Biochimica et Biophysica Acta, 503 (1978) 480—490

BBA 47546

THE MECHANISM OF REDUCTION OF THE UBIQUINONE POOL IN PHOTOSYNTHETIC BACTERIA AT DIFFERENT REDOX POTENTIALS

B.G. DE GROOTH, R. VAN GRONDELLE, J.C. ROMIJN and M.P.J. PULLES

Department of Biophysics, Huygens Laboratory of the State University, Wassenaarseweg 78, 2300 RA Leiden (The Netherlands)

(Received February 15th, 1978)

Summary

(1) A flash number dependency of flash-induced absorbance changes was observed with whole cells of *Rhodospirillum rubrum* and chromatophores of *R. rubrum* and *Rhodopseudomonas sphaeroides* wild type and the G1C mutant. The oscillatory behavior was dependent on the redox potential; it was observed under oxidizing conditions only. Absorbance difference spectra measured after each flash in the 275—500 nm wavelength region showed that a molecule of ubiquinone, R, is reduced to the semiquinone (R^-) after odd-numbered flashes and reoxidized after even-numbered flashes. The amount of R reduced was approximately one molecule per reaction center.

(2) The flash number dependency of the electrochromic shift of the carotenoid spectrum was studied with chromatophores of *Rps. sphaeroides* wild type and the G1C mutant. At higher values of the ambient redox potential a relatively slow phase with a rise time of 30 ms was observed after even-numbered flashes, in addition to the fast phase (completed within 0.2 ms) occurring after each flash. Evidence was obtained that the slow phase represents the formation of an additional membrane potential during a dark reaction that occurs after flashes with an even number. This reaction is inhibited by antimycin A, whereas the oscillations of the R/R^- absorbance changes remain unaffected. At low potentials (E = 100 mV) no oscillations of the carotenoid shift were observed: a fast phase was followed by a slow phase (antimycin-sensitive) with a half-time of 3 ms after each flash.

(3) The results are discussed in terms of a model for the cyclic electron flow as described by Prince and Dutton (Prince, R.C. and Dutton, P.L. (1976) Bacterial Photosynthesis Conference, Brussels, Belgium, September 6—9, Abstr. TB4) employing the so-called Q-cycle.

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

Introduction

During the last years, much work has been done in order to establish the identity of the reactants involved in the primary photochemical processes in photosynthetic bacteria. It has become clear that the excited bacteriochlorophyll dimer *P*-870 * transfers an electron to a bacteriopheophytin molecule within a few picoseconds [1—3]. In a subsequent dark reaction of about 200 ps, the charge separation is further stabilized by electron transfer from bacteriopheophytin to what is generally called the "primary acceptor" X, probably an iron · quinone complex. The quinone has been identified as a menaquinone in *Chromatium vinosum* [4—5] and as a ubiquinone in *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum* [6—8]. X, in its turn, reduces under normal conditions a secondary acceptor [9—11]. This secondary acceptor probably belongs to a relatively large pool of ubiquinone (UQ) molecules. Since it has been shown that X becomes reduced in a one-electron transfer reaction [9], whereas the ubiquinones of the pool act as two-electron acceptors, the exact mechanism of electron transfer between X and the UQ pool needs further clarification.

Recently, Vermeglio [12] and Wraight [13] studied flash-induced electron transfer from X to exogenous ubiquinone in reaction center preparations of the R-26 mutant of *Rps. sphaeroides*. They found that, after dark adaptation, a special molecule of ubiquinone closely bound to the reaction center was reduced to ubisemiquinone by flashes with an odd number. After the even numbered flashes, the semiquinone was reoxidized and one molecule of the exogenous ubiquinone was fully reduced to ubihydroquinone. These observations were discussed in terms of the following model, similar to one proposed earlier for spinach chloroplasts [14,15]:

$$X\,R\,UQ \xrightarrow{h\nu_1} X^-\,R\,UQ \rightarrow X\,R^-\,UQ \xrightarrow{h\nu_2} X^-\,R^-\,UQ \rightarrow X\,R^=\,UQ \xrightarrow{2\,H^+} X\,R\,UQH_2 \qquad (1)$$

where, in analogy with Photosystem II in spinach chloroplasts and algae, R is the quinone molecule functioning as the secondary electron acceptor [15,16]. In a recent communication, Barouch and Clayton [17] reported oscillations of a quinone and of proton uptake in agreement with this scheme in chromatophores of the carotenoidless mutant R-26 of *Rps. sphaeroides* but not in the green Ga mutant. In contrast to this model, which was developed to explain the oscillating absorbance changes, several schemes have been proposed to explain the cytochrome *c*, cytochrome *b* and ubiquinone redox reactions and correlate these to proton uptake and proton release [18—21]. In some of these schemes ubiquinone becomes reduced to the protonated semiquinone only and then becomes reoxidized via a cytochrome *b* chain [20,21].

Recently, Prince and Dutton [22] proposed a model in which the secondary ubiquinone upon each flash might pass through a so-called Q-cycle in which ubiquinone becomes fully reduced and takes up two protons which are subsequently released when the ubiquinone becomes reoxidized via reactions with cytochrome *b* and *c*.

In the present study, experiments with whole cells and chromatophores

of different species of bacteria will be shown and discussed in a model combining the linear scheme to explain the oscillating absorbance changes and the Q-cycle model of Prince and Dutton. Part of the results have been presented earlier in a preliminary form [23].

Materials and Methods

R. rubrum and *Rps. sphaeroides* (wild type and G1C mutant) were grown anaerobically in the light on media described by Cohen-Bazire et al. [24] and Slooten [25], respectively. After 2—3 days the cells were harvested by centrifugation and resuspended in fresh medium. For the preparation of chromatophores the cells were resuspended in 50 mM potassium hydrogen phosphate buffer, pH 7.5, and sonicated for 3—10 min with a Branson type S125 sonifier. After centrifugation for 30 min at 20 000 × g, the supernatant was centrifuged for 90 min at 110 000 × g. The pellet was suspended in buffer and stored for up to 4 days at 4° C until use.

Flash-induced absorbance changes were measured with a single beam apparatus with a flow system (see ref. 26 for details) or on a similar apparatus [27], provided with a cuvette similar to that described by Dutton [28] in which the redox potential could be measured. In this cuvette the sample was kept anaerobic with nitrogen and, in the presence of 50 μM TMPD the ambient redox potential could be varied between 100 and 350 mV by adding small amounts of ferricyanide or ascorbate. Actinic light was provided by a Xenon flashlamp ($t_{1/2}$ = 10 μs). The samples were kept in the dark (measuring beam off) for at least 5 min.

Results

Binary oscillations of the redox state of R

Figs. 1a and b show the absorbance changes at 450 nm induced by a series of short, saturating flashes given to dark adapted cells of *R. rubrum*. Under aerobic conditions, large binary oscillations are observed (recording a). If the sample was made anaerobic by bubbling with N_2 for several minutes, the oscillations were absent (recording b). With whole cells of *Rps. sphaeroides* we did not observe oscillations in either case, probably because of the fast oxygen consumption by this bacterium. With chromatophores of *Rps. sphaeroides*, however, oscillations were observed at high redox potentials (Fig. 1c), whereas they were absent at low potentials (recording d). In this case, the measurements were done at 452.8 nm, where the electrochromic carotenoid shift which is prominent in this bacterium has an isosbestic point [29]. The initial transient observed on each flash is probably due to the fast oxidation and rereduction of *P*-870. Essentially the same results were obtained with chromatophores of *R. rubrum* and those of the G1C mutant of *Rps. sphaeroides* (see below). Apparently, oscillations of absorbance changes in the blue region only occur under oxidizing conditions.

If the absorbance changes induced by the second flash are subtracted from those induced by the first flash, absorbance changes independent of the flash number should be eliminated. In Fig. 2, difference spectra obtained in this way

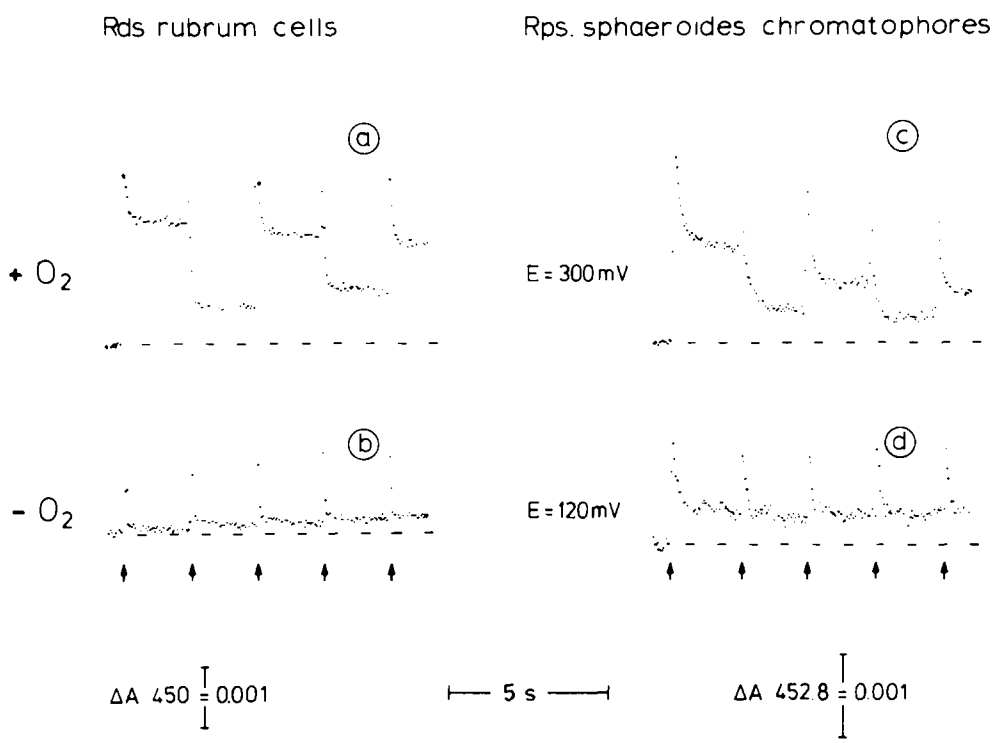

Fig. 1. Kinetics of the flash-induced absorbance changes at the indicated wavelengths of whole cells of *R. rubrum* (recordings a and b, absorbance in the cuvette at 880 nm is 0.9) and chromatophores of *Rps. sphaeroides* (recordings c and d, absorbance in the cuvette at 850 nm is 2.5). Recording a was measured under aerobic conditions, recording b under anaerobic conditions, recordings c and d were obtained at the indicated ambient redox potentials (50 μM TMPD present). Actinic flashes were given at the times indicated by the arrows. Optical pathlength: 0.9 cm.

for the absorbance changes measured 1 s after the flashes are shown for whole cells of *R. rubrum* and chromatophores of *Rps. sphaeroides* (Figs. 2a and b respectively). The latter spectrum was measured in the presence of gramicidin to prevent the generation of electrochromic absorbance changes. In Fig. 2a, the difference spectrum is compared with the difference spectrum for the reduction of ubiquinone to ubisemiquinone anion in vitro (dashed line, obtained from ref. 30). From this it seems likely that the oscillations are mainly due to a reduction of a molecule of ubiquinone (R) to its ubisemiquinone anion (R^-) on the odd-numbered flashes, and its reoxidation on the even-numbered flashes. This is in agreement with Model I given in the introduction if we assume that the UQH_2 formed is reoxidized within 1 s. The spectrum obtained with chromatophores of *Rps. sphaeroides* can be interpreted similarly, although the relatively small negative band around 280 nm may indicate that in this case part of the UQH_2 formed on the second flash is still present after one second. By comparing the amplitude of the changes due to R with that of *P*-870 (measured at 602 nm 1 ms after the flash), we calculated that approximately 1 molecule of R per active reaction center participates in the

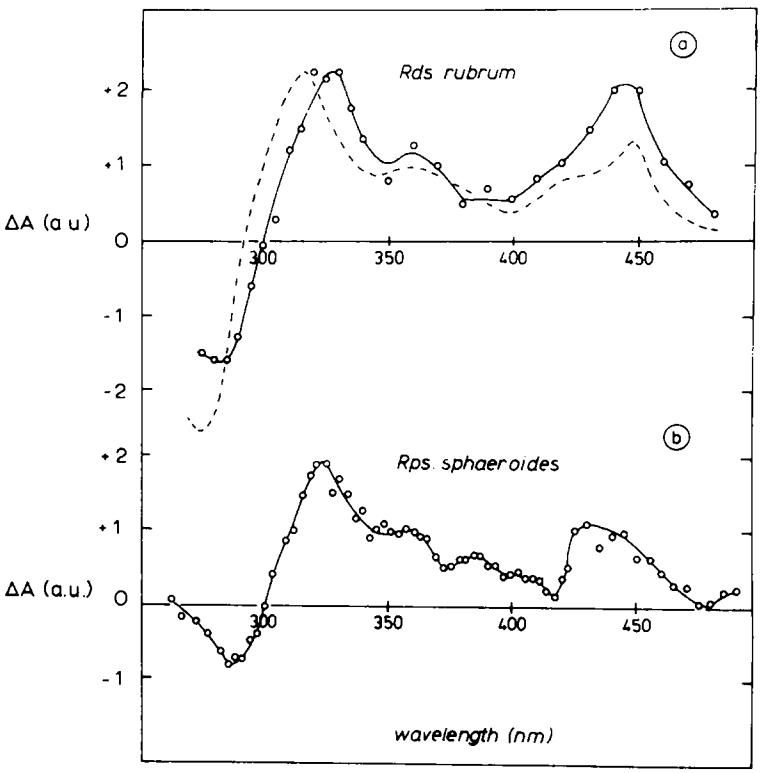

Fig. 2. Spectrum of the absorbance changes induced by the first flash minus those induced by the second flash measured in aerobic cells of *R. rubrum* (a, solid line) and in aerobic chromatophores of *Rps. sphaeroides* (b). Spectrum b was measured in the presence of 50 μM TMPD and 50 μM gramicidin. The time between the flashes was 1 s. The dotted line represents the difference spectrum of the reduction of ubiquinone to ubisemiquinone obtained from ref. 30.

oscillation, using an extinction of 5 $mM^{-1} \cdot cm^{-1}$ for R at 450 nm [30] and 15 $mM^{-1} \cdot cm^{-1}$ for *P*-870 at 602 nm [31].

In Fig. 3, the absorbance change in *Rps. sphaeroides* chromatophores at 452.8 nm induced by the second flash, is plotted against the preceding dark time. From this we may estimate that the life time of R^- under these conditions is approx. 1 min.

Oscillations of carotenoid band shift

It is generally accepted that the interaction between the (induced) dipole moment of carotenoid molecules and the delocalized electric field across the chromatophore membrane, leads to an electrochromic shift of the absorption spectra of part of the carotenoid present [32—35]. Since it has been shown that there is a linear relationship between these absorbance changes and the changes in the membrane potential [33], a study of the kinetics of the carotenoid shift may provide valuable information about the movement of charges inside the membrane, in a direction perpendicular to the surface of the chromatophore membrane [36].

With dark-adapted chromatophores of *Rps. sphaeroides*, we observed at

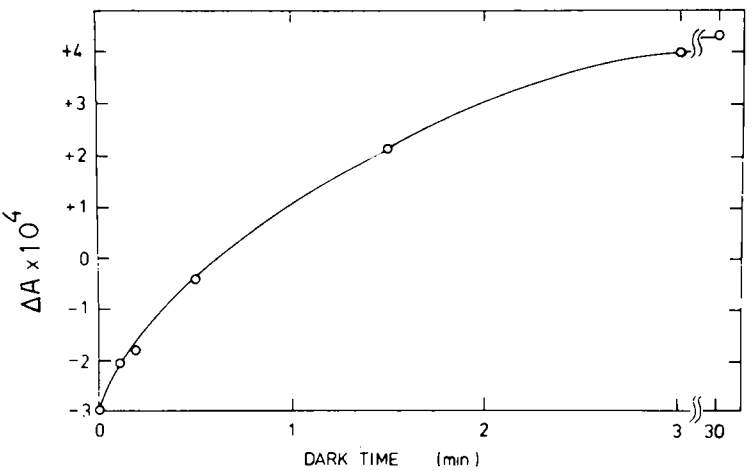

Fig. 3. Absorbance changes measured at 452.8 nm induced by the second flash as a function of the preceding dark time measured with aerobic chromatophores of *Rps. sphaeroides* in the presence of 50 μM TMPD. Optical pathlength: 1 mm; absorbance at 850 nm is 0.7.

redox potentials below 150 mV a biphasic behavior of the carotenoid shift measured at 509 nm, after each flash (Fig. 4a). A fast phase, completed within the response time of our apparatus (0.2 ms) was followed by a phase of about 3 ms; these phases might be identical to the first and third phase reported by Jackson and Dutton [36]. At high redox potentials, however, the first flash induced mainly a fast phase, whereas the second flash gave a fast absorbance decrease, followed by a slow one with a half time of about 30 ms (Fig. 4b). The amplitude of the slow phase was again lower after the third flash, whereas at still higher numbered flashes the kinetics were obscured by absorbance changes due to the decay of the membrane potential, built up by the preceding flashes. In order to investigate whether the fast and slow phases

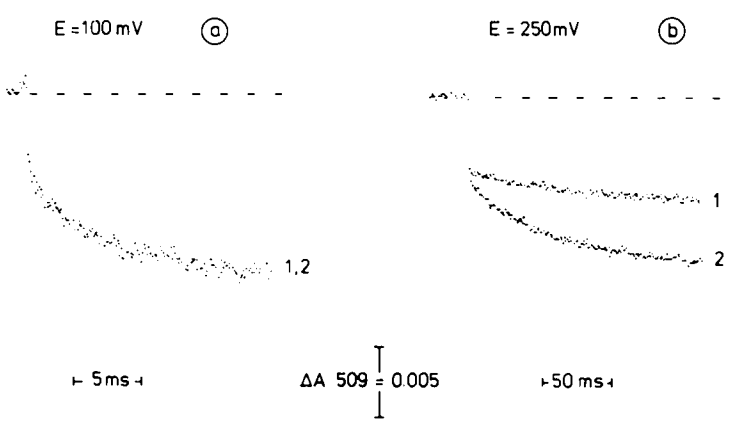

Fig. 4. Kinetics of the absorbance changes measured at 509 nm in chromatophores of *Rps. sphaeroides* at the indicated redox potentials (50 μM TMPD present). 1: first flash; 2: second flash. The time between the flashes was 2 s. The absorbance at 850 nm was 2.5 in the cuvette with an optical pathlength of 0.9 cm.

represent the same phenomena we measured the difference spectra of both phases (Fig. 5). It is clear from the spectra that both phases represent a carotenoid band shift, while the differences in the spectra may be described to absorbance changes due to *P*-870 [8]. This, together with the observation that both phases were inhibited by gramicidin, indicate that at high redox potentials after the second flash, a dark reaction with a half time of approx. 30 ms occurs which creates an additional membrane potential. In the presence of 0.5 μM antimycine A, the slow phase of the carotenoid shift was inhibited whereas the fast phase and the oscillations at 452.8 nm due to R were not affected (see also below). Oscillations of the slow phase of the carotenoid shift at higher redox potentials, were also observed in chromatophores of the G1C mutant of *Rps. sphaeroides.*

Absorbance changes due to TMPD

In the presence of TMPD at low redox potentials dark adapted chromatophores of *R. rubrum*, *Rps. sphaeroides* and its G1C mutant showed an absorbance increase around 580 nm after each flash, indicating a net oxidation of TMPD. This is shown in Fig. 6a for the G1C mutant. At high redox potentials, however, oscillating absorbance changes were observed: the odd numbered flashes induced an absorbance increase whereas the even numbered flashes brought about an initial increase followed by a decrease (recording b). This decrease was slowed down substantially in the presence of 0.5 μM antimycin, whereas the oscillations of R were not affected (recording e and f). This might indicate that the reduction of TMPD on the even-numbered flashes is related to the slow phase of the carotenoid band shift. The exact amount of TMPD that was oxidized was difficult to measure. The extent of the absorbance changes varied largely with different preparations and the spectrum showed significant deviations from the in vitro spectrum. The three absorption bands with maxima at 525, 565 and 615 nm that appear upon oxidation of TMPD were much sharper than those of the in vitro spectrum. The reason for this is

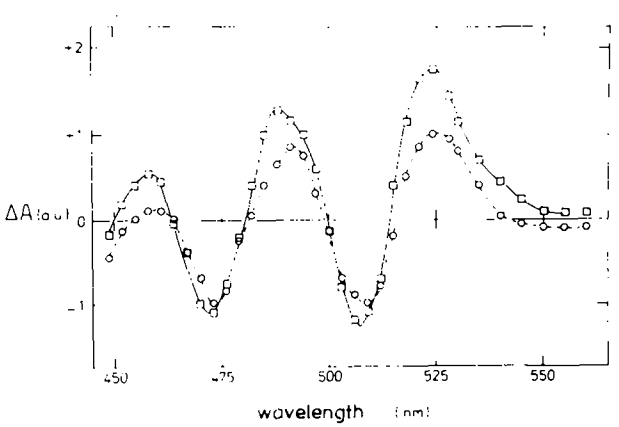

Fig. 5. Absorbance difference spectra induced by the second flash (given 1 s after the first flash) in aerobic chromatophores of *Rps. sphaeroides* in the presence of 50 μM TMPD. □———□, absorbance changes 2 ms after the flash; ○———○, absorbance changes occurring between 2 and 180 ms after the flash.

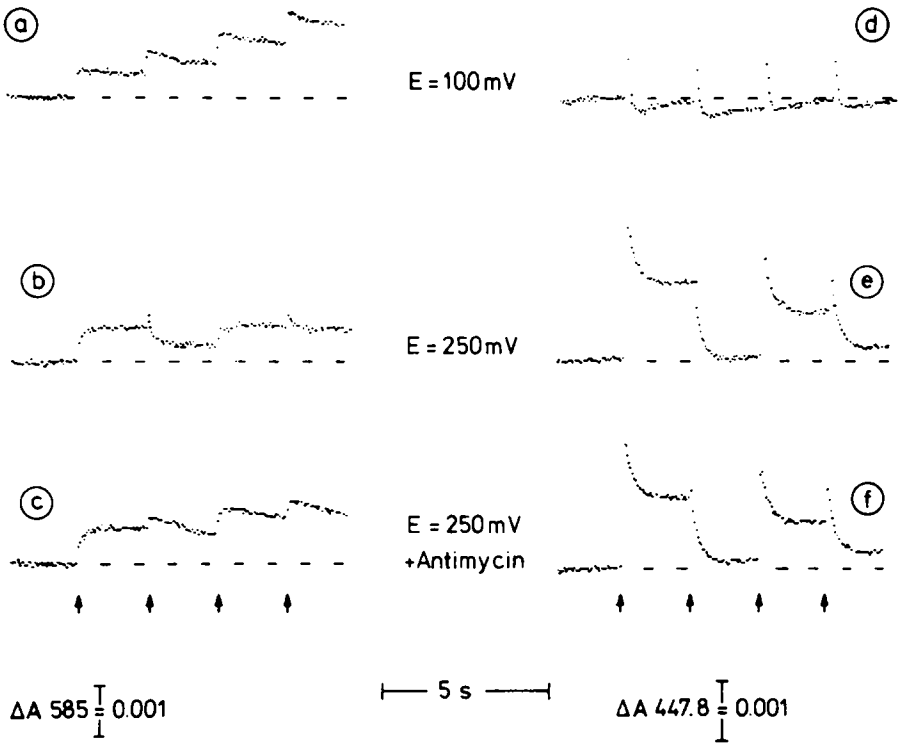

Fig. 6. Kinetics of the absorbance changes induced by a series of flashes in anaerobic chromatophores of the G1C mutant of *Rps. sphaeroides* at the indicated redox potentials and wavelengths. Additions: a—f, 50 μM TMPD; c and f, 0.5 μM antimycin. The absorbance at 850 nm was 2.4 in the cuvette with an optical pathlength of 0.9 cm.

not clear. Depending on the preparation used and the estimation of the extinction coefficient, the number of TMPD molecules per reaction center that could be oxidized by the first flash at high redox potentials varied from 0.2 to 0.8.

Discussion

The results reported in this paper strongly indicate that under oxidizing conditions a scheme as given in Model I applies to intact bacterial cells and chromatophores. However, a complete model will have to account for the observations that the oscillations of the redox state of R were not observed at reducing conditions. One might argue that at low redox potentials exactly 50% of R is in the reduced state (R^-) so that no net oscillations will be observed. This does not seem very likely, however, since on further lowering the redox potential, one would expect to reduce nearly all R and to see oscillations again starting from R^-, which were not observed. We therefore suggest that under reducing conditions an additional electron donor D is operational, which, together with R^- can reduce UQ to UQH_2:

$$X\,R\,UQ\,D \xrightarrow{h\nu} X^-\,R\,UQ\,D \rightarrow X\,R^-\,UQ\,D \xrightarrow[2\,H^+]{} X\,R\,UQH_2\,D^+$$

At high redox potentials D becomes oxidized in the dark, leading to the oscillations of R according to the scheme given in the introduction. One may consider the possibility that D is a cytochrome *b*, operating as a component of a second Mitchell loop as discussed by Prince and Dutton [22]. Such a scheme is given in Fig. 7 for intact bacteria. The reactions causing the three phases in the carotenoid shift [36] are indicated with I, II and III. In the scheme the reduction of oxidized cytochrome *c* and an unknown component of the second loop by UQH_2 is inhibited by antimycin, which explains the retardation of the rereduction of cytochrome c^+ [20,27] and the inhibition of the third phase.

Since after addition of antimycin to anaerobic cells of *R. rubrum* flash-induced oscillations of R still were not observed (data not shown), we must assume that at low redox potentials cytochrome *b* can be reduced by a compound of the medium if reaction III is blocked. In the chromatophores that we used, the role of cytochrome *c* is probably taken over by TMPD. At high redox potentials reaction III occurs only after even-numbered flashes, whereas at low potentials it is induced by every flash, which explains the behavior of the carotenoid band shift in Fig. 4.

The observation that under reducing conditions net oxidation of some TMPD occurs after each flash indicates that not all the electron transport is cyclic in chromatophores. The oscillatory behavior of the redox state of TMPD at high values of the redox potentials is in qualitative agreement with the model: after the odd-numbered flashes one electron is stored on R, whereas a positive charge is present on other components, among which is TMPD. After the even numbered flashes a second positive charge is formed as is reflected by the initial TMPD oxidation shown in Fig. 6. The two electrons formed on R^{2-} will be transported together with two protons across the membrane by ubiquinone. One of the electrons of UQH_2 reduces a component of the medium ($TMPD^+$) while the other electron will reduce cytochrome *b* causing the slow phase in the carotenoid shift (reaction III). This cytochrome *b* in turn is oxidized by the medium (TMPD). Assuming again that the action of

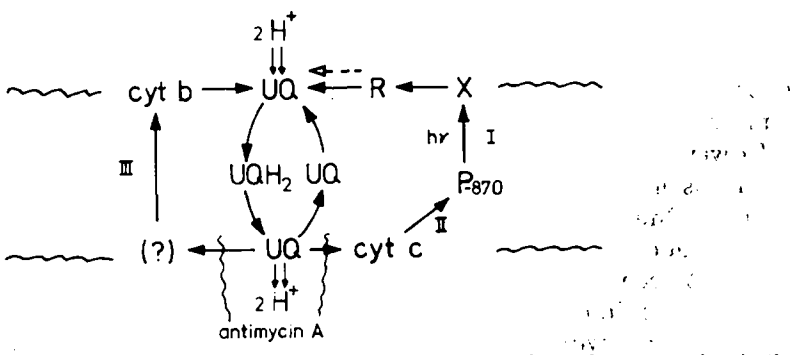

Fig. 7. Model for electron transport and localization of the electron carriers in the membrane for intact bacteria. The solid arrows indicate the direction of electron transport. The reactions causing the three phases in the carotenoid shift are labeled with I, II and III. The reduction of cytochrome *b* by UQH_2 might occur via an unknown component, indicated here with a question mark. At high redox potentials, when cytochrome *b* is oxidized in the dark, R can accumulate two electrons from X and transfer them together to UQ which is indicated by the broken arrow. For further explanation, see text.

antimycin is a strong decrease of the rate of reoxidation of UQH_2, the inhibition by this substance of both the slow carotenoid shift and the fast TMPD reoxidation after the second flash can be explained.

The model discussed here is a hypothetical one, but it may serve as a useful starting point for further experiments. Experimental results supporting at least part of the model have been reported recently. At low redox potentials Petty et al. [37] found a ratio of two between the number of protons taken up and the number of electrons transferred from *P*-870 to X upon flash illumination, whereas at high potentials (in the absence of valinomycin) this value was one, in agreement with the model of Fig. 7. Although Petty et al. did not report oscillations in the proton uptake, possibly because they may not have used dark adapted chromatophores, Barouch and Clayton [17] observed the uptake of approximately two protons on the even-numbered flashes only, under conditions where oscillations at 450 nm, presumably due to R, were also detected. However, measurements of the exact relation between proton uptake and oscillations of R at different redox potentials will be needed to test this part of the model.

Acknowledgements

We are indebted to Dr. J. Amesz for valuable discussions and for critical reading of the manuscript. This investigation was supported by the Netherlands Foundations for Chemical Research (S.O.N.) and the Netherlands Foundation for Biophysics, financed by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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