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Incorporation of reaction centers into submitochondrial particles resulting in light induced electron transfer

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SUMMARY - Conditions for the incorporation of reaction centers, isolated from *Rhodospirillum rubrum*, into submitochondrial particles have been studied. Incorporation of the reaction centers into the lipid bilayer occurs in both orientations. Electron flow from the light activated reaction center to the b-c_1 complex is demonstrated. Preliminary data on the reaction kinetics of the b cytochromes are given.

INTRODUCTION - Our knowledge of the structure and composition of electron transport chains in energy conserving systems has increased greatly in the past few years. It has been realized that striking similarities exist between respiratory electron transport components in mitochondria of animals, plants and fungi [1,2], and the ones of photosynthetic bacteria [3,4]. In all of them a membrane bound ubiquinol-cytochrome c oxidoreductase, the so called b-c_1 complex is present. The composition of this complex is similar in all organisms investigated so far. Purified preparations contain two b cytochromes, one cytochrome c_1, one Rieske type Fe-S center and 1-2 molecules of quinones associated with this complex [2,3]. Based on these structures different models for the electron transport through the complex have been proposed, they can be classified in three main groups: the linear model [5], the Q-cycles, originally proposed by Mitchell [6] and the B-cycles, proposed by Wikstrom and Krab [7].

From detailed studies of single electron turnover in the bacterial photosynthetic electron transport a modified Q-cycle has been suggested [8], which accounts for all of the oxidation-reduction reactions observed in this system. Progress in the understanding of the bacterial photosynthetic electron transport mechanisms is enhanced by the fact that the transport of single electrons can be readily induced by the use of a short flash activation of the system. By this way it became possible to introduce a known amount of reduced quinol into the system within a very short time (typically below one microsecond) and to study the consequent electron transport reactions with kinetic spectrophotometry.

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The electron transport through the mitochondrial ubiquinol cytochrome c oxidoreductase has been studied intensively [9-12]. However, due to experimental limitations, it has not been possible to follow this process with a similar time resolution as in chromatophores of photosynthetic bacteria. To overcome this limitation, a combination of isolated bacterial reaction centers with isolated mitochondrial b-c_1 complexes have been used [13, 14]. Since the quinone pool has an important role in the electron transfer between reaction centers and the b-c_1 complex [15, 16], the mitochondrial b-c_1 complex should be studied in a system which allows to control the concentration of quinones. We have therefore investigated the incorporation of isolated bacterial reaction centers into submitochondrial particles. This allows the restoration of a functional cyclic electron transfer chain consisting of the bacterial reaction center and the mitochondrial b-c_1 complex. In this system it has become possible to study the reaction mechanism of the mitochondrial ubiquinol-cytochrome c oxidoreductase in a similar way as the one in the bacterial photosynthetic membrane.

MATERIALS AND METHODS - Reaction centers were prepared from the carotinoidless mutant G-9 of *R. rubrum* according to the method of Snozzi and Bachofen [17]. The pure reaction centers were concentrated to about 5 mg protein/ml by ultrafiltration and dialyzed for at least 24 h against 10 mM Tris-HCl buffer, pH 7.5. The protein content was measured from the absorption at 802 nm using the relation of absorption to protein reported by Steiner et al. [18].

Heavy mitochondria were prepared from bovine heart using the method of Azzone et al. [19]. The final pellets were suspended in 0.25 M sucrose, 10 mM Tris-HCl pH 7.5 to a concentration of 20 mg protein per ml. Protein concentrations of mitochondrial membranes were measured using the Coomassie Blue reagent of Bio-Rad and BSA as protein standard. Sub'%mito'%chondrial particles were produced either by sonication (3 times 1 min.) or by treatment with the French press. Sonication gave about 70 % vesicle with inside-out orientation, whereas the French press yielded more right-side out membranes. The membrane vesicles were first separated from the outer membrane by centrifugation on a sucrose gradient (15 - 40 %) for 2 h at 4° C and 160 000 g. The turbid band of vesicles of the gradient was diluted with Tris buffer and the membranes sedimented by centrifugation. The resulting pellet was suspended in Tris buffer to a final concentration of 10 mg protein per ml and stored frozen at -20 ° C.

This vesicle preparation had no monoaminoxidase activity and was thus free of any contamination by the outer mitochondrial membrane. The orientation of the particles was measured by the antimycin A sensitive ferricyanide reduction by succinate [20]. Separation of the two orientations of the submitochondrial particles could be obtained by the use of a cytochrome c thiol sepharose 4B column prepared according to Bill et al. [21]. Inside-out particles (iso SMP) did not bind to the column and were eluted with Tris buffer, whereas the right-side out particles (rso SMP) eluted only in the presence of 0.2 M NaCl (fig. 1). This chromatographic separation resulted in highly uniform membrane preparations.

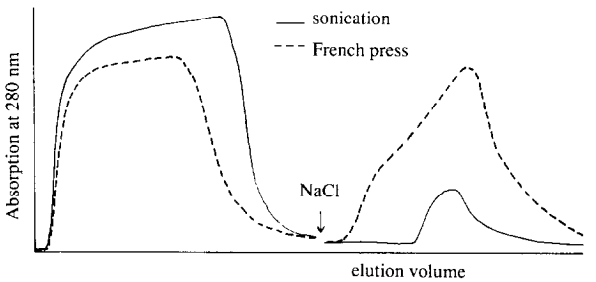


Fig. 1: Purification of submitochondrial particles by affinity chromatography on cyt c - thiol Sepharose 4B. Column (1.5 x 10 cm) prepared according to (17) was equilibrated with 10 mM Tris-HCl buffer pH 7.2. 30 ml of membrane suspension (2 mg protein /ml) were loaded on the column and washed at a flow rate of 0.5 ml/min. After a first fraction the column was eluted with 0.2 M NaCl in buffer.

The cytochrome content of the membranes was determined by redox-spectra as in [19]. For iso SMP a ratio of 1 cyt aa_3 : 2 cyt b : 1 cyt c_1 : 3 cyt c was measured, whereas rso SMP gave values of 2 cyt aa_3 : 2 cyt b : 1 cyt c_1 : 0.5 cyt c.

Potentiometric titrations of absorbance changes were done in the presence of mediators and ionophores as described in reference [8] and [22].

RESULTS AND DISCUSSION

Incorporation of reaction centers - Reaction centers and submitochondrial membranes were mixed in the dark at 4°C and kept stirred for 20 to 60 minutes. The incorporation was accomplished within 20 minutes and no additional change in the amount of reaction centers associated with membranes could be observed with a prolonged incubation time. The incubation mixture was separated on a sucrose gradient (15-40%, 160'000 g, 2 hours). Free reaction centers banded near the top of the gradient, unchanged submitochondrial membranes towards the bottom, whereas mitochondrial vesicles with incorporated reaction centers were recovered as a blue colored sediment.

No difference with respect to incorporation was observed between crude submitochondrial membrane preparations and the purified rso SMP or iso SMP fractions. In the pH range between 7 and 8.5 no influence of proton concentration on the amount of RC incorporated was detectable. Also the type of buffer used did not interfere with the incorporation of the RC, similar incorporation rates were obtained with 10 mM Tris-HCl, 10 mM Mops, 10 mM Hepes and 10 mM phosphate buffer.

The amount of incorporation of the added RC was furthermore largely unaffected by the ratio of mitochondrial membrane protein to reaction center protein used in the incubation mixture. The amount of reaction centers which can be incorporated into the inner mitochondrial membrane is apparently limited by the amount of detergent (LDAO) associated with the isolated reaction centers. It was therefore important to dialyse the reaction centers after purification against buffer to remove LDAO as much as possible. Using well dialysed RC, preparations of membranes showing a 1:2 ratio of reaction center protein to mitochondrial protein could be obtained which were still closed membrane vesicles, as judged from their content of cytochrome c. A 1:1 ratio in the incubation mixture led to a change in density on the sucrose gradient. The blue colored band was found at a lower density and showed less light scattering. Furthermore the amount of c cytochromes measured by redox differential spectra had decreased. This is interpreted as a partial solubilisation of the mitochondrial membrane due to the high amount of LDAO associated with the added reaction centers.

For optimal incorporation of reaction centers into the mitochondrial membrane vesicles, the presence of divalent cations, preferably Ca^{2+}, was obligatory (Table 1). With 5 mM Ca^{2+} complete incorporation of the reaction centers present in the incubation mixture was achieved within 60 min. With 20 mM Mg^{2+} only about 80% of the reaction centers were incorporated and with 20 mM Na^{+}, although stimulating incorporation compared to the absence of cations, gave an even lower incorporation.

It is assumed that ions are necessary to compensate the repulsion of the negatively charged membrane and reaction center proteins. Divalent cations are able to form bridges between the two and lead to an attachment of the reaction center to the membrane. Later the

Table I: Extent of incorporation of bacterial reaction centers into submitochondrial membranes. Reaction centers and mitochondrial membranes were mixed at a protein ratio of 1:4 and stirred for 60 Min. at 4°C in the dark. The cations shown in the table were added at the same time as the reaction centers to the final concentration indicated, unless otherwise stated.

Addition	% of incorporation
0	0-5
1 mM Ca^{2+}	70
5 mM Ca^{2+}	100
20 mM Ca^{2+}	100
20 mM Mg^{2+}	80
20 mM Na^{+}	40
5 mM Ca^{2+} + 20 mM EDTA	10-15
5 mM Ca^{2+}, after 30 min addition of 20 mM EDTA	100
5 mM Ca^{2+}, reaction centers added after 30 min	20-30

incorporation of the hydrophobic part of the reaction centers into the lipid bilayer seems to occur spontaneously. This sequence of incorporation is supported by the observation that addition of Calcium ions 30 min. prior to the addition of reaction centers resulted in some aggregation of the membranes but poor incorporation. When small amounts of Ca^{2+} were added simultaneously with 20 mM EDTA and reaction centers, again only a minor part of the protein complexes was incorporated. If, on the other hand the same amount of EDTA was added after 30 min of incubation of the membrane and reaction center mixture with Ca^{2+}, no decrease in incorporation was seen. This indicates, that the method used leads to a true incorporation of the reaction centers into the lipid bilayer of the membrane and not only to an association or aggregation of the bacterial protein to the mitochondrial membrane surface. Furthermore this is confirmed by the fact, that the reaction centers cannot be separated from the SMP on sucrose gradients containing 20 mM EDTA or 1 M sodium chloride.

Incorporation of the reaction centers is also demonstrated by the oxidation kinetics of the P865 with ferricyanide (figure 2). Free reaction centers show an immediate oxidation upon addition of ferricyanide, which is not resolved in time on the spectrophotometer used. Reaction centers incorporated in SMP vesicles in contrast show a clear biphasic oxidation with a fast phase followed by a slow phase. The fast phase is attributed to reaction centers having their reaction site exposed at the outside of the vesicles. This kinetic would also be expected for reaction centers only adsorbed on the surface of the membrane, but since it was not possible to remove the RC with EDTA or high salt treatment, it is concluded that this decrease in absorbance at 865 nm originates from incorporated complexes only. The slower phase was due to those reaction centers having their reaction site for ferricyanide inside the vesicle. The oxidation rate would then reflect the permeation of ferricyanide into the submitochondrial vesicles. This is supported by the fact, that addition of small amounts of detergent abolished this slow oxidation phase. Similar kinetics are also obtained using reaction centers incorporated into liposomes by the method of Meyer et. al. [23]. These authors have shown by freeze fracture micrographs that the reaction centers actually form particles within the lipid bilayer.

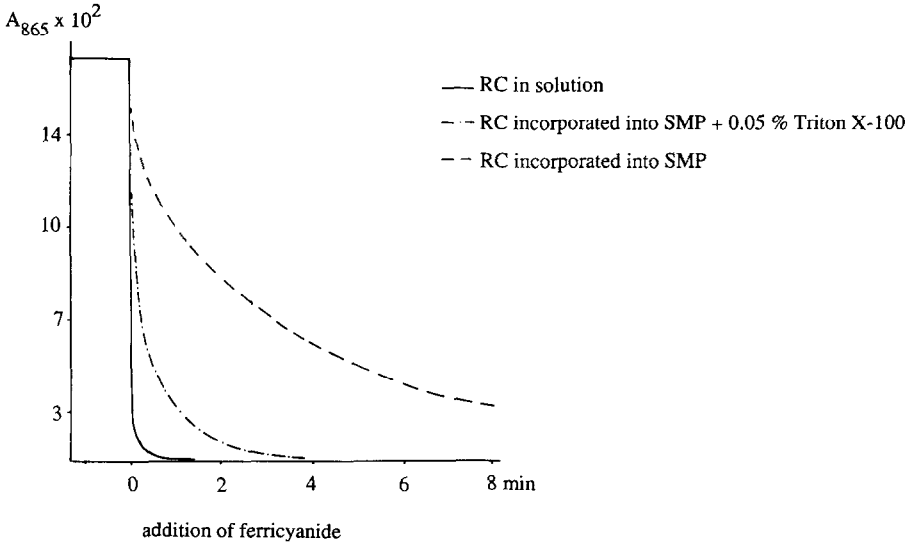
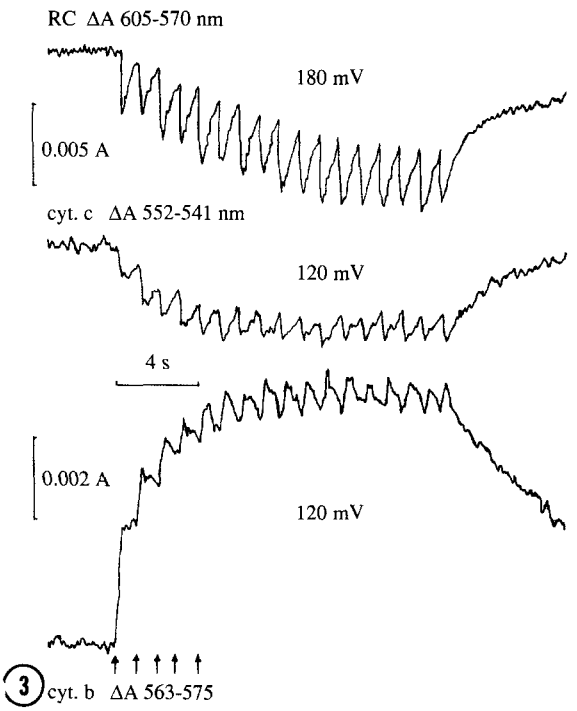


Fig. 2: Oxidation of bacterial reaction centers by ferricyanide. The reaction medium was oxidized by addition of 1 M ferricyanide to a final concentration of 2 mM.

Electron transport between reaction centers and mitochondrial components. - A reconstitution of cyclic electron flow through reaction centers and the mitochondrial b-c_1 complex seems possible when several requirements are fulfilled. First the reaction centers have to be incorporated in the membrane in a way that QH_2 produced by RC in the light reacts with the quinone pool of the membrane. Second the reaction centers must have the same transmembrane orientation as the b-c_1 complex with respect to the cytochrom c reaction site. A higher rate of electron transport from the b-c_1 complex to the reaction center through cyt c is expected in the case when the orientation of both cyt c reaction sites are on the inside of the vesicles. Diffusion distances between the two membrane bound complexes then become minimal. Furthermore the concentration of the carrier cytochrome c in the vesicles must be high as in native chromatophores or SMP. Although determinations according to [19] gave values of 3 cytochrome c per cytochrome c_1 in our iso SMP preparations the flash induced electron transport observed in our reconstituted system suggested some limitations due to restricted re-reduction of the reaction centers by cytochrome c. In our experimental system it was rather difficult to obtain saturating flash intensities. With a homebuilt Xenon flash we were not able to activate more than 70 % of the reaction centers present with one flash at the reaction center concentrations usually used.

Figure 3 shows the redox changes obtained with trains of flashes in a preparation of reaction centers incorporated into iso-SMP in the presence of antimycin A. The signal at 605-570 nm displays the oxidation and re-reduction of the bacterial reaction center chlorophyll P_{860} upon a series of flashes. About 40% of the change after the first flash is immediately reversed indicating fast re-reduction of $P860^+$ by reduced cytochrome c. The second trace (552-541 nm) shows the concomittant oxidation of cytochrome c reaching a steady state after about 5 flashes. The trace 563-577 nm is mainly the signal of the reduction of the b cytochromes. As with cytochrome c reduction of cyt b reaches saturation after the fifth flash. The two latter traces in



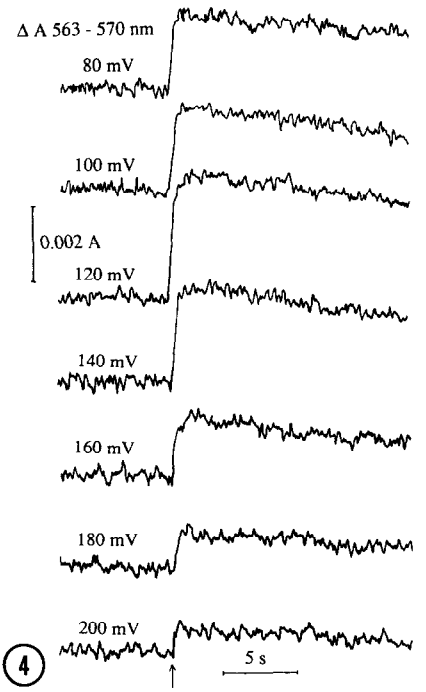


Fig. 3: Absorption changes of reaction center containing mitochondrial vesicles induced by a train of flashes in the presence of antimycin A (10 mM). Intervals between flashes 1 sec.

Fig. 4: Kinetics of reduction of cytochrome b after a single flash at different ambient redox potentials in the presence of antimycin A.

fig. 3 have been taken at an ambient redox potential of 120 mV, where part of the quinone pool is reduced before the experiment to increase the extent of reactions.

From the predictions of the Q-cycle model it is expected that the flash-induced reduction rate of cyt b is dependent on the redox potential [8]. Although the time resolution of the traces in figure 4 is rather poor, it is obvious that lower redox potentials have a greater effect on the extent of reduction after one flash than on the reduction rate. The increase in reduction of the cytochrome b in the potential range between 180 and 120 mV is probably related to a chemical reduction of part of the quinone pool. As has been demonstrated in chromatophores [15] each flash produced two positive charges and one QH_2 per pair of reaction center P860. Each positive charge is able to oxidize one cytochrome c_1 of the b-c_1 complexes mediated by cytochrome c. On the other hand one QH_2 can only interact with one complex. Additional QH_2 in the pool is therefore able to increase the amount of cytochrome b reduced in the membrane. Preliminary measurements at 446 nm indicate that a substantial fraction of the reaction center displays an oscillation with flash number in the redox state of the secondary quinone Q_B as has been observed in chromatophores at a low concentration of cytochrome c_2 [24]. This oscillation would decrease the yield of QH_2 on the first flash without effecting the positive charges. The reduction of cytochrome b would thus depend even more on the presence of chemically reduced quinol in the membrane pool.

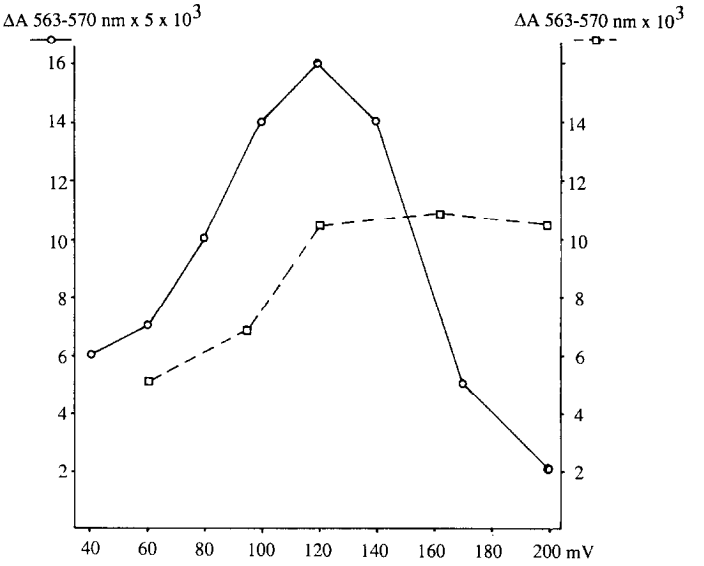


Fig. 5: Amount of cytochrome b reduction after one single flash and maximal reduction after 5 flashes at different ambient redox potentials in the presence of antimycin A.

The decrease of the extent of reduction of cyt b at potentials below 120 mV is related to the chemical reduction of the high potential cytochrome b of the b - c_1 complex at these potentials. This is obvious from Fig. 5, where the total extent of cyt b reduction is compared with the one after the first flash. Below 120 mV both curves decrease similarely.

The maximal extent of light induced reduction of cyt b in the presence of Antimycin A at potentials above 120 mV is rather constant similar to the measurements with chromatophores.

The results reported here show clearly the possibility to pump electrons through the mitochondrial b and c cytochromes by bacterial reaction centers incorporated into the mitochondrial membrane. A higher resolution of the kinetics of the different electron transfers obtained with more sophisticated equipment may give definite information on the reaction sequence of the mitochondrial ubiquinol: cytochrome c oxidoreductase.

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