Proton pumping by cytochrome oxidase as studied by time-resolved stopped-flow spectrophotometry

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ABSTRACT The H+/e− stoichiometry for the proton pump of cytochrome c oxidase reportedly varies between 0 and 1, depending on experimental conditions. In this paper, we report the results obtained by a combination of transient optical spectroscopy with a time resolution of 10 ms and a singular value decomposition analysis to follow the kinetics, separate the observed spectral components, and quantify the stoichiometry of the pump. By using cytochrome oxidase reconstituted into small unilamellar vesicles, we show that the time courses of ferrocytochrome c oxidation and phenol red acidification or alkalization fit a simple kinetic scheme. The fitting procedure leads to unbiased and objective determination of the H+/e− ratio under various experimental conditions. The proton-pumping stoichiometry was found to be 1.01 ± 0.10, independent of the number of turnovers, proton back-leak rate, or type of experiment (oxidant or reductant pulse).

Cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, is the crucial site of the energy transduction machinery in mitochondria. Evidence that cytochrome oxidase vectorially pumps protons was initially provided by Wikström (1) with rat liver mitochondria, where he observed acidification of the external medium associated with the oxidation of ferrocytochrome c by oxygen. Proton-pumping evidence was substantiated by more controlled experiments carried out with the simpler system of purified cytochrome oxidase reconstituted into small unilamellar vesicles (cytochrome c oxidase vesicles, COVs), by monitoring the external medium acidification or internal medium alkalinization either potentiometrically (pH meter) or spectrophotometrically (pH indicator) (2−6).

Present structural evidence shows that three metal sites (cytochrome a, cytochrome a3, CuA) are bound to protein ligands provided by subunit I, whereas CuA is thought to be bound to subunit II (7–9). These metals must mediate the electron-transfer processes from ferrocytochrome c leading to the breakdown of dioxygen to water and coupled proton translocation (9). The steady-state kinetics of cytochrome c oxidation by cytochrome oxidase displays nonlinear Michaelis−Menten behavior (10, 11); this nonhyperbolic steady-state behavior may be related to proton translocation if the two cytochrome oxidase conformational states providing the alternating access to protons on the two sides of the membrane are capable of accepting electrons from cytochrome c, albeit with different rates (12). On the other hand, there is growing evidence that the cytochrome a3−CuA binuclear site is involved in molecular events that provide the free energy for proton pumping (9).

Data from various laboratories indicate that the H+/e− ratio is ~1; nonetheless, the stoichiometry of the pump under different experimental conditions is under question, given that (for example) values considerably less than unity have been observed (3, 13–15). Depending on the total number of turnovers, the absolute turnover rate, and the initial state of the enzyme, either pulsed or resting (or definition, see refs. 16 and 17). Determination of the H+/e− stoichiometry under different conditions is thought to be of great significance when deciding between the two alternative mechanisms of H+ translocation—i.e., a direct or an indirect type of coupling. A decrease of the phenomenological H+/e− ratio could be due to a proton "leak" (enhanced proton permeability), for a direct type of coupling, or could be due to a proton "slip" (partial uncoupling of the H+ pumping machinery), for an indirect type of coupling (14, 18).

We have shown (19) that use of stopped-flow spectrophotometry to follow the time course of electron transfer and H+ translocation allows us to kinetically resolve proton pumping from back-leak, which may jeopardize the analysis of the kinetics and the stoichiometry of the pump; this is likely to have happened for the early experiments on subunit III-depleted cytochrome oxidase, which was incorrectly thought to have lost the proton-pumping activity (ref. 20, see also ref. 6). Most of the previous experiments (6) have been carried out by measuring external acidification by potentiometry; with this method, it is very difficult to follow the time course of proton pumping, especially during the initial turnovers. On the other hand, overlap of the absorption bands of the cytochromes and the pH indicator phenol red (pKα = 7.7) makes it very difficult to objectively analyze the time course of electron transfer and H+ translocation; the outcome relies on calibrations to set proper isosbestic points for a point-by-point correction of the time-dependent spectral overlaps. Therefore, application of stopped-flow spectrophotometry has been very limited, and the relatively slow-responding potentiometric method has been almost universally employed.

In this study, we have employed transient spectroscopy and singular value decomposition (SVD) analysis to follow the time course of the various spectral components and to assess quantitatively the stoichiometry of the proton pump in COVs, under selected conditions. A simple kinetic scheme fits the data at all wavelengths (500–650 nm) and allows us to prove (i) that the H+/e− stoichiometry (1.01 ± 0.10) is independent of the total number of turnovers (from 2 to 10 turnovers) and of the rate of proton back-leak [increased by various concentrations of carbonylcyanide m-chlorophenylhydrazone (CCCP)] and (ii) that experiments initiated either with a pulse of reductant (cytochrome c2+) or with a pulse of oxidant (oxygen) yield the same stoichiometry, proving that...

Abbreviations: COV, cytochrome c oxidase vesicle; SVD, singular value decomposition; CCCP, carbonylcyanide m-chlorophenylhydrazone.

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resting and pulsed states of cytochrome oxidase are indistinguishable in this respect.

**MATERIALS AND METHODS**

Cytochrome c oxidase was purified from ox heart as described by Yonetani (21). The final pellet was dissolved in 0.1 M potassium phosphate (pH 7.4), containing 0.5% dodecyl maltoside, at a protein concentration of 0.3 mM (total heme). Samples were frozen in liquid nitrogen, stored at −70°C, and used within 2 months of preparation. Protein concentration was determined spectrophotometrically using a Δε (reduced-oxidized, at 605 nm) of 22 mM⁻¹·cm⁻¹ per functional unit (αd₃).

Soybean phospholipids (1-α-phosphatidylcholine type II S from Sigma) were repurified by acetone/ether fractionation cycles (2). Reconstitution of COVs was achieved by the cholate dialysis method (22); the internal buffer was 0.1 M Hepes-KOH (pH 7.3); the external medium was brought to 50 μM Hepes-KOH containing 43.6 mM KCl and 46.1 mM sucrose (pH 7.3) by passage through a Sephadex G-25 column immediately before the experiment. The phospholipid/protein weight ratio was 50:1 or 70:1. The orientation of cytochrome oxidase in the reconstituted system was ≈85% cytochrome a facing outside, as described by Wriggleworth (23). The maximal stimulation of cytochrome oxidase activity by ionophores (respiratory control ratio) was 5–8, as measured spectroscopically (24).

Cytochrome c type VI was from horse heart, and valinomycin and CCCP were from Sigma.

The experiments were carried out with two different protocols:

- **Reductant pulse.** COV [1.25 μM (aa₃)] in air with 10 μM valinomycin and various concentrations of CCCP (from 0 to 2.5 μM) was mixed in the photodiode array stopped-flow apparatus (see below) with phenol red (60 μM) and various concentrations of ferrocytochrome c (from 5 to 40 μM). In this type of experiment, cytochrome oxidase is (by definition) in the resting state.

- **Oxidant pulse.** COV [1.25 μM (aa₃)] with 10 μM valinomycin and various concentrations of CCCP (from 0 to 2.5 μM) was reduced under anaerobic conditions by addition of ferrocytochrome c (from 10 to 30 μM) and 0.5 mM sodium ascorbate; after 20 or 30 min to dissipate any transmembrane gradient, the solution was mixed with an air-equilibrated solution of 60 μM phenol red. In this protocol, cytochrome oxidase is in the reduced state (as confirmed by an enhanced cytochrome c oxidation rate, see Results).

Spectra were collected by using a rapid scanning photodiode array spectrophotometer (model TN6500, Tracor Northern, Madison, WI) adapted to a Durrum–Gibson stopped-flow apparatus with a 2-cm light path. The Tracor rapid scanning spectrophotometer acquires 512 or 1024 photodiode elements in 5–10 ms and can store up to 62 spectra. The absorption spectra were recorded over a 150-nm range at various times after mixing; the time recording mode was logarithmic to properly cover several decades. Sometimes we observed a mixing artifact, seen also with COVs plus phenol red in the absence of cytochrome c, as reported and discussed (19), where necessary corrections were made for this artifact. Data were always analyzed in terms of difference spectra by taking the absorbance of the fully oxidized sample (see Fig. 1).

The TN6500 data sets were transferred to a MicroVax 3500 for spectral analysis, by using MATLAB (Math Works, South Natick, MA) for computation and graphic procedures. The data matrices were built by linking together the spectral time courses obtained at CCCP concentrations from 0 to 1.25 μM (62 spectra and three to five conditions), adding the spectral acid–base transition of phenol red (recorded in the apparatus), and reducing the observed wavelengths to 256 by averaging every four-diode data point in the spectral range of 500–650 nm. The resulting arrays (256 × 248 or 256 × 372) were analyzed by an SVD algorithm (25, 26) that transforms the spectral matrix OD (OD = m diodes × n times) into the product of three matrices; i.e., OD = U × S × Vᵀ, where U is a matrix of basis spectra, S is a positive diagonal matrix with decreasing values, and Vᵀ is the time course of the corresponding U spectrum. The S matrix contains the “singular values” of the OD matrix, thus representing the contribution of the corresponding U spectra to the observed OD spectral matrix (see Fig. 2). The OD changes of the various chromophores were thus separated by this procedure, which is essentially based on resolving processes with different temporal and spectral behaviors.

Reconstruction of the separate spectral components is possible by taking the U, S, and V columns of interest. The spectral change of cytochrome c with time, separated by those of phenol red and cytochrome oxidase, can be obtained by taking only the U column where the cytochrome c contribution is largest (U₂), multiplied by its singular value (S₂,2) and its time course (V₂). The optical transition monitoring ΔpH was calibrated internally by (i) quantitation of the extent of alkalinization observed in the presence of saturating CCCP and assuming a H⁺/e⁻ ratio of 1.0 for the scalar process:

\[ \text{O}_2 + 4 \text{e}⁻ + 4 \text{H}⁺ \rightarrow 2 \text{H}_2\text{O} \]

and (ii) correction for the ratio between the external and the internal plus external (19, 27) buffer power.

Data fitting was carried out with the MATLAB variable minimization procedure and the time courses of the kinetic mechanism shown in Scheme I (see below) were calculated by numerical integration of the ordinary differential equations that describe the mechanism.

**RESULTS**

Fig. 1 shows 62 difference spectra (baseline, fully oxidized at infinite time) recorded after mixing oxidized COVs containing 10 μM valinomycin and 0.1 μM CCCP with a solution containing reduced cytochrome c and phenol red in the presence of air; the corresponding SVD analysis is depicted in Fig. 2. The time courses of absorbance change at 550 nm (representing mainly cytochrome c oxidation) and at 556.6 nm (representing mainly phenol red acidification and close to being an isosbestic point for cytochrome c) are shown in Fig. 3. Upper, traces *. The other traces in Fig. 3 are described below. It is clear that quantitation of the extent of cytochrome c oxidation and phenol red protonation, which allows calculation of the vectorial H⁺/e⁻ stoichiometry, is dependent on how the partially overlapping spectral transitions are separated. For a two-component system, this may be achieved if (i) an isosbestic point for either optical transition can be found (i.e., 556.6 nm) and (ii) an appropriate extinction coefficient at a selected wavelength is available. In general, and specifically here, the system is much more complex, containing more than two chromophores with nonsynchronous time dependencies. Thus, the separation of chromophores must rely on some bias. The SVD technique, on the other hand, is especially suited for chromophore separation if the behavior of each component follows a different time course (see ref. 26). Therefore, unbiased separation of the various spectral components was obtained by SVD.

The SVD analysis of the experiment, depicted in Fig. 1, with the separation of the spectral contribution of phenol red (U₁) and cytochrome c (U₂), is shown in Fig. 2. Notice that the two spectral components are almost completely represented in U₁ and U₂; U₃, though containing an extremely
small residual contribution of cytochrome c and phenol red, brings out the spectral contribution of cytochrome oxidase, the third component of this system (see below); \( U_4 \) contains information not interpretable at present as well as noise. Examination of the \( V \) columns that represent the time de-

![Graph A](image)

**Fig. 1.** Sixty-two time-resolved difference spectra obtained after mixing COVs (1.25 \( \mu M \) aa3) containing valinomycin (10 \( \mu M \)) and CCCP (0.1 \( \mu M \)) with phenol red (60 \( \mu M \)) and ferrocytochrome c (20 \( \mu M \)), in air at 20°C (2-cm light path). Buffer internal to COVs was 0.1 M Hepes-KOH (pH 7.3); buffer external to COVs was 50 \( \mu M \) Hepes-KOH/43.6 mM KCl/46.1 mM sucrose, pH 7.3. (A) Spectra are superimposed. (B) Spectra are plotted on a logarithmic time scale.

![Graph B](image)

**Fig. 2.** SVD analysis of the spectra reported in Fig. 1. (A) \( U \) columns 1–4 (S values: \( S_1 \), 129; \( S_2 \), 30.7; \( S_3 \), 0.64; \( S_4 \), 0.32). (B) \( V \) columns 1–4.

![Graph C](image)

**Fig. 3.** Time course of cytochrome c oxidation (Left) and proton pumping (Right) by COVs containing 5 \( \mu M \) valinomycin and 0, 0.05, 0.15, 0.35, and 1.25 \( \mu M \) CCCP (after mixing). The asterisk-labeled traces are taken from the experiment shown in Fig. 1. The SVD analyses of these traces are shown in Fig. 2. Other conditions are as in Fig. 1. (Upper) Observed time courses at 550 and 556.6 nm are plotted from the data of Fig. 1. (Lower) Same data after SVD analysis and spectral reconstruction. Data are from Figs. 1 and 2.

...pendencies of the corresponding \( U \) columns (Fig. 2B) yields the following results. In \( V_1 \) we observe the transient acidification and subsequent alkalization of phenol red (\( U_1 \)) seen when CCCP, added to increase the rate constant of the proton back-leak, was not saturating. In \( V_2 \) the time course of cytochrome c oxidation is seen. These experiments (repeated at several concentrations of CCCP) allow efficient separation of the chromophores by the SVD algorithm, given that in the absence of the protonophore cytochrome c oxidation and phenol red protonation are synchronous (19) and thus not separated by SVD (results not shown). \( V_3 \) shows the time course of the corresponding \( U_3 \) column, which represents mainly cytochrome oxidase but also contains small contributions from cytochrome c and phenol red; the time course corresponds to oxidation of cytochrome \( a \) and reflects electron redistribution in a fraction of the enzyme molecules (≤20% of the total) when oxidation of cytochrome c is almost complete (28). This process is "catalyzed" by cytochrome c acting as an electron shuttle and seems to be associated with proton consumption. Thus, cytochrome c (≤1.5% of the total) and phenol red (<1%) signals share the same time course in \( V_3 \) as cytochrome oxidase and are represented in the same \( U_3 \) column. The slow phase in \( V_4 \) is not interpretable at present, but accounts for at most 0.2% of the total absorbance change of cytochrome c.

It is important to point out that all calculations regarding the \( \text{H}^+ / e^- \) stoichiometry and the fit to the kinetic scheme have been carried out by reconstruction of the USV columns 1 and 2, which account for >98% of the total spectral transition of phenol red and cytochrome c, respectively.

Experiments similar to those depicted in Fig. 1 were carried out at various CCCP concentrations and are shown in Fig. 3 at selected wavelengths. Fig. 3 Upper shows actual raw data, as obtained from the experiment before chromophore separation. Fig. 3 Lower shows the results obtained by the SVD analysis as shown in Fig. 2. The reconstructed time courses shown in Fig. 3 Lower were obtained by multiplying any given basis spectrum (\( U \) column) by its singular value and by its corresponding transposed \( V \) column. This yields a matrix containing the time dependence of the single spectral component of choice. When cytochrome c is separated from phenol red in the wavelength and time domains, it is possible to evaluate the apparent \( \text{H}^+ / e^- \) stoichiometry. Notice that increasing the CCCP concentration has a dramatic effect on the \( \text{H}^+ \) signal (Fig. 3 Lower right) since the proton permeability is severely increased. On the contrary, the oxidation of cytochrome c (Fig. 3 Lower left) is only slightly affected by...
the addition of the protonophore (as also deduced from the fitting procedures shown below), since as reported (29, 30), valinomycin alone in this condition accounts for release of 70–80% of the respiratory control ratio. Fig. 4 and Table 1 show the reconstructed time courses at 550 nm and 556.6 nm for experiments carried out at two cytochrome c concentrations and various CCCP concentrations. The time scale is logarithmic for the best representation of both the early (H+ pumping) and late (H+ back-leak) events. The continuous lines are the best fit to the following simple scheme:

\[
\text{cyt } c^{2+} \overset{k_1}{\rightarrow} \text{cyt } c^{3+} \quad \text{(electron transfer)}
\]

\[
H_{\text{in}}^{+} \overset{k_1}{\rightarrow} H_{\text{out}}^{-} \quad \text{(vectorial process)}
\]

\[
H_{\text{in}}^{+} \overset{k_2}{\rightarrow} H_2O \quad \text{(scalar process)}
\]

\[
H_{\text{out}}^{+} \overset{k_3}{\rightarrow} H_{\text{in}}^{-} \quad \text{(back-leak)}
\]

Scheme I

This mechanism contains the following features: (i) The oxidation of cytochrome c follows exponential behavior, \(k_1\) representing the rate-determining step of the catalytic cycle and being faster in an oxidant pulse experiment (leading to pulsed cytochrome oxidase). (ii) The time course of H+ pumping is synchronous with cytochrome c oxidation, as shown by Sarti et al. (19). (iii) The H+ back-leak, very slow in the presence of valinomycin alone, is increased by adding CCCP at various concentrations and is assumed to be the same at any given CCCP concentration in the oxidant and oxidant pulse experiments. (iv) In the presence of saturating CCCP, the total buffer power (inside + outside) is higher than in the absence of CCCP (i.e., only outside) (27). Therefore, for a given H+ pulse, the spectral transition of phenol red is smaller in the presence of CCCP (80% of that observed in the absence of CCCP); (v) the net consumption of H+ inside the vesicles (scalar reaction) is 1.0 per electron.

The variables used in the fit of the experimental data are as follows: (i) the stoichiometry of the pump (H+\text{e}− ratio), (ii) the rate of cytochrome c oxidation (\(k_1\)), and (iii) the rate of H+ back-leak (\(k_3\)). The fit of the data shown in Fig. 4 and Table 1 to the kinetic scheme illustrated above is satisfactory (with the parameters given). It leads to an unequivocal determination of the H+\text{e}− stoichiometry of the pump as a function of the number of turnovers and of the actual value of \(\Delta p\)H (given that \(\Delta p = 0\) because valinomycin is saturating). The H+\text{e}− stoichiometry in the presence of nonsaturating CCCP is unequivocally determined by the constraints used in the fit (i.e., initial and final absorbance values and \(k_1\), as determined by the corresponding rate of cytochrome c oxidation). Of course, with saturating CCCP, when no transient acidification could be observed, the H+\text{e}− stoichiometry is essentially undetermined, and in the latter case, a H+\text{e}− of 1 is one of the possible solutions of the fit. However, we rely on fitting the family of curves in Fig. 4, where only the time course at the highest CCCP concentration displays no significant spectral contribution of the vectorial process.

Table 1. Parameters for Fig. 4

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<tr>
<th>(c_{\text{yt c}}), (\mu\text{M})</th>
<th>H+\text{e}− ratio</th>
<th>CCCP, (\mu\text{M})</th>
<th>(k_1), s−1</th>
<th>(k_{3b}), s−1</th>
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(\(c_{\text{yt c}}\), ferrocytochrome c.)

Table 2. Parameters for Fig. 5

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<th>(k_1), s−1</th>
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(\(c_{\text{yt c}}\), ferrocytochrome c.)
oxidation is 2-fold higher in the oxidant pulse experiment (as expected since cytochrome oxidase is in the pulsed state) and increases slightly with increasing concentration of CCCP and that (ii) the H^+/e^- ratio is, however, 0.96 and 1.07 for the pulsed and resting enzyme, respectively, thus showing identical stoichiometry.

**DISCUSSION**

The use of rapid scanning spectrophotometry and analysis by the SVD procedure have been employed to reinvestigate the H^+ pumping properties of COVs. The remarkable advantage of this technique to separate the time courses and amplitudes of the three chromophores (cytochrome c, phenol red, and cytochrome oxidase) is shown in Figs. 2–4. Analysis allows observation and quantitation, in a single shot, of the kinetics of cytochrome c oxidation and medium acidification (or alkalization). Fits of the data with a kinetic scheme for cytochrome c oxidation and H^+ pumping, taking into account the H^+ back-leak rate, allow objective quantitation of the time courses of electron transfer, H^+ translocation, and the H^+/e^- ratio under various conditions.

In these experiments, the stoichiometry of the pump was determined to be 1.01 ± 0.10 under all conditions explored, and the optical changes related to ferrocyanochrome c oxidation and external medium acidification are synchronous at the resolution level used (5–10 ms), in agreement with Sarti et al. (19). The independence of the H^+/e^- ratio on CCCP concentration, and thus on the rate of back-leak, emerges from the fit. This implies that the absolute value of the proton chemical gradient does not affect the proton-pumping stoichiometry provided the electrical component of the gradient is collapsed by valinomycin; this should be contrasted with the fact that in the presence of a fully developed membrane potential, proton translocation by cytochrome oxidase has not been observed. The cubic model we proposed (30) for the control of cytochrome oxidase activities predicts that the electrical component of the gradient controls the rate of electron entry and of the internal electron transfer and the proton-pumping stoichiometry, by freezing the oxidase in a "slipping state." Since in the present experiments, the H^+/e^- is, within the errors, the same for pulsed and resting COVs, it follows that neither of these states of the enzyme is a slipping state.

The best fit of the time-resolved spectroscopic data to Scheme I shows that proton pumping by cytochrome oxidase has a constant stoichiometry over the whole time range and at various concentrations of cytochrome c (from 5 to 25 μM; i.e., from 2 to 10 turnovers). This indicates that the H^+/e^- ratio is the same from the first turnover onward; i.e., the proton-pumping machinery is fully active after the initial four-electron reduction of the enzyme.

A variable-pump stoichiometry has been reported by several groups under different experimental conditions. As an example, the H^+/e^- stoichiometry was reported to be 0.8 in a reductant pulse experiment but dropped to 0.3 in an oxidant pulse experiment (13). Given that the two experimental protocols yield, respectively, resting and pulsed oxidase (16, 17), it has been argued that only the resting state of the enzyme is a competent proton pump with high efficiency. Because pulsed oxidase is probably the only functional state of the enzyme in vivo, this result may have had some consequence in assessing the significance and the stoichiometry of the pump in vivo. The evidence presented above shows convincingly that the H^+/e^- ratio is the same for the resting and pulsed enzyme and implies that the variable stoichiometry must be attributed to other presently unknown experimental variables.

In conclusion, we have demonstrated that using transient spectroscopy and SVD analysis the proton-pumping activity of cytochrome oxidase reconstituted into vesicles is well resolved and, thereby, amenable to quantitative kinetic analysis. Accurate quantitation of the kinetics and stoichiometry of proton pumping by cytochrome oxidase can thus resolve some experimental discrepancies related to variable H^+/e^- ratios; in particular we have shown that pulsed and resting oxidase are both able to pump protons with the same stoichiometry.