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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XVI. CIRCULAR DICHROIC STUDY OF CYTOCHROME *c* OXIDASE AND ITS LIGAND COMPLEXES

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SUMMARY

1. CD spectra of cytochrome *c* oxidase have been determined both in the absence and presence of the extrinsic ligands CO, NO, cyanide and azide.

2. CO and NO affect the CD spectrum of cytochrome *c* oxidase in a similar way.

3. Cyanide and azide also affect the CD spectrum of cytochrome *c* oxidase in a similar way, but distinctly different from CO and NO.

4. From the CD spectra of the oxidized and reduced enzyme, in the presence and absence of extrinsic ligands, CD difference spectra (reduced minus oxidized) are calculated for the so-called cytochrome *a* and cytochrome a_3 moieties of the enzyme.

5. These spectra are largely dependent on the extrinsic ligand used. It is therefore concluded that these spectra do not represent independent cytochrome *a* and cytochrome a_3 difference spectra, but that heme–heme interactions occur within the cytochrome *c* oxidase molecule, in such a way that binding of a ligand to one of the heme *a* groups of cytochrome *c* oxidase affects the spectral properties of the other heme *a* group.

6. As a consequence, ligand-binding studies cannot give information as to the pre-existence of separate cytochrome *a* and cytochrome a_3 moieties in the absence of extrinsic ligands.

INTRODUCTION

It is almost 35 years ago now, that Keilin and Hartree [1] advanced their concept of cytochrome *c* oxidase being a complex consisting of "two distinct but closely allied and intimately connected components", cytochrome *a* and cytochrome a_3. In this concept cytochromes *a* and a_3 have the same heme *a* nucleus, but different reactivities towards respiratory inhibitors. Cytochrome a_3 is the component which in the reduced form directly reacts with molecular oxygen, this reaction being inhibited by CO. In the oxidized state cytochrome a_3 reacts with cyanide, azide, sul-

phide, fluoride or hydroxylamine. Cytochrome *a* is the component which is inert to these inhibitors.

Since Keilin and Hartree's original report, hydrazine [2–5] and NO [6–9] have been added to the list of ligands of cytochrome a_3*. For many of the ligands of cytochrome a_3 a stoichiometry of 1 molecule of inhibitor per molecule of cytochrome *c* oxidase [9–15], i.e. per two hemes *a*, has indeed been found. Despite this functional difference between the two cytochrome components of cytochrome *c* oxidase, all attempts to isolate separate cytochrome *a* and cytochrome a_3 moieties which on recombination again give rise to an active cytochrome *c* oxidase have been unsuccessful. The question has therefore been raised whether these differences in properties between cytochrome *a* and cytochrome a_3 are due to different environments of the two heme *a* groups, or only induced [16] as a result of negative cooperativity [17, 18]. In the latter case, binding of a ligand to one of the heme groups of cytochrome *c* oxidase makes the other heme group less or not reactive towards these inhibitors.

The distinction between cytochrome *a* and cytochrome a_3 has been largely based on spectroscopic work in the presence of inhibitors. Yonetani [19], Horie and Morrison [20], and Vanneste and co-workers [12, 21] determined the reduced minus oxidized difference absorbance spectra, using cyanide as a means of separating the reduction of cytochrome a_3 from that of cytochrome *a*. In our laboratory [22, 23] it has been shown that addition of cyanide or azide greatly slows down or even prevents the reduction of cytochrome a_3 by the NADH/phenazine methosulphate system. With this type of experiments the contributions of cytochromes *a* and a_3 to the spectral absorbance differences at 445 or 605 nm on reduction have been determined. It was also found that the reduction of the intrinsic copper of cytochrome *c* oxidase is inhibited by the reaction of the enzyme with these inhibitors [23].

In recent years the validity of this type of approach for obtaining the spectral contributions of cytochrome *a* and cytochrome a_3 has been criticized [17, 24–27]. Potentiometric titrations [17, 25–27], EPR spectroscopy [27–29] and CD studies [30–34] have accumulated evidence for metal–metal interactions, and for the occurrence of conformational transitions in the protein on valence-state changes of the metals. Attempts in our laboratory to calculate the reduced minus oxidized difference absorbance spectra of cytochrome *a* and of cytochrome a_3 always showed small but reproducible differences between the results obtained with cyanide or with azide, and between those found with CO or with NO, whereas larger differences were found between the cyanide or azide experiments and the CO or NO experiments. In our opinion such differences might reflect changes in protein conformation, induced by the addition of extrinsic heme ligands.

Since the optical activity of the heme *a* groups of cytochrome *c* oxidase is mainly induced by the protein [30, 35] (cf. ref. 36) changes in optical activity are an indication of transitions in the protein conformation around the heme *a* groups. The present CD study has therefore been undertaken in order to see whether it is possible to obtain the reduced minus oxidized difference spectra of the separate cy-

* For practical reasons we shall in this paper maintain the cytochrome *a* and cytochrome a_3 terminology. Cytochrome a_3 then is that part of the protein in which the heme group is less readily reduced (cyanide and azide) or oxidized (CO and NO) in the presence of ligands.

tochrome *a* and cytochrome a_3 components of the enzyme by algebraic combinations of the CD spectra recorded in the presence and absence of inhibitors, or that the contributions of the separate components to the CD spectra are affected by conformational changes. In this paper we give the complete set of the directly recorded CD spectra, and of the calculated difference CD spectra, of cytochrome *c* oxidase in the α- and Soret region, determined in the presence of CO, NO, cyanide and azide. Other respiratory inhibitors, such as hydroxylamine, sulphide, fluoride and hydrazine have been purposely omitted from this study, because of the large secondary effects of these inhibitors: they not only act as a ligand of cytochrome *c* oxidase but, as was shown in our laboratory, also either bring about a considerable amount of reduction (hydroxylamine [9] or sulphide) and oxygenation (hydrazine [4]) or dissociate readily from the enzyme–inhibitor complex (fluoride [18, 23, 37]).

EXPERIMENTAL

Enzyme

Bovine cytochrome *c* oxidase was isolated according to the methods of Fowler et al. [38], and MacLennan and Tzagoloff [39], as modified by Van Buuren [40]. The starting material was a crude heart-muscle preparation, made from fresh hearts. Crude cytochrome *c* oxidase (green residue) fractions were pooled in liquid N_2. The isolation procedure from green residue to purified cytochrome *c* oxidase was usually completed in 12 h. Concentrations, based on a catalytically active unit containing two hemes, are calculated with an absorbance coefficient [41] (reduced minus oxidized) at 605 nm of 24.0 $mM^{-1} \cdot cm^{-1}$. Protein concentrations were determined by the biuret method of Gornall et al. [42], as modified by Yonetani [43]. The preparations used in this study contained at least 5 μmoles cytochrome *c* oxidase (i.e. 10 μmoles heme *a*) per gram of protein.

Chemicals

All experiments were carried out in 100 mM potassium phosphate (pH 7.2) and 0.5% Tween 80. CO gas was either prepared by dehydration of formic acid by concentrated H_2SO_4, or purchased from Matheson; NO gas was from Matheson or Baker. Other chemicals were Analar Grade, mainly obtained from British Drug Houses. Cyanide and azide solutions were freshly prepared in Tween–phosphate buffer and adjusted to pH 7.2 with H_3PO_4 directly before use.

Visible absorbance and CD spectra

Absorbance spectra were obtained with a Cary-14 or Cary-17 registrating spectrophotometer; circular dichroic spectra were recorded with a Cary-60 spectropolarimeter, equipped with a Cary-6002 CD attachment. The CD instrument was set to a spectral bandwidth of 1.5 nm throughout the whole wavelength range. Linearity of the CD signal was checked with increasing cytochrome *c* oxidase concentrations; additivity by comparing the CD spectrum of a mixture of cytochrome *c* oxidase and cytochrome *c* with the calculated sum of the separate spectra of these two proteins. CD spectra presented here are the average of at least five experiments with three different preparations. Directly before and after recording of the CD spectra absorbance spectra were taken in order to check that no absorbance changes had taken place during recording of the CD spectra. These absorbance spectra of the

oxidized and reduced forms and of the liganded complexes were similar to those reported in literature [3, 6, 7, 15, 19, 20, 36, 37, 44–50], and have therefore been omitted for the sake of brevity. CD data are expressed as molar ellipticities, based on a catalytic unit containing two hemes.

NO and CO spectra

Incubations with NO were carried out in a Thunberg-type cuvette, equipped with a hollow stopper with two side-arms. The cuvette was several times evacuated and flushed with pure N_2 which had been freed from traces of residual O_2 by passing it through alkaline pyrogallol; this procedure was finished by evacuation of the Thunberg cuvette. After recording of the absorbance and CD spectra of oxidized cytochrome *c* oxidase, a 2–3-fold excess of solid $Na_2S_2O_4$ was added from one of the side-bulbs, and after a reduction time of 30 min the spectra of reduced cytochrome *c* oxidase were recorded. Then NO was added anaerobically, and the spectra of the reduced cytochrome *c* oxidase–NO complex were recorded after an incubation time of 5–20 min. Any brown colour in the gas phase or turbidity of the solution was taken as an indication of incomplete anaerobiosis; such samples were discarded. The last step in the experimental procedure was the anaerobic reoxidation by addition of a slight excess of solid $K_3Fe(CN)_6$ from the other side-arm. The spectra of the re-oxidized cytochrome *c* oxidase–NO complex were recorded after an incubation time of 2–3 min.

For incubations with CO the same procedure was applied as with NO.

Cyanide and azide spectra

The oxidized cytochrome *c* oxidase was incubated at room temperature for at least 2 h with either cyanide or azide. After recording of the absorbance and CD spectra, a slight excess of solid $Na_2S_2O_4$ was added. Reduced spectra were then recorded after a reduction time of 3–5 min. Cyanide and azide concentrations of 10–100 mM and 50–100 mM, respectively, were chosen in order to prevent reduction of cytochrome a_3 during the time needed to record the spectra of the reduced cytochrome *c* oxidase–inhibitor complex.

Algebraic procedure

From the absolute CD spectra obtained in the way described above, difference CD spectra for the separate cytochrome *a* and cytochrome a_3 moieties of cytochrome *c* oxidase were calculated, according to the method of Yonetani [19], Horie and

TABLE I

CALCULATION OF (CD) DIFFERENCE SPECTRA (REDUCED MINUS OXIDIZED) OF CYTOCHROME *a* AND CYTOCHROME a_3 USING A LIGAND TO PREVENT REDUCTION (CYANIDE OR AZIDE) OR REOXIDATION (CO, NO) OF CYTOCHROME a_3

Ligand present	Spectra subtracted	Calculated difference spectra
None	$a^{2+}a_3{}^{2+} - a^{3+}a_3{}^{3+}$	$a^{2+}a_3{}^{2+} - a^{3+}a_3{}^{3+}$
Cyanide CO	$a^{2+}a_3{}^{3+} \cdot CN - a^{3+}a_3{}^{3+} \cdot CN$ $a^{2+}a_3{}^{2+} \cdot CO - a^{3+}a_3{}^{2+} \cdot CO$	$a^{2+} \quad - a^{3+}$ $a_3{}^{2+} - \quad a_3{}^{3+}$

Morrison [20], and Vanneste and co-workers [12, 21]. An outline of this procedure is given in Table I.

CO or NO, bound to reduced cytochrome *c* oxidase, stabilize the reduced form and inhibit the reoxidation of the a_3^{2+} part of cytochrome *c* oxidase by small amounts of ferricyanide. Cyanide or azide bound to oxidized cytochrome *c* oxidase stabilize cytochrome a_3 in its oxidized form, and in this way inhibit its reduction by a slight excess of dithionite. Algebraic combination of these spectra in the way described in Table I then results in the separate cytochrome *a* and cytochrome a_3 reduced minus oxidized difference spectra.

RESULTS

Absolute spectra

Fig. 1 gives the γ-band CD spectra of cytochrome *c* oxidase in the presence and absence of inhibitors. The corresponding α-band spectra are shown in Fig. 2.

In the absence of inhibitors (Figs 1e and 2e) oxidized cytochrome *c* oxidase shows positive ellipticities between 407 and 525 nm, and negative values below 407 and above 525 nm. A positive ellipticity band is present at 427–428 nm and two weak but definite minima of about equal intensities at 570 and 620 nm. In the reduced form, weak negative ellipticities are found below 433 nm. Two positive extrema are present at 446 and 607–608 nm, and two minor positive bands at 525 and 570 nm.

On anaerobic incubation of reduced cytochrome *c* oxidase with CO (Figs 1a and 2a) the CD α- and γ-bands of the reduced enzyme move to shorter wavelengths. The α-band increases about 50% in peak height, and a shoulder appears at the short-wavelength side of this peak. The Soret band broadens and decreases about 50% in

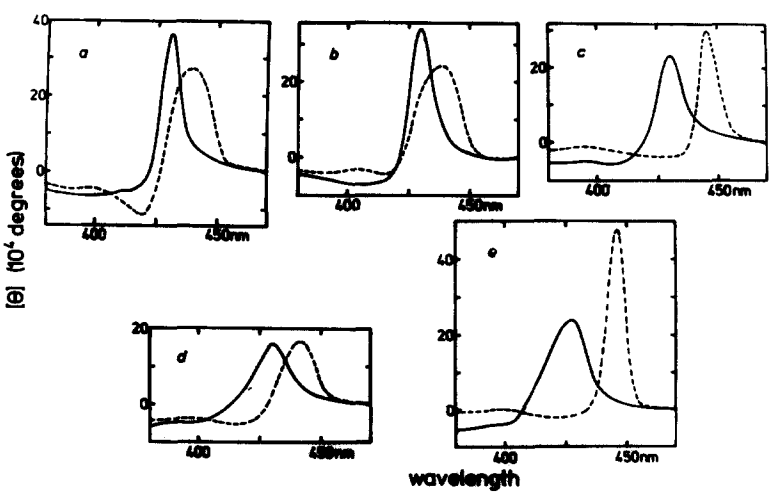

Fig. 1. γ-band CD spectra of cytochrome *c* oxidase in the presence of ligands. 8–12 μM cytochrome *c* oxidase, 100 mM potassium phosphate +0.5% Tween 80, pH 7.2, 25 °C. —, oxidized; - - -, reduced. a, in the presence of CO (1 atm.); b, NO (1 atm.); c, 10–100 mM cyanide; d, 50–100 mM azide; e, no additions. In the presence of CO or NO the oxidized spectra were measured after reoxidation of the reduced enzyme with $K_3Fe(CN)_6$, as described in Experimental.

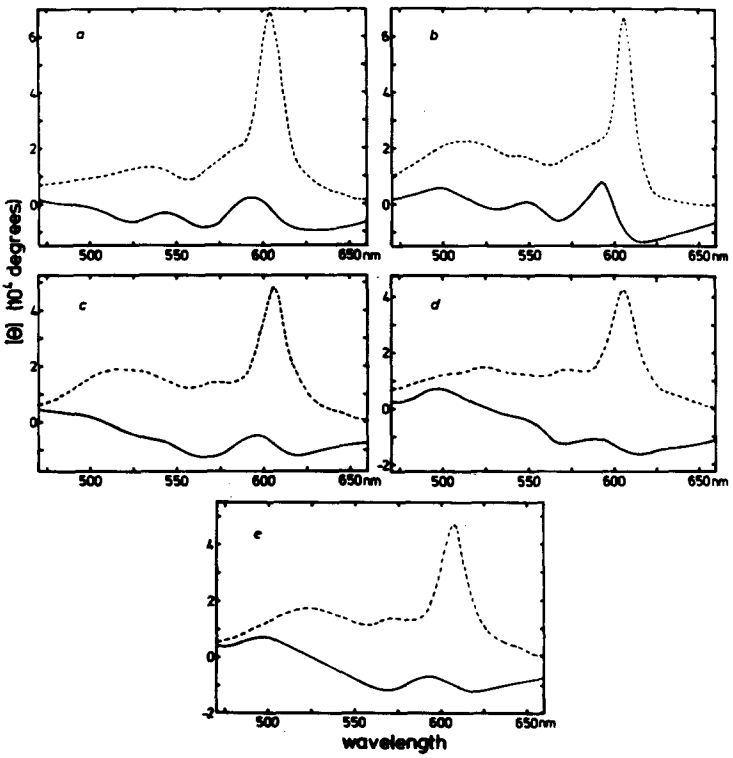

Fig. 2. α-band CD spectra of cytochrome *c* oxidase in the presence of ligands. 40–60 μM cytochrome *c* oxidase, 100 mM potassium phosphate +0.5% Tween 80, pH 7.2, 25 °C. —, oxidized; - - -, reduced. a, in the presence of CO (1 atm.); b, NO (1 atm.); c, 10–100 mM cyanide; d, 50–100 mM azide; e, no additions. The spectra of the oxidized enzyme–CO and –NO complexes were determined as described in Experimental.

peak height. Similar phenomena can be seen on anaerobic incubation of reduced oxidase with NO (Figs 1b and 2b).

Reoxidation of reduced cytochrome *c* oxidase by O_2, or by $K_3Fe(CN)_6$ in the presence of O_2, leads to the formation of 'oxygenated' cytochrome *c* oxidase [51, 52], which is characterized by about the same α-band CD spectrum as oxidized cytochrome *c* oxidase, and by a 5% increased γ-band CD peak at 430 nm. Anaerobic reoxidation, however, of reduced cytochrome *c* oxidase with $K_3Fe(CN)_6$ did not lead to the formation of the oxygenated compound, but restored the original CD spectrum of oxidized cytochrome *c* oxidase (not shown). Upon anaerobic reoxidation with $K_3Fe(CN)_6$ in the presence of CO or NO a Soret band is found at about 430 nm, which is sharper and has a 50% higher ellipticity than the band of the oxidized enzyme in the absence of these ligands. In the α-band region, in the presence of CO the two negative bands are still present at 567–568 and 630 nm, but at about 590 nm the ellipticity now reaches positive values. In the presence of NO this positive peak is even more pronounced. Moreover, both in the presence of CO and NO at 525 nm a third negative peak has appeared.

TABLE II

PEAK POSITIONS AND MOLAR ELLIPTICITIES OF CYTOCHROME *c* OXIDASE IN THE ABSENCE AND PRESENCE OF VARIOUS LIGANDS

Conditions	This paper		Literature		
	λ (nm)	$[\theta] \times 10^{-3}$	λ (nm)	$[\theta] \times 10^{-3}$	Ref.
Oxidized	427.5	240	428.5	220	30
			426	240	32
			428	115	53
			427	250	54
			425	280	56
			426		57
	570	−12	570	−8	55
	620	−12			
Reduced	446	480	445.5	460	30
			445	380–450	32
			446	165	53
			446	480	54
			445	560	56
			445		57
	607.5	47	608–611	44	55
CO/reoxidized	430.5	365	429–432	265–290	32
			430	470	56
	567	−9			
	630	−10			
Reduced/CO	439.5	270	440	240	30
			435–439	245–280	32
			438	280	56
	604	68	606	52	55
NO/reoxidized	430	340			
	567	−6			
	615	−14			
Reduced/NO	439	245			
	606	67			
Oxidized/cyanide	430	230	431	175	30
			428–430	235	32
			427	150	54
	567	−13	570	−8	55
	620	−12	620	−4	55
Cyanide/reduced	445.5	300	445	180	30
			443.5	205	32
			446	225	54
	606	48	608	40	55
Oxidized/azide	430	160			
	570	−12			
	615	−16			
Azide/reduced	441.5	165			
	605	43			

On incubation of oxidized cytochrome *c* oxidase with cyanide the spectra in Figs 1c and 2c were obtained. The Soret band narrows on addition of cyanide and moves to 430 nm, whereas in the α-band region only slight changes are found. The changes found were independent of variation of the incubation time (from 2 to 10 h) or cyanide concentration (from 10 to 100 mM).

On reduction of the oxidase–cyanide complex, a Soret band is found at 445–446 nm with a peak height about 40% lower than in the absence of cyanide. In the α-band region the peak is of about equal intensity, but has moved from 607–608 to 606 nm.

In the presence of azide (Figs 1d and 2d) oxidized cytochrome *c* oxidase shows a Soret band of 33% lower intensity with a peak at 430 nm. The reduced oxidase–azide compound has peaks at 441–442 and 605 nm, with about 66 and 10% lower height, respectively, than those of the untreated enzyme.

The peak positions and peak ellipticities of cytochrome *c* oxidase in the presence of various ligands are summarized in Table II, where these values are compared with the few values available in the literature [30, 32, 53–57]. In general there is good agreement between the values listed. It is interesting to note that the differences in peak position of the Soret CD band of the oxidized enzyme parallel these differences in the absorbance spectrum: an absorbance peak at 418 nm corresponds to a CD band at 426 nm; absorbance peaks at 423–424 nm correspond to a CD band at 428 nm.

Cytochrome a and cytochrome a_3 difference spectra

From the directly recorded CD spectra shown in Figs 1 and 2, difference

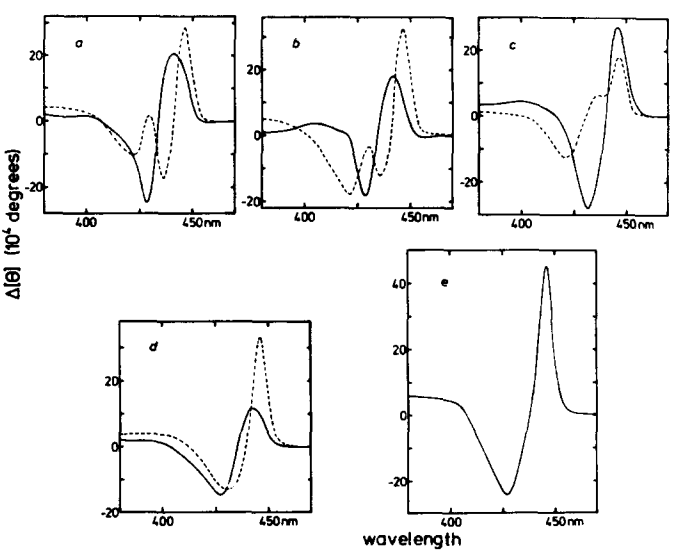

Fig. 3. γ-band CD difference spectra (reduced minus oxidized) of the cytochrome *a* (—) and cytochrome a_3 (- - -) moieties of cytochrome *c* oxidase, calculated from Fig. 1. a, determined in the presence of CO; b, NO; c, cyanide; d, azide; e, gives the summated difference spectrum [cytochrome *c* oxidase (reduced minus oxidized)] = [cytochrome *a* (reduced minus oxidized)]+[cytochrome a_3 (reduced minus oxidized)].

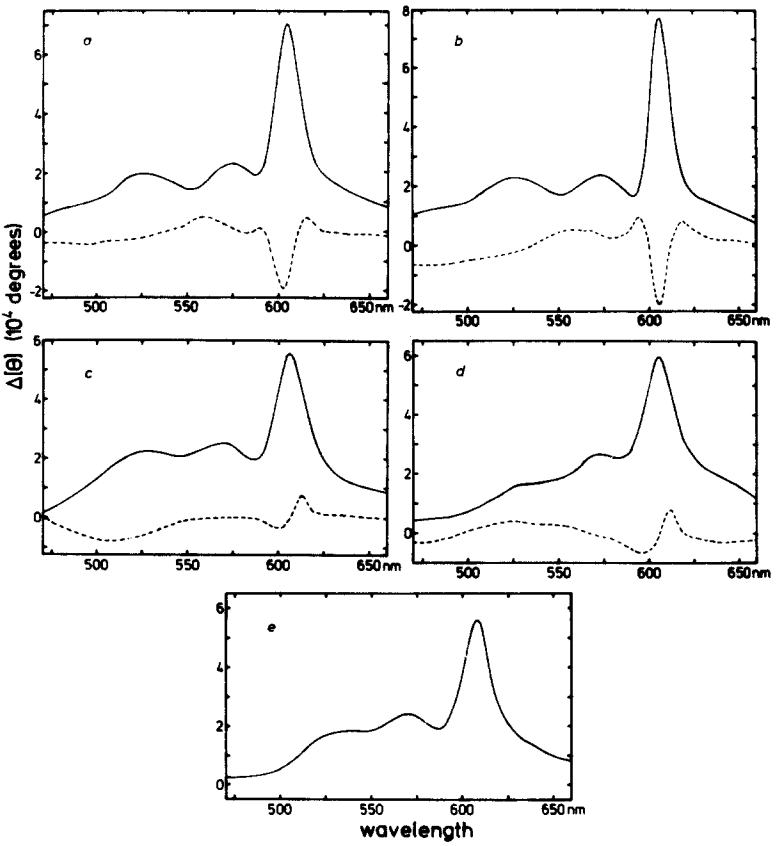

Fig. 4. α-band CD difference spectra (reduced minus oxidized) of the cytochrome *a* (—) and cytochrome a_3 (- - -) moieties of cytochrome *c* oxidase, calculated from Fig. 2. a, determined in the presence of CO; b, NO; C, cyanide; d, azide; e, gives the summated difference spectrum [cytochrome *c* oxidase (reduced minus oxidized)] = [cytochrome *a* (reduced minus oxidized)] + [cytochrome a_3 (reduced minus oxidized)].

spectra of the cytochrome *a* and cytochrome a_3 moieties were calculated in the way described above. These difference spectra are given in Figs 3 (γ-band) and 4 (α-band).

From these figures it is clear that CO (Figs 3a and 4a) and NO (Figs 3b and 4b) give rise to similar difference spectra for the cytochrome *a* and cytochrome a_3 parts of the enzyme, respectively. In the Soret region in both these cases a double minimum is found for the cytochrome a_3 part. In the α-region a strong positive peak is found for the cytochrome *a* part, and a negative peak for the cytochrome a_3 part.

The difference spectra of cytochrome *a* and of cytochrome a_3, as determined in the presence of cyanide (Figs 3c and 4c) or azide (Figs 3d and 4d) are grossly similar. The most striking finding is the very weak CD signal attributable to cytochrome a_3 in the α-region. A more careful examination of the cytochrome *a* and a_3 difference spectra, however, shows that, despite the apparent similarities, the cytochrome *a* difference spectra all differ from one another in peak positions and peak intensities, and as a consequence similar differences are also met with in the cytochrome a_3

TABLE III

PEAK POSITIONS AND MOLAR ELLIPTICITIES OF THE CALCULATED CD DIFFERENCE SPECTRA (REDUCED MINUS OXIDIZED) OF THE CYTOCHROME *a* AND CYTOCHROME a_3 MOIETIES OF CYTOCHROME *c* OXIDASE

Ligand used	γ-band		α-band			
	Cytochrome *a*	Cytochrome a_3	Cytochrome *a*		Cytochrome a_3	
			λ (nm)	$[\Phi]\times10^{-3}$	λ (nm)	$[\Phi]\times10^{-3}$
CO	428, −250 441, +205	422, −105 436, −175 446, +285	604.5,	+70	589.5, + 1 602.5, −19 615, + 5	
NO	429, −185 442, +180	421, −180 436, −125 446.5, +325	606,	+77	594, + 9 605.5, −20 618.5, + 7	
Cyanide	432, −280 446, +270	420.5, −125 436, + 60 447, +175	606,	+56	600, − 3 612.5, + 8	
Azide	427, −145 443, +120	430.5, −130 446, +330	605.5,	+60	596, − 7 611, + 8	

difference spectra. This is made clear in Table III. Depending on the ligand used for determination of these spectra, the cytochrome *a* negative Soret peak varies from 427 to 432 nm, and its positive Soret peak from 441 to 446 nm. Similar variations are found for other peaks. The peak heights too are dependent on the ligand used.

DISCUSSION

Our finding that the same algebraic procedure, applied to CD spectra obtained in the presence of four different heme group ligands, yields four different reduced minus oxidized difference CD spectra for cytochrome *a*, and as a consequence also four different reduced minus oxidized difference CD spectra for cytochrome a_3, can in our opinion only satisfactorily be explained by the occurrence of heme–heme interactions.

Perutz [58] has shown for hemoglobin that on deoxygenation of a heme group the low-spin to high-spin transition of this heme group makes its iron atom move out of the plane of the porphyrin ring. This movement of the iron atom triggers in the reacting subunit a conformational change, which is then transmitted via inter-subunit interactions to the other subunits. Similar interactions [59] may take place in the cytochrome *c* oxidase molecule. Binding of CO or azide to mitochondrial (Wilson et al. [25]) or isolated (unpublished) cytochrome *c* oxidase affects the mid-point potential and absorbance coefficient not only of the CO- or azide-bound heme *a*, but also of the other heme *a* group. EPR spectroscopy has shown the appearance of a high-spin heme *a* species upon partial reduction of cytochrome *c* oxidase, as monitored by its $g = 6$ signal [27–29]. Quantitation of the EPR spectra of the oxidized enzyme [29] showed that only about 40% of the intrinsic copper and about 30% of the hemes contribute to the EPR spectrum. These low percentages have

been taken as an indication of either heme–heme and copper–copper, or heme–copper anti-ferromagnetic coupling between part of the metals.

In some of their recent papers [31, 33, 34] King and Yong suggested that the difference spectrum $a_3^{2+} \cdot CO - a_3^{3+} \cdot CN$ is dependent on the redox state of the intrinsic copper of cytochrome *c* oxidase. Although such copper–heme interactions may well be present, and may account in part for the differences found between the cytochrome *a* or cytochrome a_3 difference CD spectra obtained in the presence of cyanide or azide on one hand, and those obtained in the presence of CO or NO on the other hand, they cannot explain the differences between the cytochrome *a* and cytochrome a_3 difference spectra found in the presence of CO and those found in the presence of NO, since in these two cases the copper atoms probably have the same redox state. An analogous argument holds for the cytochrome *a* and cytochrome a_3 difference spectra obtained using cyanide, and those obtained using azide.

According to Urry [60] and Urry and Van Gelder [30] juxtaposition of two heme groups with overlapping absorbance bands should give rise to CD bands of opposite sign, with in the ideal case about equal magnitude. The negative CD extremum at 422 nm (caused by a negative Gaussian CD band at 428 nm of the heme *a*–CO compound [30]), in combination with a positive extremum at 440 nm, might indicate juxtaposition of the hemes in the reduced cytochrome *c* oxidase–CO complex. The absence, however, of a well-defined negative extremum at about 420 nm in the CD spectrum of the reduced cytochrome *c* oxidase–NO complex makes it unlikely that such interactions occur in the case of NO binding. In the presence of cyanide or azide no indications in the CD spectra are found for the occurrence of direct heme–heme interactions.

The occurrence of direct or indirect heme–heme interactions implies that the properties of the two heme *a* groups of cytochrome *c* oxidase are mutually dependent. On binding of a ligand to one of the heme *a* groups, symmetry changes are brought about at the other heme *a* group. These symmetry changes are reflected in the CD spectra described in this paper. These changes are also reflected in the dependence of the far ultraviolet CD band around 220 nm on the presence of ligands (refs 32, 53, 61 and Tiesjema, unpublished). As a consequence, ligand-binding studies cannot decide whether cytochrome *a* and cytochrome a_3 are "ab initio" different [62] parts of the cytochrome *c* oxidase molecule, or that the differences between cytochromes *a* and a_3 are only induced by ligands. A way out of the problem of ligand binding may be the potentiometric titration, followed by CD.

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