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THE ELECTRONIC STATE OF HEME
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I. MAGNETIC CIRCULAR DICHROISM OF
THE ISOLATED ENZYME AND ITS DERIVATIVES

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Magnetic circular dichroism (MCD) spectra have been recorded for beef heart cytochrome oxidase and a number of its inhibitor complexes. The resting enzyme exhibits a derivative shape Faraday $\mathcal{C}$ term in the Soret region, characteristic of low-spin ferric heme, which accounts for 50% of the total oxidase heme $a$. The remaining heme $a$ (50%) is assigned to the high-spin state. MCD temperature studies, comparison with the MCD spectra of heme $a$–imidazole model compounds and ligand binding (cyanide, formate) studies are consistent with these spin state assignments in the oxidized enzyme. Furthermore, the ligand binding properties and correlations between optical and MCD parameters indicate that in the resting enzyme the low spin heme $a$ is due solely to cytochrome $a^{3+}$ and the high spin heme $a$ to cytochrome $a^{2+}$.

The Soret MCD of the reduced protein is interpreted as the sum of two MCD curves; an intense, asymmetric MCD band very similar to that exhibited by deoxymyoglobin which we assign to paramagnetic high spin cytochrome $a^{2+}$ and a weaker, more symmetric MCD contribution, which is attributed to diamagnetic low spin cytochrome $a^{2+}$. Temperature studies of the Soret MCD intensity support this proposed spin state heterogeneity. Ligand binding (CO, CN$^-$) to the reduced protein eliminates the intense MCD associated with high spin cytochrome $a^{2+}$; however, the band associated with cytochrome $a^{2+}$ is observed under these conditions as well as in a number of inhibitor complexes (cyanide, formate, sulfide, azide) of the partially reduced protein.

The MCD spectra of oxidized, reduced and inhibitor complexed cytochrome oxidase show no evidence for heme-heme interaction via spectral parameters. This conclusion is used in conjunction with the fact that ferric, high spin heme exhibits weak MCD intensity to calculate the MCD spectra for the individual cytochromes of the oxidase as well as the spectra for some inhibitor complexes of cytochrome $a_3$. The results are most simply interpreted using the model we have recently proposed to account for the electronic and magnetic properties of cytochrome oxidase (Palmer, G., Babcock, G.T., and Vickery, L.E. (1976) Proc. Nat. Acad. Sci. USA, in press).
Introduction

Although it is now firmly established that the active center of cytochrome oxidase contains two moles of heme a and two atoms of copper (1-3) there is considerable uncertainty regarding the functional relationships between these four metal components (4,5).

As originally proposed by Keilin and Hartree (6) the two heme moieties of the oxidase can be distinguished by their ligand binding properties. One of them, named cytochrome a₃, was postulated to react with common heme ligands such as cyanide and carbon monoxide, while the other, called cytochrome a, was unavailable to such reagents. This heterogeneity with respect to ligand binding has been amply confirmed in a number of laboratories and is most simply interpreted in terms of the so-called "classical model" for the enzyme. In this model the two hemes are asserted to be chemically and spectrally distinct species. Cytochrome a₃ is presumed to be high spin (S = 5/2 in the ferric state, S = 2 in the ferrous state) to account for its ready reaction with external ligands and is expected to exhibit weak absorbance in the a band region. Cytochrome a, on the other hand, is assigned as a typical hexacoordinate low-spin heme protein (S = 1/2 in the ferric state, S = 0 in the ferrous state) with an a band intensity roughly four times that of cytochrome a₃. Cytochrome a₃ is presumed to be the component which reacts with oxygen while cytochrome a is postulated to be the site for reaction with cytochrome c. This classical model has been summarized most forcefully in a review by Lemberg (1).

An alternative hypothesis minimizes any intrinsic spectral differences between the two hemes. In its most extreme form cytochromes a and a₃ are postulated to be indistinguishable and low-spin in the oxidized enzyme and that addition of ligands or reducing equivalents is required for the observed heterogeneity in the properties of the two hemes (7). In this model some form of heme-heme interaction exists such that the redox and/or ligand binding state of one of the hemes a has a pronounced effect on the spectral properties of the second heme a (8-11).

In a third model the two hemes a of cytochrome oxidase have independent spectral properties. However, heme-heme interactions are manifested by interdependent redox
potentials such that reduction or ligand binding by one of the hemes alters the reduction potential of the second heme (12). The arguments in favor of each of these models have been reviewed by Nicholls and Chance (4).

These models make distinct predictions of the coordination geometry around the Fe atoms of cytochromes a and a₃ so that a crucial test for differentiating between these extremes is a determination of the heme a spin-states in the enzyme in various redox states and after reaction with external ligands. EPR spectroscopy has been used extensively for this purpose and while the technique has been most fruitful in the overall characterization of the enzyme, resolution of the above questions has been complicated by the following observations: (i) in the oxidized enzyme only 50% of the heme iron contributes to the low-spin resonance at \( g = 3 \) and only ca. 40% of the copper can be accounted for by the resonance at \( g = 2 \) (13,14). (ii) During the course of a reductive titration both axial and rhombic high-spin EPR signals appear at \( g = 6 \); these resonances account for ca. 15-25% of the total iron although under some conditions the value approaches 35-45% (14-16). These observations have led to contradictory assignments with Wilson and coworkers (9,11) identifying the low-spin species as cytochrome a₃ by virtue of it having the most positive potential while Hartzell et al. (15), noting that the \( g = 3 \) resonance appears to be the site of reduction during reaction with ferro-cytochrome c, assign this resonance to cytochrome a.

Magnetic circular dichroism spectroscopy (MCD) is emerging as a powerful tool in the study of iron spin-states in heme-proteins. The versatility of the method lies in two recent observations: (i) heme iron in both ferric (\( S = 1/2 \) and \( 5/2 \)) and ferrous (\( S = 4/2 \)) valence states show temperature dependent MCD intensity in the Soret region which is indicative of the paramagnetic ground states of these species (17-21) and (ii) the intensity of this MCD in ferric heme proteins is correlated with the fraction of iron in the low-spin state irrespective of the nature of the axial ligands (19). Thus MCD spectroscopy provides spin-state data for both common valence states of heme iron. This information both extends and complements the results available from EPR. Moreover,
MCD has the particular advantage that data are routinely obtained close to ambient temperature in contrast to the extremely low temperatures (ca. 10° K) required for the satisfactory observation of all the species contributing to the EPR spectrum of this enzyme.

We have previously reported preliminary results on the MCD of cytochrome oxidase (22,23) and these data have recently been confirmed (24). Based on our observations we have proposed a model for the enzyme which is similar in many respects to the classical model described above, but has as added hypotheses (i) an antiferromagnetic interaction between cytochrome a₃ and one of the two copper moieties in the resting enzyme and (ii) the occurrence of a conformational change subsequent to partial reduction of the enzyme. This model accounts for the available EPR and magnetic susceptibility data. In the experiments described in this paper we present further data in support of this model and in a subsequent paper we will describe the behavior of the enzyme during reductive titrations.

**Materials and Methods**

Solubilized beef heart cytochrome oxidase was obtained by three different isolation techniques: (a) as described by Volpe and Caughey (25); (b) as described by Hartzell and Beinert (26), and (c) as described by Yu, et al. (27). The spectroscopic ratios for solubilized product were:

\[
\frac{\text{OD}_{\text{red}} (605 \text{ nm})}{\text{OD}_{\text{ox}} (600 \text{ nm})} = 2.10 - 2.22 \text{ and }
\]

\[
\frac{\text{OD}_{\text{red}} (443 \text{ nm})}{\text{OD}_{\text{ox}} (418 \text{ nm})} = 1.26 - 1.32
\]

which are in agreement with those cited by Lemberg (1). The observed MCD spectra for cytochrome oxidase obtained by these different isolation procedures showed little variation. Moreover, solubilized yeast cytochrome oxidase prepared by an unpublished procedure of J.N. Siedow, exhibited similar MCD properties. Enzyme concentration on a per
heme a basis was determined using $\epsilon_{\text{red}} (605 \text{ nm}) = 21.2 \text{ mM}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{ox}} (419 \text{ nm}) = 80 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{red}} (443 \text{ nm}) = 106 \text{ mM}^{-1} \text{ cm}^{-1}$. All spectra shown were recorded using the Hartzell preparation (26). The temperature was maintained at 4°C unless noted. The buffer system used is noted in the figure legends.

The resting enzyme, with no additions, is taken as fully oxidized. Reduction was carried out using a few crystals of solid dithionite (Virginia Smelting) and the absorbance monitored at either 605 nm or 443 nm until no further increase was observed, usually 30-60 min. The formate-oxidized cytochrome oxidase complex was obtained by incubating the oxidized protein in 60 mM neutralized sodium formate. The absorption spectrum of the resulting complex showed a 5 nm blue shift in the Soret region, from 422 nm for the oxidized enzyme to 417 for the inhibitor complex (28). Partial reduction of this species was carried out either by addition of 0.1 mM TMPD and 10 mM ascorbate or a few grains of solid dithionite. In both cases the resulting complex had Soret absorption maxima at 441 nm and 415 nm. The cyanide derivative of the oxidized enzyme was prepared by incubating oxidized cytochrome oxidase with neutral 6 mM KCN. The formation of the cyanide complex was followed by the increase in absorbance at 426 nm; this generally required three to five hours for completion. The cyanide complex of partially reduced cytochrome oxidase was formed by the addition of either a few grains of solid dithionite or 0.1 mM TMPD plus 10 mM ascorbate to the oxidized enzyme preincubated in the presence of 1 mM KCN. When fully formed the inhibitor complex exhibited a peak at 441 nm and a shoulder at 429 nm in the Soret optical spectrum. The sulfide and azide derivatives were obtained by an analogous procedure except that the inhibitor concentrations were 10 mM and 4 mM respectively. The cyanide complex of fully reduced cytochrome oxidase was prepared by the addition of 6 mM KCN, previously made anaerobic by bubbling the KCN stock solution with Argon gas, to the dithionite reduced enzyme. The reduced protein-cyanide complex had a Soret absorption peak at 443 nm with a slight shoulder in the 430 nm region. Carbonmonoxy-cytochrome oxidase was prepared either by bubbling the reduced protein with CO gas or, more gently, by replacing the atmosphere in an anaerobic, titration cuvette with CO gas. With both procedures, the Soret absorp-
tion exhibited the 430 nm peak characteristic of the reduced protein-carbon monoxide complex. "Oxygenated" cytochrome oxidase was obtained by bubbling air through the dithionite-reduced enzyme.

Heme a was isolated as described by Takemori and King (29) and was stored dry under Argon at -25° C. Concentrations of heme were established using an extinction of E_red (587 nm) 27.4 mM^{-1} cm^{-1} for the dithionite-reduced pyridine hemochrome (29). Heme a-imidazole complexes were prepared essentially as described by Vanderkooi and Stotz (30), except that the dried heme a was dissolved initially in a small volume of 0.01 N NaOH rather than ether. Buffer and imidazole were then added to the concentrated heme a solutions; the final pH was 7.4.

Optical spectra were recorded using a Cary 14 spectrophotometer thermostatted at 4° C. Maximal optical densities of cytochrome oxidase and the heme a solutions used in obtaining MCD spectra never exceeded 1.2. MCD spectra were recorded using the computer interfaced spectrometer described by Sutherland, et al. (31). The calibration of the instrument using D-10-camphorsulfonic acid for natural circular dichroism and potassium ferricyanide for the magnetic field determination has been described by Vickery et al. (19). For measurements at 4° C, 1 cm pathlength cells and a magnetic field of approximately 1.36 Tesla were used; for experiments in which the sample temperature was varied down to 77° K, a low-temperature optical dewar and a 3.0 mm cell with a copper constantan thermocouple incorporated were used; the applied magnetic field was 0.9 Tesla. All spectra are presented as the difference in extinction for left versus right circularly polarized light (\(\Delta \varepsilon = \varepsilon_L - \varepsilon_R\)) on a per heme a basis and are normalized to an applied magnetic field of 1 Tesla (10 kG), i.e., as \(\Delta \varepsilon/\text{Tesla}\). The use of these units allows direct comparison of all MCD spectra obtained. The data can be converted to \([\theta]_m\) units normalized to a 1 gauss field by the relation \([\theta]_m = 0.33 \frac{\Delta \varepsilon}{\text{Tesla}}\). The spectral bandwidth in recording MCD spectra was 2 nm or less. The instrument scan rate and time constant were either 0.5 nm/sec with \(\tau = 0.3\) sec. or 1 nm/sec with \(\tau = 0.1\) sec. To reduce the noise level, some spectra were subjected to a 25 point least-squares polynomial smoothing algorithm. The original and smoothed spectra were then overlayed on
a video monitor to confirm that no spectral distortion had been introduced by the digital filter. Spectra so manipulated are identified in the legends to the figures. EPR spectra were recorded at X-band using a Varian E-6 spectrometer operated at approximately 12° K.

**Results**

**Resting enzyme**

Figure 1 shows the MCD and absorption spectra for oxidized cytochrome oxidase in the Soret and visible regions. In the visible region, there is a derivative shape MCD curve associated with the 600 nm alpha-band which is reversed to that routinely observed with other heme-proteins in that the arm of negative intensity is to shorter wavelengths. The amplitude of this band is relatively insensitive to temperature (see below) over the range 130-300° K and this, taken in conjunction with the derivative shape, allows us to assign this band to a Faraday A term. A second, less intense, derivative MCD curve is present to longer wavelengths with a crossover at 665 nm; this may be associated with the optical absorption band observed at 655 nm in the oxidized protein. Although a broad positive MCD band is present at 520 nm none of the fine structure observed with other ferric heme proteins is seen in the region from 480-560 nm. This fine structure has been interpreted as arising from charge-transfer bands which exhibit MCD transitions that are highly dependent on the nature of the axial ligand (19).

In the Soret region a derivative type curve is also observed along with a shoulder at shorter wavelengths near 400 nm. The crossover for the S-shaped curve is at 427 nm with positive and negative extrema at 420.5 and 434 nm respectively; it is considerably red-shifted when compared to the absorption maximum of the oxidized enzyme at 418 nm. This red shift is anomalously large when compared with other heme proteins which exhibit this derivative shape curve (19,20,32,33). In these the MCD approximates the first derivative of the absorption spectrum and the MCD zero crossing corresponds closely to the maximum in the absorption spectrum. In addition MCD transitions of this type have been shown to be temperature dependent, therefore corresponding to Faraday $\alpha$ terms, and typical of ferric heme proteins in which all or part of the heme iron is in the $S = 1/2$, $S = 3/2$, or mixed-spin states.
low-spin state (17,19,20). Similarly if the derivative shaped MCD curve observed in the Soret region for cytochrome oxidase (Fig. 1) is due to an S = 1/2 paramagnetic ground state, strong temperature dependence for the intensity of this band is expected. The results of Fig. 2 show that this is indeed the case for the Soret MCD of resting cytochrome oxidase. At both 421 and 435 nm the amplitude of the MCD spectrum is directly proportional to 1/T (Fig. 2, inset) demonstrating for cytochrome oxidase that this MCD band arises from Faraday C terms. The data of Fig. 2 were obtained only to -145° C; however, in a second set of experiments with oxidase in which cytochrome bc₁ contamination interfered at wavelengths less than 415 nm, we were able to obtain MCD spectra to -196° C, which were consistent with the results of Fig. 2. The observation that the MCD intensity varies linearly with temperature down to liquid nitrogen temperatures indicates the absence of temperature dependent high-spin ↔ low-spin equilibria. Since these data were obtained at temperatures which overlap the temperature range for the EPR data of van Gelder and Beinert (13) we attribute the MCD in the Soret to the 50% of the heme a occurring as ferric low-spin heme, as recently indicated by careful EPR integrations of resting cytochrome oxidase (14,16).

However, a comparison of the amplitude of the trough of the Soret MCD of cytochrome oxidase (Δε₄₃₅/Tesla = 20) with those observed for metmyoglobin derivatives and cytochromes b and c (Δε₄₃₅/Tesla = 90 for 100% low-spin (19)) would indicate that by intensity criteria only 20% of the heme a is low-spin. We have resolved this apparent discrepancy by preparing heme a imidazole model compounds and comparing their MCD intensity with that observed for cytochrome oxidase. The optical properties of these derivatives were similar to those reported by Vanderkooi and Stotz (30). In the absence of SDS the complex was heterogeneous; the EPR intensity of the oxidized compound accounted for only 50-60% of the total heme a and, when reduced, the optical spectrum exhibited two maxima in the Soret region, at 429 and 454 nm. The MCD spectrum of the oxidized form in the absence of SDS had a derivative shape in the Soret; however, the intensity was low (Δε₄₂₈/Tesla = 28) and the MCD zero crossing (λ = 420 nm) is shifted relative to the 424.5 nm absorption maximum confirming the heterogeneity of the sample. Dispersing the heme by the addition of 1 mM SDS resulted in homogeneous behavior; the Soret
absorption maximum of the oxidized derivative occurred at 422 nm and upon reduction, only a single peak at 436 nm was observed. The intensity of the EPR spectrum of the oxidized compound now accounted for 100% of the heme a when compared to metmyoglobin cyanide. The MCD spectrum for this oxidized species is shown in Fig. 3 together with that observed for the resting enzyme. The cross-over for the heme a-imidazole-SDS derivatives is at 421 nm, shifted only slightly from the peak at 422 nm in the absorption spectrum while the intensity of the trough at 428 nm (Δε_{428}/Tesla ≈ 43) is about twice that observed with the resting enzyme. The decrease in the MCD intensity of heme a when compared to low-spin protoheme derivatives (19) presumably resides in the fact that the protoheme lacks both the formyl and farnesyl-ethyl substituents of heme a (5).

**Oxidized enzyme-cyanide and formate complexes**

Addition of cyanide to the oxidized enzyme shifts the Soret maximum of the optical absorption spectrum from 419 nm to 426 nm consistent with a high-spin to low-spin transition (34). The MCD spectrum of this derivative (Fig. 4) intensifies by 100% when compared with the resting enzyme but the crossover point stays at 427 nm, which is now close to the position of the absorption maximum. Assuming, with Keilin and Hartree (6), that cyanide ligates exclusively to cytochrome a₃ leads to the conclusion that the changes shown in Fig. 4 reflect the conversion of cytochrome a₃ from the high-spin to low-spin forms. A similar conclusion has been reached by van Buren, et al. from the effect of CN⁻ on the visible absorption spectrum of the oxidized enzyme (35); difference MCD spectra will be discussed below.

In contrast to the red shift induced in the absorption spectrum by CN⁻ binding, Nicholls (28) has recently reported that binding of formate shifts the Soret absorption maximum to the blue by 4-5 nm. We have confirmed this observation. However, between 360 and 500 nm the MCD spectrum of the enzyme-formate complex (Fig. 4) is identical to that of the resting enzyme. Since the MCD intensity of most ferric high-spin proteins is weak (19), the insensitivity of the MCD spectrum to formate binding indicates that the formate binding site is high-spin both in the absence and presence of this inhibitor.
Again assuming that formate binds solely to cytochrome $a^3_3$, we conclude that both the cytochrome $a^3_3$·formate complex and cytochrome $a^3_3$ in the resting enzyme are high spin. The high spin to low spin transition observed by MCD for CN$^-$ binding to cytochrome $a^3_3$ in the resting enzyme offers very strong support for these assignments.

**Reduced cytochrome oxidase**

The visible and Soret MCD and absorption spectra for reduced cytochrome oxidase are shown in Fig. 5. Comparison with Fig. 1 shows that the intensity of the MCD in the region of the α band roughly doubles on reduction although the sign of the A term at 598 nm remains unchanged and anomalous. The weak MCD band centered at 665 nm disappears on reduction, as does the corresponding band in the optical spectrum. Because of the electron withdrawing properties of the formyl side-chain the β bands are very weak (34) and we observe very little MCD intensity in the spectral range from 500-560 nm except for a negative shoulder at 568 nm and weak but complex structure between 500 and 530 nm. The weak derivative curve centered at 550 nm is due to a slight contamination of the oxidase with complex III (cytochromes $b-c_1$). The MCD of reduced b and c type cytochromes is extremely intense ($\Delta c$/Tesla = 200-300, depending on bandwidth) (20) and this region of the spectrum provides a very sensitive test for the presence of Complex III contamination.

The change in MCD upon reduction is much more dramatic in the Soret region with the spectrum changing sign and growing about four-fold in intensity (cf. Fig. 1). The crossover at 440.5 nm is slightly blue-shifted from the absorption maximum at 443 nm in the optical spectrum of the reduced protein. In general the MCD spectrum of reduced oxidase bears a striking resemblance to the spectra obtained with deoxymyoglobin (19), deoxyhemoglobin (18) and ferro-horseradish peroxidase (36). All three of these proteins are ferrous high-spin and exhibit an asymmetric derivative type Soret MCD with the trough occurring at higher energy than the larger amplitude peak; again the cross-over is blue shifted with respect to the absorption maximum. In addition all three protoheme proteins show a negative shoulder some 15-30 nm to shorter wavelength of the major trough; this secondary feature appears at 413.5 nm in the MCD spectrum of reduced cyto-
The principal difference observed with oxidase compared to the other three proteins is the larger amplitude observed for the principal trough; for cytochrome oxidase the peak-to-trough ratio is 1.95, while for deoxyhemoglobin it is 3.45 (18).

The temperature dependence of the 440 nm band of reduced oxidase (Fig. 6a) establishes the paramagnetic origin (Faraday C terms) of the Soret MCD for the reduced protein. In addition to an increase in intensity as the temperature is lowered there is also a change in the shape of the spectrum; this is most clearly seen in the difference spectrum between the MCD spectra recorded at -145 and 0° C (Fig. 6b). The peak present at 447 nm at 0° has shifted to 446 nm and the peak to trough ratio has increased from 1.95 to 2.45 over this temperature range. The corresponding CD spectra, recorded simultaneously (31), exhibited a temperature independent peak at 446 nm with only slight (<5%) changes in amplitude indicating that any intrinsic band narrowing must be small and cannot be the origin of the increase in MCD intensity. Plots of MCD intensity vs 1/T (Fig. 6a, inset) are linear at the four wavelengths shown. However, these plots show marked deviations from behavior expected from a simple, temperature dependent Boltzmann distribution. This effect is most pronounced at 447 and 452 nm: the Boltzmann factor between 273° K and 128° K is 2.13, the intensity increase \( \frac{\Delta e/\text{Tesla (128°)}}{\Delta e/\text{Tesla (273°)}} \) is 2.42 at 447 nm but only 1.66 at 4.52 nm. This behavior suggests the contribution of Faraday A and/or B terms to the spectra in the region around 450 nm. However, due to the possibility of small band shifts and narrowing as the temperature is lowered (37), and the resulting uncertainties in extrapolation to infinite temperature, we have not carried out this analysis in more detail.

**Reduced cytochrome oxidase: carbon monoxide and cyanide complexes**

The MCD spectra for reduced cytochrome oxidase and its inhibitor complexes with CO and CN\(^-\) are shown in Fig. 7. In the presence of CO the Soret MCD peak decreases in intensity by about one-half and is red-shifted by 5.5 nm compared to the untreated enzyme; the peak is at 452.5 nm with the crossover at 447 nm, a negative shoulder at approximately 434 nm, a second zero-crossing at 428 nm and a small positive peak at 423 nm.
The optical absorption spectrum of this derivative has a peak at 430 nm which is classically assigned to the $a^2_3\cdot CO$ complex on the basis of photochemical action spectra (4). In addition, there is a shoulder at 442.5 nm which is assigned to cytochrome $a^2_3$. On the basis of this absorption spectrum we assign that portion of the MCD curve with a peak at 423 nm, crossover at 428 nm and shoulder at 434 nm to the cytochrome $a^2_3\cdot CO$ complex. This weak, derivative shape curve is qualitatively similar to the spectra of low-spin ferrous complexes of hemoglobin (18) and myoglobin (19) but the intensity is much smaller than that observed for the protoheme proteins. The more intense, red-shifted MCD curve with a peak at 452.5 is assigned to cytochrome $a^2_3$. These assignments are borne out by the cyanide and formate derivatives reported below as well as by reductive titrations to be reported in a subsequent paper.

The MCD spectrum of the $CN^-$ complex of reduced cytochrome oxidase (Fig. 7) resembles the CO complex closely. The peak occurs at 452.5 nm and the zero-crossing occurs at 446 nm. The absorption spectrum of the complex showed a peak at 443 nm with a slight shoulder at ca 430 nm; this shoulder may correspond to incomplete reduction of cytochrome oxidase prior to addition of the $CN^-$ leading to the formation of the $a^2_3\cdot CN$ complex. This would result in a greater intensity between 420-435 nm (vide infra) and may account for the increased MCD around 440 nm compared to the CO complex.

**Partially reduced cytochrome oxidase: cyanide, formate, sulfide and azide complexes**

Complexes of partially reduced cytochrome oxidase with a number of inhibitors can be prepared in the aerobic steady-state. Under these conditions, cytochrome $a$ is largely reduced while the cytochrome $a^2_3\cdot$ inhibitor complex is primarily oxidized (4,38). The MCD and absorption spectra of two of these derivatives, cyanide and formate, are shown in Fig. 8.

The derivative curve with a peak at 452 nm ($\Delta \epsilon$/Tesla = 35) and crossover at 447 nm is common to both spectra and corresponds to low-spin cytochrome $a^2_3$, as observed in the MCD spectra of fully reduced cytochrome oxidase in the presence of carbon monoxide or cyanide. However, in the region 400-440 nm the spectra of the two derivatives are markedly different. The strong MCD intensity observed in this region with the $CN^-$ complex
is diagnostic of low-spin, ferric heme $a$ (cf. Fig 3, heme $a +$ imidazole hemichrome).

EPR observations of low-spin ferric resonances in the partially reduced cyanide complex are consistent with this MCD behavior (39). Therefore, as in the complex of fully oxidized cytochrome oxidase with cyanide, the binding of this inhibitor converts cytochrome $a^3$ to the low-spin state. In contrast, the MCD intensity observed in the 400-430 nm region for the formate complex is weak even though there is intense Soret optical absorption ($\lambda_{\text{max}} = 415$ nm) associated with the formate-cytochrome $a^3$ complex (Fig. 8). This weak MCD is diagnostic of high-spin ferric heme and, in agreement with the data of Fig. 4, indicates that the $a^3$-formate complex is high spin. Nicholls has reached a similar conclusion (28). Due to the weak MCD intensity for the high-spin $a^3$-formate compound and also the 25 nm separation in absorption maxima for the two heme species, we conclude that the derivative MCD curve (442 nm trough, 447 nm crossover, 451.5 nm peak) in Fig. 8 is due solely to cytochrome $a^2$. This assignment will be used subsequently in calculating MCD difference spectra.

We have also observed the MCD spectra of cytochrome oxidase in the presence of sulfide and azide with reductants (TMPD and ascorbate) added subsequent to the inhibitor. The spectra exhibit the characteristics of ferrous cytochrome $a$ with peak at 452 nm ($\Delta\varepsilon$/Tesla = 40), a crossover at 447 nm and a trough at 438 nm. As observed by Nicholls (38), we also see partial reduction of the $a^3$-inhibitor complex under these conditions so that interpretation of the spectra at wavelengths less than 435 nm is difficult.

"Oxygenated" cytochrome oxidase

We have prepared the "oxygenated" derivative of cytochrome oxidase by oxygenating the dithionite reduced enzyme. The MCD spectrum recorded 30 min after oxygenation is shown in Fig. 9. This spectrum is identical to that of the resting enzyme, apart from some additional intensity at 452 nm which we attribute to incomplete reoxidation of cytochrome $a^2$. The absorption spectrum of the species ($\lambda_{\text{max}} = 427.5$ nm) gradually changed overnight to that of the oxidized enzyme ($\lambda_{\text{max}} = 419$ nm), but the MCD remained the same.
MCD spectra of the individual cytochromes of cytochrome oxidase and their derivatives

The spectra presented above indicate that the Soret MCD intensity for native cytochrome oxidase and its liganded derivatives is highly dependent upon the oxidation and spin-states for cytochromes $a$ and $a_3$. Under certain conditions it is possible to obtain MCD bands which are due almost exclusively to one of these two cytochromes. For example, in the resting oxidase the derivative curve with crossover at 427 nm represents 50% of the heme $a$ in a low-spin state, and by comparison with EPR experiments, we have assigned this to cytochrome $a_3^{3+}$. Under these conditions cytochrome $a_3^{3+}$ is postulated to be high spin and to exhibit very weak MCD intensity. Similarly, the partially-reduced formate derivative exhibits a derivative shape curve with crossover at 447 nm which we have assigned to cytochrome $a_2^{2+}$; the weak MCD intensity in the region of the $a_3^{3+}$·formate absorption maximum (415 nm) confirms the high-spin nature of the $a_3^{3+}$·formate complex.

MCD spectra of the fully reduced carbon monoxide and cyanide derivatives of cytochrome oxidase resemble that of the partially reduced protein in the presence of formate which implies that the MCD intensity associated with both cytochrome $a_3^{2+}$·CN and cytochrome $a_3^{2+}$·CO is weak and that the dominant feature in these spectra is likewise the MCD contribution of cytochrome $a_3^{2+}$.

These arguments allow us to obtain the MCD spectra of cytochrome $a$ in both the oxidized and reduced states essentially free from any MCD contribution from cytochrome $a_3$. By preparing difference spectra in which the MCD contribution from cytochrome $a$ is subtracted from the MCD spectra of cytochrome oxidase derivatives in which both cytochromes are MCD active, we should be able to determine the intensity and bandshape due to cytochrome $a_3$. The internal consistency of this method can be established by applying the technique to a number of cytochrome oxidase derivatives.

Figure 10 shows the MCD spectrum attributed to cytochrome $a_3^{2+}$, calculated by subtracting the cytochrome $a_3^{2+}$ contribution to the MCD spectrum of the fully reduced protein. The calculated MCD spectrum for $a_3^{2+}$ has been obtained by 3 different subtraction procedures: (1) fully reduced oxidase minus partially reduced + formate (2) fully reduced oxidase minus fully reduced + cyanide (3) fully reduced oxidase minus fully
reduced + carbon monoxide. Fig. 10a shows the MCD spectra for reduced cytochrome oxidase and the formate complex of the partially reduced enzyme, the difference spectrum is shown in Fig. 10b. Figs. 10c and d show the difference spectra for the CO and CN derivatives respectively (see also Fig. 7). In each case the shape of the resulting spectrum shows very strong similarity to the direct MCD spectrum of deoxyhemoglobin, deoxy-myoglobin. The most dramatic aspect of this similarity is the anomalous sign of the derivative type spectrum (peak to longer wavelengths) observed for all three of these proteins. Treu and Hopfield (18) and Livshitz et al. (21) have considered the origins for this type of sign reversal in detail and have attributed it to a large spin-orbit coupling in the excited state of deoxyhemoglobin. In addition to this sign reversal the similarities extend to the asymmetry in the peak and trough intensities for the derivative curve. For the cytochrome $a_3^{2+}$ spectrum the intensity of the peak at 445.5 nm is approximately 3.8 times greater than the trough around 430 nm; for the deoxy-protoheme proteins the peak to trough ratios are ca 3.5. Finally all three proteins show a negative trough 15-30 nm to higher energy from the derivative crossover.

The full-width at half-height for the peak of the cytochrome $a_3^{2+}$ MCD spectrum is considerably less than that for the deoxy-protoheme proteins: 8.6 nm compared to 14-18 nm for deoxyhemoglobin. This decrease in bandwidth explains the greater MCD intensity at the peak observed for cytochrome $a_3^{2+}$. By comparing integrated intensities (obtained as the product of (full width at half height)$^2$ and amplitude) for deoxyhemoglobin and $a_3^{2+}$ we calculate that, on a per heme basis, about 45% of the heme in cytochrome oxidase contributes to the difference spectrum.

Of the three derivatives used in obtaining the difference spectra of Fig. 10, that of partially reduced enzyme plus formate yields the spectrum of cytochrome $a_3^{2+}$ in its purest form (vide supra). The effect of both CO and CN$^-$ on cytochrome oxidase appears to be analogous to the effect of CO on myoglobin and hemoglobin, viz the conversion of high spin ferrocytochrome $a_3^{2+}$ to a low-spin ferrous state.

We have proposed above that the CN$^-$ complex of ferric cytochrome $a_3$ is low spin. Accordingly, we expect a fairly strong, derivative shape Soret MCD band for this derivative.
Fig. 11 shows that this curve is indeed observed and can be determined by three different procedures. Fig. 11a shows the difference obtained by subtracting the MCD spectrum of oxidized cytochrome oxidase from that observed for the CN⁻ complex of the oxidized protein. Cytochrome a³⁺ is low spin in both species; therefore, this difference represents a³⁺-CN minus a³⁺. We have assigned weak MCD intensity to high spin cytochrome a³⁺ so the difference spectrum primarily represents the MCD contribution of cytochrome a³⁺·CN.

Fig. 11b is the difference spectrum for the CN⁻ complexes of partially and fully reduced cytochrome oxidase. Cytochrome a²⁺ is common to both species and, since we have assigned weak intensity to cytochrome a³⁺·CN, this spectrum again represents cytochrome a³⁺·CN.

Finally Fig. 11c shows the difference spectrum for the partially reduced enzyme complexes of CN⁻ and HCOOH. The weak MCD intensity of the high spin cytochrome a³⁺·formate complex (cf. Fig. 8) allows the assignment of this spectrum to the a³⁺·CN complex.

The similarity of the spectra of Fig. 11 is striking. The MCD crossover at 427 nm is common to all three and, moreover, this wavelength corresponds to the maximum absorption of the oxidized cytochrome oxidase·cyanide complex. The peak and trough wavelength positions also agree well and the observed derivative shape of the difference spectra reinforces the assignment of the a³⁺·CN complex to ferric low-spin heme a. There are differences in the MCD intensities at the extrema. These may reflect incomplete formation of the various inhibitor complexes or weak MCD contributions from the high spin a³⁺ derivatives (Figs. 11a or c) or the a³⁺·CN complex (Fig. 11b). Nonetheless the intensity at the 435 nm trough (Δε₄₃₅/Tesla = 20-28) is approximately half the intensity observed for the heme a - Imidazole - SDS model compound of Fig. 3 and indicates, in agreement with the original postulates of Keilin and Hartree (6), that about half the heme a of cytochrome oxidase is reactive with CN⁻. A further conclusion permitted by the difference spectra of Fig. 11 is that the MCD spectra of cytochrome a in both valence states are insensitive to the ligation state of cytochrome a³⁺·CN.

Table I summarizes the redox and ligation dependence for the MCD spectra of a number of cytochrome oxidase derivatives as well as the probable origin for the MCD curves and the proposed spin state of the heme under consideration. In general, ferric
low spin and ferrous high spin derivatives are MCD intense while ferric high spin species exhibit weak MCD. Ferrous low spin derivatives, in agreement with observations on cytochromes b$_5$ and c and carbonmonoxy- and oxymyoglobin (19,20,33,40,41) exhibit variable Soret MCD intensity which appears to be highly dependent on second-order effects.

**Discussion**

The MCD spectra of cytochrome oxidase and its derivatives are most simply interpreted in terms of the classical scheme of Keilin and Hartree (6) with each heme a existing in a different chemical environment in both redox states of the enzyme.

Although the theoretical bases for the origin of MCD spectra is under active investigation currently the most promising application of the method depends upon comparisons and correlations drawn from spectra of compounds of known chemical composition, valence and spin-state. By this means we have established that about 50% of the heme a in the resting enzyme is low-spin with the balance presumably in the high-spin state. Recent EPR data has established that the species with g-values of 3.03, 2.21 and 1.45, typical of low-spin heme, has an intensity equivalent to one low-spin heme/mole enzyme (14,16). Beinert and coworkers (13,15,16,42) have assigned this low-spin resonance to cytochrome a$^{3+}$ since it appears to be the site for ferrocytochrome c oxidation. Correlating these data we conclude that the MCD derivative curve centered at 427 nm in the resting enzyme arises solely from low-spin cytochrome a$^{3+}$, the contribution from high spin cytochrome a$_3$$^{3+}$ being too small to be identified. Moreover, the linearity of the peak and trough MCD intensities as a function of 1/T (Fig. 2) demonstrate the absence of significant thermal spin equilibria in the resting enzyme. However, in this spectrum the crossover occurs at 427 nm, significantly to the red of the 418 nm absorption maximum in the optical spectrum of the oxidized enzyme. This apparent discrepancy is readily resolved when one considers previous conclusions on the contributions of the two hemes to the optical absorption. By assuming no interaction between the two hemes, Yonetani (43), Horie and Morrison (44) and more recently Vanneste (45) have utilized difference optical spectroscopy to establish the spectra of cytochromes a and a$_3$. Vanneste combined these difference spectra with the photochemical action spectrum of the cytochrome oxidase-CO complex to arrive at the absolute spectra of cytochrome a$^{3+}$ and a$_3$$^{3+}$; these have maxima...
in the Soret at 427 nm and 414 nm respectively. The close correlation of the 427 nm absorption maximum for cytochrome $a_{3}^{3+}$ with the 427 nm MCD crossover we observe for the resting enzyme strongly supports our assignment of this derivative MCD curve to cytochrome $a_{3}^{3+}$. Malmstrom (3) has previously pointed out that the Soret absorption band for cytochrome oxidase is about two-fold broader than expected for a heme protein in which there is no heterogeneity in the environment of the heme. This unusual width can also be attributed to the contributions from two species, cytochrome $a$ at 427 nm and $a_{3}$ at 414 nm, the sum of the two spectra yielding the maximum for the enzyme at 418 nm. In agreement with the 13 nm difference in peak positions for cytochromes $a_{3}^{3+}$ and $a_{3}^{3+}$ calculated via absorption data are the CD difference spectra obtained by Tiesjema and van Gelder (46). These results suggest that the CD band(s) associated with cytochrome $a_{3}^{3+}$ are shifted 8-12 nm to the red of the Soret CD bands for cytochrome $a_{3}^{3+}$. Finally, Vanneste's data (45) on the position of the Soret maxima, and particularly the fractional contributions of cytochromes $a$ and $a_{3}$ to the 600 nm absorption band (80% $a$ cytochrome) are strongly supportive of our assignments of $a_{3}^{3+}$ and $a_{3}^{3+}$ to high-spin and low-spin states respectively.

Further evidence for these general conclusions are the changes found after addition of formate and cyanide to the oxidized enzyme. With formate binding, there is no significant change in the Soret MCD band shape or intensity; since this ligand stabilizes high-spin cytochrome $a_{3}^{3+}$ the conclusion that $a_{3}^{3+}$ is already high-spin prior to formate addition is easily reached. In contrast, upon CN$^{-}$ binding the MCD intensity centered about 427 nm doubles but the position of the crossover does not change, an observation which is most simply explained by assuming that cyanide binds to cytochrome $a_{3}$ and induces a high-spin to low-spin transition so that all of the heme $a$ is now low-spin and spectrally very similar. Consistent with this interpretation is Vanneste's result that the calculated absorption maximum of the cytochrome $a_{3}$·CN compound occurs at 426 nm (45); the shift in the Soret maximum absorption to 426 nm upon CN$^{-}$ binding is also consistent with a high-spin to low-spin transition. Further support for this conclusion is provided by CD experiments (46,47) which show that the ellipticity in the Soret region is composed of a number of components and that the addition of cyanide results in the near elimination of a CD component near 410 nm and a slight red-shift (2 nm) in the maximum of the CD spectrum. The CD spectra we
have recorded simultaneously with the MCD spectra throughout this work confirms this result.

The conclusion that cytochrome $a_3^{3+}$ is high spin has led us to propose a specific model for the active center of cytochrome oxidase (23). In this model cytochrome $a_3^{3+}$ and the EPR detectable copper ($Cu_A^{2+}$) are magnetically isolated while cytochrome $a_3^{3+}$ and the EPR undetectable copper ($Cu_B^{2+}$) are antiferromagnetically coupled to give an $S = 4/2$ ground state; it was suggested that the antiferromagnetic coupling is mediated by an imidazole which simultaneously coordinates the copper and iron in a manner similar to the bridging histidine in superoxide dismutase (48). With these assignments for the metals it is possible to account for the published magnetic susceptibility (49,50) of the enzyme and the behavior of the various EPR species observed during a reductive titration of the enzyme (16). The model predicts a value of 32 for $\mu_{\text{eff}}^2$ which decreases only slightly, to 24-29, on full reduction.

Because of its even spin the antiferromagnetically coupled pair will not exhibit EPR under conventional operating conditions; addition of cyanide converts $a_3^{3+}$ to a low-spin species but need not eliminate the antiferromagnetic coupling between the iron and copper. The apparent discrepancy between the typical ferric low-spin MCD spectrum observed for cytochrome $a_3^{3+}\cdot CN$ and the lack of a corresponding low-spin EPR signal in the $CN^-\cdot$ treated oxidized enzyme may reside in the extremely low temperatures required for the EPR measurements, i.e. at $0^\circ$ C the coupling between low spin $a_3^{3+}$ and $Cu_B^{2+}$ may be sufficiently weak so that both the $S = 0$ and $S = 1$ coupled states are occupied, thus allowing detection of a paramagnetic state ($S = 1$) by MCD. However, the low temperature of the EPR measurement may result in extensive occupancy of only the $S = 0$ state. We are currently exploring this possibility by MCD. Upon partial reduction of the cyanide treated enzyme both MCD and EPR measurements detect the low spin cytochrome $a_3^{3+}\cdot CN$ complex presumably since the $Cu_B$ has been reduced under these conditions and $a_3^{3+}\cdot CN$ now behaves as an isolated and typical $S = 1/2$ spin system.

Our temperature dependence data on the MCD of the oxidized enzyme was obtained over the range (77°-273°) and overlaps the magnetic susceptibility results of Tsudzuki and Okunuki (48) and the EPR data of Beinert and coworkers (10°-77°) (13,16). All of the data are consistent with the absence of a thermal spin state equilibrium in the range of
10-300°, and establishes that the magnitude of the proposed cytochrome $a_3^{3+} - CuB^{2+}$ exchange interaction is considerably larger than 200 cm$^{-1}$. This conclusion coupled with the recent data on the intensity of the low-spin EPR (14,16) argue strongly against a model in which the two hemes of cytochrome oxidase and/or one of the coppers are coupled to give rise to a $g = 3$ signal (5,7).

The MCD spectrum of the reduced enzyme is most simply interpreted as the sum of two components; an intense hemoglobin-like spectrum from high-spin ferrous cytochrome $a_3^{2+}$ with a positive peak at 446 nm, and a weaker, low-spin ferrous cytochrome-like spectrum for cytochrome $a_2^{2+}$ with a peak at 452 nm. By comparing the integrated intensities for deoxyhemoglobin and the difference spectrum of cytochrome $a_3^{2+}$, (Fig. 10) we estimate that the high and low-spin species are present in approximately equal amounts. The crossover point for cytochrome $a_3^{2+}$ is at 440 nm to be compared with the value of 442.5 nm obtained by Vanneste (45) for the wavelength maximum of this species, while the comparable values for cytochrome $a_2^{2+}$ are 447 and 444 nm respectively.

The temperature dependence of the spectrum of the reduced enzyme reveals greater paramagnetism at 447 than at 452 nm consistent with the expected contributions from a Faraday C term for the high-spin cytochrome $a_3^{2+}$ (446 nm) and a Faraday A term from the diamagnetic low-spin ferrous cytochrome $a_2^{2+}$ (452 nm). Analogous temperature dependence studies on deoxymyoglobin show that the MCD intensity for this $S = 2$ system increases with the Boltzmann factor whereas ferrocytochrome $c$ ($S = 0$) shows only slight temperature effects (19-21).

Several recent models for cytochrome oxidase have invoked a strong heme-heme interaction to explain certain redox and ligand binding properties of the enzyme (4). These interactions are seen most clearly upon ligand binding in which changes of up to 70% in extinction coefficients for cytochromes $a$ and $a_3$ are postulated to occur (8). The data we have presented here argue against the existence of any heme-heme interactions which modify spectral properties to any great extent. This is seen most clearly for the MCD peak of cytochrome $a_2^{2+}$ at 452 nm. The intensity of this peak is independent of the redox or ligation state of cytochrome $a_3$ under all experimental conditions we have
studied. For example, the partially and fully reduced cyanide derivatives of cytochrome oxidase (Figs. 7 and 8) are similar both in peak position (452 nm) and intensity. This type of MCD spectrum is also seen in the reduced cytochrome oxidase-CO complex and we see comparable behavior with the formate, azide and sulfide complexes; thus the spectral properties of reduced cytochrome \( a \) are unaffected by changes in the redox or spin state of cytochrome \( a_3 \).

The data of Fig. 11 also argue strongly against extensive spectral heme-heme interaction in cytochrome oxidase. The spectrum of the cytochrome \( a_3^{3+}\cdot CN^- \) complex was obtained by three different subtraction techniques. Two of these methods involved cytochrome \( a \) in the ferrous state (Figs. 11b and c) and, the third, cytochrome \( a \) was in the ferric state (Fig. 11a). The close similarity of these three spectra establishes that the redox state of cytochrome \( a \) has little, if any, effect on the MCD spectrum of the cyanide complex of \( a_3^{3+} \), and further weakens the credibility of "heme-heme" interaction manifested by extensive interdependence of spectral properties.

The interpretation of CD spectra of oxidase and its derivatives is controversial. Myer (47) arguing from the lack of any exitedon-type resonance interaction in the observed CD spectra could find no evidence for heme-heme interaction, while Tiesjema and van Gelder (46) using difference techniques modeled after Yonetani (43) find small differences in both wavelength extrema and ellipticity for difference spectra obtained with various ligands and conclude that heme-heme interaction must exist. However, it should be noted that CD is remarkably sensitive to variations in the symmetry of the heme environment, and that the origin of rotational strength may arise from coupling with transitions in chromophoric groups distant from the heme moiety (51), even in different subunits (52); MCD on the other hand reflects the electronic structure of the heme group itself and hence is sensitive only to those perturbations which affect the iron or porphyrin orbitals directly. In addition, some heterogeneity in ligand binding properties are invariably present with purified preparations of cytochrome oxidase (53). The small differences in both CD and MCD spectra of cytochrome oxidase are more simply attributable to effects such as these, rather than to the more profound alternative of heme-heme interaction. We do not mean
to imply, however, that the redox properties of the four metal centers are necessarily independent, and indeed it is entirely plausible that the electron affinity of each of the sites depends on the chemical status of the other species present. Rather it is our contention that the spectroscopic properties of the enzyme can be explained without invoking a heme-heme interaction so large as to modify the intrinsic optical parameters of the individual components.

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### TABLE I

| Species      | MCD Intensity | MCD Origin | Proposed spin state |
|--------------|---------------|------------|---------------------|
| **Ferric Iron** |               |            |                     |
| a_{3+}       | Strong        | C terms    | Low-spin            |
| a_{3+}       | Weak          | A & C terms| High-spin, Coupled  |
| a_{3+} \cdot CN | Strong     | C terms    | Low-spin            |
| a_{3+} \cdot CHOO | Weak   | A & C terms| High-spin           |
| **Ferrous Iron** |          |            |                     |
| a_{2+}       | Strong        | A terms    | Low-spin            |
| a_{2+}       | Strong        | C terms    | High-spin           |
| a_{2+} \cdot CN | Weak    | A terms    | Low-spin            |
| a_{2+} \cdot CO | Weak     | A terms    | Low-spin            |
Footnote: In the nomenclature for MCD features developed by Stephens and coworkers (54) temperature dependent MCD bands correspond to Faraday C terms, whereas temperature independent, derivative shape MCD curves are denoted as Faraday A terms. Since Faraday B terms are generally weak for heme proteins, we will restrict our discussion of the MCD of cytochrome oxidase to Faraday A and C terms.

Abbreviations: CD, natural circular dichroism; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; SDS, sodium dodecyl sulfate; TMPD, N,N,N',N'-tetramethylphenylenediamine.
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FIGURE CAPTIONS

Fig. 1. MCD (upper) and absorption (lower) spectra for oxidized cytochrome oxidase. The enzyme was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80. The temperature was maintained near 0° C.

Fig. 2. Temperature dependence of the Soret MCD spectrum of oxidized cytochrome oxidase. Spectra were recorded at -8° C (- - -), -73° C, -125° C and -145° C (---). The inset shows the absolute value of the MCD intensity at the indicated wavelengths plotted as a function of 1/T. The glass forming solvent consisted of 75% glycerol, 0.5 M KCl, 0.012 M potassium phosphate buffer and 0.5% Tween 80. The pH was 7.4.

Fig. 3. Soret MCD spectra for oxidized cytochrome oxidase (- - -) and heme a - imidazole (——). The enzyme was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80; heme a was dissolved in 0.1 M potassium phosphate buffer containing 5% imidazole and 0.001 M SDS, the pH was 7.4.

Fig. 4. MCD spectra of oxidized cytochrome oxidase (· · ·) and the formate (- - -) and cyanide (——) complexes of the oxidized enzyme. The buffer system contained 0.1 M potassium phosphate buffer, pH 7.4, 0.5% Tween 80. Neutralized sodium formate (60 mM) and neutralized potassium cyanide (6 mM) were added to form the respective inhibitor complexes.

Fig. 5. MCD (upper) and absorption (lower) spectra for reduced cytochrome oxidase. The buffer system consisted of 0.1 M potassium phosphate, pH 7.4, containing 0.5% Tween 80. Reduction was carried out by adding a few crystals of solid sodium dithionite.

Fig. 6. (a) Temperature dependence of the Soret MCD spectrum of reduced cytochrome oxidase. Spectra obtained at 0° C, -115° C and -145° C are shown in the figure. The inset shows the absolute value of MCD intensity at the indicated wavelengths plotted as a function of 1/T. The glass forming solvent contained 75% glycerol, 0.5 M KCl, 0.012 M potassium phosphate and 0.1% potassium cholate; the pH was 7.4. (b) MCD difference spectrum for reduced cytochrome oxidase obtained by
subtracting the MCD spectrum recorded at 0° C from that observed at -145° C. The smoothing routine was used to enhance signal to noise.

**Fig. 7.** MCD spectra of reduced cytochrome oxidase (- - -) and the CO (· · · · · ·) and CN⁻ (——) complexes of the reduced enzyme. The buffer system consisted of 0.1 M potassium phosphate, pH 7.4, containing 0.5% potassium cholate. The enzyme was reduced with a few crystals of dithionite; CO gas or neutralized potassium cyanide (6 mM) were added to form the respective inhibitor complexes.

**Fig. 8.** MCD (upper) and absorption (lower) spectra of the partially reduced complexes of cytochrome oxidase with formate (- - -) and cyanide (——). The buffer system contained 0.1 M potassium phosphate, pH 7.4, and 0.5% Tween 80. Partial reduction was achieved by the addition of 0.1 mM TMPD and 10 mM ascorbate to the oxidized enzyme preincubated with either 60 mM neutralized sodium formate or 1 mM neutralized potassium cyanide.

**Fig. 9.** Soret MCD spectrum of oxygenated cytochrome oxidase. The enzyme was dissolved in 0.1 M potassium phosphate, pH 7.4, containing 0.5% potassium cholate. Reduction was achieved using a few crystals of dithionite; reoxidation by bubbling air thru the enzyme solution. The smoothing routine was used to enhance signal to noise.

**Fig. 10.** (a) Soret MCD spectra of reduced cytochrome oxidase (——) and the formate complex of the partially reduced enzyme (- - -). Conditions as described in Figs. 6 and 8 respectively. (b) Difference MCD spectrum obtained by subtracting the spectrum of the partially reduced enzyme·formate complex from that observed for the reduced enzyme. (c) Difference MCD spectrum obtained by subtracting the spectrum of the fully reduced oxidase·CO complex from that of the fully reduced enzyme. (d) MCD difference spectrum analogous to (c) using the fully reduced enzyme·CN⁻ complex as the subtrahend.

**Fig. 11.** MCD spectra of the cytochrome a₃⁺·CN complex obtained as (a) the difference of the MCD spectra of the oxidized enzyme·CN⁻ complex and the oxidized enzyme (cf. Fig. 4): a₃⁺ a₃⁺·CN - a₅⁺ a₃⁺ = a₃⁺·CN - a₃⁺, (b) the difference of the MCD
spectra of the partially and fully reduced oxidase-CN\(^-\) complexes (cf. Figs. 8 and 7 respectively): \( \frac{a^{2+}}{a_3} \cdot \text{CN}^- - \frac{a^{2+}}{a_3} \cdot \text{CN}^- = \frac{a^{3+}}{a_3} \cdot \text{CN}^- - \frac{a^{2+}}{a_3} \cdot \text{CN}^- \) and (c) the difference of the MCD spectra of the partially reduced cytochrome oxidase complexes with CN\(^-\) and HCOOH (cf. Fig. 8): \( \frac{a^{2+}}{a_3} \cdot \text{CN}^- - \frac{a^{2+}}{a_3} \cdot \text{HCOOH} = \frac{a^{3+}}{a_3} \cdot \text{CN}^- - \frac{a^{3+}}{a_3} \cdot \text{HCOOH} \).
Figure 1
Figure 3

$\Delta \varepsilon / H$ (M·cm·TESLA)$^{-1}$

- OX. CYT. OXIDASE
- HEME $a$–Im–SDS

$\lambda$ (nm)

400 450

0

-40

-20

20

40
Figure 4

\[ \frac{\Delta \varepsilon}{H} \text{ (M \cdot cm \cdot TESLA)}^{-1} \]

\[ \lambda \text{ (nm)} \]

- OXIDIZED
- HCOOH
- CN⁻
Figure 6
Figure 7

Reduced CO

\[ \lambda (\text{nm}) \]

\[ \text{H}/\text{EV} \]
Figure 8

PARTIALLY REDUCED CYTOCHROME OXIDASE

\[ \frac{\Delta \varepsilon}{H} (\text{M} \cdot \text{cm} \cdot \text{Tesla})^{-1} \]

\[ \text{ABSORBANCE} \]

\[ \lambda \text{ (nm)} \]

- --- HCOOH
- --- CN⁻
Figure 9
Figure 11
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