Reductive titration of CoQ-depleted Complex III from Baker's yeast

Evidence for an exchange-coupled complex between QH· and low-spin ferricytochrome *b*

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1. INTRODUCTION

The role of coenzyme Q in electron transport has been intensively studied since its discovery as a component of the mitochondrial electron chain, particularly since Mitchell [1,2] postulated the CoQ loop as the mechanism for proton translocation at site II.

The anaerobic titration of Complex III from yeast mitochondria with dithionite reported in [3] shows an intense EPR signal assigned to the CoQ free radical which appears midway in the titration; this signal disappears as the complex becomes fully reduced. Siedow et al. [3] noted that the data obtained by EPR and from optical measurements during the reduction of cytochrome *b* contained some contradictions, and they proposed the formation of an exchange-coupled complex between the Q radical and the b_k component of cytochrome *b* as a rationalization for these contradictions.

Here, we present the results of an anaerobic titration with dithionite of yeast Complex III which has been substantially depleted of CoQ. In this experiment EPR and optical data obtained during the titration suggest the existence of two pools of CoQ which are isolated from one another in this complex.

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2. MATERIALS AND METHODS

Complex III was obtained from yeast mitochondria as in [3]. The preparation contained an average concentration of 9 nmol cytochrome *b*/mg protein and catalyzed the oxidation of the coenzyme Q analog 2,3-methoxy-5-methyl-6-pentylbenzohydroquinone with a turnover of 70 s^{-1}.

CoQ-depleted samples were prepared as in [4]. The suspension containing Complex III was lyophilized at 0°C and the dried residue extracted five times with hexane. Residual hexane was then removed from the extracted material under high vacuum at room temperature, and the dry residue was resuspended in the appropriate medium. CoQ was determined as in [5] by measuring the decrease in absorbance at 275 nm observed upon reduction of the chromophore with KBH_4. The concentrations of cytochromes *b* and c_1 were determined optically as in [3], and protein was determined as in [6].

The combined optical-EPR titration of Complex III was carried out as in [7]. Optical measurements were made on a Cary 17 spectrophotometer; EPR spectra recorded on a Varian E-6 spectrometer under non-saturating conditions viz. cytochromes, 40 mW power at 8.8 K with 20 G modulation; iron-sulfur center, 0.1 mW power at 18 K with 20 G modulation; Q-radical, 12 μW power at 100 K with 10 G modulation. Dithionite solutions

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for titration were prepared and standardized with lumiflavin-3-acetate as in [8].

Cholic acid, cytochrome *c* (type III), DPIP, NADH, and PMSF were from Sigma. Ammonium sulfate (Enzyme grade) was from Mann. The CoQ analog was given to us by Dr K. Folkers, University of Texas, Austin.

3. RESULTS AND DISCUSSION

Stoichiometric reductive titrations are a powerful tool in the characterization of complex oxidation reduction enzymes even though they only permit the calculation of the relative reduction potentials of the redox centers which are present. The titration of yeast complex containing about 1.5 equivalents of CoQ [3] proceeds according to expectation except that the degree of reduction of b_k determined by EPR did not agree with that found using optical spectroscopy. In the early phase of reduction, the intensity of the EPR signal at $g = 3.60$ (corresponding to b_k) decreased rapidly to a plateau at 75% reduction. In contrast, the optical change at 561 nm during this phase was only 20% of the total. It was proposed that cytochrome b_k and some other species, e.g., CoQ, form a complex so that in this part of the titration the QH· that is produced and the low-spin ferric heme of the b_k form a diamagnetic, exchange-coupled complex thus eliminating the EPR spectra of both the cytochrome and Q radical, e.g.:

$$b_k^{+3} \cdot Q \xrightarrow{+1e} b_k^{+3} \cdot QH^{\cdot}$$

EPR-detectable EPR-undetectable

As expected, about 1 electron was consumed during this phase of the titration [3].

By implication two pools of CoQ radical are necessary: the first is bound to cytochrome b_k and is undetectable by EPR, and a second which is isolated from other paramagnetic species and exhibits the free radical signal. As a means of evaluating this suggestion, we have repeated these reductive titrations using yeast Complex III in which the CoQ has been reduced to about 0.1 equivalents, i.e., less than 10% of that present in the native complex.

The optical spectra obtained at different states of reduction during the titration with dithionite are presented in fig.1. The peak which appears at 554 nm corresponds to the α-band of reduced c_1; this species is totally reduced after the addition of two electrons; the peak at 562 nm reflects the reduction of cytochrome *b* and is maximal after addition of four electrons.

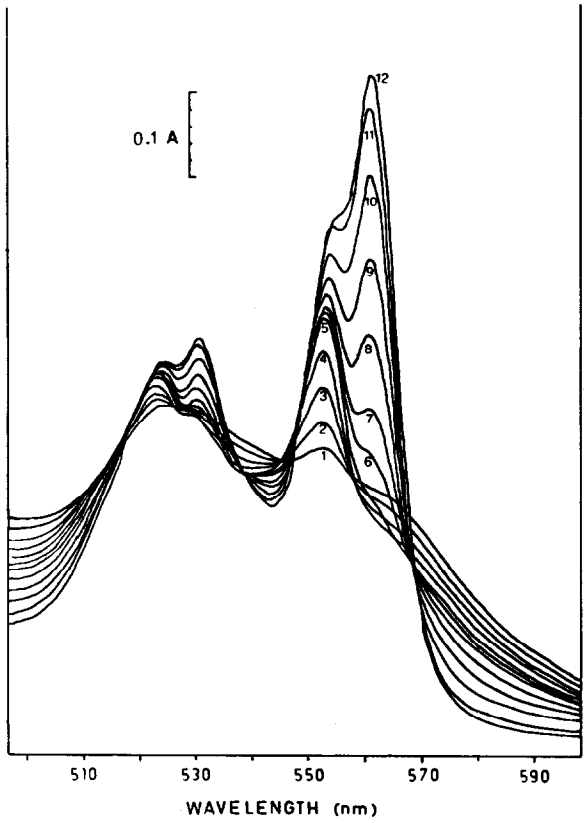

Fig.1. Absorbance spectra of cytochromes *b* and c_1 during the titration with dithionite of yeast CoQ-depleted Complex III. The preparation containing 0.12 equivalents of CoQ was suspended in 6 ml of 0.1 M potassium phosphate buffer (pH 7.4) to a cytochrome *b* concentration of approximately 100 μM, and the dithionite titration was carried out at 10°C. Spectra were recorded for each addition of dithionite and after stabilization of the absorbance. The path length was 0.2 cm.

The absorbance changes due to cytochrome *b* were resolved into two species (corresponding to b_k and b_t) and analyzed separately. In fig.2 we show the relative increase in absorption at 561.5 and 556.5 nm due to the two species; these two wavelengths are selected after examination of the

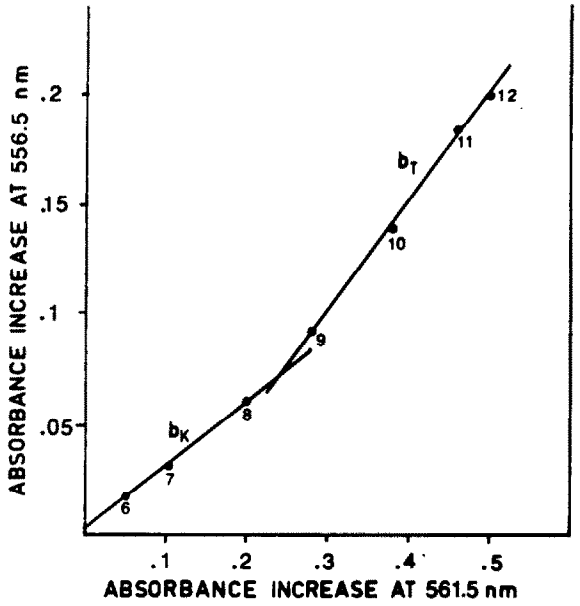

Fig.2. Different absorbance increase at 561.5 and 556.5 nm during the early and late phases of cytochrome *b* reduction in the titration with dithionite. The data are obtained from the spectra of fig.1.

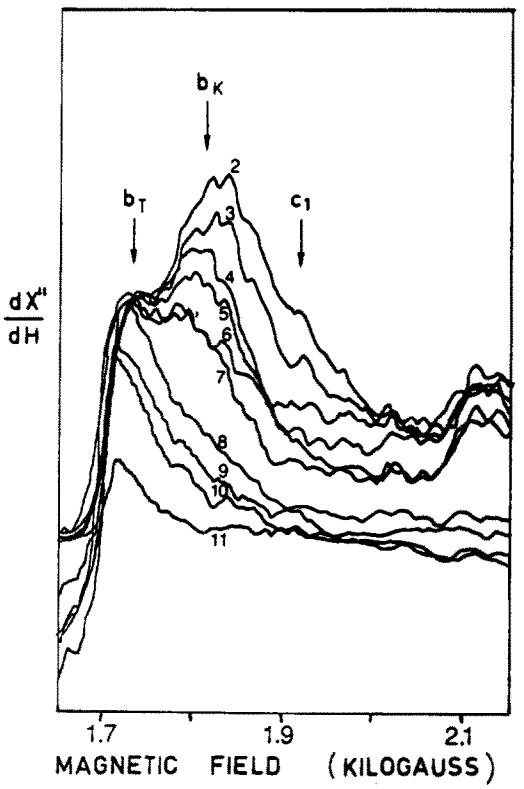

Fig.3. EPR spectra of yeast CoQ-depleted Complex III at different points of the reductive titration in the low-field cytochrome region. The number of the spectra correspond with the dithionite addition in the titration (fig.1). The reduction of cytochromes b_t, b_k and c_1 is measured by the disappearance of EPR intensity at g = 3.76, 3.60 and 3.49, respectively.

difference spectrum during the early and late phases of reduction of the cytochromes *b* [3]. b_k is reduced between addition of 0.7 and 1.35 reducing equivalents/*b* and while the reduction of b_t occurs subsequently; the inflexion point was used to separate the two compounds of cytochrome *b* with different midpoint potential (fig.4).

The quantification of the cytochromes c_1, b_k and b_t was also achieved by low temperature EPR. Fig.3 shows EPR spectra of the complex in the low-field cytochrome region recorded at different stages of the dithionite titration. The broad absorption assigned to c_1 and the two species of *b* are completely eliminated by reduction. In fig.4 the reduction of redox components obtained from optical spectra at different reducing equivalent additions are presented. The cytochrome *b* which is reduced between 0.6 and 2.1 reducing equivalents is resolved in both b_k and b_t species; each needs 0.8 equivalent for total reduction. Fig.5 presents the fractional reduction of redox components during the titration as determined by the EPR spectra. Cytochromes c_1, b_k and b_t exhibit a dependence on the degree of reduction very similar to that obtained from optical data. As expected, the iron-sulfur center has a midpoint potential more positive than c_1, and 0.7 reducing equivalent/*b* is required for complete reduction of both c_1 and iron-sulfur protein.

Of interest is the observation that the disappearance of the EPR signal attributed to b_k is linear and does not plateau at 75% reduction as observed with the normal complex [3]; the Q radical appears between 0.7–2.0 reducing equivalents/*b* and is maximal when b_k is 50% reduced; thus, most of Q radical is observed during the reduction of b_k. In contrast to the data obtained with the normal complex where it was found that the maximum value of the radical signal accounted for 10% of the total Q (0.1–0.15 equivalents), with Q-depleted complex the max-

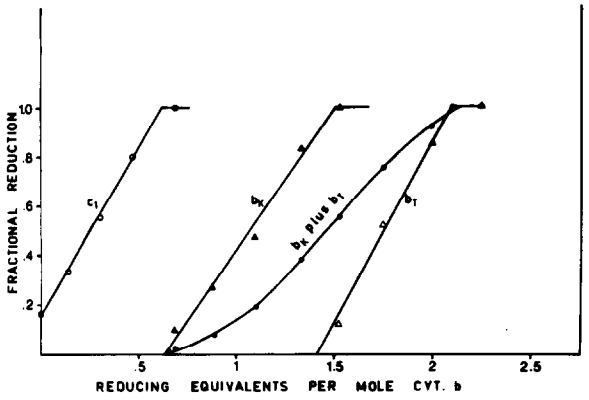

Fig.4. Fractional reduction of the components of yeast CoQ-depleted Complex III during the titration with dithionite obtained from optical spectra. The amounts of reduced cytochromes c_1 and total b were derived from the absorbance changes at 553.5 and 561.5 nm, respectively, and cytochromes b_K and b_t individually from the absorbance changes at 561.5 nm during the early and late phases of cytochrome b reduction as shown in fig.2.

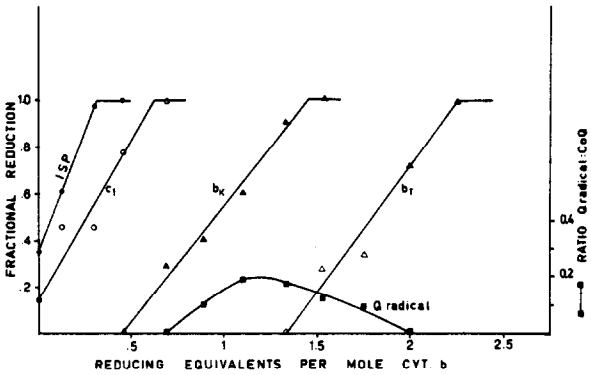

Fig.5. Fractional reduction of the components of CoQ-depleted Complex III during the reductive titration obtained from EPR spectra. The amounts of reduced iron-sulfur protein (●) and free radical (■) were obtained from the intensities of the EPR signals at g = 1.89 and 2.00, respectively. The radical was quantitated with diphenylpicrylhydrazyl as standard of EPR intensity. The fractional reduction of cytochromes c_1, b_K and b_t were derived from EPR spectra as shown in fig.3.

imum radical is equivalent to 20% of the total Q (i.e., about 0.02 equivalents).

The absence of anomalies in the EPR signal of cytochrome b_K and its agreement with the data from optical spectroscopy contrast with the titration on unextracted yeast Complex III and support the idea that in the natural complex some or all of the CoQ is forming an exchange-coupled complex with the low-spin iron of the ferricytochrome b as proposed in [3]. This interpretation requires the existence of two pools of CoQ which are independent of one another. A similar interpretation has been used to explain some anomalies in the EPR spectrum of Complex II.

Finally, our data reaffirm the absence of cryptic electron carriers in the purified yeast Complex III [9] as has been previously indicated [10–12], there being essentially no excess of reductant needed to complete the titration of the spectroscopically visible species.

ACKNOWLEDGEMENTS

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