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# REDOX TITRATIONS OF CYTOCHROME *c* OXIDASE

## AN ANALYSIS OF A MULTI-ELECTRON SYSTEM

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## Summary

1. Equilibrium redox titrations of cytochrome *c* oxidase available in the literature are discussed in terms of models with interactions both with respect to oxidation-reduction potentials and the particular property studied.

2. The interaction is restricted to be pairwise. For the present data more complicated forms of interaction are not required. In addition, with this limitation a simple matrix formulation can be used.

3. The EPR titrations require potential interaction involving the hemes and the undetectable Cu. In this way the low intensity of the *g*6 signal can be accounted for. However, it is shown that there is no unique solution to the problem.

4. The optical titrations at 605 nm can be fitted reasonably well with the potentials from the EPR data with no spectral interaction.

5. Simulated titrations of magnetic susceptibility show a sensitivity to the model chosen indicating the usefulness of future experiments in this area.

## Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase EC 1.9.3.1) plays a key role in most living systems since it catalyses the reduction of molecular oxygen to water. This reaction involves the transfer of four electrons to oxygen, but the details of the mechanism are largely unknown. Although equilibrium redox titrations give little direct information on the mechanism, they provide the necessary background for kinetic experimentation and model building. The aim of the present paper is to show how equilibrium data can be analysed.

The starting point for a discussion of equilibrium redox titrations of cyto-

chrome oxidase is the assumption of a functional unit containing four electron acceptors. Two of these are hemes going from the ferric to the ferrous state on reduction. Although not directly proven it is generally assumed that the other acceptors are cupric ions that are reduced to the cuprous state. These assumptions will be used also in the present study.

Most published data on cytochrome oxidase have been interpreted in a model where the electron acceptors are treated as independent units. However, evidence has accumulated that interaction between the electron acceptors must exist. This is most obvious from the observation that an EPR signal at $g6$ appears in the partially reduced enzyme and disappears on complete reduction [1]. Similarly, magnetic susceptibility data are most easily interpreted in a model involving ferromagnetic coupling between the acceptors [2]. It has also been proposed that the optical absorption of one heme is affected by the reduction of the others [3]. Finally, the interdependence of the oxidation-reduction potentials of the acceptors has been discussed by several authors [4—7].

Some analyses of redox data from cytochrome oxidase in terms of interacting units have already appeared in the literature [5—7]. However, these have been limited to a consideration of the coupling between two acceptors only at a time. The present paper extends the analysis to the simultaneous interaction between the acceptors in the oxidase both with respect to oxidation-reduction potentials and the measurable physical quantities. It was hoped that such an analysis would lead to a better understanding of the system and provide suggestions for further experimentation. Some difficulties existing in earlier interpretations could be resolved through the introduction of interaction but it is also shown that available data do not have an unambigous interpretation.

## Materials and Methods

The complexes I, II and III of the electron transport chain were removed from mitochondria with deoxycholate according to the method of van Buuren [8]. The cytochrome oxidase remaining after this treatment, was homogenized in Tris/histidine/sucrose buffer and used without any further purification. The reduction was brought about with excess of ascorbate and small amounts of cytochrome *c* in an EPR tube. The course of reduction, after depletion of oxygen, was followed at 605 nm in a DBS-1 Johnson Research Foundation dual wavelength spectrophotometer at room temperature. At different degrees of reduction the samples were immersed in liquid nitrogen, which gives a freezing time of a few seconds, and examined in the EPR spectrometer at 15 K. The intensity of the $g6$ signal was measured relative to the $g3$ signal in the oxidized sample.

For the simulations a program was written in BASIC and executed on a Nova 3 minicomputer. The program steps through successive values of the redox potential. The program can also allow the use of a titrant, such as cytochrome *c*, with an oxidation-reduction potential not much different from those of the oxidase.

## Results and Discussion

*Parameter choice*

Ligand binding to macromolecules has been discussed extensively in the literature (for example, see refs. 9 and 10). In particular, the binding of oxygen to hemoglobin has been given elaborate treatments. In general at least 15 parameters (microscopic constants) are required for a full description of a four-site system, since the number of distinguishable species is 16 in this case. A measurement of the average number of ligands (electrons) bound as a function of the concentration of free ligand (the solution redox potential) can only give the four stoichiometric (macroscopic) constants. In the hemoglobin case one usually greatly reduces the number of parameters needed by symmetry considerations based on structural knowledge. For cytochrome oxidase no such symmetry can be presumed, but, on the other hand, there are more spectroscopic "handles" on the individual sites than in the hemoglobin case. Nevertheless, the number of parameters has to be restricted somehow, and we have limited our treatment to pairwise interaction. Thus, interactions that require the simultaneous consideration of the oxidation states of more than two acceptors have been neglected. A priori it is difficult to justify such a limitation, in particular since the protein has been suggested to exist in different conformations depending on its redox "history" [11,12]. Titrations of a system with several conformations in equilibrium with each other do not necessarily require more parameters for their interpretation, but when conformation changes occur due to the acceptance of electrons the effects on one acceptor of the redox states of the others may not be additive. However, as will be seen from the discussion below, the ten parameters remaining (four intrinsic site constants and six pairwise interaction parameters) are more than sufficient to account for the data presently available.

In spite of the limitations in our models, the systems are complicated enough that effects can occur that might not be intuitively clear. For example, consider three acceptors such that acceptor a has a negative interaction with b which in turn is positively coupled to c. Provided the interactions are strong enough acceptor a may first be reduced and then reoxidized when reducing agents are added.

*Computational technique*

Let o and r denote the oxidized and reduced forms of a particular site in any four-electron acceptor, respectively. The intrinsic constants $E_{ii}$ and the pairwise interaction parameters $E_{ij}$ are defined such that, for example, the oxidation-reduction potential of the reaction

$$\mathrm{o\,r\,r\,o} + e^- \rightleftharpoons \mathrm{o\,r\,r\,r} \qquad (1)$$

is $E_{44} - E_{14} + E_{24} + E_{34}$. The sign of the interaction parameters is chosen so that a cooperative interaction leads to a positive sign. The interaction is defined such that $E_{ij} = E_{ji}$, and the intrinsic site constants $E_{ii}$ are the average of the potentials of all reactions in which the site $i$ is reduced. The system then can be conveniently described by a symmetric 4 × 4 "potential" matrix.

The first steps in the simulation involve the formation of a 5 × 6 matrix con-

taining the equilibrium constants for each species relative to the fully oxidized form. The $i$th row contains the constant for the species having $i - 1$ electrons, and there are 1, 4, 6, 4 and 1 non-zero elements in the successive rows. A summation of the elements in each row forms a vector from which the stoichiometric constants easily can be obtained. In general, the potential matrix can be "diagonalized", i.e. the four microscopic constants for a non-interacting system can be found that give the same titration curve as the non-diagonal matrix. The diagonal matrix gives a direct feeling for the general behaviour of the system. For example, in the simple case of two acceptors both with an intrinsic potential of 0 mV and an interaction of −60 mV the system accepts electrons as if it had two independent acceptors at ±78 mV. However, these hypothetical acceptors are no longer "pure" in that each electron is shared between the original acceptors, in the given example equally. The diagonal matrix is obtained from the roots of a fourth-order polynomial with its coefficients given as functions of the stoichiometric constants (cf. ref. 9). When not all roots are real, the oxidation-reduction behaviour cannot be described without interaction [9].

In an analogous way, for each property studied in a titration, a "property" matrix, $A$, is formed, again restricting the interaction to be pairwise only. The changes relative to the fully oxidized form are computed. For Reaction 1 the change in the observed property would be $A_{44} - A_{14} + A_{24} + A_{34}$.

*EPR titrations*

A large number of EPR signals has been reported for cytochrome oxidase [13,14]. Some, such as the two different $g2$ signals [14], may well represent heterogeneity of the preparation. The three different $g6$ signals [14] could represent some of the 16 different molecular forms that in principle exist in a four-site system. However, we have chosen to lump the different $g2$ and $g6$ signals together and also to neglect the low-spin heme signal at $g2.6$ that appears with low intensity in some titrations.

Due to the disturbing fact that the $g2$ signal of the oxidized protein usually corresponds to less than one per cytochrome $aa_3$ [13,14], the "extinction coefficient" of the EPR-detectable Cu has been taken as 0.8 for this work. No good explanation for this anomalous behaviour has been found.

In the oxidized enzyme we have designated the EPR-detectable copper $Cu_A$ [15]. The $g3$ signal has been assigned to cytochrome $a$ [16,17]. Cytochrome $a_3$ and the other copper, $Cu_B$, are assumed to be EPR-undetectable in the oxidized protein due to a magnetic coupling [1].

There are two hypothesis put forward concerning the origin of the $g6$ signal in the partially reduced enzyme. In one model cytochrome $a$ changes from low-spin to high-spin when cytochrome $a_3$ gets reduced [16]. We have discarded this hypothesis because with the potentials used in the literature [16,18] the amount of cytochrome $a_3^{3+}Cu_B^+$ produced in a titration should be rather high, and no heme signal has been detected at partial reduction with a redox behaviour substantially different from that of $g6$. In addition, Beinert and Shaw [17] have produced samples of cytochrome oxidase, for which the sum of the $g3$ and $g6$ signals significantly exceeds one per cytochrome $aa_3$. We have preferred the original suggestion [1], that the $g6$ signal comes from cytochrome $a_3$. Both

hypotheses mentioned can give rise to a certain amount of cytochrome $a_3^{2+}Cu_B^{2+}$, which in principle should show an EPR signal, since it would be an odd-electron system. However, if cytochrome $a_3$ is high-spin [2], the presumably coupled unit consisting of cytochrome $a_3^{2+}$ and $Cu_B^{2+}$ may well have a symmetry low enough that the signal would be spread over a large field interval and thereby difficult to detect.

In the potential and property matrices used below the four electron acceptors are taken in the order cytochrome *a*, $a_3$, $Cu_A$ and $Cu_B$. The property matrix for the *g*3 signal is then simply $A_{11} = -1$ with all other elements zero. For the *g*6 signal a slightly more complicated matrix is required with the non-zero elements $A_{22} = -0.5$, $A_{44} = 0.5$ and $A_{24} = A_{42} = -0.5$. In this way changes will only occur when reducing $Cu_B$ with cytochrome $a_3$ oxidized (change $A_{44} - A_{24} = +1$) or when reducing cytochrome $a_3$ with $Cu_B$ reduced (change $A_{22} + A_{42} = -1$).

The maximal quantity of the *g*6 signal obtained in a titration poses a special problem. Hartzell and Beinert [18] have reported that the maximum varies between 0.25 and 0.5 per cytochrome $aa_3$ in reductive titrations, depending on the preparation used. Wilson et al. [16] claim that in mitochondria and submitochondrial particles, in contrast to in isolated oxidase, the signal amounts to one heme per cytochrome $aa_3$. In this laboratory the *g*6 signals we obtain maximally correspond to 0.25 per cytochrome $aa_3$, using isolated cytochrome oxidase or deoxycholate solubilized mitochondria (see Materials and Methods).

Since the titrations of Hartzell and Beinert [18] are the only ones in the literature with complete integrations of all the EPR signals, we have compared their data to our simulations. Two of their titrations have been replotted in Figs. 1 and 2. The reducing agents were NADH and ferrocytochrome *c*, respectively. Note that there is a qualitative difference between the two experiments in the maximal amount of *g*6 signal obtained. The authors [18] extracted oxidation-reduction potentials for the appearance of the *g*6 signal and for the disappearances of the *g*2, *g*3 and *g*6 signals, which we have used for simulation of titration curves (Figs. 1A and 2A). The main difference between experiments and simulations is the amount of *g*6 signal produced. This is also appar-

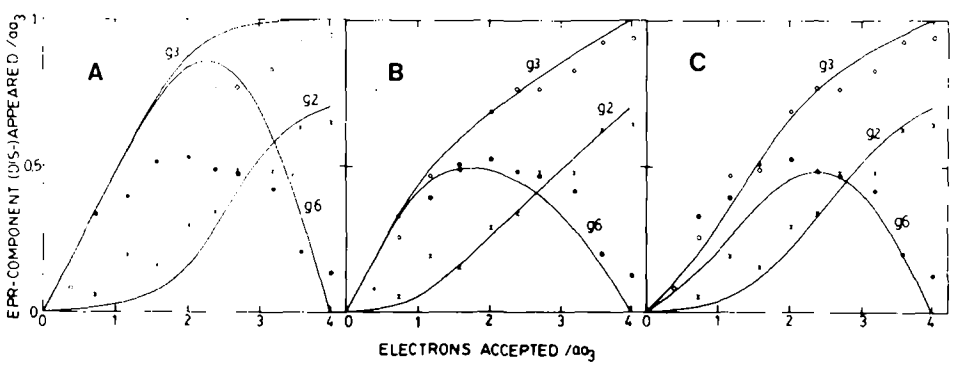


Fig. 1. Simulations of an EPR titration with NADH made by Hartzell and Beinert [18]. The symbols used are: ○, *g*3 signal disappeared; ●, *g*6 signal appeared and X, *g*2 signal disappeared. In A the oxidation-reduction potentials from ref. 18 are used (340, 210, 260 and 340 mV for cytochrome *a*, cytochrome $a_3$, $Cu_A$ and $Cu_B$). The potential matrices used in B and C are given in the text.

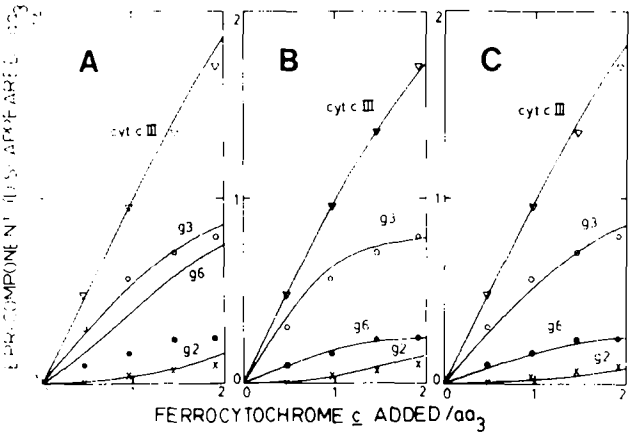


Fig. 2. Simulations of an EPR titration with ferrocytochrome *c* by Hartzell and Beinert [18]. $\nabla$, designates amount of cytochrome *c* oxidized. The other symbols are as in Fig. 1. The oxidation-reduction potentials in A are 340, 200, 260, 325 and 230 mV for cytochrome *a*, cytochrome $a_3$, $Cu_A$, $Cu_B$ and cytochrome *c* [18]. In B and C an oxidation-reduction potential of 255 mV has been used for cytochrome *c*. The potential matrices for cytochrome oxidase are given in the text.

ent from the simulations performed by Palmer et al. [15] based on the same experimental data. In order to reduce this difference and also to give a better fit to the *g*3 signal in Fig. 1 we introduced potential interaction. For each titration two different potential matrices were found. They are shown below with potentials in mV. The parentheses indicate the figure for which the matrix was used.

| | *a* | $a_3$ | $Cu_A$ | $Cu_B$ | | *a* | $a_3$ | $Cu_A$ | $Cu_B$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| *a* | 310 | 0 | 0 | −30 | | 300 | −30 | 0 | 0 | |
| $a_3$ | 0 | 250 | 0 | 0 | (1B) | −30 | 290 | 0 | −30 | (1C) |
| $Cu_A$ | 0 | 0 | 260 | 0 | | 0 | 0 | 260 | 0 | |
| $Cu_B$ | −30 | 0 | 0 | 310 | | 0 | −30 | 0 | 290 | |
| *a* | 335 | 0 | 0 | −30 | | 360 | 10 | 0 | 0 | |
| $a_3$ | 0 | 280 | 0 | 10 | (2B) | 10 | 300 | 0 | −30 | (2C) |
| $Cu_A$ | 0 | 0 | 260 | 0 | | 0 | 0 | 260 | 0 | |
| $Cu_B$ | −30 | 10 | 0 | 310 | | 0 | −30 | 0 | 290 | |

A systematic search for all possible solutions has not been performed and there may exist other solutions, in particular for the titrations with cytochrome *c* which are only carried to half reduction. The two pairs of matrices presented predict the experimental data reasonably well, although the interactions are qualitatively different. For example, in Fig. 2B a negative coupling is used between cytochrome *a* and $Cu_B$ and a cooperative interaction between cytochrome $a_3$ and $Cu_B$. In Fig. 2C, on the other hand, the negative coupling is between cytochrome $a_3$ and $Cu_B$.

We have used two methods of reducing the amount of $g6$ signal. A cooperative interaction between cytochrome $a_3$ and $Cu_B$ makes them tend to titrate together. If one uses potential matrices such that the acceptors titrate over a large potential interval the $g6$ signal may appear early in a titration and disappear late without growing too large.

*Optical titrations*

The property most thoroughly studied in redox titrations of cytochrome oxidase is the optical absorbance change at 605 nm. In summary all data have been interpreted in terms of one heme titrating at a potential higher than 330 mV and one lower than 250 mV. There is no agreement on the spectral contribution of the hemes. For example, the high-potential heme has been reported to give 50 [19], 45 [3], 35 [20] or 20 [21] percent of the total change. To facilitate a comparison the data from the given references have been replotted in Fig. 3A. The potentials given in ref. 20 have been complemented with potentials for the copper ions from the same laboratory [16]. Obviously there is a large spread in the data.

For simulations of optical titrations it seems reasonable to try the same potentials as those used for the EPR data. In principle, since the extinction coefficients of the acceptors are unknown, nine additional parameters are now available (normalizing reduced-minus-oxidized to 1), which, however, reduces to one under the usual assumption of contributions from spectrally non-interacting hemes only. In Figs. 3B and 3C the sets of potentials from Figs. 1B and 1C have been used with different spectral distribution. Since both hemes now are part of coupled systems, none of them is pure high- or low-potential in character. As a consequence the titration curves are not as sensitive to changes in spectral contributions as they would be if the hemes were well separated in potential.

A detailed comparison between experimental data and simulations may not be too meaningful at the present stage, but in no case cytochrome $a$ seems

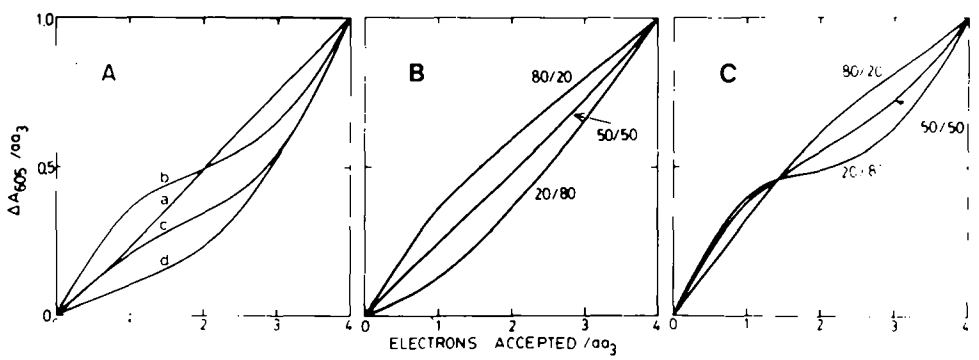


Fig. 3. Simulations of redox titrations of cytochrome oxidase followed at 605 nm. In A the following potentials for the two hemes and the two copper ions have been used: a, 340, 215, 350 and 215 (Mackey et al. [19]); b, 370, 230, 280 and 280 (Tiesjema et al. [3]); c, 360, 205, 340 and 250 (Leigh et al. [20] and Wilson et al. [16]); d, 340, 220, 340 and 240 (Schroedl and Hartzell [21]). The spectral contributions used for the first heme are 50, 45, 35 and 20% for curve a, b, c and d, respectively. In B and C the same potential matrices as in Figs. 1B and 1C have been used, with varying relative spectral contributions of cytochromes $a$ and $a_3$, 80/20, 50/50 and 20/80, respectively.

required to have a larger spectral contribution than cytochrome $a_3$. For example, the data of Tiesjema et al. [3] (45% from the high-potential heme) can be simulated with the potential matrix of Fig. 1C with cytochrome *a* contributing 20%. If one introduces heme-heme spectral interaction there will be several ways of making our potential matrices fit the experimental data. It should be noted that a priori heme-copper spectral interaction is equally probable since there are reasons to believe that $Cu_B$ and cytochrome $a_3$ are spatially close to each other [15]. If one of the cupric ions has a spectral contribution similar to that of Type 1 $Cu^{2+}$ in blue oxidases (5 $mM^{-1} \cdot cm^{-1}$), the simulated titration curves will be displaced up or down depending on which copper contributes. The magnitude of these displacements will be approximately the same as those occurring when the contribution of the hemes changes with ±15%.

*Susceptibility titrations*

Recently the magnetic susceptibility of oxidized and reduced cytochrome oxidase was measured [2]. The results were interpreted in a model with one low-spin and one high-spin heme in both oxidized and reduced enzyme. The high-spin heme was proposed to be antiferromagnetically coupled to a copper in the oxidized enzyme. On the assumption that this coupling remains also in the partially reduced states the change in the spin-only magnetic susceptibility expressed in $\mu_{eff}^2$ will lead to a matrix with the non-zero elements $A_{11} = -3$, $A_{22} = -10$, $A_{33} = -3$, $A_{44} = 10$ and $A_{24} = A_{42} = -1$. The simulations based on the potentials from Figs. 1B, 1C, 2B and 2C are shown in Fig. 4. The sets of potentials used give very different behaviour of the susceptibility during reduction. Thus, susceptibility titrations could be a powerful tool in studies of cytochrome oxidase.

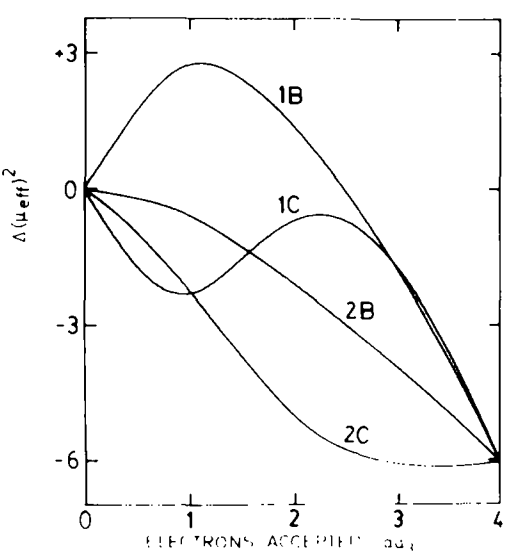


Fig. 4. Simulations of susceptibility titration of cytochrome oxidase. It is assumed that cytochrome *a* is low-spin and that this cytochrome and $Cu_A$ are magnetically isolated. Cytochrome $a_3$ is assumed to be high-spin and antiferromagnetically coupled to $Cu_B$ throughout the titration. The susceptibility and potential matrices used for the curves are given in the text.

*Concluding remarks*

There is no general agreement whether cytochrome *a* is high- or low-potential or has a larger or smaller contribution at 605 nm than cytochrome $a_3$. We have chosen the view that $g3$ originates from cytochrome *a*, mainly from the fact that this signal can be obtained even when CO is bound [22]. With the $g3$ and $g6$ signals originating from different hemes it follows, as shown above, that cytochrome *a* is essentially high-potential and that it, if anything, has a smaller contribution than cytochrome $a_3$ at 605 nm. The opposite view, that cytochrome $a_3$ is high-potential, stems mostly from optical titrations in the presence of CO [23]. However, these experiments have no unique interpretation, if one allows interactions both with respect to potentials and spectra. On the other hand, our discussion is based on the assumption that no intramolecular electron flows occur on freezing. Optical and EPR spectra recorded at the same temperature would be useful in discriminating between the two models.

Even within the framework of our assignments the simulations have demonstrated that the data available are not good enough to allow the formulation of a unique model for the equilibrium redox behaviour of cytochrome oxidase. In fact, it may turn out that the models used here are inherently too simple. The neglected higher-order terms might be necessary for a full description of cytochrome oxidase for which conformational changes could be an integral part of the mechanism.

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