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Rapid redox equilibrium between the mitochondrial Q pool and cytochrome *b* during triphasic reduction of cytochrome *b* by succinate

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(1) The reliability of monitoring the redox reactions of cytochrome *b* using the different wavelengths employed by different authors has been reexamined. It was found that 562–575 nm is suitable in succinate:cytochrome *c* reductase but not in mitochondria, in which case 562–540 nm is a better pair. (2) Direct optical measurements of the redox reaction kinetics of the mitochondrial Q pool using a commercial dual-wavelength spectrophotometer are possible when succinate is used as the electron donor. (3) Using the correct wavelength pair, and with malonate to slow down the electron input, the reduction course of cytochrome *b* was still triphasic but a plateau or a turn replaced the oxidation phase previously reported by several authors. At the same time, the reduction course of the Q pool was also triphasic, and in perfect match with that of cytochrome *b*. Destruction of the Rieske iron-sulfur cluster by British anti-Lewisite (BAL) + O_2 treatment or prereduction of the high-potential components made the reduction of both Q and *b* monophasic. (4) The plot of log (Q/QH_2) against log (b^{3+}/b^{2+}) gave a straight line with an *n* value of 1.7 for cytochrome *b* at pH 7.4. This *n* value rose to 2.0 at pH 6.5 and dropped to 1.4 at pH 8.5. On the other hand, the mid-point potential of cytochrome *b* relative to that of the Q pool remained essentially unchanged between pH 6.5 and 8.4. BAL treatment had a small effect on the midpoint potential of cytochrome *b* relative to that of the Q pool and had no effect on the *n* value. (5) Addition of quinone homologues and analogues extended the plateau phase in the reduction of cytochrome *b*, but exogenous quinones did not equilibrate rapidly with cytochrome *b*. (6) It was concluded that the appearance of the plateau between the two reduction phases of Q and *b* is caused by the rapid delivery of electrons to the high-potential components of the respiratory chain as envisaged in the Q cycle; the unexpected *n* value for cytochrome *b* suggests a concerted reduction by QH_2 of two species of cytochromes *b*-562.

Introduction

The triphasic reduction of cytochrome *b* – an initial partial reduction, followed by a net oxidation, and after that the final reduction – has aroused considerable interest and various explanations have been proposed [1–5]. De Vries et al. [6] confirmed the triphasic reduction, but, in disagreement with the previously cited reports, found

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Abbreviations: BAL, British anti-Lewisite, 2,3-dimercaptopropanol; Q, ubiquinone-10; Q_n, ubiquinone-*n*, where *n* is the number of isoprenoid units; DB(H)Q, (reduced)5-*n*-decyl-6-methyl-2,3-dimethoxyl-1,4-benzoquinone.

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a constant redox state in the second phase, that is, a plateau between the two redox phases. We have found that the difference between the different groups is explainable by the wavelength used to monitor the reduction of cytochrome *b*. At wavelengths chosen to monitor cytochrome *b* reduction without interference from redox changes in the *c*-type cytochromes, we, like de Vries et al. [6], find a plateau in the second phase. However, the extent of reduction found in the first and third phase does not agree with that reported by De Vries et al. [6], who correlated the phases with the reduction of high- and low-potential cytochrome *b*-562, respectively.

It has long been believed that cytochrome *b* is in redox equilibrium with the Q pool [7,8]. In Mitchell's Q-cycle model, Q (in different redox states) is both the reductant and oxidant of cytochrome *b* [9]. For the understanding of the peculiar kinetics of *b* reduction, it is of great importance to know the redox behavior of the Q pool while *b* undergoes multiphasic reduction. Q absorbs in the ultraviolet with a peak around 272 nm in aqueous solution or in mitochondria preparations where many mitochondrial components have high extinction coefficients and where the turbid suspension of mitochondria scatters incident light strongly. For this reason, the redox level of Q has in many cases been measured after extraction into organic solvents. This method is laborious and can hardly give an accurate kinetic curve, especially when the redox state of Q changes rapidly. We have, therefore, used the direct spectrophotometric procedure, introduced by Chance and Redfearn [10] and Szarkowska and Klingenberg [11], to follow the redox changes of Q. A modern commercially available dual-wavelength spectrophotometer was found suitable. Using this method we found that the Q pool behaves similarly to cytochrome *b* in response to succinate addition. BAL treatment or prereduction of the high-potential components of the respiratory chain removes the plateau in the reduction of *b* and the Q pool, whereas addition of quinone homologues or analogues results in a longer plateau between the two reduction phases of cytochrome *b*. Thus, a simple explanation for the triphasic reduction of cytochrome *b* can be proposed on the basis of the Q-cycle model and the Q-*b* redox equilibration. Moreover, quantitative calculation of the Q-*b* equilibrium gives the interesting value of *n* for cytochrome *b* in the Nernst equation of 2.0 to 1.4, depending on the pH, instead of 1 as usually obtained [12–15].

Materials and Methods

Pig-heart mitochondria were prepared by the method described by Crane et al. for bovine-heart mitochondria [16]. The mitochondria thus obtained were uncoupled and contained 0.31 nmol cytochrome c_1/mg protein. They were suspended in 50 mM Tris-HCl buffer (pH 7.4), containing 250 mM sucrose and 1 mM EDTA. Unless otherwise specified, this buffer was used throughout our experiments and is referred as the standard solution. The preparations of succinate : cytochrome *c* reductase used in our experiments, prepared according to the method of Yu and Yu [17], were kindly supplied by Mrs. S.L. Lee and Mr. D.C. Wang.

Activation of the succinate dehydrogenase was achieved by incubating mitochondria (10 mg protein/ml) with 2 mM malonate at 37°C for 45 min. BAL treatment was performed together with the activation by shaking the malonate-containing mitochondrial suspension in the open air with 20 mM BAL. After incubation, mitochondria were spun down, washed twice, than homogenized into the standard solution.

Kinetic measurements were performed with a Hitachi 557 dual-wavelength spectrophotometer at room temperature. The selection of detecting wavelengths for the mitochondrial Q pool and cytochrome *b* will be described in Results.

Antimycin was from Sigma and duroquinone from Ehrlich. Q_2 and DB((H)Q were the kind gifts from Prof. E.C. Slater and Dr. J.A. Berden. All these chemicals were added in ethanol solution, the concentration of which never exceeded 1% (v/v). All other reagents were of the highest purity available.

Results

Selection of wavelengths for monitoring the redox reaction kinetics of cytochrome b in succinate : cytochrome c reductase and mitochondria

Previous discussions about the wavelengths used

in measuring the redox kinetics of cytochrome *b* are mainly based on spectral considerations. We adopted a more direct approach, namely, by adding ascorbate to reduce the high-potential components of the respiratory chain (the Rieske iron-sulfur protein and cytochrome c_1 in succinate : cytochrome *c* reductase, plus cytochrome *c* and aa_3 in mitochondria) and recording at various *b*-measuring as well as c_1- or (c_1 + *c*)-measuring wavelengths. The traces thus obtained are illustrated in Fig. 1. The calculated extent of interference, as the fraction of the maximum magnitude of cytochrome c_1 (in succinate : *c* reductase) or c_1 + *c* (in mitochondria) under the same conditions, is given in Table I.

It is clear that, at least under our experimental conditions, 562–575 nm is a suitable wavelength pair for monitoring cytochrome *b* in succinate : *c* reductase, but not for mitochondria due to the presence of considerable amount of cytochrome *c* which gives an absorption decrease at this wavelength pair upon reduction. For mitochondria, 562–540 nm used by Dutton's group [18] is more suitable. Measuring *b* at the single wavelength of 562 nm (simulated by 265–700 nm) suffered the most severe interference in both cases (data not shown for mitochondria).

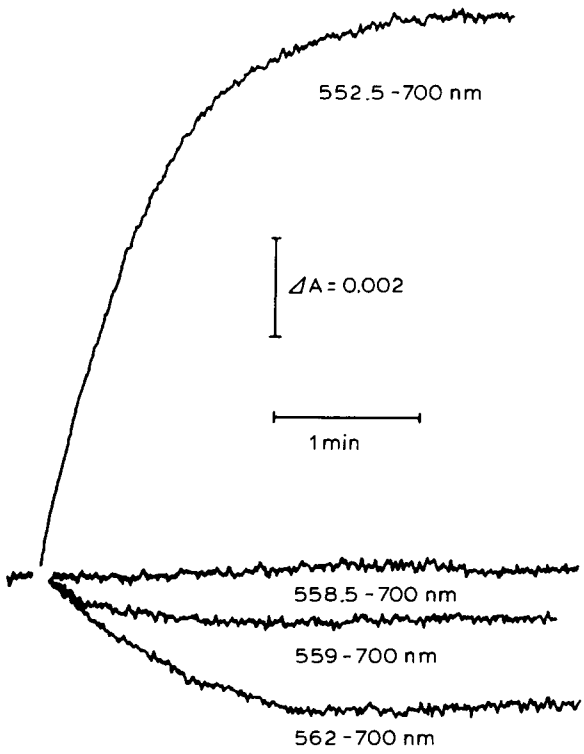

Fig. 1. Absorption change caused by the addition of ascorbate to succinate : cytochrome *c* reductase. The reaction was started by adding ascorbate (0.2 M solution at pH 6.0) to succinate : cytochrome *c* reductase sample to a final concentration of 2 mM, and absorption changes due to the reduction of the high-potential components of the enzyme were recorded at the indicated wavelength pairs. The concentration of c_1 in the sample was 0.6 μM.

TABLE I

THE ABSORBANCE CHANGE CAUSED BY THE ADDITION OF ASCORBATE RECORDED AT VARIOUS WAVELENGTH PAIRS

Data were taken when constant absorption values were reached after the addition of 2 mM ascorbate to succinate : cytochrome *c* reductase (abbreviated as SCR in this Table) and mitochondria together with 1.8 mM KCN. The absorbance changes recorded at 552.5–700 nm in succinate : cytochrome *c* reductase and at 550–540 nm in mitochondria were taken as 100%. The actual reduction courses were illustrated for one preparation of succinate : cytochrome *c* reductase in Fig. 1. The concentration of c_1 in mitochondria and three succinate : cytochrome *c* reductase samples were 0.28 μM, 1.1, 1.1 and 1.5 μM, respectively.

Preparation	Wavelength pair (nm)	Absorbance change (arbitrary units)	Percentage of the maximum value
SCR (1)	552.5–700	41	100.0
	558.5–700	3	7.3
	558.7–700	0	0.0
	559.0–700	−2	−4.8
	559.5–700	−5.5	−13.4
	562.0–700	−9.0	−22.0
SCR (2)	552.5–700	42	100.0
	558.5–700	0	0.0
	559.0–700	−3	−7.1
	562.0–700	−7	−16.7
SCR (3)	552.5–700	59	100.0
	558.5–700	1	1.7
	559.0–700	−3	−5.1
	562.0–700	−12.5	−21.2
	562.0–575	−1	−1.7
Mitochondria	550 –540	53	100.0
	562 –575	−9	−17.0
	562 –540	−1	−1.9

The triphasic reduction course of cytochrome b

Major differences in the second phases can be

seen when *b* reduction traces recorded at different wavelengths are compared (Fig. 2). The fact that the 562–700 nm traces went below the baseline in the second phase and the close correlation in time between apparent *b* oxidation and c_1 reduction strongly suggest that the absorption drop in the second phase is due to the reduction of *c*-type cytochromes. When the interference was essentially eliminated (by measuring at the isosbestic point of c_1 or 562–575 nm for succinate : cytochrome *c* reductase, at 562–540 nm for mitochondria), a plateau (Fig. 2A) or a turn (Fig. 2B) rather than an oxidation phase appeared between the two reduction phases, depending on the rate of electron input. Furthermore, correction of the 562–700 nm traces for the contribution of *c*-type cytochromes, made on the basis of data in Table I, yielded a curve almost the same as the 562–575 nm trace in Fig. 2A and 562–540 trace in Fig. 2B. In Fig. 2A, the 562–575 nm curve and the corrected curve also coincided kinetically with that recorded at the isosbestic point of c_1 (558.7 nm). These results not only demonstrate the reliability of the wavelengths selected in the previous section, but also that the so-called 'oxidation phase' is very likely an artifact caused by the reduction of the high-potential components.

Our results agree qualitatively with that of De

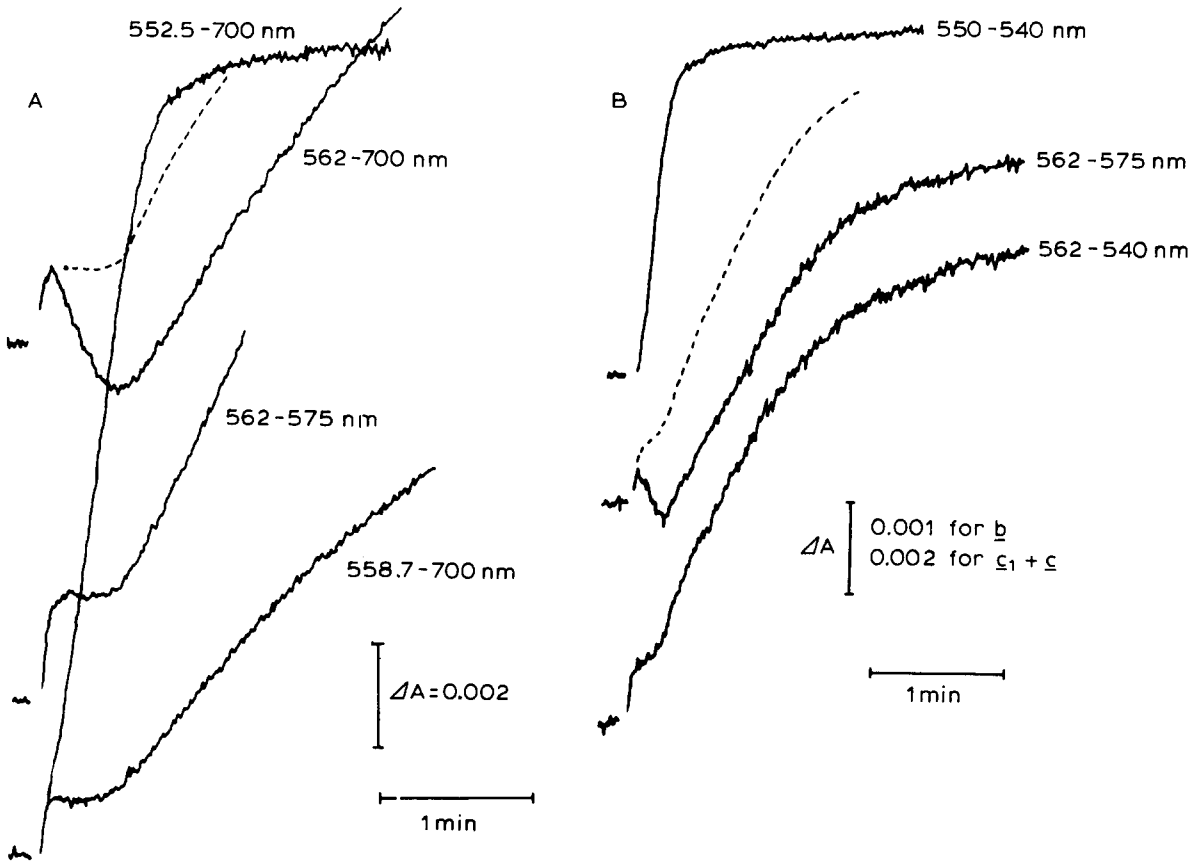

Fig. 2. Comparison of triphasic reduction of cytochrome *b* recorded at different wavelengths as indicated. The reactions were started by the addition of succinate (0.5 mM in A and 0.42 mM in B) to succinate : cytochrome *c* reductase (A) and mitochondria (B). The concentration of c_1 in mitochondria and succinate : cytochrome *c* reductase was 0.36 μM and 0.82 μM, respectively. In B 1.8 mM KCN was also added together with the succinate to inhibit the cytochrome oxidase. Malonate was used both in A (1 mM) and in B (0.42 mM) to slow down the rate of electron input. Dotted lines are corrected 562–700 nm (in A) and 562–575 nm (in B) curves for the contribution of the *c*-type cytochrome reduction according to the data in Table I.

Vries et al. in succinate: *c* reductase measured at 562–575 nm [6]. However, we did not observe the 40% of the total absorbance change in the first phase as reported by these authors which they concluded corresponds to the reduction of the high-potential cytochrome *b*-562, and the remainder to the reduction of the low-potential *b*-562. In our case, the initial absorbance increase was small, not more than 10% of the total absorbance change. We shall return to this point in the Discussion.

Optical measurement of the mitochondrial Q-pool reduction

The deuterium lamp and the monochromator of a modern commercial dual-wavelength spectrophotometer (e.g., Hitachi 557) provides well-resolved monochromatic light, and since NADH was not present in our preparations, the problems with stray light encountered by Chance and Redfearn [10] and Szarkowska and Klingenberg [11] were not apparent in our experiments as evidenced by the close resemblance between the redox difference spectra of ubiquinone in solution and in mitochondria (Fig. 3). The fidelity of this direct optical measurement is further substantiated by the close coincidence of the direction and magnitude of absorbance changes recorded at different wavelength pairs, and the spectral changes after succinate addition, with or without externally added Q. In Fig. 4, addition of cyanide led to the reduction of the high-potential components by endogenous electron donors as judged by the spectral change in the cytochrome α band region (not shown). A peak at 313 nm and a trough at 272 nm were observed. Further addition of succinate greatly increased the height of the negative peak, but only added a shoulder around 298 nm on the 313 nm peak. Since cytochromes *b* and aa_3 contribute little in this region when reduced as evidenced by their difference spectra (not shown), it is clear that the 313 nm peak results predominantly from the reduction of the *c*-type cytochromes, while the negative peak at 272 nm and the shoulder at 298 nm arise from the reduction of the Q pool. The high concentration of Q in mitochondria (about $15Q/c_1$ in our case) gave a quite acceptable signal-to-noise ratio when the protein concentration of the sample was not higher than 1 mg/ml even when a 1-cm light path cuvette was used.

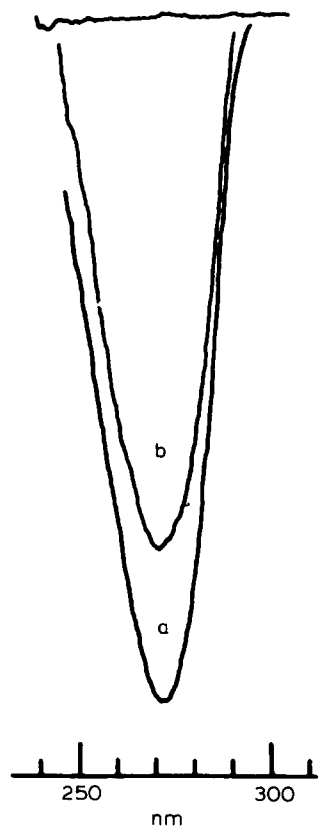

Fig. 3. Comparison of the redox difference spectra of ubiquinone in buffer and in mitochondria. Curve a, ubiquinone (in ethanol) was added to the standard solution to a final concentration of 4 μM and stirred for several minutes. When its absorption spectrum became stable, the baseline was taken, then a few granules of potassium borohydride were added to reduce the ubiquinone. The difference spectrum was scanned repeatedly until a stable spectrum was recorded. Curve b, mitochondria were suspended in the standard solution to a c_1 concentration of 0.24 μM, then the baseline recorded. The difference spectrum of the ubiquinone pool was recorded 5 min after the addition of 1 mM succinate and 2 mM KCN. In both cases, only the trough (negative peak) was shown.

Chance and Redfearn [10] used 275–300 nm to eliminate the interference by the δ peak of cytochrome *c* based on the data of Margoliash and Frohwirt [19]. In our hands, the 'mixed isosbestic point' of cytochromes lay between 289 nm and 292 nm (Fig. 4), which is close to the reference wavelength used by Szarkowska and Klingenberg

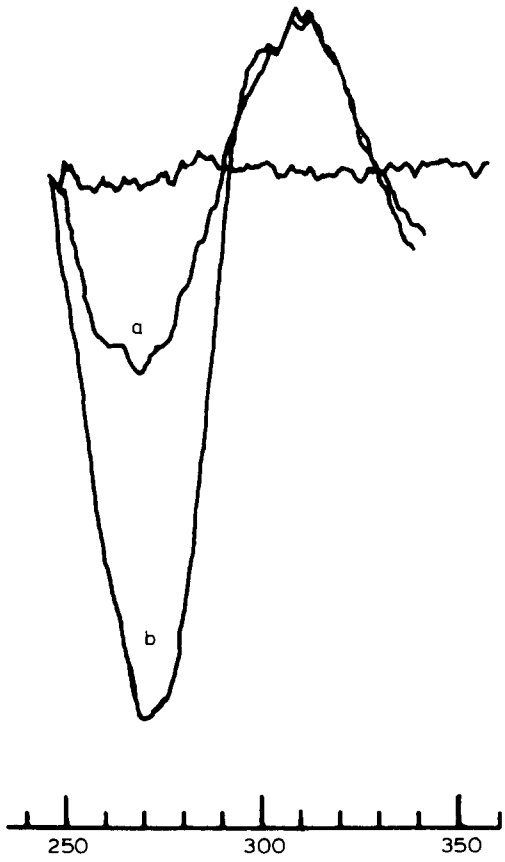

Fig. 4. The 'isosbestic point' of cytochromes and ubiquinone in mitochondria. Mitochondria (c_1 concentration, 0.3 μm) were treated first with 2 mM KCN. When the reduction of cytochrome c_1, c and aa_3 was nearly complete, the difference spectrum was recorded in the ultraviolet region (curve a). Curve b was recorded 5 min after the further addition of 1 mM succinate. The 'isosbestic point' was around 289–292 nm.

(289 nm) [11]. In order to obtain maximum sensitivity free of contributions from cytochromes, we used the wavelength pair 273–287 nm. As pointed out by these authors, the apparent extinction coefficient of the mitochondrial Q varies with protein concentration, being 6.8 $mM^{-1} \cdot cm^{-1}$ at 273–287 nm at a protein concentration of 1 mg/ml for our preparation.

When succinate was used as the electron donor, the only interfering component was fumarate, a product of succinate oxidation. The calculated absorbance change at 273–287 nm due to fumarate formation is only −3.8% of the absorbance change due to Q reduction. This small contribution of fumarate was almost synchronous with Q reduction and was partially compensated by the formation of semiquinone which has a similar absorption as hydroquinone at 273–287 pair [20]. Therefore, fumarate formation has almost no effect on the measurement of the Q reduction kinetics.

The correlation of cytochrome b reduction with that of the Q pool

Under the conditions in which mitochondrial *b* exhibited triphasic reduction behavior, the reduction course of the Q pool recorded according to the method described in the previous section was also triphasic and in perfect kinetic match with that of cytochrome *b* (Fig. 5). A plot of $\log(b^{3+}/b^{2+})$ against $\log(Q/QH_2)$ gave a straight line (Fig. 6), indicating that Q and *b* are in rapid redox equilibrium in all three phases. The second phase of the reduction of these components closely correlated in time with $c_1 + c$ reduction and the further reduction of Q and *b* came only when the reduction of $c_1 + c$ was near completion. These observations suggest that the appearance of the plateau or the turn during *b* and Q reduction is caused by the rapid delivery of electrons to the high-potential components of the chain. The cutting off of this electron delivery by BAL + O_2 treatment abolished the plateau or turn and changed the reduction course of both *b* and Q into a monophasic one (Fig. 5B). Prereduction of the high-potential components by endogenous substrate (ascorbate could not be used because of its strong absorption in the ultraviolet) in the presence of cyanide had similar effects (traces not shown). For this reason, cyanide was added at the same time as the succinate in our experiments. Earlier addition of cyanide always resulted in the reduction of the high-potential components by endogenous electron donors which are usually present in mitochondrial preparations, thus abolishing the triphasic course of *b* reduction. This may explain why in some earlier experiments [21,22], in which the mitochondrial preparations were pretreated with cyanide, cytochrome *b* and the Q pool did not show multiphasic reductions. A plot of $\log(b^{3+}/b^{2+})$ against $\log(Q/QH_2)$ calculated from the traces in Fig. 5B also produced a straight line (Fig. 6, line b), indicating that here *b* was

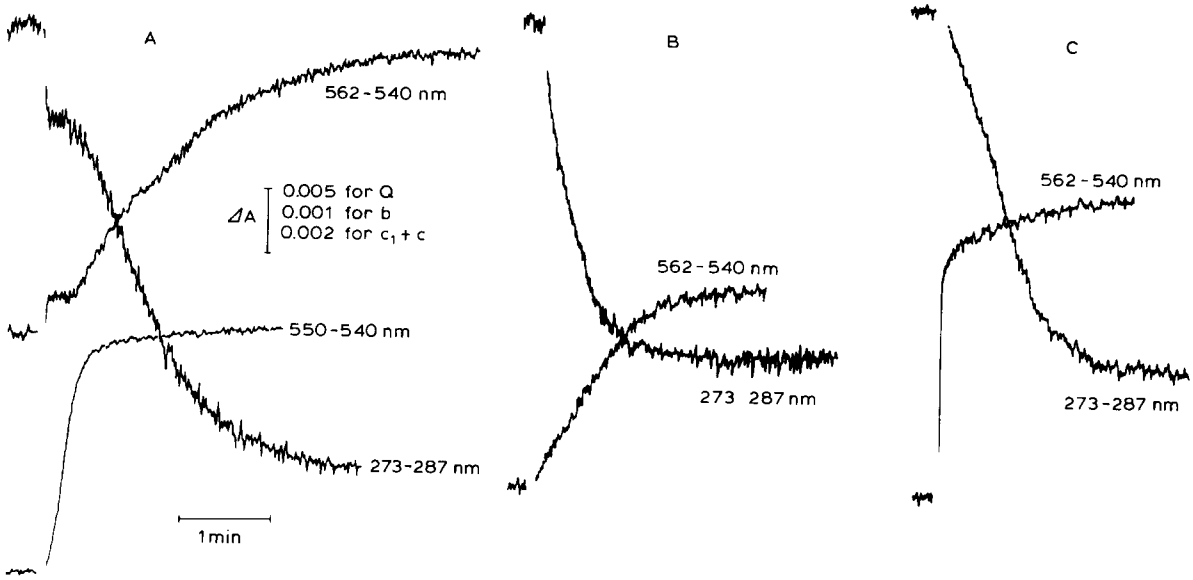

Fig. 5. (A) Correlation of the reduction courses of ubiquinone, cytochrome *b* and cytochrome $c_1 + c$ in mitochondria (0.38 μM c_1). Mitochondria were first treated with 0.625 mM malonate, then the reaction was started by adding 0.42 mM succinate together with 1.8 mM KCN. (B) Reduction of Q and *b* in BAL-treated mitochondria. The experimental conditions were the same as in (A), but with a c_1 concentration of 0.28 μM. (C) Reduction of Q and *b* in mitochondria in the presence of antimycin. The experimental conditions were the same as in (A), except that c_1 concentration was 0.28 μm and 2 μM antimycin replaced KCN. Cytochrome *b* was reduced nearly completely during the time mixing the substrate into the sample.

co-reduced with Q by means of its rapid electron exchange with Q through what is called center i, the BAL-insensitive but antimycin sensitive entry of electrons into the reductase [9,23].

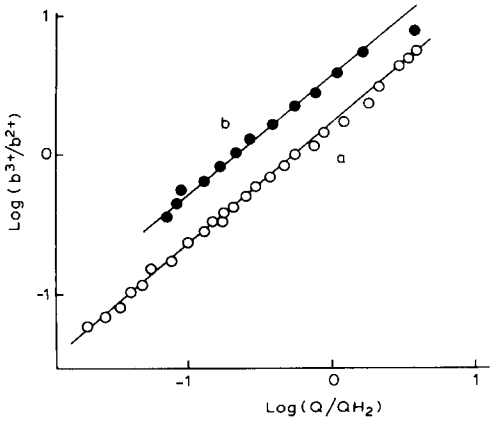

Fig. 6. The Nernst plot of Q-*b* reduction. Data were taken from Fig. 5A for normal mitochondria (line a) and from Fig. 5B for BAL-treated mitochondria (line b). The small absorbance drop due to dilution upon addition of succinate has been subtracted from the total absorbance change.

Line a in Fig. 6 can be described by the formula

$$\log(b^{3+}/b^{2+}) = 0.068 + 0.84 \log(Q/QH_2) \quad (1)$$

Since for the Q/QH_2 couple, $n = 2$ [24], Eqn. 1 can be changed into

$$34.5 \log(b^{3+}/b^{2+}) = 7.7 + 29.5 \log(Q/QH_2) \quad (2)$$

This formula indicates that at pH 7.4, *b* and Q have very similar midpoint potentials (the difference being only 7.7 mV). Furthermore, in this kinetically measured process, the RT/nF value of 34.5 in the *b* term implies that the *n* value for *b* is near two (1.7), quite different from the value of one obtained from both theoretical considerations and redox titrations in the presence of dyes under equilibrating conditions [12–15].

When the experiment was repeated at different pHs, it was found that the *n* value for cytochrome *b* is pH dependent, being exactly 2.0 at pH 6.5, decreasing to 1.4 at pH 8.5 (figures not shown).

On the other hand, pH change had little effect on the mid-point potential of cytochrome *b* relative to that of the Q pool. At pH 6.5, the E_m of cytochrome *b* was 7.1 mV lower than that of the Q pool. At pH 8.5, the difference became even smaller (4.2 mV).

Similarly, line *b* for BAL-treated mitochondria can be described by the formula

$$34.5 \log(b^{3+}/b^{2+}) = 19.2 + 29.5 \log(Q/QH_2) \quad (3)$$

This indicates that BAL-treatment lowered the midpoint potential of *b* relative to that of Q by about 10 mV, whereas the *n* value remained the same.

Q and b reduction in the presence of antimycin

When antimycin was used to cut off the interaction between *b* and Q through center i, the redox equilibrium between these two components disappeared. Fig. 5C shows that the instantaneous reduction of *b* was not accompanied by an instantaneous reduction of the Q pool. Rather, the latter was reduced monophasically at the same speed as in the case of BAL-treated mitochondria. This phenomenon can be easily explained by the widely accepted reaction mechanism at the so-called antimycin-insensitive but BAL-sensitive center o [9,23]:

$$QH_2 + (Fe\text{-}S^{2+} \cdot b^{3+}) \rightarrow Q + (Fe\text{-}S^{1+} \cdot b^{2+}) + 2H^+$$

Since this is a concerned reaction, cytochrome *b* by itself cannot equilibrate with the Q pool through center o. In the presence of antimycin, maximumly two molecules of QH_2 can be oxidized at center o. In the case of mitochondria, the two electrons reaching the iron-sulfur protein will be taken away almost completely by oxygen (even in the presence of cyanide), thus strongly driving the above reaction toward the right, leading to the full reduction of cytochrome *b* in spite of the fact that the Q pool is still largely oxidized. The full reduction of the Q pool requires many more turnovers of the dehydrogenase.

The effect of externally added quinones

The addition of Q_2, DB(H)Q or duroquinone to mitochondria affected both the duration of the second phase and the extent of the initial reduction of cytochrome *b*. The larger the amount of added quinone, the longer the plateau lasted (Fig. 7A). As to the reduction level reached in the first phase, the faster the quinone took electrons away from the respiratory chain, the lower the extent. For example, in the case of duroquinone which receives electrons much slower than Q_2, the *b* reduction level in the plateau phase was much higher (Fig. 7B and C). The final reduction of cytochrome *b* was reached only when the exogenous quinones were nearly completely reduced. When quinone was added to succinate-reduced mitochondria, cytochrome *b* became temporarily more oxidized, until the added quinone became reduced (Fig. 8). With added quinone, the $\log(b^{3+}/b^{2+})$ vs. $\log(Q/QH_2)$ plot is no longer a straight line, although in the region corresponding to the third phase of *b* reduction the slope approached that shown in Fig. 6 (Fig. 9). These results can be explained by proposing that cytochrome *b* equilibrates only with the endogenous Q pool and that externally added quinones act as an additional electron sink. The 'leak' of electrons to the exogenous quinone pool prevents temporarily the reduction of the endogenous Q pool – and hence cytochrome *b* – from reaching its final level. The faster the leak, the lower the temporary 'steady state' reduction level of the endogenous Q pool and cytochrome *b*. Their final reduction level is reached only when all electron sinks are filled. These results support our previous proposal that it is the rapid delivery of electrons to the high-potential components of the respiratory chain that results in the multiphasic reduction of cytochrome *b*.

The different redox relationship between cytochrome *b* with endogenous and exogenous quinones indicates that the latter do not form a common Q pool with the endogenous Q. This is somewhat unexpected, since, for example, Q_2 can actively interact with the respiratory chain and is regarded as a good replacement for Q_{10} in sustaining electron-transfer activities. This might be due to their different locations relative to the membrane or to different interactions with cytochrome *b* because of their structural differences. Our studies on the interaction of exogenous quinones with the respiratory chain will be reported in a following paper.

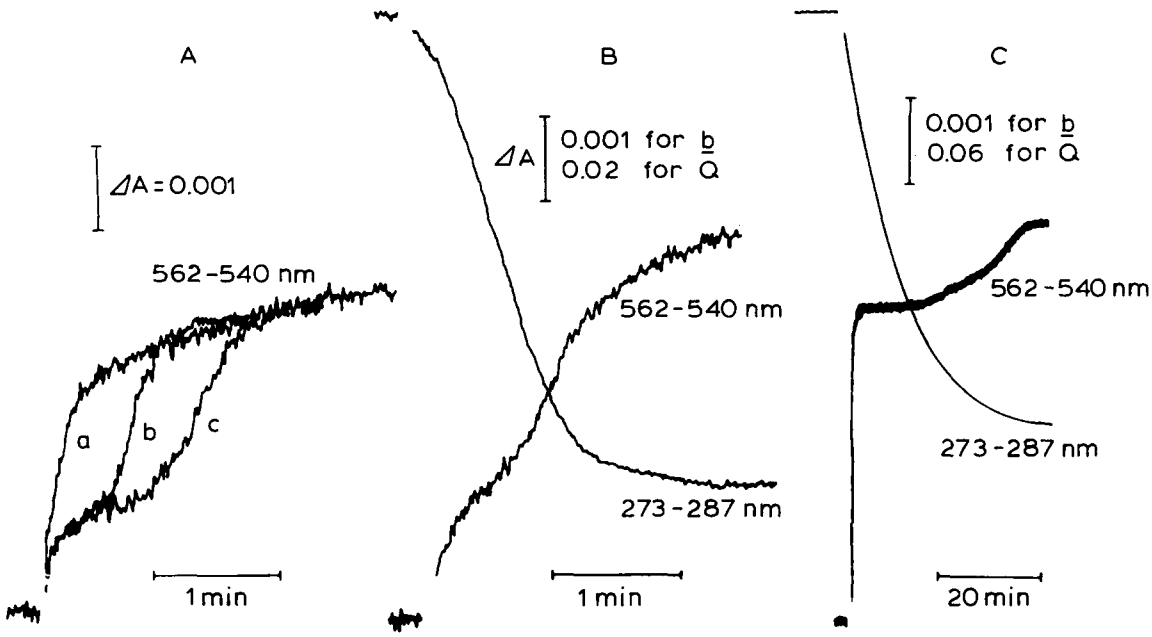

Fig. 7. Effect of exogenous quinones on *b* reduction. Mitochondria were first incubated with Q_2 (A, B) or duroquinone (C). The reactions were started by adding 2.4 mM succinate and 1.8 mM KCN. No malonate was used in these experiments in order to ensure a higher rate of electron input. (A) Cytochrome *b* reduction traces in the presence of zero (a), 26 μM (b) or 72 μM (c) Q_2. (B) Cytochrome *b* reduction in the presence of 9 μM Q_2 in comparison with the reduction course of Q_2 plus endogenous Q_{10}. (C) Same as in (B), except 20 μM duroquinone replaced Q_2.

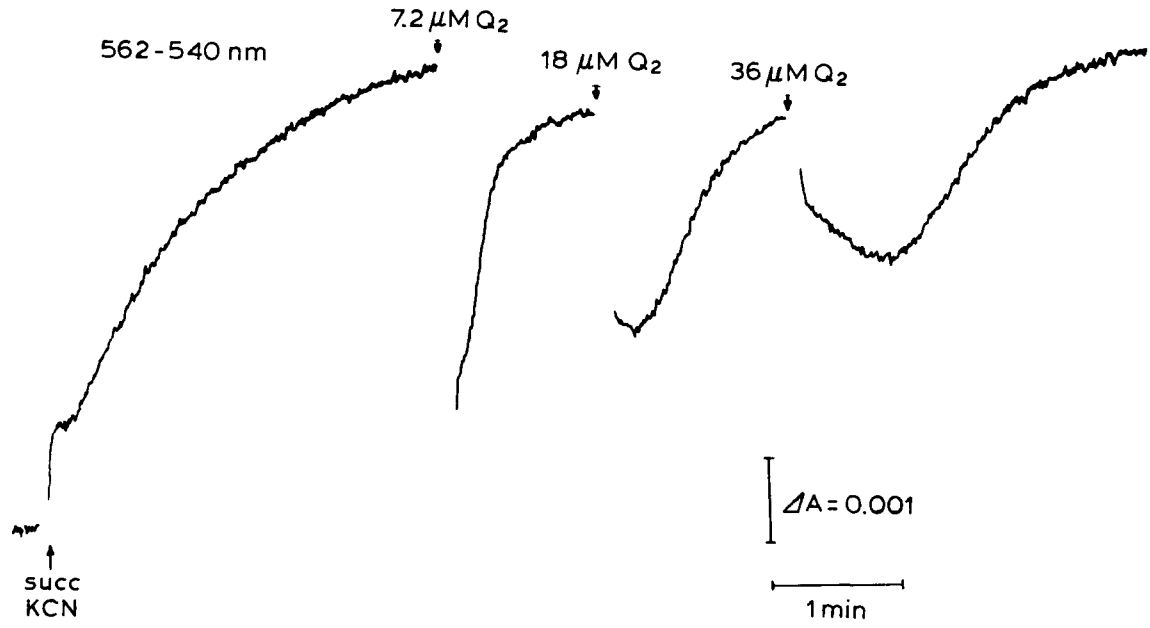

Fig. 8. Q_2-induced oxidation of the succinate-reduced cytochrome *b* in mitochondria. Cytochrome *b* in the mitochondria (0.3 μM c_1) was first reduced by 3.2 mM succinate in the presence of 0.5 mM malonate and 1.8 mM KCN. Q_2 was added as indicated in the figure. It may be noted that the rereduction of cytochrome *b* following its partial oxidation by the addition of 7.2 μM Q_2 is faster than the third phase reduction resulting from the increased rate of electron input from succinate : Q reductase with added Q_2. It may also be noted that with increasing amount of Q_2H_2 accumulated in the reaction system, the oxidation of *b* by newly added Q_2 (although in larger amounts) became slower.

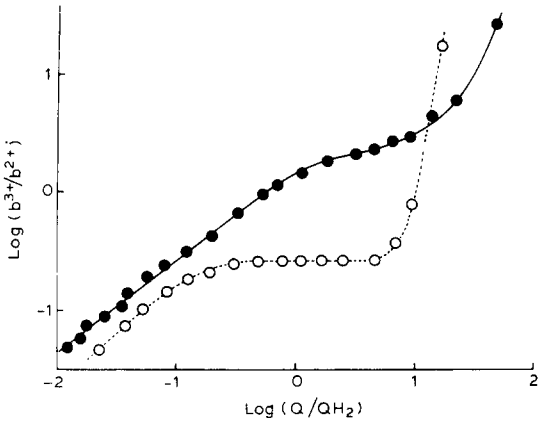

Fig. 9. The Nernst plot of Q-*b* reduction in the presence of Q_i2 (●———●) or duroquinone (O— — —O). Data were taken from Fig. 7B and C, respectively. Corrected as indicated in the legend to Fig. 6.

Discussion

Selection for proper wavelengths in detection of cytochrome b redox changes

The spectrum of cytochrome *b* closely overlaps that of *c*-type cytochromes which are always present together with cytochrome *b* in mitochondria, succinate : cytochrome *c* reductase or QH_2 : cytochrome *c* reductase. Specifically, 562 nm is near or at the trough in the redox difference spectrum of c_1 or *c*, respectively. Recording at this wavelength will certainly introduce negative signals from c_1 and/or *c* reduction which can easily account for the apparent 'oxidation phase' of *b* recorded at 562 nm.

Theoretically, a 'clean' *b* signal can be obtained with single wavelength measurements if the isosbestic point of c_1 or $c_1 + c$ is used. However, in practice, this is possible only for succinate : *c* reductase. For mitochondria, the sensitivity is too low.

In succinate : cytochrome *c* reductase, the redox difference spectrum of cytochrome c_1 shows the same absorbance change at 562 nm as that at 575 nm, so the redox change of cytochrome c_1 makes practically no contribution to the signal measured at 562–575 nm which corresponds to the maximum absorbance change of *b* reduction. In the case of mitochondria, however, the measurement of *b* reduction at 562–575 nm introduces a large negative interference due to the presence of considerable amounts of cytochrome *c*, which has a trough at 562 nm in difference spectrum, so that 562–575 nm is negative on reduction.

So far, our discussions are mainly based on spectral considerations. In practice it was found that the spectra of cytochromes to some extent depend on the source and the method of preparation due to the presence of other interfering components in these preparations. The content of cytochrome *c* also varies from preparation to preparation. These uncertainties, together with the difference in instrument setting, made it impossible to find out a 'universal' wavelength or wavelength pair for all *b*-562 measurements. A more straightforward and reliable method is, by means of ascorbate reduction, experimentally to determine, for a given preparation and instrument, which wavelength or wavelength pair can eliminate interference while retaining sufficient sensitivity to measure the redox state of cytochrome *b*.

The multiphasic reduction of cytochrome b and its mechanism

Our results clearly demonstrate that, at least under all the experimental conditions tested by us, no net oxidation of cytochrome *b* occurs after its initial partial reduction either in succinate : *c* reductase or in mitochondria. Instead, a plateau or a turn exists between two reduction phases, in agreement with the results of De Vries et al. [6] in succinate : *c* reductase measured with the wavelength pair 562–575 nm. The oxidation phase reported by several laboratories [1–5] might be due to the fact that *b* traces were recorded at a single wavelength, 562 nm.

On the basis of the results reported in this paper, a simple explanation based on the Q-cycle model can be proposed for the multiphasic reduction of cytochrome *b*: *b* and Q exchange electrons rapidly through center i so that they are always in redox equilibrium as evidenced by our quantitative results shown in Fig. 6. The addition of substrate to the 'empty' enzyme brings about rapid partial reduction of both *b* and Q, electrons entering cytochrome *b* through either center o or center

i, or both. However, due to the existence of the high-potential components of the respiratory chain. QH_2 is rapidly oxidized and a steady redox state of the Q pool is reached. During this steady state, the redox state of cytochrome *b* will also be constant. Electrons go to *b* through center o and leave it through the Q-*b* equilibrium at center i. When the reduction of these high-potential components approaches completion, center o stops operating, and *b* is reduced through the *b*-Q interaction at center i. In this respect, the high-potential components act as an electron sink.

This mechanism is supported by the following observations:

(1) The plateau or turn correlates closely in time with the reduction of the *c*-type cytochromes.

(2) Cutting off electron flow to the high-potential components, either by the destruction of the Rieske iron-sulfur cluster by BAL + O_2 treatment [23] or by prereduction of these components, abolishes the plateau and renders the reduction of Q and *b* monophasic.

(3) Provision of an additional electron sink (exogenous quinones for instance) prolongs the duration of the plateau in *b* reduction. The larger the sink, the longer the duration. With mitochondria, oxygen can provide an additional electron sink if cyanide is not used, yielding the well-known triphasic reduction traces of c_1, *c* and aa_3, as well as Q and *b*.

(4) The perfect match between Q and *b* reduction traces that is found both in normal and BAL-treated mitochondria.

(5) The linear relationship that exists between $\log(Q/QH_2)$ and $\log(b^{3+}/b^{2+})$ throughout all three reduction phases.

(6) The interruption by antimycin of the interaction between Q and *b* through center i abolishes their kinetic matching, *b* being reduced much more rapidly than the Q pool.

(7) Addition of quinones causes a temporary partial oxidation of the substrate-reduced cytochrome *b*.

The significance of the large n value and the high midpoint potential of cytochrome b-562 in redox reactions

In potentiometric titrations with dyes, two species of cytochrome *b*-562 differing in redox potential [13] and EPR spectra [6,26] have been identified. The value of *n* equal to 1 in the Nernst equation for both species indicates that each is a single-electron acceptor, acting independently of one another. Under our experimental conditions, however, a single redox potential – about midway between the two potentials measured with redox dyes – and a value of *n* close to 2 at pHs between 6.5 and 7.4 have been found. A *n* value larger than one for at least one species of cytochrome *b* has also been derived by Berden [27]. Since this value has been found with the succinate/fumarate couple, we consider that it is the more significant one under physiological conditions.

In order to explain the value of *n* close to 2, even when the operation of center o has been destroyed by BAL + O_2 treatment, we propose that two cytochromes *b*-562 cooperate at center i to bring about the rapid oxidation of one molecule of QH_2. The reaction can be described as:

$$QH_2 + \left(b^{3+}_{562} \cdot b^{3+}_{562}\right) \rightleftharpoons Q + \left(b^{2+}_{562} \cdot b^{2+}_{562}\right) + (2H^+)$$

in which the two cytochromes *b*-562 can be from the same functional unit in the dimeric model [6] or from two monomers that rapidly associate and reassociate with each other. Since the midpoint potential of *b*-562 is comparable with that of the Q pool rather than considerably lower and higher than the Q pool as found in potentiometric titrations [13], we propose that the two species of *b*-562, with $E_{m,7}$ of 150 mV and 50 mV, respectively, are involved in a concerted reaction as follows:

$$b_{150} \xleftarrow{e^-} \begin{matrix} QH_2 \\ Q^- \end{matrix}$$

$$b_{50} \xleftarrow{e^-} \begin{matrix} Q^- \\ Q \end{matrix}$$

Although this scheme involves two species of *b*-562 with quite different midpoint potentials, since the oxidation of QH_2 is a concerted reaction, they are reduced in a single phase as evidenced by the semi-log plot (not shown). In contradiction to the situation in potentiometric titrations with dyestuffs, electron transfer between the two cytochromes is not possible. The apparent

midpoint potential is just the mean value of that of the two cytochromes *b*-562, therefore close to the mid-point potential of the Q-pool. This mechanism differs from that of De Vries et al. [6] in that the two cytochromes *b*-562 are reduced simultaneously and not consecutively as proposed by these authors. In our experiments, the reduction level reached in the first phase was far less than that observed by De Vries et al. [6]. This difference may be due to the fact that in our experiments *b* interacted with the endogenous Q pool, while in those of De Vries et al. durohydroquinone was the electron donor.

The value of n decreases with increasing pH from 2.0 at pH 6.5 to 1.4 at pH 8.5. The value of 2.0 at pH 6.5 shows that the reduction of the two cytochromes *b* is perfectly in concert, that is to say, on the basis of the explanation given above, the two electrons from QH_2 reach the two cytochromes with a very low concentration of the intermediate Q_i^-. At higher pH values, however, which favor the formation of the semiquinone anion, the two reactions leading to the reduction of the two cytochromes *b*-562 are no longer very tightly coupled, part of the reaction between *b* and Q can now occur through a one-electron step. In addition to the concerted reaction, where $n = 2$, the net result is a line with a slope between 1 and 2. Interestingly, in the experiment of Meinhardt and Crofts [28] who measured the reduction of cytochrome *b* in chromatophores kinetically, n is close to one when the pH is high, but at lower pH, the $n = 2$ theoretical curve seems to give a better fit to the experimental results.

Our results also shows that at all pH values between 6.5 and 8.5 the mid-point potential of cytochrome *b* and the Q pool are almost the same. This close relationship between Q and *b* in redox properties very possibly has important physiological significance, since it might secure the rapid redox equilibrium between Q and *b* at all physiological pH's which the enzymes may encounter at different phosphate potentials.

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