Biochimica et Biophysica Acta, 680 (1982) 233–241
Elsevier Biomedical Press

BBA 41078

COULOMETRIC AND POTENTIOMETRIC EVALUATION OF THE REDOX COMPONENTS OF CYTOCHROME *c* OXIDASE IN SITU

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(Received September 22nd, 1981)
(Revised manuscript received February 3rd, 1982)

Key words: Redox component; Coulometric assay; Potentiometry; Cytochrome c oxidase

A new coulometric-potentiometric titration cuvette is described which permits accurate measurements of oxidation-reduction components in membranous systems. This cuvette has been utilized to measure the properties of cytochrome *c* oxidase in intact membranes of pigeon breast muscle mitochondria. The reducing equivalents accepted and donated by the portion of the respiratory chain with half-reduction potentials greater than 200 mV are equal to those required for the known components (cytochrome a_3 and the high-potential copper plus cytochrome *a*, 'visible copper', cytochrome c_1, cytochrome *c*, and the Rieske iron-sulfur protein). Titrations in the presence of CO show that formation of the reduced cytochrome a_3-CO complex requires two reducing equivalents per cytochrome a_3 (coulometric titration). Potentiometric titrations indicate (Lindsay, J.G., Owen, C.S. and Wilson, D.F. (1975) Arch. Biochem. Biophys. 169, 492–505) that both cytochromes a_3 and the high-potential copper must be reduced in order to form the CO complex ($n = 2.0$ with a CO concentration-dependent half-reduction potential, E_m). By contrast, titrations in the presence of azide show that the E_m value of the high-potential copper is unchanged by the presence of azide and thus azide binds with nearly equal affinity whether the copper is reduced or oxidized.

Introduction

Cytochrome *c* oxidase of the mitochondrial respiratory chain is responsible not only for the reduction of molecular oxygen to water but also for energy transduction at the third site of oxidative phosphorylation and for regulation of the rate of mitochondrial respiration. This makes it of great physiological importance as well as offering fascinating opportunity to study the molecular basis for oxidative phosphorylation and the mechanism of oxygen reduction to water.

There are only four oxidation-reduction components in cytochrome *c* oxidase (making it a relatively simple system). Three components, cytochromes *a* and a_3 and the low-potential ('visible') copper, have characteristic optical and EPR absorption properties (see, for example, Refs. 1–5) and can be readily measured. The fourth component, variously called the 'invisible copper', Cu_3 or the high-potential copper, cannot be measured by conventional techniques and extraordinary (such as X-ray absorption or fluorescence) or indirect methods must be used. The high-potential copper has, for example, been found to play a role in the binding of CO to cytochrome *c* oxidase and its state of reduction and thereby its half-reduction potential can be inferred from its effect on the redox properties of the reduced cytochrome a_3-CO complex [6,7]. Moreover, its reduction and oxidation can be observed when the stoichiometry of the reducing equivalents required to reduce and oxidize cytochromes *a* and a_3 in anaerobic suspensions of cytochrome oxidase is measured [8–11].

In the present communication, both redox potentiometry and coulometry are used to examine the properties of cytochrome *c* oxidase in situ. Particular attention is paid to the high-potential copper and its response to the binding of ligands to the active site of the oxidase.

Materials and methods

Electrometric equipment. Both coulometric and potentiometric measurements were carried out using a four-electrode system. The potentiometric sensing electrodes (platinum and calomel) were monitored using a high-impedance voltmeter with a digital readout. The working electrodes (glassy carbon and Ag/AgCl) were attached to the output of an amplifier system which could maintain either constant current through the circuit or constant voltage on the electrodes with the current and voltage displayed digitally. This circuit includes a bimodal digital coulometer (Princeton Applied Research) to measure the current flow through the working electrodes.

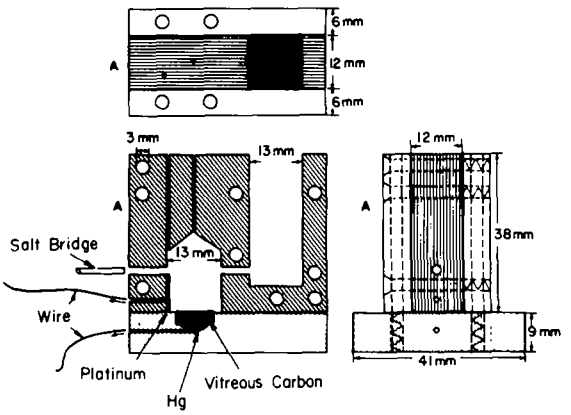


Fig. 1. A four-electrode coulometric-potentiometric titration cuvette. The cuvette is constructed of plexiglas with the central portion from which the reaction chamber is made of black plexiglas to prevent light from passing through any portion except the reaction chamber. The reaction chamber has a volume of 1.79 ml and a light path of 1.2 cm. The top of the chamber is conical with a 1.2 mm diameter hole extending 2 cm from its apex to the top of the cuvette. Solutions are added and withdrawn through a second hole 1.0 mm in diameter which is placed near the edge of the chamber. When a solution is added through the smaller hole using a syringe with a 25 gauge, 1.5 inch needle, all of the air, including bubbles, is forced out through the central hole. Filled to the top of each access hole is a 2 cm column of unstirred liquid, an effective barrier to oxygen diffusion. The counter electrode of Ag/AgCl is placed in a 1 M KCl solution in the open chamber adjacent (0.8 cm separation) to the reaction chamber. Electrical connection is through a 3 mm diameter hole, 1 cm long, between the chambers which is normally filled with a 5% agar gel containing 100 mM KCl. A flexible plastic tube (1.5 mm inner diameter) filled with 5% agar containing 1 M KCl is used to provide electrical contact between the reaction medium and an external calomel electrode.

Sample cuvette. The cuvette is constructed from plexiglas, and contains 1.79 ml of sample and has a 1.2 cm light path (Fig. 1). A 0.8 cm diameter glassy carbon electrode forms the bottom of the sample chamber while a 1 cm^2 platinum foil sensing electrode is attached to the inside surface of the chamber. Both the reference electrode (saturated KCl/calomel electrode) and the counter electrode (Ag/AgCl) are external and are connected to the chamber through 5% agar/100 mM KCl salt bridges. Black plexiglas was used for the central piece of the cuvette to minimize stray light and stirring is accomplished by a 1 cm Teflon-coated stirring bar which runs on the polished surface of the glassy carbon electrode, driven by an external magnetic stirrer.

The sample in the working cuvette may be monitored using any spectrophotometric method. In the present work transmission absorption measurements were made using either a dual-wavelength spectrophotometer for measuring the time course of the reaction or a scanning dual-wavelength spectrophotometer to determine the wavelength dependence of the absorbance changes.

Mitochondria were isolated from pigeon breast muscle as previously described [12]. These mitochondria were suspended at approx. 50 mg protein/ml in 0.25 M sucrose, frozen in liquid nitrogen and stored at −20°C until needed. The mitochondria were rapidly thawed, diluted 25-fold with 10 mM phosphate buffer, pH 7.0 and allowed to stand at 0–4°C for 10 min. They were then centrifuged for 10 min at 15000 × *g*, washed once in 0.1 M phosphate buffer, pH 7.0, and finally suspended at approx. 50 mg protein/ml in 0.1 M phosphate buffer, pH 7.0.

Inasmuch as these mitochondria are modified by freeze-thawing as well as the extraction of most (greater than 95%) of their cytochrome *c*, the preparation will be referred to as submitochondrial particles. The experiments all involve anaerobic

titrations in the absence of ATP and in no case was the final result affected by the presence of uncoupler.

Results

Quantitation of reducing equivalents using coulometric titrations

In order to test the quantitative accuracy of the coulometric system, electronics and cuvette, the system was used to titrate solutions of horse heart cytochrome *c*. methyl viologen was added to the cytochrome *c* solutions and the working electrodes polarized (glassy carbon negative) to generate reduced methyl viologen until the residual oxygen was exhausted and the potential on the sensing electrode became more negative than −400 mV. A potential (E_h) of −400 mV on the sensing electrodes was maintained for 5–10 min to remove oxygen which may be dissolved in the salt bridges and plastic in contact with the titration chamber. Ferrocene (0.5 mM, $E_{m,7.0} = 0.56$ V) was then added and the solution subjected to cycles of oxidation and reduction (Fig. 2). Changes in the state of reduction of cytochrome *c* were measured by the absorption at 550 nm minus that at 565 nm. Oxidative and reductive titrations were very similar and quantitative comparison was achieved by plotting the measured absorbance change against the number of coulombs of electricity injected or removed from the cuvette (Fig. 2). Different rates of injection of current were used, viz., $10 \cdot 10^{-6}$ and $20 \cdot 10^{-6}$ A. The resulting titration curves are indistinguishable with respect to both direction of titration (oxidation or reduction) and rate of titration. The O_2^- and H_2O_2 generated in removing O_2 do not influence the results, since neither inclusion of superoxide dismutase and catalase or preextraction of O_2 with ultrapure argon gas has any effect on the measurements. When the titration curve was fitted using a computer program the calculated extinction coefficient for cytochrome *c* at 550 nm minus 565 nm was 23.0 $mM^{-1} \cdot cm^{-1}$, the same as that reported by Margoliash and Frohwirt [13].

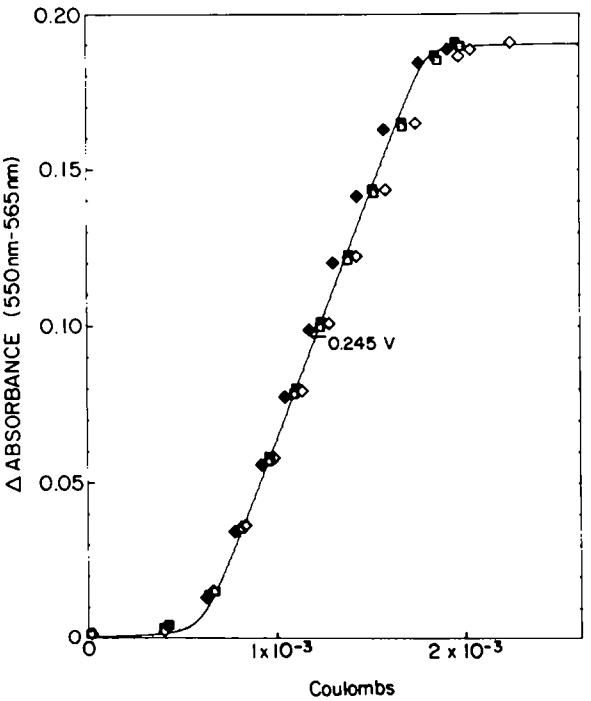


Fig. 2. Coulometric titration of solutions of cytochrome *c* from horse heart. Cytochrome *c* was dissolved at $7 \cdot 10^{-6}$ M in 0.1 M phosphate buffer, pH 7.0. Both oxidative (□) and reductive (◇) titrations were carried out using working electrode currents of $10 \cdot 10^{-6}$ A (◇, □) and $20 \cdot 10^{-6}$ A (◆, ■). The solid line is the behavior for cytochrome *c* assuming an $E_{m,7.0}$ value of 0.245 V and an extinction coefficient for the absorbance at 550 nm minus that at 565 nm of 23.0 $mM^{-1} \cdot cm^{-1}$. This extinction coefficient is the same as that reported by Margoliash and Frohwirt [12].

Titrations of solutions of potassium ferricyanide again indicated an accurate measure of the equivalents required for reduction of the ferricyanide. Moreover, because the ferricyanide-ferrocyanide couple equilibrates with the platinum sensing electrode, accurate potentiometric measurements can be made simultaneously with the coulometric measurements. An extinction coefficient of 1.0 $mM^{-1} \cdot cm^{-1}$ was obtained at 420 nm and the measured E_m value was 0.420 V.

Stoichiometry of the oxidation-reduction components of the cytochrome c oxidase region of the respiratory chain

Suspensions of submitochondrial particles from pigeon breast muscle were placed in the electrometric cuvette and 0.8 mM methyl viologen added. Reduced methyl viologen was generated electrometrically to exhaust residual oxygen and reduce the respiratory chain components. Ferrocene was then added and the particles subjected to cycles of

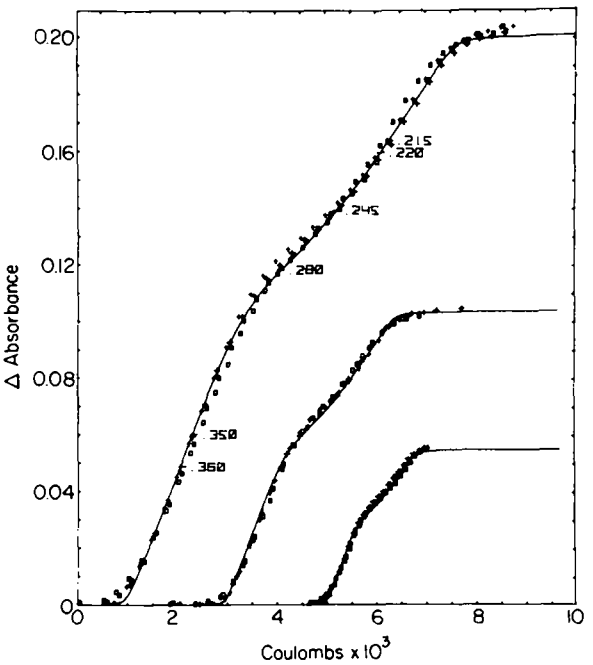


Fig. 3. Coulometric titration of cytochrome *c* oxidase in intact mitochondrial membranes. Pigeon breast muscle submitochondrial particles were suspended at approx. 6 (upper curve), 3 (middle curve) or 1.5 (lower curve) mg protein/ml in 0.1 M phosphate buffer, pH 7.0. Methyl viologen (1.1 mM), anthroquinone-2,6-disulfonate (0.67 mM), phenazine ethosulfate (11 μM) and ferrocene (55 μM) were used to catalyze electron transfer at the working electrode. Reduction and oxidation of cytochrome *a* and a_3 were measured at 605 nm minus 630 nm. At each protein concentration, both oxidative (□) and reductive (+) titrations were carried out at working electrode currents of 10 and 40 μA. The solid lines are the expected behavior of six redox components (cytochrome *a*, cytochrome a_3, high-potential copper, low-potential copper, cytochrome *c*, and the Rieske iron-sulfur protein) of equal stoichiometry and $E_{m,7.0}$ values of 0.220, 0.350, 0.360, 0.245, 0.215 and 0.280 V, respectively. The calculated extinction coefficients for cytochrome a_3 and *a* are 15.3 and 11.1 $mM^{-1} \cdot cm^{-1}$, respectively, for a combined value of 26.4 $mM^{-1} \cdot cm^{-1}$.

oxidation and reduction; the absorbance changes of the hemes of cytochrome oxidase were measured at 605 nm minus 630 nm as electrons were injected into or removed from the sample in the cuvette. The oxidative and reductive titrations are very similar and quantitative comparison is readily achieved by plotting the change in absorbance against the number of coulombs of electricity injected or removed from the cuvette (Fig. 3) where the data are given for titrations carried out using three different protein concentrations. Each titration curve shows three phases, the first (high-potential) portion accounts for approx. 50% of the absorbance change and one-third of the number of coulombs. This relative stoichiometry is consistent with the high-potential phase representing cytochrome a_3 and the high-potential copper while the low-potential phases account for the remaining two-thirds of the number of coulombs and include cytochrome *a* and the low-potential copper in addition to the Rieske iron-sulfur protein and cytochrome c_1.

Quantitative analysis of the curves requires consideration of the effect of reduction of the hemes which contribute to the absorbance change as well as that of the components which have either no or small contributions to the measured absorbance change but are stoichiometrically equal in concentration to the heme. In Fig. 3, theoretical curves are fitted to each set of experimental data. These theoretical curves are constructed for equal concentrations of cytochrome a_3, high-potential copper, cytochrome *a*, low-potential copper, cytochrome c_1 and Rieske iron-sulfur protein with respective E_m values of 360, 350, 215, 245, 220 and 280 mV. A precise fit to the data is obtained when an extinction coefficient of 26.4 $mM^{-1} \cdot cm^{-1}$ is used for cytochromes *a* and a_3, in agreement with the value reported by Van Gelder [8]. The nonlinearity of the low-potential phase is consistent with potentiometric data showing that cytochrome c_1 has a half-reduction potential slightly more negative than those of the low-potential copper and the Rieske iron-sulfur protein [14–16].

Stoichiometry of the redox component(s) of the reduced cytochrome a_3-CO complex

Suspensions of mitochondrial membranes in the presence of varying concentrations of CO were placed in the sample cuvette, and then methyl viologen and, after anaerobiosis, ferrocene were added. Coulometric titrations were carried out while measuring the absorbance changes at 589 nm minus 607 nm. When this wavelength pair is used, formation of the reduced cytochrome a_3-CO complex results in an absorbance change opposite to that which accompanies reduction of cytochrome *a*. This facilitates evaluation of the titration data. A plot of the data from a typical experi-

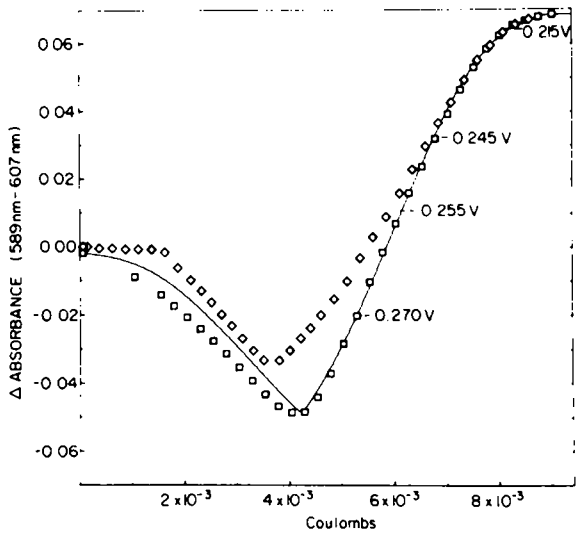
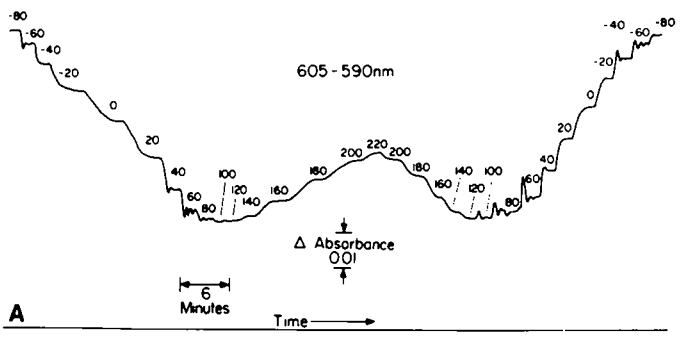


Fig. 4. Coulometric titration of cytochrome *c* oxidase in the presence of CO. The submitochondrial particles were suspended in 0.1 M phosphate buffer, pH 7.0, containing 50 μM CO. Methyl viologen (1.0 mM), ferrocene (0.5 mM), and anthroquinone-2,6-disulfonate (1 mM) were used to catalyze electron transfer from the electrodes and both oxidative (□) and reductive (◇) titrations carried out at working electrode currents of 20 μA. The solid line is a theoretical curve constructed assuming the indicated $E_{m,7.0}$ values for cytochrome c_1, visible copper, cytochrome *a* and the Rieske iron-sulfur protein at equal concentrations as determined using an extinction coefficient (reduced minus oxidized at 605 nm and 630 nm) of 26.4 mM^{-1} for cytochrome *a*. The cytochrome a_3-CO complex was assumed to have an E_m value of 425 mV and $n = 2.0$.

ment is shown in Fig. 4. The oxidative and reductive titrations are not identical, indicating the presence of a relatively slow step ($t_{1/2}$ approaching 1 min) in the reactions which result in formation of the CO complex in reductive titrations. Experiments carried out in the presence of 5 μM ferricyanide show improved equilibration but higher levels (such as for potentiometric titrations) are required for complex equilibration and this defeats the purpose of the coulometric titrations. The low-potential part of the titration curve requires the same number of coulombs as did the low-potential portion of the titration curve in the absence of CO. In oxidative titrations in the presence of CO (where the interchain equilibration is less important because CO is already bound), oxidation of the CO complex involves two equivalents per cytochrome oxidase (Fig. 4).

Potentiometric titrations of cytochrome oxidase in the presence and absence of CO

The electrometric method can equally well be used for potentiometric titrations if suitable redox mediators are added. In this case, the redox mediators are present at concentrations higher than that of cytochrome oxidase and the working electrode



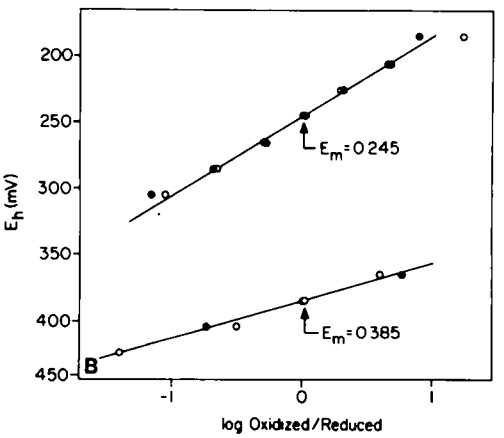


Fig. 5. Potentiometric titration of cytochrome *c* oxidase in the presence of CO. Submitochondrial particles were suspended at approx. 6 mg protein/ml in 0.1 M phosphate buffer, pH 8.0. Methyl viologen (1.1 mM) was added and the working electrode circuit polarized. After anaerobiosis was attained, ferrocene (0.5 mM), ferricyanide (0.2 mM), diaminodurene (60 μM), and phenazine methosulfate (100 μM) and CO (50 μM) were added. Titrations were carried out measuring the absorbance at 605 nm minus 590 nm and the regulating electrical circuit was set to use the working electrodes to bring the sensing electrodes to designated voltages relative to the standard calomel electrode. In A, the data are shown as the measured absorbance change as a function of time with the potential reading on the sensing electrode when $dA/dt = 0$ indicated at the appropriate places on the tracing. In B, these data are plotted according to the Nernst equation and lines drawn for a one-electron acceptor (cytochrome *a*) or two-electron acceptor (cytochrome a_3-CO complex). Both oxidative (●) and reductive (○) titration data are plotted in B.

functions primarily to reduce or oxidize the mediators. The platinum sensing electrode equilibrates with the oxidation-reduction mediators allowing the oxidation-reduction potential to be continuously measured. The regulatory electrical circuits can be set to cause the working electrode to attain and hold to within ± 1 mV any desired voltage on the sensing electrodes. Thus, potentiometric titrations can be carried out using precise increments in oxidation-reduction potential. A typical titration at pH 8.0 and in the presence of 50 μM CO is shown in Fig. 5A where steps of 20 mV was used for both reductive and oxidative titrations with the potential relative to a saturated calomel electrode given for each step. In Fig. 5B, the data are presented as the logarithm of the ratio of oxidized to reduced forms plotted against the oxidation-reduction potential relative to a standard hydrogen electrode. In the absence of CO, a sigmoid curve is obtained which resolves into two components with n values of 1.0 and half-reduction potentials of 320 and 195 mV. In the presence of 50 μM CO the curve is resolved into a high-potential component with an n value of 2.0 and a low-potential component with an n value of 1.0. These components have half-reduction potentials of 385 nV ($n = 2.0$) and 245 mV ($n = 1.0$), respectively (see also ref. 7). Spectra of the components were measured in similar potentiometric titrations and clearly identify the formation of the CO complex and reduction of cytochrome *a* (Fig. 6).

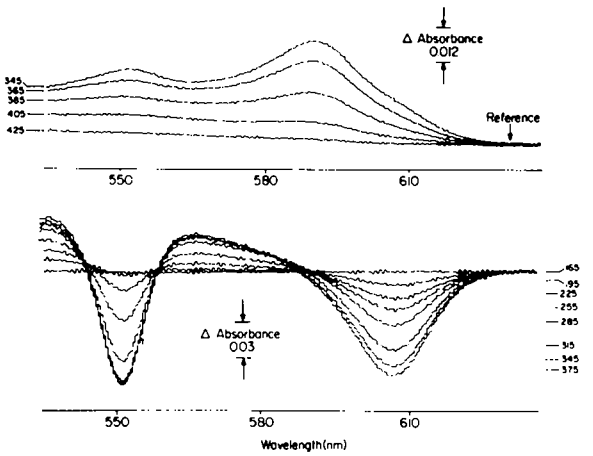


Fig. 6. Spectral properties of the high- and low-potential components of cytochrome oxidase in the presence of CO. The submitochondrial particles were treated as described in the legend of Fig. 5 except that the absorption spectra were measured when the sample was equilibrated at each potential. The titration was begun with the potential at 425 mV and a scanning dual-wavelength instrument with computer memory was used to plot the absorption at the indicated potential minus that for the sample at 425 mV. By 350 mV the high-potential component was essentially completely reduced. The sample was then fully reduced (165 mV) this absorption recorded and then the sample was oxidatively adjusted to each of the indicated potentials and the absorption subtracted from that of the fully reduced sample. The difference spectra are shown in the lower set of spectra.

Coulometric titration of cytochrome oxidase in the presence of azide

Titrations were carried out using submitochondrial particles suspended at pH 7.0 in the presence of 10 mM azide. In the presence of this

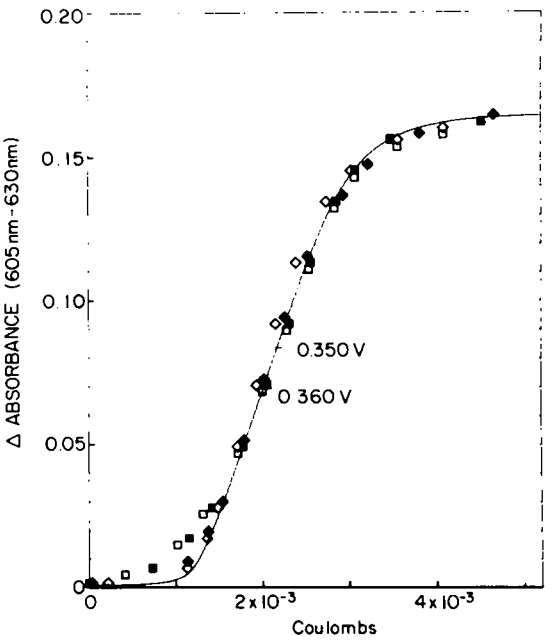


Fig. 7. Coulometric measurement of the effect of azide on the half-reduction potential of the low-potential copper. Submitochondrial particles were suspended at approx. 5 mg protein/ml in a 0.1 M phosphate buffer containing 10mM NaN_3, pH 7.0. Anthroquinone-2,6-disulfonate (1.7 mM), phenazine ethosulfate (33 μM) and ferrocene (1.4 mM) were used to catalyze the transfer of electrons between the working electrodes and the respiratory chain. The solid line is the theoretical curve for an *a* cytochrome ($E_{m,7.0} = 0.350$ V) and the low-potential copper ($E_{m,7.0} = 0.360$ V) present at stoichiometries equal to that of cytochrome *a*. Closed points, 20 μA; open points, 10 μA; (□,■) oxidative titrations, (◇,◆) reductive titrations.

concentration of azide, one of the two hemes has a measured E_m value near 360 mV while the other has a much more negative E_m value of near 130 mV [16]. Most (86%) of the absorbance change at 605 nm minus 624 nm is due to the high-potential heme [16] allowing precise titration of the high-potential components. As may be seen in Fig. 7, in which the absorbance change is plotted against the number of coulombs injected or removed from the solution, the reductive and oxidative titrations are in excellent agreement. Two equivalents are required per mole of the high-potential *a* cytochrome reduced or oxidized indicating that the high-potential copper has an E_m value approximately equal to that of the high-potential *a* cytochrome. A curve which assumes an E_m value of 360 mV for the *a* cytochrome and an additional redox component in equal concentration is fitted to the data. A good fit requires that the additional component has an E_m value of 350 ± 10 mV. It should be noted that the presence of azide does not affect the E_m value of the low-potential copper which remains at 245 mV [15]. Thus, the second reducing equivalent must be accepted by the high-potential copper.

Discussion

Isolated cytochrome *c* oxidase was first stoichiometrically titrated with reducing equivalents and shown to accept four equivalents per two *a* hemes by Van Gelder [8]. This has been confirmed [9,10] and extended to intact membranes using both titrations with chemical oxidants and reductants [11,17] and electrometric methods (present paper). In the present work, resolution of the coulometric method was sufficient to determine that in intact membranes at pH 7.0, the half-reduction potentials of cytochrome a_3 and the high-potential copper are the same, ± 15 mV, while those of the low-potential copper and the Rieske iron-sulfur protein are more positive than that of cytochrome *a*, in agreement with potentiometric data [14,15]. Analysis of the coulometric and potentiometric titration data shows that in each case, approx. 50% of the absorbance change of 605 nm minus 630 nm can be attributed to the high-potential heme (cytochrome a_3) and the remainder to the low-potential heme (cytochrome *a*). The extinction coefficient for cytochrome oxidase (cytochrome $a + a_3$) using this wavelength pair is calculated to be 26.4 mM^{-1} for a 1 cm light path assuming an active unit of two *a* hemes and two copper atoms, cytochrome c_1 and the Rieske iron-sulfur protein. This is the same value obtained for isolated cytochrome *c* oxidase using chemical reduction with NADH [8].

The formation of the reduced cytochrome a_3-CO complex requires two reducing equivalents per cytochrome a_3 as measured coulometrically, potentiometrically, and by titration with chemical reductants [11,17]. The slow step observed in the coulometric titrations could arise either from an intrinsic change in cytochrome oxidase or slow equilibration of the reducing equivalents at these high potentials. Wohlrab [18] demonstrated that in mitochondria, interchain electron transfer (oxidase to oxidase) occurs rapidly at the level of cytochrome *c* and that cytochrome *c* is required for this process. In coulometric titrations, the formation of the reduced cytochrome a_3-CO complex occurs at potentials at which cytochrome *c* is highly oxidized and unable to catalyze effectively this interchain electron transfer. Both the high-potential copper and cytochrome a_3 must be reduced in order for CO to bind with high affinity [6,7] and the statistical probability of this event is low at high potentials (above 400 mV). As CO binds to the existing doubly reduced species electron reequilibration must occur. It is this electron equilibration, with half-times of many minutes, which is responsible or the slow reaction in coulometric titrations. This conclusion is supported by the observation that rapid equilibration occurs in potentiometric titrations where high concentrations of the mediators (ferricyanide-ferrocyanide) catalyze this equilibration.

The report by Anderson et al. [19] that during coulometric titrations of cytochrome oxidase in the presence of CO three reducing equivalents could be removed without oxidizing the cytochrome a_3-CO complex was not supported. The presence of endogenous substrates in the membrane and cytochrome oxidase preparations (Refs. 11 and 20, this paper) could account for both the extra reducing equivalents observed by Anderson et al. [19] and their inability to oxidize the cytochrome a_3-CO complex. Moreover, cytochrome

oxidase can oxidize CO by an as yet unknown mechanism [20] and this may contribute some of the reducing equivalents observed in the presence of CO. The experimental apparatus used in the present experiments allowed titrations to be carried out over time ranging from minutes to hours. The endogenous donor is sufficient to interfere seriously with titrations requiring extended periods of time, adding progressively more reducing equivalents and decreasing the ability to oxidize the reduced cytochrome a_3-CO complex.

The observation that the redox state of the high-potential copper contributes to determining access of CO and oxygen to the active site [4,5] raises the possibility that binding of other ligands will also be influenced. During titrations of anaerobic suspensions of mitochondria in the presence of azide, for example, appearance of the resonance at g 2.9 characteristic of the ferric heme-azide complex is reported to be dependent on reduction of the high-potential copper [15]. However, coulometric titrations (Fig. 7) show that the half-reduction potential of this copper is the same to within 20 mV in the presence and absence of azide. Thus, the effect of the high-potential copper on the g 2.9 oxidized cytochrome-azide compound is not due to a change in affinity for the ligand but is due to a change in the properties (conformation?) of the overall heme-azide complex.

The involvement of two redox centers, iron and copper, in the active site for oxygen reduction by cytochrome oxidase is of central importance to understanding the reaction mechanism. A mechanism has been proposed [6,7,21] in which the oxygen molecule forms a bridged complex between the two metal atoms and then undergoes two-electron reduction to a bridged peroxide compound [6,7]. This proposed mechanism is consistent with the available titration data in the presence of CO [6,7] and the chemistry of molecular oxygen and its reduction products. Supporting evidence for the two-metal-atom reaction site mechanism has been obtained using several experimental approaches. (1) NO, an oxygen analogue, has been reported to form an EPR-identifiable bridged complex between heme and copper under certain selected experimental conditions [22]. (2) Infrared spectra of CO in cytochrome oxidase are reported to show that photodissociation of CO (from the heme) at temperatures below 140 K results in formation of a copper-CO complex [23], suggesting the two metal atoms are in the same active site. (3) Measurement of the X-ray edge absorption and extended fine structure of the iron and copper centers suggests that a copper atom is approx. 3.7 Å from one of the iron atoms [24].

The existence of a bridged peroxide intermediate in the reaction with oxygen has not yet been unequivocally established. Analysis of the steady-state rate of oxidation of reduced cytochrome c in phosphorylating mitochondria shows the existence of an intermediate with the expected kinetic behavior [25,26]. Presteady-state kinetic measurements of the reaction of molecular oxygen with isolated cytochrome oxidase at room temperature [27] and both isolated oxidase and mitochondria at low temperatures [28–31] show at least three identifiable intermediates in the reaction. It has proven impossible as yet to assign unambiguously structures to these intermediates or to be sure of their relevance to the physiological reaction. Rapid progress is being made, however, and we may soon be able to describe accurately the chemical events which occur during this important reaction.

Acknowledgement

This study was supported by a grant, GM 12202, from the U.S. National Institues of Health.

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