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KINETICS OF CYTOCHROME *b* REDUCTION IN SUBMITOCHONDRIAL PARTICLES

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(1) In agreement with Eisenbach and Gutman (Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 52, 107–116) the reduction of cytochrome *b* in beef-heart submitochondrial particles by succinate in the presence of antimycin was found to be biphasic, the relative amounts of fast and slow phases being dependent on the redox state of a component located on the oxygen side of the antimycin block. (2) HQNO in a concentration sufficiently large to saturate the specific antimycin- and HQNO-binding sites can substitute for antimycin in these experiments. (3) The rate of the slow phase of the reduction of cytochrome *b* is decreased under anaerobic conditions and after pretreatment with 2,3-dimercaptopropanol (BAL). (4) In the presence of antimycin and cyanide, cytochrome *b*-562 is, to some extent, preferentially reduced in the rapid phase and *b*-566 in the slow phase. (5) The previously proposed regulatory effects of redox-sensitive components X and Y on the redox level and reduction kinetics, respectively, of cytochrome *b* are ascribed to the role of the Fe-S protein, when it is oxidized, in producing the reductant of cytochrome *b* by oxidation of QH_2, and by the fact that when QH_2 is bound to it, the reduced Fe-S protein cannot be oxidized by its natural oxidant, cytochrome c_1.

Introduction

In anaerobic substrate-reduced submitochondrial particles, the *b*-cytochromes are not reduced to the extent expected from the low redox potential of the substrate couple applied [1–3]. After a pulse of oxygen, a transient full reduction of cytochrome *b* occurs [4,5]. This so-called extra reduction is stabilized when the oxidation of cytochrome *b* is inhibited by antimycin or HQNO [6–9]. With appropriate substrates and oxidants, these phenomena can be observed with submitochondrial particles, isolated succinate-cytochrome *c* reductase and isolated ubiquinol-cytochrome *c* reductase [7].

Baum and Rieske [10,7] postulated that the reducibility of the *b*-cytochromes is under the control of a component, X, that must be oxidized to promote full reduction of cytochrome *b*. Chance and coworkers [11,12] (see also Ref. 4) proposed that X is cytochrome c_1, the oxidation of which induces a high-potential form of cytochrome *b*.

Wikström and Berden [8], on the other hand, explained the extra reduction by a shift of the equilibrium $b^{3+} + QH_2 \rightleftharpoons b^{2+} + QH^{\cdot}$ brought about by the oxidation of $QH^{\cdot}$ via an antimycin-insensitive pathway. A similar proposal has been made by Mitchell [13] in a scheme that differs from that of Wikström and Berden in the electron acceptor of cytochrome *b*. Mitchell [14] and Trumpower [15] have considered variants of this scheme, in which cytochrome *b* is reduced by $QH^{\cdot}$ that is formed by the oxidation of QH_2 via an antimycin-insensitive pathway. Arguments in favour of one of the schemes considered by Mitchell [14] have been given elsewhere [16].

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; BAL, British anti-lewisite (2,3-dimercaptopropanol); HQNO, 2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide.

Eisenbach and Gutman [17] reported experiments that can be described as the inverse of an oxidant-induced reduction. In the presence of antimycin, the rate of reduction of cytochrome *b* by succinate is strongly decreased when the cytochromes *c* and c_1 are previously reduced by ascorbate and TMPD. This reductant-induced inhibition of the rate of reduction of cytochrome *b* is also found with isolated fragments of the respiratory chain [18,19]. In the presence of antimycin and cyanide, and absence of ascorbate or TMPD, biphasic reduction of cytochrome *b* is obtained. The first-order rate constant calculated from the rapid phase is identical to that obtained from the monophasic reduction of cytochrome *b* in the absence of cyanide, and the constant calculated from the slow phase is identical to that obtained from the monophasic reduction of cytochrome *b* in the presence of cyanide, ascorbate and TMPD [17]. Biphasic reduction of cytochrome *b* by succinate in the presence of antimycin is also obtained during steady-state oxidation of ascorbate in the presence of TMPD [20]. From the variation of the contributions of the rapid and slow phases to the total reduction of cytochrome *b* when the concentrations of ascorbate and TMPD were varied, it was concluded that the kinetics of cytochrome *b* is under the control of a component, Y, reducible by ascorbate [20].

Despite the obvious similarities between the component X, postulated to explain the apparently anomalous reducibility of cytochrome *b* in response to oxidant, and the component Y, postulated to explain the abnormal kinetics of reduction of cytochrome *b* in response to reductant (cf. Ref. 21), Eisenbach and Gutman [17] concluded that X and Y are not identical. One argument is that during slow-phase reduction ('Y' reduced) cytochrome *b* is fully reducible ('X' oxidized). A more serious argument is the reported different effects of antimycin and HQNO on the kinetics of reduction of cytochrome *b*, which led these authors to place the site of action of the two inhibitors on either side of Y [22]. Since both antimycin and HQNO cause extra reduction, X must be on the oxygen side of both the antimycin- and the HQNO-binding sites.

However, in the light of the extensive binding and effect studies with HQNO [23], it is highly unlikely that antimycin and HQNO exert their effects on different sites of the respiratory chain. Therefore, we repeated some of the experiments of Eisenbach and Gutman, not only to study the differences between antimycin and HQNO, but also to evaluate the possibility that X and Y are in fact identical, as has also been proposed by Trumpower and Katki [21].

Experimental

Submitochondral particles were prepared from heavy beef-heart mitochondria according to Fessenden and Racker [24] (A particles), Lee and Ernster [25] (EDTA particles) or according to Löw and Vallin [26] (Mg-ATP particles). A particles and EDTA particles were stored at −20°C; Mg-ATP particles were used on the day of preparation.

If not stated otherwise, the reaction mixture contained 250 mM sucrose/10 mM $MgCl_2$/1 mM EDTA/50 mM Tris-HCl buffer at pH 7.5 (standard medium).

Full activation of the succinate dehydrogenase of submitochondrial particles was achieved by incubating the particles (20 mg/ml) with 1 mM malonate at 30°C for 30 min, after which the preparation was diluted 5-fold with ice-cold standard medium [17].

Inhibition of the succinate oxidase of submitochondrial particles by 2,3-dimercaptopropanol (BAL) was measured with a Clark-type oxygen electrode after preincubation at 35°C of the submitochondrial particles with BAL and oxygen according to Deul and Thorn [27].

Absorbance measurements were performed with an Aminco-Chance double-beam spectrophotometer (DW2) equipped with either a thermostatically controlled cuvette holder for measurements at 2°C or with an Aminco-Morrow stopped-flow apparatus for measurements at 25°C.

Antimycin was obtained from Nutritional Biochemical Corporation and HQNO from Sigma. They were added in ethanolic solutions such that the final ethanol concentration did not exceed 1% (v/v). The concentrations were determined spectrophotometrically as described in Ref. 23.

Protein was measured using the biuret reaction after precipitation of protein with trichloroacetic acid [28].

Results

Kinetics of reduction of cytochrome b

In aerobic suspensions of submitochondrial particles, to which antimycin and KCN are added, cytochrome *b* is reduced biphasically by succinate (Fig. 1A). At 2°C, pseudo-first-order reaction constants of 0.15 and of 0.017 s^{-1} are calculated for the reduction of cytochrome *b* in the rapid and slow phases, respectively (Fig. 1C). In the course of the transition from the rapid to the slow phase, cytochrome *c* and c_1 become appreciably reduced (Fig. 1B). During the steady-state oxidation of ascorbate in the presence of TMPD, the addition of succinate

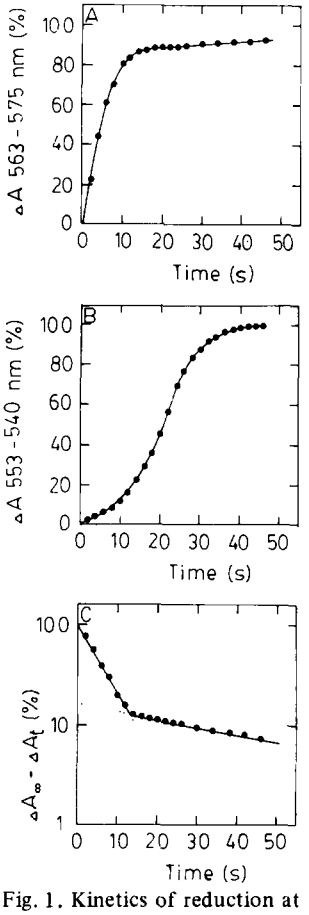

Fig. 1. Kinetics of reduction at 2°C of cytochrome *b* and the cytochromes *c* and c_1 by succinate under antimycin- and KCN-inhibited conditions. KCN (1 mM) was added to a suspension of malonate-activated Mg-ATP particles (2.6 mg/ml) in the presence of rotenone (50 ng/ml) and antimycin (5 nmol/mg). After 2 min, 5 mM succinate was added (at $t = 0$). (A) Reduction of cytochrome *b* ($\Delta A_{563-575nm}$) as percentage of the final level ($\Delta A_\infty = 0.049$). (B) Reduction of the cytochromes *c* and c_1 ($\Delta A_{553-540nm}$) as percentage of the final level ($\Delta A_\infty = 0.034$). (C) Semilogarithmic representation of the experimental points of (A). The rapid phase corresponds to a rate constant of 0.15 s^{-1}. The slow phase corresponds to a rate constant of 0.017 s^{-1}.

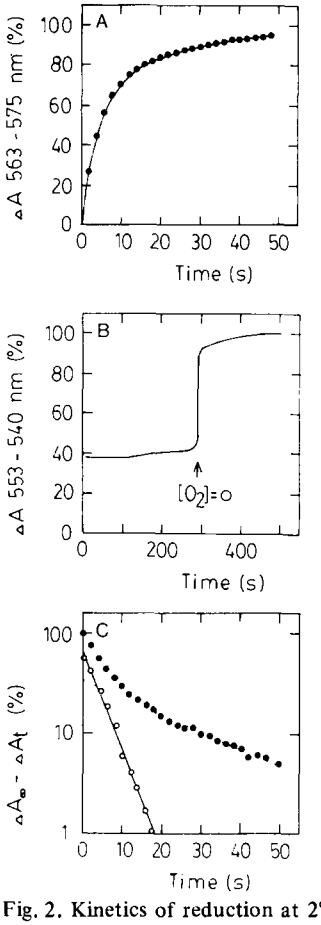

Fig. 2. Kinetics of reduction at 2°C of cytochrome *b* and the cytochromes *c* and c_1 by succinate under antimycin-inhibited ascorbate-oxidizing conditions. Antimycin (5 nmol/mg) was added to a suspension of malonate-activated Mg-ATP particles (2.5 mg/ml) in the presence of rotenone (50 ng/ml). After 2 min, successive additions were made of 5 mM ascorbate (pH 7.5), 42 μM TMPD and 5 mM succinate (at $t = 0$). (A) Reduction of cytochrome *b* as percentage of the final level ($\Delta A_\infty = 0.042$). (B) Reduction of cytochromes *c* and c_1 ($\Delta A_{553-540nm}$) as percentage of the final level ($\Delta A_\infty = 0.030$). (C) Semilogarithmic representation of the experimental points of (A). The rapid reaction (o———o) corresponds to a rate constant of 0.23 s^{-1}. The slow phase corresponds to a rate constant of 0.04 s^{-1}.

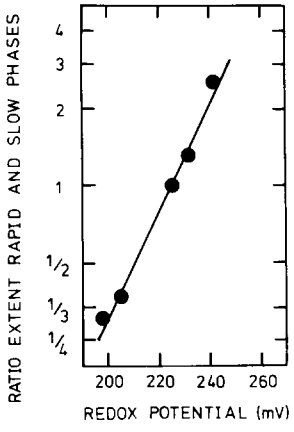

Fig. 3. Determination of the half potential for the appearance of the slow phase. Ascorbate (5 mM) and varying concentrations of KCN (0–0.33 mM) and TMPD (0–8 μM) were added to suspensions of malonate-activated EDTA particles (2.0 mg/ml) in the presence of rotenone (100 ng/mg) and antimycin (7 nmol/mg). When the redox level of the cytochromes *c* and c_1, measured at $\Delta A_{553-540nm}$, was constant, 5 mM succinate was added and $\Delta A_{563-575nm}$ was followed in time. From the semilogarithmic plot of the reduction of cytochrome *b* the extents of the rapid and slow phases were calculated. From the redox level of the cytochromes *c* and c_1 before the addition of succinate the redox potential was calculated assuming a midpoint potential of 230 mV. The temperature was 2°C.

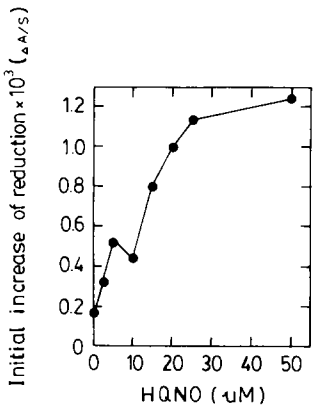

Fig. 4. Titration with HQNO of the initial reduction of cytochrome *b* by succinate at 2°C. KCN (2 mM) was added to suspensions of malonate-activated EDTA particles (1.0 mg/ml) in the presence of the indicated concentrations of HQNO. After 2 min, 5 mM succinate was added and the initial rate of increase in the level of reduction of cytochrome *b* was measured at $\Delta A_{562-575nm}$.

also causes the reduction of cytochrome *b* in a biphasic manner (Fig. 2A), but in this case, the kinetics can be analyzed as a summation of a rapid and a slow reaction (Fig. 2C), rather than as two discrete phases (cf. Fig. 1C). In this case the redox state of cytochromes *c* and c_1 was constant from $t = 0$ (Fig. 2B).

In agreement with Eisenbach and Gutman [20], the ratio of the contributions of rapid and slow reactions to the reduction of cytochrome *b* can be varied by varying the redox state of the cytochromes *c* and c_1 (Fig. 3). In our hands, the use of more than 50 μM TMPD under ascorbate-oxidizing conditions leads to spectral interference by $TMPD^+$ with the *b* and *c* cytochromes. However, in the presence of 5 mM ascorbate, different steady-state redox levels of the cytochromes *c* and c_1 could be established by prior addition of small amounts of cyanide and maximally 8 μM of TMPD. Due to the slower equilibration of cytochromes *c* and c_1 with ascorbate in the presence of these low concentrations of TMPD, sufficiently constant redox levels were only obtained within the range 200–260 mV (75% reduced–75% oxidized). When the logarithm of the ratio of the amounts of cytochrome *b* reduced in the rapid and the slow phases is plotted against the redox level of the cytochromes *c* and c_1 (Fig. 3), a half potential of +225 mV may be calculated for the appearance of the slow phase of cytochrome *b* reduction. The *n* value in this plot is 1.1.

Similar results were obtained in stopped-flow experiments at 25°C, pseudo-first-order reaction constants of 2 and 0.2 s^{-1} for the rapid and slow phases, respectively, being found.

Comparison of the effects of antimycin and HQNO on the kinetics of reduction of cytochrome b

In the absence of cyanide, rapid and monophasic reduction is observed in the presence of antimycin, but biphasic reduction of cytochrome *b* was reported by Eisenbach and Gutman [22] in the presence of HQNO. However, since antimycin prevents the binding of HQNO to any high-affinity site [23], it seemed to us unlikely that any low-concentration effect of HQNO can be exerted on a site of the respiratory chain different from that of antimycin. The effects of high concentrations of HQNO on the oxidation of ferrocytochrome *c* reported by Izzo

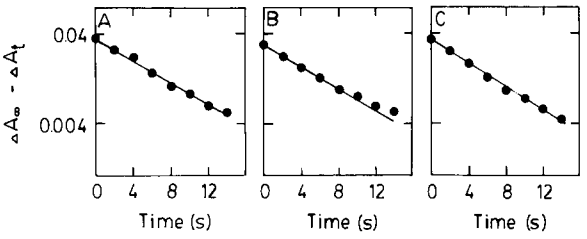

Fig. 5. Semilogarithmic plots of the rapid reduction at 2°C of cytochrome *b* by succinate. Comparison between the kinetics in the presence of 5 nmol/mg antimycin (A), 60 μM HQNO (B) and antimycin and HQNO (C). At t = 0, 5 mM succinate was added to suspensions of malonate-activated EDTA particles (2.0 mg/ml) in the presence of rotenone (100 ng/mg) and antimycin and/or HQNO, and either $\Delta A_{563-575nm}$ (A and C) or $\Delta A_{562-575nm}$ (B) followed in time.

et al. [29] can be explained by nonspecific binding [23,30].

The experiments given in Fig. 4 show that, as is to be expected, with increasing concentrations of HQNO, the initial rate of reduction of cytochrome *b* is increased. From this figure it is clear that the concentration of HQNO employed by Eisenbach and Gutman (16 μM with twice the concentration of particles) is insufficient to prevent reduction of components on the oxygen side of the HQNO site. In Fig. 5 it is shown that using a higher concentration of HQNO (60 μM), monophasic reduction of cytochrome *b* by succinate is obtained at the same

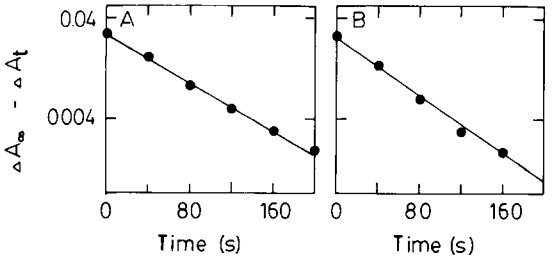

Fig. 6. Semilogarithmic plots of the slow reduction at 2°C of cytochrome *b* by succinate. Comparison between the kinetics in the presence of antimycin and in the presence of HQNO. KCN (2 mM), ascorbate (5 mM) and TMPD (0.1 mM) were added to suspensions of EDTA particles (2.0 mg/ml) in the presence of rotenone (100 ng/mg) and 5 nmol/mg antimycin (A) or 60 μM HQNO (B). After 2 min, 5 mM succinate was added (at t = 0) and either $\Delta A_{563-575nm}$ (A) or $\Delta A_{562-575nm}$ (B) followed in time.

rate as obtained when HQNO is replaced by antimycin or when HQNO and antimycin are present together. In the presence of KCN, HQNO and antimycin give rise to identical biphasic reduction of cytochrome *b* (not shown) and in the presence of sufficiently high concentrations of KCN, ascorbate and TMPD to keep cytochrome c_1 largely reduced (cf. Fig. 3), both HQNO and antimycin give rise to monophasic and slow reduction of cytochrome *b* by succinate (Fig. 6).

It is concluded that maximal inhibition, either by antimycin or by HQNO, of the oxidation of cytochrome *b* is necessary to obtain rapid and monophasic reduction of cytochrome *b* by succinate.

Spectral resolution of the reduction of cytochrome b

From the observations that, depending on the pretreatment of the submitochondrial particles, all cytochromes *b* are reduced either in a rapid or in a slow fashion, it is apparent that the mechanism that influences the rate of reduction is not limited to one of the cytochrome *b* components. However, during biphasic reduction of cytochrome *b* in the presence of antimycin and KCN, there is to some extent a preference for cytochrome *b*-562 in the rapid phase and for cytochrome *b*-566 in the slow

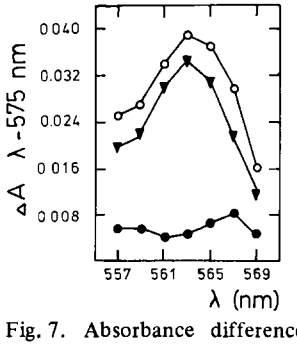

Fig. 7. Absorbance difference spectra of the kinetically distinguishable phases in the biphasic reduction of cytochrome *b*. Experimental conditions as in Fig. 3. At the indicated wavelength pairs, ΔA was followed in time. At the time of transition from rapid to slow kinetics (t = 13 s), the difference spectrum representative for the rapid phase (▼———▼) was constructed from the individual dual-wavelength measurements. When the final level of reduction had been reached, the total difference spectrum (○———○) was constructed. The difference (●———●) between the total difference spectrum and that at t = 13 s is representative for the slow phase.

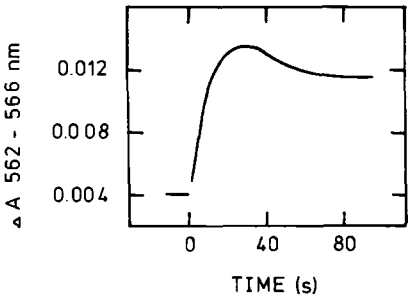

Fig. 8. Preferential reduction of cytochrome *b*-562 in the rapid phase. Experimental conditions as in Fig. 7. $\Delta A_{562-566nm}$ was followed in time.

phase. This is shown both by constructing from individual dual-wavelength measurements the difference spectra that are representative for the rapid and slow phases (Fig. 7) and by directly measuring the time resolution of the absorbance difference between 562 and 566 nm (Fig. 8). These differences in the kinetics of reduction of *b*-562 and *b*-566 are presumably a reflection of the higher midpoint redox potential of *b*-562.

Factors controlling the slow-phase reduction of cytochrome b

The above experiments on the kinetics of cytochrome *b* were carried out under aerobic conditions in the presence of antimycin, when cytochrome *b* is fully reducible, even in the presence of cyanide [8]. As shown in Fig. 9, under anaerobic conditions in the presence of antimycin, ascorbate and TMPD, the partial reduction of cytochrome *b* by succinate is monophasic but extremely slow ($k = 0.006$ s^{-1} at 2°C). As can be concluded from the experiments of Deul and Thorn [27], the effect of oxygen on the reducibility of cytochrome *b* in the presence of antimycin can be prevented by pretreatment of the submitochondrial particles with 2,3-dimercaptopropanol (BAL) [31], which has recently been shown to destroy the Rieske Fe-S protein [32]. Pretreatment of submitochondrial particles with suboptimal concentrations of BAL causes biphasic reduction of cytochrome *b* by succinate in the presence of antimycin. The rate constant of the slow phase ($k = 0.003$ s^{-1}) is similar to that obtained under anaerobic conditions. The amount of cytochrome *b* reduced in the slow phase after pretreatment with BAL increases with increasing inhibition of succinate oxidation by BAL (Fig. 10).

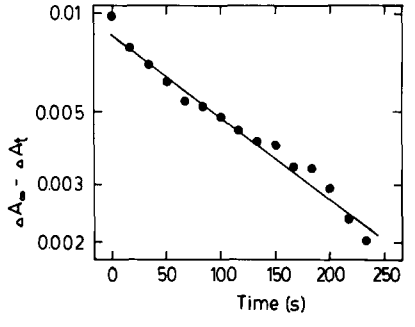

Fig. 9. Semilogarithmic plot of the reduction of cytochrome *b* at 2°C by succinate under antimycin-inhibited anaerobic conditions. A suspension of EDTA particles (1.0 mg/ml) in the presence of antimycin (5 nmol/mg) was made anaerobic in a Thunberg cuvette. From a separate compartment, 5 mM ascorbate and 0.1 mM TMDP were added. When the reduction of the cytochromes *c* and c_1 was completed, 5 mM succinate was added from a second separate compartment. $\Delta A_{563-575nm}$ was followed in time. The corresponding rate constant is 0.006 s^{-1}.

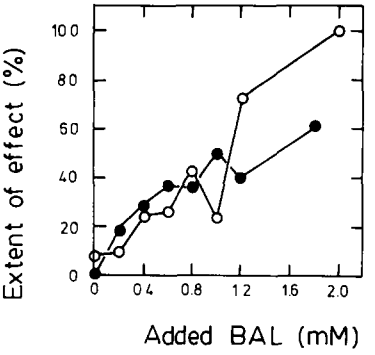

Fig. 10. Comparison of the effects of preincubation with BAL on the kinetics of reduction of cytochrome *b* by succinate and on the rate of succinate oxidation. Suspension of malonate-activated EDTA particles (2.0 mg/ml) were preincubated with the indicated concentrations of BAL. The percentage inhibition of the succinate oxidase activity (●——●) was measured at 35°C after the addition of 5 mM succinate. The percentage of cytochrome *b* reduced in the slow phase (○——○) was measured at 2°C in the presence of antimycin (5 nmol/mg) and air after the addition of 5 mM succinate and following $\Delta A_{563-575nm}$ in time.

Discussion

The key observation of Eisenbach and Gutman [17] that, in the presence of antimycin, the rate

of reduction of cytochrome *b* by succinate in sub-mitochondrial particles is dependent on the redox state of a component on the oxygen side of the antimycin block has been confirmed in this study. This is revealed by the biphasic kinetics of reduction of cytochrome *b* by succinate added to oxidized particles (Fig. 1), by the existence of two kinetically distinguishable reduction systems when the particles are reduced with ascorbate and TMPD prior to the addition of succinate (Fig. 2), and by the abolition of the rapid phase when oxidation of components on the oxygen side of the antimycin block is prevented by anaerobiosis (Fig. 9) or by destruction by BAL of the Rieske Fe-S protein [32] (Fig. 10). However, one of their experiments that led Eisenbach and Gutman to define their dynamic control mechanism could not be reproduced in our hands. We find no difference between antimycin and HQNO, provided that sufficient HQNO is added to saturate their common binding site [23]. Thus, the regulatory component proposed by these workers must be located on the oxygen side of the antimycin and HQNO-binding site.

A similar regulatory site, designated X, was previously proposed by Baum and Rieske [10,7] to explain the oxidant-induced extra reduction of cytochrome *b*. Wikström and Berden [8], however, proposed as an alternative explanation that electrons from substrate proceed alternately along two electron-transfer chains to cytochrome *c* and that the effect of oxidant is to increase the concentration of the electron donor to the chain containing cytochrome *b*. As discussed in detail elsewhere [16], Wikström and Berden's proposal and other similar proposals by Mitchell [13] provide a satisfactory explanation for the oxidant-induced reduction of cytochrome *b* without the necessity of invoking a regulatory site.

The variant of the two-pathway respiratory chain ('Q' cycle [14,15]) that we now favour is shown in Fig. 11. According to this scheme, QH_2 formed by the reduction of Q by succinate (catalysed by succinate dehydrogenase) on the inside surface of the mitochondrial inner membrane is freely diffusible. In the region of the inner surface it is oxidised by Q to form a nondiffusible semiquinone anion (designated $Q_c^{\cdot-}$). In the region of the outer surface it is oxidized by the BAL-sensitive [32]

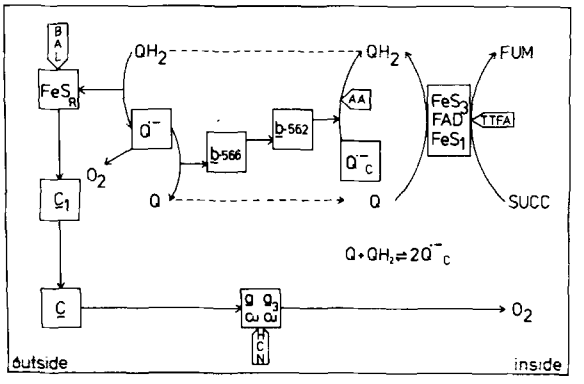

Fig. 11. Postulated two-electron, Q-cycle [14] pathway for oxidation of succinate by O_2. Curved lines with arrow heads describe chemical reactions, full straight lines with arrow heads flow of electrons, dotted straight lines pathway of diffusion. Large arrow heads indicate site of action of inhibitors. FeS_1 and FeS_3 denote the two Fe-S clusters in succinate dehydrogenase, FeS_R the Rieske Fe-S protein. Letters b, c_1, c, a and a_3 denote the corresponding cytochromes. $Q_c^{\cdot-}$ (cf. [33]) and $Q^{\cdot-}$ denote two species of ubisemiquinone anion. Components in boxes have limited mobility, others are freely diffusible.

Rieske Fe-S protein to form a second species of nondiffusible semiquinone (designated $Q^{\cdot-}$). The latter delivers electrons to the cytochromes *b* which are then oxidised by $Q_c^{\cdot-}$ in an antimycin-sensitive (cf. Ref. 14) reaction. The Q cycle is completed by diffusion of Q from the outside surface to the inside. $Q_c^{\cdot-}$ is tentatively identified with the antimycin-sensitive species of ubiquinone detected by Ohnishi and Trumpower [33] and de Vries et al. [34].

This scheme satisfactorily explains: (i) complete inhibition of the electron transfer by BAL treatment [31] which by destroying the Fe-S protein [32] cuts the main electron-transfer chain; (ii) inhibition of electron transfer by antimycin or HQNO, which prevent the oxidation of cytochrome *b* by $Q_c^{\cdot-}$ and thereby block the main pathway for the regeneration of Q; (iii) the antimycin-insensitive reduction of O_2 to $O_2^{\cdot-}$, which is thought to proceed via the auto-oxidation of $Q^{\cdot-}$ (cf. Ref. 35); (iv) the sensitivity of the antimycin-resistant respiration to cyanide [36], which by inhibiting the reoxidation of the Fe-S protein prevents the formation of $Q^{\cdot-}$; (v) the reduction of cytochrome *b* by succinate (via QH_2 on the inside surface) after inactivation [31,32] or removal [15,37] of the Fe-S protein and the inhibition

of this reaction by antimycin [27]; (vi) the incomplete reduction of cytochrome *b*, especially of low-potential cytochrome *b*, even in the presence of antimycin, unless oxygen or other oxidant is present [4–7] to oxidize QH_2 to $Q^{\cdot-}$, the direct reductant of cytochrome *b*. Indeed, it seems impossible to explain these various findings without assuming some sort of two-pathway respiratory chain of the type depicted in Fig. 11.

By taking into account some further observations, it is also possible to explain, on the basis of the scheme in Fig. 11, the observations reported by Eisenbach and Gutman [17,19,20,22] and in this paper that two kinetic species of cytochrome *b* are present – slowly and rapidly reduced – and that the proportion of the two species is determined by the redox potential of a component on the oxygen side of the antimycin and HQNO block. In an extension of the experiment shown in Fig. 10, it has been shown that, after treatment with different quantities of BAL, the extent of the fast phase measured in the presence of antimycin is proportional to the fraction of intact Fe-S cluster [38]. This is understandable, on the basis of Fig. 11, since only those assemblies of the respiratory chain with an intact Fe-S cluster will be able to form $Q^{\cdot-}$, the normal electron donor for cytochrome *b*. It is more difficult to understand why the same effect should be obtained when a component is kept reduced. Since the redox potential of this component (225 mV at pH 7.5) is in the same region as that of the Rieske Fe-S protein (about 285 mV at pH 7.0 [39]), it seems likely that this protein is involved, but it is not immediately apparent why the fraction reduced behaves just as if it has been irreversibly destroyed by BAL, that is it appears to be irreversibly reduced, and no longer able to function as an electron carrier between QH_2 and cytochrome c_1. Velthuys [40] has shown that this is precisely what happens when the analogous plastoquinol (or the inhibitor 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole) is bound to the reduced Fe-S protein. It seems then that in QH_2 : ferricytochrome *c* oxidoreductase, the normal reductant for cytochrome *b* ($Q^{\cdot-}$) is formed only when QH_2, formed by succinate dehydrogenase, reacts with assemblies containing oxidized Fe-S protein.

The magnitude of *k* of the slow phase of reduction of cytochrome *b* in the presence of antimycin declines parallel with the amount of rapidly reducible cytochrome *b* (unpublished observations). This suggests that the slow phase is largely due to a slow reaction between the $Q^{\cdot-}$ formed in respiratory assemblies containing an oxidized Fe-S cluster with cytochrome *b* in assemblies containing reduced Fe-S clusters. However, the lowest value obtained when no fast phase is present (when cytochromes *c* and c_1 are fully reduced by ascorbate) is still much greater than that found with isolated succinate : cytochrome *c* oxidoreductase [21]. This difference, which has also been pointed out by Trumpower and Katki [21], may be due to a slow direct reduction of cytochrome *b* by ubiquinol which is present in much larger amounts in particles than in the isolated enzyme.

The previously proposed regulatory effects of the redox-sensitive components X [10,7] and Y [17] on the redox level and the reduction kinetics, respectively, of cytochrome *b* are now ascribed, then, simply to the role of the Fe-S protein [41], when it is oxidized, in producing the reductant for cytochrome *b* by oxidation of QH_2, and by the fact that when QH_2 is bound to it, the reduced Fe-S protein cannot be oxidized by its natural oxidant, cytochrome c_1.

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