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Resolution of the circular dichroism spectra of the mitochondrial cytochrome bc_1 complex

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The circular dichroic spectrum of the mitochondrial cytochrome bc_1 complex isolated from bovine heart has been resolved into the contributions from the prosthetic groups: cytochrome c_1, the 'Rieske' iron-sulphur centre and the two *b* cytochromes. It is apparent that firstly, the circular dichroism (CD) properties of cytochrome c_1 within the bc_1 complex differ from those found in the isolated cytochrome c_1 and secondly, both the oxidized and reduced b cytochromes exhibit an intense spectrum of bilobic shape, with the wavelengths of the cross-over points closely corresponding to those of the maxima in the optical absorbance spectra. These latter CD features are discussed in relation to the proposed structure of cytochrome *b*.

Introduction

The spectroscopic properties of multiprotein (redox) enzymes are often complicated by overlapping contributions from the different constituent chromophores [1]. This is also evident in the CD spectra of the mitochondrial cytochrome bc_1 complex, where the spectral bands of the haemoproteins in the Soret region result from the combined dichroic absorption of all four redox groups in the enzyme [2]: two *b*-cytochromes, cytochrome c_1 and the 'Rieske' iron-sulphur centre [3,4]. Although the CD features of isolated cytochrome c_1 [5] and the 'Rieske' iron-sulphur protein [4,6] have been reported, the dichroic properties of the native *b*-cytochromes were not known, thus preventing any attempt to resolve the CD spectra of cytochrome bc_1 in order to reveal the in situ state of these components.

The investigation reports the complete resolution of the CD spectra of the chromophores of the bc_1 complex; we also report how the problem of obtaining a CD spectrum of the *b*-cytochromes may be surmounted by using guanidine-hydrochloride to cleave the two *b*-cytochromes from the cytochrome bc_1 complex, hence obtaining the in situ CD spectrum of these cytochromes by subtraction.

Materials and Methods

Cytochrome bc_1 complex was isolated from bovine heart mitochondria [7] essentially according to the procedure of Rieske [8] but with the modifications described previously [9]. The con-

Abbreviation: CD, circular dichroism.

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centration of cytochrome c_1 was determined at 553–540 nm after ascorbate reduction using an extinction coefficient of 17.5 $mM^{-} cm^{-1}$ [5], and that of cytochrome *b* after dithionite reduction at 562–575 nm using an extinction coefficient of 25 $mM^{-1} cm^{-1}$ [9].

The 'Rieske' iron-sulphur protein and cytochrome c_1 were isolated by the same procedure as that described by Shimomura et al. [10,11]. Isolated cytochrome c_1, obtained in the reduced state, exhibited a A_{417}/A_{280} ratio of 2.1—2.2, and was found to be essentially pure by SDS-polyacrylamide gel electrophoresis performed as previously described [4,9]. Cleavage of cytochrome *b* from oxidized cytochrome bc_1 complex was performed as described by Rieske [12], with 25 μM enzyme and 1.16 M guanidine-HCl. The turbid mixture was incubated at room temperature for 10 min, and then centrifuged as $5000 \times g$ for 14 min to remove aggregated cytochrome *b* [12]. The supernatant was immediately diluted to 3–4 μM cytochrome c_1 with 50 mM potassium phosphate, 1 mM EDTA (pH 7.4), containing 0.1% Tween-20, and was used directly for the CD measurements. The subcomplex obtained contained less than 6% of the original concentration of cytochrome *b*.

The enzymatic assay of the cytochrome bc_1 complex was performed with ubiquinol-2 and horse-heart cytochrome *c* (type VI Sigma) as previously described [9]. The reduction of cytochrome *c* catalyzed by the isolated 'Rieske' protein was performed in the same buffer that was used for the enzymatic assays (25 mM potassium phosphate, 1 mM EDTA (pH 7.4) [9]) with 10 μM bovine heart cytochrome *c* (type V Sigma) 36 μM ubiquinol-2 and 0.1–0.4 μM isolated iron-sulphur protein [13]. The concentration of the isolated 'Rieske' protein was determined from the difference CD spectrum, ascorbate-reduced minus ferricyanide-oxidized, at 497–482 nm using an extinction coefficient $\Delta\epsilon$ of 14 $M^{-1} \cdot cm^{-1}$ [4].

CD spectra of samples containing 2–4 μM cytochrome bc_1 complex dissolved in 50 mM potassium phosphate, 1 mM EDTA (pH 7.4), containing either 20% glycerol or 0.1% Tween-20, were recorded at room temperature using a Jasco J-500 spectropolarimeter. CD spectra of isolated cytochrome c_1 and isolated 'Rieske' protein were performed at 3–8 μM in 25 mM Tris-HCl (pH 7.5), containing 0.5% potassium cholate and 20% glycerol. The $\Delta\epsilon$ values of the CD spectra were calculated on the basis of the concentration of cytochrome c_1 determined spectrophotometrically in the same sample. The spectra were the mean of 2–4 scans in the Soret region (370–470 nm) at a bandwidth of 2 nm and no corrections were made for the insignificant interference by any of the buffers used. No significant differences were observed between the spectra of the cytochrome bc_1 complex suspended in the different buffers.

Results and Discussion

The CD spectrum of the oxidized cytochrome bc_1 complex is dominated by the cytochrome c_1 (Fig. 1A); this agrees with previous observations that isolated cytochrome c_1 and the bc_1 complex, in their oxidized form, both exhibit a single positive band of apparently similar shape [3–5], which appears to obscure the contribution of the oxidized *b* cytochromes and the oxidized iron-sulphur protein at least in the Soret region. However, the peak of the intact enzyme is blue-shifted by about 3 nm and has an extinction coefficient higher than that reported for the isolated cytochrome c_1 [5]. This difference is shown in Fig. 1A: the oxidized subcomplex from which cytochrome *b* has been removed by guanidine-HCl cleavage [12] exhibits CD features similar to those reported for the isolated cytochrome c_1. Thus, the small negative band at 426 nm which is present in the CD spectrum of the intact oxidized bc_1 complex is not exhibited by either the bc_1 subcomplex or the isolated cytochrome c_1, and further, the major peak of the bc_1 subcomplex has a maximum 2 nm red-shifted with respect to that of the native enzyme.

The difference spectrum between the whole oxidized enzyme and the subcomplex devoid of cytochrome *b* is a bilobe band with a maximum at 411 nm, minima at 374 and 426 nm and cross-overs at 402 and 416.5 nm (Fig. 1B). In principle, this bilobic spectrum may be attributed to the specific CD absorption of oxidized *b* cytochromes; this is further supported by a close correspondence between the cross-over point in the CD spectrum at 416.5 nm and the optical absorption maximum of oxidized cytochrome *b* at 417 nm [3,14].

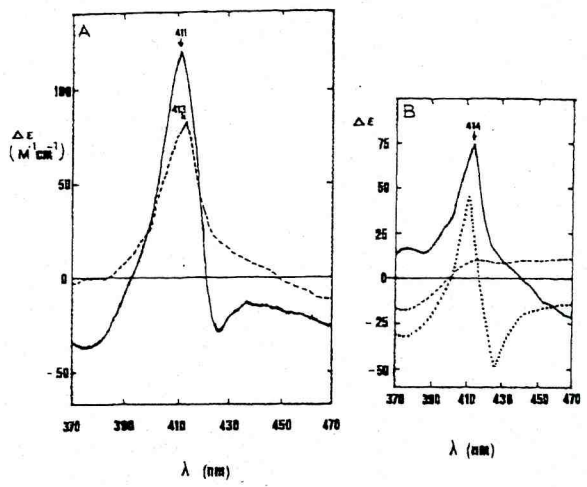

Fig. 1. CD spectra of oxidized cytochrome bc_1 complex. (A) Spectrum of the native enzyme at 3.5 μM (———) and of the subcomplex obtained after cleavage of cytochrome *b* [12] (- - - - - -) at the same concentration as that of the native enzyme. (B) Resolution of the different components comprising the CD spectrum of oxidized cytochrome bc_1 complex. (· · · · · ·), difference spectrum between the native enzyme and the subcomplex devoid of cytochrome *b* (see A). (- - - - - -), spectrum of oxidized, purified iron-sulphur protein [4] considering a 1:1 stoichiometry with cytochrome c_1 as verified experimentally [2]. (———), resolved spectrum of oxidized cytochrome c_1, obtained after subtraction of the CD absorbance of an equimolar oxidized iron-sulphur protein from the spectrum of the subcomplex devoid of cytochrome *b* (see A). The spectra were recorded in the phosphate buffer containing 0.1% Tween-20.

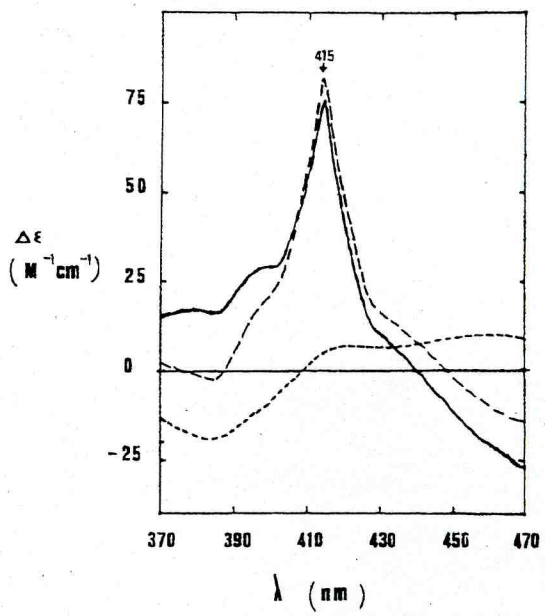

Fig. 2. Resolution of the CD spectrum of reduced cytochrome c_1. The spectrum of reduced cytochrome c_1 (———) was resolved from the spectrum of the ascorbate-reduced subcomplex which was depleted of cytochrome *b* by the guanidine cleavage (- - - - - -) after subtraction of the equimolar CD absorbance of the reduced, isolated iron-sulphur protein [4] (· · · · · ·). The experimental conditions were the same as in Fig. 1.

The native 'Rieske' protein within the cytochrome bc_1 complex and the isolated 'Rieske' protein have previously been demonstrated to possess very similar CD characteristics [4]. Hence, subtraction of the CD spectrum of oxidized 'Rieske' protein and of the oxidized cytochrome *b* from that of the whole oxidized complex should reveal the contributions specifically due to the native, oxidized cytochrome c_1 (Fig. 1B). Similarly, since ascorbate only reduces cytochrome c_1 and the 'Rieske' centre in the cytochrome bc_1 complex [2], the spectrum of reduced cytochrome c_1 can be resolved after subtraction of the contributions of the reduced 'Rieske' protein and of the oxidized cytochrome *b* from the spectrum of the ascorbate-reduced enzyme (Fig. 2). It is also interesting to note that ascorbate reduction of the enzyme produces a slight red-shift of the single positive band, which mainly reflects the corresponding red-shift in the spectrum of the reduced isolated cytochrome c_1 [3–5].

The CD extinction coefficients of the native cytochrome c_1 within the complex, whether it is oxidized or reduced, are much higher than those characteristic of the isolated cytochrome, as shown in Fig. 3A. The preparation of purified cytochrome c_1 obtained as in Refs. 11 and 13 exhibits, in the reduced state, the maximum of the Soret Cotton band at 419 nm, with $\Delta\epsilon$ of 13 $M^{-1} \cdot cm^{-1}$ (Fig. 3A). The overall shape of the spectrum is similar to that reported for many reduced *c*-cytochromes [1] and resembles the mirror image (about the wavelength maximum) of the resolved spectrum of reduced cytochrome c_1 (Fig. 2). The value of $\Delta\epsilon$ is in agreement with that found in another preparation of purified cytochrome c_1 [15], but is about one-half smaller than that reported for a preparation of cytochrome c_1 which actually consisted of two polypeptides, the apocytochrome

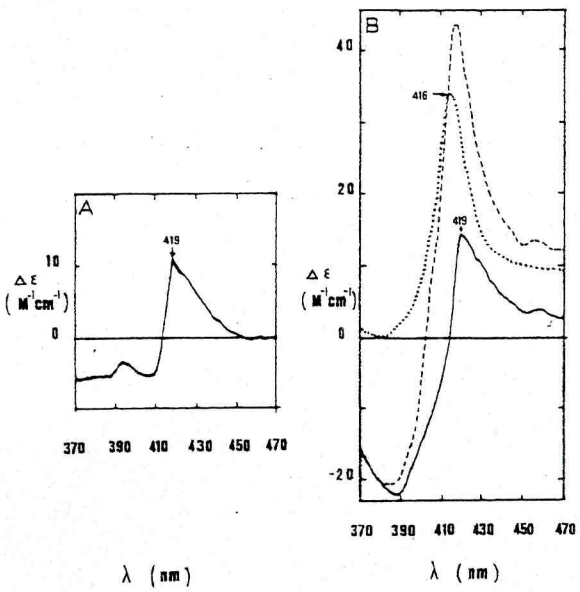

Fig. 3. The spectra of the isolated, reduced cytochrome c_1, (A) CD spectrum of isolated cytochrome c_1 at 3 μM in the reduced state, as obtained after elution from the phenyl-sepharose column [11,13]. (B) Comparison between the sum of the CD spectra of 3.7 μM isolated iron-sulphur protein and of 3.6 μM isolated cytochrome c_1 recorded in separate cuvettes (———) and the spectrum obtained when the two isolated proteins are mixed in the same cuvette and incubated for 30 min at room temperature (- - - - - -). The difference spectrum (· · · · · ·) with peak maximum at 416 nm resembles the spectrum of the native, reduced cytochrome c_1 within the complex (cf. Fig. 2). The isolated iron-sulphur protein and the iron-sulphur-cytochrome c_1 (1:1, mol/mol) mixture catalyzed the reduction of cytochrome *c* by ubiquinol-2 at a rate of 3 and 5 nmol cytochrome *c* reduced per nmol iron-sulphur centre per s, respectively. Addition of purified phospholipids up to 1 mg/ml had no effect on the spectrum of the mixture of the two purified proteins (data not shown).

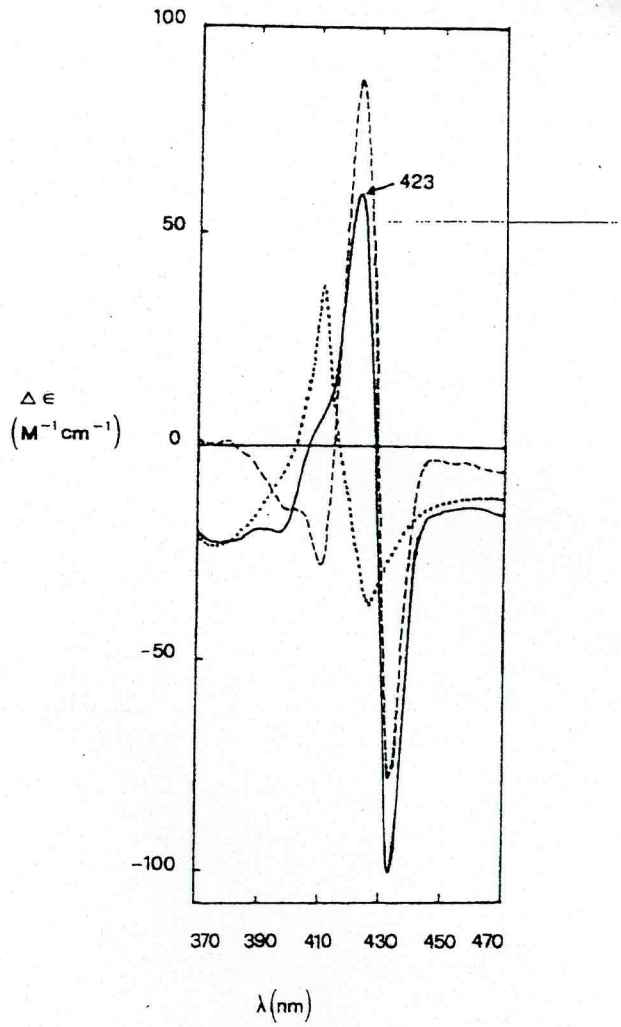

Fig. 4. Resolution of the CD spectrum of reduced cytochrome *b*. (- - - - - -), difference CD spectrum, dithionite-reduced minus ascorbate-reduced, of the native cytochrome bc_1 complex at 3.9 μM. (· · · · · ·), CD spectrum of oxidized cytochrome *b* resolved after cleavage of cytochrome from the native enzyme as illustrated in Fig. 1. (———), sum of the spectrum of oxidized cytochrome *b* and the difference spectrum, dithionite-reduced minus ascorbate-reduced, of the cytochrome bc_1 complex. The CD spectrum obtained by this summation is specifically due to reduced cytochrome *b* (see the text for further details). The extinction coefficients are calculated on the basis of the determination of cytochrome c_1 in the sample, which contained 1.8 mol of cytochrome *b* per mol of cytochrome c_1. The Δε referred to cytochrome *b*, therefore, are about one-half of those represented in the figure.

and the 'hinge' protein [15,16]. It is therefore possible that cytochrome c_1 interacts with other subunits of the cytochrome bc_1 complex in such a way as to amplify the Soret Cotton effect of the c_1-heme, as has been demonstrated upon formation of a complex between purified cytochrome c_1, purified 'hinge' protein and cytochrome *c* [16].

A functional complex has already been shown to exist between the isolated cytochrome c_1 and the isolated 'Rieske' protein [13], and it is also known that the two proteins are in rapid redox equilibrium within the bc_1 complex [17,18]. Further illustration of the existence of an interaction between cytochrome c_1 and the 'Rieske' centre can be seen from the data in Fig. 3B; the spectral sum of the CD features of isolated cytochrome c_1

and isolated 'Rieske' iron-sulphur protein cannot in total account for the greater Soret Cotton band that is seen when the two purified proteins are mixed together. Note also that the difference between the spectrum of the mixed proteins and that obtained after summation of their individual spectra shows a single positive band at 416 nm which is similar to the resolved spectrum of the reduced cytochrome c_1 within the enzyme (cf. Fig. 2). The structural interaction between cytochrome c_1 and the 'Rieske' protein, leading to a remarkable increase in the CD absorption of the c_1-haem, may largely account, therefore, for the $\Delta\epsilon$ of cytochrome c_1, being greater in situ than when it is isolated (cf. Figs. 2 and 3).

The specific CD absorption of the reduced *b*-cytochromes may also be dissected from the total spectrum of the cytochrome bc_1 complex in one of three ways. (i) By manipulating the difference spectrum, dithionite-reduced minus ascorbate-reduced, which reflects the changes in absorption due to only the redox state of cytochrome *b* [3] and may be considered to be the spectral difference between the *b*-cytochromes reduced and oxidized. Hence, simple addition of this difference spectrum to that which was previously obtained for oxidized cytochrome *b* (cf. Fig. 1B) yields the true spectrum of the reduced cytochrome *b* (Fig. 4). (ii) By directly subtracting the combined contributions of reduced cytochrome c_1 and reduced 'Rieske' protein from the spectrum of the dithionite-reduced cytochrome bc_1 complex. (iii) By obtaining the difference spectrum between the dithionite-reduced bc_1 complex and the ascorbate- or dithionite-reduced subcomplex which is lacking cytochrome *b* (cf. Fig. 1). The last method, however, might be affected due to cytochrome *b* removal from the bc_1 complex.

The spectrum of the reduced *b*-cytochromes obtained from method (i) is shown in Fig. 4; nevertheless, all three methods produced similar spectra (Fig. 5). These spectra of reduced *b*-cytochromes exhibit a bilobic shape analogous to that previously reported for intact bovine heart submitochondrial particles [19] (compare also with the spectrum of dithionite-reduced yeast cytochrome bc_1 complex in the presence of uncouplers [20]). The bilobic spectrum of the reduced *b*-cytochromes is less symmetrical about the cross-over points than that of the oxidized *b*-cytochromes (cf. Fig. 1B), although the wavelength of the central cross-over point (428.5 nm) closely corresponds to the maximum of optical absorbance of the *b*-cytochromes (429 nm [3,17,21]). Note also that the positive band ($\lambda_{max} = 423$ nm) has a shoulder around 410 nm, and possesses a $\Delta\epsilon$ which is about 60% of that of the negative peak at 434 nm. No attempt has been made to resolve the specific contribution from each of the two *b* haems (*b*-562 and *b*-566 [2]) to the total CD spectrum of the *b* cytochromes. Therefore, we shall refer to cytochrome *b* as a single chromophore containing two hemes [2,3,14].

The major characteristics of the resolved CD spectra of the cytochromes *b* and c_1 within the enzyme complex are summarized in Table I.

The CD features of cytochrome *b* incorporated within the cytochrome bc_1 complex are remarkably different from those of other *b*-type haemoproteins [1,22] (including myoglobins and haemoglobins [23]). In principle, this difference may be ascribed to the double-haem nature of the mitochondrial cytochrome *b* [2,14,24]. Hence, its CD properties may be expected to arise largely from heme–heme interactions resulting in exciton splitting of the Soret electronic transition [20], as exemplified by the flavocytochrome *c*-552 of *Chromatium vinosum* (which also contains two haems per molecule [1,25]). In apparent accordance with the existence of strong haem–haem interactions, the CD spectrum of cytochrome *b* of the cytochrome bc_1 complex exhibits the 'S-shaped' band, characteristic of exciton splitting, which is almost symmetrical about a cross-over point corresponding to the wavelength of maximal optical absorption [1,20,25,26]. However, examination of the amino acid sequences of cytochrome *b* from different sources [24,27] suggests that strong haem–haem interactions between the two *b*-cytochromes are not possible.

The primary structure of mitochondrial cytochrome *b* [24,27] predicts that the apoprotein is arranged in nine transmembrane α-helical segments, two of which contain a pair of highly conserved histidine residues separated by 13 other amino acids. These invariant histidines are believed [24,27] to coordinate the irons of the two hemes such that they lie, one in each half of the bilayer,

in a single plane which is perpendicular to the plane of the membrane and with a centre-to-centre spacing of approx. 20 Å. However, this haem separation of 20 Å is almost 3-times greater than the 7 Å which is considered to be the upper limit for heme–heme interactions to produce exciton splitting and the resultant bilobic spectra of cytochromes [1,26].

Another mechanism which determines the CD absorption of haemoproteins is the dipole–dipole interaction between the excited state of the porphyrin rings and their surrounding aromatic amino acids [1,28]. A radius of 15 Å around a haem centre is considered to be the limit for the occurrence of dipole–dipole interactions which are capable of inducing Soret Cotton effects [28]. Indeed, close examination of the primary structure and the folding of the apoprotein of cytochrome *b* [24,27,29] reveals that, in bovine heart, there are 15 aromatic residues located in the two transmembrane segments coordinating the hemes within 15 Å of each of the two porphyrin ring centres, as shown in Fig. 6. This does not include the four histidines which are believed [24] to be the ligands to the hemes. In the tertiary structure of the apoprotein, it is unlikely that aromatic amino

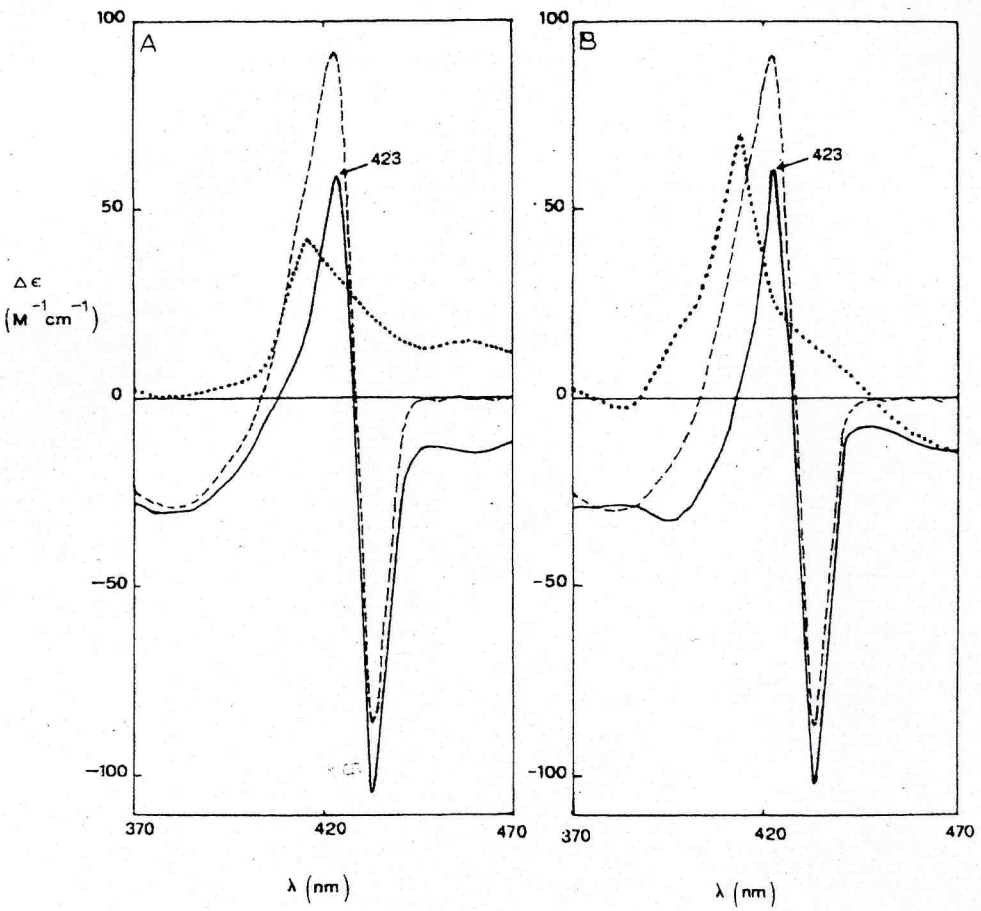

Fig. 5. Resolution of the CD spectrum of reduced cytochrome *b*. (A) (- - - - -), CD spectrum of the dithionite-reduced bc_1 complex. (· · · · · ·), CD spectrum of the mixed reduced FeS protein and reduced cytochrome c_1 (———), difference CD spectrum, dithionite-reduced bc_1 complex minus mixed reduced FeS protein and reduced cytochrome c_1. (B) (- - - - -), CD spectrum of the dithionite-reduced bc_1 complex. (· · · · · ·), CD spectrum of the ascorbate-reduced subcomplex (guanidine-treated bc_1 complex). (———), difference CD spectrum, dithionite-reduced bc_1 complex minus ascorbate-reduced subcomplex. The $\Delta\epsilon$ referred to cytochrome *b* are about one-half of those represented in the figure (see legend to Fig. 4 for details).

acids belonging to other transmembrane segments may be located at a distance shorter than 15 Å from the hemes, with the possible exception of Y_{155} in the recent model proposed by Von Jagow and colleagues [29].

More specifically, the amino acid sequencing suggests that a highly conserved–phenylalanine (F_{90} in bovine heart [24,27,29]) plus at least two other aromatic aminoacids (F_{91}, F_{187} and possibly Y_{155} in bovine heart [24]) are located midway between the two haems [29]. The orbitals of these aromatic residues are likely to overlap the orbitals of the two porphyrin rings, forming a large electron cloud that could act as an 'electron bridge' between the excited states of the two haems (Fig. 5). The haem–haem interaction of the two distant (20 Å apart [24,29]) *b*-cytochromes, therefore, could be mediated and amplified by the electron could formed by the aromatic residues between the two porphyrin rings, producing the necessary conditions for the observed exciton splitting. The existence of an 'electron bridge' between the two *b*-haems is also suggested by the rapid electron transfer which is generally considered [30] to occur between these two centres (Crofts, A. and Cramer, W. personal communications).

Alternatively, the asymmetry of the *b*-cytochromes bilobic peak (Figs. 4 and 5) may arise from diverse environments around the two constituent haems. For example, there are differing numbers

TABLE I

RESOLVED CIRCULAR DICHROISM CHARACTERISTICS OF CYTOCHROME c_1 AND CYTOCHROME *b* WITHIN THE BOVINE HEART CYTOCHROME bc_1 COMPLEX IN THE SORET REGION

Data are derived from the Figs. 1–4. The $\Delta\epsilon$ are calculated on the basis of the determination of cytochromes c_1 and *b*, considering the latter as as a single chromophore, comprising two hemes. The molar ratio of cytochrome *b* per cytochrome c_1 is 1.8, as verified experimentally (cf. Fig. 4).

Cytochrome	λ_{max} (nm)	$\Delta\epsilon$ ($M^{-1}\cdot cm^{-1}$)	$\lambda_{cross\text{-}over}$ (nm)
Oxidized cytochrome c_1	414	+74.5	441
Reduced cytochrome c_1	415	+76.0	441
Oxidized cytochrome *b*	411	+39.5	402, 416.5
	426	−37.0	
Reduced cytochrome *b*	423	+58.0	408, 428.5
	434	−101.0	

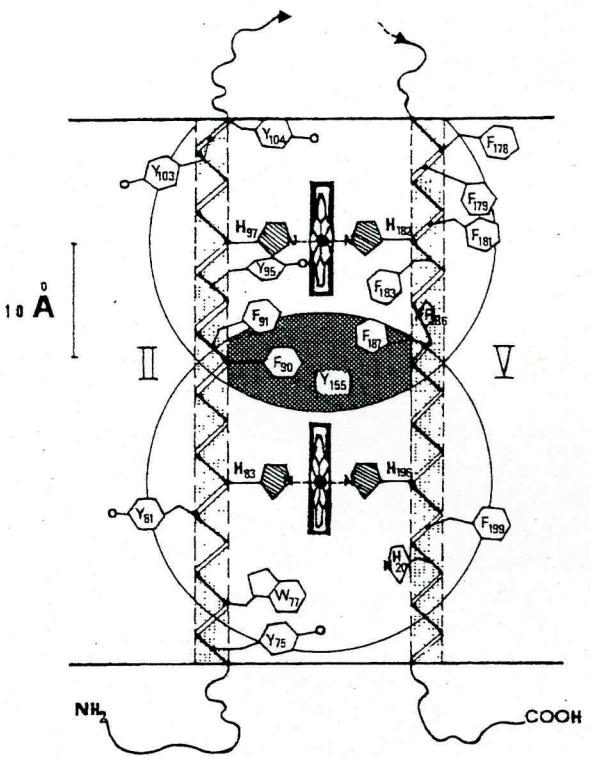

Fig. 6. Model for the interaction of the two haems of cytochrome *b* with surrounding aromatic amino acids. The two α-helix transmembrane segments II and V, which are predicted to contain the four histidines that coordinate the two *b*-haems [24,27], are drawn according to the structural model of Saraste [24]. The position of the aromatic amino acids within these segments are outlined according to the proposed folding of the two α-helices, which are chosen to be formed by about 30 amino acids. No consideration has been given to the possible bending of segment V with respect to segment II due to the presence of P_{186} [29]. The aromatic residues are drawn with arbitrary orientations in order to emphasise their possible interactions with the hemes. The relative dimensions of the α-helices diameter are not respected. The hatched area midway between the two hemes represents the common region of possible interaction within 15 Å from each of the two irons (indicated by the cycles around the haems). Such a region contains F_{90}, F_{91}, F_{187} and possibly also Y155. The latter residue belongs to the transmembrane segment III [24], but could be located within the interaction region between the two hemes in the tertiary structure of the apoprotein according to the model of Von Jagow and co-workers [29]. The hatched area may constitute the 'electron bridge' between the two hemes (see the text). Note that the heme located in the upper phospholipid layer of the membrane is surrounded by a higher number of aromatic residues than the other haem.

and types of aromatic residue in the vicinity of the two porphyrin rings (Fig. 6); these may interact with the transition dipole moments of the excited hemes in two distinct ways, resulting in an asymmetrical bilobic peak [1]. The fact that oxidized and reduced cytochrome *b* display this asymmetry to different extents (cf. Figs. 4 and 5) suggests that the protein undergoes a conformational transition upon changing its redox state. These conformational changes may alter the orientation and location of some of the aromatic residues around one or both of the two heme centres, giving rise to different interactions with the porphyrin rings [1].

In conclusion, we have shown firstly, that the CD spectra of the cytochrome bc_1 complex can be resolved into the contributions arising from each of its individual prosthetic groups. Secondly, the resolved CD features of the cytochromes *b* and c_1 depend upon the structural organisation within the complex; this is clearly shown by the dramatic difference between the CD spectra of the single isolated subunits and the CD spectra of the subunits within the whole complex. Moreover, we suggest a strong reciprocal influence of the *b* haem centres due to the presence of aromatic amino acids in their vicinity.

Acknowledgments

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