for O_2 reduction and H^+ pumping have been proposed (Brzezinski and Adelroth, 1998; Varotsis *et al.*, 1993; Wikström *et al.*, 1998). However, it is still necessary to explain why the steady state rates of electron transfer (Han *et al.*, 2000; Verkhovskaya *et al.*, 1997) and oxygen consumption (Reynafarje, 1991; Reynafarje and Davis, 1990) suddenly decrease from maximum of $\approx 1.0 \times 10^4$ s^{-1} to minimum of ≈ 1.0 s^{-1}. Recent advances in the three-dimensional crystal structure of the oxidase have contributed to clarify the role played by protons in the process of oxygen reduction (Adelroth *et al.*, 1998; Iwata *et al.*, 1995; Tsukihara *et al.*, 1996; Verkhovsky *et al.*, 1995; Yoshikawa *et al.*, 1998). Thus, the slow rates of electron flow between Fe_a and Fe_{a3} is apparently because of limitations in proton uptake rather than impairments in the tunneling event (Brunori *et al.*, 1994; Malatesta *et al.*, 1990). Limitations in the immediate availability of protons were also postulated to explain the slow rates of O_2 consumption (≈ 1.0 s^{-1}) during the second phase of the respiratory process (Reynafarje, 1991).

Here, we have studied the effect of medium pH and the concentrations of enzyme, cytochrome *c*, and O_2 on the simultaneous processes of O_2 consumption, vectorial H^+ ejection and scalar H^+ uptake as they proceed immediately after the diffusion-controlled encounter of O_2 with the fully reduced enzyme. The results show that the *extents of H^+ transfer* depend not only on the extent of O_2 consumption but also on the *concentration and degree of reduction and/or protonation* of the enzyme itself. The data also shows that the vectorial H^+/O stoichiometry is a variable that approaches the maximal value of 4.0 only when O_2 is entirely consumed during the period in which cytochrome *c* oxidase undergoes net oxidation. The study indicates that the origin, the pathways and the final destination of structural protons are intrinsically involved in the mechanism and efficiency of energy transduction.

MATERIALS AND METHODS

Source of Enzyme, Chemicals, and Materials

Cytochrome *c* oxidase from bovine heart and liposomes were prepared as previously described (Hendler *et al.*, 1991; Wrigglesworth *et al.*, 1987). Horse heart cytochrome *c* (type IV), succinate, NADH, 1 (+) ascorbate, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and *N*,*N*,*N*′,*N*′-tetraphenylenediamine (TMPD) were products of Sigma Chemical Co. All other reagents were of analytical grade. The standard reaction mixture in 1.65 mL of total volume at 24°C and pH 7.05 contained 200 mM sucrose, 50 mM KCI, 3.0 mM HEPES, 10 mM ascorbate and the indicated concentrations of cytochrome *c*, O_2, and enzyme, with 1 μM Valinomycin. The air-saturated medium had an O_2 content of 230 μM (Reynafarje *et al.*, 1985). The reaction mixture was stirred with a glass-coated magnetic bar driven at a speed of 2,000 rpm.

Changes in the O_2 concentration of the medium were measured using an oxygen electrode (Davies, 1962) that has a 90% response time of about 10 ms as compared with the conventional Clark electrode that has a respond time of near 2.0 s. To determine the thermodynamic activity of H^+ in the medium we used a combination glass electrode with a 90% response time of about 300 ms. The electrical signals of the electrodes were suitably amplified and fed into a Soltec multichannel recorder model 330 whose chart was run at a speed of 120 cm min^{-1}. The molar absorption coefficient of the 200-kDa enzyme (Tsukihara *et al.*, 1996), as measured at 605–630 nm, was 17.6×10^3 M^{-1} cm^{-1}.

Methodology Used to Monitor Changes in H^+ and O_2 Concentration

Reactions were initiated by injecting small volumes of air-saturated medium into anaerobic test-systems containing fully reduced forms of the enzyme. The following equation was used to calculate the initial rates of O_2 and H^+ uptake.

$$\text{Rate} = \Delta[\text{reactant}]/\Delta t = k[\text{reactant}]^n \qquad (1)$$

The method selected to calculate the proportionality or rate constant k and the order of the reaction n depended on the type of rate law that most precisely applied to the data obtained. As a first approximation it was assumed that the rates of reaction depended on O_2 concentration to the first power, i.e., $n = 1.0$. Therefore, the following integrated equation was used to calculate the value of k.

$$\ln[O_2]_t = -kt + \ln[O_2]_0 \qquad (2)$$

Where $[O_2]_t$ is the concentration at any time and $[O_2]_0$ the concentration at zero time. We considered that the reaction depended on oxygen concentration to the first power when a plot of $\ln [O_2]_t$ versus time (taken at intervals of 20 ms) generated a straight line within the first 700 ms of the reaction. Because the rates of H^+ *uptake* double by doubling

the amount of oxygen added ($n = 1.0$), the initial rates of H^+ and O_2 utilization were for the most part calculated by measuring the slope of the traces at steady state. Under special experimental conditions (see Fig. 1(B), however, the initial rates of O_2 uptake were calculated using differential procedures. In all such instances, the amount of O_2 consumed in the first phase was calculated by subtracting the amount of O_2 remaining at the end of the first phase from the amount of O_2 added at zero time. In this calculation, however, we used only 75% of the time elapsed in the first phase, thus excluding the time elapsed during the tailing of the trace at the end of the first phase. To determine the extent of O_2 just bound to the protein we recorded the signal of the O_2-electrode after injecting different concentrations of oxygen (from 0.092 to 230 μM) into anaerobic reaction mixtures devoid of either enzyme or cytochrome *c*.

RESULTS AND DISCUSSION

Cytochrome *c* Oxidase Catalyzes the Reduction of O_2 to Water in a Kinetically Polyphasic Process

The results presented in Fig. 1 show that the millisecond kinetics of O_2 and H^+ uptake, in reactions catalyzed by *isolated* and fully reduced cytochrome *c* oxidase, takes place in an essentially polyphasic process with the following novel characteristics. First, the *steady state rates* of O_2 and H^+ uptake during the *first phase* of the reaction are extremely fast and perfectly compatible with the rates of electron flow through the oxidase. Thus, the trace b of Fig. 1 shows that, even in the presence of only 4.6 nmols of O (1.39 μM O_2), the first phase proceeds at the respective rates of H^+ and O uptake of about 10,000 and 5,000 nmols min^{-1} mg^{-1} of protein (33.3 and 16.6 turnovers s^{-1}, respectively). Consequently, the first phase of O_2 uptake cannot be simply attributed to O_2 binding. Second, regardless of the initial concentrations of O_2 and protein, there is always an abrupt transition between the *first and second phases* of both H^+ and O_2 uptake. Although the transition was previously attributed to limitations in the immediate availability of H^+ (Reynafarje, 1991; Reynafarje and Davies, 1990), the real reason remains an open question (see however Fig. 4). Third, the rates of O_2 uptake in the *second phase* are orders of magnitude lower than in the first phase but kinetically identical to the rates of O_2 uptake in reactions catalyzed by mitochondria under State 4 conditions (Chance and Williams, 1955). The rates of O_2 and H^+ uptake during the third and following phases decrease as the amount of O_2 in the medium disappears. Fourth, regardless of experimental conditions, the H_m^+/O ***rate ratio*** is always 2.0. The

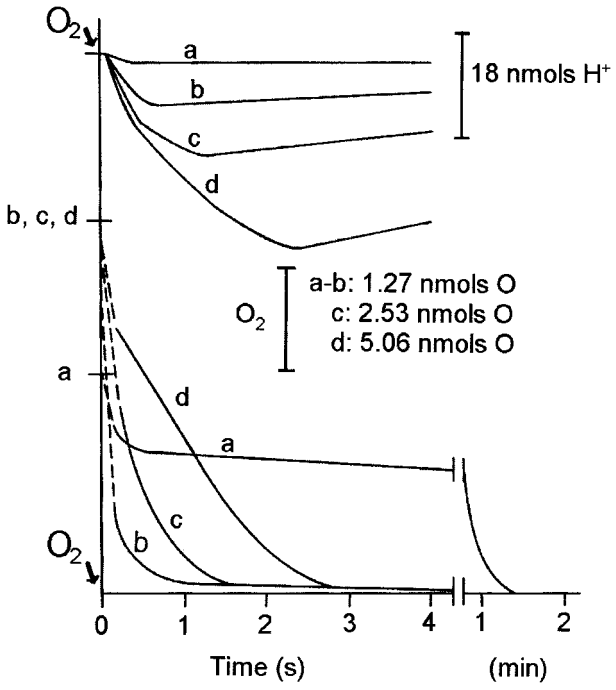

Fig. 1. Time courses of O_2 and H_m^+ uptake in reactions catalyzed by *purified* cytochrome *c* oxidase. The standard reaction medium (see Materials and Methods) contained ascorbate (10 mM), TMPD (90 μM and cytochrome *c* (60 μM), and the following amounts of cytochrome oxidase from bovine heart: 0.05 nmols in "a," and 0.9 nmols in "b," "c," and "d." Reactions were initiated at zero time by adding 2.76 nmols O in "a," 4.6 in "b," 9.2 in "c," and 18.4 in "d." The broken lines in the oxygen traces show the initial phase of the reaction during which dilution and net consumption of O_2 may overlap. The downward defection of the pen indicates uptake of O_2 and H_m^+. The electrical signals of the O_2 electrode were suitably modified to fit the recorded changes in O_2 uptake as indicated in the scale at the right-hand side of the figure and the crossing bars in the *Y*-axis. Note that the time scale in the *X*-axis change to minutes after 4 s of reaction.

H_m^+/O ***extent ratio*** *at the end of the first phase*, however, may be under certain conditions somehow lower than 2.0. This abnormality is most likely due to the overlapping between the uptake of medium H_m^+ and the release of substrate (ascorbate) H_s^+ (see scheme in Fig. 9). The possibility that a significant portion of H^+ involved in the reduction of O_2 to water proceed directly from protonated clusters around the Fe_{a3}–Cu_B center (Kannt *et al.*, 1998) is however not ruled out. Fifth, the number of molecules of O_2 and H^+ utilized in the first phase depends not on the initial concentration of O_2 alone but most importantly on the number of molecules O_2 present per molecule of enzyme. Thus, although the initial concentration of O_2 is much larger in trace "d" (18.4 nmols O) than in trace "a" (2.76 nmols O), the percent of O_2 consumed in the first phase is practically the same (≈29%) because the O_2/enzyme molar ratio is 55.2 (2.76/0.05) in trace "a"

and 20.2 (18.4/0.9) in trace "d." These results provide evidence that the first phase of the respiratory reaction—the phase most directly associated with energy transduction (personal observations)—has a K_m for O_2 that is indeed much higher than what is believed up to now (see below).

The Extent of O_2 and H^+ Uptake in the First Phase of the Reaction Depends on the Relative Concentrations of Enzyme, O_2, and Cytochrome *c*

Figures 2 and 3 show that, as shown in Fig. 1, the ***extent of enzyme*** regulates, to a large extent, the ***amount*** of O_2 reduced to water during the first phase of the reaction. Figure 2(A) shows that, at a fixed concentration of cytochrome *c* (60 μM), the ***fraction*** of O_2 consumed in the first phase depends on both initial concentration of O_2 (abscissa) and enzyme concentration. Note that in the presence of 20 nmols of O the ***fraction*** of O_2 reduced to water in the first phase increases from 26.3 to 93.3% when the amount of enzyme increases from 0.3 to 3.6 nmols. Fig. 2(B) shows that, at a fixed amount of enzyme (1.14 nmols), the dependence of O_2 consumption on cytochrome *c* concentration is less dramatic than the dependence on enzyme concentration. Above 1 turnover or 2.2 nmols of O_2 reduced to water, the amount of O_2 consumed in the first phase only increases 30% when the concentration of cytochrome *c* increases from 3 to 60 μM.

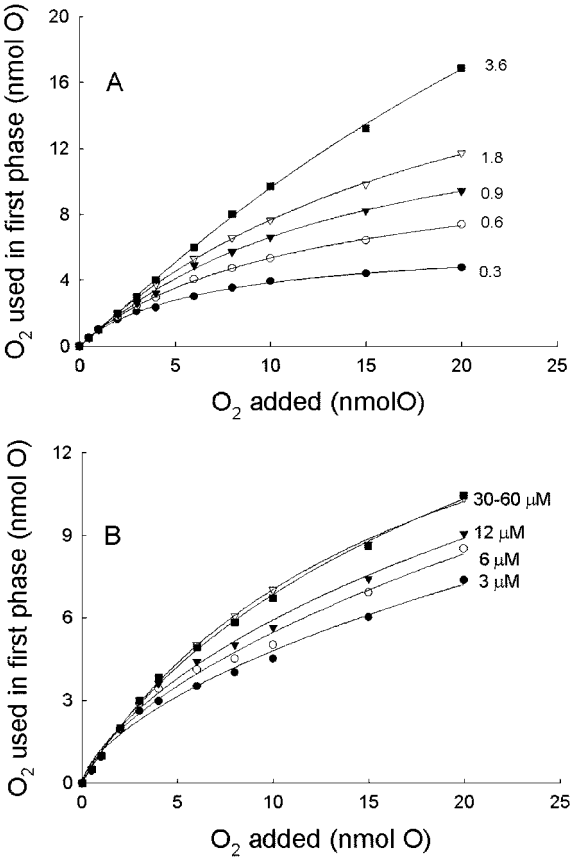

Fig. 2. Dependence of the *extent* of O_2 consumed in the fast phase on the concentrations of enzyme, O_2, and cytochrome *c*. Experimental conditions are as indicated in Fig. 1. (A) The concentration of cytochrome *c* was 60 μM and the amount of enzyme in nmols as indicated at the right side of each trace. Each point is the arithmetic mean of at least two values. (B) The amount of enzyme was the same in all experiments (1.14 nmols) but the Micromolar concentration of cytochrome *c* varied as indicated at the right side of each trace.

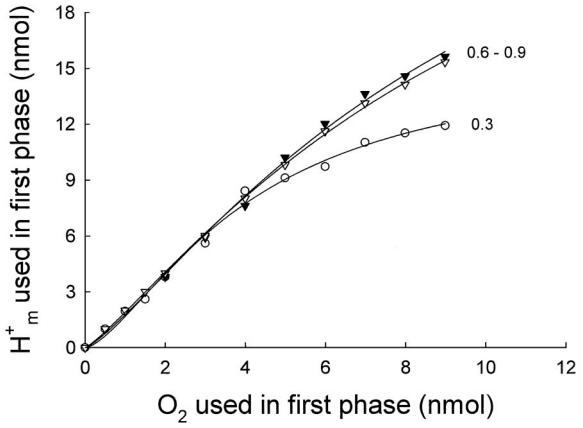

Fig. 3. Stoichiometric relationship between *extents* of H_m^+ and O_2 uptake during the first phase of the reaction. Experimental conditions are as in Fig. 1 except that the concentration of cytochrome *c* was equal to 73 μM (120 nmols). Reactions were initiated by adding O_2 (abscissa) to anaerobic suspensions of fully reduced enzyme (from 0.3 to 0.9 nmols) as indicated at the right side of each trace.

Figure 3 provides direct experimental evidence that, even in the presence of very low concentrations of O_2, the consumption of O_2 during the first 300 ms of reaction is directly related to the uptake of medium H_m^+ with a H_m^+/O stoichiometry of 2.0 (see also Fig. 1 and Table I). In other words, the fraction of O_2 that disappears in the first phase is due to the O_2 reduction to water and not to just binding that under these conditions is practically negligible. Only at high concentrations of both O_2 and enzyme the H_m^+/O stoichiometry is less than 2.0 due to overlapping with substrate protons (H_s^+) as shown in Fig. 9. Indeed, the true V_{max} and K_m of the oxidase for O_2 can be evaluated by separately measuring the rates of electrons transfer (Hill, 1991; Hill and Greenwood, 1984), O_2 consumption, or H_m^+ uptake in reaction in which the oxidase undergoes *net oxidation* in the presence of high concentration of reduced cytochrome *c* at the optimal pH (see below).

The pH of the Medium Effectively Modulates the Initial Rates of H^+ and O_2 Uptake

Consistent with the results obtained using the flow-flash procedure (Hallen and Nilsson, 1992), Fig. 4 shows that the ***initial rates*** of H_m^+ uptake are extremely sensitive to both O_2 concentration and external pH. Under current experimental conditions, the rates of H_m^+ uptake increase from 23 to 43 turnovers s^{-1} when the external pH increases from 6.15 to 6.5 and decreases from 43 to only 7.5 turnovers s^{-1} when the pH of the medium further increases from 6.5 to 7.7. The data also shows that at every pH the rates of H_m^+ uptake reach a maximum value that remains constant in spite of the fact that the concentration O_2 increases from 2.0 to 11.2 μM (6.6 to 36.8 nmols of O). The apparent lack of dependency of H_m^+ uptake on O_2 concentration does not mean, however, that the enzyme has many V_{max} or that the K_m for O_2 is lower than 2.0 μM since in these experiments the rates of reaction are limited by the concentrations of both O_2 and H_m^+ (pH of the medium). Obviously, the real V_{max} of the oxidase can only be attained when the enzyme is saturated with its three substrates (electrons, protons, and O_2). Likewise, the real K_m for O_2 can only be attained when the initial rates of O_2 or H_m^+ uptake are determined in the presence of an excess of reduced cytochrome *c*, at optimal pH and, under current conditions, during the first 200 ms of reaction. In reality the rates of reaction depend not only on the concentrations of O_2 and H_m^+ but also on the concentration of the enzyme itself. Thus, Fig. 4 shows that at an O_2 concentration of 2.3 nmols of O (0.7 μM) and at pH 7.0, the rates of H_m^+ uptake ***increase*** from 14 turnovers s^{-1} (trace with filled triangles) to 54 turnovers s^{-1} (single point at the left upper corner) when the amount of enzyme ***decreases*** from 0.9 to 0.08 nmols, i.e. when the number of molecules of enzyme per molecule of O_2 initially present increases from 2.55 (2.3/0.9) to 28.7 (2.3/0.08). Evidently, the rates of O_2 reduction to water are limited not only by substrate concentration but, in all probability, also by the *extent and state of reduction and/or* ***protonation*** *of the enzyme*.

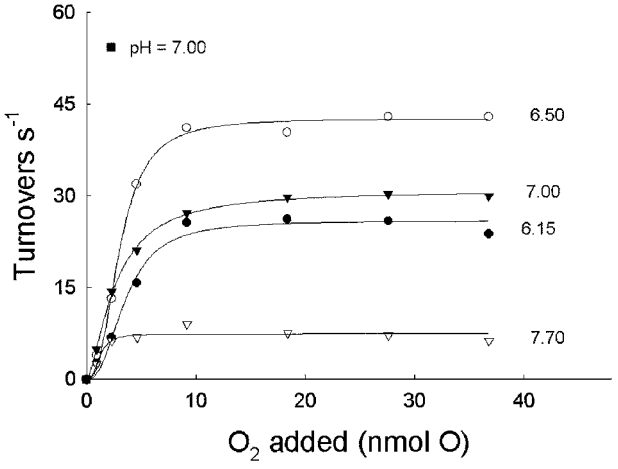

Fig. 4. Dependence of the initial rates (turnovers) of H_m^+ uptake on the pH of the medium. The experimental conditions were the same as those described in Fig. 1. Reactions were initiated by injecting oxygen (abscissa) into anaerobic suspensions of 0.9 nmols (0.54 μM) of fully reduced enzyme supplemented with cytochrome *c* (121 μM) plus ascorbate (10 mM). The pH of the medium varied between 6.15 and 7.7, as indicated at the end of each trace. The single point on the upper left corner of the figure represents the number of turnovers in an experiment carried out under identical conditions as in Fig. 4 by adding 4.6 nmols of O to an anaerobic suspension of only 0.08 (0.05 μM) of enzyme (see legend to Fig. 5 and Table I).

True Initial Rates of O_2 and H^+ Uptake Depend on O_2 Concentration Obeying Michaelis–Menten Kinetics

Although it is well known that even a mild physical exercise is performed with great difficulty when the concentration of O_2 in the arterial blood is less than 50 μM (Reynafarje, 1966), the concept that the K_m of the respiratory chain for O_2 is very low (<1.0 μM) still prevails. The results of this study provide experimental evidence that the initial rates of H^+ and O_2 uptake, in reactions catalyzed by fully reduce cytochrome oxidase in the absence of respiratory inhibitors such as CO or CN^-, depend on O_2 concentration strictly obeying Michaelis–Menten kinetics. Data presented in Fig. 5 and Table I show that the Turnover Number (V_{max}) of the oxidase for H_m^+ and O_2 uptake is respectively close to 0.52 and 0.13×10^4 s^{-1} and that the K_m for O_2 is near 75 μM, i.e. at least 75-fold higher than generally believed. The K_m value of 75 μM is identical to that obtained in few trustworthy studies using flow/flash techniques (Greenwood and Gibson, 1967; Hill, 1991; Hill and Greenwood, 1984) and very low temperatures (Chance *et al.*, 1975). Furthermore, by simultaneously determining the initial rates of O_2 uptake and ATP synthesis in reactions catalyzed by submitochondrial particles, we have recently found that the K_m for O_2 during oxidative phosphorylation is close to 70 μM (unpublished observations). Here, by determining the initial rates of both O_2 and H_m^+ uptake under closely resembling physiological conditions, we provide direct experimental evidence that the K_m of the oxidase for O_2 is in reality orders of magnitude higher then generally believed.

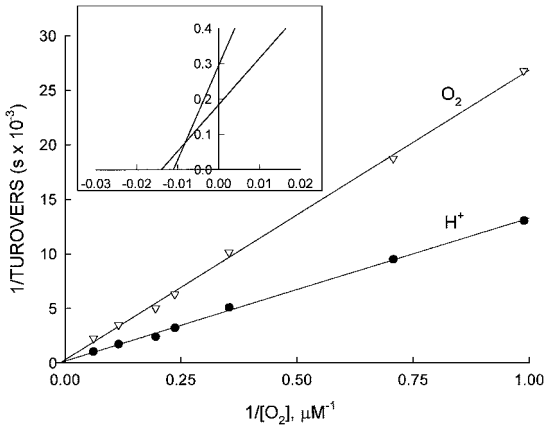

Fig. 5. Double reciprocal plots of O_2 consumption and H_m^+ uptake versus O_2 concentration in reactions catalyzed by *isolated* cytochrome *c* oxidase. Experimental conditions are as in Fig. 1, with 100 μM of cytochrome *c*, 10 mM of ascorbate and 0.05 μM (0.08 nmols) of Bovine-heart cytochrome *c* oxidase at a medium pH of 7.0. The linear regression analysis of the initial rates of H^+ and O_2 uptake has a correlation of 0.99. Values represent averages of at least two determinations in the range of O_2 concentrations between 1.0 μM (3.3 nmols O) and 13.9 μM (46.0 nmols O). The inset in Fig. 5 represents a blowup of the data near the origin of the coordinates.

The Time Course of H_v^+ Ejection by Cytochrome *c* Oxidase Embedded in Liposomes is Intrinsically Polyphasic

Consistent with the polyphasic nature of O_2 consumption (Reynafarje, 1991) and comparable to the submillisecond multiphase kinetics of electron flow (Hill, 1991; Hill and Greenwood, 1984) and H^+ transfer (Brzezinski and Adelroth, 1998), data presented in Fig. 6 shows that the polyphasic processes of O_2 uptake and H^+ transfer have the following novel characteristics. First, the rates of O_2 and H^+ uptake during the first phase are extremely fast and perfectly compatible with the rates of electron flow through the oxidase. Second, the first phase of all, vectorial H_v^+ ejection, scalar H_m^+ uptake, and O_2 consumption are abruptly interrupted in less than 300 ms to continue during a second phase in which the rates are two to three orders of magnitude slower than in the first phase. Third, although the first phase of O_2 uptake is as simple as in reactions catalyzed by the isolated enzyme (see Fig. 1), the first phases of H_m^+ uptake and H_v^+ ejection are extremely complex due to the simultaneous

Table I. The Kinetics of Oxygen and Proton Utilization in Reactions Catalyzed by Purified Cytochrome *c* Oxidase

O_2 added (nmols O)	Extent of O_2 and H^+ uptake in the first phase of the reaction		Initial rates of O_2 and H^+ consumption (turnovers per second)	
	nmols O	nmols H^+	O	H^+
2.3	0.96	1.88	26.3	54.3
4.6	1.85	3.76	53.4	107.0
9.2	3.10	6.30	99.9	198.6
13.8	4.66	8.27	155.3	311.0
18.4	5.92	11.0	202.0	408.0
27.6	7.50	15.0	281.9	588.5
46.0	12.0	24.9	457.7	917.0

Note. The experimental conditions were identical to those described in the legend of Fig. 5.

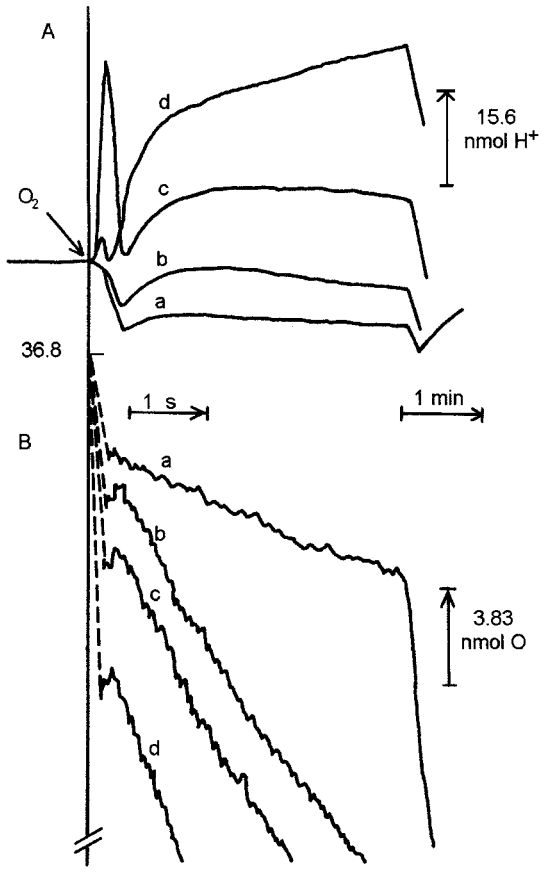

Fig. 6. Time course of O_2 consumption, H_m^+ uptake and H_v^+ ejection in reactions catalyzed by cytochrome *c* oxidase embedded in liposomes. The experimental conditions were as in Fig. 1, with 1.0 μM of valinomycin and 100 μM of cytochrome *c*. Liposomes containing 0.15 nmols of enzyme in "a," 0.6 in "b," 1.2 in "c," and 2.3 in "d" were incubated for at least 30 min prior to the addition of 36.8 nmols O (11.2 μM O_2) in all experiments. In the upper portion of the figure the downward and upward deflections of the traces indicate uptake and ejection of H^+, respectively. In the lower portion of the figure the downward deflection of the traces represents O_2 disappearance. The dashed lines represent the portion of the first phase during which dilution and actual reduction of O_2 may overlap.

ejection and uptake of vectorial protons. Thus the data shows that, for the same amount of O_2 added (36.8 nmols O), the uptake of H_m^+ catalyzed by 0.15 nmols of enzyme (trace "a") proceeds with a H_m^+/O stoichiometry of 2.0 with absolutely no apparent *net* ejection of H_v^+. As the enzyme concentration increases to 0.6 nmols (trace "b"), the extent of H_m^+ uptake decreases so that the observed H_m^+/O is lower than 2.0. At the same time the ***first phase*** of H_v^+ *ejection and its subsequent reuptake* begin to appear (traces "c" and "d"). At very high concentrations of enzyme (2.3 nmols in traces "d") the rates are so high that the *first* phase of the reaction ends in less than 150 ms with only traces of *net* ejection and *net* reuptake of vectorial H_v^+. Fourth, regardless of the initial concentration of O_2, the ***amount*** of O_2 consumed in the *first phase* (dashed lines) is directly proportional to enzyme ***concentration***. Thus, Fig. 6 shows that out of 36.8 nmols of O added, 4.4 nmols O are consumed during the first phase in the presence of 0.15 nmols of enzyme and 14.0 nmols O in the presence of 2.3 nmols of enzyme.

The results presented in Fig. 6 bring into light current discrepancies concerning the real value of the H_v^+/O stoichiometry of the respiratory chain (Brand, 1994; Brand *et al.*, 1976) and the order of events in the transfer of H^+ at the level of the oxidase; i.e., whether the pumping of H_v^+ follows or takes precedence over the uptake of medium H_m^+ (Michel, 1999; Verkhovsky *et al.*, 1999; Wikström, 1989 and 2000). Our results indicate, first, that the H_v^+/O stoichiometry is a variable and, second, that the ejection and uptake of H^+ can occur simultaneously provided the availability of all, O_2, electrons, medium H_m^+, and enzyme (structural H^+) do not limit the very fast initial phase of the reaction. Only when the enzyme/O_2 molar ratio is extremely low, and the rates of reaction are limited by the extent of conformational changes associated with the redox state of the Fe_{a3}–Cu_B center, the uptake of medium H_m^+ is not accompanied by the ejection of vectorial H_v^+ (see trace "a" in Fig. 6). Obviously, the transition from the extremely rapid first phase to the very slow second phase is not due exclusively to limitations in the availability of H^+ but also to limitations in the relative concentrations of electrons, medium H_m^+ (external pH), O_2, and *extent and state of reduction and/or protonation* of the enzyme (see below).

The H_v^+/O Stoichiometry of H_v^+ Pumping by Cytochrome *c* Oxidase Embedded in Liposomes is a Variable With a Maximal Value of 4.0

The results presented in Fig. 7 provide, for the first time, direct experimental evidence that the overall extent of H_v^+ ejection is a sensitive function of both enzyme concentration and initial concentration of O_2. The consensus is that, *independently* of enzyme concentration, the extent of H_v^+ ejection only depends on the total amount of O_2 consumed in the reaction and that the observed H_v^+/O stoichiometry at the level of the oxidase is always 2.0. Data presented in Fig. 7 provides convincing experimental evidence that the H_v^+/O stoichiometry is not a constant but that varies depending on both *O_2 and enzyme concentration*. In reality, the value of the H_v^+/O stoichiometry depends directly on the enzyme/O_2 molar ratio. Thus, in the presence of 1.5 nmols of enzyme the H_v^+/O stoichiometry is 2.7 when the enzyme/O_2 molar ratio is 0.65 (1.5/2.3) and only 0.96 when this ratio is 0.04 (1.5/36.8). These results demonstrate that it is not the number of molecules of O_2 consumed per unit of enzyme but the number of molecules of enzyme (protonated groups?) per molecule of O_2 reduced to water that actually determines the value of the H_v^+/O stoichiometry.

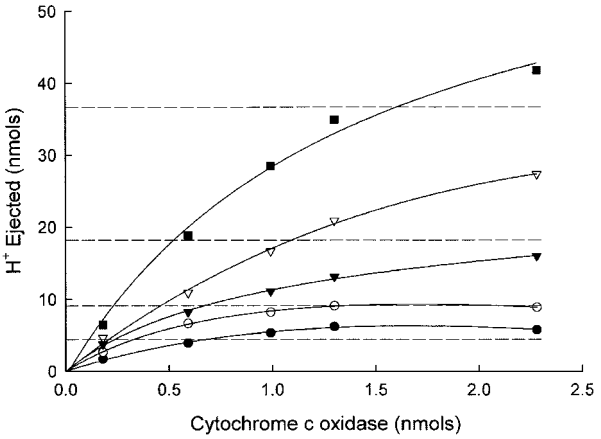

Fig. 7. Dependence of the overall extent of H^+ ejection on the extent of both enzyme and O_2 consumed in the entire reaction. The experimental conditions were as in Fig. 6. Reactions were initiated by adding 2.3 nmols of O in (●), 4.6 in (O), 9.2 in (▼), 18.4 in (▽), and 36.8 in (■) to anaerobic suspensions of enzyme as indicated in the abscissa. The horizontal dotted lines represent the assumedly constant H_v^+/O stoichiometry of 2.0 in reactions in which the total amount of O consumed varied between 2.3 and 18.4 nmols.

Figure 8 shows that, consistent with the results presented in Figs. 6 and 7, the *overall* H^+/O stoichiometry is an exponential function of both enzyme concentration and extent of O_2 consumed in the first phase of the reaction. The higher the ratio between enzyme concentration and O_2 consumed in the first phase the higher is the H_v^+/O stoichiometry and the closer to the maximal value of 4.0 as predicted by Wikström (1977). Definitely these results cannot be attributed to experimental artifacts such as those related with the release of H^+ from ascorbate since these H^+ would increase not decrease the observed H_v^+/O

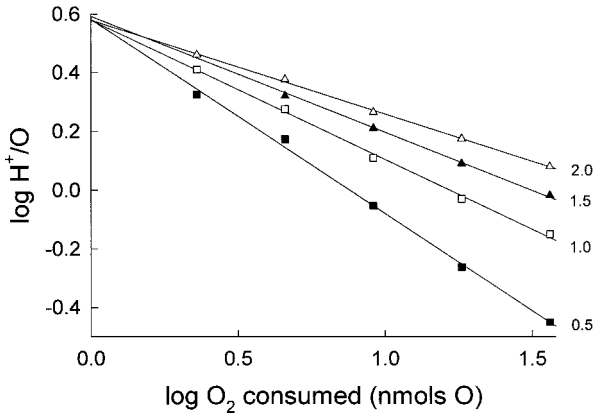

Fig. 8. Exponential dependence of the vectorial H^+/O stoichiometry on both enzyme concentration and extent of O_2 consumed in the reaction. Experimental conditions were as described for Fig. 6. Reactions were initiated by adding from 2.3 to 36.8 nmols of O (abscissa) to anaerobic suspensions of liposomes containing the fully reduced enzyme at the concentrations (in nmols/system) indicated at the right-hand side of each line.

stoichiometry in the presence of high concentrations of O_2 (see the scheme shown in Fig. 9).

The results of this study strongly suggest, in accordance with a recent report (Liebl *et al.*, 1999), that the protonation and deprotonation of the enzyme is mechanistically involved in the different phases of electron transfer, O_2 reduction, H^+ uptake, and H^+ ejection. The structure and function of the oxidase, and increasing evidence that a low barrier hydrogen bond (LBHBs) plays an important role in enzyme catalysis (Cleland *et al.*, 1998), support the above inference. It is therefore concluded that the H_v^+/O stoichiometry is, under current and in all probability normal physiological conditions, a variable that subtly depends on the constantly changing concentrations of all the substrates involved in the reduction of O_2 to water, including the *extent and state of reduction and/or protonation* of the enzyme itself.

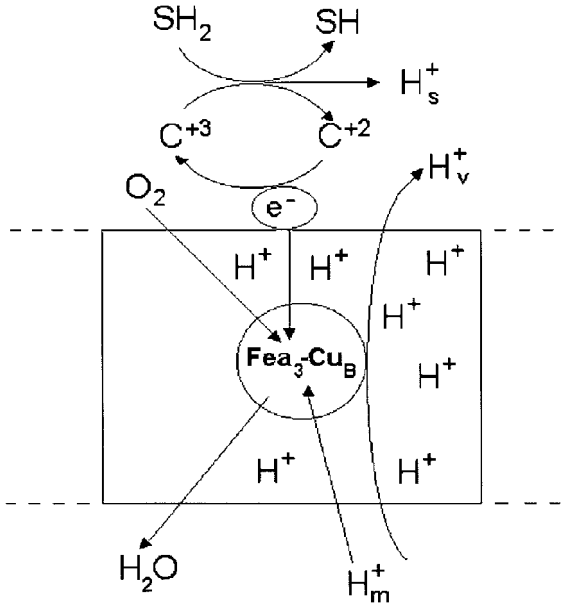

Fig. 9. Diagrammatic representation of the oxidase embedded in liposomes showing the pathways of O_2, electron, and H_m^+ uptake, together with the pathway of vectorial H_v^+ and substrate H_m^+ ejection.

ACKNOWLEDGMENTS

The authors thank Dr. Sally H. Cavanaugh, Emig Research Center of York Hospital, York, PA, for generously allowing the use of essential equipment and to Drs B. Kadenback, Philipps-Universitat, and R. Hendler, N.I.H, for providing purified bovine heart cytochrome *c* oxidase. This work was supported in part by Grants 1981066 from FONDECYT and ENL 2001/05 from DID.

REFERENCES

Adelroth, P., Gennis, R. B., and Brzezinski, P. (1998). *Biochemistry* **37**, 2470–2476.
Babcock, G. T., and Wikström, M. (1992). *Nature* **356**, 301–309.
Brand, M. D. (1994). *The Biochemist* **16**, 20–24.
Brand, M. D., Reynafarje, B. D., and Lehninger, A. L. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73**, 437–441.
Brunori, M., Antonini, E., Guiffre, A., Malatesta, F., Nicoletti, F., Sarti, P., and Wilson, M. T. (1994). *FEBS Lett.* **350**, 164–168.
Brzezinski, P., and Adelroth, P. (1998). *J. Bioenerg. Biomembr.* **30**, 99–107.
Chance, B., Saronio, C., and Leigh, J. S. (1975). *J. Biol. Chem.* **250**, 9226–9237.
Chance, B., and Williams, G. R (1955). *J. Biol. Chem.* **217**, 409–417.
Cleland, W. W., Frey, P. A., and Gerlt, J. A. (1998). *J. Biol. Chem.* **273**, 25529–25532.
Davies, P. W. (1962). *In Physiological Techniques in Biological Research: The Oxygen Cathode, Vol. 4, Chap. 3* (Nastuk, W. L., ed.), Academic Press, New York, pp. 137–179.
Gibson, Q. H., and Greenwood, C. (1963). *Biochem. J.* **86**, 541–554.
Greenwood, C., and Gibson, Q. H. (1967). *J. Biol. Chem.* **242**, 1781–1787.
Hallen, S., and Nilsson, T. (1992). *Biochemistry* **31**, 11853–11859.
Han, S., Takahashi, S., and Rosseau, L. (2000). *J. Biol. Chem.* **275**, 1910–1919.
Hendler, R. W., Pardhasaradhi, K., Reynafarje, B. D., and Ludwig, B. (1991). *Biophys. J.* **60**, 415–423.
Hill, B. C. (1991). *J. Biol. Chem.* **266**, 2219–2226.
Hill, B. C., and Greenwood, C. (1984). *Biochem. J.* **218**, 913–921.
Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995). *Nature* **367**, 660–669.
Kannt, A., Lancaster, C. R., and Michel, H. (1998). *J. Bioenerg. Biomembr.* **30**, 81–87.
Liebl, U., Lipowski, G., Negrerie, M., Lambry, J.-C., Martin, J.-L., and Vos, M. H. (1999). *Nature* **401**, 181–184.
Malatesta, F., Sarti, P., Antonini, E., Vallone, B., and Brunori, M. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7410–7413.
Michel, H. (1999). *Biochemistry* **38**, 15129–15140.
Mitchell, P. (1961). *Nature* **191**, 144–148.

Mitchell, R., West, I. C., Moody, A. J., and Mitchell, P. (1986). *Biochim. Biophys. Acta.* **849**, 229–235.
Reynafarje, B. D. (1966). *In Symposia on Artic Biology and Medicine. VI: The Physiology of Work in Cold and Altitude* (Helfferich, C., ed.), F. Wainright, Alaska.
Reynafarje, B. D. (1991). *Biochem. Biophy. Res. Commun.* **176**, 150–156.
Reynafarje, B. D., Costa, L., and Lehninger, A. L. (1985). *Anal. Biochem.* **145**, 406–418.
Reynafarje, B. D., and Davies, P. W. (1990). *Am. J. Physiol.* **258**, C504–C511 (Cell Physiol. 27).
Tsukihara, T., Aoyama, H., Yamashita, E., Tomisaki, T., Yamaguchi, H., Shinzawa- Itoh, K., Nakashima, T., Yaono, R., and Yoshikawa, S. (1996). *Science* **272**, 1136–1144.
Varotsis, C., Zhang, Y., Appelman, E. H., and Babcock, G. T. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90**, 237–341.
Verkhovskaya, M. L., Garcia-Horsman, A., Puustinen, A., Rigaud, J. L., Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1997). *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10128–10131.
Verkhovsky, M. I., Jasaitis, A., Verkhovskaya, M. L., Morgan, J. E., and Wikström, M. (1999). *Nature* **400**, 480–483.
Verkhovsky, M. I., Morgan, J. E., and Wikström, M. (1995). *Biochemistry* **34**, 7483–7491.
Wickström, M. (1989). *Nature* **338**, 776–778.
Wikström, M. (1977). *Nature* **266**, 271–273.
Wikström, M. (2000). *Biochemistry* **39**, 3515–3519.
Wikström, M., Morgan, J. E., and Verkhovsky, M. I. (1998). *J. Bioenerg. Biomembr.* **30**, 139–145.
Wrigglesworth, J. M., Wooster, M. S., Elsden, J., and Daneel, H. J. (1987). *Biochem. J.* **246**, 737–744.
Yoshikawa, S., Shinzawa-Itoh, K., and Tsukihara, T. (1998). *J. Bioenerg. Biomembr.* **30**, 7–14.