Journal of Bioenergetics and Biomembranes, Vol. 23, No. 5, 1991

Can Ferricyanide Oxidize Carbon Monoxide-Liganded Cytochrome a_3?

Richard W. Hendler[1]

Received October 11, 1990; revised January 11, 1991

Abstract

Evidence for the oxidation of CO-liganded cytochrome a_3 by ferricyanide has been published recently. These observations conflict with the long-held belief that ferricyanide is thermodynamically incapable of oxidizing the CO complex. The present paper examines the facts on which the earlier idea was based. It is concluded that the earlier evidence did not estabish that ferricyanide was incompetent as an oxidant for CO-liganded cytochorme a_3.

Key Words: Redox potentials; cytochrome oxidase; electron transport.

For the last three decades it has been an unquestioned belief among cytochrome oxidase researchers that ferricyanide cannot oxidize CO-liganded cytochrome a_3. Many important experiments have been performed, and conclusions drawn based on the belief that it is safe to assume that ferricyanide can be added to the reduced CO complex and that cytochrome *a* and Cu_A will be completely oxidized, while cytochrome a_3 and Cu_B will remain completely reduced. Therefore, when our laboratory presented evidence that ferricyanide can oxidize cytochrome a_3 within the CO complex (Hendler *et al.*, 1986; Subba Reddy *et al.*, 1986; Sidhu and Hendler, 1990), a dilemma was created. In order to resolve this apparent conflict, we decided to examine the earlier literature on which the existing concept was based to see whether the data did support the earlier conclusion. This paper shows that it was never established that ferricyanide was thermodynamically incompetent as an oxidant for the CO complex of cytochrome a_3.

Most papers in which it is assumed that CO protects cytochrome a_3 from oxidation by ferricyanide cite the work of Horie and Morrison (1963). In that

[1]Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland, 20892.

paper, the conclusion is clearly stated, "Adding ferricyanide to the reduced preparation treated with carbon monoxide results in the oxidation only of the hemoprotein that has not combined with carbon monoxide." However, quantitative evidence to support this conclusion is not presented. The only evidence pertaining to this question is a $Na_2S_2O_4$-reduced plus CO spectrum, before and after the addition of $K_3Fe(CN)_6$ (final concentration not specified, cf. Fig. 3). The Soret region shows the increase in a peak at 429 nm, which is most likely due to the absorbance of $K_3Fe(CN)_6$ itself. The α peak shows a decrease and a shift from 603 nm (peak absorbance for reduced cytochrome *a*) to 590 nm (peak absorbance for CO-liganded reduced cytochrome a_3). This peak at 590 nm is higher than that of the α peak in the enzyme as prepared at 598 nm (cf. Fig. 4) but it is decreased from its level before the addition of ferricyanide. This could best be explained as the near total oxidation of cytochome *a* and the oxidation of as much CO-liganded cytochrome a_3 as there should be considering its E_m of ~ 340 mV (Sidhu and Hendler, 1990) and the ambient voltage at the (hydrophobic) site of the heme a_3. In the Horie and Morrison work, as in most of the other papers which support the view that the E_m of CO-liganded cytochrome a_3 is raised to unspecified high values, no E_h measurements were made, and no lipid-soluble redox mediators were present to bring the local voltage at the heme a_3 site into equilibrium with the voltage in the aqueous medium, which was set by the water-soluble oxidant, ferricyanide. Considering that the E_m of $K_3Fe(CN)_6$ is ~ 430 mV, and $Na_2S_2O_4$ was present in these experiments, the E_h of the solution was most likely between 300 and 400 mV. Therefore, some reduced CO-liganded cytochrome a_3 should be present. It does not follow that no oxidation of the complex occurred nor that the E_m of CO-ligand cytochrome a_3 is higher than that of ferricyanide.

Nicholls (1963) performed essentially the same experiment as Horie and Morrison using a Keilin–Hartree heart muscle preparation. The reduced enzyme–CO complex was produced in the presence of succinate and CO. The α peak showed a maximum at ~ 605 nm and a shoulder near 590 nm. The addition of 2 mM ferricyanide removed nearly all of the 605 nm absorbance, but left a substantial part of the 590 nm absorbance. The same problems that were cited in connection with the Horie and Morrison paper apply here. It was not established that ferricyanide is incapable of oxidizing the CO complex.

The same experiment was performed by Wharton using a purified enzyme preparation, but in this study correlative absorption changes at 830 nm (believed to represent Cu_A) were recorded. When the enzyme was reduced with dithionite and exposed to a stream of CO, the usual peak at ~ 605 and shoulder at ~ 590 nm were seen as well as a decrease in the absorbance at 830 nm relative to that of the oxidized enzyme. The addition

of ferricyanide caused the virtual disappearance of the 605 nm absorbance and a lowering of the 590 nm absorbance, similarly to the observations of Horie and Morrison, and Nicholls. The 830 nm absorbance increased, but not completely up to the level of the fully oxidized enzyme, indicating that ferricyanide oxidized the Cu_A. Insofar as the question of oxidation of CO-liganded cytochrome a_3 however, none of these three papers can provide an answer.

To be properly answered, the question must be rephrased in a thermodynamically rigorous context. The following presentation relies heavily on the approaches so clearly and completely set out by others (Clark, 1960; Dutton and Wilson, 1974; Walz, 1979). At the start, it is important to restate the meaning of the thermodynamic quantities E_h, E_0, and E_m. The term E_h is the voltage difference between a medium containing one or more redox couples and that of the standard hydrogen electron arbitrarily taken as zero. The term E_0 is the standard potential of a redox couple. It represents the value of E_h that would be set if all of the reactants and products for the particular redox couple were present and maintained at unit activities. If protons are participants in the redox reaction, then the pH of the medium cell must be zero. The term E_m represents the value of E_h at which 1/2 of the redox group in the couple is in the oxidized state and 1/2 in the reduced state. When no other ligands (than the electron) participate in the redox reaction, $E_0 = E_m$. As discussed in the references cited above, when E_m is used to characterize a redox reaction in which additional ligand(s) participate, the effect of the ligand activity is used as an offset to the E_0. For example, in a one-electron redox reaction which dissociates one proton from the reduced species upon oxidation,

$$E_m = E_0 + RT/nF \ln ([H^+])$$

The question under consideration in the present work is how does the binding of the ligand, CO, to the redox center of cytochrome a_3 affect the binding affinity of the center for electrons. To simplify the answer to this particular question, we consider only the interaction between the binding of CO and of electrons. For this purpose we present Fig. 1 which shows the four necessary equilibria. The two vertical sides are electron-binding isotherms and the two horizontal are CO-binding isotherms (where L represents CO). As can be seen, the ligand is not a participant in either of the two redox reactions, and therefore, $E_m = E_0$. Because E_m is most often used in biological studies, we will use this quantity wherever possible. The electron-binding equilibria are expressed as Nernst equations. For the free a_3 we have

$$E_h = E_{mf} + Z \log ([O]/[R]) \quad (1)$$

where $Z = 60/n$, n is the number of electrons transferred, [O] is the concentration of the oxidized member of the couple, and [R] is the concentration

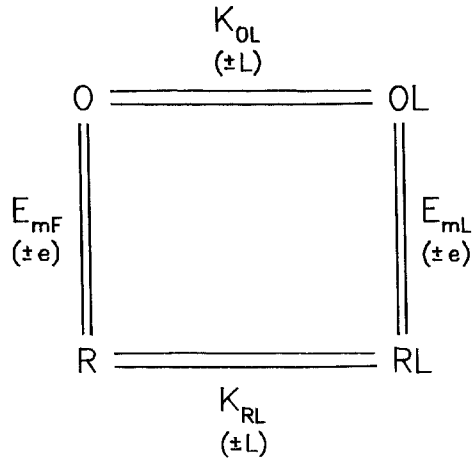

Fig. 1. General diagram showing the four equilibria which determine electron and chemical ligand binding affinities to a molecule that contains both a redox and a ligand binding center. For the analysis discussed in this paper, consider O to be ferric cytochrome a_3, R to be ferrous cytochrome a_3, and L to be CO. The midpoint potentials are E_{mF} for the free enzyme and E_{mL} for the CO-liganded enzyme. The dissociation equilibrium constants for CO binding are K_{OL} for the oxidized enzyme, and K_{RL} for the reduced enzyme.

of the reduced member of the couple. For the CO–a_3 complex, we have

$$E_h = E_{mL} + Z \log ([OL]/[RL]) \tag{2}$$

where [OL] is the concentration of oxidized CO complex and [RL] is the concentration of reduced CO complex.

The CO-binding equilibria are expressed for the ferric heme as

$$K_{OL} = [O][L]/[OL] \tag{3}$$

and for the ferrous heme as

$$K_{RL} = [R][L]/[RL] \tag{4}$$

Using Eqs. (3) and (4) in Eq. (2) leads to

$$\begin{aligned} E_h &= E_{mL} + Z \log (([O]/[R])(K_{RL}/K_{OL})) \\ E_h &= E_{mL} + Z \log ([O]/[R]) + Z \log (K_{RL}/K_{OL}) \end{aligned} \tag{5}$$

Subtracting Eq. (1) from (5) and rearranging yields

$$E_{mL} = E_{mF} + Z \log (K_{OL}/K_{RL}) \tag{6}$$

i.e., the E_m of the liganded species differs from the E_m of the free species by the log term in Eq. (6). The Z factor contains n, the number of electrons involved. It is very important to note that the concentration of the ligand is not a factor in this relationship. This same point was demonstrated by Walz (1979)

using a different approach. The relative amounts of free and liganded species, but not the individual E_m's of the two species, will change with [L]. This fact has an important bearing on a number of the studies to be discussed in this paper.

Many studies citing the work of Horie and Morrison involve the formation of a "mixed valence" state of cytochrome oxidase. The procedure is to reduce the enzyme, form the CO complex, and then to treat with ferricyanide. According to the usual interpretation of the Horie and Morrison experiment, cytochrome a and Cu_A will be oxidized, but the cytochrome a_3 and Cu_B will remain reduced. The appearances of the spectra before addition of reducing agent and CO, after their addition, and after the addition of ferricyanide do show that this is largely what happens. However, although an appreciable amount of the 590 nm absorbance remains, there is usually some decrease in its amplitude. In none of these studies has a quantitative relationship been determined between the actual change in the specific absorbance of the CO-liganded cytochrome a_3 and the effective E_h at its site in the pure enzyme or when it is submerged in a membrane. There is little doubt that a substantial amount of mixed valence enzyme is made by this procedure. However, one must consider kinetic as well as thermodynamic factors. Ferricyanide immediately raises the E_h of the aqueous medium. The E_h of the hydrophobic interior of the protein or the membrane will be more slowly raised. The immediate effect of adding ferricyanide is more quickly sensed by the cytochrome a and Cu_A. In the absence of CO, the oxidation of cytochrome a_3 by ferricyanide is always seen to lag behind that of cytochrome a. At equilibrium, when CO is present, the amounts of oxidized and reduced liganded species will be that which is predicted by Eq. (2). In none of these studies has it been shown that ferricyanide is incapable of oxidizing some of the liganded species. Nonetheless, the successful use of this procedure to form the mixed valence state has been taken as a confirmation of the conclusion of Horie and Morrison and others that no oxidation of the complex by ferricyanide occurred. Some examples of the use of the procedures described above for the formation of the mixed valence state of the enzyme are Tzagaloff and Wharton (1965), Powers *et al.* (1979), Greenwood *et al.* (1974), Wikström *et al.* (1976), and Clore *et al.*, (1980).

A substantial part of the literature on the E_m's of free and CO-liganded cytochrome a_3 comes from D. F. Wilson and his collaborators. In these studies, redox mediators are used to equilibrate the E_h throughout the entire mitochondrial suspension. The data are collected as two-point ΔA's between a peak wavelength for a particular cytochrome species and a reference point. Their analysis of the data involves a plot of the E_h vs. log (oxidized/reduced) and a graphical resolution of the data in order to resolve individual species, n values, and E_m's. We have shown (Shrager and Hendler, 1986) that two-point ΔA data are not specific to one particular species. They carry

contributions from other overlapping spectra and from a changing background. The graphical method of analysis entails a high degree of subjectivity, especially in determining total absorbances and inflection points. Computer fittings using a number of different possible models and objective "goodness of fit" criteria are not used, and it is difficult to resolve more than two species, or species that have fairly similar E_m values.

Wilson *et al.* (1972) studied cytochrome oxidase in intact pigeon heart mitochondria. In the absence of CO, the ΔA (605–630 nm) was used to determine log(ox/red) of cytochrome oxidase as a function of E_h. The data were resolved into two equally absorbing $n = 1$ components with E_m's of 375 and 230 mV. When the titration was repeated under a 50% CO/50% argon atmosphere, again monitoring the ΔA (605–630 nm), only a single component was seen with an apparent E_m of 255 mV. It should be noted that the curve for the − CO titration showed data up to ~400 mV, whereas the + CO data go to only ~340 mV with a marked deviation from the theoretical $n = 1$ slope for E_h values greater than 325 mV. According to our findings, if the monitoring wavelength pair were based on the absorbance maximum for the CO complex at ~590 nm and if the E_h data were extended to 400 mV, the deviation evident above 325 mM would have turned into the titration of the CO complex. In order to try to extract an E_m value for the CO complex from these data, it is first necessary to identify cytochrome a_3 from the titration in the absence of CO. Wilson *et al.*, without any independent experimental tests, identified the 375-mV component as cytochrome a_3. The rationale, given on pages 282 and 283 of their paper, is essentially the following. By definition, cytochrome a_3 is the one that combines with CO and O_2. Therefore, the high-E_m component mut be a_3. The theoretical basis for concluding that the E_m of the complex is so much above that of ferricyanide is that it is well known that the reduced enzyme binds CO strongly, and the oxidized form weakly. This would tend to raise the E_m of the free form by 60 mV per decade difference in the ratios of the dissociation constants (at higher CO concentrations). For instance, a 1000-fold difference could raise the E_m to ~560 mV. These findings and considerations were said to be consistent with the demonstration of Horie and Morrison that "in the presence of excess CO the cytochrome a_3 is not oxidized by ferricyanide." The identification of the higher of the two E_m's with cytochrome a_3 has not been universal. Hartzell and Beinert (1976) identify the higher E_m component as cytochrome *a*. In our studies which employed much more powerful techniques of data collection and analysis, the ~340 mV transition is also identified with cytochrome *a*. The lower voltage transition has been resolved into two components, one of which is a low potential form of cytochrome a_3 (E_m ~200 mV) identified on the basis of its ability to form the CO complex (Hendler *et al.*, 1986; Subba Reddy *et al.*, 1986; Sidhu and Hendler, 1990).

A very serious flaw in the evaluation of the thermodynamic effects of ligands on the E_m's of the free redox components they bind to, is apparent in this paper of Wilson *et al.* This flaw carries through in all of the other papers from this group (and others) and we will discuss it at this point. The equation below is given by the authors to describe the effects of ligand binding on E_m. Using our terminology,

$$E_{mL} = E_{mF} + 0.06 \log (K_{OL}/K_{RL}) + 0.06 \log ((K_{RL} + [L])/(K_{OL} + [L])) \quad (7)$$

This is not the equation (6) we derived above. It has an extra term showing a dependence of E_{mL} on ligand concentration. This equation cannot be used to characterize the thermodynamic entity E_{mL} for a liganded species, which while it may be dependent on external variables like pressure or temperature, may not be dependent on ligand concentration. The E_m of the ligand complex must be the same at any [L]. What then, is the meaning of this equation? The derivation below answers this question. Let E_{mM} be the voltage at which the mixture of free and CO-liganded oxidized species is equal to the mixture of free and CO-liganded reduced species. Therefore,

$$E_h = E_{mM} + Z \log (([O] + [OL])/([R] + [RL])) \quad (8)$$

Using Eqs. (3) and (4) for [OL] and [RL], we can derive

$$E_h = E_{mM} + Z \log ([O]/[R]) + Z \log (K_{RL}/K_{OL}) + Z \log ((K_{OL} + [L])/(K_{RL} + [L])) \quad (9)$$

Subtracting Eq. (1) from Eq. (9) and rearranging produces Eq. (7), except that instead of E_{mL}, the midpoint for the CO-liganded species, we find E_{mM}, the weighted average for a mixture of free and liganded species.

This is the equation used by Wilson *et al.* in this and all the other investigations on the effects of CO on the E_m of cytochrome a_3. It is clearly stated that the E_m specified is the E_m for the liganded species, but this is not true. It is a weighted average of the two E_m's that describe the electron-binding affinities of the free and liganded species. The concentration of ligand definitely affects this value because it shifts the relative amounts of the two components in the mixture. There is no thermodynamic species that has an $E_m = E_{mM}$. This equation describes what would happen in an experiment in which the spectral measurements cannot distinguish between the free and liganded forms of the oxidized and reduced species.

Lindsay (1974) reported that ATP can lower the E_m of cytochrome a_3 from ~ 580 mV in the presence of saturating amounts of CO (the paper of Wilson *et al.* discussed above was cited for this E_m value) to ~ 350 mM, so that it then could be oxidized by ferricyanide. The wavelength pair

605–630 nm is used to monitor the reduced, uncomplexed heme centres and 590–630 nm to monitor the CO-liganded reduced cytochrome a_3. Figure 4B of the Lindsay paper clearly shows that the addition of 0.2 mM ferricyanide, in the absence of ATP, did result in the oxidation of a chromophore reporting at 590–630 nm. The wrong equation (7)[2] is used to simulate the data obtained in the presence of 6 mM ATP, which shows an effect of [CO] on the E_m of the complex. In the simulation, the E_m for free cytochrome a_3 in the presence of 6 mM ATP is taken as 160 mV, the K_{RL} as 0.6 μM (Wohlrab and Ogunmola, 1971), and K_{OL} as 600 μM (a guess). The E_m vs. [CO] plot seemed to show a 60 mV per decade [CO] dependence in the range of 10^{-6} M to 1.2 mM. It was implied that this was consistent with the theoretical equation (7), and the simulated line was extended in their Fig. 9 to show this linear slope continuing to beyond 350 mV. However, inspection of Eq. (7) shows that the curve should be sigmoidal. The [CO] term is both in the numerator and the denominator. At high concentrations, this term should disappear. A linear slope with increasing [CO] should not result. Using 160 mV for the E_m of the unliganded species, a value of 60 mV for Z, and a ratio of 1000 for the two dissociation constants, the curve should asymptotically approach a maximum of 340 mV. A further point of confusion is that the paper states that the CO–a_3 compound always behaves as an $n = 2$ electron acceptor (page 710). If this is true, then the value of Z is 30 mV and the maximum value of the E_m should be $160 + 90 = 250$ mV. This complication could be removed, however, by simply assuming a ratio of 1,000,000 for the two dissociation constants rather than 1000.

The experiments of Lindsay (1974) were further pursued by Lindsay and Wilson (1974). In this work, the $n = 2$ nature of the titration of the CO complex was firmly established, and in the absence of ATP, a 30-mV change in the "E_m" (i.e., E_{mM}) of the CO complex per decade change in [CO] was seen.

A rather complicated study was published by Lindsay *et al.* (1975). This paper introduced a completely new mathematical method to study the effects of CO on the E_m of the liganded species. It is important to note that at the start of the paper, the mistaken idea that the E_m is dependent on [CO] is again stated. Reviewing the earlier two papers described above, it was stated that in the presence of ATP, ferricyanide could oxidize the CO complex *provided low concentrations of CO are employed.* The conclusions of this new study

[2]The equation (7) used for simulation by Lindsay (1974) is given incorrectly on both pages 705 and 710 of his article. The correct representation of this inappropriate equation requires the E_h to be replaced by E_{mM} for the mixture of free and liganded species, and the E_m to be replaced by E_{mF} for the free species. The equation as shown in the paper shows only a dependence of the E_h of the solution on [CO]. In the simulation shown in Fig. 9 of the Lindsay paper, the correct form of the inappropriate equation was used.

depend entirely on the new mathematical treatment that is introduced. Because I find many serious problems with the assumptions made, the mathematical simplifications used in the derivations, and the conclusions drawn, it is necessary to review the new method in some detail.

To begin with, the model ignores any cooperative interactions exerted by electron binding to either cytochrome a or Cu_A. It also ignores evidence (discussed below) that CO may bind to cytochrome oxidase when the cytochrome a_3 is in the ferric state. All of the a_3 is considered to be conserved in five states: (I) $a_3^{3+} \cdot Cu^{2+}$; (II) $a_3^{3+} \cdot Cu^{1+}$; (III) $a_3^{2+} \cdot Cu^{2+}$; (IV) $a_3^{2+} \cdot Cu^{1+}$; (V) $a_3^{2+} \cdot Cu^{1+}$–CO. The total a_3 expressed by the term A, is

$$A = [\mathrm{I}] + [\mathrm{II}] + [\mathrm{III}] + [\mathrm{IV}] + [\mathrm{V}] \tag{10}$$

The dissociation constant K_d can be used in the form

$$[\mathrm{CO}]/K_d = [\mathrm{V}]/[\mathrm{IV}] \tag{11}$$

to solve for the total concentration of unbound a_3:

$$\begin{aligned} A - [\mathrm{V}] &= [\mathrm{IV}][1 + \exp((F/RT)(E_\mathrm{h} - E_{a_3}))] \\ &\quad \times [1 + \exp((F/RT)(E_\mathrm{h} - E_\mathrm{Cu}))] \end{aligned} \tag{12}$$

Substituting (11) in (12), rearranging, and noting that $A - \mathrm{V}$ = unbound, and V = bound yields:

$$\begin{aligned} [\mathrm{CO}]/K_d &= \{[\text{bound}]/[\text{unbound}]\} \\ &\quad \times [1 + \exp((F/RT)(E_\mathrm{h} - E_{a_3}))] \\ &\quad \times [1 + \exp((F/RT)(E_\mathrm{h} - E_\mathrm{Cu}))] \end{aligned} \tag{13}$$

or, taking natural logarithms

$$\begin{aligned} \ln([\mathrm{CO}]/K_d) &= \ln\{[\text{bound}]/[\text{unbound}]\} \\ &\quad + \ln[1 + \exp((F/RT)(E_\mathrm{h} - E_{a_3}))] \\ &\quad + \ln[1 + \exp((F/RT)(E_\mathrm{h} - E_\mathrm{Cu}))] \end{aligned} \tag{14}$$

This equation is simplified by noting that for high values of E_h relative to E_{a_3} or E_Cu,

$$\ln[1 + \exp((F/RT)(E_\mathrm{h} - E_{a_3\,\mathrm{or}\,\mathrm{Cu}}))] \sim (F/RT)(E_\mathrm{h} - E_{a_3\,\mathrm{or}\,\mathrm{Cu}}). \tag{15}$$

In the voltage range that this equation is applied (i.e., rom ~ 200 to ~ 450 mV), the error introduced into the $\ln([\mathrm{CO}])/K_d)$, just for the term involving E_{a_3}, ranges from -2.135 at $E_\mathrm{h} = 205$ mV to -0.117 at 445 mV. Using this simplification for both the a_3 and Cu terms in (14) and rearranging

yields

$$E_h = ((E_{a_3} + E_{Cu})/2) + (RT/2F) \ln ([CO]/K_d) + (RT/2F) \ln \{[\text{unbound}]/[\text{bound}]\} \quad (16)$$

At very low voltages, all of the unbound is in the form IV, and so

$$(\text{bound}/\text{unbound}) = [CO]/K_d$$

Then, Eq. (16) reduces to

$$E_h = (E_{a_3} + E_{Cu})/2 \quad (17)$$

If there is a discrete E_h where (17) becomes true, and if we know E_{a_3}, then the E_{Cu} for the "invisible" copper is known directly from Eq. (17). But, in Nernstian titrations, there is no discrete E_h where all of any redox form appears or disappears. Changes in concentration occur at factors of 10 for each $60/n$ mV increment in E_h. The rationale for the particular graphic extrapolation shown in Fig. 4 of this work to determine a particular E_h for Eq. (17) at each different [CO] was not provided and is not apparent. Furthermore, the E_{a_3} and E_{Cu} are defined in terms of the E_m's of the unliganded species. Why should they change with different levels of [CO]? Most importantly, the simplification cited in Eq. (15) used to derive Eq. (16) is not valid at the "very low voltages" where Eq. (17) is said to be applicable.

Equation (16), which is an approximation of a theoretically derived equation, resembles a Nernst equation but it is not a Nernst equation. Therefore, it is not clear why the E_m of the CO complex is taken as the E_h where the concentrations of the bound and unbound species are equal. Setting [unbound] = [bound] in Eq. (16) results in $E_h = (E_{a_3} + E_{Cu})/2 + (RT/2F)(\ln ([CO]/K_d)$. Setting [unbound] = [bound] in Eq. (14) leads to a much more complicated expression that is not simply $E_h = E_{CO\,complex}$.

In plots of E_h vs. log (unbound/bound), a 30-mV per decade change in unbound/bound was interpreted to mean that there is an $n = 2$ character to the titration. Because Eq. (16) is not a Nernst equation, the significance of the slope at high values of E_h is not clear. The "apparent" $n = 2$ character in Eq. (16) is a result of the fact that $n = 1$ values were assumed for the a_3 and Cu titrations. When the simplified approximation in expression (15) was used in Eq. (14) to obtain Eq. (16), there was an intermediate expression obtained:

$$\ln ([CO]/K_d) = \ln \{[\text{bound}]/[\text{unbound}]\} + (F/RT)(E_h - E_{a_3}) + (F/RT)(E_h - E_{Cu}) \quad (14a)$$

Multiplying both sides by RT/F yields

$$(RT) \ln ([CO]/K_d) = (RT/F) \ln \{[\text{bound}]/[\text{unbound}]\} + E_h - E_{a_3} + E_h - E_{Cu} \quad (14b)$$

Rearranging to

$$2E_h = (RT/F) \ln ([CO]/K_d) + (RT/F) \ln \{[\text{unbound}]/[\text{bound}]\} + (E_{a_3} + E_{Cu}) \quad (14c)$$

dividing by 2, then produces Eq. (16). The $RT/2F$ slope in the plot of E_h vs. log {[unbound/[bound]} comes from specifying $n = 1$ for the a_3 and Cu titrations, each at a different E_m. Its relation to a Nernstian value of $n = 2$, which means that two electrons must be added or removed at a single E_m in each redox step, is not apparent.

Other questions about this work remain. How was the ΔA (590–624) used to obtain [unbound]/[bound]? Why is this method used instead of the usual one to follow the titration of the CO species according to the Nernst equation? It seems that in addition to the usual problems associated with the use of two-point ΔA data to follow titrations of discrete species among other titrating species and a changing background, more assumptions and uncertainties have been introduced. What new advantages have been gained to offset the apparent disadvantages? In summary, new and independent thermodynamic data to support the notion that the E_m of CO-liganded cytochrome a_3 is much higher than that of ferricyanide is not in this paper of Lindsay *et al.* (1975).

The final paper from this group to be reviewed is Wilson and Nelson (1982). Surprisingly, evidence in support of our findings that ferricyanide can oxidize the CO complex of cytochrome a_3 can be found in this paper. Although the usual two-point ΔA data are used for the analyses in the paper, a figure is presented which shows a series of intact spectra of the α region taken at known values of E_h during titration of submitochondrial particles from pigeon breast muscle in the presence of 50 μM CO. Their Fig. 6 (top) shows five spectra taken in the voltage range 425 to 345 mV. The peak is at $\sim$590 nm and so these data relate more specifically to the CO-liganded species rather than a mixture of both the free and liganded species. Using a millimeter ruler, the amplitudes at the peak can be measured. When these data are fitted according to the Nernst equation, using n values of 1 and 2, the E_m's obtained are 379 and 382 mV, respectively. A somewhat better fit is obtained at $n = 1.5$ with an E_m of 381 mV. Considering that only five points were available and a millimeter ruler was used, this E_m is surprisingly close to the value of 340 mV that we have found in much more extensive titrations of the CO-liganded species (Sidhu and Hendler, 1990). The titration seen in this range is not consistent with the value of $\sim$580 mV presumed by this group. The E_m value of 380 mV obtained is not low because of the rather low [CO] in this titration. Although a relatively small fraction of liganded cytochrome a_3 may have been present, its E_m is independent of [CO]. These

observations add weight to the comment made above in connection with the paper of Wilson *et al.*, that if the monitoring pair of wavelengths included the 590 nm absorbance and if data to 425 mV were shown, the deviations evident above 325 mV would have indicated the titration of the CO-liganded species.

The E_{mL} of the liganded species is described by Eq. (6) above. It is higher than the E_m of the free compound by the factor $Z \log(K_{OL}/K_{RL})$. For an $n = 2$ reaction, Z is 30 mV. The value of K_{RL} is $\sim 0.6\,\mu M$. It has generally been assumed that when cytochrome a_3 is oxidized, virtually no CO can be bound by the enzyme. For the thermodynamic effect of CO to be exerted, however, it does not have to be bound to the oxidized cytochrome a_3, but only to a molecule of cytochrome aa_3 while a_3 is in the oxidized state. There are independent studies which have concluded that such binding of CO to the molecule does occur when a_3 is in the oxidized state. Shaw *et al.* (1978) provide strong EPR evidence that the fully oxidized enzyme binds CO. They consider that the binding may be to a gas channel or pocket in the protein leading to the heme prosthetic group. A very similar conclusion was drawn by Brzezinski and Malmström (1985) on the basis of other findings. They studied the mechanism and kinetics of the reaction in which CO is added to the fully oxidized cytochrome aa_3 producing the mixed valence state in which the cytochrome a_3–Cu_B centre becomes reduced and cytochrome a and Cu_A remain oxidized. They conclude that there must be a heme pocket with an affinity for CO even when cytochrome a_3 is oxidized. Using the lower value for the E_m of the CO-binding form of cytochrome a_3 found in our work (Hendler *et al.*, 1986; Subba Reddy *et al.*, 1986; Sidhu and Hendler, 1990), or the somewhat higher values found by Hartzell and Beinert (1976), a value of 30 mV for Z, and a not insignificant binding affinity of the molecule containing ferric a_3 for CO, Eq. (6) does predict that the E_{mL} for cytochrome a_3 in the CO-liganded molecule should allow the center to be oxidizable by ferricyanide ($E_m = \sim 430$ mV).

There is another sound thermodynamic consideration that has been generally ignored in the literature reviewed in this paper. The reasoning that has been employed is that if CO is bound very strongly to the reduced species and very weakly to the oxidized species, the E_m of the liganded species must be greatly elevated. However, if cytochrome aa_3 is a proton pump, binding affinities for protons must also be considered. Using the same kind of derivation that produced Eq. (6), it can be shown that when both a ligand like CO and a proton can be bound, the E_m of the cytochrome a_3 (i.e., E_{mLH}) can be described as

$$E_{mLH} = E_{mF} + Z \log (K_{OL}/K_{RL}) + Z \log (K_{OLH}/K_{RLH}) \qquad (18)$$

where K_{OLH} and K_{RLH} represents the binding affinities of the oxidized and reduced liganded species for a proton. Models for a redox-driven proton

pump have been developed based on the Bohr principle that a decrease in binding affinity for L which occurs when cytochrome a_3 is oxidized results in an increase in binding affinity for a proton (Hendler and Westerhoff, unpublished). In this manner, the effects of the binding of the two ligands (CO and H^+) on the E_m of cytochrome a_3 are anticooperative. This view, which is at least as justifiable as the one which considers only the binding of CO, does not lead to a prediction that the E_m of cytochrome a_3 is markedly elevated in the presence of CO.

In conclusion, there is convincing experimental data that the CO complex of cytochrome a_3 is oxidizable by ferricyanide. This view is thermodynamically consistent. Previous work thought to show that the complex could not be oxidized by ferricyanide actually did not demonstrate this.

Acknowledgments

The author thanks Barry Bunow, Richard Shrager, and Hans Westerhoff for helpful suggestions during the preparation of the manuscript.

References

Brzezinski, P., and Malmström, B. G. (1985). *FEBS Lett.* **187**, 111–114.
Clark, W. M. (1973). *Oxidation–Reduction Potentials of Organic Systems*, Waverly Press, Baltimore, Maryland.
Clore, G. M., Andreasson, L.-E., Karlsson, B., Aasa, R., and Malmström, B. G. (1980). *Biochem. J.* **185**, 155–167.
Dutton, P. L., and Wilson, D. F. (1974). *Biochim. Biophys. Acta* **346**, 165–212.
Greenwood, C., Wilson, M. T., and Brunori, M. (1974). *Biochem. J.* **137**, 202–215.
Hartzell, C. R., and Beinert, H. (1976). *Biochim. Biophys. Acta* **423**, 323–338.
Hendler, R. W., Subba Reddy, K. V., Shrager, R. I., and Caughey, W. S. (1986). *Biophys. J.* **49**, 717–729.
Horie, S., and Morrison, M. (1963). *J. Biol. Chem.* **238**, 1855–1860.
Lindsay, J. G. (1974). *Arch. Biochem. Biophys.* **163**, 705–715.
Lindsay, J. G., and Wilson, D. F. (1974). *FEBS Lett.* **48**, 45–49.
Lindsay, J. G., Owen, C. S., and Wilson, D. F. (1975). *Arch. Biochem. Biophys.* **169**, 492–505.
Nicholls, P. (1963). *Biochim. Biophys. Acta* **73**, 667–670.
Powers, L., Blumberg, W. E., Chance, B., Barlow, C. H., Leigh, J. S., Smith, J., Yonetani, T., Vik, S., and Peisach, J. (1979). *Biochim. Biophys. Acta* **546**, 520–538.
Shaw, R. W., Hansen, R. E., and Beinert, H. (1978). *Biochim. Biophys. Acta* **504**, 187–199.
Shrager, R. I., and Hendler, R. W. (1986). *Biophys. J.* **49**, 697–691.
Sidhu, G. S., and Hendler, R. W. (1990). *Biophys. J.* **57**, 1125–1140.
Subba Reddy, K. V., Hendler, R. W., and Bunow, B. (1986). *Biophys. J.* **49**, 705–715.
Tzagoloff, A., and Wharton, D. C. (1965). *J. Biol. Chem.* **240**, 2628–2633.
Walz, D. (1979). *Biochim. Biophys. Acta* **505**, 279–353.
Wharton, D. C. (1964). *Biochim. Biophys. Acta* **92**, 607–609.
Wikström, M. K. F., Harmon, H. J., Ingledew, W. J., and Chance, B. (1976). *FEBS Lett.* **65**, 259–276.
Wilson, D. F., and Nelson, D. (1982). *Biochim. Biophys. Acta* **680**, 233–241.
Wilson, D. F., Lindsay, J. G., and Brocklehurst, E. S. (1972). *Biochim. Biophys. Acta* **256**, 277–286.
Wohlrab, H., and Ogunmola, G. B. (1971). *Biochemistry* **10**, 1103–1106.