min will lead to decomposition of approximately 50% of the UDPGal initially added. If the enzymatic conversion being studied results in utilization of a small proportion of the UDPGal during the incubation, the substrate concentration assumed will be seriously in error.

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Spectroelectrochemical Investigations of Stoichiometry and Oxidation–Reduction Potentials of Cytochrome *c* Oxidase Components in the Presence of Carbon Monoxide: The "Invisible" Copper[†]

James L. Anderson,[‡] Theodore Kuwana,* and Charles R. Hartzell[§]

ABSTRACT: Spectroelectrochemical studies are presented for the carbon monoxide complex of isolated, purified cytochrome *c* oxidase (EC 1.9.3.1) in solutions saturated with carbon monoxide. The results indicate a stoichiometry of three equivalents per oxidase-carbon monoxide complex molecule. Formal reduction potentials ($E^{\circ\prime}$) of the two copper and one heme component at pH 7.0 were obtained by means of quantitative absorbance-charge titrations in the absence and presence of cytochrome *c*, and by means of a Nernstian "Minnaert" plot in the presence of cytochrome *c*. Analysis of the absorbance-charge curves from these titrations gave an indirect determination of the high potential, "invisible" copper component. The copper potentials in the carbon monoxide complex were found to be relatively unchanged with respect to those of the native enzyme. The $E^{\circ\prime}$ values obtained were: high potential ("invisible") copper (340 ± 20 mV (NHE)), low potential copper (190 ± 20 mV), and low potential heme (250 ± 10 mV).

Numerous papers have appeared concerning the carbon monoxide complex of cytochrome *c* oxidase (EC 1.9.3.1) since the early work of Keilin and Hartree (1939). Both equilibrium properties, such as redox potentials (Tzagoloff and Wharton, 1965; Tsudzuki and Wilson, 1971; Wilson et al., 1972; Wilson and Leigh, 1972; Lindsay and Wilson, 1972, 1974; Cusanovich and Wharton, private communication, 1973; Lindsay, 1974; Lindsay et al., 1975), and kinetic properties (Gibson and Greenwood, 1963, 1964; Greenwood et al., 1974) have been investigated.

This work was initiated in order to consider the effects of CO on the formal reduction potentials at pH 7 of the two copper and two heme prosthetic groups in cytochrome *c* oxidase. Special consideration was given to the potentials of the copper centers, and in particular, the "invisible" copper, to ascertain how they were affected by CO addition. The absorbance-charge approach (Heineman et al., 1972, 1973; Hawkridge and

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Kuwana, 1973; Mackey et al., 1973; Fujihira et al., 1974; Kuwana and Heineman, 1974) is particularly useful as a probe of the potentials of "invisible" components. Several workers have reported potentials for the low-potential heme (Tzagoloff and Wharton, 1965; Tsudzuki and Wilson, 1971; Wilson et al., 1972; Wilson and Leigh, 1972; Lindsay and Wilson, 1972, 1974; Cusanovich and Wharton, private communication, 1973; Lindsay, 1974; Lindsay, et al., 1975) and low-potential copper (Tsudzuki and Wilson, 1971; Wilson and Leigh, 1972; Cusanovich and Wharton, private communication, 1973) in the presence of CO. Direct evidence of the potential of the high-potential copper has been lacking, although an estimate of its value has been indirectly inferred (Lindsay and Wilson, 1974; Lindsay, et al., 1975). In this work the potential of the "invisible" copper has been obtained from quantitative coulometric titrations of cytochrome *c* oxidase in the presence of CO. Consideration is also given to recent reports of the oxidizability (Cusanovich and Wharton, private communication, 1974; Lindsay, 1974; Lindsay and Wilson, 1974; Lindsay et al., 1975) of the oxidase-CO complex (oxCO).[1]

Experimental Procedures

Materials

Cytochrome *c* oxidase was purified according to the method of Hartzell and Beinert (1974), and stored in liquid nitrogen. The preparation contained 13–14 mg of heme iron/g of protein, 1.1:1 Cu:heme ratio and ≤5% lipid. Cytochrome *c* was purchased from Miles Research Laboratory, rechromatographed after the manner of Margoliash and Walasek (1967), and stored at 5 °C. 1,1′-Bis(hydroxymethyl)ferrocene (BHMF) (Strem Chemicals) was purified by recrystallization from ethanol. The water solubility and stability of BHMF make it an attractive one-electron mediator. The formal reduction potential at pH 7.0 is 465 ± 19 mV vs. NHE, as determined by both oxidative and reductive potentiometric titrations (R. Szentirmay and T. Kuwana, unpublished results, 1974). Triton QS-30 (Rohm and Haas) stock solutions were prepared by neutralization of appropriate quantities of 90% solution to pH 7 with NaOH, prior to dilution to final volume. Tween 20 (Sigma) was purified on alumina prior to use. Benzylviologen (BV) chloride (K & K Laboratories) was used as received. Carbon monoxide (Matheson Gas Products) was passed through a Ridox (Fisher Scientific) column to remove contaminating oxygen. All experiments were performed in 0.15 ionic strength phosphate buffer at pH 7.0. The temperature for most experiments was 24 ± 1 °C.

Titration cell design, optical and electrochemical equipment, and general procedure were similar to those previously discussed (Hawkridge and Kuwana, 1973). In some of the cells, ground glass joints (greased with Apiezon N) replaced Hamilton valves for reference and counter electrode compartments, and the working electrode was sealed to the cell with epoxy (Elmer's) in place of the previously used O-ring seal to minimize the possibility of oxygen leakage into the cell. Oxygen levels less than 5×10^{-7} M were conveniently attained by vacuum degassing. Titration current was integrated digitally by means of a voltage-to-frequency converter (Vidar Corp., Model 240) and an events counter (Beckman/Berkeley Model 5311 DR EPUT meter). The total number of counts was proportional to the electrochemical charge added. Precision and accuracy of the integrator were better than 1%. Spectra were acquired at slow speeds (ca. 250 nm/30 s) on a Houston *x–y* recorder using a previously described double-beam spectrometer (Hawkridge and Kuwana, 1973). Photometric accuracy and reproducibility for measurement of absorbance changes was better than ±0.005 absorbance units in all cases. Total absorbance changes during titrations were typically in the range 0.3–0.5 absorbance units.

Wavelengths were calibrated against a holmium oxide filter standard. A glass filter was used to remove higher grating orders of wavelengths less than 340 nm. With a 600 line/mm grating, spectral band-pass was 1.2 mm at half-height.

Methods

Total charge passed during a titration was the time-integrated sum of two current components: that due to electrolysis of the enzyme under study (titration current); and charging current, defined as current required to change the electrode potential from its resting value to the electrolysis potential, plus any currents due to extraneous reactions.

Charging current corrections were obtained by performing the titration on blank supporting electrolyte solutions in the absence of enzyme and coulometric titrants. Stirring was identical for both blank and enzyme titrations. In blank experiments, the initial potential was varied and charge recorded as a function of time at a fixed electrolysis potential used during the coulometric titrations. Potentials for oxidation and reduction were chosen to afford diffusion-controlled generation of the appropriate mediator titrant.

Experimental charging corrections (generally less than 6% of total electrolysis charge for each charge increment) were obtained by the following procedures for the enzyme titrations. The potential of the working electrode was noted prior to addition of a charge increment. (During enzyme titrations, the working electrode is disconnected from the electrolysis circuit except during addition of charge increments.) The electrode was then reconnected and a given charge increment was added by applying the desired electrolysis potential. At the end of this interval, the electrode was switched out of the circuit, and the time was noted.

The charge correction, applicable to the initial potential and electrolysis time at the fixed electrolysis potential, was obtained by interpolation from the charging data for blank supporting electrolyte.

Experimentally, the first several charge correction increments were largest when changing from an oxidative to a reductive titration (or vice versa). Subsequent charge corrections were of nearly constant magnitude. Uncertainties in the corrected titration charge resulting from this procedure were estimated to be between 2 and 5%.

Enzyme solutions were prepared aerobically and quickly vacuum degassed and pressurized under nitrogen by previously described procedures (Hawkridge and Kuwana, 1973), to minimize the extent of catalytic oxidation of the high-potential mediator titrant (BHMF) by the oxidase. This oxidation results from the Nernstian equilibrium (unfavorable, but finite) between BHMF and oxidase, and the availability of oxygen to catalyze turnover of reduced oxidase. Generally, the enzyme was electrochemically cycled to a fully reduced state, reoxidized, and returned to the fully reduced state, while spectrally monitoring the redox state to establish native enzyme absorbance-charge behavior. The fully reduced preparations

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[1] Abbreviations used are: BHMF, 1,1′-bis(hydroxymethyl)ferrocene [$(HOCH_2C_5H_4)_2Fe$]; BV, benzylviologen; cytochrome a_H, high potential heme prosthetic group; cytochrome a_L, low potential heme prosthetic group; cytochrome Cu_H, high potential copper prosthetic group; cytochrome Cu_L, low potential copper prosthetic group; oxCO, complex of cytochrome *c* oxidase with carbon monoxide; EPR, electron paramagnetic resonance.

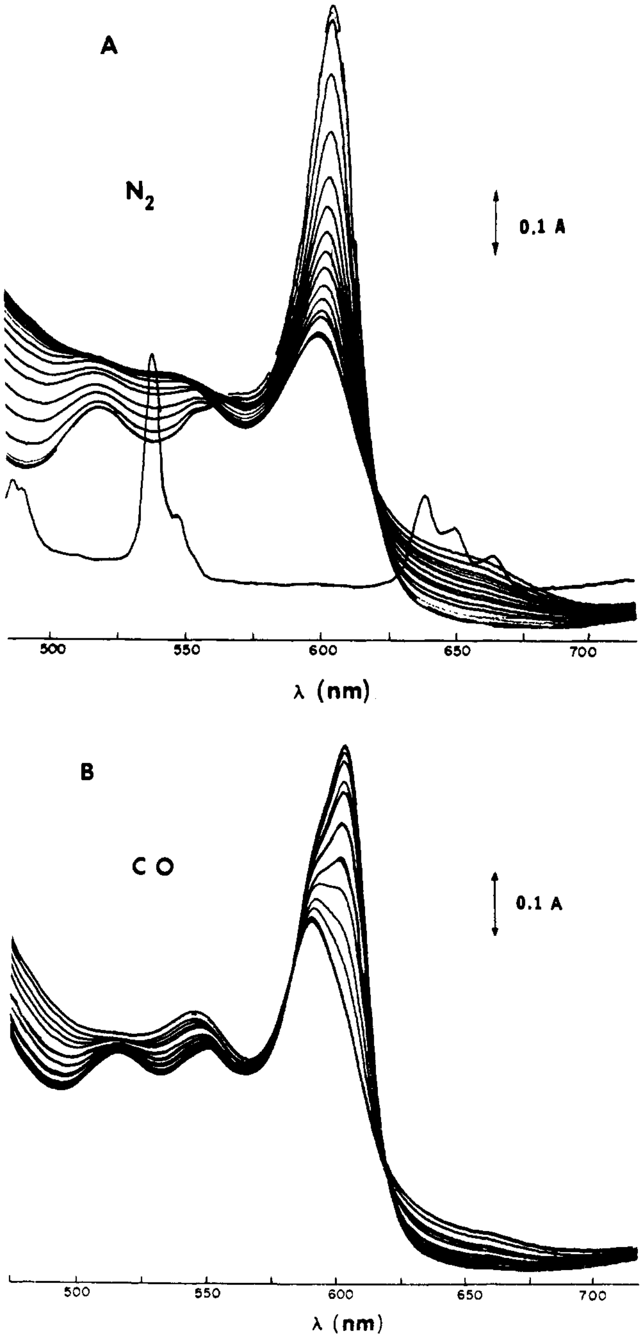

FIGURE 1: Spectra of cytochrome *c* oxidase during incremental coulometric titration. Optical path length 1.225 ± 0.002 cm; cell volume 0.851 ml. 36.0 μM total heme *a*, 0.38 mM BHMF, 0.65 mM BV, pH 7.0, phosphate (0.15 ionic strength), 0.19% Triton QS-30. Absorbance peak at 602–603 nm increases as enzyme is reduced, in all spectra. (A) First oxidative titration of initially reduced native oxidase (charge increments, 0.6 mC/ml of solution, except first and last two increments; dashed line, excess BV^+). Lower trace: holmium oxide wavelength calibration spectrum. (B) Second reductive titration of initially oxidized oxidase-CO complex (0.4 mC/ml of charge increments). Solution saturated with CO.

TABLE I: Characteristic Wavelengths of Cytochrome *c* Oxidase in the Absence and Presence of Carbon Monoxide.[a]

	CO Absent	CO Present
Oxidized peak (nm)	598	590[b]
Reduced peak (nm)	603	602
Isosbestic points (nm)		
Short wavelength	551 (high potential) 562 (low potential)	582
Long wavelength	620	619

[a] Wavelengths determined relative to a holmium oxide filter standard, by linear interpolation between the 536.4- and 637.5-nm filter peaks. [b] The oxidized form of the CO complex contains one reduced species (the cytochrome a_3-CO complex) and three oxidized species (cytochrome *a* and two coppers).

were then saturated with carbon monoxide (ca. 1.2 atm) and the titration repeated through several complete oxidation–reduction cycles.

The rapidity and convenience of the anaerobic coulometric titration approach made it possible to look at a single enzyme sample under constant solution conditions in the absence and presence of CO. Carbon monoxide did not significantly affect the absorbance–charge behavior of the mediator titrants (BV, BHMF) or the cytochrome *c* used in some experiments. $E^{\circ\prime}$ values reported (at pH 7.0) are referenced to the normal hydrogen electrode (NHE). For oxidase and cytochrome *c* components, the formal reduction potential is experimentally defined as the electrode potential that would be observable when the absorbance due to that component is midway between the values obtained for that component in fully reduced and fully oxidized states, respectively. The potentials of invisible components are obtained indirectly by correlating the charge and the absorbance.

Results

Titration spectra are presented in Figure 1A,B for cytochrome *c* oxidase under nitrogen, and after formation of the CO complex, respectively, under otherwise identical solution conditions. Formation of the "oxCO" complex shifts characteristic absorbance maxima and isosbestic points from the native enzyme values, as summarized in Table I.

Absorbance–charge plots were constructed by plotting the absorbance change at a wavelength of 602 nm as a function of the electrochemical charge. An absorbance–charge plot is presented in Figure 2A for a series of reduction–oxidation cycles of a single enzyme sample. Both reductions and oxidations are plotted on the graph; charge increments from reduced to oxidized state are plotted from left to right.

Superimposed on the experimental points is a theoretical curve simulated by previously described techniques (Heineman et al., 1973; Mackey and Kuwana, 1973; Kuwana and Heineman, 1974) assuming three titratable one-electron redox centers in the enzyme and Nernstian equilibrium among all redox species. The satisfactory agreement between experiment and theory supports previous results (Greenwood et al., 1974) indicating a stoichiometry of 3 equiv/mol of the CO oxidase complex. The curvature at both ends of the titration curve is indicative of the titration of the two copper centers (not directly detectable in the wavelength region employed) with midpoint potentials distinct from the midpoint potential of the heme. Comparison of the three electron behavior in the presence of CO, with previously reported four electron behavior for native oxidase in the absence of CO (Heineman et al., 1972; Mackey et al., 1973; Kuwana and Heineman, 1974) indicates significant inhibition of one redox center by CO.

The absorbance–charge (*A*–*q*) method is capable of detecting the presence and potentials of the two invisible components whenever redox potentials of the invisible components are sufficiently close to the potential of the visible component that substantial fractions of the visible and invisible compo-

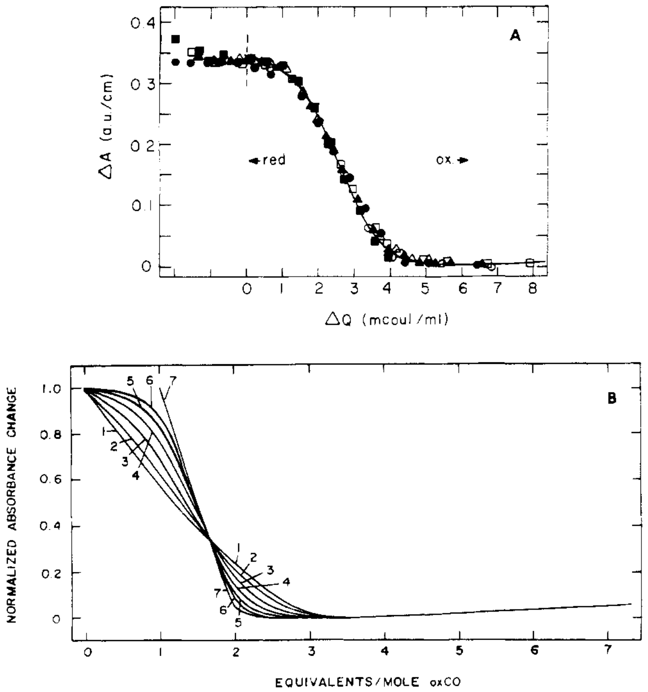

FIGURE 2: (A) Composite absorbance-charge plot for multiple oxidative and reductive coulometric titrations of oxidase-CO complex in CO-saturated solution. Points are experimental values; line is theoretical for three one-electron components with midpoint ($E^{\circ\prime}$) potentials: $E^{\circ\prime}$ (Cu_H) = 340 mV; $E^{\circ\prime}$ (Cu_L) = 190 mV; $E^{\circ\prime}$ (a_L) = 260 mV. The high-potential heme remains reduced ($E^{\circ\prime}$ (a_H) $\geq$ 465 mV). $E^{\circ\prime}$ (BHMF) = 465 mV. Plotted with reduced conditions at left, oxidized at right. Excess reductant at left of dashed line. Concentrations as in Figure 1. Points: (● ▲ ■) successive reductions; (○ △ □) successive oxidations. (B) Theoretical A-q curves for simultaneous variation of $E^{\circ\prime}$ of low and high potential "invisible" components, with fixed $E^{\circ\prime}$ of visible component (three titratable components). Concentrations: 32.0 μM total heme a, 0.32 mM BHMF. $E^{\circ\prime}$ (BHMF) = 0.465 V; $E^{\circ\prime}$ (a_3-CO complex) > 0.465 V; $E^{\circ\prime}$ (uncomplexed a) = 0.250 V. Curves 1-7 have $E^{\circ\prime}$ (Cu_H) values of 0.28, 0.30, 0.32, 0.34, 0.36, 0.38, and 1 V, respectively, and $E^{\circ\prime}$ (Cu_L) values of 0.25, 0.23, 0.21, 0.19, 0.17, 0.15, and −1 V, respectively. (Titrations of coppers are not detectable under conditions of this plot.)

nents accept charge concurrently. This criterion is met for invisible components whose potentials are not separated by more than ca. 120 mV from the potential of the visible component. Figure 2B illustrates the sensitivity of the absorbance-charge curve to variations in $E^{\circ\prime}$ of two invisible components and a visible component of fixed intermediate $E^{\circ\prime}$. When the $E^{\circ\prime}$ values of invisible components differ by less than 100 mV from the $E^{\circ\prime}$ of the visible component, 20 mV variations in the invisible components' potentials are readily determined. A generalized discussion of experimental errors in the A-q and other methods of potential determination will be presented elsewhere (Anderson and Kuwana, manuscript in preparation).

Figure 3 illustrates spectra obtained during titration of a mixture of cytochrome c oxidase with cytochrome c in the absence and presence of CO under otherwise identical solution conditions. All isosbestic points hold rigorously, except the short-wavelength oxidase isosbestic in absence of CO (551 nm oxidized; 562 nm reduced; each holds for half of the titration), as noted previously (Heineman et al., 1973).

Experimental absorbance-charge data for oxidase (602 nm) and cytochrome c (550 nm) in the presence of CO are shown in Figure 4. It is important to note that absorbance data plotted are *raw data*. No corrections of any kind have been made on the absorbance axis. Charge data have been corrected for the

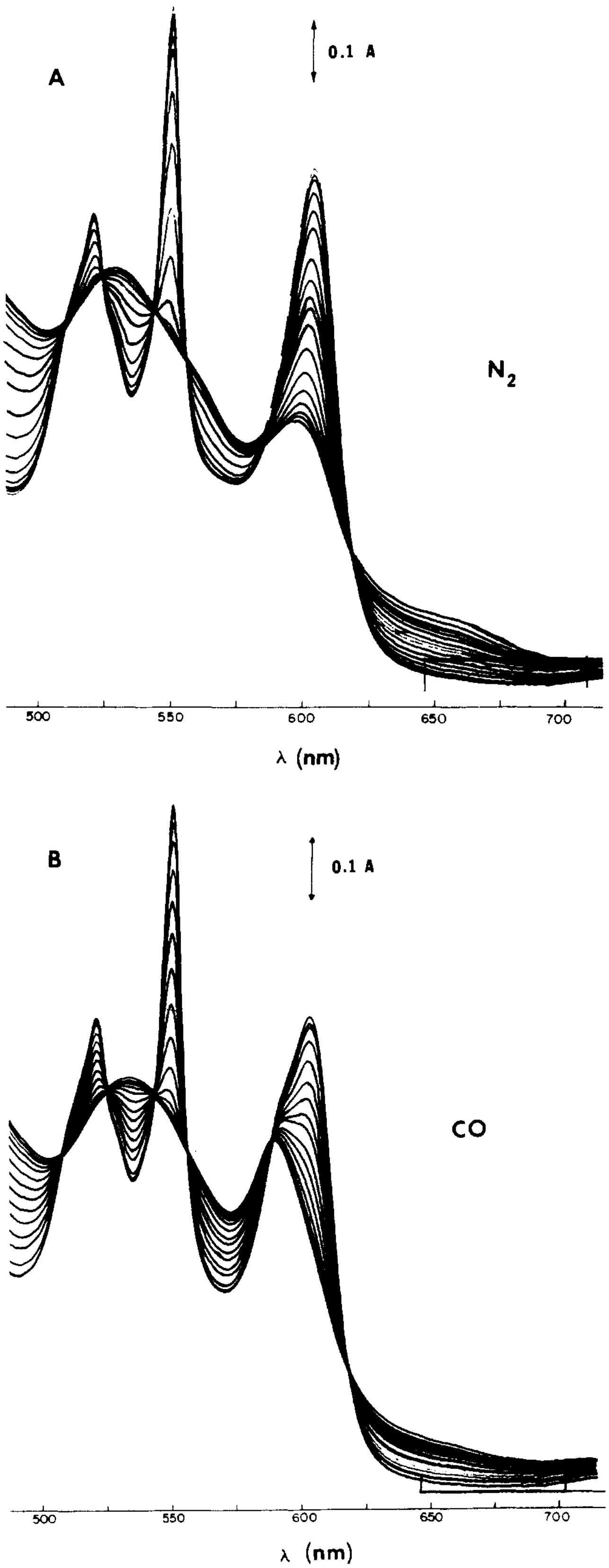

FIGURE 3: Spectra of mixtures of cytochrome c oxidase with cytochrome c during incremental coulometric titration. Cell parameters same as in Figure 1. 32.0 μM total heme a, 21.5 μM cytochrome c, 0.26 mM BHMF, 0.45 mM BV, pH 7.0, phosphate (0.15 ionic strength), 0.13% Triton QS-30. (A) Second reductive titration of initially oxidized native oxidase (first 12 charge increments 0.6 mC/ml; last 6 increments 0.3 mC/ml); dashed line, excess BV^+). (B) First oxidative titration of initially reduced oxidase-CO complex (0.4 mC ml^{-1} charge increment^{-1}, except last 2). Solution saturated with CO.

experimental blank; a correction of less than 6%, with total charge uncertainty of ca. 2–5%. The effects of spectral overlap were explicitly considered theoretically, by taking into account the behavior of *isolated* components. However, corrections were made only in the *theoretical* calculations to avoid biasing raw data. Theoretical absorbance–charge curves are superimposed on the experimental points.

Theoretical curves were generated using the following assumptions: (1) Molar absorptivity changes $\Delta\epsilon$ (reduced minus oxidized) at *602 nm* are due to: (a) cytochrome *c* oxidase as CO complex, based on one heme ($\Delta\epsilon = +19\,500\ M^{-1}\ cm^{-1}$, 100% due to uninhibited heme *a*, no contribution from copper or the inhibited component(s); determined from the ratio of maximum extinction changes (reduced minus oxidized) of isolated oxidase samples before and after addition of CO to the same sample, and assuming $\Delta\epsilon = 24\,000\ M^{-1}\ cm^{-1}$ or 12 000 $M^{-1}\ cm^{-1}$ per heme at 602 nm in native oxidase; (b) cytochrome *c* ($\Delta\epsilon = -1640\ M^{-1}\ cm^{-1}$; based on the ratio of absorbance changes at 602 and 550 nm for isolated samples, and assuming $\Delta\epsilon = 21\,000\ M^{-1}\ cm^{-1}$ at 550 nm); (c) BHMF (oxidized BHMF$^+$: $\epsilon = 300\ M^{-1}\ cm^{-1}$; reduced BHMF^0: $\epsilon = 0$; $\Delta\epsilon = -300\ M^{-1}\ cm^{-1}$. (2) Molar absorptivity changes (reduced minus oxidized) at *550 nm:* (a) cytochrome *c* oxidase (low potential heme component) in CO complex ($\Delta\epsilon = -2460\ M^{-1}\ cm^{-1}$; based on the ratio of absorbance changes at 550 and 602 nm for isolated samples, and assuming $\Delta\epsilon = 19\,500\ M^{-1}\ cm^{-1}$ at 602 nm); (b) cytochrome *c* ($\Delta\epsilon = +21\,000\ M^{-1}\ cm^{-1}$); (c) BHMF (BHMF$^+$: $\epsilon = 200\ M^{-1}\ cm^{-1}$: BHMF^0: $\epsilon = 0$; $\Delta\epsilon = -200\ M^{-1}\ cm^{-1}$). In the wavelength range employed, the oxidized form of BV (BV^{2+}) does not absorb. The appearance of absorption due to an excess of the reduced form (BV$^+$; molar absorptivity ca. 11 000 at 602 nm (Steckhan and Kuwana, 1974)), readily indicates completion of a reductive titration. In the potential range of the titratable components, the absorption changes due to BHMF$^+$ formation have a negligible influence on the shape of the titration curve, and can be ignored, as seen in Figure 2B.

A–q titrations for the isolated oxCO complex unambiguously define *relative* potentials differences (estimated uncertainty of ±20 mV) between the two coppers and uncomplexed heme if two assumptions are correct. (1) The oxCO absorbance change at 602 nm ($\Delta\epsilon = 19\,500\ M^{-1}\ cm^{-1}$) is due primarily (≥95%) to the uninhibited heme. (2) The charge taken up during the enzyme titration is due primarily (≥90%) to enzyme components rather than adventitious redox components. No evidence was found to contradict these assumptions. Freedom of the enzyme from adventitious redox components is evidenced by observed stoichiometry of 4.0 electron equiv/mol in native enzyme (Heineman et al., 1972). *A–q* titration of the oxCO–cytochrome *c* mixture defines oxCO potentials more precisely and supports these assumptions, in two respects. Potentials obtained by the independent titrations agree well. Theoretical and experimental *A–q* slopes agree well for both oxCO and cytochrome *c* simultaneously. Serious failure of either assumption listed above would tend to preclude simultaneous fitting of oxCO *and* cytochrome *c* curves using parameters obtained in the absence of cytochrome *c*.

Potentials for the two coppers and the non-CO-complexed heme *a* in the presence of cytochrome *c* are in good agreement with those found in the absence of cytochrome *c*. In the CO complex, the potential of the titratable heme (260 ± 10 mV and 250 ± 10 mV, in the absence and presence of cytochrome *c*, respectively) is in good agreement with those reported by other workers (Tzagoloff and Wharton, 1965; Tsudzuki and Wilson, 1971; Wilson, et al., 1972; Wilson and Leigh, 1972; Lindsay and Wilson, 1972; Lindsay, 1974). The 10-mV change in apparent heme potential upon cytochrome *c* addition is within the limit of experimental uncertainties. The heme complexed by CO (traditional cytochrome a_3) is not readily oxidized by BHMF (midpoint potential $E^{\circ\prime} = +465$ mV) in solutions saturated with CO (1.2 mM CO). This result is consistent with observations of others that ferricyanide ($E^{\circ\prime} = +420$ mV) ordinarily cannot oxidize the a_3CO complex (Wharton, 1964; Greenwood et al., 1974; Lindsay, 1974). The potential of the other heme *a* at 250 ± 10 mV (NHE) is distinctly different from the value of 213 ± 10 mV observed for the low-potential heme in the same oxidase sample prior to CO addition.

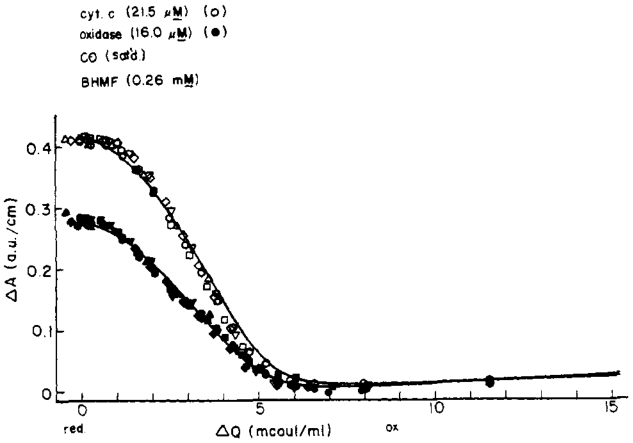

FIGURE 4: Composite absorbance–charge plot for multiple oxidative and reductive coulometric titrations of a mixture of cytochrome *c* + oxidase–CO complex in CO-saturated solution. Plotted with reduced conditions at left, oxidized at right. Points are experimental values; lines are theoretical for three one-electron oxidase components. ($E^{\circ\prime}$ (Cu_H) = 340 mV; $E^{\circ\prime}$ (Cu_L) = 190 mV; $E^{\circ\prime}$ (a_L) = 250 mV; the fourth component remains reduced—$E^{\circ\prime}$ (a_H) ≥ 465 mV); $E^{\circ\prime}$ (cytochrome *c*) = 255 mV; $E^{\circ\prime}$ (BHMF) = 465 mV. Concentrations as in Figure 3. Open circles, ΔA_{550} (due primarily to cytochrome *c*). Filled circles, ΔA_{602} (due primarily to uncomplexed heme in oxCO). Points: (△ ▽ ◇) successive reductions; (○ □ ○) successive oxidations.

It is important to emphasize that the same *three-electron stoichiometry* is observed for the oxCO complex for titrations in the presence and absence of an added redox component, cytochrome *c*. The good agreement between titrations illustrates the feasibility of determining the potentials of two "invisible" components, and strongly suggests that the observations do not merely reflect experimental artifacts.

The $E^{\circ\prime}$ value for the low-potential heme was further evaluated by means of Minnaert plots (Figure 5) of log $(C_R/C_O)_{\text{cyt}\ c}$ vs. log $(\text{ox}_R/\text{ox}_O)_{\text{oxidase}}$ in the absence and presence of CO. Here, R is the reduced and O the oxidized form of an enzyme. The data were corrected for the mutual absorbance contributions of cytochromes *c* and oxidase at 550 and 602 nm before plotting the points. Correction of absorbance due to BHMF$^+$ was made in the theoretical curve simulation. Also shown for comparison purposes is the curve that would be obtained for the same potentials if data had been corrected for BHMF$^+$ absorption prior to plotting.

The results for the CO complex ($E^{\circ\prime}$ = 250 ± 10 mV) are in good agreement with potential values obtained from absorbance–charge plots (Figure 4) and with potential values reported by other workers (Tzagoloff and Wharton, 1965; Tsudzuki and Wilson, 1971; Wilson et al., 1972; Wilson and Leigh, 1972; Lindsay and Wilson, 1972; Lindsay, 1974).

If an *n* value of 1.0 is assumed for cytochrome *c*, a slightly low *n* value is obtained for heme *a* (0.80 ± 0.02) from the

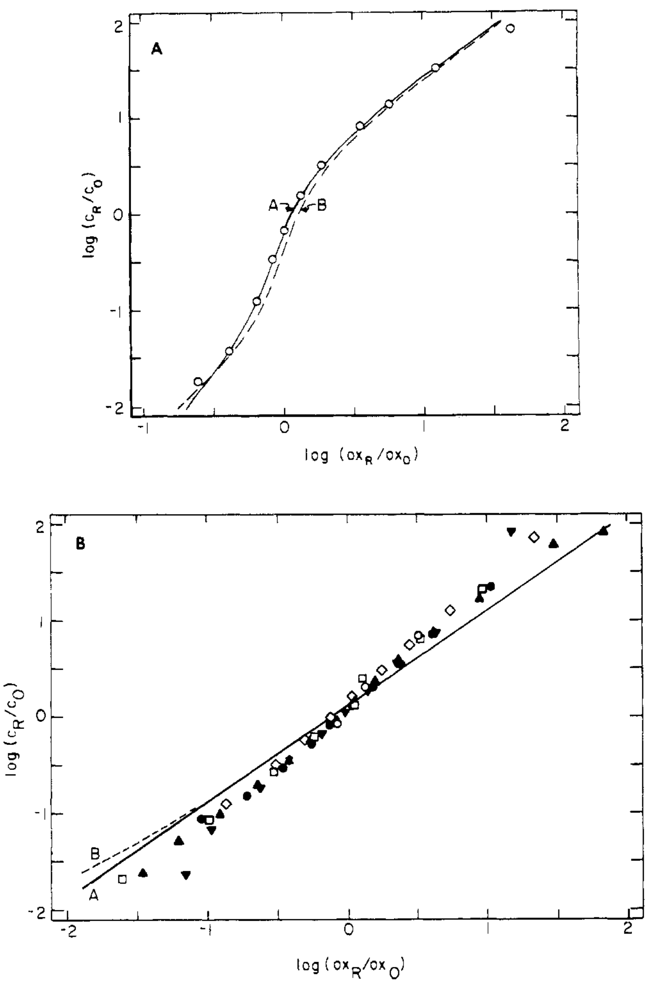

FIGURE 5: Minnaert plots of mixture of cytochrome *c* with oxidase in absence and presence of CO. Concentrations as in Figure 3. (A) Single titration of cytochrome *c* + oxidase mixture in absence of CO. Points are experimental values corrected for mutual absorbance of cytochrome *c* at 602 and oxidase at 550 nm; assuming $\Delta\epsilon$ (oxidase red − ox, 602) = +24 000 M^{-1} cm^{-1}; $\Delta\epsilon$ (oxidase red − ox, 550) = 2180 M^{-1} cm^{-1} (low potential heme); other absorbance corrections as described in text. Data not corrected for $BHMF^+$ absorbance. Lines are theoretical for two one-electron heme components ($E^{\circ\prime}$ (a_H) = 350 mV; $E^{\circ\prime}$ (a_L) = 213 mV) of equal $\Delta\epsilon$ (602). Line A includes correction for $\Delta\epsilon$ ($BHMF^+$); line B would be obtained if data rather than theoretical line were corrected for effect of $BHMF^+$ absorbance. (B) Composite plot of multiple reductive and oxidative coulometric titrations of cytochrome *c* + oxidase mixture in CO-saturated solution. Points are experimental values. Apparent oxidase *n* value 0.80, assuming *n* = 1.0 for cytochrome *c*. Lines are theoretical for $E^{\circ\prime}$ (a_L) = 250 mV, $E^{\circ\prime}$ (cytochrome *c*) = 255 mV (*n* = 1.0). Line A, data corrected for $BHMF^+$ absorbance; (*n* = 1.0) line B: data uncorrected for $BHMF^+$ absorbance—theory corrected. Points: (○ □ ◇) successive reductions; (▲ ▼ ●) successive oxidations.

Minnaert plot. Similar behavior has been observed for the CO complex in presence of cytochrome *c* by Tzagoloff and Wharton (1965) (0.8 ≤ *n* ≤ 1.0 from Minnaert plots), and by Cusanovich and Wharton (private communication, 1973) (*n* = 0.83 ± 0.04 from potentiometry). The heme potential obtained from our data with Minnaert slope corresponding to an apparent value of *n* = 0.80 differs by less than 2 mV from the value expected for a line with theoretical slope corresponding to a value of *n* = 1.0.

The anomalous Minnaert *n* value could reflect a copper contribution at either 550 or 602 nm. A copper contribution at 602 nm could account for "invisible" copper effects alluded to by Lindsay and Wilson (1974) and Lindsay et al. (1975). Yong and King (1972), have also suggested copper contributions at 603 nm in cyanide-inhibited oxidase. Alternatively, slight underestimation of ΔA_{602} of cytochrome *c* or ΔA_{550} of oxCO could account for the apparent deviation from *n* = 1 behavior. Small absorbance errors can produce large errors in Minnaert slopes, which depend on the logarithm of a ratio of absorbance changes: log $[\Delta A/(\Delta A_{max} - \Delta A)]$ (Anderson and Kuwana, manuscript in preparation). Stoichiometry obtained from *A*–*q* plots is *much* less susceptible to small errors in absorbance or charge (estimated charge uncertainty <5% in these titrations).

The excellent fit of the theoretical oxCO *A*–*q* curve and the slightly less exact fit of the theoretical cytochrome *c A*–*q* curve suggest that any absorbance error may lie at 550 rather than 602 nm. In any event, a *one-electron process* is indicated, rather than a recently reported two-electron process (Lindsay, 1974; Lindsay and Wilson, 1974; Lindsay et al., 1975).

Absorbance–charge titrations indicate that the high-potential copper in the CO complex (340 ± 20 mV) is unchanged in potential relative to the native enzyme. The low potential copper (190 ± 20 mV) also is little affected relative to potential values determined for samples of the same enzyme batch in absence of CO.

Cusanovich and Wharton (private communication, 1973), Lindsay (1974), Lindsay and Wilson (1974), and Lindsay et al. (1975) have recently reported the oxidizability of the CO-inhibited high-potential heme both in mitochondrial preparations and in isolated oxidase. Lindsay and Wilson (1974), and Lindsay et al. (1975), followed the titration behavior by difference measurements at the wavelength pair (590–624 nm). In contrast, the spectra reported here suggested that the absorbance change at 590 nm was due entirely to a shoulder of the 602-nm absorbance assigned to the uncomplexed heme *a*. To test this hypothesis, difference spectra were obtained by coulometrically generating the fully reduced CO complex of cytochrome *c* oxidase in identical oxidase sample solutions in electrochemical cells with matched (±0.2%) optical paths. The sample solution was then subjected to coulometric oxidation and reduction while the reference solution was maintained in either fully oxidized or fully reduced form. A peak of 603 nm was maintained for all redox states of the sample, as seen in Figure 6. For comparison, difference spectra are shown for the native enzyme, which also exhibits the 603-nm peak in all redox states.

For the CO complex, the slightly asymmetric 603-nm difference peak accounts completely for the absorbance changes at 590 nm during titration. Thus, the ratio of $(\Delta A_{590}/\Delta A_{603})$ remains constant as the redox state is varied. Consequently, for CO saturated solutions, no evidence was found for the *n* = 2, high-potential component reported by Lindsay (1974), Lindsay and Wilson (1974), and Lindsay et al. (1975).

Discussion

The indirect coulometric titration method allowed the repetitive redox cycling of the oxidase–CO complex by the generation of the reductant, the benzylviologen radical cation, and of the oxidant, the bis(hydroxymethyl)ferricinium ion. Analysis of the resultant absorbance–charge (*A*–*q*) curves for these cyclings gave a stoichiometry of three electrons (*n* = 3) per oxidase–CO complex and $E^{\circ\prime}$ values of 340, 250, and 190–200 mV vs. NHE. Assuming one of the hemes was complexed by CO and "inhibited" from participation in electron transfer with these mediator titrants, the three electron stoichiometry, which is consistent with Greenwood's chemical titration results (1974), implies electron transfer involving the two coppers and the one heme centers.

The $E^{\circ\prime}$ values are listed and compared with literature values in Table II. The $E^{\circ\prime}$ value of the titratable heme in presence of CO is distinct from the heme of native oxidase. The independence of copper potentials on CO supports the hypothesis (Wharton, 1964; Volpe et al., 1975) that copper does not participate as intimately as the hemes in CO complex formation.

Lindsay (1974; mitrochondrial particles in presence of ATP); Lindsay and Wilson (1974; submitochondrial particles and isolated oxidase); and Lindsay et al. (1975) have reported an $n = 2$ for the potentiometric titration of a high-potential heme and an "invisible" copper. The potential for the copper was inferred from the dependence of the potentiometric slope with varying CO concentrations. Concentration changes were determined by monitoring absorbance differences at two wavelengths of 590 and 624 nm during oxidative titrations using ferricyanide. Present spectral results as shown in Figures 1A,B or the difference spectra of Figures 6A,B exhibit *changes* of *similar magnitude* in the 590–624-nm region for oxidase in the *presence* and *absence* of CO. The question remains whether there is a finite optical absorbance by copper in the 590–624-nm region. If not, the potentiometric method can yield an $E^{\circ\prime}$ value for the "invisible" copper *only if the concentrations of all redox species can be simultaneously measured.* Only directly or indirectly *detectable* ("visible") species influence the course of potentiometric or Minnaert (1965) plots (see Figure 5A or consult other works: Heineman et al., 1973; Kuwana and Heineman, 1974; Leigh et al., 1974; Lindsay and Wilson, 1972; Mackey et al., 1973; Muijsers et al., 1972; Tiesjema et al., 1973, 1974; Tsudzuki and Wilson, 1971; Wilson et al., 1972) where concentrations are spectrally determined. At present, there is no conclusive evidence that copper or a related high-potential species significantly contributes to absorbance changes at the 590–624-nm region, although Gibson and Greenwood (1965) have reported kinetic evidence suggesting an absorption change due to copper in this region, opposite to the changes due to cytochromes a and a_3.

There is also another possible source of error in potentiometry. That is, the deleterious effect of CO adsorption on Pt (Breiter, 1968; Roethlein and Maget, 1969) in the potential region of interest; CO adsorption has been shown to inhibit electron transfer (Lau and Hubbard, 1971). As a consequence of this adsorption, a nonequilibrium condition may exist where the measured electrode potential (Pt indicator electrode) may not reflect the equilibrium redox state of the bulk solution.

The concentration ratios of reduced native oxidase to the oxidase–CO complex vary from 10^{-3} to 0.3 as the CO concentration is reduced from 300 to 3 μM in the 6 μM total heme solution of Lindsay and Wilson (1974) if their value of 4×10^{-7} M is assumed for the CO complexation constant for reduced oxidase. Thus, substantial uncomplexed (native) oxidase remains present when the CO concentration is at 3 μM. A suggested explanation for observation of a 350 mV component in the 3 μM CO solution is that the absorbance change at 590–624 is partially, if not totally, due to titration of the high-potential heme of "uncomplexed" oxidase. It should also be mentioned that inconsistent results have been obtained when ferricyanide was employed as the oxidant for the titration of cytochrome c oxidase (Mackey, 1975, Schroedl and Hartzell, 1974, private communication).

The difference of a heme potential in the presence of CO can be accounted for qualitatively on the basis of either heme–heme interaction or the report of Volpe et al. (1975), that two CO molecules are complexed per oxidase molecule (i.e., one per heme). However, CO binding studies by others on native oxidase (Gibson and Greenwood, 1963; Gibson et al., 1965; Vanneste, 1966) indicate one CO molecule per native oxidase molecule. A stoichiometry of one CO molecule per heme has been reported for a denatured oxidase "monomer" (Chan et al., 1970).

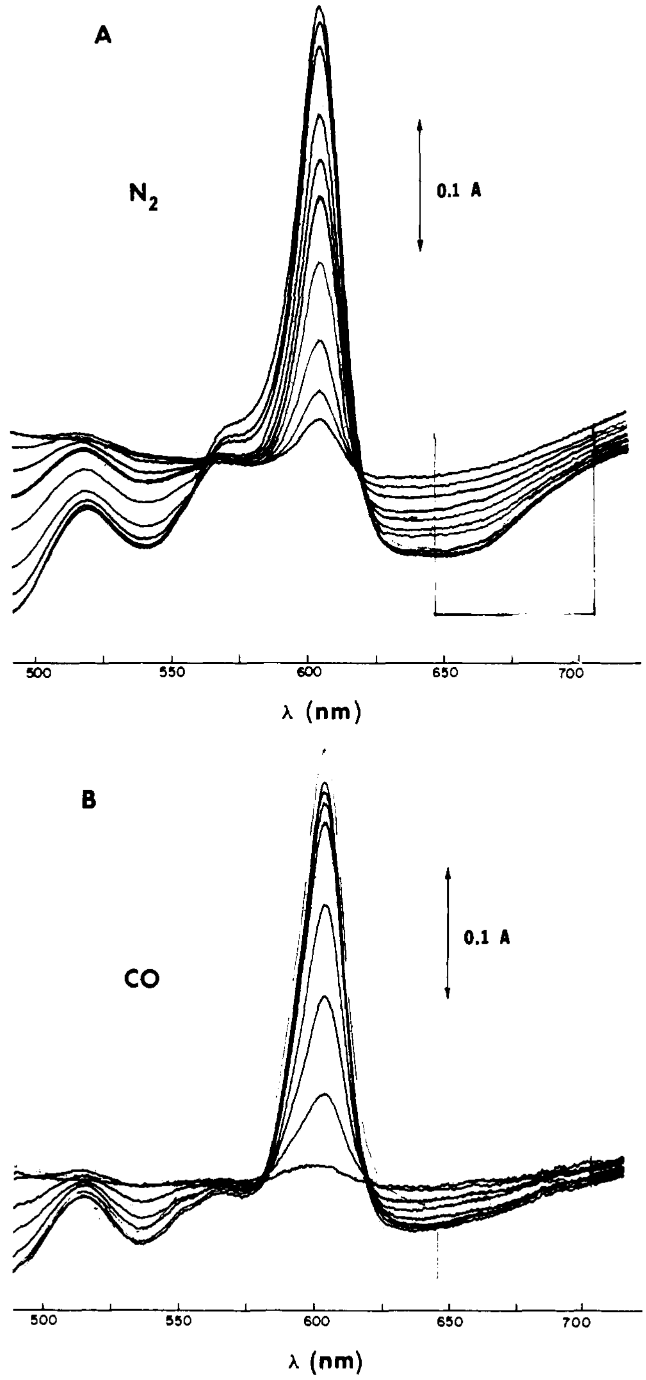

FIGURE 6: Difference spectra of cytochrome c oxidase during incremental coulometric titration. Cell parameters same as in Figure 1. Dashed line excess BV^{+}. 31 μM total heme a, 0.38 mM BHMF, 0.65 mM BV, pH 7.0, phosphate (ionic strength 0.15), 0.19% Triton QS-30. (A) First reduction of initially oxidized native oxidase in absence of CO (0.5 mC/ml of charge increment; initial two reductive charge increments not shown). (B) Third reduction of initially oxidized oxidase–CO complex. (0.4 mC/ml of charge increments, except in no. 2, 0.54 mC/ml).

The reported variation of the apparent midpoint potential of the high-potential heme by 60 mV/decade CO concentration change in ATP-coupled mitochondria (Lindsay, 1974) is consistent with one CO molecule per oxidase molecule. A re-

TABLE II: Representative Values Reported for Redox Midpoint Potentials of Components in the Carbon Monoxide Complex of Cytochrome *c* Oxidase.[a]

	Apparent Redox Potential (mV, NHE)[i]				
	Component				
	Heme		Copper		
Source	a_H	a_L	Cu_H	Cu_L	Titration Conditions (Wavelength, nm)
This work (pH 7.0)	>465 mV	260 ± 10 mV (1.0)	340 ± 20 mV (0.1)	190 ± 20 mV (0.1)	Coulometric (602 nm)
	>465	250 ± 10 (1.0)[b,c]	340 ± 20 (1.0)	190 ± 20 (1.0)	Coulometric (602)
	–	250 ± 10 (0.80)[b,c]	–	–	Minnaert plot (550, 602)
Tzagoloff and Wharton (1965)	–	250 (1.0)[b]	–	–	Potentiometric (605)
(pH 8.0)	–	242 (0.8–1.0)[b–d]	–	–	Minnaert plot (550, 605)
Tsudzuki and Wilson (1971)					Potentiometric
(pH 7.0)	–	260 (1.0)	–	230 (1.0)	(heme, 444–460; Cu, 830)
(pH 7.2)	–	–	–	225 (0.74)[e]	(EPR)
Cusanovich and Wharton (1973) pH 7.0	342 ± 10	246 ± 20 (0.83	–	274 ± 6 (0.63)	Potentiometric (heme, average of 590, 605, 650; Cu, 830)
Lindsay (1974)					Potentiometric
(pH 7.2, mitochondria)	~580[f]	260	–	–	(satd CO, 604–610)
	350 ± 10 (2.0)	220	–	–	(ATP + satd CO; a_H, 590–630; a_L, 604–610)
	290 ± 10	220	–	–	(ATP + 120 μM CO; 602–608)
Lindsay and Wilson (1974) (pH 7.8)	315 ± 10	–	–	–	Potentiometric (isolated enzyme or submitochondrial particles; no CO, 590–624)
	355 ± 10 (1.5–1.8)	–	350 ± 20[g]	–	(3 μM CO)
	385 ± 10 (2.0)	–	350 ± 20[g]	–	(30 μM CO)
	410 ± 10 (2.0)	–	350 ± 20[g]	–	(300 μM CO)
Lindsay et al. (1975) (pH 8.3, mitochondria)	–	–	340[h]	–	Potentiometric

[a] Isolated oxidase in CO-saturated solution unless otherwise noted. [b] Cytochrome *c* added. [c] Potentials calculated relative to cytochrome *c*, assuming $E^{\circ\prime}$ (cyt *c*) = 255 mV. [d] Calculated from spectra shown in Figure 6 of Tzagoloff and Wharton (1965). [e] *n*-value recalculated from best fit to potentiometric slope of data. [f] Calculated assuming $K_d \approx 0.6\ \mu M$. The equation used for this estimate (Clark, 1960) assumes that only cytochrome a_3 is directly oxidizable. The affinity of CO for reduced a_3 shifts the equilibrium so that the sum of native and CO complexed a_3 in reduced form is equal to the sum in oxidized form at 580 mV, rather than the lower potentials observed in absence of CO. This does *not* imply that the a_3 CO complex itself can be *directly* oxidized at 580 mV. The thermodynamic $E^{\circ\prime}$ value of the a_3 CO complex may be much more positive. [g] Estimate, inferred from $n = 2.0$ behavior of high-potential heme, a_H. [h] Estimate, inferred from $n = 2.0$ behavior and pH dependence of a_H potential. [i] Apparent number of electrons in parentheses.

ported 30 mV/decade CO concentration dependence in submitochondrial particles and isolated oxidase (Lindsay and Wilson, 1974, Lindsay et al., 1975) could be interpreted as consistent with two CO molecules per oxidase molecule. However, it is surprising that binding of two CO molecules per oxidase molecule, at two dissimilar sites (a strongly inhibited and a slightly affected site) would give rise to the single infrared absorbance band observed (Volpe et al., 1975). The alternative hypothesis of heme–heme interaction is supported in part by EPR results in the presence of CO (Wilson and Leigh, 1972; Leigh et al., 1974; Schroedl and Hartzell, private communication, 1974; and Hartzell and Beinert, 1975), and would appear to be preferable.

The report of Volpe et al. that the infrared spectrum of CO bound to oxidase is characteristic of terminal rather than bridging attachment, if correct, provides evidence against the proposed bridging linkage of CO between "invisible" copper and the high-potential heme.

The observance of invariant copper potentials, despite the CO-induced difference in the heme potential may be interpreted to signify the importance of heme–heme interactions as compared to heme–copper interactions. Observation of copper potentials in the presence of other heme inhibitors such as cyanide or azide may provide valuable information on the structure and interactions between the centers.

Previous results have given $E^{\circ\prime}$ values of one heme and one copper to be 345 ± 15 mV and the other heme and copper to be 215 ± 15 mV vs. NHE in native oxidase. As stated previously, one cannot infer anything about the identity or similarities of the two hemes or two coppers from these $E^{\circ\prime}$ values. As electrons are added or removed, similar to the effects of ligands, the entity called oxidase is perturbed thermodynamically, and further additions or removals may occur at different potentials, especially if the redox centers interact strongly. Thus, in the present case of CO complexation, one cannot necessarily say that "the low-potential heme with $E^{\circ\prime}$ of 215 mV is shifted to a higher potential of 250 mV by CO". One can only say that when one heme is complexed by CO, the other heme titrates with a value of $E^{\circ\prime}$ equal to 250 mV. What about the $E^{\circ\prime}$ value of the complexed heme? The only certain conclusion is that it could not be oxidized or reduced by the titrants employed in this study.

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