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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XVIII. POTENTIOMETRIC TITRATIONS OF CYTOCHROME *c* OXIDASE FOLLOWED BY CIRCULAR DICHROISM

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SUMMARY

1. Potentiometric circular dichroism titrations of cytochrome *c* oxidase, carried out in the absence of cytochrome *c*, confirm the potentiometric equivalence of the two heme *a* groups of cytochrome *c* oxidase. In the presence of cytochrome *c*, two different midpoint potentials are found for the two heme *a* groups of cytochrome *c* oxidase.

2. Circular dichroism difference spectra (reduced minus oxidized) of the two heme *a* components of cytochrome *c* oxidase have been obtained by means of this potentiometric titration. On reduction of the first heme *a* group a circular dichroism difference spectrum is obtained with peaks at 425, 442 and 602.5 nm; the second heme *a* group shows difference peaks at 434, 447 and 608 nm. Whereas both heme *a* groups contribute about equally to the absorbance difference spectrum, the second heme *a* group reduced contributes about twice as much to the circular dichroism difference spectrum as does the first heme *a* group.

3. From these spectral and circular dichroism differences it is concluded that, on reduction of or ligand binding to cytochrome *c* oxidase, conformational changes occur which affect the symmetry of the environments of the heme *a* groups.

INTRODUCTION

Circular dichroism (CD) is a most useful method in the physico-chemical study of cytochrome *c* oxidase, since the optical activity of this enzyme in the visible region is largely induced [1–6]. Changes in optical activity therefore reflect changes in the environments of the heme *a* groups, and in this way CD can be helpful in elucidating the changes in protein conformation that occur on oxidation or reduction of the enzyme or on ligand binding.

In a previous paper of this series [7] we have reported on the CD changes occurring on binding of ligands to cytochrome *c* oxidase, and an attempt was made

to calculate the separate CD difference spectra (reduced minus oxidized) of what are usually called the cytochrome *a* and a_3 components of this enzyme. It was concluded that the CD spectra of both heme *a* groups are mutually dependent, and that binding of a ligand to one of the heme *a* groups affects the spectrum of the other heme *a* group. Potentiometric titrations of particulate [8–12] and of isolated [13–15] cytochrome *c* oxidase, as well as EPR studies [9, 11, 12, 16, 17] also revealed this interdependence of the two heme *a* groups.

The present study combines potentiometric titrations and CD measurements. The combination of these methods makes it possible to obtain the CD difference spectra (reduced minus oxidized) of the separate heme *a* components of cytochrome *c* oxidase, and to correlate the CD changes directly or via a potential scale to absorbance changes.

EXPERIMENTAL

Enzymes

Cytochrome *c* was isolated from horse heart according to the method of Margoliash and Walasek [18], and cytochrome *c* oxidase was prepared from beef heart by the Van Buuren [19] modification of the combined methods of Fowler et al. [20] and of MacLennan and Tzagoloff [21]. Cytochrome *c* concentrations were calculated from the absorbance difference (reduced minus oxidized) at 550 nm, using an absorbance coefficient [22] of 21.1 $mM^{-1} \cdot cm^{-1}$. For cytochrome *c* oxidase an absorbance coefficient [23] (reduced minus oxidized) of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm was used. Protein concentrations were determined by the biuret method of Gornall et al. [24] in the modification of Yonetani [25]. A ratio of 5 μmoles cytochrome *c* oxidase (or 10 μmoles heme *a*) per g of protein was judged as a minimal criterion of purity. Cytochrome *c* oxidase preparations were stored in liquid N_2 until use.

Chemicals

All experiments were carried out in 100 mM potassium phosphate, 0.5 % Tween-80 (pH 7.2). Chemicals were Analar Grade obtained mainly from British Drug Houses. NADH was Boehringer's grade 2.

Spectra

Visible absorbance spectra were obtained with a Cary-17 recording spectrophotometer, thermostatted at 27 °C; circular dichroic (CD) spectra with a Cary-60 spectropolarimeter, equipped with a Cary-6002 CD-attachment. The CD instrument was thermostatted at 27 °C and set to a spectral bandwidth of 1.5 nm.

Potentiometric titrations

Comparative titrations with a mixture of cytochrome *c* oxidase and cytochrome *c* were carried out in evacuated Thunberg-type cuvettes as described elsewhere [13]. Absorbance and CD spectra were alternately recorded. A potential scale was calculated from the degree of reduction of cytochrome *c*, determined from the absorbance at 550 nm after correction for the small contribution of cytochrome *c* oxidase at this wavelength.

Direct potentiometric titrations were performed in an all-glass titration cell

provided with a platinum and a calomel electrode, calibrated as described earlier [13]. Potentials were measured with a Philips-PW9408 digital pH/mV meter. The titration cell was kept anaerobic by purging with N_2 gas, made by evaporation of liquid N_2 and freed from traces of O_2 by passing through a column filled with *Azotobacter vinelandii* particles in the presence of 50 mM malate. All connecting lines were made of copper, stainless steel or glass, in order to exclude contamination with O_2; to prevent back diffusion of O_2 the outlet of the titration cell was vented against a pressure of 10 cm saturated KCl. As has been reported earlier from our laboratory [13, 26] the presence of even traces of O_2 induces differences in midpoint potential between the two heme *a* groups. Reduction was brought about by the addition of 3–5-μl aliquots of concentrated NADH (about 20 mM); after each addition of reductant the contents of the titration cell were mixed by passing a stream of O_2-free N_2 through the solution.

Both in the direct and in the comparative potentiometric titrations midpoint potentials and n values were calculated for cytochrome *c* (*c*) and cytochrome *c* oxidase (*a*) with the Nernst formula:

$$E = E_{m(a)} + \frac{60}{n_a} \log \frac{[a_{ox}]}{[a_{red}]} = E_{m(c)} + \frac{60}{n_c} \log \frac{[c_{ox}]}{[c_{red}]}$$

When a curved line was found in the Nernst plot by the interfering reduction of two groups with overlapping absorbance bands and different midpoint potentials, it was resolved into two straight lines for the separate groups, taking into account the spectral contributions of these two groups.

RESULTS

Fig. 1A shows a comparative titration of a mixture of cytochrome *c* oxidase and cytochrome *c*, carried out in the Soret region, and a similar titration in the α-band region is shown in Fig. 1B. These figures correlate the absorbance changes at

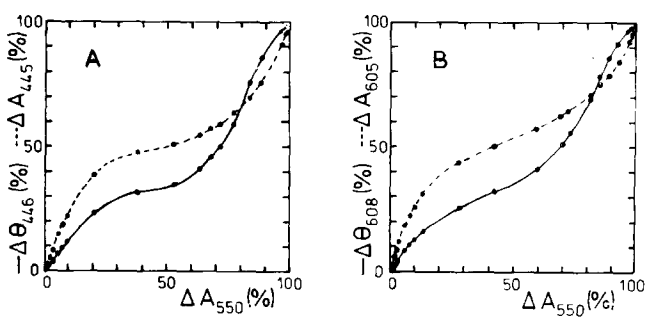

Fig. 1. Comparative potentiometric CD titration of a mixture of cytochrome *c* and cytochrome *c* oxidase. A. Soret region. The absorbance increase at 445 nm (- - -) and the corresponding ellipticity increase at 446 nm (———) of cytochrome *c* oxidase are plotted against the absorbance increase of cytochrome *c* at 550 nm. 8 μM cytochrome *c* oxidase, 16 μM cytochrome *c*, 100 mM potassium phosphate, 0.5 % Tween-80, pH 7.2. B. α-band region. The absorbance increase at 605 nm (- - -) and the corresponding ellipticity increase at 608 nm (———) are plotted against the absorbance increase of cytochrome *c* at 550 nm. 40 μM cytochrome *c* oxidase, 70 μM cytochrome *c*, 100 mM potassium phosphate, 0.5 % Tween-80, pH 7.2. Reductant NADH.

550 nm, which are due to reduction of cytochrome *c*, and those at 445 and 605 nm, caused by reduction of cytochrome *c* oxidase. As was found earlier [13–15, 27, 28], first one of the hemes *a* is reduced, followed by the reduction of cytochrome *c*; in the final stage of the titration the second heme *a* is reduced. The contributions of the two heme *a* groups to the absorbance increase at these wavelengths are about equal.

The same figures also correlate the increase of ellipticity measured at the α- and γ-bands of cytochrome *c* oxidase, and the redox state of cytochrome *c*. It is clear that the same sequence of heme reduction is found from the ellipticity changes as from the absorbance changes, namely heme *a*–heme *c*–heme *a*. The contributions, however, of the two heme *a* groups to the ellipticity changes differ markedly. The first heme *a* group reduced contributes only about 30 % to the total ellipticity changes, whereas the second heme *a* group contributes as much as 70 %. In Figs 1A and 1B, the upward inflection in the CD data at high reduction levels suggests that more than two components contribute to the CD changes. However, since no indication for the contribution of more than two components is present in the optical titration data, and since high optical absorbances lead to an increase in noise in CD spectra, this inflection is probably due to a less accurate determination of the ellipticity of the fully reduced enzyme.

The CD data of Figs. 1A and 1B can be replotted as a Nernst graph. When the two heme *a* groups of cytochrome *c* oxidase are not separately treated, a sigmoidal line is obtained (not shown). In Fig. 2, this line is resolved into two lines for the separate high- and low-potential heme *a* groups (open symbols), by taking into account the 30 and 70 % contributions, respectively, of these components to the CD changes. Furthermore, in the same figure the results of a direct potentiometric

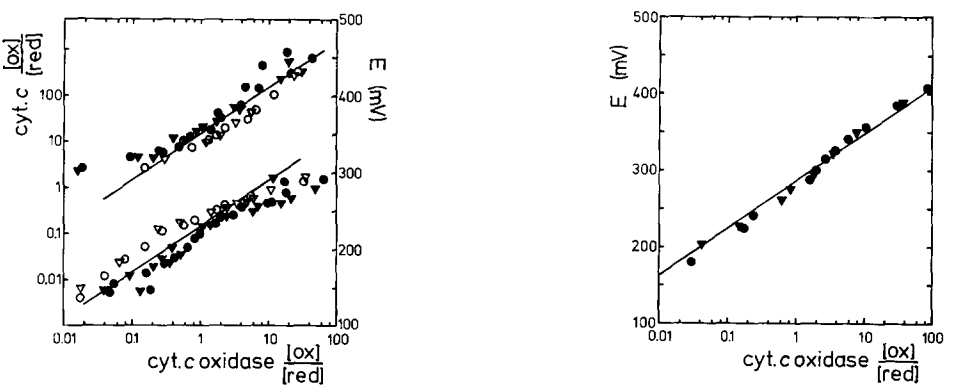

Fig. 2. Nernst type plot of a potentiometric CD titration of cytochrome *c* oxidase in the presence of cytochrome *c*. ▽ and ▼, 446 nm; ○ and ●, 608 nm. Open symbols, obtained from the comparative titration in Fig. 1. A potential scale was calculated from the redox state of cytochrome *c* measured at 550 nm, taking $E_{m(c)} = 280$ mV. Filled symbols, obtained in a direct potentiometric titration of 6.9 and 34 μM cytochrome *c* oxidase respectively, in the presence of 14 and 70 μM cytochrome *c* respectively, 30 μM phenazine methosulphate, 30 μM diaminodurene, 120 μM $K_3Fe(CN)_6$, 100 mM potassium phosphate, 0.5 % Tween-80, pH 7.2.

Fig. 3. Nernst-type plot of a potentiometric CD titration of cytochrome *c* oxidase in the absence of cytochrome *c*, ▼, 446 nm, 6.9 μM cytochrome *c* oxidase; ●, 608 nm, 37.5 μM cytochrome *c* oxidase. 30 μM phenazine methosulphate, 30 μM diaminodurene, 120 μM $K_3Fe(CN)_6$, 100 mM potassium phosphate, 0.5 % Tween-80, pH 7.2.

titration of a mixture of cytochrome *c* oxidase and cytochrome *c* have been plotted (filled symbols). It is clear that this type of experiment leads to similar results as does the comparative titration, although the values of the midpoint potentials of the high- and low-potential heme *a* groups obtained in the direct potentiometric titration are somewhat farther apart than those found in the comparative titration. This difference is probably due to the less accurate way of determining a potential scale from the degree of reduction of cytochrome *c*. The lines in Fig. 2 correspond to single-electron acceptors ($n = 1$) with midpoint potentials of 350 and 225 mV, in good agreement with values reported earlier [10, 13–15].

Fig. 3 shows the results of a direct potentiometric CD titration of cytochrome *c* oxidase, carried out in the absence of cytochrome *c*. A straight line is found, the slope of which corresponds to an *n* value of 0.95. This straight line suggests, in agreement with earlier observations [13–15], that in the absence of cytochrome *c* the heme *a* groups of cytochrome *c* oxidase are potentiometrically equivalent. From the line a midpoint potential of 285 mV is obtained.

Since the midpoint potentials of the heme *a* groups of cytochrome *c* oxidase in the presence of cytochrome *c* are far apart [8–15], it is possible to record the CD difference spectra (reduced minus oxidized) of the separate high- and low-potential heme *a* groups. These difference spectra in the Soret- and α-band region are shown in Figs 4A and 4B, respectively. It is clear from these figures that the peak ellipticities of the low-potential heme *a* group are greater than those of the high-potential heme *a* group, and that the peaks are at a higher wavelength (by about 5 nm). The difference CD spectrum fully reduced minus fully oxidized shows a negative extremum at 427.5 nm and positive ones at 446 and 607 nm, that of the high-potential heme *a* group at 425, 442 and 602.5 nm and that of the low-potential heme *a* group at 433, 447 and 608 nm.

In the absence of cytochrome *c* (Figs 5A and 5B) essentially the same peak positions and peak shapes are found as in its presence: 427, 445 and 607.5 nm for the complete CD difference spectrum, 425, 442 and 603 nm for the spectral difference

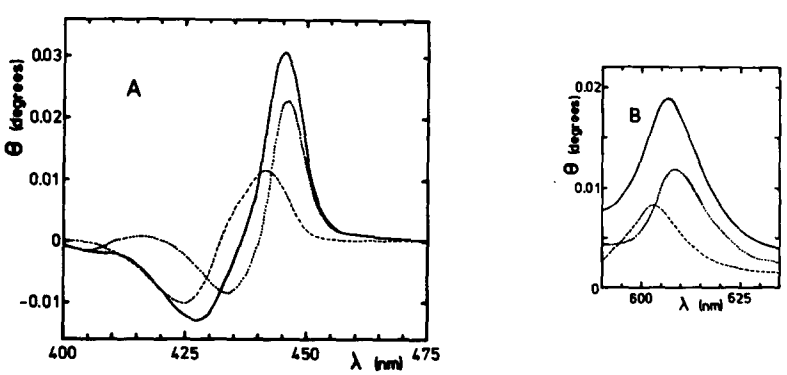

Fig. 4. CD difference spectra (reduced minus oxidized) of cytochrome *c* oxidase obtained in the direct potentiometric titration in the presence of cytochrome *c*, in Fig. 2. A. γ-band region. - - -, 294 minus 480 mV; · · ·, 98 minus 294 mV; ———, 98 minus 480 mV. B. α-band region. - - -, 288 minus 483 mV; · · ·, 117 minus 288 mV; ———, 117 minus 483 mV. The spectra have not been corrected for the small contributions of cytochrome *c* at these wavelength regions.

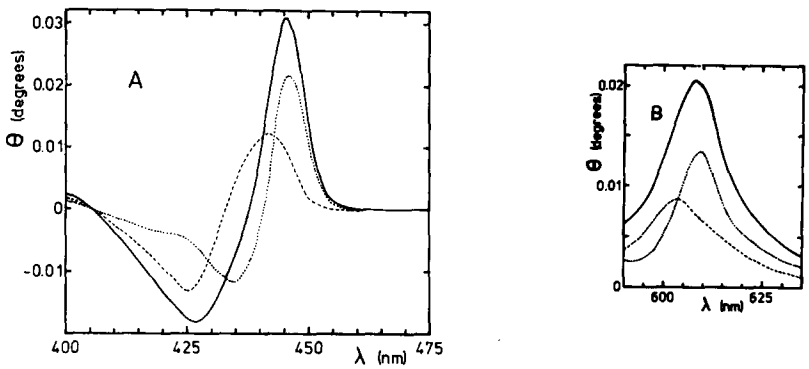

Fig. 5. CD difference spectra (reduced minus oxidized) of cytochrome *c* oxidase obtained in the direct potentiometric titration in the absence of cytochrome *c*, in Fig. 3. A. γ-band region. - - -, 288 minus 455 mV; · · ·, 126 minus 288 mV; ———, 126 minus 455 mV. B. α-band region. - - -, 286 minus 450 mV; · · ·, 138 minus 286 mV; ———, 138 minus 450 mV.

obtained at high redox potentials and 434, 447 and 608 nm for that obtained at low redox potentials. Differences are only found around 410 nm, but these are due to the CD contribution [29–31] of cytochrome *c* in that region of the spectrum in Fig. 4A.

DISCUSSION

The single line with $n = 0.95$ and $E_m = 285$ mV for the two heme *a* groups obtained from the Nernst plot of the CD titration of cytochrome *c* oxidase in the absence of cytochrome *c* in Fig. 3 supports our finding [13] that the two heme *a* groups of cytochrome *c* oxidase are potentiometrically equivalent. Since simulations of Nernst plots show that differences in midpoint potentials between the two heme *a* groups up to about 20 mV still do not give rise to essential deviation from a straight $n = 1$ line in a Nernst graph, small differences in midpoint potentials between the two heme *a* groups cannot be completely excluded.

The peak positions of CD difference spectra presented in this paper (442 and 602.5 nm for the first heme *a* group and 447 and 608 nm for the second) determined both in the presence and absence of cytochrome *c*, agree well with those described earlier by Wilson et al. [10], by Wikström [32] and by us [14] for the absorbance difference spectra (444 and 603.5 nm and 446 and 607 nm, respectively). The contributions, however, of the single heme *a* groups to the difference absorbance spectrum are little different [14], whereas the CD difference spectrum of the heme *a* group that is first reduced contributes about half as much as does the second heme *a* group.

To explain the large differences between the difference spectra (reduced minus oxidized) of the so-called cytochromes *a* and a_3, determined with the use of cyanide or CO, it has been proposed that the heme *a* group of cytochrome *a* is of the low-spin, hemochrome type, whereas the heme *a* group of cytochrome a_3 is of the high-spin, open type (cf. refs 5 and 6). In potentiometric titrations, however, much smaller spectral differences are found between the high- and low-potential heme *a* components of particulate cytochrome *c* oxidase [10] or of isolated cytochrome *c* oxidase plus cytochrome *c* [14]. Therefore it has been suggested [7, 10] that binding of a

ligand to one of the heme *a* groups of cytochrome *c* oxidase influences the spectral behaviour of the other heme *a* group.

The observation of different peak positions in the difference absorbance and CD spectra obtained at high and low redox potentials in potentiometric titrations in the absence of cytochrome *c*, where two independent heme *a* groups with the same midpoint potentials should titrate without preference, indicates that in cytochrome *c* oxidase no independent cytochrome *a* and cytochrome a_3 moieties are present. Although larger differences between the interacting heme *a* groups cannot be excluded, it is quite possible that the differences between the heme *a* components are comparable to the differences between the α- and β-chains of hemoglobin [33]. The possibility is even open that the two heme *a* groups are completely equivalent, and that cytochrome *c* oxidase is, at least with respect to the heme *a* groups, a completely symmetric dimer [14].

The (near) equivalence of both heme *a* groups is further worked out in the scheme presented in Table I. In this scheme midpoint potentials are not related to separate heme *a* groups, but to changes in redox state of the two heme *a* groups taken

TABLE I

SCHEMATIC REPRESENTATION OF THE TITRATION BEHAVIOUR OF THE HEME *a* GROUPS OF CYTOCHROME *c* OXIDASE

In the absence of cytochrome *c* or extrinsic ligands, $E_{m(1)} = E_{m(2)}$; in the presence of cytochrome *c* or extrinsic ligands, $E_{m(1)} > E_{m(2)}$. Azide and cyanide bind to an oxidized heme *a* group, and restrict the primary redox shuttle to the one indicated by $E_{m(1)}$; CO or NO bind to a reduced heme *a* group and restrict the primary redox shuttle to the one indicated by $E_{m(2)}$.

Enzyme species	Midpoint potential	(red−ox) difference absorbance peaks (nm)	(red−ox) difference CD peaks (nm)	EPR signal
ox · ox				$g = 3$
⇅	$E_{m(1)}$	444; 603.5	442; 602.5	
ox · red = red · ox				$g = 6$
⇅	$E_{m(2)}$	446; 607	447; 608	
red · red				

as a whole. Reduction of either of the two heme *a* groups leads to the same half-reduced molecule. Difference spectra in this scheme then represent "one heme reduced, one heme oxidized" minus "both hemes oxidized" at high redox potentials and "both hemes reduced" minus "one heme reduced, one heme oxidized" at low redox potentials. This means that the difference spectrum of a heme *a* group depends on the redox state of the other heme *a* group, as a result of conformational changes occurring at one of the heme *a* groups on reduction of the other heme *a* group. This point of view is strengthened by our observation that the component reduced at high redox potentials contributes only about a third to the CD difference spectrum (reduced minus oxidized), but about a half to the absorbance difference spectrum. This is an indication of the occurrence of conformational changes in half reduced cytochrome *c* oxidase, that affect the symmetry of one or both heme *a* groups. These

changes also result in a shift of the maxima in the difference absorbance spectrum for reduction of the second heme *a* group from 444 and 603.5 nm to 446 and 607 nm, and in a concomitant shift of the difference CD spectrum from 442 and 602.5 to 447 and 608 nm.

The EPR behaviour [9, 11, 16, 17, 34] of cytochrome *c* oxidase can also be explained by the occurrence of conformational transitions in half-reduced cytochrome *c* oxidase. In the fully oxidized enzyme, both heme *a* groups give the same low-spin $g = 3$ EPR signal, which presumably because of the presence of metal–metal antiferromagnetic interactions accounts only for about 30 % of the heme *a* present [16, 17]. On reduction of either of the heme *a* groups, a low-spin to high-spin transition occurs at the other heme *a* group, which is thereupon reduced at lower redox potentials. It can be calculated that this results in the bell-shaped formation and vanishing of an intermediate half-reduced species. Such a bell-shaped behaviour of the $g = 6$ signal has indeed been found by Van Gelder and Beinert [16, 17] and has been confirmed by Wilson and coworkers [9, 11, 12]. The apparent midpoint potentials of the formation and vanishing of the $g = 6$ signal and the maximal intensity of this signal will be dependent on $E_{m(1)}$ and $E_{m(2)}$.

Preliminary experiments in our laboratory have shown that the separation of the midpoint potentials of the high- and low-potential heme *a* components ($E_{m(1)}$ and $E_{m(2)}$ in Table I) by the addition of cytochrome *c* leads to an about 50 % increase of the maximal intensity of the intermediate $g = 6$ signal, and to a corresponding change in the apparent midpoint potentials for the disappearance of the $g = 3$ signal, and for the formation and decrease of the $g = 6$ signal.

Further support for the scheme presented in Table I can be found in the effect of extrinsic ligands of cytochrome *c* oxidase on the midpoint potentials of cytochrome *c* oxidase. Wilson and coworkers [8, 11] recently showed that binding of CO or azide to one of the heme *a* groups of cytochrome *c* oxidase in mitochondria affects the midpoint potentials and spectral contributions of both heme *a* groups. We have recently confirmed this observation on binding of azide to isolated cytochrome *c* oxidase. Both in the absence and presence of cytochrome *c*, a high-potential heme *a* group ($E_m = 360$ mV) then contributes 80 % to the spectral difference at 605 nm, whereas a low-potential heme *a* group ($E_m = 220$ mV or below, depending on the azide concentration) accounts for the remaining 20 %.

In the scheme presented in Table I ligands like azide and cyanide which bind to an oxidized heme *a* group and stabilize its oxidized form restrict the primary redox shuttle to the upper one (indicated by $E_{m(1)}$, 280 mV or higher in the case of cyanide [35] and 360 mV in case of azide [10]). The other redox shuttle will then be directly dependent on the cyanide or azide concentration. In the EPR spectrum, the presence of azide or cyanide at the heme *a* group that stays oxidized prevents its low-spin to high-spin transition: a $g = 2.9$ low-spin heme *a*–azide signal [10, 17] or a $g = 3.6$ low-spin heme *a*–cyanide signal [36] is then found at intermediate stages of reduction.

Ligands like CO and NO which bind to a reduced heme *a* group and stabilize its reduced form, restrict the primary redox shuttle to the lower one in Table I ($E_{m(2)} = 250$ mV in the case [8, 11] of CO; for NO no experimental data are available). Because of the high affinity of CO or NO for reduced heme *a*, the other redox shuttle ($E_{m(1)}$) then will be raised to well over 400 mV. In the EPR spectrum at intermediate stages of reduction only a $g = 3$ signal is found in the presence of NO

or hydroxylamine [37] or CO in the dark (refs 11, 12 and unpublished), but no $g = 6$ signal.

The fact that these inhibitors of electron transport by cytochrome *c* oxidase all prevent the low-spin to high-spin transition of the oxidized heme *a* group at intermediate stages of reduction may have a bearing on the mechanism of action of and energy conservation in cytochrome *c* oxidase [15, 38].

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