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KINETICS OF FLASH-INDUCED ELECTRON TRANSFER BETWEEN BACTERIAL REACTION CENTRES, MITOCHONDRIAL UBIQUINOL: CYTOCHROME *c* OXIDOREDUCTASE AND CYTOCHROME *c*

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Ascorbate-reduced horse heart cytochrome *c* reduces photo-oxidized bacterial reaction centres with a second-order rate constant of $(5–8) \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at an ionic strength of 50 mM. In the absence of cytochrome *c*, the cytochrome c_1 in the ubiquinol: cytochrome *c* oxidoreductase is oxidized relatively slowly ($k = 3.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$). Ferrocytochrome *c* binds specifically to ascorbate-reduced reductase, with a K_d of 0.6 μM, and only the free cytochrome *c* molecules are involved in the rapid reduction of photo-oxidized reaction centres. The electron transfer between ferricytochrome *c* and ferrocytochrome c_1 of the reductase is rapid, with a second-order rate constant of $2.1 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at an ionic strength of 50 mM. The rate of electron transfer from the Rieske iron-sulphur cluster to cytochrome c_1 is even more rapid. The cytochrome *b* of the ubiquinol: cytochrome *c* oxidoreductase can be reduced by electrons from the reaction centres through two pathways: one is sensitive to antimycin and the other to myxothiazol. The amount of cytochrome *b* reduced in the absence of antimycin is dependent on the redox potential of the system, but in no case tested did it exceed 25% of the amount of photo-oxidized reaction centres.

Introduction

Considerable progress has been made during the last few years in understanding the electron-transfer pathway within the mitochondrial ubiquinol: cytochrome *c* oxidoreductase and the analogous enzyme in photosynthetic bacteria [1]. The studies on the mitochondrial enzyme have been focussed on the role of ubiquinone and its semiquinone, the Q-binding sites and Q-binding proteins (see various chapters in Ref. 1), the Rieske Fe-S cluster [2,3], the pre-steady-state kinetics [4,5] and the site of inhibition of various recently introduced inhibitors [6–9]. All these studies have resulted in the formulation of a Q-cycle model for electron transfer, in which the two protomers of the dimeric complex co-operate to make the complex a self-contained ubiquinol: cytochrome *c* oxidoreductase [4,5]. At the same time many studies have been performed with the bacterial system, also leading to detailed models of electron transfer [10–12], but only recently [13] has a Q-cycle model become favoured over a linear model.

Dutton and co-workers [14–16] have demonstrated that electron transfer is possible between isolated bacterial reaction centres, mitochondrial

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Abbreviations: (oxido)reductase, ubiquinol : cytochrome *c* oxidoreductase; Q, ubiquinone-10; HMHQQ: 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazol; Mops, 4-morpholinepropanesulphonic acid.

ubiquinol : cytochrome *c* oxidoreductase and horse heart cytochrome *c*. In the present study we have used a similar system to investigate the functioning and kinetics of the mitochondrial enzyme and its interaction with cytochrome *c* and reaction centre. In this paper we report data on the functioning of the system itself, i.e., how the electrons leave the mitochondrial enzyme and how they enter it. In a subsequent paper we shall report data concerning the electron transfer within the oxidoreductase.

The combined system has some advantages over both the isolated mitochondrial system and the intact bacterial chromatophores. (1) Within the time of the flash, a specific amount of oxidizing equivalents is generated at one site and the same amount of reducing equivalents at another site. (2) The reactions can be started without addition of reactants, thereby avoiding mixing problems and baseline changes. (3) The experiments can be carried out at any desired redox potential, provided that the bacteriochlorophyll dimer of the reaction centre ($E_m \approx 450$ mV) is not oxidized and the primary electron acceptor Q_A ($E_m \approx -100$ mV) is not reduced. During the process under observation, the potential of the system is fixed; this in contrast to the isolated mitochondrial system where the potential is affected by the addition of a substrate or electron acceptor. (4) The amount of oxidizing or reducing equivalents can be accurately measured and controlled. (5) The concentration of all reactants can be easily varied. (6) Each experiment can be repeated many times with the same sample. (7) The transmission of the sample in the desired wavelength region is not limited by the presence of large amounts of antenna pigments.

In this paper we shall focus on the properties of the system as a whole, i.e., the oxidation of cytochrome *c* by oxidized reaction centres, its re-reduction by the cytochrome *c* reductase, the reduction of oxidized reaction centres by the reductase in the absence of cytochrome *c*, the binding of cytochrome *c* to the reductase and the reduction of cytochrome *b* by reducing equivalents originating from the reaction centre. It will be shown that with the flash-activated reaction centre as an electron donor, the same pathways for the reduction of cytochrome *b* are available as revealed in the studies in which oxidizable substrates were added to the mitochondrial enzyme.

Materials and Methods

Reaction centres were prepared from cells of the photosynthetic purple bacterium *Rhodopseudomonas sphaeroides* mutant R26 following the procedure of Kendall-Tobias and Seibert [17]. The concentration of the reaction centres was determined using an absorbance coefficient of 288 $mM^{-1} \cdot cm^{-1}$ at 802 nm [18]. The preparations contained 2 mol Q per mol reaction centre.

Bovine heart mitochondrial ubiquinol : cytochrome *c* oxidoreductase was isolated by treatment at high pH of succinate : cytochrome *c* oxidoreductase according to the method of Yu and Yu [19]. The preparation was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5% deoxycholate. It contained 0.8 mol Q per mol cytochrome c_1. The concentration of the enzyme was determined by either optical measurement of cytochrome c_1 or titration of the duroquinol : cytochrome *c* oxidoreductase activity with antimycin.

The reaction mixture contained 1 μM reaction centres, 0–7 μM oxidoreductase, 0–30 μM horse heart cytochrome *c*, with or without 1–2 mM ascorbate, and 0.1 μM phenazine methosulphate or 10 μM naphthoquinone in 50 mM Tris-HCl buffer (pH 8.0). When a titration with reductase was performed, an extra 0.02% deoxycholate was added to the mixture to prevent the precipitation of low concentrations (was less than 1 μM) of reductase.

Absorption changes induced by a flash were measured in a home-built spectrophotometer in single-beam mode using a cuvette with an optical path length of 0.3 cm. The actinic illumination was provided by a xenon flash of 20 μs duration through Scott RG 750 and RG 715 filters, which transmitted only the near-infrared component of the flash. Stray light from the flash was kept from the measuring photomultiplier by two sets of filters. During the measurement of the absorbance change of the reaction centres at 604 nm, the set Corning CS 4-97 and Schott AL 601 was used; for the measurement of the absorbance change of the cytochromes, at 551, 561 and 566 nm, the set Schott BG 38-4 and Corning CS 4-96 was used.

From the measured absorbance changes, the changes of the redox state of the cytochromes were calculated with the aid of a PDP 11/44 computer. The measured kinetics of the cytochromes were corrected for changes due to the reaction centres and the other cytochromes as described in Ref. 20. The reduced-minus-oxidized difference extinction coefficients of the reaction centres used in the correction were: $\epsilon^{604}_{red-ox} = +25\ mM^{-1} \cdot cm^{-1}$ [21], $\epsilon_{red-ox} = +11\ mM^{-1} \cdot cm^{-1}$ at 551, 561 and 566 nm (values derived from the spectrum). The extinction coefficients of cytochromes *c* and c_1 used were: $\epsilon^{551}_{red-ox} = 17\ mM^{-1} \cdot cm^{-1}$, $\epsilon^{561}_{red-ox} = -4\ mM^{-1} \cdot cm^{-1}$ and $\epsilon^{566}_{red-ox} = -5.6\ mM^{-1} \cdot cm^{-1}$ (values derived from the spectrum). For cytochrome *b*-561 and *b*-566 it was assumed that the coefficient at 566 and 561 nm, respectively, was 50% of the coefficient at 561 and 566 nm, the latter being 21 $mM^{-1} \cdot cm^{-1}$ [22].

Antimycin (obtained from Sigma) and myxothiazol (a gift from Dr. G. Thierbach) were added in ethanolic solution, HMHQQ (a gift from Dr. K. Folkers) was added in ethanol/2 M Tris-HCl buffer, pH 8.0 (9:1, v/v). The total amount of ethanol in the reaction mixture never exceeded 1% (v/v). After each addition, a dark period of 15 min was used to allow re-equilibration of the sample.

Results

The reaction between reaction centres and horse heart cytochrome c and the effect of addition of ubiquinol: cytochrome c oxidoreductase

Prince et al. [23] have made a detailed study of the interaction of reaction centres isolated from *Rps. sphaeroides* R26 with horse heart cytochrome *c* as well as with native cytochrome c_2. They showed that these reactions are first order with respect to each reactant. Although the isoelectric point of horse heart cytochrome *c* (greater than 10) is very different from that of cytochrome c_2 (6.1), the second-order rate constants are not very different, viz., $3.8 \cdot 10^8$ and $8.2 \cdot 10^8\ M^{-1} \cdot s^{-1}$, respectively. Crofts et al. [13] have reported a somewhat smaller value for cytochrome c_2 in intact chromatophores ($1.67 \cdot 10^8\ M^{-1} \cdot s^{-1}$). In our system the re-reduction of photo-oxidized reaction centres by ascorbate-reduced horse heart cytochrome *c* is pseudo-first order (Fig. 1) due to the fact that the concentration of reaction centres oxidized by each flash is much less than that of ferrocytochrome *c* in the presence or absence of the cytochrome *c* reductase. If the pseudo-first-order rate constant

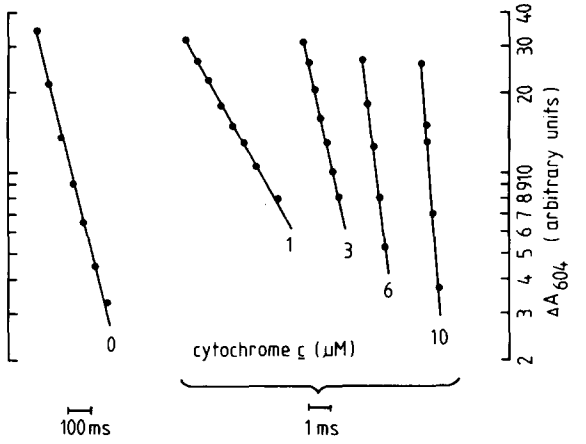

Fig. 1. Semi-logarithmic plot of the re-reduction of photo-oxidised reaction centres. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 1 μM reaction centres, 3 μM ubiquinol: cytochrome *c* oxidoreductase, 2 mM ascorbate, 0.1 μM phenazine methosulphate and 0–40 μM horse heart cytochrome *c* as indicated by numbers in the figure.

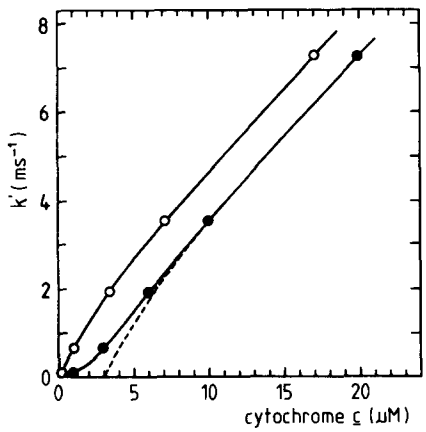

Fig. 2. The pseudo-first-order rate constant of the re-reduction of photo-oxidised reaction centres in the presence of 3 μM ubiquinol: cytochrome *c* oxidoreductase and variable amounts of cytochrome *c*. The experimental conditions were the same as in Fig. 1. (●——●) The abscissa represents total cytochrome *c* added; (○——○) the abscissa represents free cytochrome *c*. The concentration of free cytochrome *c* is obtained by correcting total cytochrome *c* for cytochrome *c* bound to the reductase, assuming a 1:1 complex with $K_d = 0.6$ μM.

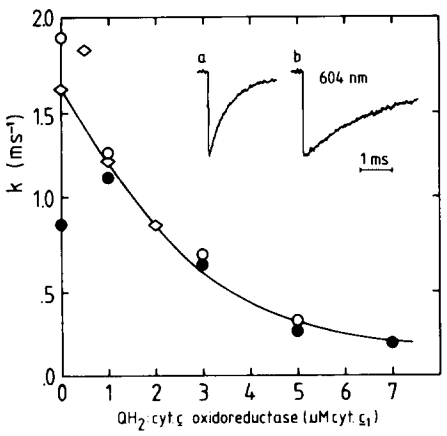

Fig. 3. The pseudo-first-order constant of the re-reduction of photo-oxidised reaction centres in the presence of 3 μM cytochrome *c* and variable amounts of reductase. The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0 (◇——◇), or 50 mM Mops-NaOH buffer, pH 7.9 (●——●), or pH 7.0 (○——○). Also present were 2 mM ascorbate, 10 μM naphthoquinone, 0.02% deoxycholate and enough antimycin to saturate the reductase. The curve is a theoretical curve assuming binding of cytochrome *c* to the reductase with a K_d of the 1 : 1 complex of 0.6 μM. The inset shows the actual traces (at 604 nm) in the absence (trace a) and presence (trace b) of 7 μM reductase.

in the presence of the reductase is plotted against the total amount of cytochrome *c* added, the curve (●——●) in Fig. 2 is obtained. The shape of the initial part of the curve suggests some kind of specific binding of cytochrome *c*. When the pseudo-first-order rate constant at a constant total cytochrome *c* concentration is plotted against the concentration of reductase, it is clear that increasing concentrations of enzyme slow down the reaction (Fig. 3). The experimental points fit reasonably well a theoretical curve based on the assumption that there is a specific binding between the ascorbate-reduced reductase and cytochrome *c*, with a K_d of 0.6 μM, and that only free cytochrome *c* can rapidly reduce the flash-oxidized reaction centres. If the points in Fig. 2 are corrected for the specific binding of cytochrome *c* to the reductase, the curve (○——○) is no longer sigmoidal. The bending of the curve at increasing cytochrome *c* concentration indicates that possibly also some weak binding sites are present on the reductase, since in the absence of enzyme this plot is linear (see also Ref. 23). From the pseudo-first-order rate constant in the absence of reductase and the concentration of cytochrome *c*, a second-order constant of $5.6 \cdot 10^8$–$8.0 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ can be calculated depending on the reaction centre preparation. This value is very close to those reported previously [23].

Since only about 20% of the reaction centres in the system are excited by each flash and there is no 'concentration lag' in the corrected curve in Fig. 2, it can be concluded that there is no specific binding between reaction centres and cytochrome *c*, leading to a decreased availability of cytochrome *c* for electron transfer. If the strong 1 : 1 binding between cytochrome *c* and reaction centres found by Rosen et al. ($K_d = 0.4$ μM at 10 mM Tris-HCl [24]) were taken into account, the first two open circles in Fig. 2 would lie on the ordinate. It appears, then, that our preparation of reaction centres, which is made in a different way from that of Rosen et al. [24], does not bind cytochrome *c* strongly (cf. Ref. 23).

Variation of the buffer (Tris-HCl or Mops-NaOH at constant ionic strength) and the pH between 7 and 8 have little effect on the reaction between reaction centres and cytochrome *c* provided enough reductase (greater than 1 μM) is present. The reason for the observed scatter in the absence of reductase is not clear.

The reaction between reaction centres and cytochrome c_1

The direct oxidation of cytochrome c_1 by the reaction centres, in the absence of cytochrome *c*, is very slow and cannot be detected under normal conditions, since the re-reduction of cytochrome c_1 is more rapid than its oxidation by reaction centres. The intrinsic rate of oxidation of cytochrome c_1 can be measured in two ways: directly, in the presence of inhibitors that prevent the re-reduction of cytochrome c_1, and indirectly, via the oxidant-induced reduction of cytochrome *b*. Both methods are shown in Fig. 4. For the direct measurement the re-reduction of cytochrome c_1 was inhibited by antimycin plus HMHQQ under which conditions cytochrome *b* is neither oxidized nor reduced, since HMHQQ [7], like myxothiazol [6], inhibits the oxidant-induced reduction of cytochrome *b* in the presence of antimycin (see also below) and antimycin inhibits the oxidation of cytochrome *b*. As-

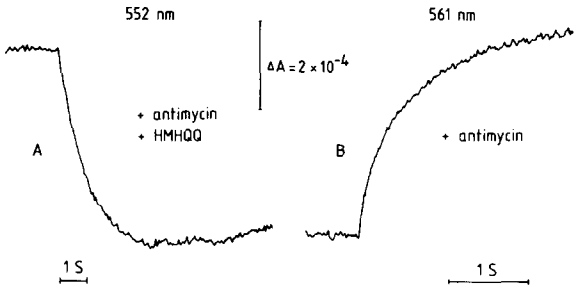

Fig. 4. (A) The oxidation of cytochrome c_1 by photo-oxidised reaction centres. The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0, 1 mM ascorbate, 0.1 μM phenazine methosulphate, 1 μM reaction centres, 3 μM ubiquinol: cytochrome *c* oxidoreductase, 20 μM HMHQQ and 5 μM antimycin. (B) The oxidant-induced reduction of cytochrome *b* when HMHQQ is omitted from the system described in A.

suming that HMHQQ, like UHDBT, as proposed by Bowyer et al. [25], inhibits the oxidation of the Rieske Fe-S cluster by cytochrome c_1, trace A of Fig. 4 shows the rate of oxidation of cytochrome c_1. From experiments similar to that of trace A in Fig. 4 at various concentrations of the reductase, a second-order rate constant of $3.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ could be calculated. If, on the contrary, we suppose that HMHQQ inhibits the reduction of the Fe-S cluster, not its oxidation, then the Fe-S cluster is also partially oxidized, and the rate constant for oxidation of cytochrome c_1 is greater than the calculated value. From the extent of the oxidation we may conclude that this is the case. This conclusion is confirmed by the indirect measurement shown in trace B of Fig. 4, where antimycin is present as the only inhibitor. The rate of reduction of cytochrome *b* is now determined by the rate at which cytochrome c_1 is oxidized (and re-reduced). This reaction is indeed slightly faster ($k = 5 \cdot 10^5$ $M^{-1} \cdot s^{-1}$).

The interaction between cytochrome c and cytochrome c_1

The extent of the very rapid net oxidation of cytochrome *c* by the reaction centres, immediately after the flash, is decreased when ascorbate-reduced ubiquinol: cytochrome *c* oxidoreductase, together with the inhibitor myxothiazol, is also present (Fig. 5). Since the extent of the oxidation of the reaction centres is not changed by the addition of the enzyme and the reaction centres are com-

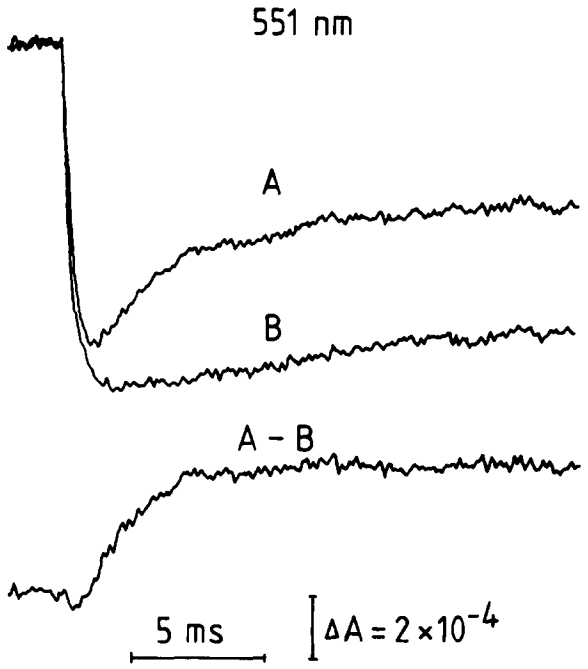

Fig. 5. Effect of ubiquinol: cytochrome *c* oxidoreductase on the re-reduction of cytochrome $c + c_1$. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 2 mM ascorbate, 10 μM naphthoquinone, 0.02% deoxycholate and 1 μM reaction centres. In A 1 μM reductase, 3,83 μM cytochrome *c* (3.0 μM free cytochrome *c*) and 3 μM myxothiazol were present; in B only 3 μM cytochrome *c* was present. The difference A − B represents the reduction of cytochrome $c + c_1$ due to the presence of reductase.

pletely re-reduced in 1–2 ms (as shown by spectrophotometric observation at 604 nm), it follows that a partial re-reduction of cytochrome *c* has occurred. Since at 551 nm cytochrome *c* and cytochrome c_1 are indistinguishable, the reducing equivalents originate from another component in the reductase. Since myxothiazol which inhibits electron flow to the Rieske Fe-S cluster and cytochrome c_1 [5,26] was present, it is concluded that the electron donor is the Rieske Fe-S cluster. Measurements at different wavelengths (not shown) further reveal that cytochrome *c* is largely rereduced but cytochrome c_1 is more oxidized. Thus, both the Fe-S cluster and cytochrome c_1 are partly oxidized by the cytochrome *c*. These results indicate that the apparent E_m of the Rieske Fe-S cluster at pH 8.0 is lower than that of cytochrome *c* and not much different from that of cytochrome c_1.

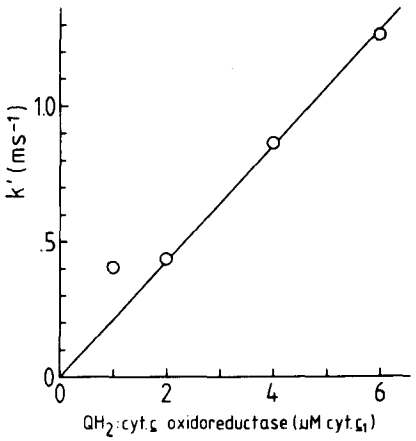

Fig. 6. Re-reduction of cytochrome *c* by ubiquinol : cytochrome *c* oxidoreductase. From traces as in Fig. 5 (A − B) the pseudo-first-order rate constant for the re-reduction of cytochrome *c* was determined at various concentrations of reductase. The concentration of free cytochrome *c* was kept constant, so that the rate of oxidation by photo-oxidised reaction centres was constant. From the straight line a second-order rate constant of $2.1 \cdot 10^8\ M^{-1} \cdot s^{-1}$ can be calculated for the reduction of cytochrome *c* by cytochrome c_1.

Thus, the difference between the two traces in Fig. 5 (indicated as trace A − B) reflects the electron transfer from the Rieske Fe-S protein to cytochromes *c* and c_1. Analysis of such traces at different concentrations of ubiquinol : cytochrome *c* oxidoreductase (Fig. 6) shows that the rate of reduction of cytochrome $c + c_1$ increases with increasing concentrations of the reductase, indicating that this rate is not determined by the electron transfer within the reductase from the Rieske Fe-S cluster to cytochrome c_1 but by the rate of electron transfer from cytochrome c_1 to cytochrome *c*, since only this rate is dependent on the concentration of the reductase. We may conclude, then, that electron transfer between the Fe-S cluster and cytochrome c_1 is much faster than that between cytochrome c_1 and cytochrome *c*. The second-order rate constant for the electron transfer from cytochrome c_1 to cytochrome *c*, calculated from data as shown in Fig. 6, is $2.1 \cdot 10^8\ M^{-1} \cdot s^{-1}$, in excellent agreement with the value obtained by extrapolation to 50 mM ionic strength of the curve reported by König et al. [27] for the second-order rate constant of electron transfer between isolated cytochrome c_1 and cytochrome *c* ($1.5 \cdot 10^8\ M^{-1} \cdot s^{-1}$). This value is much higher than the $8.3 \cdot 10^6\ M^{-1} \cdot s^{-1}$ reported by Crofts et al. [13] for electron transfer from bound cytochrome c_1 to soluble cytochrome c_2 in *Rps. sphaeroides*. An explanation for this large difference may be the fact that in intact chromatophores most of cytochrome c_2 not bound to cytochrome c_1 is bound to the reaction centres [24].

Electron transfer from the reaction centres to the ubiquinol : cytochrome c oxidoreductase

When cytochrome *c* is omitted from the system and the ubiquinol : cytochrome *c* oxidoreductase is reduced by ascorbate, a flash causes the rapid reduction of cytochrome *b*-562 with a $t_{1/2}$ of about 3 ms (Fig. 7A, cf. Ref. 16). This fast phase is followed by a much slower phase ($t_{1/2} \approx 200$ ms) which contributes about 30% of the maximum absorption change. The maximum amount of cytochrome *b* reduced is about 10% of the amount of reaction centres that are photo-oxidized. The rapid reduction of cytochrome *b* under these conditions is antimycin sensitive. The slow reduction is the consequence of the slow oxidation (and re-reduction) of cytochrome c_1. The level is stable for several seconds.

Similar results are obtained in the presence of cytochrome *c* if myxothiazol is added. In this case more cytochrome *b* can be reduced, up to 25% of

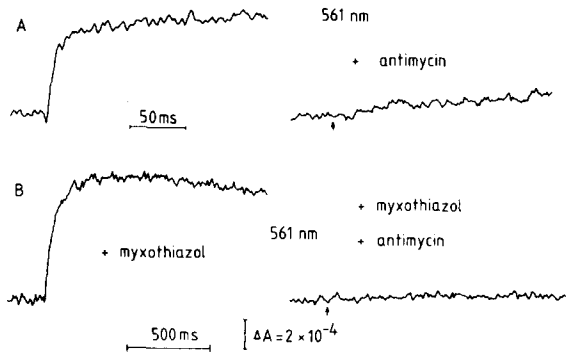

Fig. 7. Reduction of cytochrome *b* by light-activated reaction centres in the absence of an oxidant. (A) The system contained 1 μM reaction centres, 3 μM reductase, 1 mM ascorbate, 0.1 μM phenazine methosulphate and 50 mM Tris-HCl buffer, pH 8.0. In the presence of 5 μM antimycin only a slow phase of reduction is present. (B) As in A, but, in addition, 2 μM cytochrome *c* and 10 μM myxothiazol were present.

the photo-oxidized reaction centres, but no slow phase is present, since re-reduction of cytochrome $c + c_1$ is inhibited (Fig. 7B). The further addition of antimycin abolishes the reduction of cytochrome *b*.

Also, in the absence of ascorbate, with all components of the reductase in the oxidized form, a flash causes a rapid ($t_{1/2} \approx 3$ ms) reduction of cytochrome *b* (Fig. 8A). The amount of rapidly reduced cytochrome *b*, however, is only 6% of the photo-oxidized reaction centres, comparable with an observed reduction of cytochrome c_1 equal to about 4% of the photo-oxidized reaction centres. The reduction of cytochrome *b* is followed by a slow oxidation. The addition of antimycin increases the extent of cytochrome *b* reduction (up to about 10% of the concentration of photo-oxidized reaction centres) but has little effect on the reduction of cytochrome c_1 (5% of the reaction centres) (results not shown). When the redox potential of the system is further increased by the addition of 30 μM ferricytochrome *c*, the flash-induced reduction of cytochrome *b* decreases even further (3% of photo-oxidized reaction centres, also in the presence of antimycin). Both with and without added ferricytochrome *c*, antimycin and myxothiazol are required to prevent the reduction of cytochrome *b*; either inhibitor alone does not prevent reduction.

In the experiments described in Figs. 7 and 8, the electrons that rapidly reduce cytochrome *b* and cytochrome c_1 can only come from reaction centres and not from QH_2 already present. In Fig. 7, the oxidation of QH_2 is prevented either by keeping its specific oxidant reduced by addition of ascorbate, or by the presence of myxothiazol. In Fig. 8, no reductant is present. From the small extent of reduction of cytochrome *b* and cytochrome c_1 at different potentials (cf. Ref. 14), it seems that most of the reducing equivalents generated by the light-activated bacteriochlorophyll dimer cannot reach the cytochrome *c* reductase even if we assume that also the bound ubiquinone is partly reduced by the flash. In support of this conclusion, we observe a successive decrease of the reaction centre signal (P-870 oxidation) if the time interval between the flashes is shorter than 20 s in the presence of ascorbate, indicating that reducing equivalents remain in the reaction centre at the primary and secondary acceptor site after a flash and that dissipation of these equivalents needs tens of seconds. Since the saturation of the light used is only 20%, the observed 10% decrease of the reaction centre signal means that 50% of the reaction centres that are excited by a previous flash are still inactive after 20 s.

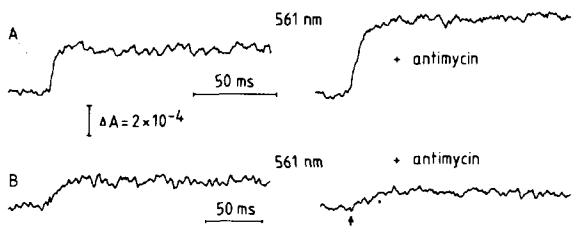

Fig. 8. Reduction of cytochrome *b* by light-activated reaction centres in the absence of ascorbate. (A) The conditions were similar to those in Fig. 7A but neither ascorbate nor phenazine methosulphate was present. In B 30 μM cytochrome *c* was added to establish a high potential in the system.

The electrons from the light-activated reaction centres apparently reduce cytochrome *b* in the reductase through two pathways only: one being sensitive to antimycin and the other to myxothiazol or the lack of oxidized cytochrome c_1. A direct reduction of cytochrome *b* by the reaction centres by-passing the antimycin-sensitive and myxothiazol-sensitive steps cannot be detected.

Discussion

The experimental results presented in this paper support the conclusion of Matsuura et al. [15,16] that despite differences in isoelectric points, horse heart cytochrome *c* is an equally effective electron donor to the flash-oxidized reaction centre as bacterial cytochrome c_2. The value for the second-order rate constant of the reduction of photo-oxidized reaction centres by cytochrome *c* is even higher than that calculated by Crofts et al. [13] for cytochrome c_2 in vivo, and is equal to the value reported by Prince et al. [23] for cytochrome c_2 and isolated reaction centres. In intact chromatophores there is a second phase of oxidation of cytochrome c_2, 100-times slower than the oxidation of free cytochrome c_2. According to Meinhardt and Crofts [28] this slow phase is not due to oxidation of bound cytochrome c_2, but to

oxidation of cytochrome c_1. However, although the measured absorbance change will be due to cytochrome c_1, the rate of oxidation will be determined by the oxidation of bound cytochrome c_2.

The reaction of cytochrome c_1 (in bovine heart ubiquinol : cytochrome c oxidoreductase) with oxidized reaction centres is 3 orders of magnitude slower than that of free cytochrome c (cf. Ref. 29). This difference is too large to be explained by the lower rate of diffusion of the reductase but may be due to an unfavourable charge at the site of interaction with the electron acceptor.

The finding that the rate of electron transfer from cytochrome c to the reaction centres is decreased by the presence of ubiquinol : cytochrome c oxidoreductase enabled us to determine the binding constant between the reduced reductase and reduced cytochrome c. The dissociation constant of 0.6 μM, calculated from our data, is significantly lower than the value of 10 μM determined by M.J. Tervoort (personal communication) at similar ionic strength for the dissociation constant of the complex between reduced cytochrome c and a preparation of isolated reduced cytochrome c_1 containing an acidic subunit [30,31] in substoicheiometric amounts. This difference indicates that the environment of cytochrome c_1 in the oxidoreductase influences its binding to cytochrome c.

The requirement for free cytochrome c in the reaction between cytochrome c and reaction centres extends the observations of Speck et al. [32] and König et al. [33], using cytochrome c oxidase as acceptor, that the site of interaction of cytochrome c with the electron acceptor is the same as that with its electron donor cytochrome c_1.

The observation that the rate of re-reduction of cytochrome c in the presence of myxothiazol-inhibited QH_2 : cytochrome c oxidoreductase depends linearly on the concentration of reductase shows that the rate of re-reduction of oxidized cytochrome c is not determined by electron transfer between the Rieske Fe-S cluster and cytochrome c_1, but by the rate of electron transfer between cytochrome c_1 and cytochrome c. The maximally measured first-order rate constant in Fig. 6 (1.2 ms^{-1}) is indeed smaller than the lower limit (2 ms^{-1}) for the reaction between the Fe-S cluster and cytochrome c_1, calculated by T'sai et al. [34]. The value obtained for the second-order rate constant ($2.1 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) for the reaction between cytochrome c_1 and cytochrome c is nearly 2 orders of magnitude higher than that reported by Crofts et al. [13] for the reaction between bacterial cytochrome c_1 and cytochrome c_2. Our value for the rate constant agrees with the observation that (in mitochondria) the rate of oxidation of cytochrome c_1 by cytochrome c is of the same order as the oxidation of cytochrome c by cytochrome c oxidase ($k = 2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at similar ionic strength [35]).

A comment has to be made concerning the conclusion from our data that the mid-point potential of the Rieske Fe-S cluster is of the same order as that of cytochrome c_1 and lower than that of cytochrome c. This conclusion has been drawn from the observation that after (partial) oxidation of cytochrome c by photo-oxidized reaction centres, cytochrome c is largely re-reduced while both cytochrome c_1 and Rieske Fe-S cluster become partly oxidized (in the presence of myxothiazol, see Fig. 5). When, however, the same experiment is performed at pH 7 instead of pH 8, hardly any oxidation of the Rieske Fe-S cluster is observed (not shown); at this pH only cytochrome c_1 becomes oxidized by cytochrome c. These findings can be explained by assuming that the pK of the oxidized Fe-S cluster is about 7.5 (cf. Ref. 5), since in this case the mid-point potential at pH 8 is about 30 mV lower than the value at pH $\leqslant$ 7.5 (260–280 mV [36,37]) and similar to the value of cytochrome c_1 (225 mV [38]). The conclusion that the (apparent) mid-point potential of cytochrome c is higher than that of cytochrome c_1 (probably by about 30 mV), although potentiometric titrations have not revealed such a difference [39], can be explained by the assumption that oxidized cytochrome c binds more strongly to reduced cytochrome c_1 than reduced cytochrome c. Such a stronger binding of the oxidized form of cytochrome c, resulting in an increase of 40 mV of the apparent mid-point potential, has indeed been observed by Tervoort (personal communication). Although generally a stronger binding of the oxidized form of a redox component to a ligand results in a lowering of its E_m by the ligand, in this case the ligand is the electron donor and the preferential

binding of the oxidized form to the electron donor results in a higher level of reduction, i.e., an increased E_m.

Matsuura and Dutton [16] have suggested that in the system containing bacterial reaction centres, bovine heart ubiquinol : cytochrome *c* oxidoreductase and horse heart cytochrome *c*, reduction of cytochrome *b* by the Q_B of the reaction centres is possible without involvement of the antimycin-sensitive or myxothiazol-sensitive steps. From our data with ascorbate-reduced oxidoreductase in the absence of cytochrome *c* or in the presence of both cytochrome *c* and myxothiazol – under these conditions cytochrome *b* cannot be reduced via centre o of the complex, the site where Q_ZH_2 reacts according to the model of Matsuura and Dutton [16] – it is clear that the reduction of cytochrome *b* is fully antimycin sensitive under these conditions. The Q_B of the reaction centres, therefore, cannot reduce cytochrome *b* except via one of the two pathways available in the oxidoreductase for ubiquinol. The component with E_m at pH 7.0 of 150 mV, called Q_y by Matsuura and Dutton, has to be the ubiquinone responsible for the reduction of cytochrome *b* at centre i (with an E_m of about 85 mV at pH 7.0) and for the antimycin-sensitive semiquinone [38]. The higher E_m, found by Matsuura and Dutton, could be due to the fact that this component is further reduced – after a flash – by Q_B. The finding of Matsuura and Dutton that antimycin slows down the reduction of cytochrome *b* is due to a full inhibition of the reduction via centre i (or Q_y) while a slow reduction still occurs via centre o (Q_ZH_2) where cytochrome c_1 is slowly oxidized by the reaction centres and re-reduced by Q_ZH_2, coupled to a reduction of cytochrome *b*. Whether Q_BH_2 can react directly with the Q-binding site of the reductase, by-passing the bound ubiquinone, or can dissociate from the reaction centre [13] cannot be determined.

The low level of reduction of cytochrome *b* in the absence of antimycin when cytochrome c_1 and the Fe-S cluster are reduced is partly due to the presence in the reductase of bound ubiquinone that accepts some of the reducing equivalents from the reaction centres. Another reason is that at increasing potentials more and more Q_B will be in the oxidized form while only the semiquinone form is reduced (after a flash) to the fully reduced form, the species that functions as reductant of the ubiquinone of the oxidoreductase. The finding that in intact chromatophores no antimycin-sensitive reduction of cytochrome *b* can be seen in the presence of myxothiazol [40,26] cannot be explained by the presence of a high concentration of ubiquinone relative to the situation in our system where only bound Q is present, since also at a high reduction level of ubiquinone after several flashes cytochrome *b* is not reduced in the presence of myxothiazol [26]. In the presence of antimycin, the reducing equivalents for reduction of cytochrome *b* and re-reduction of cytochrome *c* originate largely from the ubiquinol present. Our data do not give any evidence for the formation of a complex between reaction centre and reductase. The low level of flash-induced rapid reduction of cytochrome *b* does not suggest the presence of such a complex. It is possible, however, that the presence of detergent inhibits complex formation.

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