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FUNCTION OF THREE CYTOCHROMES IN PHOTOSYNTHESIS OF WHOLE CELLS OF *RHODOSPIRILLUM RUBRUM* AS STUDIED BY FLASH SPECTROSCOPY

EVIDENCE FOR TWO TYPES OF REACTION CENTER

RIENK VAN GRONDELLE, LOUIS N. M. DUYSENS and HENK N. VAN DER WAL

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

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SUMMARY

1. Changes in the absorption spectrum induced by 10-μs flashes and continuous light of various intensities were studied in whole cells of *Rhodospirillum rubrum* in the presence and absence of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide(HOQNO) and antimycin A.

2. Three cytochromes, *c*-420 (cytochrome c_2), *c*-560 (cytochrome *b*) and *c*-428 were photoactive with γ and α peaks at 420 and 550, 428 and 560, and 428 and 551 nm, respectively; they were photooxidized following the flash with half times of 0.3, 0.6 and 7 ms in the approximate ratios of 1/100, 1/300 and 1/1000 (cytochrome oxidized/antenna chlorophyll) and became reduced with half times of 12 ms, 60 ms and 0.7 s, respectively. *c*-428 and *c*-560 have not been distinguished before.

3. From a detailed analysis of the kinetics of P^+ (oxidized reaction center chlorophyll) and the cytochromes, we conclude that 5 % of the P^+ (P_2^+) oxidizes *c*-428, whereas the remaining 95 % of P^+ (P_1^+) oxidizes *c*-420. At actinic light intensities low enough to keep *c*-420 fully reduced, approx. 4–5 % of P becomes oxidized, accompanied by all *c*-428. The $P_2^+ - P_2$ difference spectrum induced by this weak light is, when corrected for a shift to longer wavelengths of the bacteriochlorophyll absorption band at 878 nm, identical to the difference spectrum caused by the photooxidation of the remaining P_1. At low flash intensity, *c*-428 becomes preferentially photooxidized, which suggests that the reaction centers where *c*-428 functions as a secondary donor contain much more antenna pigments compared to the centers where *c*-420 serves this purpose.

4. c^+-420 is reduced in a competitive way by reduced *c*-560 ($t_{\frac{1}{2}} = 7$ ms), and by an electron donor pool, ($t_{\frac{1}{2}} = 15$ ms). HOQNO inhibits both pathways; antimycin A only the first. In the presence of HOQNO, *c*-560 is in the oxidized state in the dark, and is reduced in a light flash ($t_{\frac{1}{2}} = 100$ ms), indicating that *c*-560 acts in a cyclic electron transport chain connected to P_1.

Abbreviation: HOQNO, 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide

5. The ratio of numbers of molecules P_1 and antenna bacteriochlorophyll, transferring excitation energy to P_1, is P_1/bacteriochlorophyll$_1$ = 1/30; P_2 : bacteriochlorophyll$_2$ = 1/300; *c*-420/P_1 = 1 : 2; *c*-560/P_1 = 1/6; *c*-428/P_2 = 1/1; bacteriochlorophyll$_1$: bacteriochlorophyll$_2$ = 7 : 3. If P_2 is oxidized, excitation energy is transferred from bacteriochlorophyll$_2$ to bacteriochlorophyll$_1$.

INTRODUCTION

The first discovered photoreaction of a cytochrome in photosynthetic cells was the photooxidation of *c*-428 [1] in anaerobic whole cells of *Rhodospirillum rubrum* at low intensity actinic light. Nevertheless, the electron transfer reactions and the function of this cytochrome have not yet been elucidated.

The photooxidation of another *c*-type cytochrome, *c*-420, was observed in aerobic cells [2, 3] or at high light intensity in donor-depleted cells [4, 5]. Absorbance changes induced by short flashes showed the oxidation of both *c*-420 and *c*-428 [6, 7]. Ke and Ngo [6] estimated the half time of *c*-420 oxidation to be 1–2 ms. In their experiments, the reduction of P^+ was markedly slower ($t_{\frac{1}{2}}$ = 10 ms). Kihara [8] suggested a much faster rate for P^+ reduction ($t_{\frac{1}{2}}$ = 0.1 ms) by *c*-420, but up to now no exact description of the P^+ and cytochrome kinetics in *R. rubrum* has been presented.

The role of different *c*-type cytochromes in *Chromatium vinosum* has been extensively studied. On basis of low temperature experiments and other indirect evidence, Duysens [9] concluded that in *Chromatium*, P^+ first oxidized a *c*-type cytochrome *c*-423.5 (or *c*-552) and after this cytochrome was oxidized another *c*-type cytochrome *c*-422 (or *c*-555). The reduction of *c*-423.5 in the dark was extremely slow at room temperature. For *Chromatium*, Parson and coworkers [10, 11] confirmed Duysens' conclusion by showing that P^+, oxidized in a laser flash, was reduced with a half time (1.0 μs) similar to the half time of *c*-423.5 oxidation. When *c*-423.5 was oxidized before the flash, P^+ was reduced with a half time of 2.0 μs, similar to the half time of *c*-422 oxidation.

In a similar way it was shown in many other photosynthetic bacteria that P^+ can be reduced by a low and a high potential cytochrome [12–14]. However, the mechanism lying behind such an arrangement remains unexplained at this moment. In many cases, it was found that the pool of reduced cytochrome *c* was much larger than the concentration of reaction centers [15–17]. We will show that in whole cells of *R. rubrum* there is approximately one *c*-420 per two reaction centers.

It has been concluded by several authors that there is more than one type of reaction center functioning in *R. rubrum* [5, 7, 18]. The reasoning leading to these conclusions was mainly based on different action spectra for the photooxidation of *c*-420 compared to *c*-428 and is of doubtful validity [19, 20]. By means of a different method, we will show that the photooxidation of *c*-428 is coupled to approx. 5 % of the reaction centers present in the cell, and that each of these reaction centers is associated with a much larger amount of antenna chlorophyll molecules than those associated with *c*-420.

A cytochrome of the *b*-type has been isolated from cells of *R. rubrum* [21, 22] and the role of such a cytochrome *b* has been the subject of many investigations.

Already in 1964, Nishimura et al. [23] measured the light-induced reduction of cytochrome *b* (which we have called *c*-560, because of the location of the α peak in the difference spectrum) in whole cells of *R. rubrum* in the presence of HOQNO. In chromatophores of the same bacterium, Parson [24] measured the reduction of *c*-560 after a saturating laser flash in the presence of antimycin A. Recently Prince and Dutton [25] found that in *Rhodopseudomonas sphaeroides*, a *b*-type cytochrome is involved in cyclic electron transport. This *b*-type cytochrome was reduced via ubiquinone after a flash and became subsequently oxidized by cytochrome c_2. The reaction between cytochrome *b* and cytochrome c_2 was strongly inhibited by antimycin A. A role in an energy-transducing process for a *b*-type cytochrome and *c*-420 in *R. rubrum* has been suggested by Dutton and Baltscheffsky [26] who showed an energy-dependent reduction of *c*-560 by *c*-420. We shall give evidence that in whole cells of *R. rubrum*, cyclic electron transport occurs from the photoreduced acceptor via *c*-560 and *c*-420 to the photooxidized donor P_1^+.

So far no convincing evidence has been given for cyclic electron transport in intact cells of purple bacteria.

Most of the experiments concerning the role of cytochromes in photosynthetic bacteria were done with chromatophores. In intact cells, the reactions may be different, since in chromatophores substances may be removed from or brought in contact with reaction sites. In *R. rubrum* this certainly is the case, since most of the cytochromes diffuse out of the chromatophores; therefore, in contradistinction to other workers, we have done most of our studies with intact cells.

MATERIALS AND METHODS

Cells of *R. rubrum* strain S-1 were grown anaerobically on a medium described by Slooten [27], under constant illumination, at a temperature of 30 °C. After 2–3 days, the cells were harvested and separated from the growth medium by centrifugation, suspended in fresh medium and bubbled in the dark with a mixture of N_2 and CO_2 until 10 min before the experiments were begun. Most of the experiments were done on a split beam spectrophotometer, which was on-line connected to a PDP-9 computer where the results were stored. The analysis and plotting of the spectra was in most cases done by means of this computer. During the measurements, the results could be displayed via a T.V. monitor connected to the computer. When the PDP-9 was used, the complete system had a minimum time constant of 15 μs. For measurements on a faster (2 μs) timescale we used a datalab 200 point averager DL 102.S. Xenon ($t_{\frac{1}{2}} = 10$ μs) or laser ($t_{\frac{1}{2}} = 0.3$ μs) flashes and/or continuous light (shutter opening or closing time about 3 ms) were used as actinic illumination. Absorbance difference spectra and kinetics in the infrared (750–1000 nm) were performed on a double beam spectrophotometer described earlier [28].

RESULTS AND INTERPRETATION

(*1*) *Cytochrome c-420*

(*1.1*) *Analysis of the absorbance difference spectrum 380–620 nm induced by short saturating flashes.* Absorbance changes induced by a dye laser or xenon flash show that within 5 μs after the flash, reaction center chlorophyll (P) has been photo-

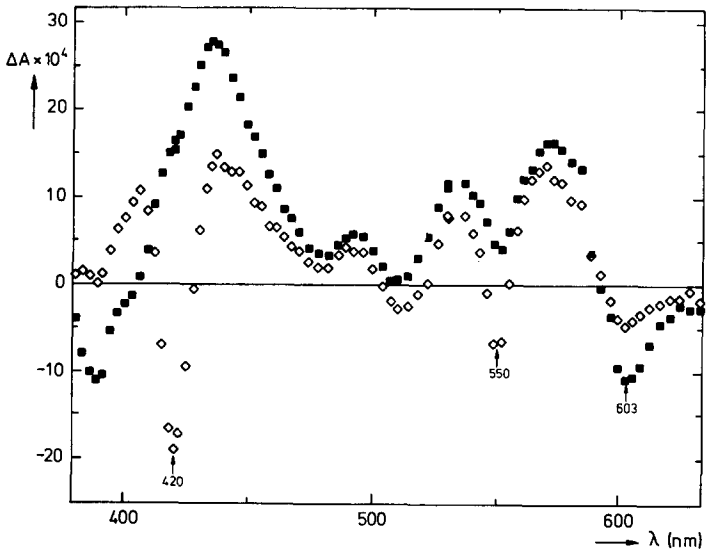

Fig. 1. Difference spectra (light-dark) induced by a saturating (100 %) xenon flash ($t_{\frac{1}{2}} = 10\,\mu s$) in whole cells of *R. rubrum*. Measurements were done under anaerobic conditions with the cells suspended in fresh medium. Repetition rate of the flashes varied from 0.03–0.06 Hz, the measuring beam ($I < 0.01$ mW/cm^2 at 420 nm) was put on 0.5 s before the experiment. Bacteriochlorophyll concentration was 7 μM, optical pathlength 5 mm, absorbance at 880–960 nm is 0.5. ■, 0.05 ms after the flash; ◇, 4 ms after the flash.

oxidized. Fig. 1 shows the difference spectrum 50 μs after the flash (solid squares). P^+ formation causes the negative peaks at 389 and 603 nm, while also a large part of the broad band around 435 is due to P^+. This band is mixed with the difference spectrum caused by a fast rising absorbance change, reflecting the electric potential generated across the membrane after the charge separation $PX \rightarrow P^+X^-$. The peaks at 490, 535 and 570 nm are characteristic for this membrane potential [6, 29]. Absorption difference spectra obtained after weak continuous illumination show the same peaks in the difference spectrum; however, a large contribution around 445 nm was also observed under these conditions.

Within 4 ms after the flash (Fig. 1, open squares) part of the absorption change at 604 nm disappears and a *c*-type cytochrome becomes oxidized (α peak at 550 nm, γ peak at 420 nm). From Fig. 1, it cannot be seen whether other pigments show absorption differences. We shall give evidence that under anaerobic conditions, two cytochromes, of which the second is present in a low concentration, are photooxidized within 4 ms after the flash. From the absorbance difference spectrum, 4 ms after the flash, it can be seen that part of the P^+ remains oxidized. This slow phase in the P^+ reduction has a half time of approx. 10 ms. A similar biphasic reduction of P^+ was observed in *R. sphaeroides* [30, 16] and has been explained by assuming that in part of the reaction centers the cytochrome c_2 occurs in different position relative to the reaction center [16]. Our studies provide quite a different explanation.

(1.2) Analysis of the redox kinetics of c-420 and P^+ upon illumination with 10-μs flashes of different intensity. Fig. 2 shows that P becomes rapidly oxidized in a flash and is subsequently partly reduced with a half time of 0.3 ms and that after the flash, a

c-type cytochrome becomes oxidized with a similar half time. We have measured the cytochrome oxidation kinetics at 406 nm because this is an isosbestic point for the initial fast absorbance change (Fig. 1); P was studied at 604 nm. There is a close

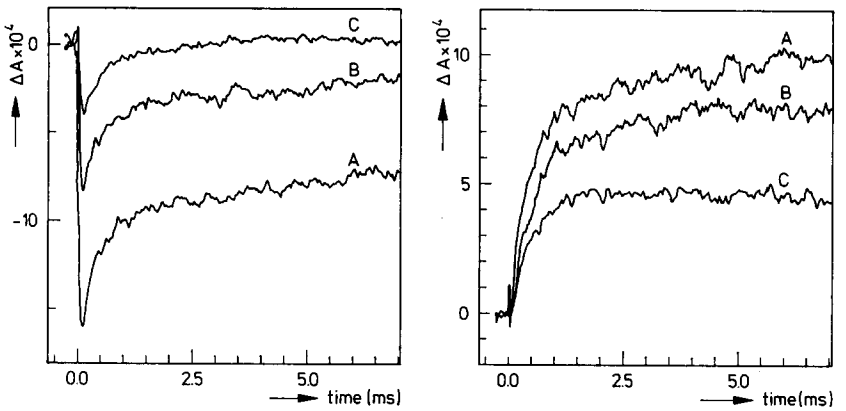

Fig. 2. Kinetics of light-induced absorption changes in whole cells of *R. rubrum*. Conditions as in Fig. 1. Left, measuring wavelength 604 nm (P): trace A, 100 % (maximum) flash intensity; trace B, 18 % flash intensity; trace C, 8 % flash intensity; Right, measuring wavelength 406 nm (*c*-420): otherwise identical to the left part.

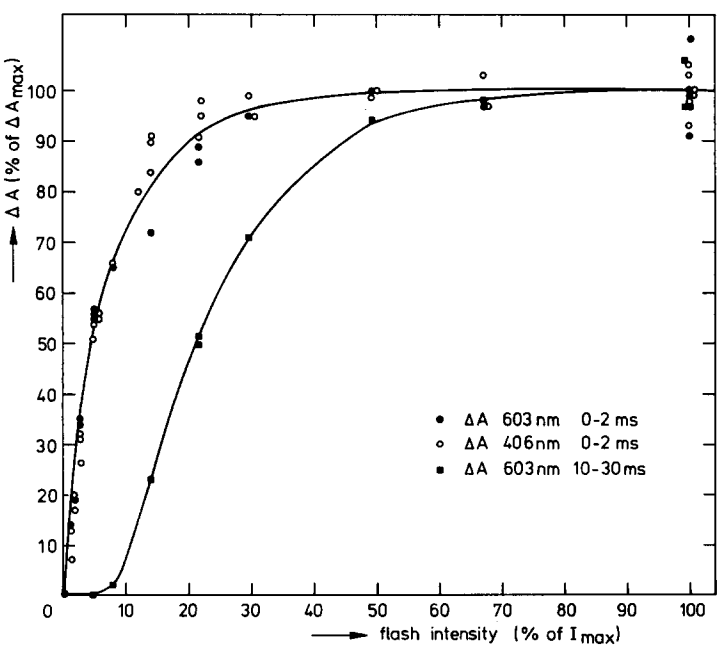

Fig. 3. Flash intensity dependence of absorption changes in whole cells of *R. rubrum*. Conditions as in Fig. 1. ●, measuring wavelength 603 nm, fraction of P^+ reduced between 0.03 and 2.0 ms after the flash; ○, measuring wavelength 406 nm, fraction of *c*-420 oxidized between 0.03 and 2.0 ms after the flash; ■, measuring wavelength 603 nm, fraction of P^+ reduced between 10.0 and 30.0 ms after the flash. All fractional absorbance changes are normalized to 100 % at maximum flash intensity.

correspondence between the fast phase of *c*-420 oxidation and P^+ reduction. The reaction seems to be approximately second order with $t_{\frac{1}{2}} = 300\ \mu s$ (this is the half time of the fast component obtained under conditions such that *c*-420 was fully reduced before a flash which oxidized all P). If we assume an extinction coefficient $\varepsilon_{\text{red-ox, 604}} = 15\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ [31] for reaction center chlorophyll at 604 nm and an extinction coefficient $\varepsilon_{\text{red-ox}} = 20\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ [32] for *c*-420 at 550 nm, we find that between 30 μs and 5 ms after the flash, equal amounts of P^+ and *c*-420 have been reduced and oxidized, respectively.

To obtain information about the slow component of the P^+ reduction we compared both P^+ and *c*-420 kinetics at different flash intensities .Fig. 2 (traces B and C) shows the kinetics of P^+ reduction and *c*-420 oxidation at flashes with 18 and 8 % of maximum flash intensity. It can be seen that at 8 % flash intensity (45 % of the saturation amplitude for *c*-420), only the fast component is apparent in the reduction of P^+. As a function of flash intensity, the slow component of the P^+ reduction (Fig. 3, $\Delta A_{604\ \text{nm}}$ 10–30 ms) decreases much faster than the rapid component (Fig. 3, $\Delta A_{604\ \text{nm}}$ 0.03–2 ms). At various intensities, the amount of *c*-420 oxidized between 0.03 and 2 ms after the flash, measured at 406 nm, is proportional to or presumably the same as the amount of P^+ reduced in the same time (Fig. 3).

The biphasic reduction of P^+ and in general the kinetics given in Fig. 2 (after 30 μs) and in Fig. 4 can be described by a model in which one assumes that the *c*-420 present can react with at least two P^+ molecules and that the concentration of ($P+P^+$) is twice that of (*c*-420+c^+-420). We have studied the two extreme possiblities of this hypothesis.

(1) The *c*-420 present can freely diffuse between the P's, which can be described by the second-order reaction:

$$P^+ + c\text{-}420 \xrightarrow{k_a} P + c^+\text{-}420 \qquad \text{(a)}$$

Furthermore we assume that c^+-420 reacts with a donor present in excess after one flash which gives us the first-order reaction:

$$c\text{-}^+420 \xrightarrow{k_b} c\text{-}420 \qquad \text{(b)}$$

(2) *c*-420 occurs in a complex with two P's and can react only with these, resulting in the first-order equations:

$$P^+P^+c\text{-}420 \xrightarrow{k_c} PP^+\ c\text{-}420 \qquad \text{(c)}$$

$$c^+\text{-}420 \xrightarrow{k_d} c\text{-}420 \qquad \text{(d)}$$

$$PP^+\ c\text{-}420 \xrightarrow{k_e} PP\ c^+\text{-}420 \qquad \text{(e)}$$

Computer simulations of the kinetics for the two sets of Eqns. a, b and c, d, e are given in Figs. 4A and 4B, respectively; the *k* values were derived from the experiments. The measured *c*-420 and P^+ kinetics are displayed in Fig. 4C. We shall point at two significant differences between both descriptions. First, the reduction rate of P^+ after a saturating flash, approximately at a time when about 25 % P^+ is in the oxidized state, differs markedly for both models (Table I, first row). Second, the amount of oxidized *c*-420, at a time when P^+ is almost completely (95 %) rereduced after a 100 % flash

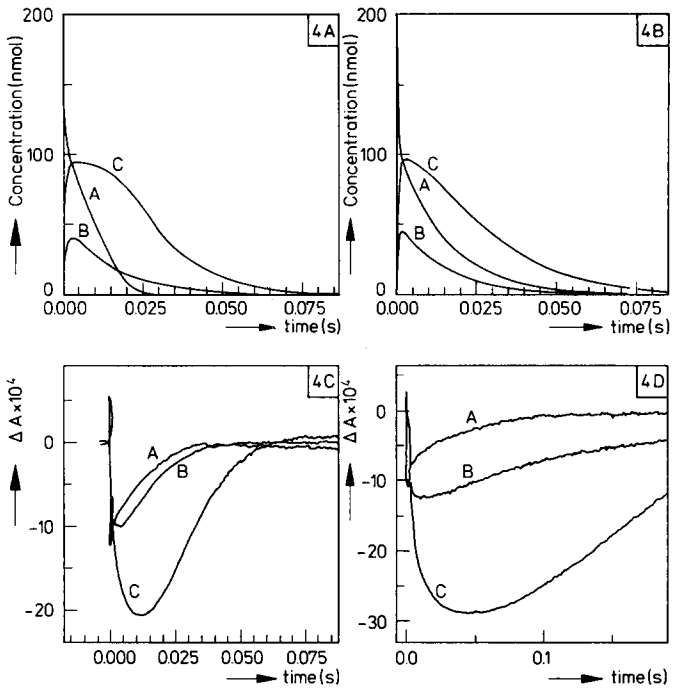

Fig. 4. Comparison of the two models (see text) for the reduction of P^+ by *c*-420 with in vivo measured kinetics. Conditions as in Fig. 1. (A) Diffusable cytochrome model, which is based on the assumption that each *c*-420 molecule can diffuse freely in the cell and can react with any P^+. The actual concentrations of P and *c*-420 at the reaction site are unknown. However, the result of the simulation of Eqn. a and Eqn. b is defined by the ratio of the initial concentrations of *c*-420 and P^+ and the initial rate of P^+ reduction. From this initial rate, we calculated $k_a = 12.5\ \text{nmol}^{-1} \cdot \text{s}^{-1}$, assuming the concentrations $P_{t=0}{}^+ = 200$ nmol and $c\text{-}420_{t=0} = 100$ nmol. Furthermore, we took $k_b = 65\ \text{s}^{-1}$. Trace A, P^+ after a 100 % flash; trace B, C^+-420 after a weak (8 %) flash; trace C, c^+-420 after a 100 % flash. The ordinate refers to the concentration of each component in nmol. (B) Bound cytochrome model, which is based on the assumption that one reduced *c*-420 molecule is bound to two reaction centers. The values for the reaction centers were: k_c (Eqn. c) = k_e (Eqn. e) = $25 \cdot 10^2\ \text{s}^{-1}$, $k_d = 65\ \text{s}^{-1}$. Otherwise identical to A. (C) In vivo-measured kinetics of absorbance changes of P^+ (at 604 nm) after a 100 % flash (trace A), c^+-420 after a weak (8 %) flash (trace B) and c^+-420 after a 100 % flash (trace C). (D) Same as C, except for the addition of 10^{-5} M HOQNO.

(Table I, second row) is relative large in the diffusable cytochrome model. If we compare both descriptions with the experimental results, it is obvious that both models show noteworthy deviations. Analogous simulations in which we varied the number of reaction centers each *c*-420 can react with, indicated that our kinetics are consistent with the hypothesis that each *c*-420 can react with approximately four P^+ molecules.

The proposed scheme, in which the *c*-420 can diffuse among a set of P^+ molecules, is also in accordance with the observation that all the *c*-420 disappears in the solution when the cell membrane is disrupted by French pressing or sonicating the cells.

Our description predicts approximately equal reaction half times for the slow phase of P^+ reduction after a 100 % flash and for c^+-420 reduction after a weak (8 %)

TABLE I

COMPARISON OF EXPERIMENTAL KINETICS OF c^+-420 AND P^+ WITH THE SIMULATED MODELS

The columns represent: model 1 (Eqns. a and b), model 2 (Eqns. c, d and e) and the experimental data. The first row gives the slope of P^+ reduction at 25 % oxidation level of P^+ (A.U.), the second row the fraction of c^+-420 at the time that P^+ is 95 % reduced. $P_r = P^+/P^+_{max}$, $C_r = c^+\text{-}420/c^+\text{-}420_{max}$.

	Diffusable cytochrome (Fig. 4A)	Bound cytochrome (Fig. 4B)	Experimental results (Fig. 4C)
$\frac{dP_r}{dt}$	23	10	17
C_r	85 %	32 %	60 %

flash (Fig. 4C, traces A and B). This is generally true as long as c^+-420 reduction is quite fast. If, however, c^+-420 reduction is slowed down to a reaction half-time greater than 80 ms (e.g. by the addition of HOQNO, Fig. 4D, or by lowering the temperature), the slow component of the P^+ reduction remains approx. 50 ms. Under these conditions the remaining P^+ is presumably reduced via another pathway, possibly by the acceptor side in a back reaction. However, this is not the direct back reaction $P^+X^- \rightarrow PX$ because the recovery of the photooxidation of P at a second flash is only determined by the amount of reduced P, suggesting that the X^- oxidation is faster. This 50 ms decay of P^+ after a saturating flash, under conditions where c^+-420 reduction is inhibited, is more or less temperature-independent (−20 °C–+20 °C), suggesting a back reaction between P^+ and a secondary acceptor pool.

(2) Cytochrome c-428

(2.1) Absorbance difference spectra induced by non-saturating flashes. As we have indicated already in paragraph 1.1, it is very difficult to see from absorbance difference spectra obtained shortly after a saturating flash (Fig. 1) whether or not other cytochromes have been photooxidized after the flash. However, by analyzing the absorbance difference spectra induced by weak flashes in the α and γ region of the cytochrome bands, we were able to identify other cytochrome bands.

If we look at the difference spectrum (Fig. 5, solid squares) measured 4 ms after a weak flash (approx. 50 % saturating for *c*-420), the oxidation of *c*-420 is again evident. However, the absorbance difference spectrum after 4 ms is not identical to the reduced-oxidized difference spectrum as measured with pure *c*-420 [32] or to the *c*-420 difference spectrum measured by Sybesma and Kok [7] in flashing light. Careful analysis of the difference spectrum indicates that another cytochrome (γ peak at 428 nm) has been photooxidized. Background illumination of very low intensity was sufficient to keep this cytochrome photooxidized. Under these conditions, we measured the exact *c*-420 difference spectrum 4 ms after the flash (Fig. 5, open oblique squares).

The absorbance difference spectrum induced by this weak actinic light (Fig. 6) shows clearly the photooxidation of a cytochrome with reduced-oxidized peaks at 428

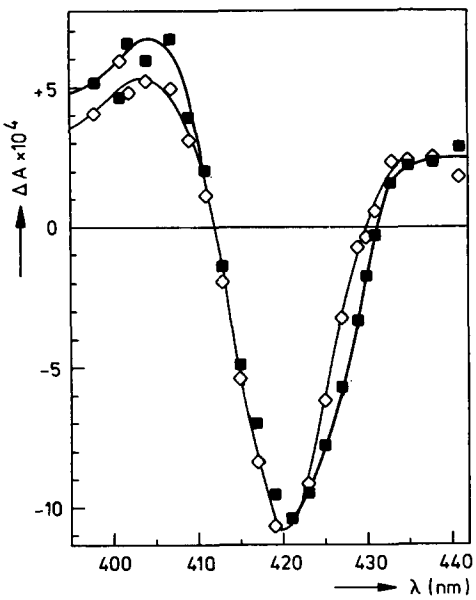

Fig. 5. Absorbance difference spectrum (light-dark) in whole cells of *R. rubrum* induced by a non-saturating (8 %) flash. Conditions as in Fig. 1. ■, 4 ms after the flash; ◇, 4 ms after the flash in the presence of 0.05 mW/cm^{+2} (λ = 880 nm) background illumination.

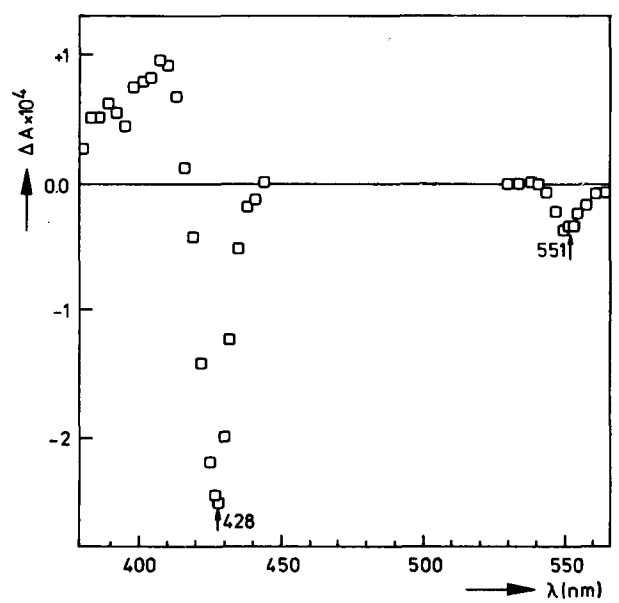

Fig. 6. Difference spectrum (light-dark) in whole cells of *R. rubrum*, induced by weak continuous light (I = 0.05 mW/cm^2, λ = 880 nm), 1 s after the onset of the illumination. Conditions as in Fig. 1.

and 551 nm. This absorbance difference spectrum is identical to the one obtained by Duysens [1] in anaerobic *R. rubrum* with weak continuous illumination. The maximum amount of *c*-428 photooxidized after a flash is about 1 per 1000 antenna chlorophylls. The half time for *c*-428 photooxidation is approx. 0.6 ms, which we estimated from the kinetics at 430 nm after a weak (8 %) flash.

(*2.2*) *Flash intensity dependence of c-428 photooxidation.* Fig. 7 (solid circles) shows the fraction of *c*-428 photooxidized 350 ms after flashes of varying intensity. The reduction of c^+-428 is slow ($\pm$0.7 s half time), so this gives 80 % of the maximum value, which was $3 \cdot 10^{-4}$ ΔA (100 % for solid circles in Fig. 7). Fig. 7 shows an identical experiment for the fraction of *c*-420 photooxidized after flashes of varying intensity (open circles, 100 % for the open circles corresponds to a ΔA of $20 \cdot 10^{-4}$). From this experiment it is clear that *c*-428 photooxidation occurs at much lower flash intensities than *c*-420 photoxidation. From the maximal absorbance change after a flash for both components, we calculated that the amount of *c*-420 photooxidized after a saturating flash is approx. 10 times the amount of *c*-428. The same ratio of the amplitudes for both cytochromes was obtained in experiments with continuous light. Fig. 7 (open squares) shows the flash intensity dependence for *c*-420 photooxidation if *c*-428 is kept in the oxidized state before the flash by weak background illumination. The slope of this curve is 1.4 times greater than in the case where *c*-428 is in the reduced state before the flash.

The results displayed in Fig. 7 can be explained by assuming that *c*-428 functions as an electron donor to P^+ in a reaction center where *c*-420 cannot play this role.

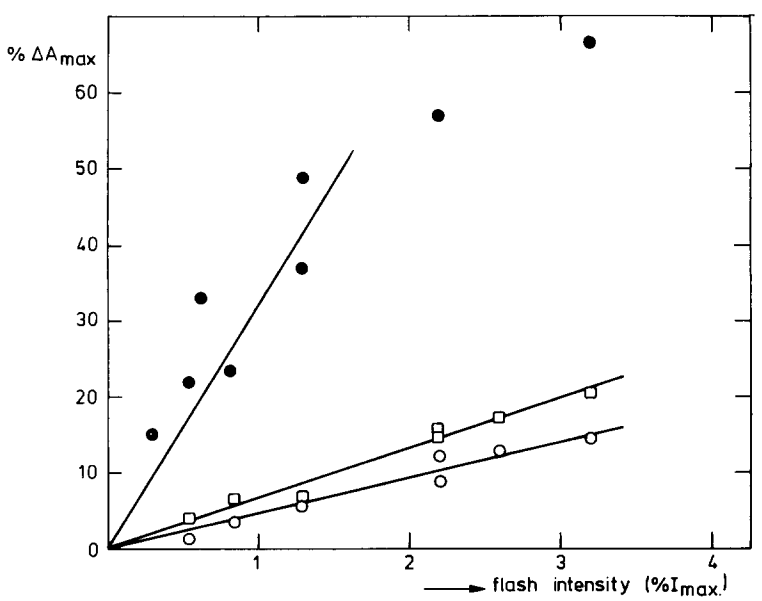

Fig. 7. Flash intensity dependence of *c*-420 and *c*-428 photooxidation in whole cells of *R. rubrum*. Conditions as in Fig. 1. All absorbance changes are normalized at maximum flash intensity to 100 %. ○, λ = 419 nm, fraction of c^+-420 reduced between 10 and 100 ms after the flash; □, λ = 419 nm, fraction of c^+-420 reduced between 10 and 100 ms after the flash in the presence of weak background illumination (I = 0.05 mW/cm², λ = 880 nm); ●, λ = 430 nm, amount of c^+-428 350 ms after the flash.

The *c*-428 reaction center differs from the *c*-420 center in that it is associated with approx. 10–12 times more antenna bacteriochlorophyll as estimated from the slopes in Fig. 7. If we assume that there is one *c*-428 per reaction center, and we make use of our knowledge that there is approximately one *c*-420 per two reaction centers (section 1.2), we can estimate that the number of reaction centers in which *c*-428 functions as a donor is about 5 % of the total. In case we oxidize *c*-428 before the flash by weak background illumination, sufficient to keep the 5 % special traps closed, the incident energy is efficiently transferred to the remaining centers, which results in a steeper slope for the amount of *c*-420 photooxidized versus flash intensity. Ratios of amounts of bacteriochlorophyll, reaction centers and cytochrome are summarized in Fig. 14.

(*2.3*) *Reaction center chlorophyll oxidation in weak continuous light.* To find more evidence for our assumption (2.2) that *c*-428 is connected to a reaction center where reduced *c*-420 is unable to function as an electron donor, we have tried to measure P^+ formation in the infrared part of the spectrum in low intensity actinic light, low enough to keep *c*-420 completely reduced. The light-induced infrared absorption changes (actinic light $I = \leqq 2.0$ mW/cm^2 at 600 nm) were biphasic. Fig. 8 (solid circles) shows the light-dark difference spectrum 1 s after the onset of the

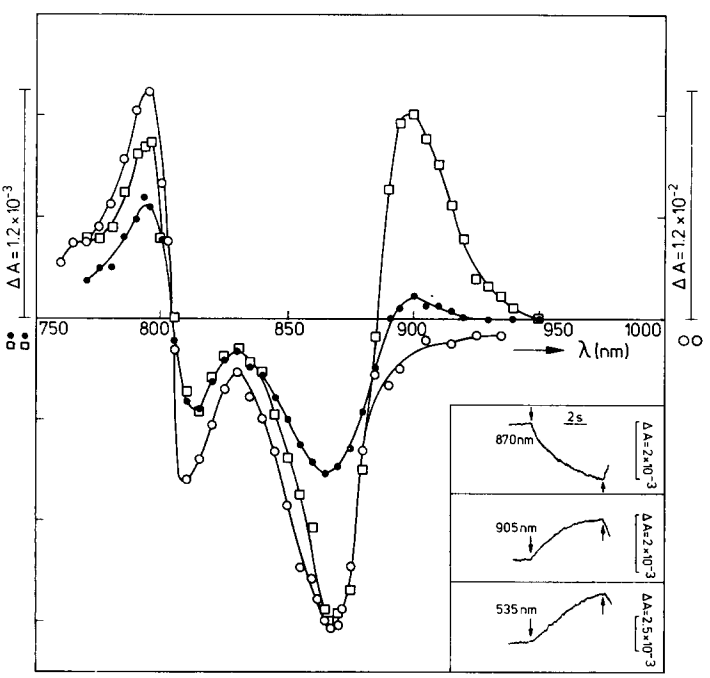

Fig. 8. Absorbance difference spectra (light-dark) induced by continuous light in whole cells of *R. rubrum*, anaerobic conditions, bacteriochlorophyll concentration 35 μM, optical pathlength 1 mm. ●, $I = 1.0$ mW/cm^2, $\lambda = 600$ nm, 1.0 s after the onset of illumination; □, $I = 1.0$ mW/cm^2, $\lambda = 600$ nm, 10.0 s after the onset of the illumination; ○, $I = 10.0$ mW/cm^2, $\lambda = 600$ nm, 3 s after the onset of the illumination. N.B. The lefthand scale applies to the ● and □ symbols, while the right-hand scale applies to the ○ symbols. Insert lower right: absorbance changes at 870, 905 and 535 nm induced by low intensity continous light in whole cells of *R. rubrum*. $I = 1.0$ mW/cm^2 ($\lambda = 600$ nm).

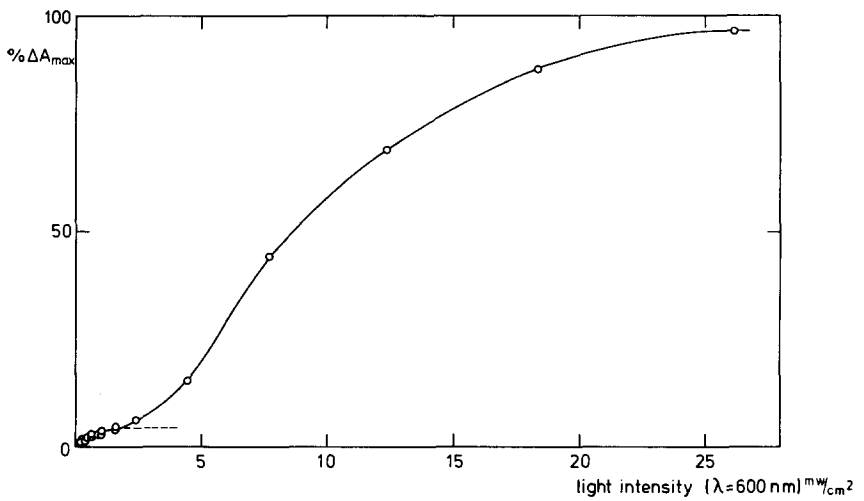

Fig. 9. Reaction center chlorophyll photooxidation as a function of continuous light intensity in whole cells of *R. rubrum*. Conditions as in Fig. 8. Measuring wavelength 793 nm, wavelength of actinic illumination 600 nm. Absorbance changes are normalized to 100 % at maximum actinic light intensity.

weak illumination. This difference spectrum indicates the bleaching of a pigment around 870 nm, accompanied by a band shift around 800 nm. The spectrum is similar to the difference spectrum published by Duysens [33] and attributed to P oxidation, but in our spectrum a small contribution of a shift of a band around 878 is superimposed on the *P*-870 bleaching. After 10 s of illumination (open squares) a much larger contribution of the shift, of the absorption band at 878–892 nm is apparent. The kinetics of this band shift are identical to the kinetics at 490, 535 and 570 nm (Fig. 8, insert) and we conclude that this band shift is also associated with the transport of charges across the membrane. Fig. 8 (open circles) shows the difference spectrum induced by 3 s of strong light, and again we see the *P*-870 bleaching accompanied by the *P*-800 band shift. Again the shift of the *P*-878 bacteriochlorophyll band is superimposed on the P^+ difference spectrum.

Fig. 9 gives the amount of P^+ formed 0.2 s after the onset of continuous illumination as a function of actinic intensity. This curve consists of two parts, one part represents approx. 4–5 % of the reaction centers and is saturated (i.e. reaches a stationary level) at an intensity of about 2.0 mW/cm^2 (λ = 600 nm), the other part is saturated at an intensity of 30–40 mW/cm^2 (λ = 600 nm).

Cytochrome *c*-428 (saturation intensity 1.0 mW/cm^2) is associated with 5 % of the reactions centers, *c*-420 (saturation intensity 25.0 mW/cm^2) with the remaining 95 %.

This experiment is consistent with our hypothesis that approx. 5 % of the reaction centers are different from the others in having a slower reaction leading to the reduction of P^+. Finally, we have measured the amount of P^+ formed on a second flash 100 ms after a first flash. Because *c*-428 is still in the oxidized state at the moment of the second flash, but *c*-420 is not, the reaction centers associated with *c*-428 will remain in the oxidized state (reduction half time 0.7 s). We found again that 4–5 % of P^+ was reduced with this half time after the second flash.

(3) Cytochrome c-560

(3.1) Absorption difference spectra induced by short flashes in the α and γ region of the cytochrome bands. Fig. 10 shows that between 4 and 25 ms after a non-saturating flash, c^+-420 becomes partly rereduced as indicated by the disappearance of the band at 420 nm, while in the same time interval a band around 428 nm appears, which is, as we will show, due to the oxidation of a cytochrome different from *c*-428, described in the previous section. This is confirmed by the difference spectrum (Fig. 11) taken 100 ms after the flash minus darkness. After 100 ms, c^+-420 has been rereduced. The spectrum is rather typical for a cytochrome with a negative peak at 428 nm. However, the α peak is double, one peak at 551 nm and the other at 560 nm, indicating the presence of two oxidized cytochromes. To prevent confusion about the notation, we have called this second cytochrome (α peak at 560 nm, γ peak 428 nm) *c*-560.

Fig. 12 shows the oxidation and reduction kinetics at 430 nm, the isosbestic point for the *c*-420-oxidized-reduced difference spectrum, after a weak (8 %) flash. The kinetics at this wavelength also reflected partly the decay of the membrane potential ($t_{\frac{1}{2}}$ = 20 ms) but the contribution of this component is small [6]. The cytochrome reduction kinetics (Fig. 12, middle trace A) are biphasic. The very slow phase ($t_{\frac{1}{2}}$ = 0.7 s) is caused by c^+-428 (α peak at 551 nm), the rapid phase ($t_{\frac{1}{2}}$ = 60 ms) stems presumably from the reduction of c^+-560. The oxidation kinetics are also biphasic and can be explained in a similar way. The fast decreasing part reflects the oxidation of *c*-428 ($t_{\frac{1}{2}}$ = 0.6 ms). *c*-428 oxidation can be prevented by giving weak background illumination (section 2.1, Fig. 5). The slowly increasing part is due to the oxidation of *c*-560 ($t_{\frac{1}{2}}$ = 7 ms). These kinetics and the absorbance difference spectrum indicated

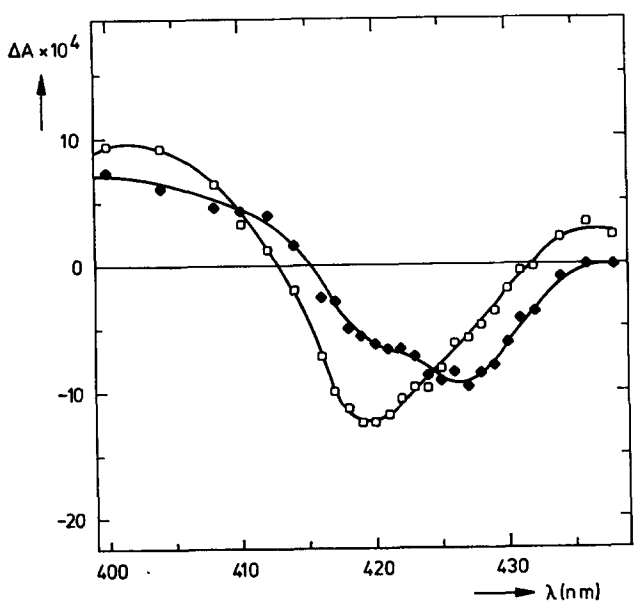

Fig. 10.Absorbance difference spectra (light-dark) induced by non-saturating flashes (8 % of maximum intensity) in whole cells of *R. rubrum*. Conditions as in Fig. 1. □, 4 ms after the flash; ◆, 25 ms after the flash.

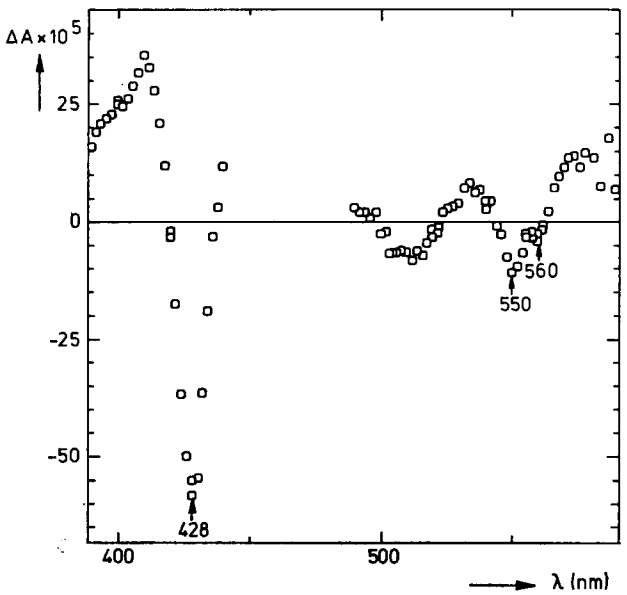

Fig. 11. Absorbance difference spectrum (light-dark) induced by saturating flashes in whole cells of *R. rubrum*, 100 ms after the flash. Conditions as in Fig. 1.

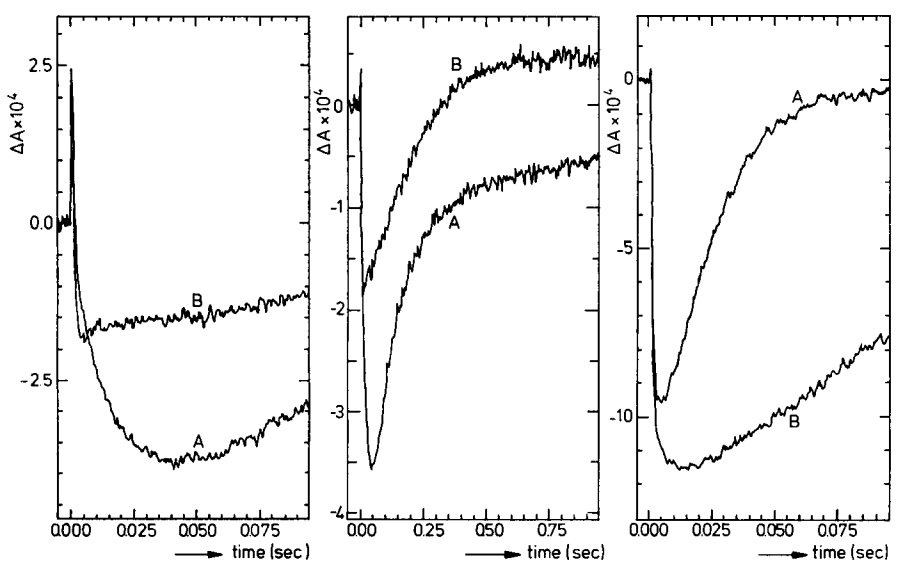

Fig. 12. Kinetics of *c*-560 and *c*-420 redox reactions induced by non-saturating flashes (6 % of the maximum intensity) in whole cells of *R. rubrum* in the absence (trace A) and presence of 10^{-5} M HOQNO (trace B). Other conditions as in Fig. 1. Left and middle; $\lambda = 430$ nm, different time scales; right: $\lambda = 419$ nm.

strongly that the cytochrome peak at 428 nm, which up to now has been interpreted as one component, is in fact caused by the oxidation of two cytochromes, *c*-428 and *c*-560.

(3.2) The effect of HOQNO and antimycin A on c-420 and c-560. Fig. 12 (left, trace B) shows the absorbance change at 430 nm in the presence of HOQNO. After the first initial oxidation of *c*-428 no oxidation of *c*-560 takes place. From Fig. 12 (middle, trace B) we can see that the reduction of c^+-560 still occurs, the half time having increased to approx. 100 ms by the addition of HOQNO. These experiments suggest that *c*-560 is normally reduced in the dark, but that in the presence of HOQNO, *c*-560 becomes oxidized. The reduction of c^+-560 after a flash ($t_{\frac{1}{2}} = 60$ ms, normal conditions) probably occurs via a secondary acceptor because the reoxidation time of X^-, as estimated from P^+ formation on a second flash, was at least faster than P^+ reduction ($t_{\frac{1}{2}} \leqq 0.3$ ms). Fig. 12 (right) shows c^+-420 reduction in the absence (trace A) and presence (trace B) of HOQNO. The reduction of c^+-420 is slowed down at least six times by HOQNO ($t_{\frac{1}{2}} = 80$ ms, in the presence of 10^{-5} M HOQNO).

Antimycin A has more or less the same effect as HOQNO on the *c*-560 reactions. Again c^+-560 becomes reduced after a flash with a half time of 50–80 ms, the reoxidation being slow ($t_{\frac{1}{2}} = 2$ s). The peak in the difference spectrum (not shown) shifts 4 nm to longer wavelengths (from 428 to 432 nm). This has been observed before with antimycin-treated mitochondrial cytochrome *b* [34]. HOQNO has an identical effect on the shape of the difference spectrum. Antimycin A increases the half time of c^+-420 reduction by only 30 %.

These results can be explained by the following scheme. *c*-560 which is present in a low concentration reduces c^+-420. Assuming an extinction coefficient of $\varepsilon = 20\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ at 560 nm [35], we estimate from the absorbance difference spectrum, that one *c*-560 is present per 350 antenna chlorophyll molecules, or one per 3–4 molecules *c*-420. Furthermore, we assume that c^+-420 is not only reduced by *c*-560 ($t_{\frac{1}{2}} = 7$ ms), but also by a large reduced pool ($t_{\frac{1}{2}} = 15$ ms). Both pathways are inhibited by HOQNO, which is known to inhibit electron transport from cytochrome *b* to cytochrome *c* in mitochondria [36] and also prevents the functioning of the cytochrome *c*-succinate reductase [37]. From this, we conclude that the pool which reduces c^+-420 may be succinate. Antimycin A stops the reduction of c^+-420 by *c*-560, and causes the oxidation of *c*-560 in the dark. A similar antimycin-induced reduction of a *b*-type cytochrome has been observed in chromatophores of *R. rubrum* [24]. Possibly *c*-560 functions as an electron and proton carrier across the membrane [38]. Antimycin and HOQNO presumably keep *c*-560 on the acceptor side of the membrane, where it remains in the oxidized state in the dark.

(3.3) c-560 and c-420 oxidation at different flash intensities. If the assumption we made in section 3.2 is correct, i.e. that c^+-420 can be reduced by *c*-560 ($t_{\frac{1}{2}} = 7$ ms) and by a reduced donor pool ($t_{\frac{1}{2}} = 15$ ms), we would expect that at low flash intensities a relatively large amount of *c*-560 will become photooxidized. Fig. 13 shows the flash intensity dependence of the 60 ms component at 430 nm, and this experiment indicates that *c*-560 becomes preferentially photooxidized by c^+-420 at low flash intensities. This curve can be explained if we assume that there is one *c*-560 per three molecules *c*-420, which agrees reasonably with our estimation in section 3.2. Furthermore, the half time of c^+-420 rereduction decreases with the flash intensity to values

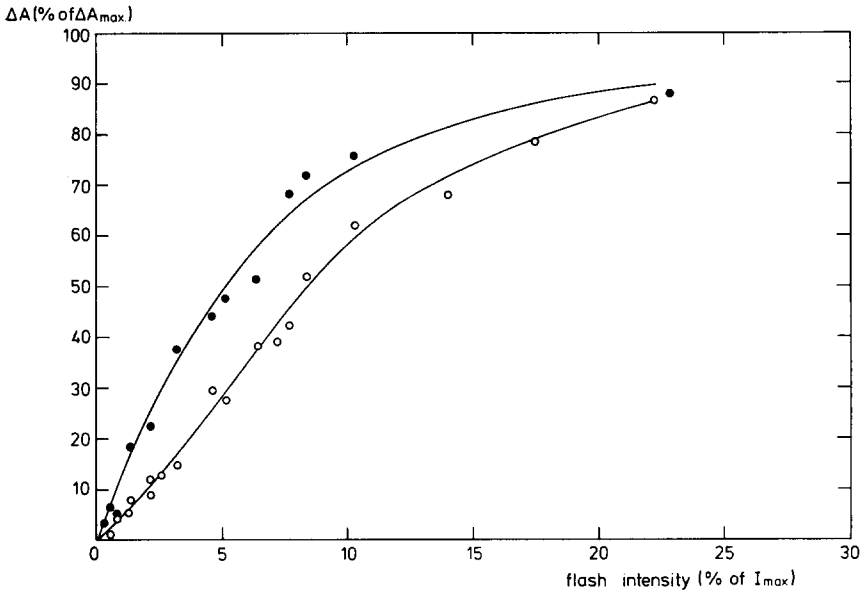

Fig. 13. Flash intensity dependence of *c*-560 and *c*-420 photooxidation in whole cells of *R. rubrum*. Conditions as in Fig. 1. All absorbance changes are normalized at maximum flash intensity to 100 %. ●, measuring wavelength 430 nm, fraction of c^+-560 reduced between 50 and 200 ms after the flash; ○, measuring wavelength 419 nm, fraction of c^+-420 reduced between 10 and 100 ms after the flash.

in the order of 8 ms (at high intensity, this is about 12 ms). Again, this agrees reasonably with the assumptions we made for the *c*-560 and *c*-420 redox reactions.

DISCUSSION

We have summarized our conclusions concerning the electron transport chain of *R. rubrum* in Fig. 14. *c*-420 functions as the secondary electron donor in approx. 95 % of the reaction centers. The reduction kinetics of P^+ can be explained if we assume that there is one *c*-420 per two reaction centers. The cytochrome is present in a relative low concentration compared to other photosynthetic bacteria [15–17, 39]. In other photosynthetic organisms, it has been found that the cytochrome concentration depended strongly on the culture conditions [40, 41]. We varied these conditions by culturing cells on different growth media, or under low or high light intensity, but were unable to increase the amount of *c*-420 significantly.

From the kinetics, it was concluded that *c*-420 can diffuse among a small number of P molecules. This is consistent with the observation that all the cytochrome disappears in the supernatant after disrupting the cell membrane by sonication or French pressing. When we analyzed the supernatant obtained after such a treatment, we found that the total amount of *c*-420 released is again about 1 per 80–100 bacteriochlorophyll molecules, which is about equal to the fraction photooxidized in the intact cell. The location of *c*-420 in the cell may be similar to that recently suggested for cytochrome c_2 in *Rhodopseudomonas capsulata* [42].

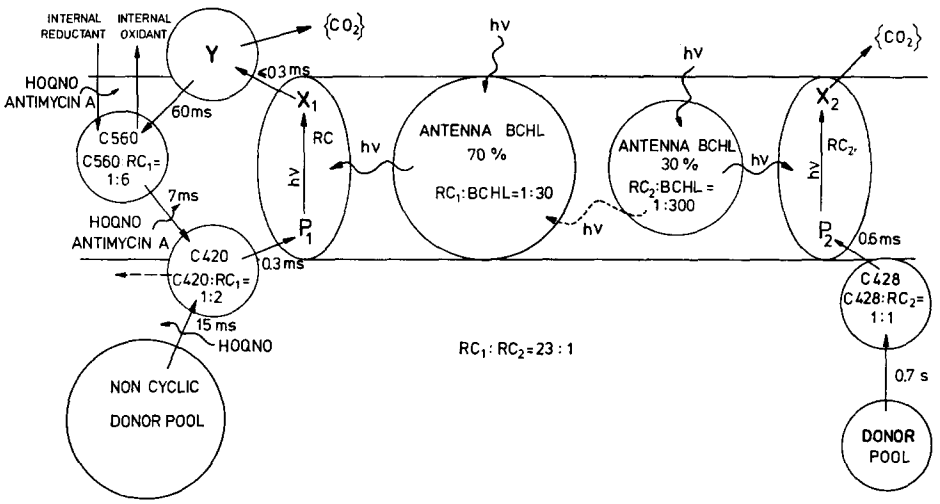

Fig. 14. Scheme for electron transport in whole cells of *R. rubrum* and the localization of the different components in the membrane. Solid arrows give the direction of electron transport, dashed arrows indicate that molecules can move to other reaction centers. RC_1 is the reaction center which contains approx. 30 molecules antenna chlorophyll. There is one *c*-420 molecule per two reaction centers and one *c*-560 molecule per six reaction centers. The non-cyclic reduced pool contains many reducing equivalents per reaction center. RC_2 is the reaction center which contains about 300 molecules antenna chlorophyll, there being one *c*-428 per reaction center. BCHL, bacteriochlorophyll.

The photoreduction of c^+-560, the rereduction of c^+-420 by *c*-560 and the photooxidation of *c*-420 show that *c*-420 is involved in cyclic electron transport via *c*-560. The relatively high rate of reduction of c^+-420 remaining after the oxidation of *c*-560 by a saturating flash and the inhibition of this reaction by HOQNO indicates that c^+-420 is also reduced by another donor via a cytochrome *c* reductase. A similar situation is encountered in *Chromatium* when cytochrome *c*-555 (*c*-422), the presumed analogue of *c*-420, is involved in cyclic and non-cyclic electron transport [43]. The observation that the presumed non-cyclic rereduction of c^+-420 is rather fast (10–20 ms half time) and that this high reduction rate is hardly influenced by long periods of strong continuous illumination indicates that a large donor pool is present, which is replenished by external donors.

c-560, which functions with *c*-420 in this cyclic electron transport chain, could be an electron carrier moving across the membrane. The midpoint potential of this cytochrome may be energy dependent [26] and in that case the amount of reduced *c*-560 could depend on the ATP and ADP concentration. This might provide a tool for the cell to regulate the rate of cyclic phosphorylation.

c-428 functions in 5 % of the reaction centers. Because of their large amount of antenna chlorophylls, these reaction centers are photooxidized preferentially at very low light intensities. *c*-428 probably has a low midpoint potential [44], and its reduction is slow ($t_{\frac{1}{2}} = 0.7$ s). To find a physiological explanation for such an arrangement, we recall that a photoelectric device which is constructed to operate at very low light intensity with a high efficiency, will not operate at high light intensity with maximal efficiency unless the collector is exchanged for one with a lower work function [45].

An analogous arrangement may permit *R. rubrum* to convert energy with optimal efficiency at very low intensity by means of *c*-428 and at high light intensity by *c*-420.

Previous publications by several authors [5, 7, 18] already suggested the existence of more than one type of reaction center in *R. rubrum*. These suggestions were mainly based on the different action spectra obtained for the photooxidation of *c*-420 and *c*-428. We did not check these action spectra extensively but a control experiment at some wavelengths indicated that the action spectra for *c*-428 and *c*-420 photooxidation may be identical. Furthermore the positive peak in the absorption difference spectrum at 905 nm, ascribed to the photooxidation of this special reaction center [44], can be explained in our view as the shift of the bacteriochlorophyll absorbance band at 878 nm to longer wavelengths, induced by the transport of charges across the membrane. A similar suggestion was made by Barskii and Samuilov [46] who measured this band shift and the carotenoid shift in the presence and absence of uncouplers and gave evidence that both might be related to proton transport. The oxidation of the 5 % of P associated to *c*-428 induces a difference spectrum identical to the difference spectrum caused by the oxidation of the remaining 95 % of P. Whether or not the scheme we have presented is applicable to other photosynthetic bacteria is not clear at this moment. In our experiments we could photooxidize even in weak flashes both *c*-420 and *c*-428. Parson and Case [11] and Seibert and DeVault [39] showed for example in *Chromatium* that the first and the second flash always resulted in the photooxidation of cytochrome *c*-552. Only when *c*-552 was oxidized before the flash, *c*-555 could be photooxidized. This suggests that in *Chromatium* both cytochromes are connected to the same reaction center. However, evidence has recently been presented [47, 48] suggesting that in *Chromatium* *c*-555 and *c*-552 function in different reaction centers, which differed also in other aspects, such as the primary acceptor. Our own results indicate that *c*-420 and *c*-428 are present in different reaction centers, and that the reaction centers oxidizing *c*-428 are associated with more antenna bacteriochlorophyll than those oxidizing *c*-420.

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