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THE REDOX PROPERTIES OF THE CYTOCHROMES OF PURIFIED COMPLEX III

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SUMMARY

Purified Complex III from beef heart contains two *b* cytochromes: a high-potential ($E_{m\ 7.2} = +93$ mV) cytochrome *b*-562 which can be enzymatically reduced, and a low-potential ($E_{m\ 7.2} = -34$ mV) cytochrome *b*-565 which is reduced only by dithionite. The two components each contribute approximately 50 % to the total cytochrome *b* of Complex III. Cytochrome c_1 of Complex III titrates with a half-reduction potential of +232 mV.

INTRODUCTION

Complex III is an isolated section of the inner mitochondrial membrane which is enriched with respect to cytochrome *b* and cytochrome c_1, and which catalyzes the reduction of cytochrome *c* by reduced forms of lower ubiquinone analogs [1, 2]. It has been suggested on the basis of these properties that Complex III represents a purified form of that section of the respiratory chain containing the electron transfer reactions at the second phosphorylating site [3], and perhaps the components required for energy conservation [4, 5]. Redox potential measurements of the cytochromes in purified Complex III, using Q_1H_2/Q_1 as the titrating couple, have shown that the complex contains but a single potentiometrically detectable cytochrome *b* [5]. In contrast, it is now well established that cytochrome *b* in mitochondria [6–8], submitochondrial particles [7] and purified fragments of the inner membrane (succinate–cytochrome *c* reductase) [9] is heterogeneous with respect to its half-reduction potentials. Cytochrome *b* in purified Complex III does, however, show spectral heterogeneity [10–13].

In view of the importance of Complex III as a model system for the electron transfer reactions between cytochromes *b* and c_1 [3], we have reinvestigated the redox potentials of the cytochromes of Complex III and their possible relevance to the cytochromes of intact mitochondria.

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulfate.

MATERIALS AND METHODS

Complex III was isolated from beef heart mitochondria by the method of Hatefi et al. [1] as modified by Rieske et al. [2]. This preparation contains approximately eight nmoles cytochrome *b* and four nmoles of cytochrome c_1 per mg of protein. The *V* for the Q_2H_2–cytochrome *c* reductase varied with different preparations between 500 and 1000 μmoles cytochrome *c* reduced $\times min^{-1} \times mg^{-1}$ protein. The complex contains a small amount of succinate-cytochrome *c* reductase (less than 0.5 % of the Q_2H_2–cytochrome *c* reductase), and EPR analysis (kindly measured by Dan Bäckström) revealed enrichment of an ascorbate-reducible nonheme iron at $g = 1.90$ [2].

The cytochrome content of Complex III was determined at 562 minus 575 nm (mM absorbance = 20) [1] for cytochrome *b*, and 554 minus 540 nm (mM absorbance = 19) [1] for cytochrome c_1 after reduction with $Na_2S_2O_4$. Q_2H_2–cytochrome *c* reductase activity was assayed by measuring the initial rate of cytochrome *c* reduction at 550 minus 540 nm. Protein was measured by the method of Lowry et al. [14].

Redox titrations were carried out using the methods of Dutton [15] and Dutton et al. [7]. The apparatus is of the same design as described by Dutton [15].

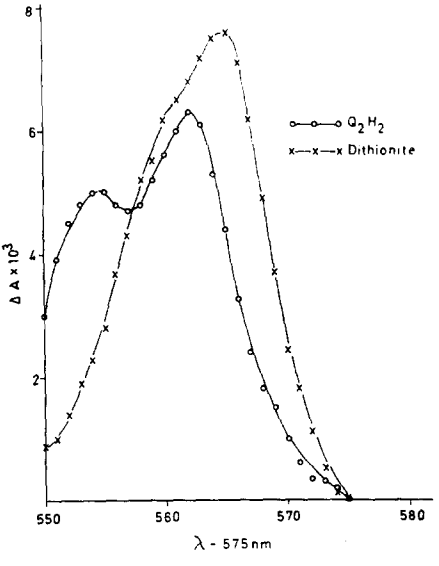

Fig. 1. Spectral analysis of the cytochromes in purified Complex III after reduction with Q_2H_2 and $Na_2S_2O_4$. The reaction mixture consisted of 170 mM sucrose, 50 mM Tris-acetate, pH 7.5, and 0.3 mg protein of Complex III; final volume was 3 ml. Spectra were obtained after the addition of 22.5 μM Q_2H_2 (final concentration) and again after the addition of $Na_2S_2O_4$. The $Na_2S_2O_4$ spectrum is the difference spectrum: $Na_2S_2O_4$ reduced minus Q_2H_2 reduced.

RESULTS

Cytochrome *b* with an absorption maximum in the α region at 562 nm, and cytochrome c_1 are reduced by Q_2H_2 (Fig. 1) as well as by ascorbate+*N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD), ascorbate + phenazine methosulfate

(PMS), and, more slowly, by succinate. Depending upon the preparation, between 35 and 55 % of the total cytochrome *b* of Complex III can be enzymatically reduced. The remaining cytochrome *b* can be subsequently reduced by $Na_2S_2O_4$. In contrast to the enzymatically reduced cytochrome *b*, that reduced by $Na_2S_2O_4$ has an absorption maximum in the α region at 565 nm and a shoulder near 560 nm. The exact

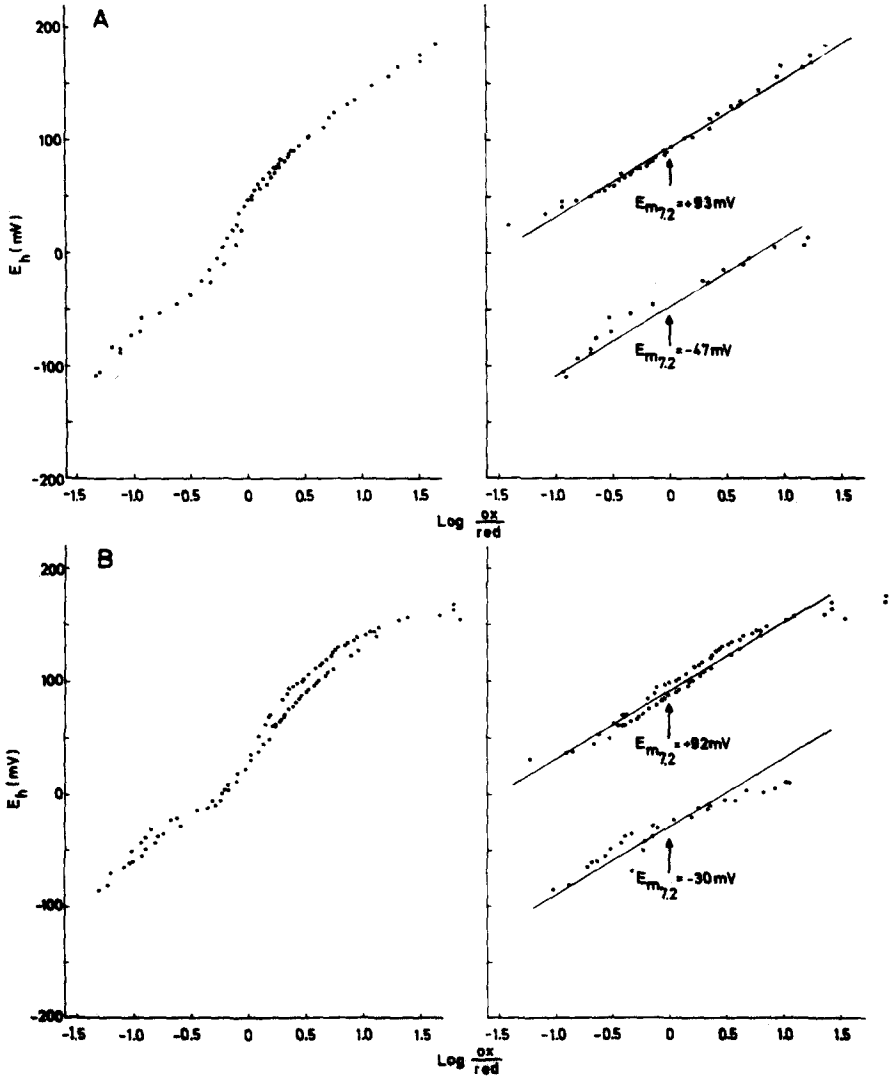

Fig. 2. Potentiometric titrations of the *b* cytochromes in purified Complex III. (A) No addition. (B) 2 nmoles antimycin/nmole cytochrome *b*. The reaction media contained 170 mM sucrose, 50 mM Tris–HCl, pH 7.2, 40 μM diaminodurene, 40 μM PMS, 40 μM phenazine ethosulfate, 40 μM duroquinone, 5 μM pyocyanine, 5 μM 2-hydroxyl-1,4-naphthoquinone and 0.17 mg Complex III protein. Approx. 50 % of the points on the curves were obtained by oxidative titration with ferricyanide and 50 % by reductive titrations with dithionite. Cytochrome *b* was measured at 562 minus 575 nm. Theoretical $n = 1$ lines are drawn through the experimental points for the separated components.

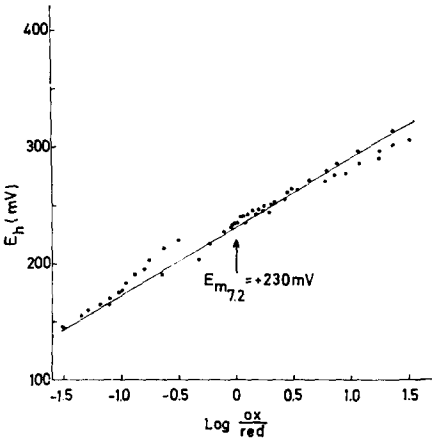

Fig. 3. Potentiometric titrations of cytochrome c_1 in purified Complex III. Conditions were as described in Fig. 2. Measurements were made at 554 minus 540 nm. Complex III concentration was 0.54 mg protein/ml.

spectrum of the lower-wavelength component could not be resolved at room temperature, and could represent either a further reduction of cytochrome *b*-562 or the reduction of cytochrome *b*-558. The latter is often associated with cytochrome *b*-565. The spectrum of the $Na_2S_2O_4$ reducible cytochrome *b*-565 can be shifted to slightly lower wavelengths in Complex III preparations in which the amount of enzymatically reducible cytochrome *b* is low. In these preparations little or no CO binding is detected.

Titrations of the half-reduction potentials of the *b* cytochromes of Complex III are shown in Fig. 2 both in the presence and absence of antimycin. Two *b* cytochromes can be separated, each contributing about 50 % of the total absorbance at 562 minus 575 nm. The half-reduction potentials ($E_{m\,7.2}$) of the two are near +93 and −35 mV, and are unchanged in the presence of antimycin (cf. refs. 16, 17). Preliminary investigations suggest that antimycin induces a slight red shift in the α band of the high potential cytochrome *b*-562 of Complex III, in agreement with

TABLE I

HALF-REDUCTION POTENTIALS OF CYTOCHROME *b* AND CYTOCHROME c_1 IN PURIFIED COMPLEX III

The numbers are expressed as the $\bar{x}\pm$S.D. n = the electron transfer number

Measuring wavelengths (nm)	Addition	$E_{m\,7.2}$ (mV)	n	Approximate contribution (%)
562 minus 575	–	+92± 5	1	51±5
562 minus 575	–	−34± 7	1	49±5
554 minus 540	–	+240±10	1	100
562 minus 575	Antimycin	+92± 8	1	51±5
562 minus 575	Antimycin	−35± 5	1	49±5
554 minus 540	Antimycin	+232	1	100

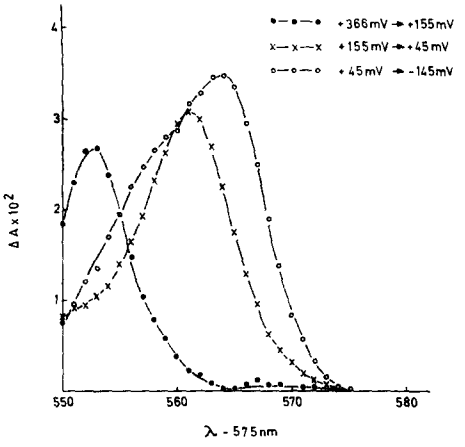

Fig. 4. Spectral analysis of the cytochromes of purified Complex III as a function of the redox potential. Conditions were as described in Fig. 2. Reduced minus oxidized difference spectra (between the potential values shown in the figure) were obtained after adding appropriate amounts of $Na_2S_2O_4$.

results obtained in submitochondrial particles [18]. An antimycin-induced alteration in the half-reduction potentials might therefore by expected to occur in the high potential cytochrome [17]. Results from 15 titrations on four different preparations of Complex III are presented in Table I. The mean values for the half-reduction potentials of the *b* cytochromes are +93 and −34 mV. The single half-reduction potential (+50 mV) for the cytochrome *b* of Complex III reported by Rieske [5] thus appears to be an average value for the two cytochromes reported here.

Cytochrome c_1 of Complex III titrates with a half-reduction potential of +230 mV (Fig. 3, Table I), in good agreement with previously published values for purified cytochrome c_1 [7, 19].

Fig. 4 shows the reduced minus oxidized difference spectra of Complex III between various fixed potentials. The redox potentials were chosen to give maximum separation of the two *b* cytochromes (+93 and −34 mV) in Complex III. From the data it can be seen that the high-potential cytochrome *b* has an absorption maximum at 562 nm. This is undoubtedly the *b* cytochrome which is reduced enzymatically (Fig. 1). The low-potential cytochrome *b* (reduced between +45 and −145 mV), on the other hand, shows an absorption maximum at 565 nm with a shoulder near 560 nm. This shoulder, also observed after $Na_2S_2O_4$ reduction (Fig. 1), was not present in all preparations of Complex III. However, in preparations where it is not clearly distinguishable the spectra of the low-potential cytochrome *b* was usually red-shifted to approx. 564 nm, suggesting a mixture of cytochrome *b*-565 and a component absorbing at a lower wavelength.

Aging of Complex III at −20 °C for several weeks leads to loss of enzymatic activity, to a decrease in the amount of cytochrome *b* which can be reduced enzymatically, and to a decrease in the amount of cytochrome *b* undergoing reduction after addition of ferricyanide to antimycin-blocked Complex III. These findings confirm the report of Rieske [5]. We have not, however, observed any significant changes in the redox potentials [5] or the CO-binding properties of the *b* cytochromes

in aged Complex III. These results suggest that loss of enzymatic activity of aged Complex III may be due to the alteration of a component other than the cytochromes. The results do not, however, allow assessment of the role of the cytochromes per se in the enzymatic activity of the isolated complex.

It is thus clear from the present results that purified Complex III contains two *b* cytochromes which can be distinguished both potentiometrically and spectrally (compare ref. 5). One has a half-reduction potential of +93 mV with an absorption maximum at 562 nm. This cytochrome *b* is reduced enzymatically and may represent either a slightly altered cytochrome b_K [4, 6–8], or a mixture of cytochrome b_K and the high-potential cytochrome *b* (+120 to +160 mV) previously reported in mitochondria [20] and submitochondrial particles [7, 21]. The latter cytochrome *b* is believed to be distinct from cytochrome b_K [21]. The high-potential cytochrome *b* reported here is probably identical to that previously reported to be enzymatically reducible in purified Complex III [11–13] and Complexes I+III [13].

Complex III also contains a cytochrome *b* component with a half-reduction potential of −34 mV. This component contains predominantly cytochrome *b*-565 and appears to be spectrally similar to the fraction of cytochrome *b* which cannot be enzymatically reduced in Complex III (Fig. 1) [11–13]. This low-potential cytochrome *b*-565 is probably identical to cytochrome b_T of mitochondria [7, 21]. The redox potentials of the *b* cytochromes of Complex III are thus generally similar to those in mitochondria [6–8, 20], submitochondrial particles [7, 21] and succinate–cytochrome *c* reductase [9], the major difference being the slightly greater half-reduction potential of the high-potential cytochrome *b*-562.

Maintenance of the redox properties of the two *b* cytochromes during purification of Complex III, coupled to the extensive enrichment of the cytochromes of this preparation (3–4 times greater heme concentration per mg protein than in the succinate–cytochrome *c* reductase [9]), makes Complex III an ideal preparation in which to study the molecular basis for the spectral and potentiometric heterogeneities of the *b* cytochromes.

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