*Biochimica et Biophysica Acta* 809 (1985) 260–270
Elsevier

BBA 41836

# Kinetics of the *c*-cytochromes in chromatophores from *Rhodopseudomonas sphaeroides* as a function of the concentration of cytochrome $c_2$. Influence of this concentration on the oscillation of the secondary acceptor of the reaction centers $Q_B$

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(Received April 9th, 1985)

Key words: Electron transport; Cytochrome *c*; Bacterial photosynthesis; Reaction center; Redox potential; (*Rps. sphaeroides*)

**The oxidation kinetics of Cyt $c_1$ and $c_2$ have been measured in normal chromatophores and in chromatophores fused with liposomes in order to increase the internal volume. The kinetics of Cyt $c_1$ oxidation were found to be dependent on Cyt $c_2$ concentration. The initial rate of Cyt $c_1$ oxidation decreased after fusion by a factor of about two, indicating a process dependent on diffusion. The results do not allow a clear distinction between a diffusion of Cyt $c_2$ along the inner membrane surface or through the inner volume of the vesicle; two- and three-dimensional models are discussed. In contrast to Cyt $c_1$, the kinetics of oxidation of Cyt $c_2$ were not influenced by changes in concentration. It is concluded that reduced Cyt $c_2$ is preferentially bound to the reaction centers. A binary pattern as a function of flash number from the dark-adapted state was measured in the turn-over of the two-electron gate of the reaction center. In chromatophores with more than 0.5 cytochrome $c_2$ molecules per reaction center, this binary pattern titrated out with a midpoint around 340 mV on reduction of the suspension. In experiments with chromatophores with a low Cyt $c_2$ content, or with spheroplast-derived vesicles which had lost Cyt $c_2$, the binary oscillation in the two-electron gate could be observed at much lower potentials. The results suggest that the binding of reduced cytochrome $c_2$ modifies the behavior of the two-electron gate. A model in which reaction center dimers are stabilized by Cyt $c_2$ is proposed to explain the effect.**

## Introduction

The involvement of the water-soluble cytochrome $c_2$ in the cyclic electron transport of photosynthetic bacteria has been known for a long time [1–3]. Bowyer et al. [4] showed that approx. one *c*-type cytochrome per reaction center was involved in the cyclic electron transfer. They confirmed earlier findings [1,2] which showed two phases of oxidation of *c*-cytochromes of roughly equal magnitudes. Based on these kinetics, and the fact that the in vivo spectrum of the *c* cytochromes was shifted to the red compared to isolated cytochrome $c_2$ [5,6] Bowyer and Crofts [7] concluded that there were at least two different populations of cytochrome *c* in the chromatophore mem-

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Abbreviations: MOPS, 4-morpholinopropanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; P-860, reaction center primary donor; $Q_B$, secondary quinone acceptor of the reaction center; Cyt, cytochrome; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

branes. Wood [8,9] was able to show the presence of a membrane-bound cytochrome $c_1$ in addition to the soluble cytochrome $c_2$ in chromatophores. There was a 2–2.5 nm difference in the absorption maximum of the alpha band (552 ± 0.5 nm for Cyt $c_1$, 550 ± 0.5 nm for Cyt $c_2$) as well as a difference in molecular weight (30 kDa for Cyt $c_1$, 14.1 kDa for Cyt $c_2$). Wood also suggested that cytochrome $c_2$ is the electron donor for the oxidized reaction center and becomes rereduced by cytochrome $c_1$. Meinhardt and Crofts [10] confirmed this sequence and gave evidence that the fast phase of cytochrome $c$ oxidation is due to cytochrome $c_2$, whereas the slower phase can be attributed to cytochrome $c_1$. On this new background the kinetics of the two cytochromes have been reinvestigated with special interest in the variations caused by changes in the concentration of cytochrome $c_2$. Changes in the concentration of cytochrome $c_2$ were obtained either by taking advantage of the variability in yield of this cytochrome in the chromatophore vesicles during normal preparation, by preparing vesicles from spheroplasts from which cytochrome $c_2$ was removed by washing, or by fusion of chromatophores with liposomes and consequent dilution of Cyt $c_2$ in the increased internal aqueous volume.

In the second part of this paper, the influence of the concentration of cytochrome $c_2$ on the oscillation with flash number of the semiquinone form of the secondary acceptor ($Q_B^-$) of the reaction center is discussed. It has been shown [11,12] that isolated reaction centers display an oscillation in the redox states of the secondary acceptor $Q_B$, with the acceptor being reduced after the first flash to the semiquinone anion, giving rise to an absorption at 446 nm. This absorption disappears after the second flash, which reduces the semiquinone to the fully reduced quinol. A similar oscillation has been shown in chromatophores by De Grooth et al. [13], by Bowyer et al. [4] and by O'Keefe et al. [14]; but in the case of chromatophores the oscillation of $Q_B^-$ was only observed at high ambient redoxpotential. In the experiments of Bowyer et al. [4], the oscillations titrated out with a midpoint potential of about 340 mV on reduction of the suspension. Since this corresponds roughly to the midpoint potential of cytochrome $c_2$ the influence of this component on the titration of the oscillations was also studied.

## Materials and Methods

Cells of *Rhodospeudomonas sphaeroides* GA were grown for about 18–20 h at high light intensity using the medium of Sistrom [15]. Chromatophores were prepared as previously described [4,16]. Spheroplast-derived vesicles were prepared by the method of Takemoto and Bachmann [17] which was slightly modified as described in Ref. 18. Fusion of chromatophores with liposomes was done by a modification of the procedure by Schneider et al. [19] as described in Ref 16. Kinetic measurements and redox titrations were carried out as in Ref. [4], except when a large number of traces was averaged. In this case a flow system providing a fresh dark-adapted sample for each kinetic measurement was used [20]. For activation, a xenon flash with a duration of about 3.5 μs at half-maximal amplitude [21] was used. The reaction center concentration in the cuvette was adjusted to give a saturation of 90–94% for the flash [21].

Cytochrome $c_2$ was isolated from the supernatant remaining after the first ultracentrifuge spin of the chromatophore preparation procedure using a modification of the method of Bartsch [22]. The supernatant was first fractionated by ammonium sulphate precipitation (60, 80, 100% saturation). The Cyt $c_2$ which remained in solution at 100% saturation was collected by addition of DEAE-cellulose to the solution and filtering through a Buchner funnel. Cyt $c_2$ was eluted with 50 mM MOPS buffer (pH 7.0) containing 100 mM KCl. This fraction was combined with the precipitate obtained between 80 and 100% saturation of ammonium sulphate. After dialysis the cytochrome was further purified on a DEAE-cellulose column. A step gradient of sodium chloride (0, 20, 40 mM) in 20 mM Tris-HCl (pH 7.3), was used to elute the pure Cyt $c_2$. It was usually concentrated on a small DEAE-cellulose column and dialysed against 50 mM MOPS buffer containing 100 mM KCl. The concentrated pure Cyt $c_2$ was stored at −20°C.

*Variation in the cytochrome c content of vesicles*

During the normal preparative procedure the membrane vesicles always loose some Cyt $c_2$ so that the isolated chromatophores usually contained between 0.8 and 1.0 total cytochrome $c$

(Cyt $c_1$ and Cyt $c_2$) per reaction center as measured by flash oxidation at ambient potentials around 200 mV. In those preparations four flashes with 32 ms between the flashes led to maximal oxidation of the *c* cytochromes in the presence of antimycin A. This amount of cytochrome *c* was taken to represent the total cytochrome ($c_1 + c_2$) available for cyclic electron transport. The determination of the *c* cytochromes by a redox titration in the dark usually gave values a little higher than those obtained by flash photooxidation as above. In some preparations the concentration of the *c* cytochromes as determined by flash oxidation was as low as 0.5 cytochromes per reaction center. In these cases, the determination of total cytochrome *c* by titration in the dark gave significantly higher values. It is therefore assumed that in this case only part of the Cyt $c_1$ was oxidized by the flash regime used. In these Cyt $c_2$-depleted preparations, when the dark time between the flashes was increased, the amount of *c* cytochrome oxidized also increased, but when the concentrations of *c* cytochromes were very low, the concentration values obtained by dark titration were always somewhat higher than the ones obtained by flash oxidation even with very long dark-times between flashes and more than four flashes in a train. The higher values for concentration of cytochrome ($c_1 + c_2$) when measured by redox titration rather than by the flash assay probably reflect a population of ubiquinol : cytochrome $c_2$ oxidoreductase complexes which are not linked to the photosynthetic apparatus. A similar discrepancy was observed when assaying Cyt *b*–561 concentration; the ratio of values for Cyt *b*-561 found by the two methods was roughly equal to the ratio of total Cyt *c* found using the two methods. This was also true in spheroplast-derived vesicles where only Cyt $c_1$ was present. The ratio of amounts found by titration and flash assays varied between 1 and 2.5 in different preparations, and we are presently investigating the effect more fully. In the present work, when concentrations of cytochrome ($c_1 + c_2$) or of cyt *b*-561 are discussed, the values refer to those assayed by the flash method, which are unambiguously kinetically competent in the cyclic chain.

It is not completely clear which factors control the amount of Cyt $c_2$ lost during preparation of the chromatophores. We found that the yield of Cyt $c_2$ in the membrane vesicles was higher when a dense suspension of cells was used in the French Press. Chromatophores from cells harvested at the end of growth had usually a higher Cyt $c_2$ content than those prepared from cells harvested in their early logarithmic-growth phase.

## Results and Discussion

*Oxidation kinetics of the cytochromes $c_1$ and $c_2$*

Fig. 1 shows the oxidation kinetics of Cyt $c_1$ in the presence of UHDBT, which inhibits the rereduction by the Rieske-type FeS center [4,23]. Cyt $c_1$ was measured at 552–548 nm as described by Meinhardt and Crofts [10]. The chromatophore preparation with a high concentration of Cyt $c_2$ (0.96 *c*-type cytochromes per reaction center, trace a) displays an initial oxidation rate of 2.0 Cyt $c_1$ per reaction center per ms. This rate fell to 0.91 Cyt $c_1$ per reaction center per $ms^{-1}$ in membranes contained only about 0.65 total Cyt *c* per reaction center (as measured by flash oxidation, trace b). This is a clear indication that the oxidation of Cyt $c_1$ by Cyt $c_2$ depends on the concentration of the two reactants and is therefore a diffusion coupled second order reaction.

Both chromatophore preparations used for the experiments in Fig. 1 (traces a and b) were fused with liposomes which increased their phospholipid content by a factor of about 2.5. Using the assumptions for the size of a chromatophore, its content of reaction centers and the percentage of membrane volume occupied by the lipids as discussed in a previous paper [16], it can be calculated that such a fusion would lead to an increase of the inner membrane surface area by a factor of 1.9, whereas the inner volume is increased by a factor of 2.6. The determination of the *c* cytochrome content of the fused chromatophores showed an effect similar to that described for membrane vesicle preparations with a low yield of Cyt $c_2$. Dark titrations gave Cyt $c_2$ per reaction center values which corresponded to the ones before fusion, whereas the measurement of total Cyt *c* by flash oxidation showed an apparent loss between 8 and 35%. In addition, there was a shift in the midpoint potential of Cyt $c_1$ after fusion.

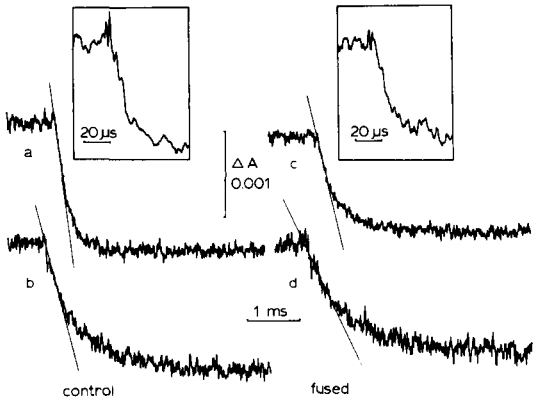


Fig. 1. Oxidation kinetics of cytochromes $c_1$ and $c_2$. The traces were taken at an ambient redox potential of $170 \pm 5$ mV in the presence of the inhibitors antimycin A (10 µM) and UHDBT (60 µM) to prevent any rereduction of the *c* cytochromes by the cyclic electron transport. Cyt $c_1$ was measured at 552–548 nm, Cyt $c_2$ at 551–542 nm using the flow system described in Ref. 18. Instrument response time was 10 µs for Cyt $c_1$, and about 0.5 µs for Cyt $c_2$. The traces have been normalized to a reaction-center concentration of 0.29 µM. The following compounds were present as mediators: 1 µM each of phenazine methosulphate, phenazine ethosulphate and pyocyanin; 10 µM each of 1,2 naphthoquinone, 1,4 naphthoquinone, duroquinone, *p*-benzoquinone; 2 µM DAD. 20 mg/ml gramicidin was added immediately to collapse the membrane potential. (a) Oxidation of Cyt $c_1$ in chromatophores with about 0.96 total Cyt *c* per reaction center as measured by flash oxidation (see text). The trace is the average of 64 measurements, initial rate of oxidation is 2.0 Cyt $c_1$ per reaction center and ms. (b) Oxidation of Cyt $c_1$ in chromatophores with about 0.65 total Cyt *c* per reaction center. The trace is the average of 64 measurements, initial rate is 0.91 Cyt $c_1$ per reaction center per ms. (c) Oxidation of Cyt $c_1$ as in (a) but measured after fusion of the chromatophores with liposomes to increase the phospholipids by a factor of 2.5. The trace is the average of 128 measurements, the initial oxidation rate is 0.93 Cyt $c_1$ per reaction center per ms. (d) Oxidation of Cyt $c_1$ as in (b) but measured after fusion of the chromatophores with liposomes to increase the phospholipids by a factor of 2.4. The trace is the average of 128 measurements, the initial oxidation rate is 0.46 Cyt $c_1$ per reaction center per ms. The inserts show the initial rate of total Cyt *c* oxidation measured at 551–542 nm, which can be entirely attributed to Cyt $c_2$ within the first 30 µs (left side: preparation (a), average of 256, right side: preparation (c), average of 512).

Computer analysis of titrations in the dark gave midpoint potentials between 270 and 290 mV for Cyt $c_1$ instaed of the 260 mV normally found in chromatophores [10]. A similar shift was found by Wood [8] and by Meinhardt [18] in spheroplasts or spheroplast-derived vesicles and by Gabellini et al. [24] for the cytochrome $c_1$ in the isolated *b*-$c_1$ complex. The reason for this shift is not known, but there might be an influence of the cytochrome $c_2$ concentration.

For membranes with a high content of cyt $c_2$, the oxidation rate of Cyt $c_1$ was decreased after fusion by a factor of 2.0–2.2 (Fig. 1, trace c). Slightly smaller values (1.9–2.1) were measured for those preparations which had a low *c*-cytochrome content from the beginning (Fig. 1, trace d). Assuming a model in which oxidized Cyt $c_2$ is released from the reaction center and reaches its reaction site with Cyt $c_1$ by diffusion, the slower rates in liposome-fused chromatophores are as expected. The values do not allow us to distinguish between a diffusion of Cyt $c_2$ on the inner surface of the chromatophore membrane [25,26] or diffusion through the inner volume, since they are higher than the factor expected for a two-dimensional diffusion, but not large enough to correlate with the theoretical value for a three-dimensional model. Yet, the two-dimensional diffusion seems to be more likely, since the amount of flash oxidizible Cyt $c_1$ is decreased after fusion, which would lead to an overestimation of the decrease in the initial oxidation rate. It should also be noted, that traces b and d in Fig. 1 show a small transient at the time of the flash. This jump was always observed in chromatophores with a low Cyt $c_2$ content, and is probably due to the fact that the wavelength pair used (552–548 nm) did not compensate exactly for the reaction center absorbance change. The presence of this jump, however, makes the measurement of the initial oxidation rate ambiguous, and it cannot be ruled out that this results in an overestimation of the oxidation rate of Cyt $c_1$ after fusion.

The insets of Fig. 1 show the initial kinetics of oxidation of Cyt $(c_1 + c_2)$ in normal and fused membranes with a high content of Cyt 2 (preparations a and c). In the range below 30 µs, these kinetics reflect only Cyt $c_2$ oxidation [10]. Since the oxidation kinetics shown in the insets are not affected by fusion, which would be expected to decrease the concentration of unbound Cyt $c_2$ by a factor of 1.9 (two-dimensional diffusion model) or 2.6 (three-dimensional diffusion model), this process must be first-order. From this it can be concluded that the reduced Cyt $c_2$ must be preferentially bound to the reaction center, so that

changes in the volume or surface area of the chromatophores do not affect its rate of oxidation. This is somewhat in contrast to findings by Wraight and co-workers [25–27] who had observed that isolated reaction centers as well as reaction centers reconstituted in phospholipid vesicles showed mainly a second order reaction with Cyt $c_2$ in the presence of 100 mM KCl (as used in the experiments described above). This difference might be due to the relatively high concentration of Cyt $c_2$ normally present in chromatophores as is discussed in more detail later.

From the results reported so far we conclude that reduced Cyt $c_2$ in chromatophores is preferentially bound to reaction centers. It can rereduce flash oxidized P-860 in a first-order reaction with a half-time around 3 $\mu$s. After dissociated from the reaction center, Cyt $c_2$ can react in a second-order process with the Cyt $c_1$ of the ubiquinol : cytochrome $c_2$ oxidoreductase (the $b$-$c_1$ complex). This model is in agreement with results by Vermeglio et al. [28,29] who demonstrated that oxygen uptake by intact cells of photosynthetic bacteria is inhibited by flash or continuous light, probably due to a preferential oxidation of the reduced Cyt $c_2$ by the reaction centers. The results also show the importance of the concentration of Cyt $c_2$ in the chromatophores for the turnover of cyclic electron flow. Since the oxidation of the Rieske-type iron sulfur center initiates the oxidation of ubiquinol at the quinol binding site of the $b$-$c_1$ complex, and oxidation of the FeS center occurs through Cyt $c_2$ via Cyt $c_1$ [21], it is quite clear that a low concentration of Cyt $c_2$ can limit the turnover of the quinol oxidation site. Even in native chromatophores with a full complement of Cyt $c_2$, the turnover of the quinol oxidase site is limited by the rate of oxidation of the FeS center under conditions in which the ubiquinol pool is sufficiently reduced to saturate the second order reaction between $QH_2$ and the $UQH_2$ : Cyt $c_2$ oxidoreductase complex [16,21].

Although the experiments reported here show clearly that Cyt $c_2$ is oxidized by a process independent of local concentration (and therefore presumably first-order), and that Cyt $c_1$ oxidation depends on Cyt $c_2$ concentration (and is therefore presumably second-order), it should be noted that the latter process occurs with an apparent second-order rate constant (about $10^7$ $M^{-1} \cdot s^{-1}$ [16]) which is slower by more than an order of magnitude than the rate constant measured for reduction of Cyt $c$ by Cyt $c_1$ in the mitochondrial ubiquinone: cytochrome $c$ oxidoreductase complex [30]. This may represent a species difference, or as suggested by Zhu et al. [30], it may alternatively indicate that the oxidized Cyt $c_2$ is also relatively tightly bound to the reaction center [31], so that its kinetic concentration is much lower than that assumed in the previous calculation [16]. This latter explanation is more consistent with the fact that the measured $E_m$ for Cyt $c_2$ in situ is similar to that for the isolated cytochrome in solution, indicating no strong preferential binding of the reduced over the oxidized form.

*Influence of cytochrome $c_2$ on the oscillations of the secondary acceptor $Q_B$ of the reaction centers*

Fig. 2 shows characteristic traces for cyt $b$-561 reduction, total cyt $c$ oxidation, oxidation of P-860 and the absorption changes at 446 nm obtained with two chromatophore preparations which differ in their content of Cyt $c_2$. Fig. 2A shows chromatophores with high content of Cyt $c_2$. The top row in Fig. 2A shows a characterization of the chromatophores. Flash-reducible Cyt $b$-561 and total flash-oxidizible Cyt $c$ were measured in the presence of antimycin A with four flashes spaced at 32 ms. The last trace in the top row is the oxidation of the reaction centers, measured with eight flashes. From these traces the amount of Cyt $b$-561 was calculated to be 0.54 per reaction center. The amount of Cyt $c$ per reaction center was 1.03, with approx. 0.54 Cyt $c_1$ and 0.49 Cyt $c_2$. With such a high content of Cyt $c_2$ the reaction center trace showed an almost complete rereduction of $P^+$-860 within a few ms of the first flash.

Although these chromatophores showed a pronounced oscillation in the extent of Cyt $b$-561 reduction and in the absorbance change at 446 nm at high ambient redox potential (more than 350 mV) as previously observed [4], they did not show any oscillations at 446 nm when poised at an ambient potential below 300 mV (Fig. 2A, second row). In addition, at these lower $E_h$ values the reduction of Cyt $b$-561 was maximal after the first flash and decreased with flash number as shown in the bottom traces of Fig. 2A. Fig. 3 shows the

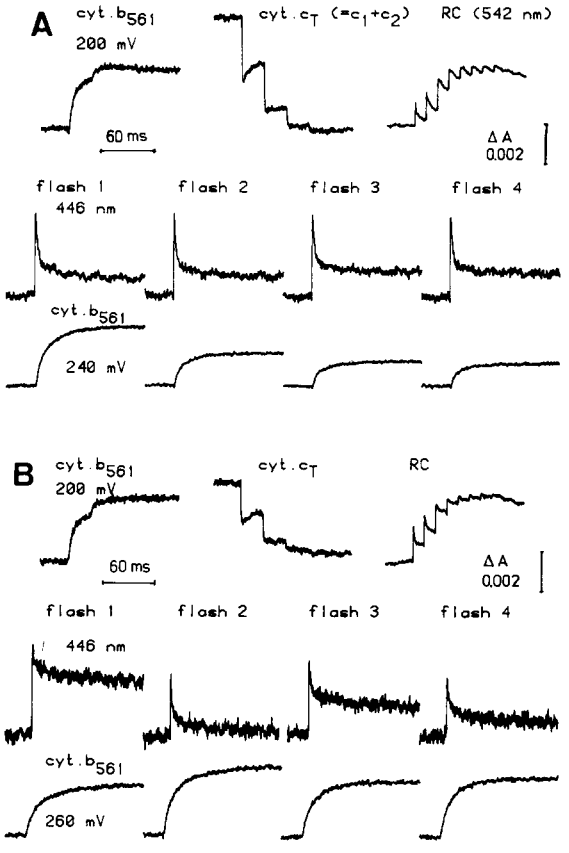


Fig. 2. Changes in the redox state of electron-transport components after flash activation of chromatophores. Chromatophores were suspended in 50 mM MOPS buffer (pH 7.0) containing 100 mM KCl to a reaction-center concentration of 0.29 μM (Fig. 2A) and 0.31 μM (Fig. 2B), respecitvely, in the presence of 10 μM antimycin A. Part A: a preparation with high Cyt *c* concentration (about 1.03 Cyt *c* per reaction center); Part B: preparation with extremely low Cyt *c* content (about 0.53 Cyt *c* per reaction center). Top rows: total reduction of Cyt *b*-561 (561-569 nm), total oxidation of the *c* cytochromes (measured at 551–542 nm with four flashes 32 ms apart, average of 2) and total oxidation of the reaction centers (measured at 542 nm with eight flashes, 16 ms apart, average of 2). Middle rows: traces at 446 nm taken with four flashes with 5 s between the flashes. They are the average of eight measurments with a dark-time of 6 min between trains of flashes. Bottom rows: Cyt *b*-561 reduction in the same flash regime as used for the middle rows. These traces are the average of four measurements. All traces were taken with a sweep of 200 ms and an instrument response time of 200 μs. Mediators were the same as in Fig. 1, ferricyanide at 1 mM concentration was added, too. 2 μM each of valinomycin and nigericin were used instead of gramicidin.

titration of these oscillations as measured from the difference between the first and the second flash at 446 nm. The midpoint of this titration is around 340 mV. The disappearance of the oscillations at 446 nm below a midpoint of about 340 mV in such titrations, together with the fact that ametrine affects only 50% of the reaction centers at these potentials [7], was explained by a model, in which about half of the reaction centers were presumed to have a bound semiquinone ($Q_B^-$), and the remainder to react with oxidized quinone. Flash activation would result in reduction of the semiquinone to quinol and of the quinone to semiquinone, leading to a net yield of one $QH_2$ per pair of reaction centers and a restoration of the preflash distribution. Effectively, a pair of reaction centers would cooperate, so that each flash would produce one $QH_2$ per two reaction centers, and no net production of $Q_B^-$ would occur [4,21].

The results using chromatophores with low Cyt $c_2$ concentration are shown in Fig. 2B. From the top row, values of 0.49 Cyt *b*-561 per reaction center and 0.53 total Cyt *c* per reaction center were calculated. This apparent cytochrome concentration is lower than the actual concentration, since under these conditions not all of the Cyt $c_1$ is oxidized, as already outlined in Materials and Methods. In this case the reaction center trace showed an incomplete rereduction after the first flash, with about 25% of the $P^+$-860 remaining oxidized 16 ms after the first flash. These chromatophores displayed some oscillations with flash number in the 446 nm traces at potentials well below 300 mV. Over the same range, a flash

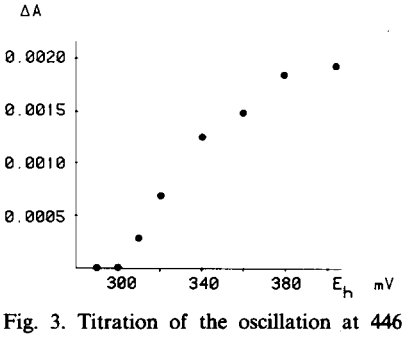


Fig. 3. Titration of the oscillation at 446 nm in chromatophores. The points represent the difference in absorption change at 446 nm between the first and the second flash from traces similar to the middle row of Fig. 2A determined 5 ms after the flash. The ambient redox potential was adjusted to the value represented by each point and held within ±2 mV by small additions of ferricyanide.

number dependent reduction of Cyt *b*-561 was observed. The traces shown in the bottom row of Fig. 2B have been taken at an ambient potential of 260 mV, since the oscillations were less pronounced at lower potentials, where the reduction of Cyt *b*-561 is limited by the incomplete oxidation of the Rieske type iron sulfur center by Cyt $c_2$ via Cyt $c_1$.

These results demonstrate that in the case of low concentration of reduced Cyt $c_2$, a net production of $Q_B^-$ after the first flash occurs and its disappearance after the second flash can be observed. This is not only obvious from the direct measurement of the semiquinone at 446 nm, but also from the reduction of Cyt *b*-561 which is more effective after the second flash (which produces more $QH_2$ than the first flash in this case).

The extent of oscillation at 446 nm in the chromatophores with low Cyt $c_2$ concentration was increased by changing the ambient redox potential to values above 300 mV (traces not shown). From this behavior it is concluded that these membranes have a fraction of their reaction centers which are controlled in the same way as those in chromatophores with a high Cyt $c_2$ concentration (e.g., the oscillation in the secondary quinone disappears below 300 mV due to a cooperation of two reaction centers). On the other hand, another fraction of the reaction centers, in membranes with low Cyt $c_2$, continues to act independently, and their secondary quinones show oscillations at redox potentials below 300 mV. Assuming that the only difference between the two preparations is the amount of Cyt $c_2$ present in the vesicles, it can be further concluded that reduced Cyt $c_2$ controls the apparent cooperation of the reaction centers. It is interesting to note that differential chemical modification of Cyt $c_2$ of *R. rubrum* in the free and the reaction-center-bound state showed a shielding of several lysine residues on the surface opposite to the heme cleft upon formation of the Cyt $c_2$-reaction center complex [32]. Since it is believed that Cyt *c* reacts with other components through its heme cleft, this shielding might reflect a binding to the reaction center at a non reactive site in addition to the reactive binding.

The influence of the concentration of Cyt $c_2$ on the oscillations at 446 nm was further tested in

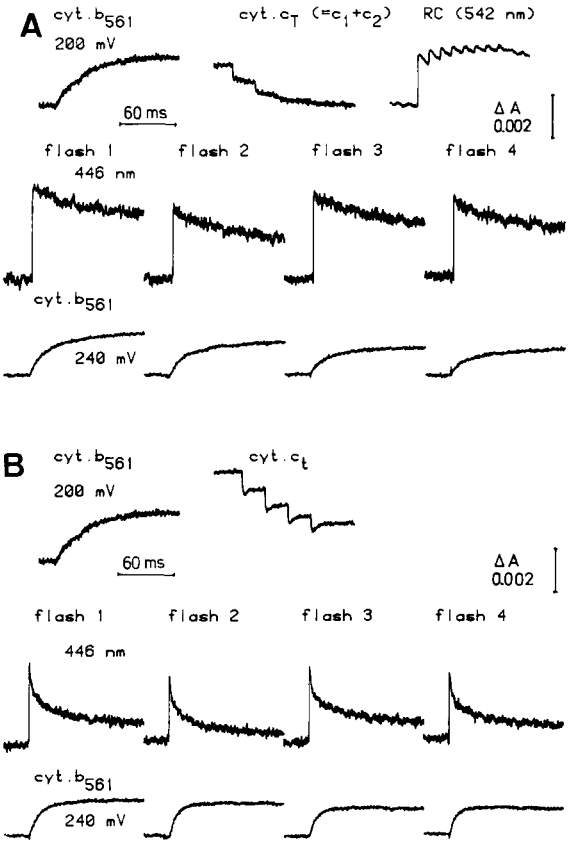


Fig. 4. Changes in the redox state of electron transport components after flash activation of spheroplast-derived vesicles. The conditions for the different traces were the same as in Fig. 2, except that spheroplast-derived vesicles were used instead of the chromatophores (reaction center concentration, 0.28 μM). Part B: pure Cyt $c_2$ was added to a final concentration of 3 μM.

spheroplast-derived vesicles. This preparation leads to the formation of membrane vesicles in which a large fraction have an opposite orientation compared to chromatophores [17,33]. The cyt $c_2$ binding site of the reaction centers is exposed to the external medium when spheroplasts are formed and most of the Cyt $c_2$ is therefore lost during preparation. Fig. 4A shows traces for the light-induced kinetics of oxidation and reduction of cytochromes and reaction centers in spheroplast-derived vesicles in the presence of antimycin A, from experiments similar to the ones in Fig. 2. Following a group of flashes, the reduction of Cyt *b*-561 is very slow and incomplete, due to poor oxidation of the Rieske iron sulfur centers by the *c*-cytochro-

mes. The oxidation of the residual Cyt *c* (predominantly Cyt $c_1$) was slower than in chromatophores. The slow oxidation also masked any rereduction of the *c*-cytochromes. Oscillations as a function of flash number in the traces at 446 nm could be observed in the spheroplast-derived vesicles at ambient redox potentials down to 200 mV. A redox titration of these oscillations is given in Fig. 5. Below 330 mV the oscillations started to decrease without showing a clear midpoint of the titration. This might be due partly to the slow rereduction of the reaction center, because it is necessary to rereduce $P^+$-860 between flashes so that the reaction center can turn over again on the second flash. This explanation is supported by the fact that addition of Cyt $c_2$ at low concentration to the suspension of spheroplast-derived vesicles increased both the rereduction of $P^+$-860, and the difference of the absorption change at 446 nm between the first and the second flash (Fig. 4B, second row). The extent of Cyt *b*-561 reduction after addition of Cyt $c_2$ to a final concentration of 3 $\mu$M (0.28 $\mu$M reaction center concentration) was increased in velocity as well as in extent. At the same time the kinetic trace of the cytochromes ($c_1 + c_2$) change displayed a rapid oxidation followed by some rereduction (Fig. 4B, top row). This clearly demonstrates that the added Cyt $c_2$

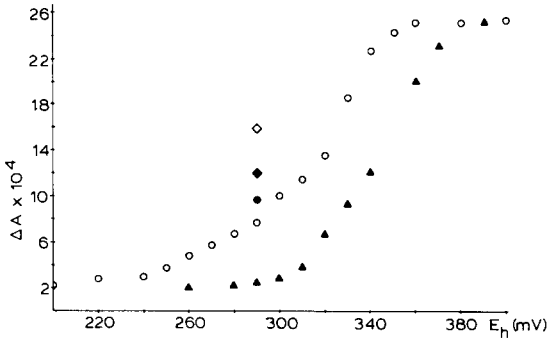


Fig. 5. Titration of the oscillation at 446 nm in spheroplast-derived vesicles. The open circles represent the difference of absorption change between the first and the second flash obtained from traces as in Fig. 4A (middle row) at the corresponding redox potentials. The filled triangles are the same values obtained from the same spheroplast-derived vesicle preparation in the presence of 76 $\mu$M Cyt $c_2$. The filled diamond represents the value at 290 mV after addition of 3 $\mu$M Cyt $c_2$, the open diamond is for 12 $\mu$M Cyt $c_2$ and the filled circle represents 36 $\mu$M Cyt $c_2$.

was able to react with the reaction centers as well as with the ubiquinol : cytochrome $c_2$ oxidoreductase (*b*-$c_1$ complex) thus reconstituting a cyclic electron transport.

Addition of more Cyt $c_2$ further increased the stimulatory effects on Cyt *b*-561 reduction, on the *c*-cytochrome oxidation and rereduction, and on the oscillations at 446 nm, but above 30 $\mu$M Cyt $c_2$ the oscillations started to become smaller again. Addition of more than 60 $\mu$M Cyt $c_2$ to the spheroplast-derived vesicles led to a marked decrease in oscillation. The titration curve in this case (Fig. 5) was very similar to the one for chromatophores (Fig. 3) except for the fact that there was always some residual oscillation at 446 nm which could be observed at ambient redox potentials around 200 mV, even in spheroplast-derived vesicles with a large excess of Cyt $c_2$ added. This behavior is very likely due to the fact that not all of the membrane vesicles were oriented with the same polarity, and therefore a fraction of the reaction centers was not able to react with the added Cyt $c_2$. Since the absorption change at 446 nm consists of several different components besides the semiquinone (reaction centers, cytochromes, carotenoids) spectra of the difference of the absorption changes between the first and the second flash were taken to make sure that the measurements used in Figs. 3 and 5 really correlate with an oscillation in the semiquinone form of the secondary quinone ($Q_B^-$) of the reaction centers. Fig. 6A shows such a spectrum derived from the difference of the absorption change after the first flash and that after the second flash, and obtained using a chromatophore at an ambient redox potential of 360 mV. Although the spectrum is a little noisy, it is still possible to correlate it with the absorption spectrum of ubisemiquinone in methanol [34]. The spectrum of Fig. 6A shows some deviation around 430 nm, which is due to the influence of the oscillations of Cyt *b*-561. Since the reduction of this cytochrome is faster and larger in extent after the second flash a negative value for the difference in absorption change for the first-minus-the-second flash resulted.

A similar difference spectrum can be shown using spheroplast-derived vesicles at an ambient redox potential of 280 mV (Fig. 6B). In this case the effect of the oscillation of Cyt *b*-561 reduction

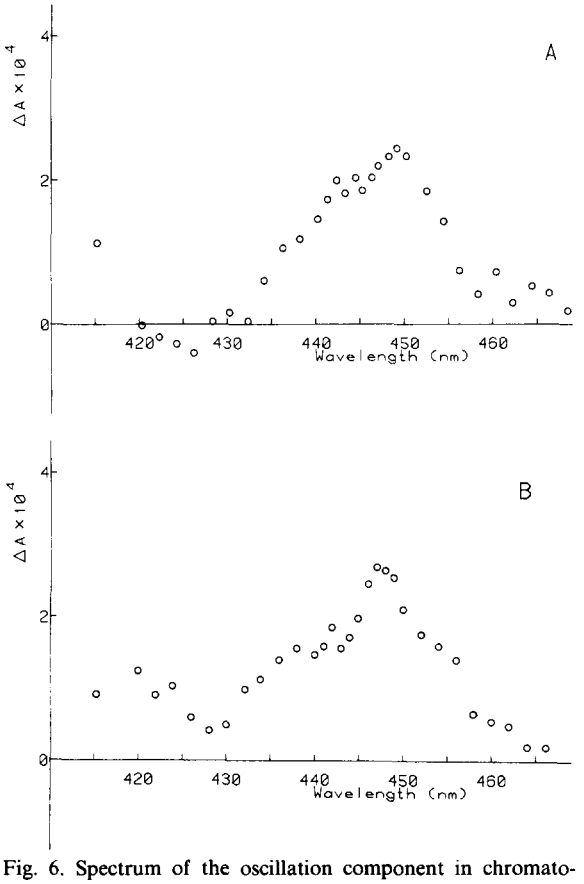


Fig. 6. Spectrum of the oscillation component in chromatophores and in spheroplast-derived vesicles. The shown spectra are composed of the difference in absorption change after the first and the second flash at different wavelengths. The conditions were the same as for Figs. 3 and 5. (A) Spectrum of the oscillating component in chromatophores at an ambient potential of 360 mV. (B) Spectrum of the oscillating component in spheroplast-derived vesicles at an ambient redox potential of 280 mV.

is less obvious than in Fig. 6A as expected from the traces of this cytochrome given in Fig. 4A. The similarity of the spectrum of the oscillation of chromatophores at 360 mV with that of spheroplast-derived vesicles at 280 mV indicates that the oscillations measured from the difference of the absorption change of the first and the second flash at 446 nm originated from the same component ($Q_B^-$) in both membrane systems.

## Conclusions

From the oxidation kinetics of Cyt $c_2$ and Cyt $c_1$ in normal chromatophores and chromatophores after fusion it is concluded that Cyt $c_2$ in its reduced form is bound to the reaction center. The reaction of the oxidized Cyt $c_2$ with Cyt $c_1$ is a second-order reaction. Diffusion of the Cyt $c_2$ from the reaction center to the $b$-$c_1$ complex is probably two-dimensional along the inner membrane surface.

The concentration of reduced Cyt $c_2$ seems to control the oscillations of the secondary quinone acceptor of the reaction centers. The net formation of semiquinone ($Q_B^-$) on the first flash is suppressed if the reduced Cyt $c_2$ is above a certain level, corresponding to about one Cyt $c_2$ per two reaction centers or more than 80 $\mu$M. In this case, two reaction centers appear to be cooperating so that each flash results in the formation of one fully reduced quinol per pair. Since this cooperation is controlled by reduced Cyt $c_2$ it seems possible that the cytochrome is able to bind to two reaction centers, thus forming a cooperating dimer.

In the experiments with spheroplast-derived vesicles, a concentration of more than 25 $\mu$M Cyt $c_2$ was needed to give a measurable decrease in oscillations of $Q_B^-$ at 280 mV (reaction center concentration in the cuvette between 0.27 and 0.32 $\mu$M). The effects of Cyt $c_2$ seemed to saturate in these experiments with a concentration around 80 $\mu$M. If our model of a reaction center dimer formation through the binding of Cyt $c_2$ were correct, the results would indicate that this dimer formation must require a Cyt $c_2$ concentration between 30 and 80 $\mu$M. In chromatophores with about 0.5 Cyt $c_2$ per reaction center, a concentration of Cyt $c_2$ in the inner volume of the vesicle of about 1 mM can be calculated using the dimensions of a chromatophore as discussed in Ref. 21. This concentration is well above the value which was required for the disappearance of the oscillations in spheroplast-derived vesicles, so that no oscillations are expected in chromatophores at ambient redox potentials where Cyt $c_2$ is reduced. The shift in the titration curve for the oscillations observed in chromatophores with a low Cyt $c_2$ content was presumably not due to the lower concentration itself, but to the fact that for stoichiometric reasons there is not enough Cyt $c_2$ for formation of all the potential reaction center pairs.

It is also interesting to note that Wraight and

co-workers [25–27] could only observe the rapid oxidation of Cyt $c_2$ by reaction centers in solution or reconstituted in liposomes above a certain concentration of the two components. At the same time not more than 50% rapid oxidation could be achieved. These facts could be well accommodated by our reaction center dimer model.

While the dimer model suggested here provides an explanation for the behavior of the system, the ad hoc postulate is not entirely satisfactory, and clearly will require further experimental support. Thus, there is no obvious explanation in the model for the presence after prolonged dark adaption of $Q_B^-$ in half of the reaction centers at ambient redox potentials ($E_h$ between 200 and 300 mV) well above those at which any quinol is present in the pool. Nevertheless, it is clear from the reduction of Cyt *b*-561 that quinol is formed by reaction centers after a single flash under these conditions [4,21], while the behavior towards ametryn inhibition [7] strongly suggests a heterogeneity of reaction centers, with only approx. 50% responding as if the secondary acceptor were fully oxidized after dark adaptation. These experiments, and the fact that oscillations as a function of flash number are observed at $E_h$ values above 340 mV, appear to rule out alternative explanations based on formation of $Q_B^-$ in all centers, followed by dismutation. In addition, the kinetics of Cyt *b*-561 reduction observed over this potential range show a pronounced initial lag, attributed in part to diffusion of quinol from the reaction center [21]. The rate after the lag is determined by a well-characterized second-order rate constant, and a concentration of quinol in the pool of close to 1 per two reaction centers [16,21]. These kinetics make a mechanism based on direct oxidation of $Q_B^-$ by the ubiquinol : Cyt $c_2$ oxidoreductase [14] seem highly implausible. Furthermore, since the kinetics are measured in the presence of antimycin A, a collaborative reduction of quinone, by the reaction center and the quinone reductase site of the $UQH_2$ : Cyt $c_2$ oxidoreductase, can be ruled out. Since the ubiquinone : cytochrome $c_2$ oxidoreductase complex behaves as if it were completely independent of direct cooperation with the reaction center, any explanation of the paradoxical redox behavior of the two-electron gate must be sought in the properties of the reaction center itself, or its interaction with some other component. The titrations previously reported, and the dependence on concentration shown here, strongly indicate an essential interaction with reduced Cyt $c_2$.

## Acknowledgements

We are grateful to Ms. Mary Hadden for expert technical assistance and to Dr. Steve Meinhardt for useful discussions. This research was supported by a grant from the National Institutes of Health, PHS 5 R01 GM26305. M.S. was in part supported by a grant from the Schweizerische Nationalfonds, No. 83.897.1.81.

## References

1 Kihara, T. and Chance, B. (1969) Biochim. Biophys. Acta 189, 116–124
2 Ke, B., Chaney, T.H. and Reed, D.W. (1970) Biochim. Biophys. Acta 216, 373–383
3 Dutton, P.L. and Jackson, J.B. (1972) Eur. J. Biochem. 30, 495–510
4 Bowyer, J.R., Tierney, G.V. and Crofts, A.R. (1979) FEBS Lett. 101, 201–206
5 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556
6 Bowyer, J.R., Meinhardt, S.W., Tierney, G.V. and Crofts, A.R. (1981) Biochim. Biophys. Acta 635, 167–186
7 Bowyer, J.R. and Crofts, A.R. (1981) Biochim. Bophys. Acta 636, 218–233
8 Wood, P. (1980) Biochem. J. 189, 385–391
9 Wood, P. (1980) Biochem. J. 192, 761–764
10 Meinhardt, S.W. and Crofts, A.R. (1982) FEBS Lett. 149, 223–227
11 Vermeglio, A. (1977) Biochim. Biophys. Acta 459, 516–524
12 Wraight, C.A. (1977) Biochim. Biophys. Acta 459, 525–531
13 De Grooth, B.G., Van Grondelle, R., Romijn, J.C. and Pulles, M.P.J. (1978) Biochim. Biophys. Acta 503, 480–490
14 O'Keefe, D.P., Prince, R.C. and Dutton, P.L. (1981) Biochim. Biophys. Acta 637, 512–522
15 Sistrom, W.R. (1960) J. Gen. Microbiol. 22, 778–785
16 Snozzi, M. and Crofts, A.R. (1984) Biochim. Biophys. Acta 766, 451–463
17 Takemoto, J. and Bachman, R.C. (1976) Arch. Biochem. Biophys. 195, 526–534
18 Meinhardt, S.W. (1984) Ph. D. Thesis, University of Illinois at Urbana-Champaign
19 Schneider, H., Leemasters, J.J., Hoechli, M. and Hackenbrock, C.R. (1980) Proc. Natl. Acad. Sci. USA 77, 442–446
20 Meinhardt, S.W. and Crofts, A.R. (1983) Biochim. Biophys. Acta 723, 219–230

21 Crofts, A.R., Meinhardt, S.W., Jones, K.R. and Snozzi, M. (1983) Biochim. Biophys. Acta 723, 202–218
22 Bartsch, R.G. (1971) Methods Enzymol. 23, 344–383
23 Bowyer, J.R., Dutton, P.L., Prince, R.C. and Crofts, A.R. (1980) Biochim. Biophys. Acta 592, 445–460
24 Gabellini, N., Bowyer, J.R., Hurt, E., Melandri, B.A. and Hauska, G. (1982) Eur. J. Biochem. 126, 105–111
25 Overfield, R.E., Wraight, C.A. and Devault, D. (1979) FEBS Lett. 105, 137–142
26 Overfield, R.E. and Wraight, C.A. (1980) Biochemistry 19, 3322–3327
27 Overfield, R.E. and Wraight, C.A. (1980) Biochemistry 19, 3328–3334
28 Vermeglio, A. and Joliot, P. (1984) Biochim. Biophys. Acta 764, 226–232
29 Vermeglio, A. and Carrier, J.M. (1984) Biochim. Biophys. Acta 764, 233–238
30 Zhu, Q.S., Van der Wal, H.N., Van Grondelle, R. and Berden, J.A. (1983) Biochim. Biophys. Acta 725, 121–130
31 Rosen, D., Okamura, M.Y. and Feher, G. (1980) Biochemistry 19, 5687–5692
32 Rieder, R., Wiemken, V., Bachofen, R. and Bosshard, H.R. (1985) Biochem. Biophys. Res. Comm. 128, 120–126
33 Lommen, M.A.J. and Takemoto, J. (1978) J. Bact. 136, 730–741
34 Bensasson, R. and Land, E.J. (1973) Biochim. Biophys. Acta 325, 175–181