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FLASH-INDUCED PHOTOPHOSPHORYLATION IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

I. THE RELATIONSHIP BETWEEN CYTOCHROME *c*-420 CONTENT AND PHOTOPHOSPHORYLATION

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Summary

The content of cytochrome *c*-420 in *Rhodospirillum rubrum* chromatophores prepared by grinding with alumina is 5—10% of that in whole cells, and 20—40% in chromatophores by 'French' pressing.

Flash-induced phosphorylation of various chromatophores which varied in cytochrome content from 7 to 40% is proportional to the cytochrome content. Extrapolating the cytochrome *c*-420 content to that observed in whole cells, a ratio ATP/P^+X^- near 1 is calculated. At low flash intensity the phosphorylation per flash is proportional to flash energy.

Photophosphorylation in flashes given after a time of several minutes is only slightly dependent on the number of flashes. If the flashes are spaced from 0.1 to 10 s, relative phosphorylation in the first flash is about 70% and in the second 90% of that observed in the following flashes. Proton binding is not affected by the cytochrome *c*-420 content and a ratio of H^+/P^+X^- of 2.3 was found.

These results can be explained by a working hypothesis in which charge separation occurring at one reaction centre and the resulting electron transport mediated amongst others by *c*-420, results in the injection of two protons into an ATPase, this in contrast to a chemiosmotic mechanism, where the protons are released in the chromatophore inner space.

Abbreviations: Bchl, bacteriochlorophyll; *P* or *P*-870, reaction centre bacteriochlorophyll dimer; X, primary acceptor.

Introduction

The primary reaction in photosynthetic bacteria involves the oxidation of the reaction centre bacteriochlorophyll dimer *P*-870 [1] and the reduction of the long wavelength bacteriopheophytin, I [2,3]; I^- reduces an iron · quinone complex, X [4], whereas $P\text{-}870^+$ is re-reduced by a high potential cytochrome *c* [5].

Both in whole cells of *Rhodospirillum rubrum* and *Chromatium vinosum* it has been demonstrated that a soluble cytochrome *c*, which functions as a mobile electron carrier, plays an important role in cyclic and non-cyclic electron transport [6,7]. In *Rhs. rubrum* the mobile cytochrome *c*-420 is the direct reductant of oxidized *P*-870 (P^+); it is present in a ratio of 0.5 cytochrome *c*-420 to reaction centre bacteriochlorophyll and one cytochrome *c*-420 molecule reduced by cyclic or non-cyclic electron transport is capable of reducing at least two oxidized *P* molecules [6].

In *Chr. vinosum* the mobile cytochrome *c*-551 reduces the oxidized high potential cytochrome *c*-555, which in its turn is capable of reducing P^+ in 2 μs. Cytochrome *c*-551 is present in a ratio of approx. 0.7 cytochrome *c*-551 to reaction centre and is, analogous to cytochrome *c*-420, also capable of reducing at least two oxidized cytochrome *c*-555 molecules bound at different reaction centres [7].

These conclusions based on an analysis of the kinetics of the mobile cytochromes agreed very well with the assumed location of the mobile cytochrome in the periplasmic phase between the cell wall and the cell membrane [8] and with the fact that both cytochromes *c*-555 and *c*-420 are easily liberated into the solution when chromatophores are prepared by solic oscillation or French-pressing [6,9].

As already mentioned, in whole cells, cytochrome *c*-420 is involved in the cyclic and non-cyclic flow [6,7] and so in chromatophores the loss of cytochrome *c*-420 or *c*-551 during cell breakage may be expected to alter the physiological functions of the cyclic system.

To find out the possible importance of this mobile cytochrome carrier we have studied flash-induced photophosphorylation (without cofactors) in chromatophores of *Rhs. rubrum* strain S1 with different cytochrome *c*-420 content, i.e. different cytochrome *c*-420 to *P*-870 ratios.

The following experimental results were obtained: (1) Flash-induced photophosphorylation is strictly dependent on cytochrome *c*-420 content and a ratio ATP/(cytochrome *c*-420 turnover) of close to 1 is calculated. (2) Proton uptake (H^+/e^- near 2) is independent of cytochrome *c*-420 content. (3) After the first flash, given after a dark period of many minutes, the photophosphorylation per 'active' electron was nearly maximal, even at low flash intensity.

Our results suggest a direct relationship between electron transport occurring close to the reaction centre, and flash-induced phosphorylation. Therefore, we suggest that the two protons, taken up from the external medium are injected into an ATPase system associated with the reaction centre. Our experiments suggest that ATP formation only occurs if cytochrome *c*-420 is present and functions as in whole cells. The experiments seem to suggest a time limit of approx. 20 ms (two cytochrome *c*-420 turnovers) before the protons which

have been taken up disappear, via another pathway leading to none or a much less efficient phosphorylation.

Methods

Cultures of *Rhodospirillum rubrum* Madrid strain S1 were grown under incandescent lamps in the medium described by Slooten [10] till the late exponential phase. Cells were collected by centrifugation at 8000 × *g* for 10 min, washed with 50 mM Tricine/100 mM KCl (pH 7.5) and centrifuged again. The cells were disrupted by alumina grinding or by a French press at 3 tons/cm^2. Chromatophores were prepared according to a previously published procedure [11].

Bacteriochlorophyll concentrations were estimated from the absorption at 880 nm using the extinction coefficient value in vivo given by Clayton [12]. Reaction centre bacteriochlorophyll and P^+X^- (charge separation), were measured from the bleaching of the band around 604 nm using a differential extinction coefficient of 20 $mM^{-1} \cdot cm^{-1}$ [13].

Light-induced ATP formation in chromatophores was determined in a 3.0 ml reaction mixture containing 50 mM Tricine (pH 7.5), 100 mM KCl, 8.25 mM K_2HPO_4, 8.25 mM $MgCl_2$, 0.825 mM ADP and 3—4 μM bacteriochlorophyll (as chromatophores). Illumination was provided by 720 flashes ($t_{1/2}$ = 10 μs) at 4 Hz and photophosphorylation was stopped by addition of 1.5 ml $HClO_4$. The amount of ATP synthesized was assayed by the luciferin-luciferase method [11].

In another type of experiments illumination was provided by a single flash or a sequence of flashes (0.1 Hz) and the ATP synthesis in separate flashes ($t_{1/2}$ = 10 μs) was determined immediately by the light emission from the firefly luciferin-luciferase. The reaction mixture contained in 30 ml 30 mM Tricine (pH 7.5), 3.3 mM KCl, 20 μM ADP, 6 mM $MgCl_2$, 3.3 mM sodium phosphate, approx. 7 μM bacteriochlorophyll (as chromatophores) and 2.0 ml firefly solution. The luciferin-luciferase preparation was obtained by the extraction of dried firefly lanterns (Sigma Chemical Co) with 0.1 M sodium phosphate buffer (pH 7.5) (10 mg lanterns/ml) in a glass homogenizer, followed by centrifugation of the resulting suspension at 35 000 × *g* for 10 min. About 9 ml of this solution could be illuminated by the short (10 μs) flash and the luminescence emitted after each flash by the luciferin-luciferase was detected with an EMI 6958R photomultiplier screened from actinic illumination by a Schott CS-496 filter. The photomultiplier signal was amplified and recorded. The luminescence was calibrated by adding known amounts of ATP. This method is in principle similar to that published by Lundin et al. [14]. The differences are that we did not use the purified luciferin-luciferase and, more important, that our method was sensitive enough to record phosphorylation under single turnover flashes.

Flash-induced absorption changes were measured using a single beam spectrophotometer described before [6]. The short Xe flash ($t_{1/2}$ = 10 μs) was intense enough to induce essentially one turnover of each reaction centre in the light path. Infrared actinic light was selected by Schott filters RG 715 and RG 780.

Proton uptake following flash excitation was measured in unbuffered chromatophore suspensions by the pH indicator phenol red [15]. Chromatophores were prepared as described above, but a 1 mM Tricine/100 mM KCl (pH 7.5) buffer was used. The reaction mixture contained in 40 ml 100 mM KCl, 50 μM phenol red and 12 μM bacteriochlorophyll (as chromatophores), but only 9.0 ml were illuminated by a short Xe flash ($t_{1/2}$ = 10 μs). The measuring wavelength was 586 nm. Background changes were subtracted electronically by means of a signal averager using a reaction mixture without phenol red as a reference.

Results

(1) Redox reactions of cytochrome c-420 and reaction centre bacteriochlorophyll in whole cells and chromatophores of Rhs. rubrum

P-870 photooxidation and reduction induced by short actinic flashes in whole cells of the Leiden strain of *Rhs. rubrum* S1 have been studied extensively [6] and the rapid reduction phase of *P*-870$^+$ ($t_{1/2} \approx$ 150—250 μs, apparent first order kinetics) has been ascribed to cytochrome *c*-420 oxidation. Fig. 1 shows the flash-induced kinetics at 604 nm in whole cells of the Madrid strain of *Rhs. rubrum* S1. The rapid absorption decrease represents the formation of *P*-870$^+$; the decay is again biphasic, one part (about 60%) decays with a half time of approx. 0.2 ms, and the remaining part (40%) decays with a half time of approx. 10 ms. Lowering the intensity of the flash first affects the slow phase. This figure can be compared with a similar experiment in ref. 6 where the Leiden strain of *Rhs. rubrum* S1 was used. There seems to be a small but systematic difference between the two strains in their cytochrome *c*-420 : *P*-870 ratio which for the Leiden strain is 0.5 and for the Madrid strain is 0.6—0.7. In both strains the behaviour of *c*-420 is qualitatively as described in ref. 6 for the Leiden strain.

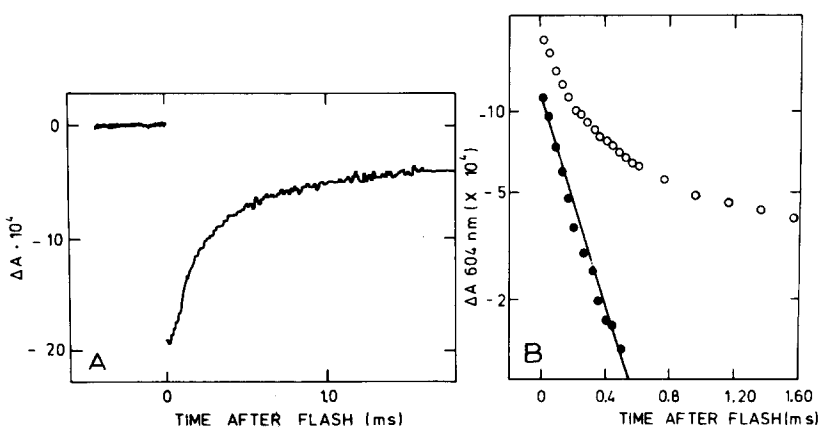


Fig. 1. (A) Kinetics of the flash-induced absorption changes at 604 nm in whole cells of *Rhs. rubrum*. Measurements were done in anaerobic conditions with the cells suspended in fresh culture medium. Bacteriochlorophyll concentration was 7.14 μM and the optical pathlength 0.5 cm. Average of 64 saturating flashes at o.1 Hz. (B) Semi-log plot of the absorption changes from (A). ○, total change; ●, rapid component only.

The explanation of the kinetics observed in Fig. 1 is as follows. Reduced cytochrome *c*-420 can reduce *P*-870$^+$ rapidly with a half time of 0.2 ms. Because there is only 0.6—0.7 cytochrome *c*-420 per reaction centre, approx. 30—40% of *P*-870$^+$ remains. This is reduced by a second turnover of cytochrome *c*-420. This means that cytochrome *c*-420 is a mobile electron carrier, which can visit at least two *P*-870 molecules. An analysis of the kinetics observed in whole cells of the Leiden strain suggested that cytochrome *c*-420 was mobile among at least 8 reaction centres (ref. 6 and van der Wal, H.N., personal communication).

In chromatophores of *Rhs. rubrum* the intact reduced cytochrome *c*-420 is again able to reduce *P*-870$^+$ with a half time of approx. 0.2 ms (variable from preparation to preparation between 0.15 and 0.25 ms).

The direct observation of the unperturbed cytochrome *c*-420 kinetics in the 540—560 nm region is unreliable because of the interference of carotenoid and bacteriochlorophyll absorption bands with the cytochrome *c*-420 absorption spectrum. If, however, in whole cells the complete difference spectrum is measured for times 0.02 and 4 ms after the flash, then the amount of cytochrome *c*-420 oxidized between 0.02 and 4 ms, as estimated from this spectrum, equals the amount of *P*-870$^+$ reduced in the same time interval [6]. The same is true for all preparations of chromatophores with different contents of cytochrome *c*-420. The kinetics at 406 nm, the isosbestic point of the *P*-870$^+$/*P*-870 difference spectrum, still gave the best approximate cytochrome *c*-420 kinetics, but in chromatophores a long-lived absorption change interfered with the cytochrome *c*-420 kinetics.

We therefore used the amplitude of the rapid phase (half time 0.15—0.25 ms) of *P*-870$^+$ reduction as an estimate of the amount of functioning *c*-420 present. The amount thus measured gave the total concentration of cytochrome connected (in a physiological way) to the reaction centre. A second flash given 4 ms after the first always oxidized a small amount of cytochrome which, however, did not contribute significantly to the total amount. The *P*-870 redox changes could easily be measured at 604 nm [16] where no other

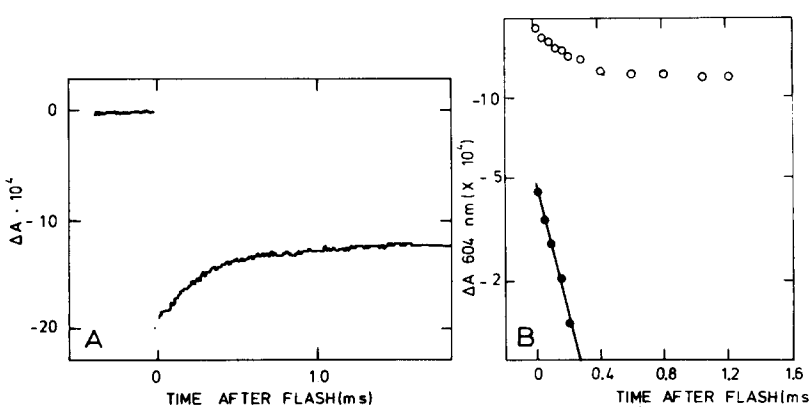


Fig. 2 (A) Kinetics of the flash-induced absorption changes at 604 nm in *Rhs. rubrum* chromatophores prepared by French-pressing. The reaction mixture contained 7.14 μM bacteriochlorophyll, 1 mM sodium ascorbate, 100 mM KCl, 50 mM Tricine/N_2OH (pH 7.5). Other conditions as in Fig. 1A. (B) Semi-log plot of the absorption changes from (A). ○, total change; ●, rapid phase only.

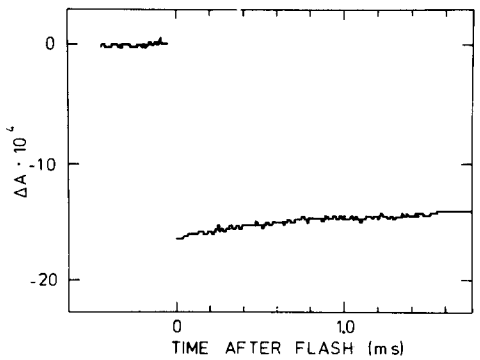


Fig. 3. Flash-induced absorption changes at 605 nm in *Rhs. rubrum* chromatophores prepared by grinding with alumina. The bacteriochlorophyll concentration was 6.87 μM. Conditions as in Fig. 2A.

changes overlap with the *P*-870 absorption changes. We will now describe the results obtained with different chromatophore preparations.

Chromatophores obtained by French-pressing yielded preparations in which cytochrome *c*-420 oxidation shows a kinetic pattern identical to that of whole cells (Fig. 2). Maximum cytochrome *c*-420 content obtained so far was 40% of that of whole cell cytochrome *c*-420 per reaction centre, but normally values between 20—40% were found. The amount of antenna bacteriochlorophyll molecules per *P*-870 is 40, as in whole cells. This means that cytochrome *c*-420 is partially lost during cell breakage but the photosynthetic unit remains the same.

Cell disruption by grinding with alumina gives chromatophores with only 5—10% cytochrome *c*-420 with respect to whole cells. The kinetic pattern of cytochrome *c*-420 photooxidation (Fig. 3) is difficult to evaluate but cytochrome *c*-420 oxidation after a flash occurs in less than 1 ms as in whole cells. Alumina grinded chromatophores again have also the same bacteriochlorophyll : *P*-870 ratio as intact cells. Cytochrome *c*-420 content is variable from preparation to preparation although chromatophores prepared in a French press always have a higher cytochrome *c*-420 content than those prepared by grinding with alumina.

Similar results have been reported in *Rhodopseudomonas sphaeroides* [17]. In chromatophores prepared by French-pressing, 70% of the reaction centres photooxidized cytochrome c_2 but after alumina grinding only 40% of the reaction centres oxidized cytochrome c_2 with normal kinetics.

(2) Photophosphorylation under short actinic flashes

ATP synthesis following short actinic flashes was measured by the luminescence from the firefly luciferin-luciferase system, after the addition of $HClO_4$. In this method low concentrations of chromatophores, less than 5 μM bacteriochlorophyll, must be used, otherwise high background luminescence was observed and unreliable results were obtained. In order to get an accurate assay, ATP concentrations higher than 0.5 mM were necessary. Because the ATP yield per flash varied from $1 \cdot 10^{-3}$ to $8 \cdot 10^{-3}$ ATP/Bchl, photophosphorylation was measured using a series of flashes. The amount of ATP/flash was calculated by

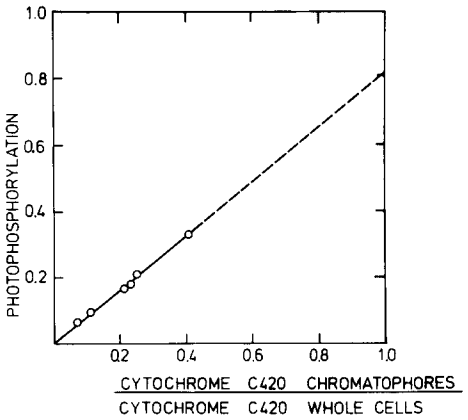


Fig. 4. Photophosphorylation (ATP/flash per reaction centre bacteriochlorophyll) plotted against the relative cytochrome *c*-420 content. Photophosphorylation: 720 saturating flashes at 4 Hz were used and the ATP formed was assayed as described in Methods. Reaction centre bacteriochlorophyll and cytochrome *c*-420 content were measured at 604 nm.

dividing the total amount of ATP formed by the number of flashes given.

At 3 μM bacteriochlorophyll and with 720—480 saturating flashes at 4 Hz, photophosphorylation was a linear function of bacteriochlorophyll concentration and the number of flashes in all types of chromatophores.

Photophosphorylation in chromatophores obtained by grinding with alumina vary from $1.5 \cdot 10^{-3}$ to $2.2 \cdot 10^{-3}$ ATP/flash per Bchl. If cells were broken with a French press chromatophores were obtained with a higher photophosphorylation yield, varying from $4 \cdot 10^{-3}$ to $8 \cdot 10^{-3}$ ATP/ flash per Bchl. Fig. 4 shows the number of ATP/flash per reaction centre bacteriochlorophyll versus cytochrome *c*-420 concentration in several chromatophore preparations. It is clear that photophosphorylation per flash is proportional to cytochrome *c*-420 content. A possible explanation of the results shown in Fig. 4 might be that photophosphorylation is caused by reaction centres which became reduced in the time between the flashes. Increasing the cytochrome *c*-420 content might then increase the number of reaction centres which are in the form *P* X before each

TABLE I

EFFECT OF DARK TIME BETWEEN FLASHES ON PHOTOPHOSPHORYLATION AND *P*-870 RE-REDUCTION

Photophosphorylation was assayed by the extraction method; 720 (10—4 Hz), 380 (2 Hz) and 180 flashes (1 Hz) were employed. *P*-870 re-reduction was measured by the absorption change at 604 nm; 64 flashes were averaged; cytochrome *C*420/cytochrome *C*420 (whole cells) = 0.41. See Methods for further details.

Flash frequency (Hz)	Photophosphorylation (ATP/flash per Bchl)	*P*-870 re-reduction (%)
10.0	$8.69 \cdot 10^{-3}$	41
6.66	$8.31 \cdot 10^{-3}$	52
5.0	$9.10 \cdot 10^{-3}$	58
4.0	$8.44 \cdot 10^{-3}$	62
2.0	$8.70 \cdot 10^{-3}$	70
1.0	$8.70 \cdot 10^{-3}$	80

flash in a sequence of flashes. In such a scheme once a flash has created P^+X^- it would be sufficient for the reducing equivalent to enter the cyclic electron transport chain to drive photophosphorylation. This interpretation is, however, excluded from the results in Table I. Both phosphorylation and the reduction level of *P*-870 were measured at several flash frequencies. The second row displays the amount of *P*-870 in the reduced form before the flash and this decreases with flash frequency (and so the number of electrons which flow per flash through the photosynthetic cyclic system also decreases) but photophosphorylation is constant at the flash frequencies used from 10 to 1 Hz, the same is true for the amount of *c*-420 photooxidized per flash (not shown). This means that photophosphorylation is probably associated with reaction centres which are reduced by the cytochrome *c*-420 directly and after one turnover of the mobile cytochrome. It appears that the P^+ which is additionally reduced (possibly via another path than that of cytochrome *c*-420) does not contribute to phosphorylation.

The good linear relationship between cytochrome *c*-420 content and photophosphorylation in Fig. 4 permits extrapolation to the photophosphorylation yield ATP/flash per reaction centre bacterchlorophyll at the same concentration of cytochrome *c*-420 as in whole cells. This ratio ATP/flash per reaction centre bacteriochlorophyll is 0.82, which indicates that in whole cells about one ATP molecule is formed per 'active charge separation' or, in other words, one ATP per cytochrome *c*-420 turnover.

Photophosphorylation in *Rhs. rubrum* chromatophores under flashing light has been measured by Geller [18] and Nishimura [19]. The photophosphorylation rate reported by Geller [18] was $2.2 \cdot 10^{-3}$ ATP/flash per Bchl and it agrees with our phosphorylation rate at low cytochrome *c*-420 content. However, cytochrome *c*-420 was not estimated by Geller. The ATP yield published by Nishimura [19] was rather high ($47 \cdot 10^{-3}$ ATP/flash per Bchl) and this high phosphorylation activity will be discussed later.

The high photophosphorylation rate in French-pressed chromatophores allowed us to study the effect of flash intensity on ATP synthesis. In chromato-

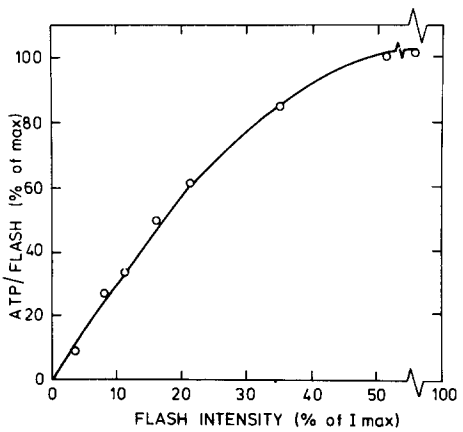


Fig. 5. Photophosphorylation as a function of flash intensity. Conditions as in Fig. 4. Photophosphorylation is normalized at maximum flash intensity ($4.72 \cdot 10^{-3}$ ATP/flash per Bchl).

phores prepared by grinding with alumina, with low phosphorylation activity, it was not possible to measure accurately the small amount of ATP formed by non-saturating flashes. Photophosphorylation against flash intensity is presented in Fig. 5. ADP phosphorylation is proportional to flash intensity up to 20% of the maximum intensity.

(3) Photophosphorylation induced by single actinic flashes

Phosphorylation in mitochondria [20] and chloroplasts [21] has been estimated by the light emission from luciferin-luciferase. We applied a similar method to study photophosphorylation in *Rhs. rubrum* chromatophores after one short flash only. This method is analogous to the one used by Lundin et al. [14].

Flash illumination of *Rhs. rubrum* chromatophores induced firefly luminescence (Fig. 6). The initial rapid change is due to a disturbance caused by the actinic flash but it was checked that the photomultiplier sensitivity had not been affected. The light emission shows an initial lag of 150 ms and then a slow rise of the luminescence occurs which requires about 1 s to reach a maximum. This kinetic pattern is similar to that previously reported [14,22]. Luminescence decay reflects the ATP consumption by the ATPase and probably the effect of adenilate kinase. ATP formation in a single flash was calculated by extrapolation of the luminescence back to the time that the flash was fired. The luminescence was calibrated by the addition of known amounts of ATP. The sensitivity of this method is high: an amount of 10^{-9} M ATP can be detected.

After six minutes dark adaptation a series of flashes separated 10 s from each other was given and the light emission is displayed in Fig. 6. The first flash induced about 75% of the maximum phosphorylation ($ATP/P^+X^- = 0.16$), the second about 95% ($ATP/P^+X^- = 0.20$) and the next flashes gave constant maximum ATP formation ($ATP/P^+X^- = 0.21$). The firefly luminescence induced by flashing light is due to ATP formation, since in the absence of exogenous ADP the luminescence is inhibited for 95% and the addition of 10 μM oligomycin or 0.2 μM gramicidin to the reaction mixture gave 90% inhibition of the firefly luminescence after a single flash.

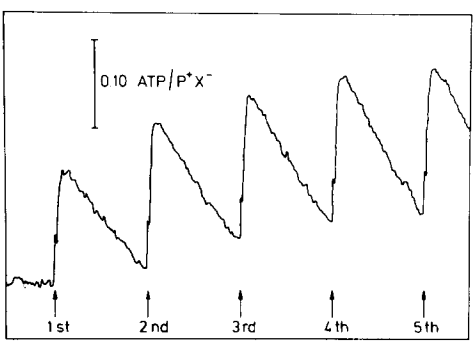


Fig. 6. Flash-induced luciferin-luciferase luminescence in a series of 5 flashes, 10 s apart. Sample is incubated 6 min in the dark. Bacteriochlorophyll concentration 7.14 μM, flash intensity 80—90% saturating. P^+X^- (charge separation) is measured as P^+ at 604 nm after 64 flashes at 0.1 or 0.005 Hz. See Methods for details.

ATP formation by a single saturating flash (under conditions that the yield is at maximum) is again proportional to cytochrome *c*-420 content, in a similar way as displayed in Fig. 4, using a high number of flashes. The extrapolated ratio ATP/P^+X^- is 1.0 at the same cytochrome *c*-420 content as in intact cells.

Flash intensity against phosphorylation by single flashes (flash rates 0.1 and 0.005 Hz) shows exactly the same pattern as Fig. 5.

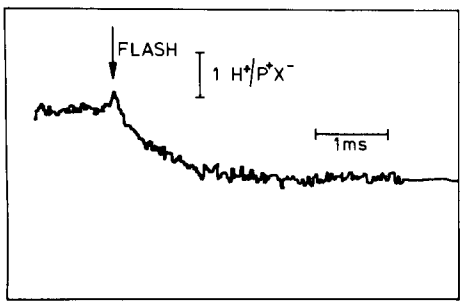
Preliminary experiments indicate that cytochrome turnover induced by flashes after a long dark period is not dependent on the flash number. We are currently investigating phosphorylation as a function of flash number, especially the difference observed between the first flash and the subsequent ones.

(4) Proton uptake

It has been demonstrated in Section 2 that flash-induced photophosphorylation is limited by the cytochrome *c*-420 concentration in the chromatophore preparation and therefore probably another electron transferring component of the cyclic system such as *c*-560 and perhaps also proton transfer is dependent on cytochrome *c*-420 photooxidation. Absorption changes associated with cytochrome *c*-560 [6] are difficult to observe in chromatophores from the wild type *Rhs. rubrum* due to interference of carotenoids.

Rapid proton uptake induced by actinic flashes has been studied in chromatophores of photosynthetic bacteria [14,23,24] and it was concluded that the rapid pH decrease in the external medium had to be attributed to ubiquinone reduction [15,25,26].

We have measured proton uptake in preparations with different cytochrome *c*-420 content by the absorption changes of the pH indicator phenol red. Protons are taken up from the suspending medium after flash illumination with apparent first order kinetics ($t_{1/2}$ approx. 300 μs) and $H^+/e^- = 1.25$. As shown in Figs. 7 and 8 rapid proton uptake is independent on cytochrome *c*-420 photooxidation. Proton binding is a complex process and has at least one other phase ($t_{1/2}$ approx. 15 ms; $H^+/e^- = 1.1$). Also this slow phase is not related to cytochrome *c*-420 content. We assume that both phases in the H^+ uptake are related



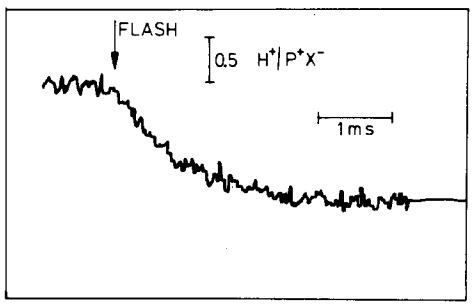


Fig. 7. Kinetics of proton uptake in chromatophores prepared by French-pressing. 64 flashes, 10 s apart, were averaged for measuring proton uptake and P^+X^-. Cytochrome *c*-420/cytochrome *c*-420 (whole cells) = 0.41. For other conditions see Methods.

Fig. 8. Kinetics of proton uptake in chromatophores prepared by alumina grinding. 32 flashes, 10 s apart were averaged for proton uptake and charge separation P^+X^-. Cytochrome *c*-420/cytochrome *c*-420 (whole cells) = 0.15. See Methods for other experimental conditions.

to the transport of the electron across the membrane. The mechanism for the uptake of two H^+ could be analogous to that given by Dutton et al. [30] or Crofts et al. [31].

Discussion

In the present work ATP formation under flash illumination has been estimated by two methods. In the first one the sample was illuminated and after addition of $HClO_4$ ATP was assayed by the firefly luminescence. The sensitivity is low and ATP formation was measured after a series of flashes (10—1 Hz). In the second method we used the firefly luciferin-luciferase which was added to the reaction mixture and phosphorylation was estimated by the light emission. Phosphorylation induced by a single flash is measured but flashes must be spaced more than 4 s to allow an analysis of the data. Both methods are, as shown in Results comparable with each other.

The results presented in Sections 2 and 3 show that cytochrome *c*-420 is a limiting factor in cyclic phosphorylation induced by flashing light. ATP formation by flash illumination is proportional to the cytochrome *c*-420 content and in the authors' view this indicates that only electrons flowing through cytochrome *c*-420 are associated to ATP formation. The alternative explanation that the phosphorylation is related to the total number of electrons delivered from *P*-870 has been excluded on basis of the experimental results presented in Table I.

The extrapolated ratio ATP/P^+X^- under a series of flashes is 0.82 and after a single flash 1.0 (mean 0.91). This implies that maximally 1 mol ATP is formed per electron passing through the cyclic electron transport chain.

An ATP/P^+X^- ratio of approx. 2 has been reported by Nishimura [19] in *Rhs. rubrum* chromatophores. Photophosphorylation was excited by a long actinic flash (0.5 ms duration), ATP formation was estimated with a sensitive pH meter and ATP yields (ATP/flash per Bchl) of $47 \cdot 10^{-3}$ were obtained. This high photophosphorylation raises some questions: (1) During the long flash duration more than one electron per reaction centre bacteriochlorophyll may be delivered, and (2) the redox potential difference (ΔE) necessary to form 1 mol ATP is given by

$$\Delta G = n\mathrm{F} \cdot \Delta E$$

where ΔG = phosphate potential, n = number of electrons involved, F = Faraday constant. The value of ΔG under the conditions of the experiments is not known, but it is between 8.2 and 15.5 kcal $\cdot$ mol^{-1} [27]; this implies that a ΔE between 1.3 and 0.7 V is required to form 2 mol ATP per electron: there is not enough free energy in the system as judged from the midpoint potentials of the primary acceptor (midpoint potential —145 mV) [28] and the reaction centre bacteriochlorophyll (midpoint potential 440 mV) [29].

The phosphorylation dependence on flash intensity has been studied at several flash rates and flash numbers. In all cases extrapolation to zero flash intensity passes through the origin and this indicates that no threshold effect is present under our experimental conditions.

Our observations that the amount of ATP produced in a flash is always

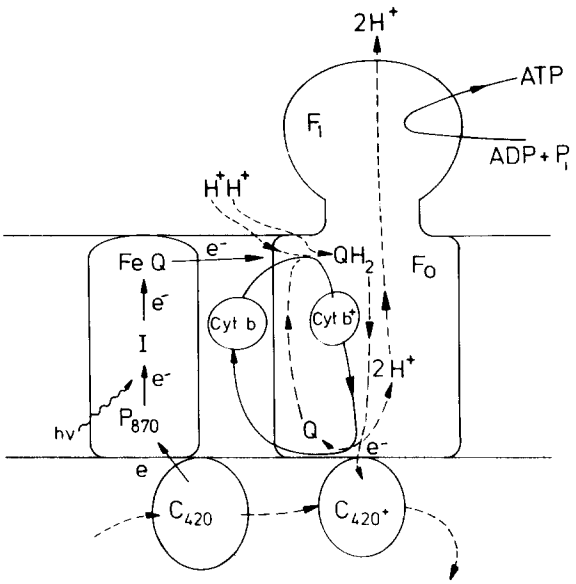


Fig. 9. Tentative scheme for electron transport and photophosphorylation in chromatophores of *Rhs. rubrum*. The arrows give the direction of the electron transport from the reaction centre bacteriochlorophyll, $P \rightarrow I \rightarrow X \rightarrow$ quinone $\rightarrow$ ubiquinone $\rightarrow$ cytochrome *c*-420 (C). The two protons taken up from the external medium upon subsequent re-oxidation of X^- and a *b*-type cytochrome (*b*) are injected into the ATPase system (F_0, F_1).

stoichiometrically related to the content of cytochrome *c*-420 and not to the amount of protons transported, and that photophosphorylation occurs with high yield at low flash intensities and at the first flash after darkness, may be interpreted in terms of a non-chemiosmotic type of coupling between electron transport and ATP formation. The tentative scheme (Fig. 9) is consistent with these observations. Two protons are taken up from the external medium upon subsequent reoxidation of X^- and a *b*-type cytochrome. These protons are injected into the ATPase system which is closely related to the reaction centre only when an oxidized cytochrome *c*-420 molecule can be re-reduced by the cyclic system. This scheme has a similar sequence of electron transfer reactions as schemes suggested on basis of Mitchell's proposals by Dutton et al. [30] and Crofts et al. [31] but in the latter schemes the protons are injected according to the chemiosmotic hypothesis first into the inner space of the chromatophores. (For a further discussion of the scheme see ref. 32.)

At the moment we do not have direct evidence for the hypothesis that the protons are not released during the first flashes in the internal aqueous space of the chromatophores. Petty et al. [26] have reported chlorophenol red absorption changes that agree with the interpretation that protons are liberated inside the chromatophore but it is also discussed by the authors that this result was not a demonstration because the dye could be in the membrane and in that case the absorption changes cannot easily be interpreted. In addition, it is necessary to test the effect of the long dye incubation on the chromatophores because a similar incubation, using phenol red and cresol red showed that both dyes modified the Bchl/*P* ratio and cytochrome *c*-420 redox reaction (results not shown).

The scheme given in Fig. 9 is at present only a working hypothesis, consistent with our experimental results. We have not yet been able to prove or disprove the prediction that in the first flash after darkness no protons, correlated with cytochrome *c*-420 reduction, are released in the internal space.

Appendix

Recently the potential between the internal and the outside space and the ΔpH was measured in *Rhs. rubrum* chromatophores by the flow dialysis technique [33]. The maximum proton motive force observed was 100 mV while the phosphorylation potential was 14 kcal/mol. The authors conclude that if Mitchell's hypothesis is correct, five protons translocated per ATP formed are necessary. If, however, in their preparation, as we have concluded for ours, the number of protons translocated per ATP would be 2, this would be inconsistent with the chemiosmotic hypothesis and a more direct coupling between electron transport and phosphorylation, as pictured in Fig. 9, would be a more likely hypothesis.

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