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CIRCULAR DICHROISM OF CYTOCHROME OXIDASE, CYTOCHROME b_{566}, AND CYTOCHROME *c* IN BEEF HEART MITOCHONDRIAL MEMBRANE FRAGMENTS

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SUMMARY

Circular dichroism spectra of the cytochromes in membrane fragments derived from sonicated beef heart mitochondria have been obtained in the wavelength region 400–480 nm in which the major absorbance maxima of the heme prosthetic groups are found.

2. Cytochrome oxidase in the mitochondrial membrane fragments has a band of positive ellipticity at 426 nm in the oxidized form and a pronounced band of positive ellipticity at 445 nm in the reduced form. The reduced-*minus*-oxidized difference molar ellipticity at 445 nm, $\Delta[\theta]_{445}$ is $3.0 \cdot 10^5$ degree$\cdot$cm$^{-2}\cdot$dmole^{-1} heme *a* for membrane-bound oxidase compared to $1.6 \cdot 10^5$ degree$\cdot$cm$^{-2}\cdot$dmole^{-1} heme *a* for the purified oxidase. The membrane-bound oxidase in the reduced form also appears to have a band of negative ellipticity at 426 nm not found in the purified oxidase.

3. When reduced with succinate in the presence of cyanide and oxygen, cytochrome oxidase in the membrane fragments has a positive band at 442 nm very similar to that observed with the purified oxidase.

4. Cytochrome *c*, which has a positive band at 426 nm in the purified form when reduced, appears to have a negative band at this wavelength in the mitochondrial membrane fragments which contributes to the pronounced negative band at 426 nm observed in the membrane fragments reduced with succinate in anaerobiosis. There is no evidence for a contribution to the CD spectra of the membrane fragments from cytochrome c_1 or from cytochrome b_{561} in either the oxidized or the reduced form.

5. Cytochrome b_{566} in the mitochondrial membrane fragments has no detectable CD spectrum in the oxidized form, but has a small positive band at 427 nm and a small negative band at 436 nm in the reduced form. The same CD spectrum is observed with cytochrome b_{566} reduced with succinate in the presence of antimycin A or 2-heptyl-4-hydroxyquinoline-*N*-oxide. The same increase in positive ellipticity is observed at 427 nm in the mitochondrial membrane fragments, treated with oligomycin to restore energy coupling, when cytochrome b_{566} is reduced with

Abbreviations: HOQNO, 2-heptyl-4-hydroxy quinoline-*N*-oxide; PMS, phenazine methosulfate.

succinate in the energized membrane, as is observed in the inhibitor-treated membrane fragments. The absence of a pronounced conformational change in cytochrome b_{566} on energization, as revealed by its CD spectrum, favors the concept that its reduction by succinate in the energized state is due to reversed electron transport rather than an intrinsic shift in the cytochrome's midpoint redox potential.

INTRODUCTION

Circular dichroism provides a most useful technique for studying the responses of the hemoprotein electron transport carriers of the mitochondrial respiratory chain, since it can measure the changes in induced optical activity of the heme group on oxidation, reduction, or energization of the respiratory chain and hence record the changes in protein conformation which affect this induced optical activity[1–3]. The positive and negative extrema of ellipticity in the CD spectrum are located near the absorbance maxima attributable to the heme group. The positive and negative CD bonds observed in the Soret region should therefore be readily correlated with the absorbance bands of the cytochromes in this region. This is evident from the extensive CD studies undertaken with purified cytochrome oxidase, cytochrome *c* and cytochrome c_1[4–11]. In the Soret region, these cytochromes all show positive ellipticity maxima near their γ-band absorbance maxima in both oxidized and reduced forms. This technique should be particularly useful in examining the conformational changes accompanying oxidation and reduction of the cytochromes bound to their native mitochondrial membrane, both in the energized and de-energized forms. To the best of our knowledge, no such study has been reported. In this paper, we report a study of the CD changes observed in the Soret region for cytochrome in beef heart mitochondrial membrane fragments which retain energy conservation coupled to electron transport.

MATERIALS AND METHODS

Membrane fragments (submitochondrial particles) were isolated from beef heart mitochondria by sonication in the presence of EDTA, followed by differential centrifugation, as described by Lee and Ernster[12]. All reactions were carried out at pH 7.5 in 50 mM Tris–acetate buffer. Cytochrome *c* (horse heart) was purchased from Boehringer Mannheim Corp.; isolated purified cytochrome oxidase was generously provided by professor E. Racker; the heme *a* content was 10 nmoles/mg protein. CD curves were obtained with a Jasco-Durrum J-10 Spectropolarimeter (most kindly made available by Dr Robert C. Davis of the Chemistry Department, University of Pennsylvania) utilizing a cell with a 1-cm optical path. The slit width was programmed to give a spectral half-band width of 6–10 nm from 520 to 400 nm. Differential absorbance changes were measured with the dual wavelength spectrophotometer[13] compensated for light source fluctuations[14].

RESULTS

The CD curve for oxidized mitochondrial membrane fragments, suspended in air-saturated Tris–acetate buffer in the absence of substrate, is the top trace shown

in Fig. 1A. The curve for the same particle suspension reduced with succinate in anaerobiosis is the bottom trace shown in Fig. 1A. The two traces are separated by an arbitrary interval for clarity. The two curves referred to a common baseline are shown in Fig. 1B, and the reduced-*minus*-oxidized difference CD spectrum calculated from Fig. 1B is plotted in Fig. 1C. A small positive band at 426 nm is seen with the oxidized mitochondrial membrane fragments, while a substantial positive band at 445 nm is seen in the reduced membrane fragments. These bands correspond to the ellipticity maxima reported for purified cytochrome oxidase[5,6,8,9]. The reduced membrane fragments also show a distinct negative band at 426 nm, which does not correspond to any ellipticity maximum reported for the oxidase.

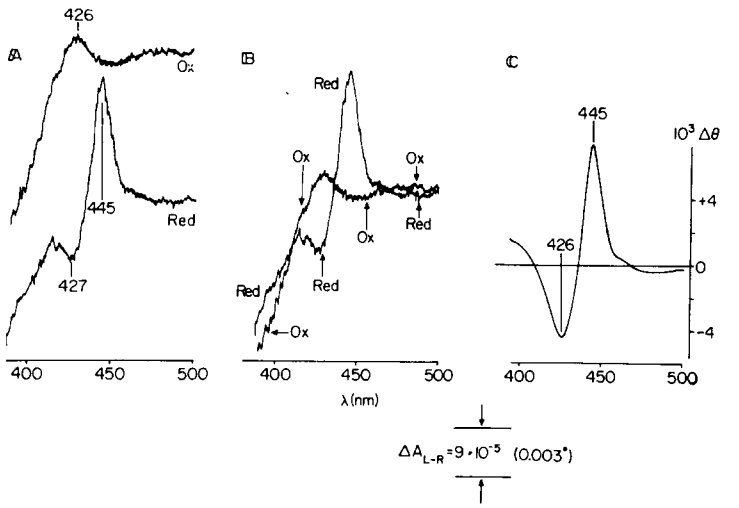

Fig. 1. CD spectra of mitochondrial membrane fragments (2.8 mg protein/ml) suspended in 50 mM Tris–acetate buffer (pH 7.5). Ox= as is *plus* O_2; Red= 8 mM succinate to $O_2 = 0$. (A) Spectrum of oxidized (top) and reduced (bottom) particles; the spectra are separated by an arbitrary interval for clarity. (B) Spectra of (A) obtained with common baseline. (C) Reduced-*minus*-oxidized difference CD spectrum calculated from B.

The reduced-*minus*-oxidized difference CD spectrum shown in Fig. 1C is obtained when the particles are reduced in anaerobiosis with succinate or with ascorbate *plus* phenazine methosulfate (PMS) in the presence of antimycin A. Under the former conditions, cytochromes aa_3, c_1, c, and b_{561} are reduced while b_{566} remains oxidized; under the latter conditions, cytochromes aa_3, c_1, c are reduced but both b_{561} and b_{566} remain oxidized. (Antimycin A has no effect on the CD spectrum of oxidized mitochondrial membrane fragments in the absence of substrate.) These results imply that the CD spectra shown in Fig. 1 are those of the two *c* cytochromes and cytochrome oxidase alone, there being no contribution from the *b* cytochromes.

For comparison with the CD spectra obtained with the particles, the CD curves for a mixture of purified cytochrome *c* *plus* purified cytochrome oxidase were obtained under conditions as nearly equal to those of Fig. 1 as possible. The ratio of the two cytochromes was 1:1 on a heme basis, which is their approximate ratio in mitochondrial membrane fragments. The CD curve relative to the instrument baseline for the oxidized cytochrome mixture is shown in the top trace of Fig. 2A;

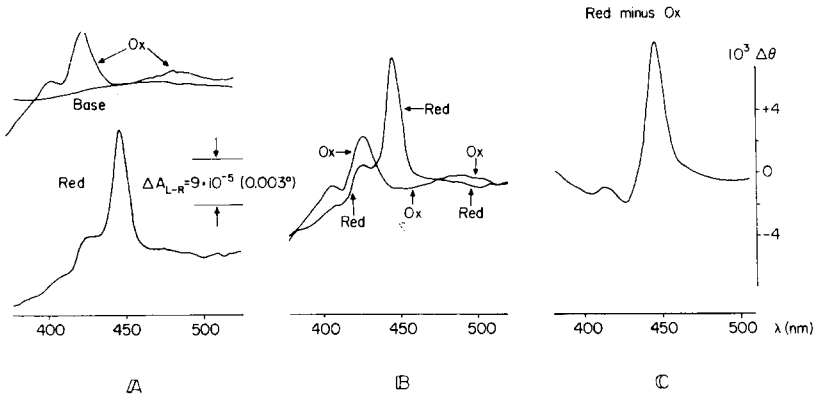

Fig. 2. CD spectra of purified cytochrome oxidase *plus* purified cytochrome *c* in 50 mM Tris-acetate buffer (pH 7.5) used for the experiment recorded in Fig. 1. Each component is at 6 μM on a heme basis. Ox= as is *plus* O_2; Red= 8 mM succinate to O_2= 0. (A) Spectrum of oxidized (top) and reduced (bottom) cytochrome *c plus* cytochrome oxidase; the baseline is shown in the top trace. The reductant is 8 mM ascorbate. (B) Spectra of A referred to a common baseline. (C) Reduced-*minus*-oxidized difference CD spectrum calculated from (B). The purified oxidase contained 10 nmoles heme *a* per mg protein.

that for the reduced mixture is shown in the bottom trace, displaced by an arbitrary distance for clarity. The CD spectra for the reduced and oxidized cytochrome mixture referred to a common baseline are in Fig. 2B; from these the reduced-*minus*-oxidized difference CD spectrum in Fig. 2C is calculated.

The individual contributions of cytochrome *c* and cytochrome oxidase to the curves shown in Fig. 2 may be assessed by comparing the CD spectra of the two purified cytochromes separately under identical experimental conditions. The CD spectra for reduced and oxidized cytochrome *c* are shown in Fig. 3A; that for oxidized cytochrome oxidase referred to the instrumental baseline is shown in Fig. 3B. Sub-

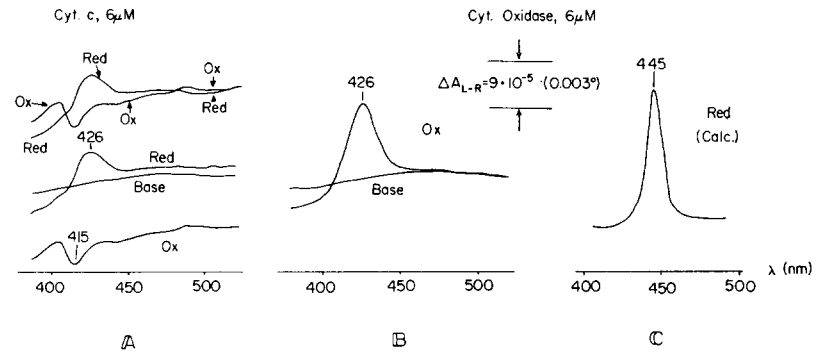

Fig. 3. CD spectra of purified cytochrome *c* and purified cytochrome oxidase as separate components. Experimental conditions as in Fig. 2. (A) Cytochrome *c* in the oxidized and reduced forms; the curves are marked "Ox" and "Red", respectively. The reductant is 8 mM ascorbate. (B) Cytochrome oxidase in oxidized form; instrument baseline is also shown. (C) Cytochrome oxidase in reduced form; CD spectrum is calculated by subtracting CD curve for reduced cytochrome *c* from CD curve for reduced cytochrome *c plus* cytochrome oxidase in Fig. 2A. All curves are then obtained under the same conditions for reduction.

tracting the contribution of reduced cytochrome *c* from the CD curve of reduced cytochrome *c plus* cytochrome oxidase in Fig. 2A gives the CD spectrum of reduced cytochrome oxidase shown in Fig. 3C. These CD spectra are very similar to those already published for these cytochromes under different experimental conditions[4-9,11], demonstrating that the choice of conditions most useful for observing the reactions of mitochondrial membrane fragments has little effect on the CD spectra of the cytochromes *per se*.

The suspension of submitochondrial particles is turbid and CD measurements are vulnerable to light scattering artifacts. It is therefore essential to check the linearity of the CD band with particle concentration. The transition from oxidized particles in the absence of substrate to particles reduced by succinate in anaerobiosis is accompanied by a positive ellipticity change at 445 nm, as is evident from the difference spectrum in Fig. 1C. The magnitude of this change is plotted as a function of particle concentration in the medium in Fig. 4. The plot is linear up to about 3 mg membrane protein per ml with a light path of 1 cm. All measurements in this study were carried out within this range of concentrations at this optical path length. From the slope of the line in Fig. 4 the reduced-*minus*-oxidized difference molar ellipticity at 445 nm, $\Delta[\theta]_{445}$, is calculated to be $3.0 \cdot 10^5$ degrees$\cdot$cm$^{-2}\cdot$dmole^{-1} heme *a* for cytochrome oxidase in the mitochondrial membrane fragments. It is of interest that $\Delta[\theta]_{445}$ for membrane-bound cytochrome oxidase is nearly twice that observed with the purified cytochrome oxidase under the same experimental conditions: from Fig. 2C $\Delta[\theta]_{445}$ is calculated to be $1.6 \cdot 10^5$ degrees$\cdot$cm$^{-2}\cdot$dmole^{-1} heme *a* for the purified oxidase.

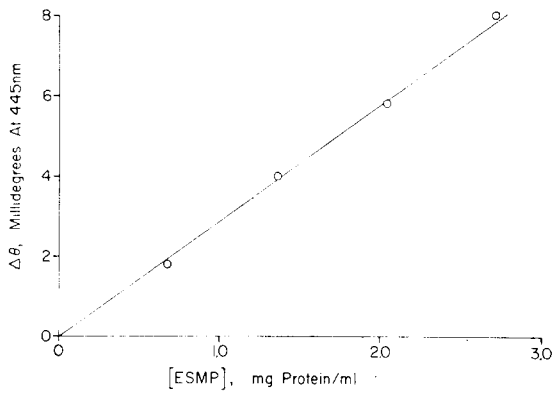

Fig. 4. Plot of the positive ellipticity change observed at 445 nm for the transition from fully oxidized mitochondrial membrane fragments (ESMP) to reduction with succinate in anaerobiosis as a function of protein concentration in the suspension. Buffer: 50 mM Tris–acetate (pH 7.5). [Heme *a*]= 0.94 nmole/mg protein.

The CD spectrum for the oxidized form of the isolated, purified cytochrome oxidase is remarkably similar to the CD spectrum of oxidized membrane fragments. In both preparations, a broad positive band with a maximum at 426 nm is observed. In both preparations, there is also an increase in negative ellipticity with decreasing wavelength to 400 nm; the decrease is greater in the mitochondrial membrane

fragments, however. This may be in part due to contributions from cytochrome *c* since the increase in negative ellipticity is greater when both isolated, purified cytochromes are present (Fig. 2A). Probably the greater contributor to this increase in negative ellipticity is differential scattering of left and right circularly polarized light by the particulate suspension of the membrane fragments which contain optically active components, as discussed by Urry and Krivacic[15]. While their analysis was confined to the ultraviolet region of the spectrum, it should be equally valid for systems scattering light at longer wavelengths. Since the membrane fragment suspensions in both the oxidized and reduced form show an increase in negative ellipticity below 415 nm, it seems reasonable to attribute this increase in large part to the differential scattering described by Urry and Krivacic[15].

Comparison of Fig. 1 and Fig. 2 reveals that the prominent negative CD band at 426 nm observed in substrate-reduced mitochondrial membrane fragments is missing from the mixture of purified cytochrome *c plus* purified cytochrome oxidase. This negative band is observed directly in the CD spectrum of the reduced particles. It is made more prominent in the reduced-*minus*-oxidized difference CD spectrum because of the positive band at 426 nm in the oxidized particles. The question of which cytochromes contribute to this band was examined further by treating aerobic mitochondrial membrane fragments with cyanide, then with succinate. Addition of cyanide has no effect on the CD spectrum of oxidized membrane fragments. Subsequent addition of succinate, which causes reduction of cytochromes *c*, c_1, and *a* in the cyanide-liganded oxidase, gives the CD spectrum in Fig. 5B. Comparison of this spectrum with the CD spectrum of the oxidized particles (Fig. 5A) shows that the positive band at 426 nm of the oxidized cytochrome oxidase has disappeared, to be replaced by a shallow negative band, and that a positive band at 442 has appeared. The reduced-*minus*-oxidized difference molar ellipticity, $\Delta[\theta]_{442}$, is $1.2 \cdot 10^5$ degrees$\cdot$cm$^{-2}\cdot$dmole^{-1} heme *a* for this band. The same positive band at 442 nm is observed with the purified oxidase *plus* cytochrome *c* in the presence of cyanide and ascorbate under the experimental conditions of

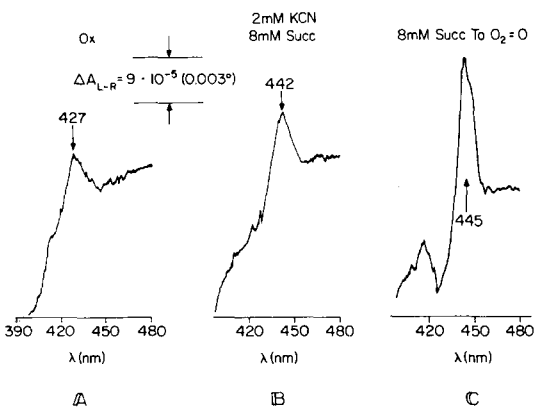

Fig. 5. CD spectrum of the same mitochondrial membrane fragment (2.5 mg protein/ml, 50 mM Tris–acetate, pH 7.5) suspension in the fully oxidized form (A); reduced with succinate in the presence of cyanide and oxygen (B); and reduced with succinate in anaerobiosis (C).

Fig. 2A, but there is no negative band at 426 nm. A positive band is observed as a shoulder at this wavelength in the purified system, corresponding to reduced cytochrome *c* (Fig. 3A); the same shoulder is observed in the reduced purified system in the absence of cyanide (Fig. 2A).

These observations suggest that the negative band at 426 nm seen in reduced mitochondrial membrane fragments is in greater part due to cytochrome oxidase, and in lesser part due to cytochrome *c*. Reduced cytochrome *c* has, as shown in Fig. 3A, a band of positive ellipticity at 426 nm; reduced cytochrome c_1 has a similar band at 417 nm[10]. It is suggested that cytochrome c_1 makes no contribution to the CD spectrum of oxidized or reduced membrane fragments, and that the positive band of reduced cytochrome *c* retains its position at 426 nm but inverts the sign of ellipticity as a consequence of being bound to the mitochondrial membrane. Further work is required to verify this suggestion. The increase in the band of negative ellipticity observed at 426 nm in mitochondrial membrane fragments for fully reduced cytochrome oxidase (Fig. 5C) as compared to the oxidase partially reduced in the presence of oxygen and cyanide (Fig. 5B) suggests that a negative band at 426 nm can be attributed to cytochrome a_3 and arises as a consequence of the incorporation of cytochrome oxidase in the mitochondrial membrane. The recently demonstrated heme–heme interaction in cytochrome oxidase[16,17], however, particularly in the presence of ligands such as cyanide, requires that caution be exercised in attributing this band to cytochrome a_3 alone based only on this comparison.

The CD spectra for oxidized and reduced membrane fragments shown in Fig. 1A are well accounted for by contributions from cytochrome *c* and cytochrome oxidase; there is no detectable contribution from cytochrome b_{561} in either the oxidized or reduced form. Under conditions where cytochrome b_{566} is also reduced, an ellipticity change due to this cytochrome can be observed. The CD spectra recorded for antimycin-treated membrane fragments in the absence of substrate (Curve A), aerobically reduced with succinate (Curve B), anaerobically reduced with succinate (Curve C), and anaerobically reduced with succinate followed by PMS (Curve D), are shown on the left hand side of Fig. 6. The difference CD spectra calculated from the measured ones are on the right hand side of Fig. 6. The same difference CD spectrum with a positive band at 427 and negative band at around 436 nm is obtained from antimycin-treated membrane fragments aerobically reduced with succinate *minus* oxidized (Curve B *minus* Curve A) and antimycin-treated membrane fragments anaerobically reduced with succinate *minus* anaerobically reduced with succinate *plus* PMS (Curve C *minus* Curve D). Both sets of conditions yield the reduced-*minus*-oxidized difference absorbance spectrum of cytochrome b_{566}, from which we conclude that the difference CD spectrum is indeed that for cytochrome b_{566}, most of the contribution being from the reduced form. Exactly the same CD spectra are obtained when the respiratory inhibitor 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) is substituted for antimycin A. These two inhibitors affect in like manner the conformation of reduced cytochrome b_{566} as reflected by the induced optical activity of its heme group. This result is in accord with those reported by Brandon *et al.*[18] who showed that the difference absorbance spectrum of cytochrome b_{566} was the same in the presence of antimycin A or HOQNO, but that the peak of the difference spectrum of cytochrome b_{561} was shifted 2 nm to the red by antimycin A and not by HOQNO. The latter cytochrome has no de-

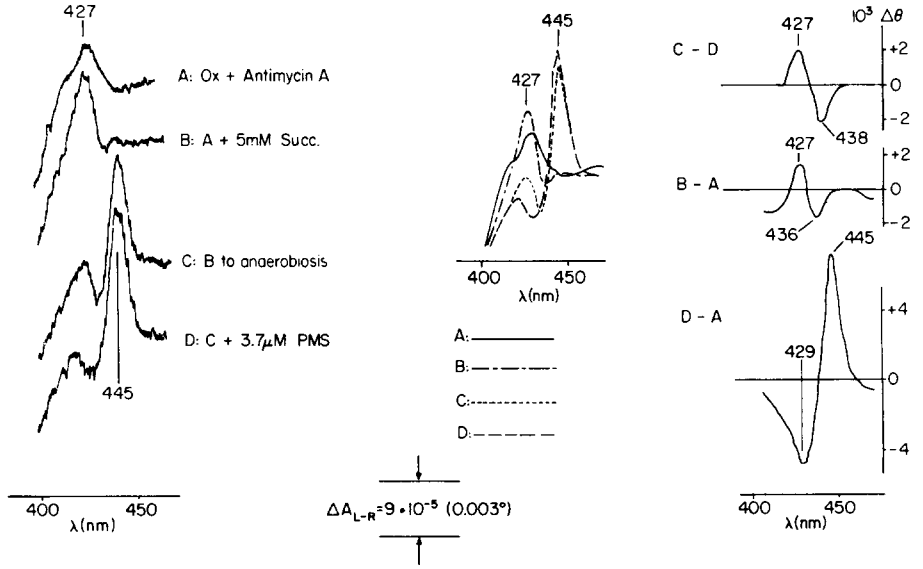

Fig. 6. Reduced-*minus*-oxidized difference CD spectrum of cytochrome b_{566} in mitochondrial membrane fragments (2.1 mg protein/ml, 50 mM Tris–acetate, pH 7.5) obtained in the presence of antimycin A (2.4 nmoles/mg protein). The experimental traces on the left are separated by an arbitrary interval for clarity; their positions relative to a common baseline are shown in the middle set of spectra. The calculated CD difference spectra for cytochrome b_{566} are shown on the right, corresponding to spectrum C *minus* spectrum D in the top trace, and spectrum B *minus* spectrum A in the middle trace. Both sets of conditions yield absorbance difference spectra corresponding to cytochrome b_{566}. The lower right hand CD difference spectrum, corresponding to spectrum D *minus* spectrum A, is the same as that of Fig. 1C, within the error of the calculation.

tectable CD curve and none is induced by antimycin A which causes the red shift.

An answer to the question of whether energization of the mitochondrial membrane causes specific conformational changes in cytochrome b_{566} – also designated cytochrome b_T[19–22] – was sought by comparing changes in ellipticity at 426 nm in mitochondrial membrane fragments treated with oligomycin on the one hand and HOQNO or antimycin A on the other. The experimental traces are shown in Fig. 5. Because of the difficulty of adding reactants in the cell chamber of the spectropolarimeter the membrane fragments were pretreated with antimycin A (Fig. 7A) or HOQNO (Fig. 7B) with proper mixing. Addition of succinate to the treated particles causes a change towards positive ellipticity which is about the same for both inhibitors. From the spectra of Fig. 6, this can be attributed to reduction of cytochrome b_{566}. On anaerobiosis, the change is towards negative ellipticity which is further increased by addition of PMS. The ellipticity changes obtained in the absence and presence of oligomycin are shown in Figs 7C and 7D, respectively. There is no net change towards positive ellipticity when succinate is added to untreated mitochondrial membrane fragments (Fig. 7C) or to oligomycin-treated membrane fragments (Fig. 7D). The change is towards negative ellipticity but is less in the oligomycin-treated, or energized, membrane fragments than in the untreated, or de-energized membrane fragments. On anaerobiosis, the same change towards negative ellipticity is seen in both cases. This result is most readily explained by little, if any, reduction

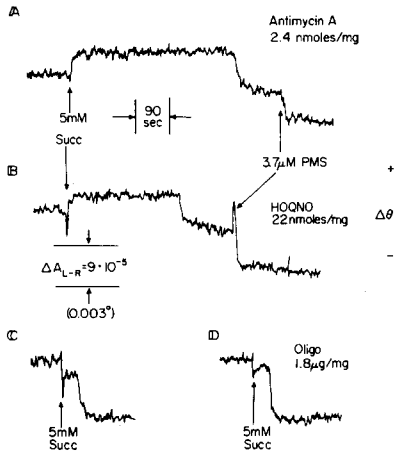

Fig. 7. Steady-state changes in ellipticity recorded in mitochondrial membrane fragments (2.1 mg protein/ml, 50 mM Tris–acetate buffer, pH 7.5) at 426 nm. (A) Membrane fragments treated with antimycin. (B) Membrane fragments treated with HOQNO. (C) Membrane fragments as is, hence uncoupled. (D) Membrane fragments treated with oligomycin, hence recoupled. At the point indicated in each trace, 5 mM succinate was added, and the sample was allowed to become anaerobic. In (A) and (B), 3.7 μM PMS was added in anaerobiosis at the point indicated.

of cytochrome b_{566} during the aerobic steady state in the de-energized particles and partial reduction in the energized particles. The change towards negative ellipticity in the de-energized state is due to partial reduction in the aerobic state of cytochrome c and cytochrome oxidase, which both give a negative CD band at 426 nm, as discussed above. The diminution in negative ellipticity seen in the energized particles is caused by the positive ellipticity contribution at 426 nm from reduced cytochrome b_{566}. The redox state changes, as measured by absorbance, of cytochromes b_{561}, b_{566}, and $c+c_1$ are shown in Fig. 8 for comparable conditions of the experiments in Fig. 7. More cytochrome b is reduced in the aerobic steady state in the oligomycin-treated membrane fragments than in untreated particles, but the effect is much more pronounced with the wavelength pair 566–574 nm, which has a larger contribution from cytochrome b_{566}, than at 562–574 nm with a smaller contribution from this cytochrome. Aeration of the anaerobic particles and addition of HOQNO is accomplished by stirring in the inhibitor; this results in the aerobic steady state with the cytochromes b fully reduced and cytochromes $c+c_1$ fully oxidized. On anaerobiosis, the cytochromes b remain fully reduced while cytochromes $c+c_1$ become reduced; addition of PMS in anaerobiosis then causes oxidation of cytochrome b_{566} and completes the reduction of cytochromes $c+c_1$. Assignment of a negative ellipticity change to cytochrome oxidase and cytochrome c, a positive ellipticity change to cytochrome b_{566}, and zero ellipticity change to cytochrome b_{561} on transition from the oxidized to reduced state readily accounts for all the ellipticity changes observed at 426 nm in the four experiments of Fig. 7.

These results show that energization of the membrane causes no conformational changes in cytochrome b_{566}, as reflected by the CD curve of its heme group, that cannot be accounted for simply by its change in redox state.

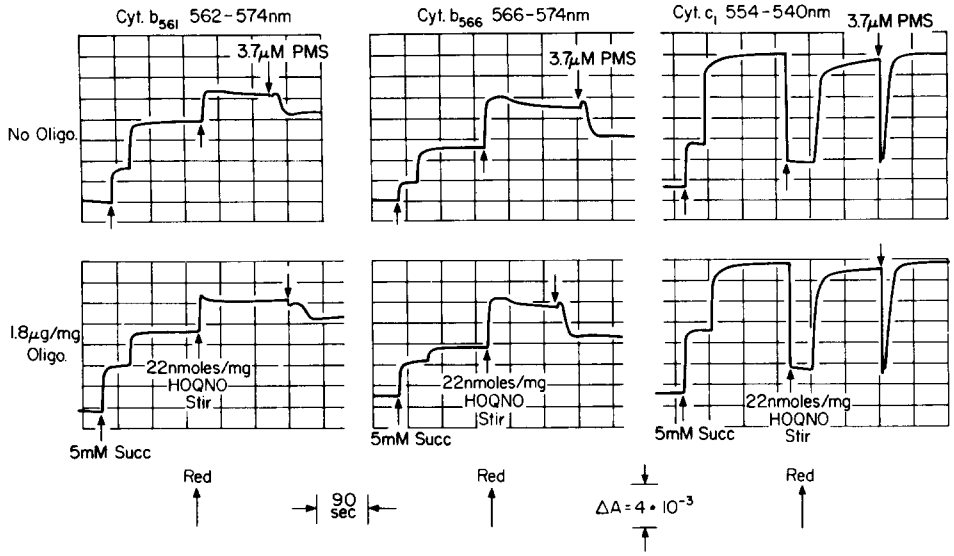

Fig. 8. Steady-state changes in absorbance corresponding to cytochromes b_{561}, b_{566} and $c+c_1$ in mitochondrial membrane fragments (2.1 mg protein/ml, 50 mM Tris–acetate, pH 7.5), recorded with the dual wavelength spectrophotometer at 562–574 nm, 566–574 nm, and 554–540 nm, respectively. In the top set of traces, the membrane fragments are untreated, hence uncoupled; in the bottom set, the membrane fragments are treated with oligomycin to recouple them. Addition of succinate as indicated gives an aerobic steady state followed by anaerobiosis; HOQNO and PMS are then added in anaerobiosis as indicated on the traces.

DISCUSSION

CD measurements in the Soret region on beef heart mitochondrial membrane fragments reveal that cytochromes aa_3 and b_{566}, and probably cytochrome c, show characteristic changes in ellipticity associated with the heme moiety on changing redox state. There are no detectable ellipticity maxima in either oxidized or reduced mitochondrial membrane fragments which can be attributed to either cytochrome b_{561} or cytochrome c_1. The CD spectrum of cytochrome oxidase is similar for the purified, solibilized enzyme and for the enzyme still bound to its native membrane, but the bound enzyme appears to have a negative CD band at 426 nm not seen in the solubilized enzyme. The specific ellipticity of the positive band at 445 nm in the reduced enzyme is higher when it is bound to the membrane. Cytochrome c possibly shows a negative band at 426 nm in the reduced state when bound to the membrane, in contrast to the positive band at 426 nm observed with the purified electron transport carriers. The inversion of the sign of ellipticity represents the effect of membrane binding and requires further study. Such an inversion is not unprecedented, however; Luzzati and co-workers[23] have demonstrated such effects in CD spectra of cytochrome c and lyosozyme in protein–lipid–water mixtures.

Reduced cytochrome b_{566} shows a CD spectrum with two bands; a positive one at 426 nm and a negative one around 436 nm. There seems to be no detectable difference in this CD spectrum when the cytochrome is reduced in the presence

of antimycin A or HOQNO and when it is reduced in the energized membrane by reversed electron transport[24] or by shift in midpoint potential[25]. This result implies that: either the conformation change accompanying a midpoint potential shift has little effect on the heme moiety, or that the midpoint potential shift is actually an apparent shift due to reverse electron transport[26]. The latter seems more probable than the former, since a substantial shift in midpoint potential would be expected to be accompanied by a substantial change in quaternary structure of the protein in the vicinity of the redox group, namely the heme.

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