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Detection of Conformational Changes in Complex III of the Respiratory Chain by a Maleimido Spin Label

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Abstract

Changes in the conformation of Complex III ($CoQH_2$-cytochrome *c* reductase) of the mitochondrial respiratory chain were detected upon oxidoreduction using the nitroxide spin label, 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl. EPR spectra of the spin label show a transition from a greater to a lesser degree of immobilization when the labeled enzyme, reduced either with ascorbate or sodium dithionite, is oxidized with potassium ferricyanide or ferricytochrome *c*. These observations are interpreted to indicate that Complex III is more compact in the reduced state at least in the locality of the spin label. An apparent increase in the concentration of total spins during oxidation of the complex suggests change in the interaction between the spin label and other paramagnetic centers and not an oxidation of spin label, itself, since reduced free spin label could not be reoxidized. Addition of antimycin A had no effect on the EPR spectrum of the spin-labeled enzyme, indicating that this inhibitor does not initiate a conformational change in the region of the spin label. Experiments in which N-ethyl-[2-^{3}H] maleimide was bound to Complex III show that binding occurs primarily to a subunit with a molecular weight of 45,000. Although no qualitative differences were observed, it was found that less radioactivity appears in samples reduced with dithionite than in those reduced with ascorbate. This difference appears to be caused by decomposition products of dithionite.

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Introduction

Complex III, also known as the "bc_1-complex," is the enzyme that catalyzes electron transfer from reduced coenzyme Q to cytochrome *c* in the mitochondrial electron transport chain (1). The enzyme, when attached to the mitochondrial membrane, is capable of generating a molecule of ATP during transport of a pair of electrons. Purified Complex III is inhibited stoichiometrically by antimycin A and is composed of eight or more polypeptide subunits (2).

Although it is generally accepted that conformational changes take place in enzymes during catalysis, to date there is no direct evidence that such transitions accompany electron transport across this segment of the respiratory chain. However, a number of observations by several investigators strongly suggest that such conformational transitions indeed occur in Complex III. Rieske et al. (3) observed that the cleavage of Complex III by dissociating reagents is blocked by reduction of the complex. This phenomenon was explained by assuming that the accessibility to dissociating reagents of certain key quaternary linkages between polypeptide components of the complex is blocked by a conformational change associated with the reduction of the complex. Antimycin A, the respiratory inhibitor of Complex III, also protects the enzyme from such an attack by the dissociating agents. Therefore, it appears that this apparent conformational transition is an integral part of the respiratory function of Complex III. Wakabayashi et al. (4) have reported that, upon reduction, the paracrystalline arrays of Complex III observed in Complex III-liposome membranes undergo significant changes in the arrangement of individual complexes. This clearly indicates a perturbation of the quaternary structure of the complex upon reduction. Berden and Slater (5) observed that CD spectra of oxidized and reduced Complex III are different near 220 nm. This observation suggests a polypeptide conformational transition in Complex III during electron transfer. The same authors also detected a difference in fluorescence-quenching between antimycin A bound to the reduced enzyme and to the oxidized enzyme (6). This observation suggests a different spatial positioning or local environment of the enzyme-bound inhibitor in different redox states of the enzyme. Finally Rieske et al. (7) have reported a significant shift in S–H stretching frequency of a sulfhydryl group, apparently located in a hydrophobic region of the complex, when the enzyme is reduced.

Visualization of conformational changes in polypeptides also can be observed by the use of reporter molecules such as nitroxides, better known as spin labels (8,9). This technique has been used successfully to detect conformational changes in cytochrome oxidase (Complex IV) (10).

DasGupta and Rieske (11) first analyzed the polypeptides of purified

Complex III by SDS[1] polyacrylamide gel electrophoresis and observed at least seven polypeptides. In recent years several other investigators have obtained similar results by SDS gel electrophoresis of Complex III obtained from mammalian as well as microbial sources. It now appears that there may be more than seven polypeptide components in Complex III (2).

In the present work we sought to preferentially label one of the subunits of Complex III with a maleimido spin label and to study the EPR spectra of the spin label under different redox states of the labeled enzyme. In addition, we wanted to determine if there is any correlation between the EPR spectra of the labeled enzyme and the corresponding optical spectra of the enzyme in different redox states.

Materials and Methods

Materials

All reagents were of reagent grade. Ascorbic acid was purchased from Merck and Co., while sodium dithionite was a gift of Professor Q. H. Gibson and was a product of Hardman and Holden, Manchester, England. Hydrogen peroxide (30% in H_2O_2) was obtained from Mallinckrodt; Sephadex G-25, *p*-chloromercuriphenylsulfonate, trypsin, ribonuclease, and horse heart cytochrome *c* were obtained from Sigma; sodium dodecyl sulfate was procured from Fisher Scientific; Tris, guanidine hydrochloride, and bovine serum albumin were products of Schwarz/Mann. Deoxycholic acid and cholic acid were purchased from MCB Manufacturing Chemists and were recrystallized from 70% ethanol before use. The spin label 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was procured from Synvar Associates; PPO, POPOP, and N-ethyl-(2-^{3}H)maleimide (161 mCi/mmol) were products of New England Nuclear. Acrylamide and N,N′-methylenebisacrylamide were obtained from Miles Laboratories, and Coomassie Brilliant Blue R-250 was purchased from Colab. Ovalbumin was a product of Worthington. A sample of coenzyme Q-2 analog (2,3-dimethyl-5-methyl-6-decyl-1,4-benzohydroquinone) was kindly provided by Professor Bernard Trumpower.

Purified Complex III was prepared according to the procedure described by Rieske (12) and without further purification routinely contained 3.0 nmol of cytochrome c_1 per mg of protein.

[1]The following abbreviations are used: SDS, sodium dodecyl sulfate; MalNEt, N-ethylmaleimide; CMS, *p*-chloromercuriphenylsulfonate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

Polyacrylamide Gel Electrophoresis

The separating gel was prepared by polymerizing a 15% solution of acrylamide in 0.1 M Tris-acetate buffer, pH 8.2, containing 0.1% SDS in glass tubes 10 cm long with an internal diameter of 4 mm. A 5% solution of acrylamide was used for preparing the stacking gel. The reservoir buffer contained 0.1 M Tris-acetate, pH 8.2, and 0.1% SDS. Protein samples were prepared by incubating 0.1 ml of the proteins (10 mg/ml) and 0.1 ml of 3% SDS and 1% β-mercaptoethanol for 2 hr. Approximately 10-70 μg of the protein so prepared were subjected to gel electrophoresis at 0–5°C and at a constant current of 1.5 mA per tube for approximately 5 hr. Gels were stained with Coomassie Brilliant Blue R-250 and, after destaining in a Canalco gel destainer for 20 min, were scanned for absorbance at 550 nm using a Gilford gel scanner.

Reaction of Complex III with N-ethylmaleimide

Samples (4 mg protein) of Complex III were reduced separately by titrating with ascorbic acid or sodium dithionite; the pH was adjusted to 7.5 in each case with small volumes of 6 N KOH. Both the ascorbate-reduced and dithionite-reduced samples were then incubated with 0.3 mg of N-ethyl-(2-^{3}H)maleimide (161 mCi/mmol) for 15 min at 23°C under a gentle stream of N_2 gas. The incubated solution was then chromatographed on a column (0.5 × 7.0 cm) of Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 7.5, containing 0.2% potassium deoxycholate. All the buffer solutions were bubbled with N_2 gas for several hours before use. About 70 μg of labeled protein were subjected to gel electrophoresis. After staining and destaining the gels were scanned at 550 nm. The individual protein bands were isolated by slicing the gels with a sharp blade, after which the gel slices were digested in 0.1 ml of 30% H_2O_2 overnight at 50°C in tightly capped glass liquid scintillation vials. After allowing the vials to cool to 23°C, 20 ml of toluene-base counting mixture (PPO–POPOP) were added to each vial and the radioactivity was counted in a Beckman Model LS-230 liquid scintillation spectrophotometer.

Spin-Labeling Technique

Samples of Complex III were reduced with ascorbic acid or sodium dithionite, rexpectively, after which 1 ml of the reduced enzyme (20 mg protein) was incubated for 15 min at 23°C with 3 mg of 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl. The reaction mixture was then chromatographed on a column (1.5 × 7.0 cm) of Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 7.5, containing 0.22% potassium deoxycholate. The eluate was collected in fractions of approximately 0.5

ml each and the most concentrated were combined and used for EPR spectroscopy.

Instrumentation

Electron paramagnetic resonance spectra were recorded at 23°C using a Varian Model E-4 X-band spectrometer. The sample was contained in a quartz aqueous sample cell of 0.25 mm internal width (Scanco, S-813) with a syringe attachment as described by Smith (8). Spectra were recorded at the following instrument settings: microwave power, 50 mw; filter time constant, 1.0 sec; modulation amplitude, 2.5 G; microwave frequency, approximately 9.5 GHz; scanning speed, 4 min. A power input of 50 mw was nonsaturating. In fact, a lower power input of 5 mw produced exactly the same spectra except that a higher receiver gain had to be used to obtain equivalent amplitudes of the signals. Optical absorption spectra were obtained at 23°C in a Beckman Model DK-2A ratio recording spectrophotometer.

Assays

Protein was determined by the biuret method of Gornall et al. (13). The concentration of cytochrome c_1 was estimated by the procedure of Rieske (12). Reduced coenzyme Q-cytochrome *c* reductase activity was measured spectrophotometrically at 23°C essentially as described by Rieske (12) except that 2,3-dimethyl-5-methyl-6-decyl-1,4-benzohydroquinone was used as the reducing substrate in place of coenzyme QH_2-2. In this assay the rate of reduction of cytochrome *c* using the saturated Q-2 analog was comparable with the rate obtained with Q-2.

Results

Binding of N-ethylmaleimide to Ascorbate- and Dithionite-Reduced Complex III

When excess [^{3}H]MalNEt was reacted with reduced Complex III at 23°C for 15 min, most of the radiolabel appeared to concentrate in the highest molecular weight subunit (45,000) of the complex. This was found to be true whether the enzyme was reduced with ascorbate or dithionite. Figure 1 is SDS-polyacrylamide gel electrophoretograms of [^{3}H]MalNEt-treated Complex III where the enzyme was reduced with a minimum quantity of ascorbate (Fig. 1A) and dithionite (Fig. 1B) before being labeled with [^{3}H]MalNEt. Complex III separated into eight protein bands of apparent molecular weights 45,000, 33,000, 29,000, 25,000, 23,000, 11,750, 8,900, and 6,500, when subjected to SDS gel electrophoresis in 15% acrylamide gel. The

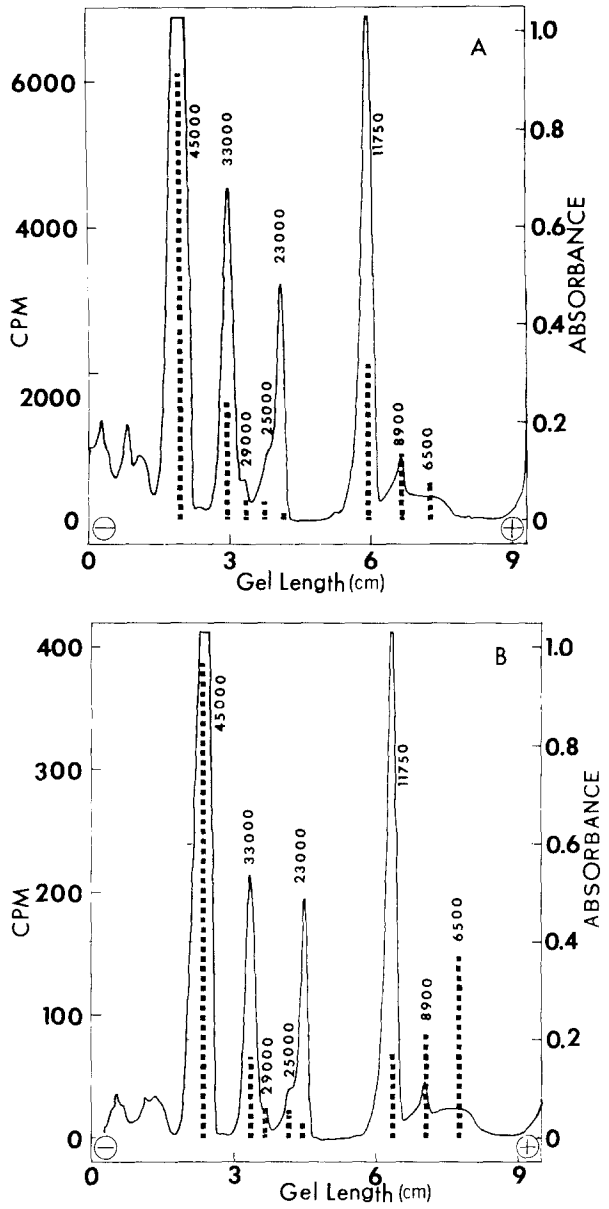

Fig. 1. SDS-polyacrylamide gel electrophoresis pattern of ascorbate-reduced (A) and dithionite-reduced (B) Complex III labeled with N-ethyl-[2-^{3}H]maleimide (161 mCi/mmol). Labeling was performed as described under Materials and Methods. The gels were stained with Coomassie Brilliant Blue R-250 and destained in a solution made of 10% acetic acid and 5% methanol in water. The gels were scanned for absorbance at 550 nm in a Gilford gel scanner. The protein bands were isolated by slicing the gels and the radioactivity was measured in a Beckman LS-230 scintillation counter as described under Materials and Methods. The solid line represents the absorbance due to staining with Coomassie Blue, and the dotted lines represent the radioactivity of the protein bands. The molecular weights of the subunits are indicated near or above the absorption peaks.

standards used for molecular weight determinations by this technique were bovine serum albumin, ovalbumin, trypsin, and ribonuclease. In all probability the 45,000 molecular weight subunit is a combination of the two core proteins (11) which do not separate in 15% acrylamide gel. The fastest moving broad protein band is obviously a mixture of polypeptides whose mean molecular weight is estimated to be 6,500.

Although the binding patterns of [^{3}H]MalNEt in the ascorbate-reduced and dithionite-reduced enzyme are quite similar qualitatively, the ascorbate-reduced enzyme accumulated substantially more radioactivity than the dithionite-reduced enzyme. We have studied the effect of dithionite decomposition products on the binding of [^{3}H]MalNEt to cytochrome oxidase and find that, although dithionite decomposition products do not react with [^{3}H]MalNEt, they severely limit its binding (unpublished data).

Spin-Labeled Complex III

The maleimido spin label, 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, binds to both the ascorbate-reduced and dithionite-reduced Complex III. Table I shows the results of spin labeling of ascorbate-reduced and dithionite-reduced Complex III, each in three separate experiments. To estimate the amount of spin label bound to reduced Complex III, the respective samples of spin-labeled enzyme were denatured in 6 M

Table I. Binding of Maleimido Spin Label to Ascorbate-Reduced and Dithionite-Reduced Complex III[a]

Enzyme sample	Experiment	Concentration of spin label (nmol/mg of protein)	Activity (% control)
Ascorbate-reduced	1	9.4	85
	2	13.0	84
	3	11.0	101
Dithionite-reduced	1	7.3	91
	2	6.4	89
	3	4.0	98

[a]Samples (20 mg protein) of Complex III were reduced with ascorbic acid or sodium dithionite and then spin-labeled with 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl as described in Materials and Methods. For quantitation of the bound spin label, samples of spin-labeled Complex III were denatured in 6M guanidine hydrochloride at pH 6.0 in the presence of 15 mM CMS before recording EPR spectra. The first derivative EPR spectra were then doubly integrated. The enzymic activities of the labeled enzyme samples were recorded as described in Materials and Methods. The activity of the native enzyme was taken as the control

guanidine hydrochloride at pH 6.0 in the presence of 15 mM CMS for 15 min prior to recording their EPR spectra. All EPR spectra were recorded at exactly the same instrument settings and then doubly integrated manually to obtain the values shown in Table I. On an average, approximately 11 nmol of

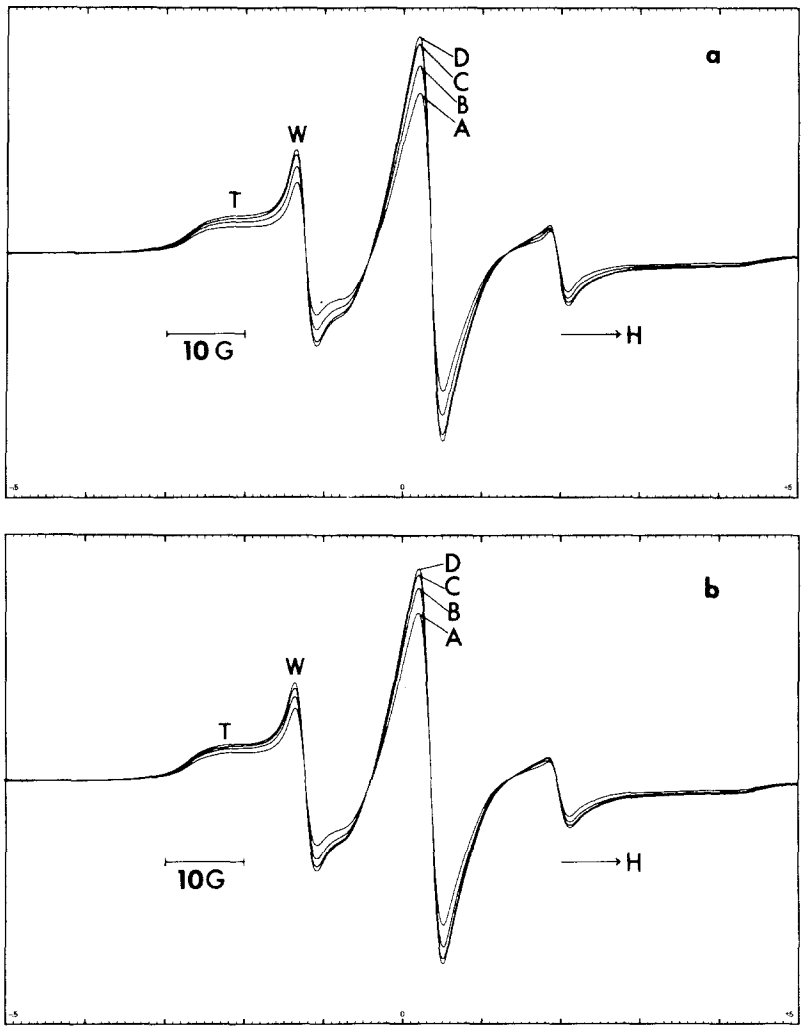

Fig. 2. EPR spectra of ascorbate-reduced, spin-labeled Complex III titrated with $K_3[Fe(CN)_6]$ (a): A, no $K_3[Fe(CN)_6]$; B, 28.5 mM $K_3[Fe(CN)_6]$; C, 57.0 mM $K_3[Fe(CN)_6]$; D, 85.5 mM $K_3[Fe(CN)_6]$; and with ferricytochrome *c* (b): A, no ferricytochrome *c*; B, 2.0 mM ferricytochrome *c*; C, 4.0 mM ferricytochrome *c*; D, 6.0 mM ferricytochrome *c*. The protein concentration of the enzyme was 9.4 mg/ml.

spin label were bound to 1 mg of ascorbate-reduced Comples III, while approximately 6 nmol were bound to 1 mg of dithionite-reduced Complex III. This difference in binding is due to the interference of dithionite decomposition products in the reaction of the maleimido moiety of the spin label and the available sulfhydryl groups of Complex III. As illustrated in Table I, at least 85% of the enzymic activity of the native enzyme is retained by spin-labeled Complex III. Whether this slight loss in activity is due to perturbation by the attached spin labels is not known. The fact that some of the spin-labeled samples of Complex III retained as much enzymic activity as the native enzyme would argue against this possibility.

The EPR spectra of both the ascorbate-reduced and dithionite-reduced spin-labeled Complex III indicate that the spin label is moderately immobilized when it is covalently bound to the enzyme. When the spin-labeled enzyme is oxidized there is a change in the spectra as shown in Fig. 2 where the ascorbate-reduced, spin-labeled enzyme was titrated with 0.01 M potassium ferricyanide (Fig. 2a) and 0.82 mM horse heart ferricytochrome *c* (Fig. 2b). It was found that in control experiments, where free spin label was reduced with ascorbate or dithionite, the spin label could not be reoxidized by potassium ferricyanide or ferricytochrome *c*. Therefore, it seems unlikely that the changes observed in Fig. 2 reflect changes in the oxidation state of the spin label.

The EPR spectra of the spin-labeled enzyme show two components in the low field region, component W and component T. According to Hamilton and McConnell (9), W is a weakly immobilized signal which reflects the spin label bound to an external site, whereas T is a strongly immobilized signal which reflects the spin label restricted in its local motion. Changes in the relative intensities of these signals, as expressed by the ratio, W/T, can be used to monitor transitions from one component to another (14). As seen in Fig. 2, as the ascorbate-reduced enzyme is oxidized, both W and T change. Furthermore, Table II shows that the ratio of W/T increases upon oxidation, indicating a lesser degree of immobilization. Another feature of the changes in W and T is that both increase in amplitude upon oxidation. Also, the amplitudes of the other two primary signals increase. Double integration of these derivative curves indicates an increase of the total spin upon oxidation. However, on the basis of experiments which show that neither potassium ferricyanide nor ferricytochrome *c* oxidize the free, reduced spin label, it seems unlikely that the increase of the total spin is due to an oxidation of bound spin label. More likely, the apparent increase in the total spin is due to a change in the interaction between the nitroxide spin label and other paramagnetic centers in the enzyme complex. Such interactions have been observed by Cohn et al. (15) in creatine kinase and have been treated theoretically by Leigh (16).

Table II. Ratios of Weakly Immobilized Component (W) to Tightly Immobilized Component (T) in the EPR Spectra of Spin-Labeled Complex III[a]

Concentration of oxidant (μM)		W/T	
$K_3Fe(CN)_6$	Cytochrome *c*	Ascorbate-reduced	Dithionite-reduced
0.0		2.698	1.688
28.5		2.917	2.032
57.0		3.077	2.090
85.5		3.085	—
	0.0	2.867	2.000
	2.0	2.904	2.150
	4.0	3.000	2.182
	6.0	3.011	—

[a]The value W/T is the ratio of signal intensities of weakly immobilized component in the low field region of the EPR spectra. It is calculated directly from the corresponding EPR spectrum.

Figure 3 illustrates the changes in the optical spectrum of the ascorbate-reduced Complex III as it is titrated with 0.002 M potassium ferricyanide (Fig. 3a) and 0.16 mM horse heart ferricytochrome *c* (Fig. 3b). The figure shows a general correspondence between the changes in the EPR spectra and the optical spectra of spin-labeled Complex III as the enzyme is titrated with oxidizing agents. It has been estimated by optical spectroscopy that cytochrome c_1 of Complex III remains completely reduced after the ascorbate-reduced enzyme is reacted with a spin label and chromatographed in a Sephadex G-25 column. Therefore, the EPR spectra of Fig. 2 and the optical spectra of Fig. 3 reflect changes concomitant with the oxidation of initially completely reduced cytochrome c_1 and iron–sulfur protein but not other components of Complex III (15).

Figure 4 illustrates the changes in the EPR spectrum of dithionite-reduced, spin-labeled Complex III titrated with 0.01 M potassium ferricyanide (Fig. 4a) and 0.82 mM horse heart ferricytochrome *c* (Fig. 4b). It is estimated by optical spectroscopy that approximately 50% of the cytochrome *b* in the complex remains in the reduced form after the dithionite-reduced enzyme is spin-labeled and chromatographed in a Sephadex G-25 column. Therefore, changes conmitant with the oxidation of initially partially reduced cytochrome *b* and initially completely reduced cytochrome c_1 and iron–sulfur protein are reflected in the EPR spectra of Fig. 4. Corresponding changes in the optical spectra of spin-labeled Complex III are shown in Fig. 5 where the labeled enzyme is titrated with 0.002 M potassium ferricyanide (Fig. 5a) and 0.16 mM horse heart ferricytochrome *c* (Fig. 5b). Again a correlation

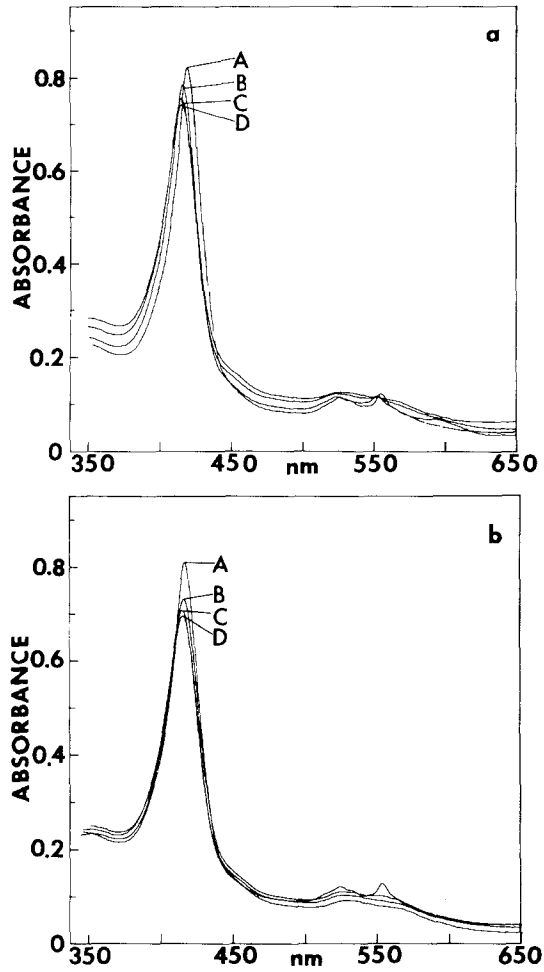

Fig. 3. Absorption spectra of ascorbate-reduced, spin-labeled Complex III titrated with $K_3[Fe(CN)_6]$ (a): A, no $K_3[Fe(CN)_6]$; B, 4 μM $K_3[Fe(CN)_6]$; C, 8 μM $K_3[Fe(CN)_6]$; D, 12 μM $K_3[Fe(CN]_6]$; and with ferricytochrome *c* (b): A, no ferricytochrome *c*; B, 0.3 μM ferricytochrome *c*; C, 0.6 μM ferricytochrome *c*; D, 0.9 μM ferricytochrome *c*. The protein concentration was 0.67 mg/ml.

between the EPR spectra and the optical spectra of the labeled enzyme is observed. This indicates that, in Complex III, conformational changes accompany electronic transitions. As in the case of ascorbate-reduced, spin-labeled Complex III, the EPR spectra of dithionite-reduced, spin-labeled enzyme reveal a lesser degree of immobilization for the spin labels as the enzyme is gradually oxidized. This is clear from the values of the W/T ratio, which change as the oxidizing agent is added to the reduced enzyme.

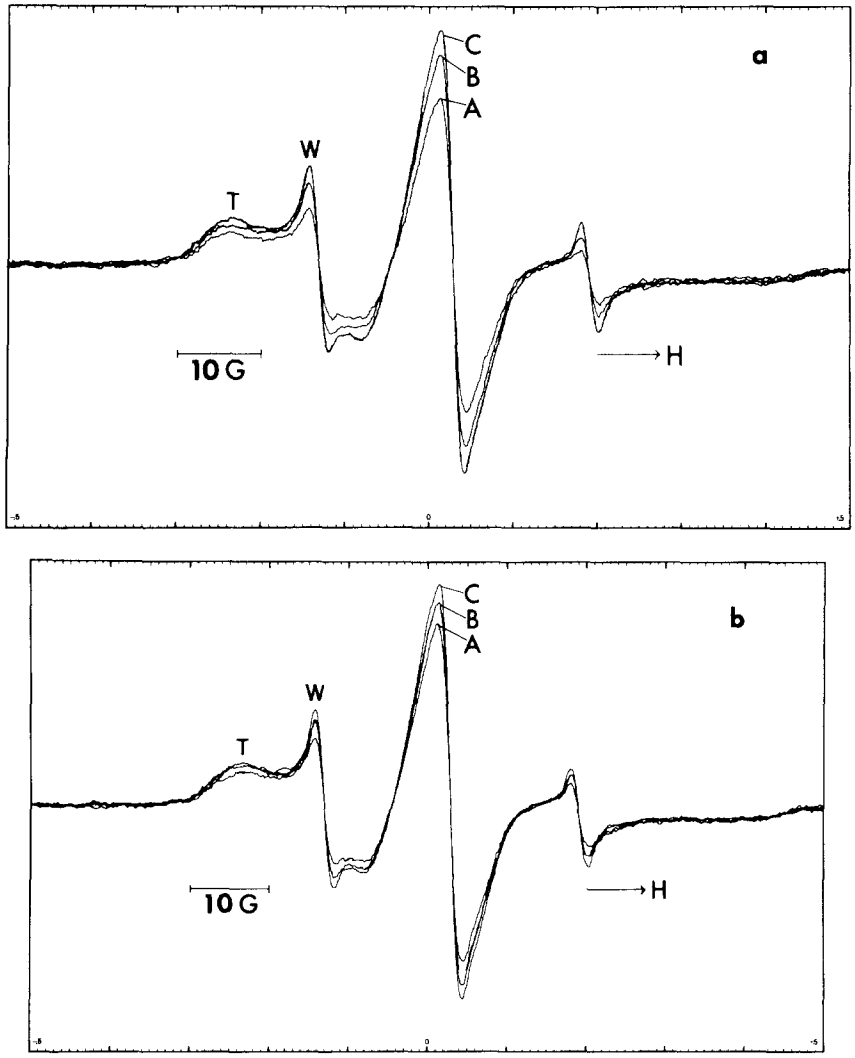

Fig. 4. EPR spectra of dithionite-reduced, spin-labeled Complex III titrated with $K_3[Fe(CN)_6]$ (a): A, no $K_3[Fe(CN)_6]$; B, 28.5 μM $K_3[Fe(CN)_6]$; C, 57.0 μM $K_3[Fe(CN)_6]$; and with ferricytochrome *c* (b): A, no ferricytochrome *c*; B, 2.0 μM ferricytochrome *c*; C, 4 μM ferricytochrome *c*. The protein concentration of the enzyme was 9.0 mg/ml.

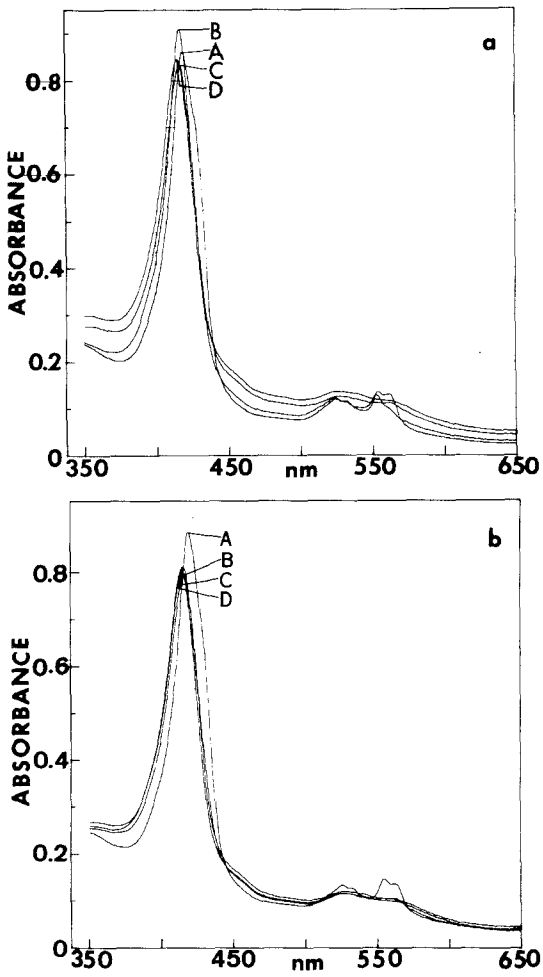

Fig. 5. Absorption spectra of dithionite-reduced, spin-labeled Complex III titrated with $K_3[FeCN)_6]$ (a): A, no $K_3[Fe(CN)_6]$; B, 4 μM $K_3[Fe(CN)_6]$; C, 8 μM $K_3[Fe(CN)_6]$; D, 12 μM $K_3[Fe(CN)_6]$; and with ferricytochrome *c* (b): A, no ferricytochrome *c*; B, 0.3 μM ferricytochrome *c*; C, 0.6 μM ferricytochrome *c*; D, 0.9 μM ferricytochrome *c*. The protein concentration was 0.7 mg/ml.

Discussion

In designing experiments to study conformational changes in Complex III using a spin label as a probe, several difficulties had to be overcome. For example, it was not possible to label the oxidized enzyme and study the EPR signal of the labeled enzyme upon reduction simply because the reducing

agents usually employed to reduce the enzyme (dithionite, ascorbate, reduced coenzyme Q) also reduce the spin label. To overcome this problem the enzyme was first reduced by titrating with just equivalent amounts of the reducing agents and then spin-labeled in an anaerobic environment. Both ascorbate and dithionite were used to reduce the enzyme because they provided an opportunity to examine the enzyme when only some of its components are reduced (cytochrome c_1 and iron–sulfur protein) and when all are reduced. Of the spin labels tried, 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl appeared to work best. Other homologues of this spin label were either too strongly immobilized or too weakly immobilized when bound to the enzyme and therefore were unsuitable for the purpose of our study. We were able to spin label Complex III with little or no loss in enzymic activity by incubating the enzyme with excess spin label for 15 min.

Potassium ferricyanide and horse heart cytochrome *c* were used to oxidize the reduced spin-labeled enzyme. This was done to compare the course of oxidation of Complex III with a laboratory reagent (potassium ferricyanide) and with the enzyme's natural substrate (cytochrome *c*). There was no visible distinction between the courses of oxidation of Complex III by potassium ferricyanide and ferricytochrome *c*.

The experimental results obtained with spin-labeled Complex III offer further evidence that the enzyme undergoes conformational changes during transitions from one redox state to another (2). When the enzyme is in the reduced state, the spin labels experience a stronger restriction in their motion than when the enzyme is in the oxidized state. This is the case for both the partially reduced (ascorbate-reduced) and the completely reduced (dithionite-reduced) Complex III. The gradual increase in the values of the ratios of the weakly immobilized component (W) to the tightly immobilized component (T) in the low field region of the EPR spectra as the enzyme is gradually oxidized supports this conclusion. In terms of the conformation of Complex III we can say that it is relatively more compact in its reduced state than in its oxidized state, at least in the locality of the spin label. This conclusion is in accord with the interpretation of Rieske et al. (17) of the observed protective effect of reduction on the cleavage of Complex III by chaotropic reagents, that a tighter conformation of the reduced enzyme blocks the entry of chaotropic agents into the interior of the complex, thus preventing cleavage. Since antimycin A, the respiratory inhibitor of Complex III, also protects the enzyme from cleavage by dissociating reagents, the conformational transitions detected using spin labels may be of significance with regard to the respiratory function of Complex III. It is well known that the effects of antimycin A on Complex III are similar to energization, namely, inhibition of respiration, enhanced reduction of cytochrome *b* by reducing substrates, and a redshift of the cytochrome *b* α-band. These similarities have led to

suggestions that antimycin A inhibits respiration by stabilizing the energized state in Complex III (18,19). Results obtained in the present work indicate a definite conformational change in Complex III during electron transport across this segment of the respiratory chain. The more compact or ordered structure in the reduced form suggested by these results implies a higher free energy content in that state of the enzyme. It is possible that antimycin A stabilizes a conformational form of Complex III and interferes with any further change in the conformation of the enzyme, thereby blocking electron transport and the coupled energy transduction. Addition of stoichiometric amounts of antimycin A to Complex III did not produce any change in the EPR spectra of the spin-labeled enzyme; therefore, binding of antimycin A to Complex III, as such, may not by itself initiate a conformational change in Complex III, at least in the region of the spin label.

Both dithionite-reduced and ascorbate-reduced, spin-labeled Complex III exhibit sharp isosbestic points at the base line in their EPR spectra during transition from the reduced to the oxidized state. Because of the sharpness of these isosbestic points, the height of the EPR derivative spectra at any point can be used directly as a measure of the concentration of the two components, W and T. The values of W and T are obtained by measuring the peak heights from the base line at two fixed values of magnetic field corresponding to the maxima of these two signals. Therefore, W/T ratios obtained with dithionite-reduced and ascorbate-reduced, spin-labeled Complex III can be used to compare the conformation of Complex III when cytochrome c_1 and the iron-sulfur protein only are reduced with that of the completely reduced enzyme. This is particularly true since the spin label is preferentially bound to the largest subunit. The following equations can be derived from Table II:

$$\Delta(W/T)_{[Ox(Fe(Cn)_6^{3-})\ \text{minus Red (ascorbate)}]} = 0.387 \quad (1)$$

$$\Delta(W/T)_{[Ox(Fe(CN)_6^{3-})\ \text{minus Red (dithionite)}]} = 0.402 \quad (2)$$

$$\Delta(W/T)_{[Ox(\text{cytochrome c})\ \text{minus Red (ascorbate)}]} = 0.144 \quad (3)$$

$$\Delta(W/T)_{[Ox(\text{cytochrome } c)\ \text{minus Red (dithionite)}]} = 0.182 \quad (4)$$

where $\Delta(W/T)_{Ox\ -\ Red}$ denotes the difference in the value of W/T between the oxidized and the reduced, spin-labeled Complex III. The bracketed subscripts indicate the type of reagent used for oxidation or reduction. Since W/T values are ratios obtained from the same EPR spectra at two fixed magnetic field values, they internally compensate for experimental error. Because of the use of a syringe for mixing and because the increase in sample volume due to titration is extremely small (less than 0.9%), the experimental error can be considered as minimal. The value W/T is a ratio and is independent of concentration terms. Therefore, $\Delta(W/T)$ reflects changes in the conforma-

tion in the vicinity of the spin label during the transition of Complex III from one redox state to another. Thus, the $\Delta(W/T)$ values for oxidation of ascorbate-reduced, spin-labeled Complex III can be compared with those of the $\Delta(W/T)$ values for the oxidation of dithionite-reduced, spin-labeled Complex III. A comparison of $\Delta(W/T)$ values of Eqs. (1) and (2) reveals that the conformational change detected by the spin labels during the complete oxidation of ascorbate-reduced enzyme is 96% of that of the dithionite-reduced enzyme when the oxidizing agent used is ferricyanide. The $\Delta(W/T)$ values of Eqs. (3) and (4) indicate that the conformational change detected by the spin labels during complete oxidation of ascorbate-reduced Complex III by ferricytochrome *c* is 80% of that of the dithionite-reduced enzyme. These results suggest that the major change in the conformation of Complex III is triggered by the reduction of cytochrome c_1 and/or the iron–sulfur protein. An additional 50% reduction of cytochrome *b* in Complex III does not alter the conformation of the enzyme by more than 20% of the total change. These results could imply that either the *b* cytochromes undergo little conformational change upon oxidation or that their allosteric influence on the conformation of the core proteins, where most of the spin label is attached, is small.

The observation that the apparent total spin concentration increases upon oxidation of Complex III either by potassium ferricyanide or ferricytochrome *c*, whether the enzyme was reduced initially by ascorbate or dithionite, suggests an interaction with other paramagnetic centers. We have already cited our reasons for discounting oxidation of some reduced spin label as the basis for this increase. Since changes in the oxidation state of the *b* cytochromes apparently exert little effect on the spectra, this interaction may involve cytochrome c_1 and/or the iron of the iron–sulfur protein.

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