Biochimica et Biophysica Acta, 461 (1977) 218–230

BBA 47329

REACTION OF CO WITH CYTOCHROME *c* OXIDASE

TITRATION OF THE REACTION SITE WITH CHEMICAL OXIDANT AND REDUCTANT

DAVID F WILSON and YURIKO MIYATA

Department of Biochemistry and Biophysics, University of Pennsylvania, Medical School, Philadelphia, Pa 19174 (U S A)

(Received December 30th, 1976)

SUMMARY

The number of reducing equivalents required to form the reduced cytochrome a_3-CO compound has been determined for suspensions of submitochondrial particles and for isolated cytochrome *c* oxidase Anaerobic preparations were titrated reductively with NADH and oxidatively with O_2 in the presence of high concentrations of CO (0 4 to 0.8 mM) while monitoring reduction of cytochrome *a* and the formation of the reduced cytochrome a_3-CO compound by their characteristic absorbance changes Analysis of the titration data show that 2.0 ± 0 3 and $2\ 1 \pm 0$ 2 reducing equivalents per mol of cytochrome oxidase (per cytochrome *a*) are required for formation of the reduced cytochrome a_3-CO compound in submitochondrial particles and isolated cytochrome *c* oxidase, respectively. In each case, the formation of the CO compound is proportional to the number of equivalents accepted by the preparation, indicating that the two equivalents are equal and the effective *n* value for the reaction is 2.0 Potentiometric titrations of cytochrome *c* oxidase using the cobalt orthophenanthrolene complex ($E_{m,7\,0} = 0.37$ V) as mediator give the same half-reduction potential values for cytochrome *a* and a_3 as those obtained using the ferro-ferricyanide couple The formation of the reduced cytochrome a_3-CO compound at pH 7.0, in the presence of 0 6 mM CO and with CO-orthophenanthrolene as mediator occurs with a half-reduction potential of 0.45 V and requires two electrons These data confirm and extend the observation of Lindsay et al. (Arch Biochim Biophys (1975) 169, 492–505) that both the "invisible" copper and cytochrome a_3 must be reduced in order for CO to bind with high affinity.

INTRODUCTION

Mitochondrial cytochrome *c* oxidase is responsible for the transfer of reducing

Abbreviations $Co(oph)_3$ is the cobalt complex of orthophenanthroline Mn^{3+}CyDTA is the manganese complex of trans-1,2-diaminocyclohexanetetraacetic acid

equivalents from cytochrome *c* to molecular oxygen with concomitant synthesis of adenosine triphosphate (ATP) at site 3 of the respiratory chain. Thus, this hemeprotein carries out two reactions essential to the existence of higher organisms: the reduction of molecular oxygen to water and the transduction of the free energy available in the redox reactions into a form suitable for ATP synthesis. The reduction of molecular oxygen to water as carried out by cytochrome *c* oxidase occurs at redox potentials greater than 0.6 V relative to a hydrogen electrode [1, 2] and is responsible for the control of mitochondria respiration [1, 3]. Although the site of the reaction of molecular oxygen and CO has early been recognized [4–6], the mechanism of the reaction has remained obscure. One electron reduction of the O_2 to bound O_2^- would be expected to have an E_m value near −0.3 V [7, 8] and could not be expected to occur as an essential intermediate in a reaction which occurs at near 0.6 V. Mechanisms involving a two electron reduction are more feasible as the E_m value for O_2 reduction to bound O_2^{2-} is near 0.8 V [7]. The two electrons can either come from a single heme iron (oxidizing it to Fe^{4+}) or from a heme iron and a second redox component, such as the "invisible" copper.

The cytochrome a_3-CO compound titrates potentiometrically as a two electron acceptor [9, 10] and one CO molecule is bound for each active site [9–12]. This provides evidence that both the "invisible" copper and cytochrome a_3 are involved in the active site for oxygen reduction. This evidence has been challenged [13] on the basis of coulometric titrations of isolated cytochrome oxidase in the presence of CO, in which 3 equivalents per cytochrome *a* were removed from the fully reduced preparation without oxidizing the CO compound. Since only 4 equivalents per cytochrome *a* were required to fully reduce the preparation in the absence of CO, it was concluded that the formation of the reduced cytochrome a_3-CO compound required only one equivalent/cytochrome *a*.

In the present communication, we report the titration of cytochrome *c* oxidase in the presence of CO with chemical reductant (NADH) and chemical oxidant (O_2) which confirm our previous observation [9, 10] that reduction of both cytochrome a_3 and the "invisible" copper are required for formation of the reduced cytochrome a_3-CO compound at CO concentrations less than 1 mM.

MATERIALS AND METHODS

Submitochondrial particles were prepared from pigeon breast mitochondria isolated as described in references 14 and 15. Cytochrome *c* oxidase purified to a heme content of 12 nmol/mg protein was prepared from pigeon breast mitochondria by a modification of the procedure of Sun et al. [16] and was generously supplied by Dr. Maria Erecinska.

All titrations were carried out in an anaerobic cuvette without a gas phase. A solution of 50 mM phosphate buffer, pH 7.0, containing 0.2 mM EDTA was saturated with CO gas at atmospheric pressure, the submitochondrial particles or cytochrome *c* oxidase then added, and the anaerobic cuvette sealed. The remaining O_2 was removed by respiration with NADH (submitochondrial particles) or 20 μM phenazine methosulfate plus NADH (cytochrome oxidase). After the suspension became anaerobic, the preparations were titrated reductively using NADH solutions and oxidatively with O_2 using medium saturated with oxygen at 0–4 °C. When oxidase was titrated, an

equimolar concentration of cytochrome *c* was added in order to facilitate equilibration between the NADH and the redox components of the oxidase. The concentration of NADH was determined enzymatically with lactate dehydrogenase for each solution used in titrations. An extinction coefficient of 6.22 mM^{-1} at 340 nm was used for calculations. The oxygen concentration of the O_2-saturated medium was measured by titration with NADH in the presence of submitochondrial particles.

Spectra of the absorbance changes which occurred during oxidation and reduction were measured using a Johnson Research Foundation scanning dual wavelength spectophotometer. A spectral half-bandwidth of the measuring light beam was less than 1 nm. The spectrophotometer has a digital memory unit which retains any selected absorption spectrum, subtracts it from subsequent spectrum and then plots the resulting difference spectrum

Titrations using the wavelength pairs 590–575 nm, and 605–575 nm were carried out using a 4-beam spectrophotometer with 1.5 nm half-bandwidth interference filters mounted in an air driven turbine (for details, see ref. 17).

The trans-1,2-diaminocyclohexanetetraacetic acid complex of Mn^{3+} was generously supplied by Dr. Scot Wherland of the Chemistry Department of the California Institute of Technology in Pasadena. The cobalt orthophenanthrolene complex was prepared by adding a 3 3-fold molecular excess of orthophenanthrolene to a 50-mM aqueous solution of $Co(NO_3)_2$ and stirring until the orthophenanthrolene was completely in solution. Aliquots of this solution were then used as required.

RESULTS

Potentiometric analysis of the half-reduction potentials and n values for the components of cytochrome oxidase

Potentiometric analysis of the redox properties of the components of cytochrome oxidase has been hampered by the limited number of the available redox mediators. Only the ferro/ferricyanide couple has the desired E_m value, electrochemical activity, water solubility and optical properties suitable for titrations in the potential region of approx. 0 3–0.5 V. Because any redox mediator may interact with the component being measured (leading to erronous measured E_m value of the latter), controls which involve measurements in the presence of a completely different mediator system are necessary to establish with full confidence the half-reduction potential value of any redox carrier.

The $Co(oph)_3$ (the cobalt complex of orthophenanthroline) reacts readily with cytochrome oxidase, has the $E_{m7\,0}$ of 0.37 ($n = 1$) and only weak absorbance in the visible region of the spectrum (ref. 18, personal communication from S Wherland) Mn^{3+} CyDTA (the manganese complex of trans-1,2-diaminocyclohexanetetraacetic acid) has an $E_{m4\,7}$ of 0.81 V, and although its oxidized complex has a deep red color, the reduced form is colorless [19]. At redox potentials more negative than 0 6 V, the Mn^{3+} CyDTA is essentially quantitatively reduced and thus can be used conveniently as an oxidant in potentiometric titrations of cytochrome *c* oxidase with $Co(oph)_3$ as a redox mediator in place of ferricyanide. The half-reduction potentials for cytochrome *a* and a_3 obtained in these titrations are indistinguishable from those reported previously [20–22]. Since $Co(oph)_3$ has either a +2 charge (reduced form) or +3 charge (oxidized form) while ferrocyanide has a −4 charge, and ferricyanide a − 3 charge, it

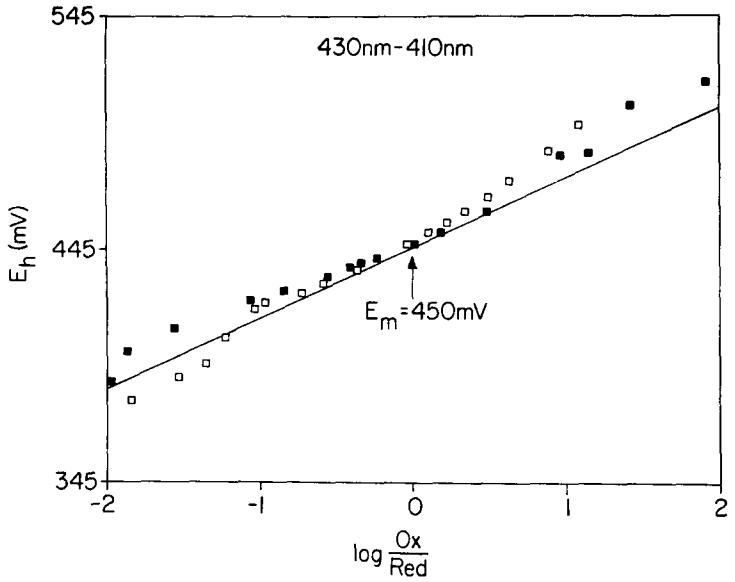

Fig 1 Potentiometric titration of the formation of the reduced cytochrome a_3-CO compound as measured at 430 nm minus 410 nm Submitochondrial particles from pigeon heart muscle were suspended at 2.5 mg protein in a 50 mM phosphate buffer, pH 7 0, and placed in a sealed cuvette CO was present at a final concentration of 600 μM Phenazene methosulfate (40 μM) diaminodurene (20 μM) and Co(oph)$_3$ (400 μM) were added and anaerobiosis obtained by addition of aliquots of a dithionite solution. After anaerobiosis, the titration was carried out reductively with dithionite (□) and oxidatively with Mn^{3+} CyDTA solution (■) The solid line is a theoretical titration curve for the titration of a component with an n value of 2 0 and an E_m of 0 450 V The measured E_h is plotted on the ordinate and the logarithm of the ratio of [reduced form] to [oxidized form] is plotted on the abscissa At this high CO concentration, it is assumed that essentially all of reduced form is bound to CO and that the only species present in significant amounts are the reduced form bound to CO and the oxidized form (see ref 10)

is unlikely that the two different mediators could give rise to similar errors in potentiometric measurements.

Moreover, because of its lack of absorbance in the Soret region, Co(oph)$_3$ can be used as a redox mediator in titrations of the reduced cytochrome a_3-CO compound by monitoring the absorbance changes at 430–410 nm. Data obtained for a suspension of submitochondrial particles in the presence of 0.6 mM CO are given in Fig. 1. In agreement with previous measurements using the 590 nm absorption band [9, 10], the formation of the CO compound occurs with an $E_{m7\,0}$ of 0 45 V when the CO concentration is 0.6 mM and the n value for the titration is 2.0. The data for the oxidative and reductive titrations fall on the same straight line (Fig. 1) and are independent of the Co(oph)$_3$ concentrations at values above 50 μM (to greater than 1 mM).

The concentration of the Co(oph)$_3$ used in the potentiometric titrations is high relative to that of cytochrome c oxidase. Since the Co(oph)$_3$ accepts more than 95 % of the reducing equivalents during reduction, the rate of formation of the reduced cytochrome a_3-CO compound by endogenous donor is insignificant (less than 5 % of that shown in Fig. 2). The measured formation of the cytochrome a_3-CO compound at a given E_h value, is the same in both oxidative and reductive titrations

and is independent of the concentration of $Co(oph)_3$ above 50 μM (to greater than 1 mM).

Attempts to determine the number of reducing equivalents in the reduced cytochrome a_3 CO compound by titration with Mn^{3+} CyDTA were unsuccessful Addition of aliquots of the Mn^{3+} CyDTA solution to fully reduced enzyme in the presence of CO caused stepwise oxidation of cytochrome *a* (and the "visible" copper), but the reduced cytochrome a_3-CO compound remains reduced even when a more than 50-fold excess of the oxidizing agent was added Addition of either $Co(oph)_3$ or ferrocyanide facilitated the oxidation of the CO-compound, but the oxidation was followed by a rereduction by an endogenous donor This indicates that in the absence of redox mediators, Mn^{3+} CyDTA does not interact rapidly enough with the cytochrome a_3-CO compound in order to accomplish its oxidation (a kinetic problem) in the presence of the endogenous electron donor(s). This endogenous donor must be present in relatively large concentrations as all efforts to exhaust it by repeated oxidation cycles were unsuccessful Moreover, its potential must be high because the rate of reduction of the cytochrome a_3-CO compound was much greater when the CO-compound was highly oxidized than when it was more than half reduced.

Titration of submitochondrial particles and cytochrome oxidase in the presence of CO

Cytochrome oxidase, both in the submitochondrial particles and in isolated state can be titrated reductively with NADH and reoxidized with its natural oxidant molecular oxygen which, in contrast to Mn^{3+}CyDTA never fails to find rapid access to its active site. An example of the titration of an anaerobic suspension of submito-

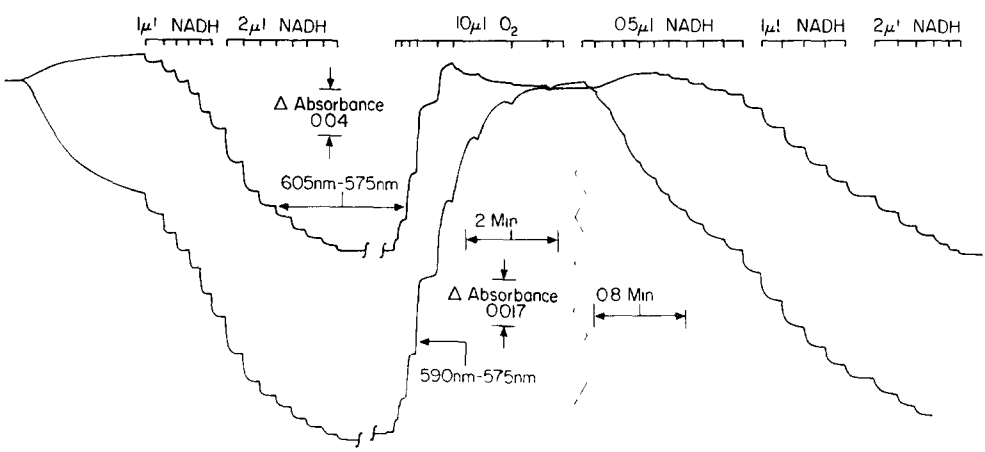

Fig 2 Titration of a suspension of submitochondrial particles with NADH and oxygen The submitochondrial particles were suspended at room temperature in a 7-ml sealed cuvette at 12 mg protein/ml in a medium containing 50 mM phosphate buffer, pH 7 0, and 600 μM CO After reduction with NADH (to establish anaerobiosis), the sample was reoxidized with aliquots of a saturated solution of oxygen (approx 4 °C, final concentration 1 6 mM) The measurements presented were begun when a slight excess of oxygen was present and the traces represent measurements at 605 –575 nm and 590–575 nm respectively. Aliquots of NADH solution (approx 12 mM) or oxygen solution were added as indicated The instrument sensitivity for each wavelength pair and the chart speed are indicated on the figure Note that he total absorbance changes at 605–575 nm are much larger than those at 590–575 nm and different instrument gain settings were used to emphasize the latter

chondrial particles supplemented with CO is presented in Fig. 2. The absorbance changes due to the reduction of cytochrome *a* (605–575 nm) and those due to the reduction of the cytochrome a_3-CO compound (590–575 nm) were measured simultaneously using a 4 beam, time-shared filter spectrophotometer. After anaerobiosis and reduction with NADH, the preparation was reoxidized with oxygen saturated medium (see Methods). A slight excess of O_2 was added to ensure 100 % oxidation of the cytochrome a_3-CO compound. After the excess of oxygen was consumed a partial reduction of the cytochrome a_3-CO compound was observed, manifested by a slow increase in absorbance at 590–575 nm This reduction occurred in every preparation examined and was caused by the presence of the endogenous high potential donor(s) described above with respect to the experiments with Mn^{3+}CyDTA The extent of the more rapid phase of the spontaneous reduction with this endogenous donor was never greater than 50 % of the cytochrome a_3-CO compound which indicates that the E_m of the former is greater than approximately 0 48 V.

Following the partial reduction of the cytochrome a_3-CO compound caused by the endogenous donor, aliquots of an NADH solution induced rapid stepwise reduction, first of the remaining CO compound and then of cytochrome *a*. A second cycle of oxidation with oxygen and reduction with NADH shown in Fig. 2 was recorded using a chart speed 2.5 times faster than that used previously. It can be seen that the reduction with endogenous donor is slower than that with NADH. After the initial approx. 25 % reduction of the CO-compound, it contributes negligibly (less than 10 %) to the stepwise increases in absorbance caused by the addition of NADH.

In suspensions of both submitochondrial particles and cytochrome oxidase, the formation of the reduced cytochrome a_3-CO compound precedes reduction of cytochrome *a* as evidenced by the increase in absorbance at 590–575 nm and decrease in absorbance at 605–575 nm. As more reducing equivalents are added and cytochrome *a* becomes reduced, the absorbance increases at both 590–575 nm and at 605–575 nm.

Fewer points were taken during the oxidative titration with molecular oxygen both because it is a secondary standard and because at the high concentration of CO used in the experiments, the rate of oxidation of the reduced cytochrome a_3-CO compound by oxygen is slower than its rate of formation on addition of NADH.

Absorption spectra of components being oxidized and reduced in the titrations

Identification of the components being oxidized and reduced can be made by comparing their spectra and absorption maxima with those of cytochrome *a* and cytochrome a_3-CO compound. Titrations were therefore carried by scanning the absorbance from 510 to 630 nm, using 575 nm as the reference wavelength (for the details see methods). A reductive titration of purified cytochrome *c* oxidase with NADH is shown in Fig 3 The baseline was obtained for a fully oxidized sample and reduction induced by stepwise addition of NADH. The first additions of NADH cause formation of the reduced cytochrome a_3-CO compound with its characteristic absorbance maximum at 590 nm. The CO compound is nearly completely reduced (> 90 %) before cytochrome *a* and *c* become reduced (< 10 %). Cytochrome *a* and *c* are reduced almost to the same extent, which indicates that their E_m values are within ± 0 01 V.

Particular attention is drawn to the fact that the reduced cytochrome a_3-CO compound contributes approx. 50 % of the 590–575 nm absorbance change (see also

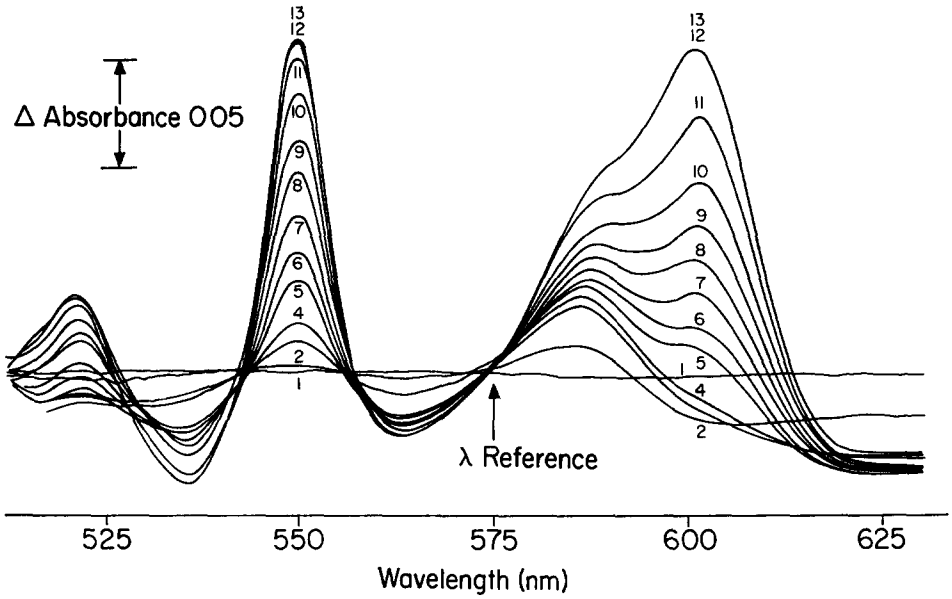

Fig. 3 The absorption spectra of the components being reduced in the titration of cytochrome oxidase with NADH Purified cytochrome *c* oxidase was suspended in a sealed cuvette at 7 6 μM cytochrome *a* in a medium containing 50 mM phosphate, pH 7 0, and 600 μM CO Cytochrome *c* (8.4 μM) and phenazine methosulfate (40 μM) were added and anaerobiosis achieved by adding aliquots of NADH After anaerobiosis, the preparation was reoxidized with aliquots of oxygen solution and the spectrum of the fully oxidized preparation recorded as a baseline The difference spectra were then measured at each stage of a reductive titration (see Fig 2) and are numbered in sequence starting with the fully oxidized baseline

Figs. 4 and 5) and a small negative absorbance change at 605–575 nm. The remaining absorbance changes measured using these wavelength pairs are contributed by cytochrome *a* reduction.

The dependence of the observed absorbance changes on the reducing equivalents present in cytochrome oxidase

Data taken from experiments of the type shown in Fig. 1 were evaluated as plots of the measured absorbance change against the number of reducing equivalents per cytochrome *a* present in the system and are shown in Fig. 4 for cytochrome oxidase and in Fig. 5 for submitochondrial particles. The fully oxidized system is considered to have zero reducing equivalents. Addition of NADH increased the number of reducing equivalents until complete reduction was reached and then addition of oxygen decreased the number of reducing equivalents until complete oxidation was again achieved. The end point for the formation of the reduced cytochrome a_3-CO compound was considered to coincide with the beginning of reduction of cytochrome *a* as determined by the increasing absorbance at 605–575 nm. The reduction of cytochrome *a* is then considered to require the equivalents added or subtracted during the change in absorbance at 605–575 nm. Since the absorbance changes are linear functions of the equivalents added during most of the titration, we

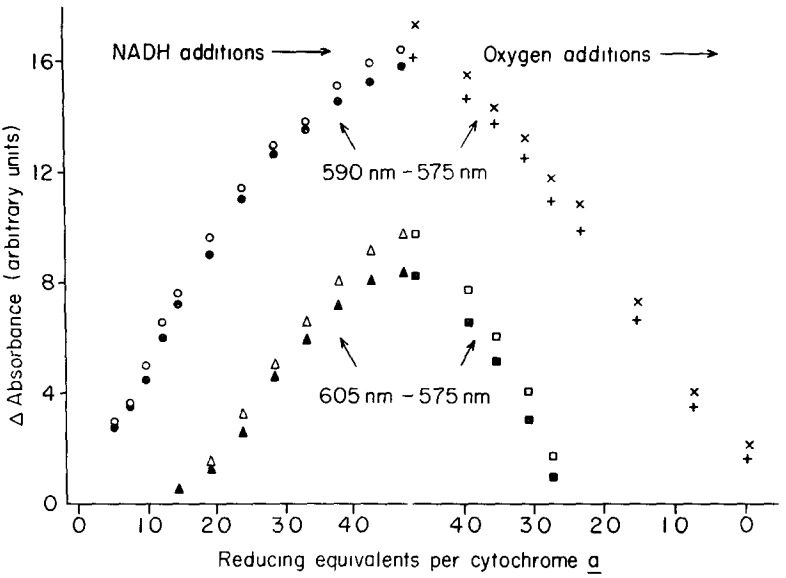

Fig 4 The stoichiometry of the reducing equivalents accepted by isolated cytochrome *c* oxidase during reduction with NADH and oxidation by O_2 The preparation of isolated cytochrome oxidase was suspended as described in the legend to Fig 1 and titrated both reductively with NADH and oxidatively with O_2 Two complete cycles of reduction and oxidation were analysed and the measured absorbance change plotted on the ordinate The abscissa is the number of reducing equivalents per cytochrome *a* present in the preparation as calculated from the reducing equivalents added (NADH) or removed (O_2)

have extrapolated the linear portion to 0 and 100 % formation of the reduced cytochrome a_3-CO compound or reduction of cytochrome *a*. The reducing equivalents required for each are indicated in Table I. A series of seven different preparations of isolated cytochrome oxidase were titrated and the results in the table are the averages data for each preparation. Analysing the data from the NADH titrations, the formation of the reduced cytochrome a_3-CO compound was found to require 2.1 ± 0.2 reducing equiv /cytochrome *a*, while the reduction of cytochrome *a* required $2\,0 \pm 0.3$ reducing equiv./cytochrome *a*. In three different preparations of submitochondrial particles, the formation of the reduced cytochrome a_3-CO compound required $2\,0 \pm$ 0.3 equiv./cytochrome *a* to form the CO compound. Analysis of the oxygen titration data is in good agreement with the values from the NADH titrations. The number of equivalents which accompanies the reduction of cytochrome *a* is much more uncertain in submitochondrial particles than in isolated oxidase because of contributions due to cytochromes *c* and c_1 and the Rieske iron-sulfur protein, in addition to cytochrome *a* and the "visible" copper. Cytochrome c_1 ($E_{m\,7\,0} = 0.215$ V), bound cytochrome *c* ($E_{m\,7\,0} = 0.235$ V), and "visible" copper ($E_{m\,7\,0} = 0.245$ V) have half-reduction potentials more negative than does cytochrome *a* ($E_{m\,7.0} = 0.26$ V in the presence of CO). Therefore, cytochrome *a* titrations are non-linear and extrapolation of the linear portion includes only approximately 70 % of the equivalents required for total reduction.

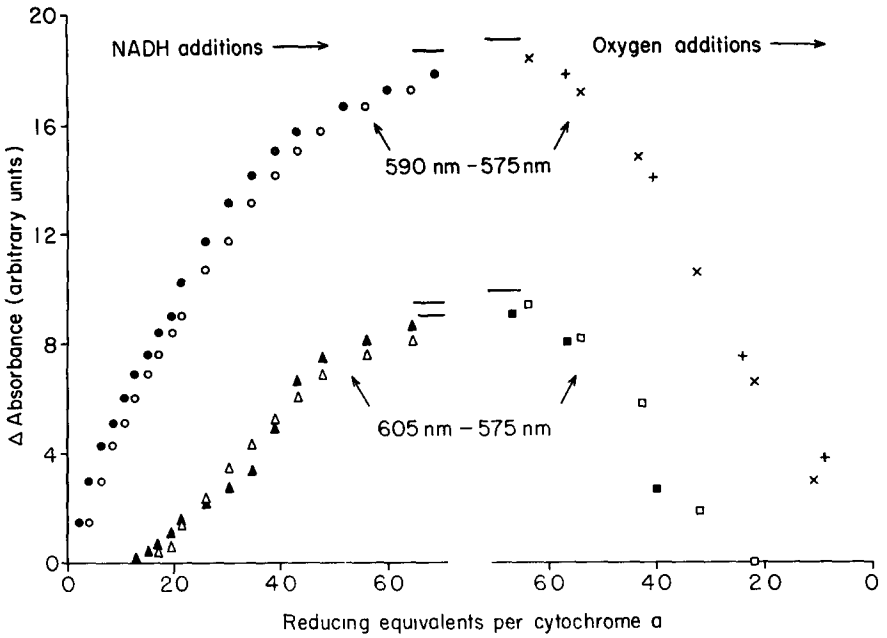

Fig 5 The stoichiometry of the reducing equivalents acpected by a suspension of submitochondrial particles during reduction with NADH and oxidation by O_2 The submitochondrial particles were suspended as described in the legend to Fig 2 and titrated reductively with NADH and oxidatively with O_2 Two complete cycles of reduction and oxidation were analysed and presented in the figure The measured absorbance change is plotted on the ordinate and the number of reducing equivalents per cytochrome *a* present in the preparation plotted on the abscissa

DISCUSSION

Titrations of cytochrome oxidase in submitochondrial particles and in isolated state with NADH and oxygen presented in this work show that two reducing equivalents are required for the formation of the reduced cytochrome a_3-CO compound. This confirms the results obtained using potentiometric titrations with either ferri/ferrocyanide [9, 10] or $Co(oph)_3$ (this work) as redox mediator. Furthermore, since the absorbance change due to the reduced cytochrome a_3-CO compound is proportional to the amount of the reducing equivalents added or subtracted throughout the entire titration curve, the two equivalents are formally identical i e. the two redox components have equal midpoint potential and *n* values in the presence of CO.

Titration of cytochrome oxidase from fully oxidized to fully reduced requires 4 reducing equivalents per cytochrome *a* both in the absence (23–25) and in the presence of CO (4 1±0 3, this paper) Thus, there is no evidence for the existence of any additional redox components in the presence of CO In the absence of CO, the four redox components are identified as cytochromes *a* and a_3, the "visible" copper and the "invisible" copper, each with an *n* value of 1 0 In the presence of 0 4–0 8 mM CO, both cytochrome *a* and the "invisible" copper can be independently measured to have an *n* value of 1 0 and $E_{m\,7\,0}$ value of approx 0.25 V This leaves the invisible copper as the redox component which, in addition to cytochrome a_3, is involved in CO binding

TABLE I

THE REDUCING EQUIVALENTS REQUIRED FOR THE FORMATION OF THE REDUCED CYTOCHROME a_3-CO COMPOUND AND THE REDUCTION OF CYTOCHROME *a*

The titrations were analysed by plotting the data as shown in Figs 4 and 5 The concentrations of NADH and O_2 required for each portion of the titration curve were obtained by extrapolating the linear portion of the titration curve were obtained by extrapolating the linear portion of the titration curve to 0 and 100 % formation of the reduced cytochrome a_3-CO compound or reduction of cytochrome *a* The observation that only reduction of cytochrome *a* contributes to the positive absorbance change at 605–575 nm helps to establish the value for 100 % formation of the CO compound and 0 % reduction of cytochrome *a*

Sample Cytochrome oxidase	Total cytochrome *c* (μM)	Total cytochrome *a* (μM)	Total O_2* (μM)		Total NADH (μM)		Equivalents/cytochrome *a***			
			cytochrome a_3-CO	cytochrome *a*	cytochrome a_3-CO	cytochrome *a*	cytochrome a_3-CO		cytochrome *a*	
							NADH	O_2	NADH	O_2
1	29 81	11 96	9 06	12 19	12 57	21 73	2 1	3 04	1 62	1 82
2	28 06	6 71	4 49	8 68	10.30	16 48	2 08	2 94	1 58	1 68
3	14 51	14 57	8.5	9 0	16 59	24 72	2 36	2 33	2 26	1 64
4	9 51	5 75	3 2	5 6	5 67	12 00	1.98	2 23	2 28	2 13
5	7 51	8 43	6 59	7 08	9.00	11 84	2.14	2 60	1 98	1 91
6	7 51	5 27	–	–	5 53	10 56	2 10	–	2 34	–
7	8 76	13 23	7 21	7 71	11 85	16 48	1 80	2 18	1 88	1 76
Average	–	–	–	–	–	–	2.08± 17	2 55± 37	1 99± 31	1 82±.18
Submitochondrial particles 1	17 03	8 43	4 68	8 80	7 60	16 17	1 80	2 22	3 84	4 17
2	18 02	8 35	2 86	7 21	7 73	14 95	1 85	1 85	3 58	3 45
3	10 51	4 41	2 97	3 46	5 13	7 70	2 33	2 69	3 49	3 15
Average	–	–	–	–	–	–	2 00±.28	2 25± 42	3 64± 18	3 59± 52

* Oxygen is a secondary standard (see Methods) with its concentration very sensitive to the temperature of the solution (approx 4 °C) and the handling during titration This accounts for the large variability in its titration values relative to those for NADH.

** The data for the isolated cytochrome oxidase has been corrected by subtracting the concentration of added cytochrome *c* The cytochrome *c* titrates parallel to cytochrome *a* (Fig 3) and the correction affects only that region of the titration There have been no corrections in the cytochrome *a* region for submitochondrial particles and all of the redox components, including cytochrome *c*, contribute to the measured equivalents/cytochrome *a*

(Note that the experimentally measured E_m value of the cytochrome a_3-CO compound of 0.45 V at 0.4–0.8 mM CO agrees well with that calculated from the E_m values of cytochrome a_3 (0.36 V), the invisible copper (0.34 V), and the K_d for CO of approx 0.4 μM (see ref. 10). One assumes that both must be reduced in order to bind CO with high affinity.)

The role of the invisible copper in the binding of CO has been questioned recently by Anderson et al. [13]. The apparent difference in experimental results presented in this work and those of Anderson et al., lies in the experimental approach In the potentiometric analyses (refs. 9, 10, and this paper) and in titrations with chemical oxidants and reductants (this work), the CO compound was oxidized and reduced and the equivalents were directly measured; while in the coulometric titrations, the CO compound was not oxidized and reduced and the reducing equivalents were calculated indirectly from the difference between the number of equivalents

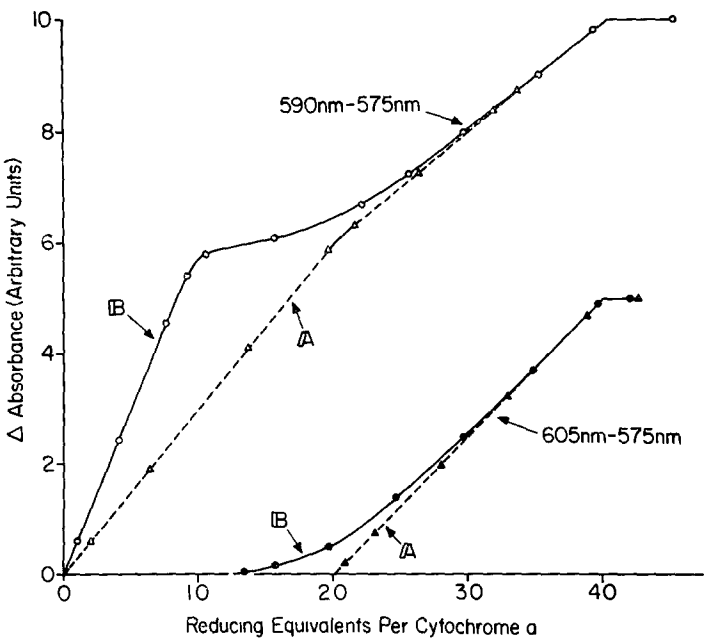

Fig 6 The expected pattern of the titration of cytochrome *c* oxidase in the presence of CO according to two different models Theoretical curves have been calculated for the absorbance changes at 590–575 nm (0 and Δ) and 605–575 nm (● and ▲) during titrations of cytochrome *c* oxidase assuming two different models Model A CO binds with high affinity only when both the invisible copper and cytochrome a_3 are reduced The formation of the CO compound would then have an n value equal to 2 0 and $E_{m7\ 2}$ of 430 mV ($E_{m7\ 2}$ = 0 34 V for the invisible copper, 0 36 V for cytochome a_3 and the K_d for CO is approx 0 4 μM) while both cytochrome *a* and the "visible" copper have E_m values of 0 25 V Model B CO binds with high affinity to reduced cytochrome a_3 and this reaction is essentially independent of the invisible copper. The formation of the CO compound would then have an n value of 1 0 and an $E_{m7\ 2}$ of 0 54 V ($E_{m7\ 2}$ = 0 36 V for cytochrome a_3 and the K_d for CO is approx 0 4 μ M) while the invisible copper, the visible copper, and cytochrome *a* would be 0 34, 0 25, and 0 25 V respectively The calculated functions are for the cytochrome *c* oxidase in the absence of added cytochrome *c* The reduced cytochrome a_3-CO compound is considered to contribute 60 % of the 590–575 nm absorbance change and 0 % to the 605–575 nm absorbance change. The symbols are placed on the calculated curves for identification purposes only, as the curves are continuous functions.

required to oxidize cytochrome oxidase in the absence (4 equiv.) and presence (3 equiv.) of CO. The failure to oxidize the CO compound in experiments of Anderson et al. [13] may arise from a combination of the slow rate of interaction of the reduced cytochrome a_3-CO compound with added non-physiological oxidants (compare our results with Mn^{3+}CyDTA) and the presence of a high potential endogenous donor which, because of its high potential, affects only the titrations in the presence of CO. The presence of this endogenous donor may also account easily for the extra reducing equivalent in the experiments of Anderson et al. [13].

The two postulated structures for the CO binding site of cytochrome oxidase, one involving two components, cytochrome a_3 and the invisible copper (refs. 9, 10, and this work), and the other involving only a single component [13], give rise to quite different titration curves Mathematical simulations for the two models are given in Fig. 6. In A, the curves represent the simulated absorbance changes for the case in which the formation of the CO compound requires 2 equivalents, while in B, the curves illustrate the simulated absorbance changes for the situation in which the formation of the CO compound requires only 1 reducing equivalent. (All the information used to compute the curves is presented in detail in the legend of Fig. 6.) It can be seen that the experimental curves shown in Figs. 4 and 5, fit well to a two-electron model represented by the curves shown in A.

ACKNOWLEDGEMENT

The authors are indebted to Dr. Scot Wherland and Dr. Harry Gray of the Department of Chemistry, California Institute of Technology, Pasadena, California, for their many suggestions and discussion of suitable oxidation-reduction mediators for the potentiometric titrations. At the meeting on copper proteins at Manziani, July 18–24, 1976, Dr. R. Wever and Dr. B. F. Van Gelder presented results of reductive titrations of cytochrome oxidase by NADH which indicated that two reducing equivalents were required to form the reduced cytochrome a_3-CO compound. (The manuscript appeared in the European Journal of Biochemistry 73, 149–154, (1977) following submission of this paper for publication.) This work was supported by U.S. Public Health Service Grant GM 21524.

REFERENCES

1 Wilson, D F , Erecinska, M. and Dutton, P L (1974) Annu Rev Biophys Bioeng 3, 203–230
2 Wilson, D F , Dutton, P L and Wagner, M (1973) Curr Top. Bioenerg 5, 233–265
3 Owen, C S and Wilson, D F (1974) Arch. Biochem Biophys. 161, 581–591
4 Warburg, O and Negelein, E (1929) Biochem Z. 214, 64–100
5 Keilin, D and Hartree, E. F (1938) Nature Lond 141, 870–871
6 Keilin, D and Hartree, E F. (1939) Proc Roy Soc B 127, 167–191
7 George, P (1965) in Oxidases and Related Redox Systems I (King, T E , Mason, H S and Morrison, M , Eds.) 1, pp. 3–33, Wiley, New York
8 Wood, P. M (1974) FEBS Lett 44, 22–24
9 Lindsay, J G. and Wilson, D F. (1974) FEBS Lett 48, 45–49
10 Lindsay, J G , Owen, C. S and Wilson, D F. (1975) Arch Biochem. Biophys 169, 492–505
11 Mansley, G. W , Stanbury, J T and Lemberg, R (1965) Biochim Biophys. Acta 113, 33–40
12 Wharton, D. C and Gibson, Q H (1976) J Biol Chem. 251, 2861–2862
13 Anderson, J L., Kuwana, T and Hartzell, C R. (1976) Biochemistry 15, 3847–3855

14 Erecinska, M , Oshino, R , Oshino, N and Chance, B (1973) Archiv Biochem Biophys 157, 431–445
15 Lindsay, J G , Dutton, P. L and Wilson, D F (1972) Biochemistry 11, 1937–1942
16 Sun, F. F , Prezbindowski, K S , Crane, F L and Jacobs, E E (1968) Biochim Biophys Acta 153, 804–818
17 Chance, B , Ligallais, V , Sorge, J. and Graham, N (1975) Analyt Biochem 66, 498–514
18 Farina, R and Wildins, R G. (1968) Inorg Chem 7, 514–518
19 Randall, E , Hamm, E , and Suwyn, M A (1967) Inorg Chem 6, 139–145
20 Wilson, D F and Dutton, P L (1970) Arch Biochem Biophys 136, 583–584
21 Wilson, D F , Lindsay, J G , and Brocklehurst, E S (1972) Biochim Biophys Acta 256, 277–286
22 Lindsay, J G and Wilson, D F (1972) Biochemistry 11, 4613–4620
23 Van Gelder, B. F and Beinert, H. (1969) Biochim. Biophys Acta 189, 1–24
24 Wohlrab, H (1970) Biochemistry 9, 474–479
25 Fujihira, Y , Kuwana, T and Hartzell, C R (1974) Biochem Biophys Res Commun 61, 488–493
26 Wilson, D F , Erecinska, M , Lindsay, J G , Leigh, Jr , J S and Owen, C S (1975) Proc 10th FEBS Meeting, pp 195–210
27 Wilson, D F , Erecinska, M and Owen, C S (1976) Arch Biochem Biophys 175, 160–175