Biochimica et Biophysica Acta, 722 (1983) 349–363
Elsevier Biomedical Press

BBA 41253

POTENTIOMETRIC STUDIES ON YEAST COMPLEX III

AH-LIM T'SAI and GRAHAM PALMER

Department of Biochemistry, Rice University, Houston, TX 77005 (U.S.A.)

(Received September 6th, 1982)

Key words: ESR; MCD; Complex III; Redox potential; Cytochrome; (Bakers' yeast)

Potentiometric measurements have been performed on Complex III from bakers' yeast. The midpoint potentials for the *b* and *c* cytochromes were measured using room-temperature MCD and liquid-helium temperature EPR. A value of 270 mV was obtained for cytochrome c_1, regardless of temperature, while the midpoint potentials found for the two species of cytochrome *b* varied with temperatures, viz., 62 and −20 mV at room temperature (MCD) compared to 116 and −4 mV at about 10 K (EPR). The midpoint potential of the iron-sulfur center obtained by low-temperature EPR was 286 mV. An abrupt conformational change occurred immediately after this center was fully reduced resulting in a change in EPR line shape. The potentials of the two half-reactions of ubiquinone were measured by following the semiquinone radical signal at 110 K and 23°C. Potentials of 176 and 51 mV were found at low temperature, while values of 200 and 110 mV were observed at room temperature. The midpoint potential of cytochrome c_1 was found to be pH independent. The potentials of cytochrome *b* were also independent of pH when titrations were performed in deoxycholate buffers, while a variation of −30 mV per pH unit was observed for both cytochrome *c* species in taurocholate buffers. These two detergents also produced different MCD contributions of the two *b* cytochromes. A decrease in E_m of greater than 300 mV was found in potentiometric measurements of cytochrome c_1 at high ratios of dye to Complex III. Antimycin does not affect the redox potentials of cytochrome c_1 but appears to induce a transition of the low-potential *b* heme to a high-potential species. This transition is mediated by ubiquinone.

Introduction

Complex III is an enzyme system located in the inner membrane of mitochondria, which contributes fundamentally to electron transfer and energy transduction in this organelle. The potentiometric behavior of this complex has been characterized while present in mitochondria and as purified preparations isolated from bovine heart [1,2], pigeon heart [3] and other sources (see, for reviews, Refs. 2 and 4). A highly purified, enzymatically active Complex III preparation from yeast mitochondria has been available only recently [5], and the chemical composition, spectral properties and redox characteristics have been reported [5]. In particular, optical and EPR measurements were used to monitor the changes in cytochrome *b*, cytochrome c_1, iron-sulfur protein and ubiquinone radical during stoichiometric reductive titrations. 7.5 electron equivalents were required to reduce completely one equivalent of Complex III. This stoichiometry is only slightly larger than the value of 6.5 ex-

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ada, 2-acetamidoiminodiacetic acid; Taps, 3-tris(hydroxymethyl)methylaminopropanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxymethyl)glycine; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol.

pected on the basis of the presumed complement of redox centers, and the extra equivalent was assumed to reduce the small amount of adventitious iron detected in the preparation. This result implies that there are no other important oxidation-reduction centers present in this yeast preparation.

The redox centers in Complex III can be divided into two groups, components of high potential, namely, cytochrome c_1 and the iron-sulfur center and components of low potential, the cytochromes *b* and coenzyme Q. The relative redox potentials of these components estimated from the stoichiometric titration fell within the range of the values previously reported for the enzyme from bovine heart and other sources.

As part of our program to characterize the internal electron-transfer sequence in Complex III, the absolute potentials of each redox component are needed. These are not simply obtained from a stoichiometric titration. Consequently, we have undertaken a potentiometric study of yeast Complex III and its isolated components. Our data includes room-temperature MCD potentiometric titrations on cytochrome *b* and c_1; liquid-helium EPR potentiometric titrations on cytochromes *b*, c_1 and the iron-sulfur center and liquid nitrogen and room-temperature EPR potentiometric titration of ubiquinone. A number of parameters which might influence the potentiometric properties of each redox component were also investigated. These include the effect of temperature of observation, variation in pH, detergent and mediator dyes and the presence and absence of the inhibitor, antimycin A.

Materials and Methods

Complex III was purified from bakers' yeast (Red Star) according to the procedure of Siedow et al. [5]. Preparations used for this work contained 9–11 nmol cytochrome *b*/mg protein and reduced 70 nmol cytochrome *c*/s per nmol cytochrome c_1 in the activity assay described below. Coenzyme Q-depleted Complex III was prepared essentially as described by Szarkowska [6]. An aqueous sample of Complex III was extracted five times with *n*-hexane, lyophilized to remove residual solvent, and redissolved in an appropriate buffer. Samples partially depleted of coenzyme Q were prepared following the same procedure but samples were removed after each extraction cycle. The coenzyme Q remaining in each sample was determined by subtracting the amount of coenzyme measured in the extracting solvent from the total coenzyme Q present in the original intact Complex III. The coenzyme Q concentration was determined as described by Szarkowska [6] using a different extinction coefficient between the oxidized and reduced quinone species of 12.25 $mM^{-1} \cdot cm^{-1}$ at 275 nm.

Cytochrome *b* was purified from Complex III by a procedure involving treatment with 3,4,5,6-tetrahydrophthalic anhydride followed by chromatography on hydroxyapatite [7]. The purified cytochrome *b* had a heme content of 37 nmol *b*/mg protein.

The zwitterionic buffers, Mes, Ada, Taps, Ches and Bicine were obtained from Sigma. Taurocholate and cholic acid were purchased from Sigma; the latter was recrystallized from ethanol before use. Triton QS-30 was obtained from Rohm and Haas. Antimycin A (Sigma) was prepared as a 1 or 10 mM solution in dimethyl sulfoxide. Other chemicals were reagent grade.

Enzyme activity was obtained by mixing 50 μM ferricytochrome *c*, 10 μM 2,3-dimethoxy-5-methyl-6-pentylbenzohydroquinone in 0.1 M potassium phosphate, pH 7.4, containing 1 mM EDTA, and following reduction of cytochrome *c* spectrophotometrically after the addition of Complex III at 25°C.

Room-temperature MCD potentiometric titrations were performed either in a Brinkmann 5 ml glass titration vessel provided with a side arm to which a 1 cm light-path quartz cuvette was attached or, alternatively, in a custom-made glass titrator with a 1 cm light-path pyrex cuvette attached directly to the base, such that the potential could be monitored continuously during spectroscopic measurements. The Brinkmann titration vessel was fitted with a hard-plastic top with standard taper holes for insertion of (i) the electrode, (ii) a stopcock for degassing and (iii) syringes containing either oxidant or reductant. The custom-made titrator has similar standard taper joints to mate with these accessories. A platinum Ag/AgCl electrode (Metrohm, type EA 234) was used together with a digital voltmeter to indicate

solution potential. This electrode was calibrated using saturated aqueous quinhydrone solutions at several pH values. The reaction mixture contained 5 μM Complex III or coenzyme Q-depleted Complex III and, unless otherwise stated, 20 μM of the following dyes: *p*-aminophenol sulfate, TMPD, DCIP, 1,2-naphthoquinone, phenazine methosulfate, methylene blue, indigotetrasulfonic acid and 2-hydroxy-1,4-naphthoquinone. This reaction mixture was made anaerobic by using techniques previously described by Beinert et al. [8]; repeated cycles of evacuation and equilibration with an atmosphere of argon were conducted before a titration was commenced. The potential was adjusted using dithionite as reductant and dry air or ferricyanide as oxidant. Equilibration of the system usually required about 5 min after each addition. The equilibrated solution was then transferred to the quartz cuvette via the side arm on the Brinkmann titration vessel or used in place in the custom-made vessel.

MCD spectra were obtained using a Jasco-500C spectropolarimeter equipped with an electromagnet and a Jasco DP-500 data processor for data accumulation and manipulation. The instruments were interfaced with a minicomputer for further data processing and presentation. A blank titration in the absence of enzyme indicated that the mediator dyes did not make any contribution to the MCD signal in the wavelength range of interest.

Liquid-helium temperature EPR potentiometric titrations were performed according to a procedure very similar to that of Dutton [9]. A 1 ml Brinkmann glass titration vessel was used and the titration was carried out with the vessel directly connected to the anaerobic train [8]. The reaction mixture contained 50 μM Complex III and 20 μM of each of the eight dyes described above. Before initiating the anaerobiosis, the solution was exposed to air and 50–100 μg bovine heart cytochrome oxidase were added in order to raise the starting potential above 350 mV; the apparatus was then closed and subjected to several cycles of evacuation and gas replacement. The titration was subsequently carried out exactly as described by Dutton [9] using a bent stainless-steel needle for initially flushing the EPR tubes with argon, transferring sample aliquots from the titrator to EPR tubes and flushing the top of EPR tubes before freezing the EPR sample in liquid nitrogen. EPR spectra were obtained using a Varian E-6 EPR which was interfaced with the laboratory data acquisition system for data storage and manipulation. The temperature was controlled by an Air Products' transfer system and a heater.

The liquid-nitrogen temperature EPR potentiometric titration for the coenzyme Q radical was carried out in much the same fashion except that cytochrome oxidase, *p*-aminophenol sulfate and TMPD were omitted and the other dyes were present at a concentration of 20 μM except for methylene blue which was reduced to 5 μM.

Room-temperature EPR potentiometric titration on Q radical was conducted in a 1 ml Brinkmann titration vessel, from which a standard taper side arm projected horizontally near the bottom. A sample transfer tube connected the side arm to a quartz flat cell positioned in the EPR cavity. The transfer tube was constructed of a 0.042 inch inner diameter polyethylene tubing with standard taper joints cemented to both ends for connection to titration vessel and the flat cell. The polyethylene tubing was enclosed by a 3/16 inch inner diameter piece of tygon tubing with 1/16 inch wall thickness. The annular space between the two tubes was constantly purged with nitrogen to minimize oxygen contamination of the sample. The whole titration system was previously flushed with N_2 for at least 20 min. The reaction mixture, which was the same as that described for liquid-nitrogen temperature EPR, was transferred between the titration vessel and the EPR flat cell by positive nitrogen pressure either on the end of the flat cell opposite the sample transfer tube or on the titration vessel.

Data analysis

In MCD titrations, the redox state of cytochrome c_1 was followed using the change in amplitude of the peak at 548.5 nm relative to the 553.5 nm trough, while the redox state of cytochrome *b* was monitored using the amplitude of 561.5 nm trough. The data for cytochrome c_1 yielded a linear Nernst logarithmic plot and the midpoint potential was obtained directly from the graph. For cytochrome *b*, the Nernst plots were biphasic and the midpoint potentials of the two compo-

nents were obtained by extrapolation of the theoretical $n = 1$ line from each arm of the titration curve to the horizontal line drawn at 50% reduction (as illustrated in Fig. 2).

The reduction of iron-sulfur center was monitred by the intensity of the g 1.89 EPR signal recorded at liquid-helium temperatures. The reduction of each cytochrome was quantitated by locating empirically the experimental spectrum which led to the best line shape of each species as judged by visual examination of difference spectra displayed on a graphics monitor. Difference EPR spectra calculated relative to this reference were required to exhibit a systematic decrease in EPR amplitudes as the potential was reduced. This procedure facilitated correction of the EPR contribution of each cytochrome for overlap from the other components present. The midpoint potential of each cytochrome was obtained by the same method as described in MCD potentiometric titration. Oxidized cytochrome c_1 was quantitated using the amplitude of the g 3.49 EPR signal, and the high- and low-potential species of oxidized cytochrome b were quantified using the EPR intensity at g 3.60 and g 3.76, respectively.

The quantitation of coenzyme Q radical at both ambient and liquid-nitrogen temperature was done using the g 2.0 radical signal after correction for the contribution from the mediator dyes; this contribution was obtained in a separate experiment from a reaction mixture containing dyes alone at the same potential. The midpoint potential values for the two half-reactions: $Q/QH^{\cdot}$ and $QH^{\cdot}/QH_2$ were obtained by fitting the data to the following equation:

$$[SQ] = (1 + 10^{(E-E_1)/59} + 10^{(E_2-E)/59})^{-1} \quad (1)$$

where [SQ] indicates the fraction of the maximum radical signal relative to the total coenzyme Q present in the original Complex III. E_1 and E_2 are the midpoint potential values for the two half-reactions, $Q/QH^{\cdot}$ and $QH^{\cdot}/QH_2$, respectively. The absolute spin concentration was determined from double integral which was then compared with that of a cupric sulfate standard at liquid-nitrogen temperature or a standard solution of potassium nitrosodisulfonate at room temperature.

Results

MCD characteristics of Complex III

The MCD spectra of yeast Complex III between 530 and 590 nm at different redox states are shown in Fig. 1. The spectrum of the oxidized enzyme is extremely weak with a peak at 550 nm, a trough at 570 nm and a crossover at 559 nm. Ascorbate-reduced Complex III has intense extrema at 548.5 and 553 nm and a crossover at 551 nm. The difference spectrum of ascorbate-reduced Complex III minus oxidized Complex III is essentially that of reduced cytochrome c_1 (data not shown) and because of the small intensity of the oxidized complex it is very similar to the absolute spectrum of the ascorbate-reduced sample. The MCD spectrum of the dithionite-reduced complex exhibits additional intense features due to the formation of reduced cytochrome b. The difference spectrum of dithionite-reduced minus ascorbate-reduced Complex III has a peak at 556.5 nm, a trough at 562 nm and a crossover at 559 nm. These extrema are very close to the corresponding values for purified reduced cytochrome b [7]. The MCD spectra of cytochrome b closely resemble those of submitochondrial particles isolated from bovine heart [10]. The calculated MCD intensities per protoheme are 197 $(M \cdot cm \cdot T)^{-1}$ at 556 nm and -262 $(M \cdot cm \cdot T)^{-1}$ at 562 nm for the complexed cytochrome b; these are higher than the corresponding values of 160 and -190 $(M \cdot cm \cdot T)^{-1}$ for purified cytochrome b. The shape and the large intensities of these spectra arise from A-terms and are typical of low-spin reduced protohemes [10]. The difference spectra of the dithionite-reduced minus ascorbate-reduced samples and the dithionite-reduced minus oxidized Complex III are identical above 557.5 nm. Importantly, the trough at 562 nm has the same amplitude in these two derivatives; this feature is attributable to cytochrome b alone. The Soret MCD of oxidized, ascorbate-reduced and dithionite-reduced Complex III do not have exclusive features to b and c_1 cytochromes, instead, the spectral contributions of cytochrome b and c_1 to the Soret MCD have significant overlap with each other.

Because of the large MCD intensities for both cytochrome b and c_1 and the well separated redox potentials of these species, we were able to per-

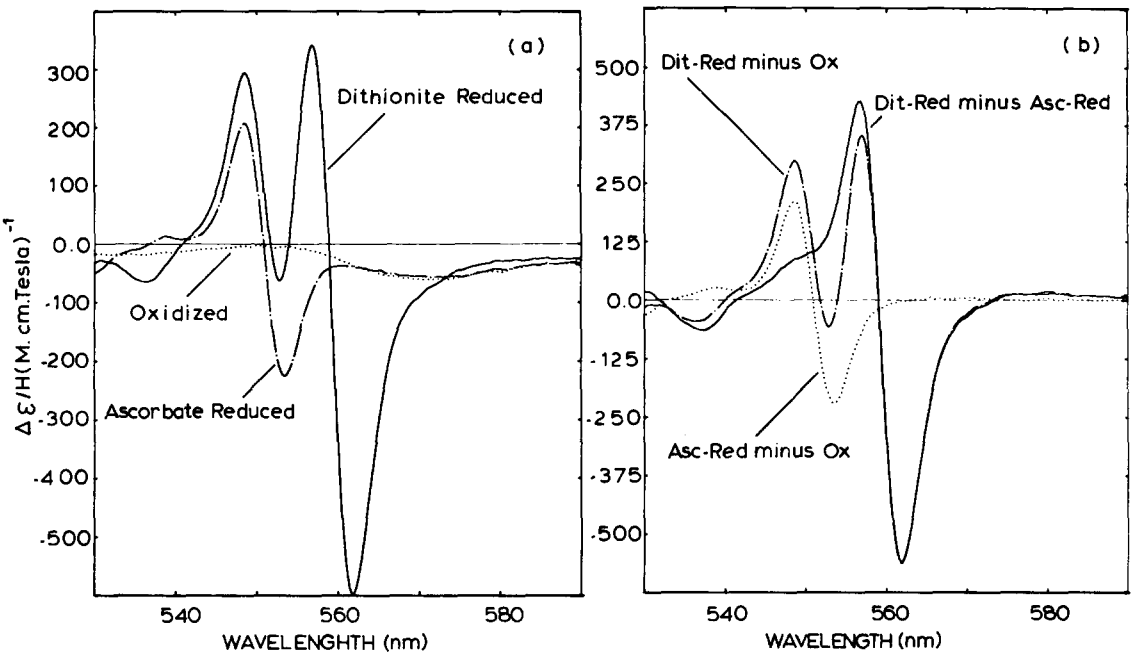

Fig. 1. Absolute (a) and different (b) MCD spectra of Complex III. The sample contained 3.9 μM cytochrome c_1, 8.3 μM cytochrome *b* and was solubilized in 0.1 M potassium phosphate, pH 7.4, containing 0.1% deoxycholate and 0.1% Triton QS-30. The absolute spectra of ascorbate-reduced and dithionite-reduced sample were recorded after the addition of solid sodium ascorbate and sodium dithionite, respectively. The difference spectra were obtained from their absolute spectra by arithmetic subtraction using the laboratory data system.

form MCD potentiometric titrations with a relatively small amount of material. However, the intense dye absorbance precludes a direct comparison of the MCD and absorbance techniques. We therefore evaluated the MCD approach by comparing MCD and optical data during a stoichiometric titration following the MCD intensities at 562 and 548–553 nm and the absorbance changes at 561.5 and 553.5 nm for the *b* and c_1 cytochromes, respectively. The two sets of measurements were in excellent agreement.

Room-temperature MCD potentiometric titrations

Since the highly colored mediator dyes make no contribution to the MCD spectrum in this wavelength range, it was possible to use a relatively large number of dyes to cover satisfactorily the wide potential span necessary to observe the oxidation-reduction of both cytochrome [12]. In these experiments, eight mediator dyes were used, including the three classes of dyes described by Walz [13]. It should also be noted that, in contrast to the absorbance properties, the MCD characteristics of the *b* and *c* cytochromes do not overlap.

A typical log-plot of data from an MCD potentiometric titration of Complex III is shown in Fig. 2. Cytochrome c_1 behaves as a simple one-electron carrier with a midpoint potential at room temperature of 270 mV; however, the MCD parameter characteristic of the *b* cytochrome(s) exibits the well known nonlinear behavior which can, in this instance, be analyzed as the sum of two one-electron redox processes. This nonlinearity can be seen clearly when titrations are carried out in either taurocholate-containing or deoxycholate-containing buffer. The derived midpoint potentials for the two *b* cytochromes are 62 and −20 mV in deoxycholate buffers; however, in taurocholate both potentials are significantly more positive, 98 and 40 mV, respectively.

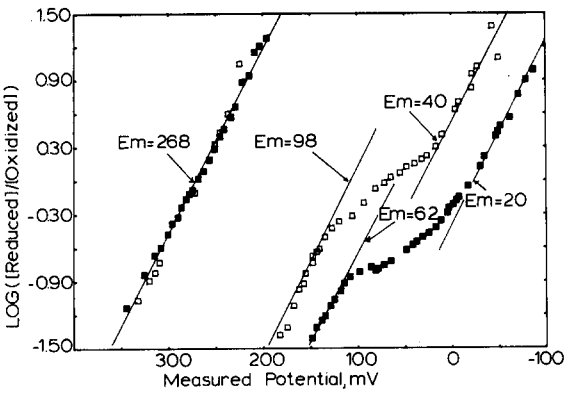

Fig. 2. Room-temperature MCD potentiometric titrations of Complex III. The titrations were performed at 23°C on 0.1 M potassium phosphate, pH 7.4, containing 1 mM EDTA, 0.1% Triton QS-30 and either 0.1% taurocholate (□) or deoxycholate (■) buffer. 5 μM Complex III and 20 μM dyes were present in the reaction mixture. The reduction of cytochrome c_1 (data at left) was monitored by a change in the difference between the MCD intensities at 548.5 and 553 nm; and the concentration of reduced cytochrome *b* (data at right) obtained from the MCD intensity at 562 nm. The midpoint potential was obtained by determining the intersection of the theoretical $n = 1$ lines of the two side arms of the titration curve with the horizontal line drawn at 50% reduction (([reduced form]/[oxidized form]) = 0).

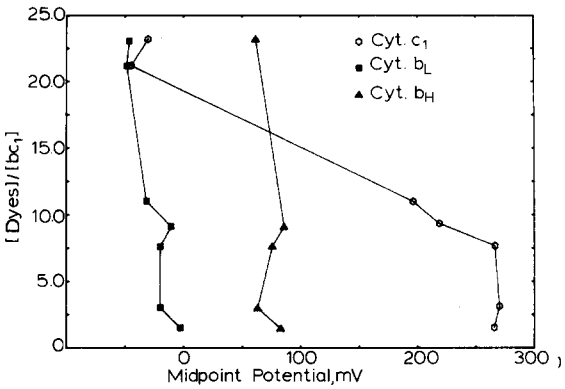

Fig. 3. The effect of the ratio of dye to protein on the midpoint potentials of the components of Complex III. All titrations were carried out at pH 7.4, in 0.1 M potassium phosphate, 0.1% deoxycholate, 0.1% Triton QS-30 and 1 mM EDTA at 23°C, 2.5 μM or 5.0 μM Complex III was used in each titration.

Dye artefact observed in potentiometric titrations

MCD potentiometric tritrations were carried out at pH 7.4 using ratios of mediator dye to enzyme complex which varied from 2 to 25. When this ratio was less than 10 the midpoint potential of cytochrome c_1 was found to be 270 mV. However, on increasing the ratio above 10 the midpoint potential decreased markedly and at a ratio of 25 the potential of cytochrome c_1 was found to be −40 mV. This amounts to a decrease of over 300 mV (Fig. 3) in response to a modest variation in relative dye concentration. Consecutive additions of each of the eight dyes at a dye/cytochrome c_1 ratio of 20 : 1 to a solution containing ascorbate-reduced Complex III led to a stepwise disappearance of the MCD signal of reduced cytochrome c_1 implying oxidation of reduced cytochrome c_1 by the dye additions. Subsequent addition of excess ascorbate did not restore the original reduced cytochrome c_1 MCD signal, consistent with the midpoint potential of cytochrome c_1 being more negative in the presence of concentrated dyes. This dye effect does not seem to be specific for any particular dye used, a simple additive effect being apparent. The *b* cytochromes, however, do not exhibit this sensitivity to the mediator dyes, at least in the concentration range that we have studied. Consequently, all titrations were performed using a dye/cytochrome c_1 ratio of less than 10, a condition which results in a traditional value for the cytochrome c_1 potential [4]. As an additional control, a titration was performed using the following alternative set of dyes (dye/cytochrome $c_1 = 7 : 1$): quinhydrone, duroquinone, 1,2-naphthoquinone, thionine, methylene blue, 2,5-dihydroxy-1,4-benzoquinone and indigocarmine. The midpoint potentials for both c_1 and *b* cytochromes obtained using this alternative system were similar to the values obtained using the original dye combination at the low dye/protein ratios.

Low-temperature EPR potentiometric titrations

Unfortunately, we were not able to identify any feature in the CD spectrum of Complex III that we could attribute to the [2Fe-2S] cluster, even though such iron-sulfur clusters typically have intense and characteristic CD [35,42]. Consequently, we were unable to obtain data on this component at room temperature. Thus, a series of potentiometric titrations were evaluated at liquid-helium

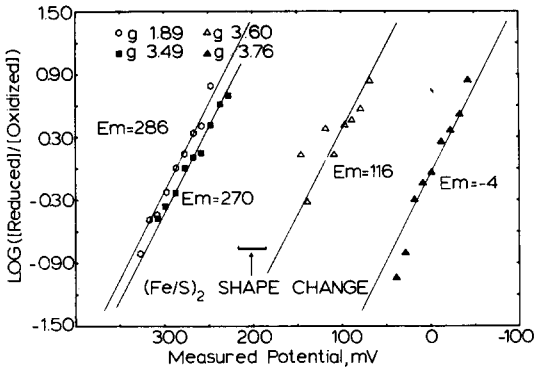

Fig. 4. Liquid-helium EPR potentiometric titration at pH 7.4. The measurements were made in 0.1 M potassium phosphate, 0.1% deoxycholate, 0.1% Triton QS-30 and 1 mM EDTA. 60 μM Complex III and 20 μM dyes were present in the reaction mixture. The midpoint potential values of the cytochromes and the iron-sulfur center were obtained by following the amplitude change of the corresponding EPR signals as a function of applied potential. The g values of the EPR signals arising from different redox centers are: iron-sulfur center, 1.89; cytochrome c_1, 3.49; high-potential cytochrome b, 3.60; low-potential cytochrome b, 3.76. The EPR conditions were: modulation amplitude, 20 G; microwave power, 0.1 mW for iron-sulfur center and 40 mW for the cytochromes; microwave frequency, 9.238 GHz; temperature, 11.8 K for the iron-sulfur center and 8.7 K for the cytochromes.

temperature using EPR spectroscopy to follow the signals of the reduced iron-sulfur center and of the three oxidized cytochromes; this latter data provide a connection with the results obtained at room temperature. This redox center exhibits a potentiometric behavior typical of a one-electron carrier and the midpoint potential of the iron-sulfur center was found to be 286 mV (Fig. 4). The midpoint potential of cytochrome c_1 deduced from the low-temperature data, 270 mV, is precisely that obtained at room temperature (Fig. 2), and the difference of 16 mV between the midpoint potentials of iron-sulfur cluster and cytochrome c_1 agrees reasonably well with the estimate of 30–40 mV obtained from combined optical and EPR stoichiometric titrations [5]. However the midpoint potentials of the b cytochromes obtained at 12 K are clearly diffrent from the values obtained at 296 K; the value of 62 mV found for the high-potential cytochrome b in deoxycholate buffer increases to 116 mV at cryogenic temperatures while the value of the low-potential cytochrome b is increased only slightly, from −20 to −4 mV. This difference in potential measurements obtained at the two temperatures can be explained in part by the difficulty in determining unambiguously the EPR contribution of the two b cytochromes from their highly overlapped EPR signals; however, the potential shift of the high-potential b cytochrome is too large to attribute to analytical error and demonstrates that the temperature differential of the spectroscopic measurements leads to electron redistribution.

The conformational change of the iron-sulfur center reported previously [5] was observed to occur in a potential range of approx. 210–220 mV, immediately following the reduction of cytochrome c_1 and the iron-sulfur cluster. It thus appears that this change of the iron-sulfur cluster is not a secondary result of changes in the redox state of the b cytochromes as previously suggested [5,34], since these components are still oxidized at the completion of the EPR transition. De Vries et al. [16] found the shape change of Rieske iron-sulfur center of bovine heart Complex III to end at 40–60 mV (pH 7.2), yet they also demonstrated that this change in line shape is unrelated to the redox behavior of cytochrome b-562. In our experiments the change in spectral shape occurs over a potential span of less than 30 mV; this abrupt behavior is inconsistent with the presence of two iron-sulfur centers with different midpoint potentials [15].

The potentiometric properties of coenzyme Q

The determination of potentiometric behavior of coenzyme Q was complicated by the presence of an EPR signal generated by the semiquinone forms of the mediator dyes which have EPR parameters similar to those of the Q radical. The EPR spectrum of the coenzyme Q radical obtained at liquid-nitrogen temperature exhibits a line width of 9 G (peak to trough) (Fig. 6a) and exhibits nonhomogeneous power saturation at about 0.1 mW. The contribution of the mediators was minimized by judicious selection of dyes and by keeping the dye concentration as low as was consistent with acceptable equilibration. By these means the total contribution of the dye signal was kept to a few percent of the size of the coenzyme Q radical

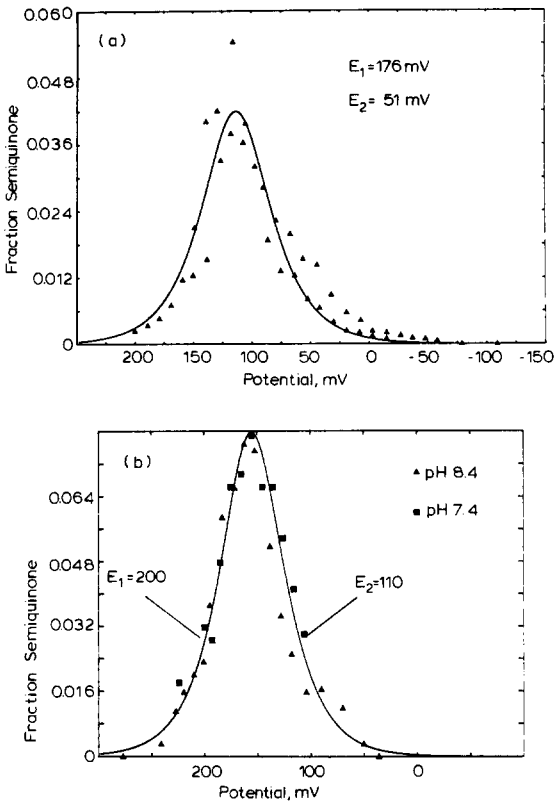

Fig. 5. Liquid-nitrogen temperature (a) and room-temperature (b) EPR potentiometric titration of coenzyme Q radical. (a) 40 μM Complex III and 20 μM of DCIP, 1,2-naphthoquinone, phenazine methosulfate, indigotetrasulfonic acid and 5 μM methylene blue were present in 0.1 M potassium phosphate, pH 7.4, containing 0.1% deoxycholate, 0.1% Triton QS-30. The EPR spectra of coenzyme Q radical were recorded through the whole titration after each addition of reductant or oxidant. The g 2.0 signal amplitude was corrected for the dye contribution and normalized against total coenzyme Q concentration. This correction assumes that the presence of the enzyme did not modify the potentials of the dyes present. The midpoint potentials for the two half-reactions were calculated according to a least-squares fit to Eqn. 1 as described in Data analysis. The EPR conditions were: modulation amplitude, 10 G; microwave power, 0.1 mW; microwave frequency, 9.238 GHz; temperature, 110 K. (b) The reaction mixture was the same as in a except that 0.1 M Taps was used in the experiments performed at pH 8.5. The EPR conditions were: modulation amplitude, 10 G; microwave power, 20 mW; microwave frequency, 9.505 GHz; temperature, 296 K.

at all but the lowest concentration of this semiquinone. Fig. 5a shows a titration profile of Q semiquinone measured at liquid-nitrogen temperature. The maximum radical concentration was found to be only 5% of the total coenzyme Q present; this corresponds to a stability constant ($[QH^{\cdot}]^2/([Q][QH_2])$) of $1.1 \cdot 10^{-2}$ assuming that the coenzyme Q present in Complex III is homogeneous. However, if the Q radicals arise from a pool of Q which is equimolar with cytochrome c_1, then the stability constant is $2.6 \cdot 10^{-2}$. Both values are very close to those found for bovine hart succinate : cytochrome c reductase by Ohnishi and Trumpower [17]. The midpoint potentials estimated by fitting the data to Eqn. 1 are 176 and 51 mV, respectively.

Similar experiments were carried out at room temperature at pH 7.4 and 8.4 (Fig. 5b). At this temperature, the stable semiquinone in Complex

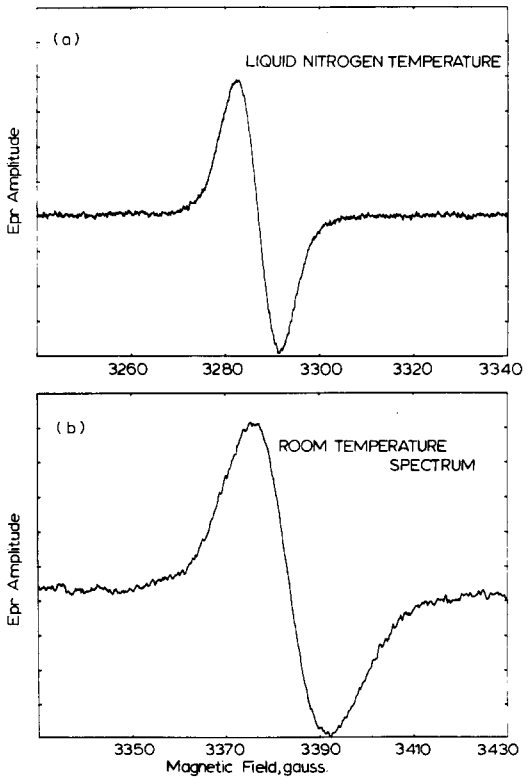

Fig. 6. Typical EPR spectra of stable coenzyme Q radical of Complex III. (a) EPR spectrum at a redox potential of 149 mV, pH 7.4 and 100 K. The EPR condition and the reaction mixture are the same as those in Fig. 5a. The signal contributed from dye molecules was corrected by a blank titration on dyes only. (b) EPR spectrum of semiquinone at pH 8.4 at 23°C. The spectrum was obtained as described in Fig. 5b. The redox potential of the sample was 151 mV.

III exhibits an EPR spectrum with a line width of 15 G (Fig. 6b), almost double that found in bovine heart mitochondria [18] and succinate : cytochrome *c* reductase [17]; this Q radical displays nonhomogeneous saturation at 10 mW, similar to that reported for bovine heart [19]. The maximum amount of stable semiquinone radical observed is about 8% of the total coenzyme Q present at both pH values; this value is comparable to that obtained at liquid-nitrogen temperatures. However, the derived midpoint potentials for the two half-reactions, 200 and 110 mV at both pH values, are somewhat larger than those obtained at liquid-nitrogen temperature. As the increase in pH from 7.4 to 8.4 does not produce any change of the potentiometric characteristics, it would appear that the p*K* values of the two half-reactions are either greater than 8.4 for the oxidized species or smaller than 7.4 for the reduced species.

Effect of pH and detergents on the midpoint potentials

The influence of pH on the midpoint potentials of cytochromes *b* and c_1 was examined at room temperature using MCD. In a preliminary experiment, a series of assays were performed to find the optimum pH for enzyme activity and to establish the dependence of enzyme stability on pH. The pH optimum was determined in two different ways: (a) Complex III was incubated in 0.1 M potassium phosphate, pH 7.4, containing 0.1% deoxycholate and 0.1% Triton QS-30 and assayed at various pH values using assay mixtures of appropriate buffers; (b) Complex III was incubated in buffers of various pH values and assayed in those same buffer solutions, each containing 0.1% deoxycholate and 0.1% Triton QS-30. Both methods indicate an optimum pH value for enzyme activity of about pH 7.5–8.0.

To study its stability, Complex III was incubated at 4°C in a series of buffers having pH values ranging from 5.0 to 10.0. Samples were removed at intervals up to 48 h and assayed in the standard system. In the pH range 5.5–9.0, about 90% activity remained after 4 h incubation, 70–85% after 24 h, and 50–80% after 2 days. Enzyme incubated at pH values greater than 9.0 or lower than 5.5 lost activity much faster; 50–60% loss of activity was observed in the first 4 h and another 10–20% activity loss occurred during the next 20 h. Less than 30% of the original activity remained after 2 days.

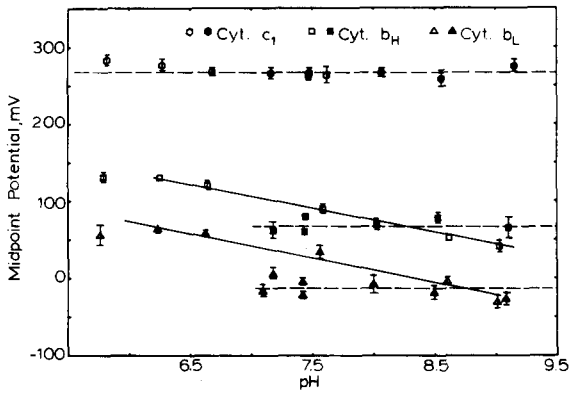

Fig. 7. The effect of pH on the midpoint potentials of cytochrome *b* and c_1. All titrations were conducted at 23°C in buffers of different pH values (pyrophosphate, pH 5.5; Mes, pH 6.0, 6.5; Ada, pH 7.0, 7.5; potassium phosphate, pH 7.5; Tris, pH 8.0; Bicine, pH 8.5; Ches, pH 9.0). The closed symbols represent data taken in 0.1% Triton QS-30 and 0.1% deoxycholate. The open symbols correspond to values obtained using 0.1% Triton QS-30 and 0.1% taurocholate. Cyt. c_1, cytochrome c_1; Cyt. b_H, high potential cytochrome *b*; Cyt. b_L, low-potential cytochrome *b*. The vertical bars represent the range of values obtained in the oxidative and reductive directions of a single titration.

Consequently, titrations were conducted at selected pH values in the range pH 5.5–9.0. The variations of the midpoint potentials of cytochromes *b* and c_1 were measured and the variation of these values with pH is presented in Fig. 7. It is clear that cytochrome c_1 has a midpoint potential of about 260–270 mV which does not change in this pH range. A similar result was observed for both *b* cytochromes when the titrations were carried out in buffers containing 0.1% deoxycholate and 0.1% Triton QS-30 as detergents.

However, when 0.1% taurocholate was substituted for 0.1% deoxycholate, the *b* cytochromes exhibited pH-dependent midpoint potentials between pH 6.0 and 9.0. This pH dependency does not appear to be a result of variability in the data, since the value of the midpoint potential of cytochome c_1 shows little variation in the same experiments. The pH dependence of both *b* cytochromes was approx. −30 mV/pH. The p*K* values of the

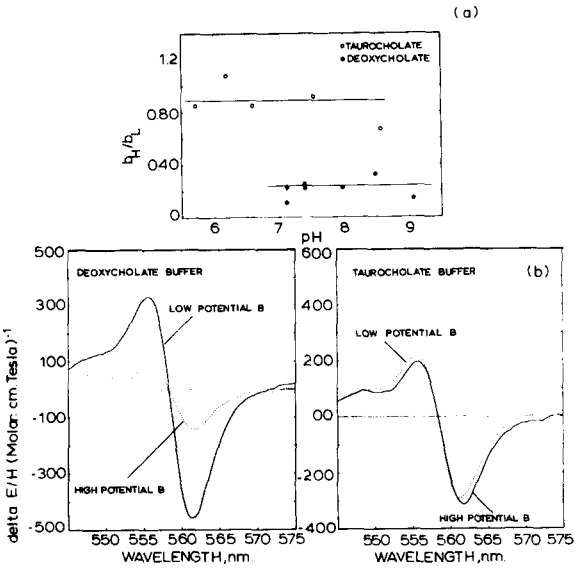

Fig. 8. Effect of the nature of the detergent on the ratio of the MCD absorbance contributions of the *b* cytochromes. (a) The difference between the MCD spectrum corresponding to the titration end point and the spectrum associated with the inflection point of the titration curve is assigned to low-potential cytochrome *b* (b_L); and the difference between the inflection point and the start of the titration of the *b* cytochromes is assigned to high-potential *b* (b_H). The ratio of the signal amplitude at 562 nm between each pair of spectra assigned to cytochrome b_H and b_L was calculated for titrations carried out at different pH values and plotted. (b) MCD spectra contributed by two cytochrome *b* species with different midpoint potentials as described in (a) in 0.1 M potassium phosphate, pH 7.4 and 7.5, containing either taurocholate or deoxycholate.

oxidized form is about pH 6.0 − 6.5 as judged from the lack of variation of E_m with pH at low pH values; the p*K* value of the reduced form was not established due to the increasing instability of the enzyme at pH values above 9.0. There was no obvious difference in the MCD or EPR spectra of ascorbate-reduced Complex III solubilized in a buffer containing either deoxycholate or taurocholate at both pH 7.5 or 8.5 (data not shown).

The change in detergent also produced a change in the fraction of cytochrome *b* that titrated at each of the two midpoint potentials (Fig. 8). The individual contributions of the high- and low-potential cytochromes *b* were calculated from the fractional change in MCD intensity associated with the inflection point of Nernst plots of the cytochrome *b* signal (see Fig. 2). The relative contributions of the high-potential cytochrome *b* to the low-potential cytochrome *b* is 0.9 : 1 in taurocholate-containing buffers and 0.1 : 1 in deoxycholate buffers. This phenomenon can be seen clearly when titrations performed at pH 7.5 in deoxycholate and taurocholate-containing buffer solutions are juxtaposed (Fig. 2). Thus, the relative contribution of the two cytochrome *b* species to the MCD spectrum at 562 nm can be affected markedly by the nature of the detergents even though the total MCD intensity is unaffected by such a change.

Changing the detergent had no obvious effect on the envelope of the low-field EPR spectrum recorded at 12 K. However, it should be noted that, unlike the bovine heart system, the individual contributions of the two cytochrome *b* components are difficult to resolve [5].

Effect of Q depletion

The presence or absence of coenzyme Q does

TABLE I

ANTIMYCIN A EFFECT ON THE MIDPOINT POTENTIALS OF CYTOCHROMES

	E_m (c_1)	E_m (b_H)	E_m (b_L)	b_H/b_L
Cytochrome bc_1	268 ± 5	62 ± 5	−20 ± 5	0.24 ± 0.01
Coenzyme Q-depleted cytochrome bc_1	256 ± 10	64 ± 8	−12 ± 8	0.16 ± 0.02
Coenzyme Q-depleted cytochrome bc_1 + Antimycin A	260 ± 5	95 ± 5	8 + 5	0.32 ± 0.02
Cytochrome bc_1 − 70% coenzyme Q Antimycin A	–	100 ± 10	16 ± 5	0.50 ± 0.01
Cytochrome bc_1 − 30% cytochrome Q + Antimycin A	256 ± 5	92 ± 5	16 ± 8	0.76 ± 0.01
Cytochrome bc_1 + Antimycin A	260 ± 10	120 ± 15	26 ± 8	0.82 ± 0.02

not seem to have any effect on the midpoint potential values for the cytochromes as indicated in Table I. The midpoint potential values for cytochromes *b* and c_1 obtained from native Complex III and Q-depleted sample are essentially the same for the cytochromes within experimental error.

Effect of antimycin A on midpoint potentials

As we reported previously [7], antimycin does bind to Complex III with a 1 : 1 stoichiometry and is a potent inhibitor of catalytic activity. However, it should be noted that, in marked contrast to Complex III from heart, antimycin has no effect on the absorbance or MCD characteristics of yeast Complex III. The influence of antimycin A on the midpoint potentials of the cytochromes was tested by including a 5-fold excess of antimycin A with Complex III and coenzyme Q-depleted Complex III during MCD potentiometric titrations. A summary of the titrations involving antimycin are presented in Table I. Clearly, the presence of antimycin A at this concentration had little effect on the midpoint potential of cytochrome c_1. However, an increase in the midpoint potential of both species of cytochrome *b* was observed. After each addition of reductant or oxidant, the cytochrome *b* MCD signal increased and then relaxed to the original value(s) over 20 min; these changes are not due to sluggish equilibration but reflect some internal transition. As each MCD potentiometric measurement took 5–15 min after each addition of reductant or oxidant, this transition could be incomplete.

Antimycin A appears to cause an increase in the ratio of high-potential cytochrome *b* to low-potential cytochrome *b*. Furthermore, this ratio is largest for intact Complex III and smallest for the sample from which coenzyme Q was almost completely removed.

Discussion

Table II summarizes the results of a number of potentiometric studies on the components of Complex III. Comparison of data obtained using submitochondrial particles, Complex III and purified *b* or c_1 cytochromes from a variety of sources, documents the close resemblance for the midpoint potentials of the individual redox components. All data for the Fe-S center and cytochrome c_1 are in close agreement while the values for cytochromes *b* and coenzyme Q exhibit only a modest variation (Table II). Furthermore, the difference reported in the number of species of cytochome *b* and the variation in the midpoint potentials observed in the different systems points to a marked sensitivity of the structure of the cytochromes *b* to their environment.

We have recently obtained highly purified preparations of yeast cytochrome *b* [7]. These preparations typically contain about 37 nmol protoheme/mg protein which corresponds to a minimum molecular weight of 27 000. However, Nobrega and Tzagoloff [36] have concluded that the three exons of the cytochrome *b* gene code for a polypeptide consisting of 385 amino acid residues. This polypeptide would have a molecular weight of 44 000. Taken together, these facts imply that yeast mitochondrial cytochrome *b* contains 2 mol heme and that our cytochrome *b* preparation is about 80% pure (or has lost part of the protoheme during purification). The presence of two heme centers in this protein subunit provides an obvious explanation for our observation of two different species of cytochrome *b* in yeast Complex III with one heme center now being identified as cytochrome *b*-566 and the second as cytochrome *b*-562. A competing interpretation is based on the finding from low-resolution X-ray diffraction measurement [32,33] that Complex III exists as a dimer in semicrystalline membrane preparations. In the latter interpretation, one subunit of this dimer provides the first species of cytochrome *b* and the other subunit provides the second species. However, as this dimer possesses apparent 2-fold symmetry, it is not obvious what structural feature could lead to the discrimination between the two cytochrome *b* subunits. As the polypeptide sequence of cytochrome *b* has no obvious internal symmetry, the differentiation of the two heme centers occurs naturally and we consequently favor the former interpretation.

The variations in enzyme preparation and the nature of the detergents in the titration mixture appear to control significantly the redox behavior of the *b* cytochrome(s). In particular, the choice of mediator dyes for this very hydrophobic protein could well have a critical influence on the

TABLE II

MIDPOINT POTENTIAL VALUES OF THE REDOX COMPONENTS IN THE CYTOCHROME bc_1 REGION OF THE MITOCHONDRIAL ELECTRON-TRANSFER CHAIN

R.T., room temperature, exact value unknown.

Materials	Component(s)	E_m (mV)	Temperature (K)	pH	Ref.
Succinate:cytochrome *c* reductase, pigeon heart	c_1	245	R.T.	7.0	45
Complex III, bovine heart	c_1	232	R.T.	7.2	1
Complex III, yeast	c_1	268	297	7.4	this work
Complex III, yeast	c_1	270	9	7.4	this work
Purified cytochrome c_1, bovine heart	c_1	225	296	7.2	40
Purified cytochrome c_1, bovine heart	c_1	250	R.T.	7.0	37
Yeast mitochondria	3*b*	-65 ± 15	R.T.	7.0	14
		5 ± 15			
		-50 ± 15			
Complex III, bovine heart	2*b*	105	298	7.0	37
		5			
Complex III, bovine heart	3*b*	85	R.T.	7.4	38
		85			
		−45			
Complex III, yeast	2*b*	62	297	7.4	this work
		−20			
Complex III, yeast	2*b*	116	9	7.4	this work
		−6			
Complex III, *Neurospora crassa*	2*b*	60	R.T.	7.0	41
		−40			
Purified cytochrome *b*, yeast	1*b*	−44	297	7.4	7
Purified cytochrome *b*, *Neurospora crassa*	1*b*	−70	R.T.	7.0	41
Purified cytochrome *b*, bovine heart	2*b*	−5	R.T.	7.0	37
		−85			
Submitochondrial particles, bovine heart	CoQ	204	293	7.0	18
		−36			
Submitochondrial particles, bovine heart	CoQ	184	77	7.0	18
		−30			
Complex III, bovine heart	CoQ	83	296	8.0	19
		51			
Complex III, yeast	CoQ	200	297	7.4 and 8.4	this work
		110			
Complex III, yeast	CoQ	176	110	7.4	this work
		51			
Mitochondria, pigeon heart	Fe/S	280	11	7.0	43
Complex III, bovine heart	Fe/S	180	100	7.4	44
Complex III, yeast	Fe/S	286	12	7.4	this work

equilibration between heme centers and the measuring electrode [12]. Similar reasoning may explain the variation of the midpoint potentials of coenzyme Q. Prince et al. [23] have assessed the physical and chemical properties of a variety of mediator dyes and based on their conclusions, several shortcomings of our dye system are to be noted. These include the instability of TMPD, the alkaline degradation and possible photodecomposition of phenazine methosulfate by the measur-

ing beam and the anticipated strong binding of some rather hydrophobic quinone dyes to Complex III.

In this paper, we present a dramatic example of an experimental artefact generated by mediator dyes, with a shift of midpoint potential by more than 300 mV observed for cytochrome c_1. This large change in midpoint potential is most simply explained if the binding of dye molecules to oxidized cytochrome c_1 is much stronger than to the reduced form. However, the dye dependence cannot be fitted by assuming that a single dye species is bound; rather, it appears that binding sites for several dye species are present. This assumption is substantiated by the demonstration of the oxidation of ascorbate-reduced Complex III by consecutive addition of the individual components of the dye mixture. However, at low dye concentrations, we were able to obtain plausible values for the midpoint potential of all the redox components of the Complex III. The consistency between the data from stoichiometric titrations [5] and potentiometric titrations using two different dye systems and low dye concentration implies that the values for the midpoint potentials we report are reliable. Unfortunately, the interference of high concentration of mediator dyes on the midpoint potential of cytochrome c_1 has limited the versatility of MCD potentiometric titrations with this component.

Substituting deoxycholate by taurocholate in the reaction mixture not only changes the pH dependency of the midpoint potentials of cytochrome *b* but also modifies the relative contribution of the two cytochrome *b* species to the total MCD signal. There is, however, no observable difference in the absolute MCD spectra of fully reduced samples containing one or other detergent. These two observations imply that the high-potential cytochrome *b* center can be modified to a form with substantially reduced potential but with unchanged MCD characteristics at 562 nm. Earlier, Leigh and Erecinska [3] observed that cytochrome *b*-566 present in succinate : cytochrome *c* reductase exhibited a different pH dependence when titrated as a phospholipid-supplemented sample as compared to a Triton-deoxycholate-solubilized sample [3] and an effect of phospholipid on the redox behavior of *b* cytochromes was also found by Yu et al. [38] when studying bovine heart Complex III. It seems clear that the potentiometric behavior of cytochrome *b* is extremely sensitive to the detergent used to solubilize the sample.

Because the entropy changes of the redox centers need not be the same, varying temperature dependencies of the redox potentials are to be expected. However, the data obtained using room-temperature EPR and MCD, and low-temperature EPR are in reasonable agreement. Thus, the temperature artefacts discussed previously [21,22] do not appear to be a serious complication in this experimental system. Although significant differences are seen for high-potential cytochrome *b* and coenzyme Q, the midpoint potential of cytochrome c_1 is apparently temperature insensitive and the midpoint potentials for the two half-reactions of coenzyme Q measured at liquid-nitrogen temperature and room temperature fall in between the high-potential components (iron-sulfur cluster and cytochrome c_1) and the low-potential *b* cytochromes. By contrast, the midpoint potential values of the *b* and c_1 cytochromes of pigeon heart succinate : cytochrome *c* reductase appear to be independent of measuring temperature (Table II and Ref. 3).

We consistently find a stability constant of about 10^{-2} for the semiquinone radical at pH 7.4 regardless of the temperature of measurement. This value, which corresponds to 0.1–0.2 spin cytochrome c_1, is similar to that obtained by Ohnishi and Trumpower [17] with succinate : cytochrome *c* reductase from bovine heart, but is smaller than the value of 0.5 reported by De Vries et al. [18] with bovine heart mitochondria at pH 7.4. Nagaoka et al. [19] also found a maximum semiquinone concentration of 0.1 spin/c_1 with beef heart Complex III using room temperature EPR at pH 8.0. However, in contrast to our observation, Nagaoka found the concentration of semiquinone to be pH dependent and maximal at pH 9.0. This pH dependence was also observed with the bovine heart succinate : cytochrome *c* reductase [17] and submitochondrial particles [18,24]; pK values of 6.4 and 8.0 were obtained with the former and 6.4 and greater than 9.0 with the latter system. It appears that in our enzyme preparation the pK values of the reduced and oxidized forms of Q lie outside the pH range 7.4–8.4. It is not known whether or

not this property is inherent to yeast mitochondria or whether the lack of pH dependence of the midpoint potential we observe is a consequence of the preparative procedure. Measurements on yeast submitochondrial particles should answer this question.

The stoichiometry of proton translocation accompanying electron transfer by Complex III is not established. Recent data from two different groups [27,28] indicates a stoichiometry of $4H^+/2e^-$, with coenzyme Q assumed responsible for the translocation of two protons; however, the mechanism of translocation of the remaining two protons has not been defined and there is no indication that cytochrome c_1 plays a role in proton translocation (Fig. 7). Prince and Dutton [29] find that the midpoint potential of the iron-sulfur center of pigeon breast mitochondria is only dependent on pH in a nonphysiological pH range. In contrast, the pH dependence of the redox behavior of cytochrome *b* is well established and of significant magnitude [3,30,31]. In this study, we found a dependence of -30 mV/pH for both *b* cytochromes when measured between pH 6.5 and 9.0 in buffers containing taurocholate. It must be emphasized, however, that no dependence on pH was observed when titrations were carried out in buffers that used deoxycholate as detergent. Apparently, taurocholate induces a structural change exposing an ionizable group which on deprotonation affects the midpoint potentials of the *b* heme. The pH dependency of the midpoint potentials of the two *b* cytochromes, -30 mV/pH, imply that there are two electron equivalents involved for each equivalent of proton consumed.

Antimycin A does not have an obvious effect on the midpoint potential of cytochrome c_1 while the measured increase in the midpoint potential of the *b* cytochromes might simply be a consequence of the retarded equilibration observed for this species when antimycin is present. Even though we increased the equilibration period substantially, the absence of complete equilibration cannot be ruled out.

Antimycin A clearly influences the relative contribution of the two species of cytochrome *b* to the total MCD signal at 562 nm. This seems to be mediated through coenzyme Q and provides another example of the transition between different species of cytochrome *b* being affected by a chemical reagent. The regulation of the redox potential of cytochrome *b* by coenzyme Q was suggested by Kok and Slater [39] from a comparative study of the oxidant-induced reduction of cytochrome *b* in wild-type and coenzyme Q-deficient strains of yeast. However, replenishing Q-depleted Complex III with coenzyme Q has no influence on the midpoint potential of the *b* cytochromes [38] and the effects we describe are fully demonstrable in Complex III with its regular complement of Q. It appears that coenzyme Q only modulates the redox potential of *b* cytochromes in the presence of antimycin A, and thus the extra reduction of cytochrome *b* observed by Kok and Slater [39] may well arise from a shift in the equilibrium between the two cytochrome *b* species which is induced by antimycin A and mediated by coenzyme Q.

Acknowledgements

This work was supported by grants from the NIH (GM 21337) and the Welch Foundation (C 636).

References

1 Nelson, B.D. and Gellerfors, P. (1974) Biochim. Biophys. Acta 357, 358–364
2 Rieske, J.S. (1975) Biochim. Biophys. Acta 456, 195–243
3 Leigh, J.S. and Erecinska, M. (1975) Biochim. Biophys. Acta 387, 95–106
4 Wikstrom, M.K.F. (1973) Biochim. Biophys. Acta 301, 155–193
5 Siedow, J.N., Power, S., De la Rosa, F.F. and Palmer, G. (1978) J. Biol. Chem. 253, 2392–2399
6 Szarkowska, L. (1966) Arch. Biochem. Biophys. 113, 519–525
7 Tsai, A. and Palmer, G. (1982) Biochim. Biophys. Acta 681, 484–495
8 Beinert, H., Orme-Johnson, W.H. and Palmer, G. (1979) Methods, Enzymol. 54, 111–132
9 Dutton, P.L. (1971) Biochim. Biophys. Acta 226, 63–81
10 Vickery, L., Nozawa, T. and Sauer, K. (1976) J. Am. Chem. Soc. 98, 351–357
11 Arutjunjan, A.M., Konstantinov, A.A. and Sharonov, Y.A. (1974) FEBS Lett. 46, 317–320
12 Dutton, P.L. (1978) Methods Enzymol. 54, 411–435
13 Walz, D. (1979) Biochim. Biophys. Acta 505, 299–353
14 Sato, N., Ohnishi, T. and Chance, B. (1972) Biochim. Biophys. Acta 275, 288–297
15 Dutton, P.L., Wilson, D.F. and Lee, C.P. (1970) Biochemistry 9, 5077–5082

16 De Vries, S., Albracht, S.P.J. and Leeuqerik, F.J. (1979) Biochim. Biophys. Acta 546, 316–333
17 Ohnishi, T. and Trumpower, B.L. (1980) J. Biol. Chem. 255, 3278–3284
18 De Vries, S., Berden, J.A. and Slater, E.C. (1980) FEBS Lett. 122, 143–148
19 Nagaoka, S., Yu, L. and King, T.E. (1981) Arch. Biochem. Biophys. 208, 334–343
20 Rieske, J.S. (1971) Arch. Biochem. Biophys. 145, 179–193
21 Williams-Smith, D.L., Bray, R.C., Barber, M.J., Tsopanakis, A.D. and Vincent, S.P. (1977) Biochem. J. 167, 593–600
22 Palmer, G. and Olson, J.S. (1980) in Molybdenum and Molybdenum Containing Enzymes (Coughlas, M., ed.), pp. 187–220, New York
23 Prince, R.C., Linkletter, S.J.G. and Dutton, P.L. (1981) Biochim. Biophys. Acta 635, 132–148
24 Salerno, J.C. and Ohnishi, T. (1980) Biochem. J. 192, 769–781
25 Erecinska, M., Oshino, R., Oshino, N. and Chance, B. (1973) Arch. Biochem. Biophys. 157, 431–445
26 Wilson, D.F., Erecinska, M., Leigh, J.J.S. and Koppleman, M.C. (1972) Arch. Biochem. Biophys. 151, 112–121
27 Guerrieri, F., Izzo, G., Maida, I. and Papa, S. (1981) FEBS Lett. 125, 261–265
28 Alexandre,A., Galiazzo, F. and Lehninger, A.L. (1980) J. Biol. Chem. 235, 10721–10730
29 Prince, R.C. and Dutton, P.L. (1976) FEBS Lett. 65, 117–119
30 Straub, J.P. and Colpa-Boostra, J.P. (1962) Biochim. Biophys. Acta 58, 307–313
31 Urban, P.F. and Klingenberg, M. (1969) Eur. J. Biochem. 9, 519–525
32 Lepault, J., Weiss, H., Homo, J.-C. and Leonard, K. (1981) J. Mol. Biol. 149, 275–284
33 Leonard, K., Wingfield, P., Arad, T. and Weiss, H. (1981) J. Mol. Biol. 149, 259–274
34 Orme-Johnson, N.R., Hansen, R.E. and Beinert, H. (1974) J. Biol. Chem. 249, 1928–1939
35 Palmer, G., Brintzinger, H. and Eastabrook, R.W. (1967) Biochemistry 6, 1658–1664
36 Nobrega, F.G. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 9828–9837
37 Von Jagow, G., Schagger, H., Engel, W.D., Hachenberg, H. and Kolb, H.J.C. (1978) in Energy Conservation in Biological Membranes (Schafer, G. and Klingenberg, M., eds.), pp. 43–52
38 Yu, C.A., Yu, L. and Kling, T.E. (1979) Arch. Biochem. Biophys. 198, 314–322
39 Kok, J.D. and Slater, E.C. (1975) Biochim. Biophys. Acta 376, 27–41
40 Chiang, Y.L. and King, T.E. (1979) J. Biol. Chem. 254, 1845–1853
41 Weiss, H. and Ziganke, B. (1974) Eur. J. Biochem. 53, 212–221
42 Stephens, P.J., Thomson, A.J., Dunn, J.B.R., Keiderling, T.A., Rawlings, J., Rao, K.K. and Hall, D.O. (1978) Biochemistry 17, 4770–4778
43 Prince, R.C., Lindsay, J.G. and Dutton, P.L. (1975) FEBS Lett. 51, 108–111
44 Rieske, J.S., Hansen, R.E. and Zaugg, W.S. (1964) J. Biol. Chem. 239, 3017–3022
45 Ross, E. and Schatz, G. (1976) J. Biol. Chem. 251, 1991–1996