Allosteric Cooperative Interactions among Redox Sites of Pseudomonas Cytochrome Oxidase†

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ABSTRACT: Anaerobic reductive spectrophotometric titrations of *Pseudomonas aeruginosa* cytochrome oxidase were performed. Both types of hemes (C and D) of the dimeric enzyme were monitored. The reduction process was found to involve cooperative allosteric and spectroscopic interactions between the two subunits. The model fitting the data best involves the following features. (1) The redox potential of heme C is about 60 mV higher than that of heme D. (2) In the electron uptake, a positive cooperativity of about 30 mV exists between the two D-type hemes residing in the two subunits. (3) A negative cooperativity of the same magnitude (30 mV) is found between the two C-type hemes bound to the two subunits. (4) No interaction was found between heme C and D in the same subunit or in the different subunits. (5) It is suggested that the reduction of the first heme, of each kind, has about twice the spectral change compared to that observed upon reduction of the second one. The possible significance of this model for the mechanism of action of the enzyme is discussed.

Pseudomonas cytochrome oxidase (ferrocytochrome *c*-551:oxygen oxidoreductase, EC 1.9.3.2) was suggested to be the terminal respiratory enzyme of *Pseudomonas aeruginosa* (Horio et al., 1961b), when the bacteria are grown anaerobically in the presence of nitrate. This enzyme catalyzes the electron transfer from cytochrome *c*-551 or azurin to molecular oxygen or to nitrate, its probable physiological acceptor (Yamanaka et al., 1961). This water-soluble protein of 120000 molecular weight is a dimer of identical subunits, each containing one heme C and one heme D (Kuronen & Ellfolk, 1972; Kuronen et al., 1975). The two kinds of hemes differ in the way they are bound to the protein, in their spectral properties, and in their ability to interact with external ligands. Heme C is covalently bound to two cysteine residues of the enzyme, while heme D is bound noncovalently and can be extracted out and reconstituted back (Yamanaka & Okunuki, 1963b; Hill & Wharton, 1978). Heme D, when oxidized, has an absorption peak at 640 nm and, when reduced, at 660 and 460 nm. The other heme has a typical heme C spectrum. These features allow convenient separate monitoring of the redox states and interactions among the hemes. pH changes and ligands such as CN^-, CO, and NO affect mainly the heme D bands (Yamanaka & Okunuki, 1963a).

Some oxidases reduce molecular oxygen by four electrons to produce two molecules of water in a complex mechanism, which is not yet fully understood. In this process, the oxidases overcome the thermodynamically unfavorable step of introducing the first electron to dioxygen and the energetic barrier of breaking the oxygen–oxygen bond (George, 1965; Malmstrom, 1973). The four redox centers of pseudomonas oxidase are capable of accepting, storing, and discharging four electrons needed to reduce molecular oxygen to water.

Extensive kinetic studies of the enzyme's reactions with low molecular weight reductants and oxidants, with its physiological donors cytochrome *c*-551 and azurin, and with oxygen and nitrite revealed quite complicated reaction patterns. Most of these studies show that the heme C is the primary electron-accepting site, while the reduction of heme D occurs by internal electron transfer (Parr et al., 1977; Wharton et al., 1973; Barber et al., 1977). The reaction with oxygen is much more complex, suggesting only heme D as the interaction site (Wharton & Gibson, 1976). In a more recent paper it was suggested that both types of hemes (Greenwood et al., 1978) are directly involved in this reaction.

Rather little information is available on the thermodynamic-equilibrium behavior of this enzyme. The only report describing reductive titrations (Horio et al., 1961a) is very limited and involved no attempt to analyze the results. In another paper a "quasi-equilibrium" is suggested to exist between heme C and D during the reduction of the protein (Shimada & Orii, 1976).

Spectrophotometric redox titrations are expected to reveal relationships among the different sites and the pattern of their changes as the oxidation state of the protein is varied. Also, potential allosteric cooperative effects may be identified. In this paper we present spectroscopic reductive titrations of pseudomonas cytochrome oxidase by several reductants. The results confirm that heme C has a higher redox potential than heme D and establish the existence of cooperative interactions among the four redox sites which may relate to the function of the enzyme as dioxygen and nitrite reductase.

Materials and Methods

Bacteria. *Pseudomonas aeruginosa* were grown anaerobically for 40–48 h in a 400-L fermentor containing 16 g/L nutrient broth, 4 g/L beef extract (Difco Laboratories, Detroit, MI), 20 g/L KNO_3, 6.4 g/L KH_2PO_4, 3.6 g/L Na_2HPO_4, 10 mg/L $CuSO_4 \cdot 5H_2O$, and 10 mg/L $FeCl_3$ at 37 °C, giving 5 g/L of cells. Acetone powder was prepared by washing the cells for 20 min in acetone (at 0 °C) (13 L of acetone per 1 kg of cells).

Protein. Cytochrome oxidase (pseudomonas cytochrome *c*-551:oxygen oxidoreductase, EC 1.9.3.2) was extracted according to the procedure of Kuronen & Ellfolk (1972), except that after dialysis the crude extract was concentrated seven- to tenfold by using a DC-2 hollow-fiber dialyzer–concentrator with H1DX50 fibers (50000 molecular weight retention) (Amicon Corp., Lexington, MA). From the effluent of the fibers cytochrome *c*-551 and azurin were extracted (Ambler, 1963; Ambler & Brown, 1967; Ambler & Wynn, 1973), while the oxidase was purified from the concentrate by the above-mentioned procedure. No crystallization was done. Spectral purity of the final product was at least $OD_{411}/OD_{280} = 1.1$. The protein was stored at a concentration of 100 mg/mL at 90% saturation of $(NH_4)_2SO_4$, in 0.1 M potassium phosphate buffer, pH 7.0, at 4 °C. The ammonium sulfate was dialyzed out against the same buffer before each

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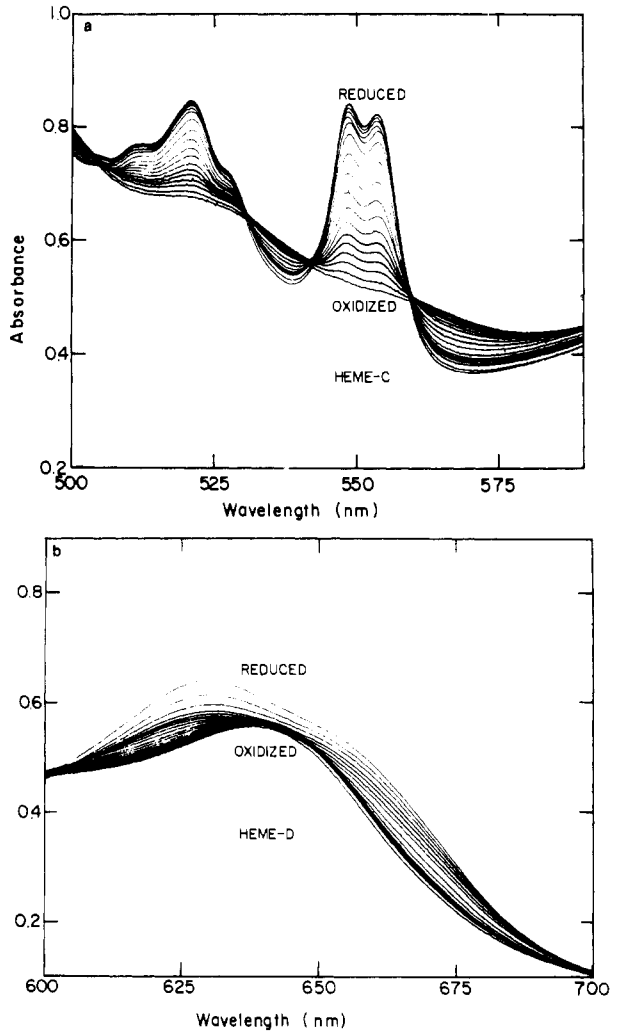


FIGURE 1: Anaerobic spectrophotometric reductive titration of pseudomonas cytochrome oxidase with $Fe^{II}EDTA$. Optical path length was 1 cm. Protein concentration was 2.87×10^{-5} M, in 2.2 mL of 0.1 M potassium phosphate buffer, pH 7.0. $Fe^{II}EDTA$ of 4.2×10^{-3} M was added in aliquots varying between 0.5 and 5.0 μL. For further details see Materials and Methods. (a) Heme C absorption bands. (b) Heme D absorption bands.

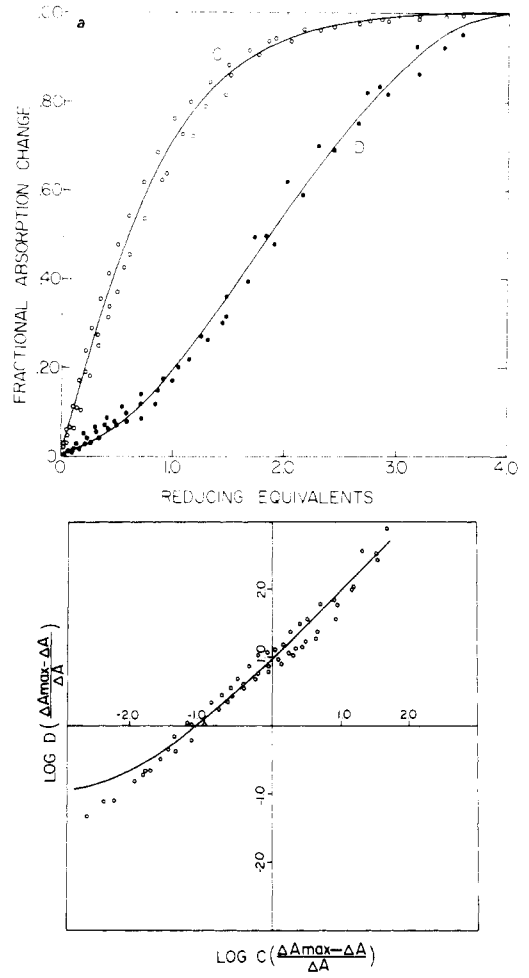


FIGURE 2: Data from three different spectrophotometric reductive titrations of pseudomonas cytochrome oxidase with $Fe^{II}EDTA$ presented as follows. (a) Fractional absorption changes ($\Delta A/\Delta A_{max}$) of the two hemes, C at 553 nm and D at 665 nm, vs. reducing equivalents. (b) Log heme D [$(\Delta A_{max} - \Delta A)/\Delta A$] vs. log heme C [$(\Delta A_{max} - \Delta A)/\Delta A$]. Corrections for the changes in volume were done on the absorption changes according to the volume of reductant added. The lines are drawn according to the parameters presented in Table II.

titration. The concentration of the protein was determined by using ϵ_{553}(red) = 2.91×10^4 L/(mol cm).

Reductants. Ferrous EDTA was freshly prepared before use by dissolving $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 20% molar excess of EDTA in the titration buffer (Wherland et al., 1975). Its concentration was determined by reducing $K_3Fe(CN)_6$ by using ϵ_{420} = 1020 L/(mol cm) for the ferricyanide formed (Schellenberg & Hellermann, 1958). Durohydroquinone was prepared by reducing duroquinone (Aldrich, Milwaukee, WI) with sodium borohydride in methanol. The white product was purified by sublimation and stored under argon in a desiccator. Ascorbic acid (BDH chemicals, Poole, England) was used without further purification.

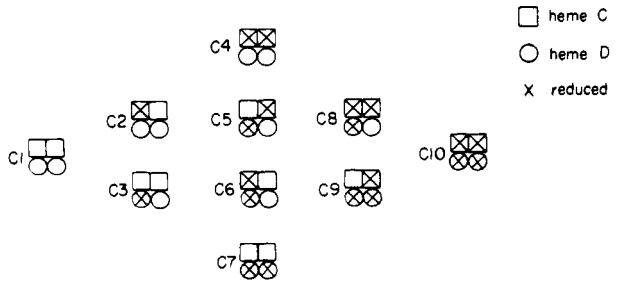
Reductive Titrations. All experiments were carried out in 0.1 M potassium phosphate buffer, pH 7.0, at 25 ± 2 °C in an anaerobic 1-cm optical path, 1-mL volume cuvette (Hellma, West Germany). Spectra were recorded on a Cary 118 spectrophotometer. Solutions were made anaerobic by the bubbling of argon (purified from traces of oxygen by passing it through methyl viologen (Sweetser, 1967)) for at least 30 min. During the titration (12–15 h), solutions were kept under a constant flow of argon similarly purified. Protein concentrations ranged between 10 and 50 μM. Reductant was added to the oxidized native protein with a Hamilton microsyringe through a serological rubber cap, and the solution was mixed by a small magnetic bar placed inside the cuvette. After each addition, spectral changes at a single wavelength were followed for 15–30 min until equilibrium was achieved, and then the full spectrum was recorded (500–700 nm).

Computation. Analysis of the results was done on an IBM 370/165 computer. For fitting the data to the models, we used a nonlinear least-squares subroutine (E04FAF) from the NAG library (Peckham, 1970). Another subroutine (NS03AD) of the Harwell library (Fletcher, 1971) was used to solve a set of nonlinear equations in each iteration of the fitting subroutine. Both subroutines needed initial guesses of the parameters (see Results and Interpretation), but only the second required the first derivative of the functions with respect to the parameters.

Results and Interpretation

A spectrophotometric reductive titration of *Pseudomonas aeruginosa* cytochrome oxidase by $Fe^{II}EDTA$ is presented in Figure 1a,b. Reduction with ascorbate or durohydroquinone resulted in the same spectral and titration pattern. The oxidation state of heme C was monitored at 553 nm and that of heme D was monitored at 665 nm. Changes measured at

Chart I



548 or 520 nm for heme C gave identical results, and the same was found at 625 nm for heme D. Figure 2a shows fractional absorbance changes ($\Delta A/\Delta A_{max}$) of the two hemes plotted as a function of added reduction equivalents obtained from three different titrations. The same data are presented on a log heme D $[(\Delta A_{max} - \Delta A)/\Delta A]$ vs. log heme C $[(\Delta A_{max} - \Delta A)/\Delta A]$ scale in Figure 2b. Two features are immediately apparent from the titration curve (Figure 2a). The reduction of heme D lags behind that of heme C, and thermodynamic interactions among the four redox sites cannot fully account for the titration pattern and, therefore, spectroscopic interactions have to be involved as well. For example, it can be seen that when 50% of the reduction equivalents were added to the system, the sum of the optical changes of the two hemes is more than 50% of the total optical change at full reduction. This interaction is expressed in the extinction coefficients as the isosbestic points are invariant throughout the titration (Figure 1a,b).

The behavior of the protein during its reductive titrations was interpreted on the basis of a model involving the following considerations.

(a) Pseudomonas cytochrome oxidase is composed of two identical subunits, each containing one heme C and one heme D, altogether four redox centers per molecule (Kuronen et al., 1975). In principle, an enzyme with four redox sites can form during its reduction 16 different species. In our case the number of distinguishable species is reduced to 10 by the symmetry properties arising from the structure of the enzyme, by assuming that the two hemes of each kind are initially identical (Chart I). The concentrations of these 10 different species are changing during the titration, but the sum has to be constant (normalized to one) (see eq 1).

$$[C1] + 2[C2] + 2[C3] + [C4] + 2[C5] + 2[C6] + [C7] + 2[C8] + 2[C9] + [C10] = 1 \quad (1)$$

(b) We assume that every reducing equivalent introduced into the solution reacts completely with the protein (i.e., that the potential difference is greater than 150 mV). In spectrophotometric redox titrations, one monitors only the behavior of the redox centers of the protein, and the solution potential is not measured. This is expressed in eq 2, where Q is the

$$2[C2] + 2[C3] + 2[C4] + 4[C5] + 4[C6] + 2[C7] + 6[C8] + 6[C9] + 4[C10] = Q \quad (2)$$

number of reducing equivalents introduced into the system.

(c) Five oxidation/reduction potentials are defined (1–5 below). These determine the concentrations of the 10 different species of the enzyme. As the zero point for the potential scale is arbitrary, the redox potentials we get are relative and not absolute (see also Table Ib). (1) $E_C - E_D$ = difference in redox potential between heme C and D. (2) E_{CC} = interaction potential between the two C-type hemes in the two subunits (the positive or negative potential difference required to reduce the second heme C in a molecule, having the first one reduced). (3) E_{DD} = interaction potential between the two D-type hemes in the two subunits (the potential required to reduce the second heme D in a molecule, having the first one reduced). (4) E_{CD1} = interaction potential between heme C and heme D in the same subunit (the potential required to reduce the heme C in a molecule, having the heme D in the same subunit reduced, or vice versa). (5) E_{CD2} = interaction potential between heme C and heme D in two different subunits (the potential required to reduce the heme C in a molecule, having the heme D in the second subunit reduced, or vice versa).

Table I

(a) Potential Difference between All Species and C1

(1) $\Delta E_{12} = E_C$
(2) $\Delta E_{13} = E_D$
(3) $\Delta E_{14} = 2E_C + E_{CC}$
(4) $\Delta E_{15} = E_C + E_D + E_{CD_2}$
(5) $\Delta E_{16} = E_C + E_D + E_{CD_1}$
(6) $\Delta E_{17} = 2E_D + E_{DD}$
(7) $\Delta E_{18} = 2E_C + E_D + E_{CC} + E_{CD_1} + E_{CD_2}$
(8) $\Delta E_{19} = E_C + 2E_D + E_{DD} + E_{CD_1} + E_{CD_2}$
(9) $\Delta E_{110} = 2E_C + 2E_D + E_{CC} + E_{DD} + 2E_{CD_1} + 2E_{CD_2}$

(b) Oxidation/Reduction Potential Defined According to Species Relations

(10) $E_C - E_D = (RT/F) \ln ([C2]/[C3])$
(11) $E_{CC} = (RT/F) \ln [([C1][C4])/[C2][C2])]$
(12) $E_{DD} = (RT/F) \ln [([C1][C7])/([C3][C3])]$
(13) $E_{CD_1} = (RT/F) \ln [([C1][C6])/([C2][C3])]$
(14) $E_{CD_2} = (RT/F) \ln [([C1][C5])/([C2][C3])]$

(c) Concentration Relationships Used in the Derivation of the Model

(15) $[C2]/[C3] = \exp[(F/RT)(E_C - E_D)]$
(16) $[C4]/[C7] = \exp[(F/RT)(2E_C - 2E_D + E_{CC} - E_{DD})]$
(17) $[C5]/[C6] = \exp[(F/RT)(E_{CD_2} - E_{CD_1})]$
(18) $[C8]/[C9] = \exp[(F/RT)(E_C - E_D + E_{CC} + E_{DD})]$
(10) $([C10][C7])/([C9][C9]) = \exp[(F/RT)E_{CC}]$
(20) $([C2][C8])/([C5][C5]) = \exp[(F/RT)(E_C - E_D + E_{CC} + E_{CD_1})]$

On the basis of these definitions one can compute the potential differences between the different species. Such potentials between all the species and C1 (Chart I) are listed in Table Ia. These relations yield the definitions of the different potentials in terms of species concentrations, as listed in Table Ib. From these definitions, some relations between certain species, which have been used in the derivation, are tabulated in Table Ic. One can express all these relations and conditions as two nonlinear equations with two unknowns (two of the species concentrations) and solve it numerically (assuming values for the five potentials).

(d) The interactions among the redox centers of the enzyme also result in the situation in which the spectral change of each heme type is not linear with its degree of reduction. To account for this we defined two spectroscopic parameters, O_C and O_D, which are factors required for multiplying the extinction coefficient of species that have only one heme reduced, in order to get the spectral change upon reduction, for heme C and D, respectively.

Using the concentrations calculated in the way explained above, together with these spectral parameters, we compared the observed changes measured in the titration (normalized $\Delta A/\Delta A_{max}$) with eq 3 and 4.

$$\text{heme C red(obsd)} = O_C([C2] + [C5] + [C6] + [C9]) + [C4] + 2[C8] + [C10] \quad (3)$$

$$\text{heme D red(obsd)} = O_D([C3] + [C5] + [C6] + [C8]) + [C7] + 2[C9] + [C10] \quad (4)$$

The fit was obtained by minimizing the difference between the observed spectral change and the calculated values in eq 3 and 4, while iterating the five redox potentials and two spectral factors as free parameters. The best fit values are

Table II: Best Fitted Parameters for the Model

$E_C - E_D = 58.5$ mV
$E_{CC} = -26.6$ mV
$E_{DD} = 26.6$ mV
$E_{CD_1} = 0.0$ mV
$E_{CD_2} = 0.0$ mV
$O_C = 2.07$
$O_D = 2.07$
$\text{rms} = [(\sum_{i=1}^{N} r_i^2)/N]^{1/2} = 0.0269$[a]

[a] Where r_i is the difference between the observed and the calculated values in each experimental point and N is the number of points.

presented in Table II, and the curves simulated by using them are shown in Figure 2a,b. A large number of different initial guesses of the parameters converge either on the same results, on physically unaccepted values, on a very poor fit, or the program was not able to reach a minimum. One should note that the best fit values that are presented here can be achieved, with only a very small increase in root mean square value (Table II), by three free parameters only ($E_C - E_D$, E_{CC}, and O_C), while holding the others fixed ($E_{DD} = -E_{CC}$, $E_{CD1} = E_{CD2} = 0$, and $O_D = O_C$) (for different models see Discussion).

The resulting values show that the midpoint redox potential of heme C (E_C; cf. Table Ia) is higher by about 60 mV than that of heme D (E_D). There is a negative cooperative interaction between the two hemes C of the two subunits, amounting to 27 mV, while that between the two D-type hemes is positively cooperative and of the same magnitude. No direct interaction between hemes C and D has to be assumed for explaining the results. The introduction of the first electron to each type of heme results in twice the spectral change compared to that occurring upon reduction of the second heme of the same type. All these findings show that the interactions among the redox centers of the enzyme occur between the two subunits and not within them.

Discussion

Our results confirm the earlier observation obtained by spectroscopic titration (Horio et al., 1961a) that heme C has a redox potential higher than that of heme D (Table II). This is also in line with kinetic results showing that heme C is reduced first in the reaction of the oxidized enzyme with reduced azurin (Wharton et al., 1973; Parr et al., 1977) or with chromous ions (Barber et al., 1977). The "quasi-equilibrium", described by Shimada & Orii (1976) in their rapid-scanning study of the reduction of the oxidase with excess ascorbate, can in our opinion also be explained in terms of the potential difference between hemes C and D, as the intramolecular electron distribution equilibrates much faster than the very slow bimolecular reduction by ascorbate.

Magnetic circular dichroism spectra of pseudomonas cytochrome oxidase were recently measured by two different groups, arriving at contrary conclusions in terms of redox-site interactions. Orii et al. (1977) claimed that their results show heme C–heme D interactions in the reduced enzyme, whereas Vickery et al. (1978) concluded that no evidence for such interactions can be found. Our data are explained by a model involving interactions between the two subunits of the enzyme exhibited in mutual influence between the two C-type hemes and between the two D-type hemes. We did not find any direct interactions between heme C and heme D either in the same subunit or in the two subunits. It is noteworthy that the interaction potential between the C-type hemes is negative; i.e., it is more difficult to reduce the second heme C (in the second subunit) of the same molecule. Thus, when the first heme C is reduced, the midpoint potential of the second is lowered by 27 mV. In contrast, positive cooperativity exists between the two D-type hemes. This increase in the midpoint potential of the second heme D upon reducing the first, by the same magnitude (27 mV), means that reduction of the second heme D is favored by a factor of 2.8. Thus, the interactions between the subunits of this enzyme are such that opposite cooperative effects of the same energy are exerted on the two heme pairs.

Another expression of the symmetry of the enzyme is the equal spectroscopic factors for the extinction of the two hemes. For each pair of identical hemes, reduction of the first causes an increase in the extinction coefficient, which is about twice as large compared to that observed upon reduction of the second. Such a difference between the extinction coefficients of the two hemes of the same kind seems to be rather large. However, since the pattern of the spectrophotometric titrations shows no wavelength dependence, a best fit to the above data is obtained for a model allowing the change in extinction coefficients.

Two alternative models were also examined. In the first we assumed that the spectral factor multiplies the extinction of reduced heme C when heme D in the same subunit is oxidized. It means that the spectral interaction occurs between heme C and heme D in the same subunit. In the second model we similarly assumed that the spectral interaction exists between heme C and heme D residing in two different subunits. However, in spite of their good fit to the data, these two possibilities were ruled out. In the first, the interaction potential between hemes C and D of the same subunit (E_{CD1}) is found to be zero, while in the other case E_{CD2} was found to be zero. In a system where no thermodynamic interactions exist, we considered it unreasonable to find spectral interactions.

Support for our observation of heme D–heme D interaction is found in the binding studies of ligands to this site. Carbon monoxide binds to reduced heme D with a Hill coefficient of 1.44 and an average binding constant of 6.3×10^4 M^{-1} (Parr et al., 1975). Sodium methabisulfite forms a complex with reduced heme D with an affinity of 3.2×10^2 M^{-1} and a cooperative Hill slope of 1.2 (Parr et al., 1974). Studies of cyanide binding show that in the reduced state of the protein, only heme D binds this ligand with an average binding constant of 3.8×10^4 M^{-1} and a cooperative Hill slope of 2.6 (Barber et al., 1978). Although this last Hill slope seems to be too high for an enzyme with only two binding sites, the cooperativity in the binding of the three different ligands to reduced heme D is clearly manifested. The binding of a first ligand enhances the binding of a second, probably by breaking the interaction between the two reduced D hemes and making the second site more available.

An interesting insight into the behavior of the oxidase is obtained by examining the changes in the enzyme throughout the reduction by simulating the titration by using the best fit potentials yet without the spectroscopic interaction (Table II). The changes in the oxidation state of the hemes and of the different species as a function of the added reduction equivalents are shown in Figure 3a,b. The relative reduction of the two hemes is shown on a logarithmic scale in Figure 4. Again, it is clear that heme C precedes heme D throughout the titration and that the prevailing species in the solution during reduction are C1, C2, and smaller amounts of C4, C8, C9, and C10. Rather small amounts of C3 and C7 are formed. It can be said that the reduction proceeds from C1 mainly to

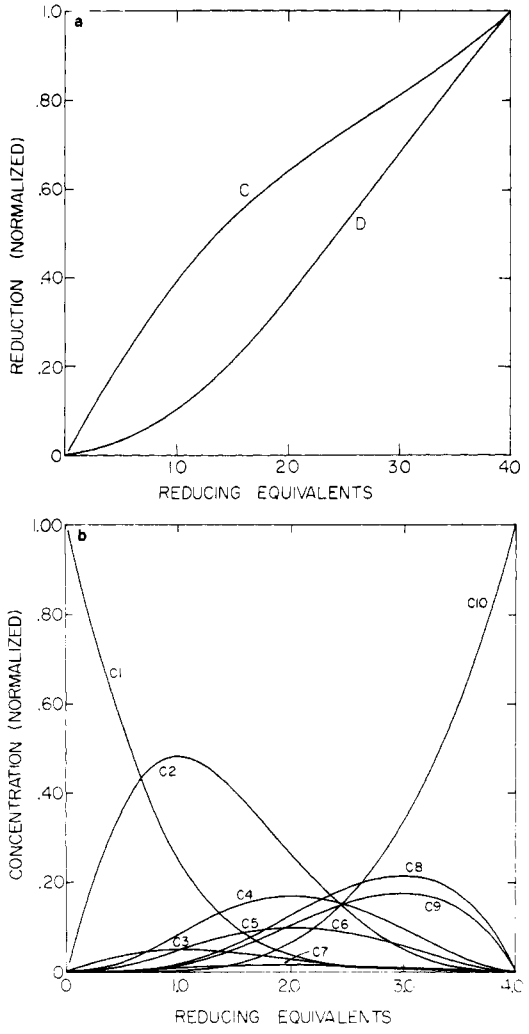


FIGURE 3: Simulation of the reductive titration according to the parameters presented in Table II without the spectral factors (O_C = O_D = 1). (a) Fractional reduced hemes C and D as a function of reducing equivalents. (b) Normalized concentrations of the 10 different species (Chart I) plotted as a function of reducing equivalents.

C2, to C4, to C8 and C9, and to C10. Thus, electrons are first accumulated in the two C hemes and then, in a cooperative manner, the two other electrons are taken up by the D-type hemes, producing the fully reduced enzyme. The relations between hemes C and D can be seen from the slope of the logarithmic plot (Figure 4). In the beginning and in the end of the titration the slope equals unity, while in the midrange the relation between the two pairs of hemes becomes positively cooperative with a slope of 1.6 (out of the theoretical maximum of 2.0).

Mammalian cytochrome *c* oxidase, which also catalyzes the reduction of molecular oxygen to water, contains four metal–redox centers, two hemes and two copper ions. Some research groups believe that the two hemes (*a* and a_3) are indistinguishable and strongly interacting. Reduction of or ligand binding to one of them modifies the properties of the second, while other groups favor the hypothesis of two chemically distinct cytochromes *a* and a_3 [Malmstrom, 1973; Lanne & Vanngard, 1978; Tiesjema et al., 1973; Schroedl & Hartzell, 1977; Babcock et al., 1978; Palmer et al., 1976; for an updated summary see Erecinska & Wilson (1978)]. Common to all these hypotheses is the concept of interactions among hemes and coppers, which is expressed in the magnetic properties and absorption spectra. Recently, Lanne &

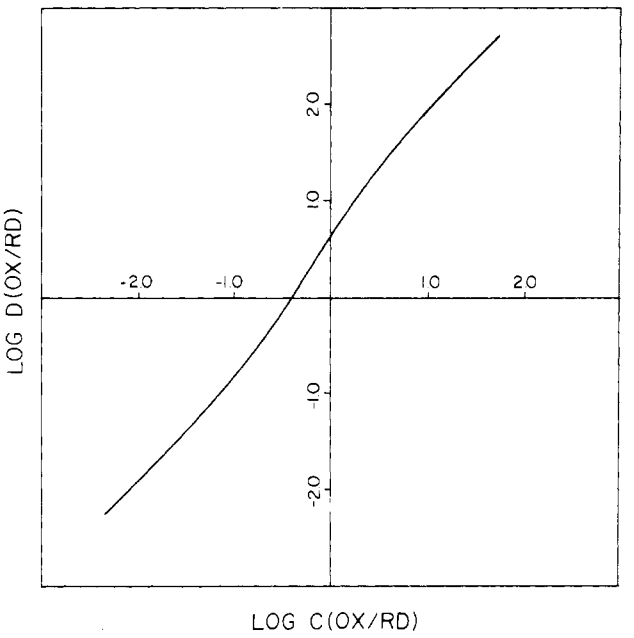


FIGURE 4: Simulated data of Figure 3a presented as log heme D (oxidized/reduced) vs. log heme C (oxidized/reduced).

Vanngard (1978) analyzed the interactions among the acceptor sites in the mammalian cytochrome oxidase, both with respect to redox potentials and with respect to the measurable physical quantities, in a way similar to that described here. They interpret the equilibrium redox titration by a minimal pairwise interaction. However, they show that there is no unique solution to the data yet.

Another oxidase that catalyzes the same reaction is laccase. This enzyme contains four copper ions, at least two of them strongly interacting with each other (Fee, 1975; Farver et al., 1978). It seems that the four redox centers found in all oxidases are needed for storing four electrons, which are transferred to dioxygen, probably in a pairwise manner. The cooperativity we found between the two pairs of hemes may be important for the reduction of oxygen and nitrite by the fully reduced enzyme.

Finally, we want to stress that the picture we drew is not the only one that fits the available data. We tried to construct a minimal model by taking into account the symmetry of the molecule, the identity of the hemes, and the confidence in assigning the spectral wavelengths and behavior to the different hemes. More data, primarily from magnetic studies, are needed in order to confirm these results and the proposed model and to better understand the behavior of this protein.

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