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PHOTOINACTIVATION OF PHOTOPHOSPHORYLATION AND DARK ATPase IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

LUIT SLOOTEN and CHRISTIAAN SYBESMA

Vrije Universiteit Brussel, Department of Biophysics, Pleinlaan 2, B-1050 Brussels (Belgium)

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SUMMARY

Preillumination of *Rhodospirillum rubrum* chromatophores with strong, far-red light in the presence of phenazine methosulfate under non-phosphorylation conditions results in a selective, irreversible inactivation (typically about 70 %) of photophosphorylation and of uncoupler-stimulated dark ATPase. The time course of the photoinactivation is similar to the light-on kinetics of the light-induced proton uptake in the absence of ADP. Only little photoinactivation occurs when the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone is present or when phenazine methosulfate is absent during the preillumination, indicating that the reaction occurs only when the membrane is energized.

Phosphorylation conditions offer a practically complete protection against the photoinactivation. Inorganic phosphate, Mg^{2+} or ADP do not provide a significant protection against the photoinactivation, nor does ATP. The pH-dependence of the reaction(s) leading to photoinactivation may indicate that a partial reaction of the photophosphorylation process (perhaps only a conformational change of the coupling factor) precedes the photoinactivation.

INTRODUCTION

The ATPase complexes of chloroplasts, mitochondria and bacteria have many functional and structural properties in common [1, 2]. It is now generally agreed that conformational changes in the enzyme are essential for its catalytic action [3–7]. However, Mitchell [7–10] and Williams [11, 12] maintain that proton gradients, or local high concentrations of protons, are the primary driving force for ATP synthesis, the conformational changes being secondary effects [7], whereas Boyer [6] and Slater [5] do not exclude the possibility that the conformational changes required for ATP synthesis are coupled directly to electron transport. Furthermore, there is disagreement on the molecular coupling mechanism between proton gradients and ATP synthesis [4, 7–12].

Conformational changes in the ATPase coupling factors have been demonstrated in chloroplasts by the light-induced changes in reactivity of the membrane-bound

Abbreviations: PMS: phenazine methosulfate; CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone.

coupling factor towards *N*-ethylmaleimide [13, 14], sulfate [15] or permanganate [16, 17], and by the light-induced exposure of hydrogen-exchanging groups on the membrane-bound coupling factor [18, 19]. In chromatophores from *Rhodopseudomonas capsulata*, *N*-ethylmaleimide appeared to have effects comparable to those found in chloroplasts [20]. Conformational changes in the coupling factors of State 3 respiring mitochondria were indicated by changes in the fluorescence yield of bound aurovertin [21]. Mitochondrial ATPase coupling factor contains a specific inhibitor protein, which was shown to dissociate from the coupling factor during substrate oxidation [22].

The present report deals with an irreversible inhibition of the photophosphorylation and ATPase reaction in *Rhodospirillum rubrum* chromatophores. The inactivation occurs after exposure of the chromatophores to strong light in the presence of phenazine methosulfate (PMS) under non-phosphorylation conditions. The inactivation may be the result of a "side" reaction of the coupling factor enzyme which occurs when the main reaction, ATP synthesis, is inhibited for lack of substrates, while the membrane remains strongly energized. A future determination of the nature of the reactions leading to photoinactivation might give a clue to the kind of conformational changes of the coupling factor which lead to photophosphorylation.

MATERIALS AND METHODS

Preparation of chromatophores

Cells of *Rhodospirillum rubrum*, strain 1, were obtained from the Biophysical Laboratory of the State University, Leiden (The Netherlands). The cells were grown in the light on a medium according to that of Cohen-Bazire et al. [23], supplemented with yeast extract and peptone, in completely filled Roux bottles. After 3 days growth, the cells were harvested and washed twice with 55 mM sodium phosphate, pH 7.5. The washed cells were resuspended in 60 ml of a medium containing sucrose (0.29 M), KCl (0.1 M) and glycylglycine/KOH buffer, pH 7.5 (0.1 M). The cells were disrupted by sonic treatment during 1 min at maximum output, with a Braun sonifier type 300 S. The suspension was cooled in an ice bath during sonication. The suspension was centrifuged and the fraction sedimenting between 20 000 $\times g$ (30 min) and 100 000 $\times g$ (1 h) was collected and washed twice in a washing medium containing 0.1 M KCl, 3 mM $MgCl_2$, 3 mM sodium phosphate, 0.1 mM EDTA and 10 mM glycylglycine/KOH, final pH 8.0. The sediment after the second washing was resuspended in a minimal volume of a storage medium of the same composition. The suspension was stored in completely filled 0.5 ml tubes under nitrogen at reduced pressure, at 5 °C. The phosphorylation activity of these preparations remained constant for 3 weeks of storage. Usually the activity declined thereafter.

Washing of chromatophores

When necessary, the chromatophores were freed from Mg^{2+} and inorganic phosphate, by washing in a medium containing 0.1 M KCl, 0.1 mM EDTA and 10 mM glycylglycine/KOH, pH 8.0. This was done twice. The chromatophores were allowed to equilibrate for 30 min with the washing medium at 5 °C prior to centrifugation. After the second centrifugation, the chromatophores were stored as described in the washing medium.

Phosphorylation measurements

The measurements were carried out with the pH method [24]. The standard reaction mixture contained 0.2 M sucrose, 3.1 mM $MgCl_2$, 0.1 mM EDTA, 3 mM sodium phosphate, 0.1 mM PMS and 0.75 mM ADP, final pH 8.0. The final volume was 5.6 ml. When measurements were done at variable pH values, the chromatophore stock suspension of pH 8.0 was diluted into the reaction medium of the desired pH. This procedure resulted in an extensive "drift" in medium pH, owing to a slow equilibration of protons across the chromatophore membrane. This problem was minimized by adding KCl (30 mM) to the reaction medium, as indicated in the legends. KCl presumably accelerated the dark equilibration of protons across the chromatophore membrane.

The experiments were carried out at room temperature in a lucite vessel (volume, approx. 10 ml) with a stainless steel lid screwed hermetically onto it. The lid contained a rubber septum to allow additions by means of syringes. A combination pH electrode (Beckman Futura 39501) was attached to the lid, and three stainless steel injection needles inserted through the lid served as gas inlet and outlet. All experiments were carried out anaerobically. A nitrogen gas stream was used to bubble through the sample solution during 25 min before the addition of chromatophores. The nitrogen gas stream was blown over the solution afterwards. Chromatophores were added with a microliter syringe. The solution was stirred with a magnetic stirring bar.

Light from a 125 W quartz-iodine lamp was focussed onto the cuvette. The light was filtered through an aqueous solution of $NiSO_4$ (15 mg/ml, 10 cm thickness) and through a far-red cut-off filter, unless otherwise indicated.

The number per s of quanta of 419 nm light incident on the sample was determined with the ferrioxalate actinometer procedure [25]. The 419 nm light was selected by an interference filter. The intensity of far-red light relative to that of 419 nm light was measured with a thermopile.

The output of a Beckmann SS-1 pH-meter was fed into a Heath-Kit model SR-255B recorder. Full scale deflection of the recorder pen corresponded to a pH-change of 0.04 or 0.14 pH units. The pH change recorded upon injection of a small amount of acid or base was 50 % complete in 1.5 s and 90 % complete in 4.2 s.

The photophosphorylation rate, expressed in μM P_i esterified per mg bacteriochlorophyll and per min, was independent of the bacteriochlorophyll concentration up to 20 μg/ml.

The concentration of bacteriochlorophyll was estimated spectrophotometrically, using the in vivo extinction coefficient given by Clayton [26].

RESULTS

A typical experiment is shown in Fig. 1. The solid line is an actual recorder tracing; the dashed line shows the time course of the photophosphorylation rate. The initially high photophosphorylation rate slowed down to a steady state level which was maintained until the reaction was about 90 % complete; then the rate dropped to zero in a few min. The initial decrease in photophosphorylation rate down to a steady level, occurred only in the presence of PMS, and at pH values of 8 and higher; it was more extensive at higher pH values (not shown). We also observed a photobleaching of PMS at pH-values above 8.0. The extent of the photobleaching likewise increased

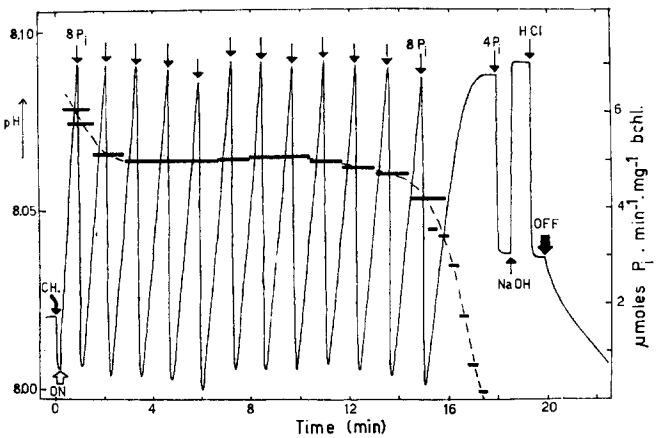

Fig. 1. Kinetics of PMS-stimulated photophosphorylation. Solid line: actual recorder tracing, including the noise (left hand ordinate). The arrow marked CH indicates addition of chromatophores containing 49 μg bacteriochlorophyll to the standard reaction medium (at $t = 0$). The light was switched on at the time marked by the arrow. The incident light energy was 54 mW. Arrows marked 8 P_i and 4 P_i indicate addition of 8 and 4 μl. respectively, of a 40 mM sodium phosphate solution, pH 5.5. This was added to compensate for the alkalinization of the medium; also, the P_i concentration was kept virtually constant in this way. The distance along the time axis of two successive, upgoing parts of the recorder tracing gives the time for neutralization of 8 μl of the acid phosphate solution. This yields the photophosphorylation rate, averaged over that time period (thick horizontal bars, right hand ordinate). The system was calibrated at the end of an experiment, by measuring the recorder deflection caused by addition of an aliquot of a 10-mM HCl solution. The buffering capacity did not change measurably during an experiment. It was assumed that 0.96 μmol H^+ are consumed per μmol of ADP esterified at pH 8.0–8.1 [24]. Dashed line, right hand ordinate: time course of the photophosphorylation rate.

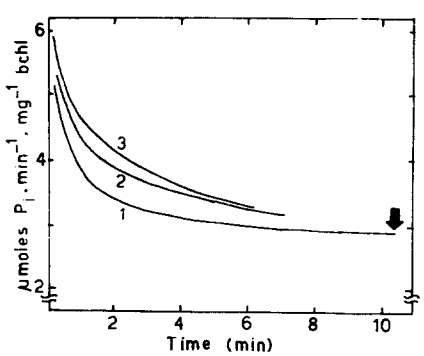

Fig. 2. Photophosphorylation rate as a function of time. The light was switched on 0.6 min after addition of the chromatophores to the standard reaction mixture. Incident light energy, 45 mW. Curve 1, 55 μg bacteriochlorophyll. The experiment was terminated by switching off the light at the time marked by the arrow. Curve 2, the chromatophores used in the experiment of curve 1 were sedimented by centrifugation at 100 000 $\times g$ for 30 min. The sediment was taken into approx. 0.1 ml of reaction medium (without ADP and PMS), and the experiment of curve 1 was repeated. Bacteriochlorophyll, 18 μg. Curve 3, 24 μg bacteriochlorophyll. The chromatophores were centrifuged, but not illuminated, prior to this experiment.

with increasing pH-values. The yellow color returned upon aeration of the sample (not shown). Presumably, the initial decrease in photophosphorylation rate (Fig. 1) was due to a pH-dependent photoreduction of PMS [27] to the leuco-compound [28]. This type of inhibition was completely reversible, as shown in Fig. 2. After termination of the experiment shown in curve 1, the chromatophores were centrifuged from the reaction medium and resuspended in fresh reaction medium. Photophosphorylation was measured again, and the result (curve 2) was practically the same as the result obtained in curve. 1 Curve 3 shows a control experiment, in which chromatophores were used which had been centrifuged, but not illuminated prior to centrifugation.

In several experiments similar to the one shown in Fig. 1, the light was kept on after photophosphorylation of all added ADP (illumination with far-red light in the presence of PMS completely inhibited ATPase, in agreement with ref. 29). When subsequently additional ADP was added in the light, photophosphorylation continued at a constant rate, which was however approximately 30 % of the steady state rate observed before exhaustion of the first supplement of ADP. This, and other results, led us to the experiments described below.

Chromatophores were preilluminated with strong, orange light in the standard reaction medium without ADP. (In this case, far-red light was avoided during the preillumination, since this would have resulted in considerable "shielding" effects because of the high concentration of bacteriochlorophyll). After preillumination, the chromatophores were centrifuged from the reaction medium and resuspended in a minimal amount of fresh reaction medium, without ADP and PMS. A stock of control chromatophores used in these experiments was prepared in the same way, except that the chromatophores were not illuminated prior to centrifugation.

Table I shows that both endogenous and PMS-stimulated photophosphorylation were inactivated irreversibly, and to a similar extent, as a result of the preillumination. The inhibition was 50–60 % in experiment 1, but it was more extensive in experiments in which the bacteriochlorophyll concentration was lower during the preillumination, so that less "shielding" occurred, e.g. experiment 2 of Table I.

The rather low rate of endogenous photophosphorylation in comparison to PMS-stimulated photophosphorylation (Table I), deserves some comment. Low rates of endogenous photophosphorylation were usually observed when chromatophores, after a period of storage of a few days or more, were subject to an extra washing, as required in this type of experiment. Washing at this time apparently removes an endogenous electron transfer component (cf. ref. 30), which is bypassed in PMS-stimulated photophosphorylation. As shown below, higher rates of endogenous photophosphorylation were observed in chromatophores which were not subject to the extra washing.

The dark ATPase reaction, with or without PMS, in the absence of uncoupler, was little or not at all affected by the preillumination (Table II). PMS stimulated the dark ATPase reaction in the absence of uncoupler; in the control experiment, this stimulation was 40 %, like usually. (This effect of PMS is not related to the photoinactivation reported above. The PMS-stimulation of the dark ATPase in untreated chromatophores might be due to the redox potential dependence of the dark ATPase [31], or alternatively to PMS acting as an uncoupler, as has been shown for oxidized DCIP [32]. CCCP provided a further stimulation of the dark ATPase

TABLE I

EFFECT ON PREILLUMINATION ON PHOTOPHOSPHORYLATION ACTIVITY IN *R. RUBRUM* CHROMATOPHORES

Experiment 1: Chromatophores containing 260 μg bacteriochlorophyll were preilluminated for 10 min in standard reaction mixture without ADP, with light filtered through 10 cm water and through an orange cut-off filter. (Schott OG550/2). The incident light energy was 240 mW. Subsequently, the chromatophores were sedimented by centrifugation and resuspended in approx. 0.3 ml of standard reaction medium supplemented with 30 mM KCl, but without ADP and PMS. A batch of control chromatophores was treated similarly, except that they were kept in the dark prior to centrifugation. Measurements were done in standard reaction medium supplemented with 30 mM KCl. The light was switched on 0.4 min after the addition of preilluminated or control chromatophores to the reaction medium. The incident light energy was 45 mW. Measurements with and without PMS were done in the same sample. The samples of preilluminated and control chromatophores contained 24 and 27 μg bacteriochlorophyll, respectively. Experiment 2: As experiment 1, except that chromatophores containing 135 μg bacteriochlorophyll were preilluminated in standard reaction medium. Phosphorylation measurements were done in standard reaction medium. Samples of preilluminated and control chromatophores contained 14 and 18 μg bacteriochlorophyll, respectively. Bchl, bacteriochlorophyll.

Experiment No.	Additions during assay	Maximum photophosphorylation rate (μM P_i esterified · mg · Bchl^{-1} · min^{-1})		Inhibition %
		Control	Preilluminated	
1	None	0.38	0.19	50
	+PMS	2.30	0.82	65
2	None	0.90	0.25	72
	+PMS	5.10	0.63	88

TABLE II

EFFECT OF PREILLUMINATION ON ATPase ACTIVITY IN *R. RUBRUM* CHROMATOPHORES

Conditions as described in the legend to experiment 1 of Table I, except that ADP was replaced by 2.2 mM ATP during the measurement. The reaction was initiated by addition of chromatophores to reaction medium without PMS; the latter was added 5 min later. Subsequently, CCCP was added in increasing amounts (as shown in Fig. 4) after 4-min intervals in which the ATPase activity was recorded. The data given were taken from the last 2 min prior to each new addition. Oligomycin (final conc., 3 μg/ml) was added 4 min after the last addition of CCCP. The samples of preilluminated and control chromatophores contained 47 and 38 μg bacteriochlorophyll (Bchl), respectively.

Additions during assay	ATPase rate μM hydrolyzed · mg Bchl^{-1} · min^{-1}		Inhibition (%)
	Control	Preilluminated	
None	0.20	0.20	0
PMS	0.28	0.24	15
PMS+CCCP*	0.90	0.46	49
PMS+15 M CCCP+oligomycin	–	0.05	–

* Optimal concentration, i.e. 2 μM in the preilluminated, and 6 μM in the control sample

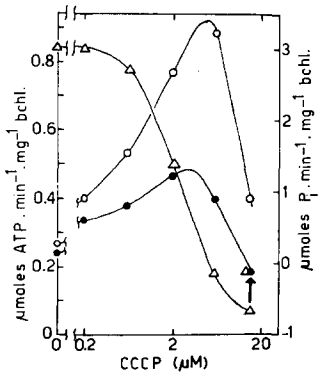

Fig. 3. Effect of CCCP on PMS-stimulated photophosphorylation (△, right hand ordinate) and on dark ATPase in the presence of PMS (○, left hand ordinate). The data concerning the ATPase rates were collected in the experiments shown in Table II. Open and solid circles represent experiments carried out with control and preilluminated chromatophores, respectively. The photophosphorylation experiments (△) were done with a different batch of non-preilluminated chromatophores. The light was switched on at 0.6 min after addition of chromatophores (containing 48 μg bacteriochlorophyll) to the standard reaction medium. Incident light energy, 45 mW. The addition of CCCP started after the photophosphorylation rate had reached a steady state (compare Fig. 1). The light was kept on while CCCP was added in increasing amounts. The arrow marks the irreversible decrease in ATPase activity after switching off of the light (see Discussion).

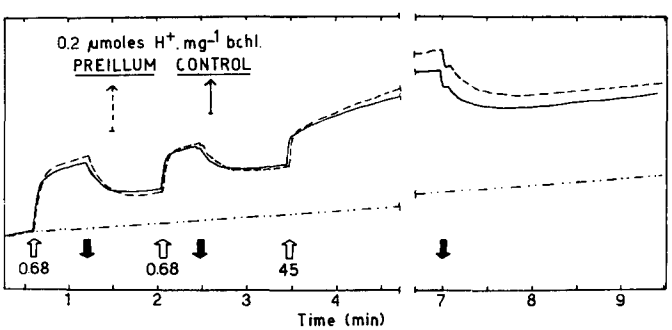

Fig. 4. Kinetics of light-induced proton uptake in the presence of PMS, but without ADP, in control (—) and in preilluminated (----) chromatophores. The preillumination and centrifugation was carried out as described in the legends to Exp. 1 of Table I. Light-induced proton uptake was measured in standard reaction mixture supplemented with 30 mM KCl (ADP omitted). The chromatophores were added at zero time. The light was switched on at times indicated by the upward pointing arrows; the light energy is expressed in mW. The samples of preilluminated and control chromatophores contained 29 and 24 μg bacteriochlorophyll, respectively. Dash-dotted line: base-line, obtained as explained in the text.

reaction in the presence of PMS. The concentration of CCCP required for maximal stimulation of the dark ATPase reaction seemed to be slightly lower in preilluminated, than in control chromatophores (Fig. 3). The CCCP-stimulated dark ATPase reaction was inhibited nearly as much as the phosphorylation reaction by preillumination without ADP (Table I and Fig. 3).

Fig. 4 shows experiments on light-induced proton uptake in the presence of PMS, at pH 8.0, and in the absence of ADP. The extent of the "base-line drift"

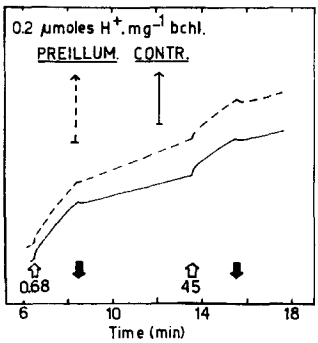

Fig. 5. Kinetics of light-induced proton uptake in the absence of PMS and ADP, in control (—) and in preilluminated (-- -) chromatophores. The experiments were carried out as described in the legends to Fig. 4, except that the measurements were done in a medium containing 0.2 M sucrose, 30 mM KCl, 0.1 mM EDTA, 3.1 mM $MgCl_2$, 0.5 mM sodium phosphate; final pH, 6.0.

(dash-dotted line) was estimated from the slope of the recorder tracing obtained in the dark on the time span of 8–12 min (not shown completely in this figure). The base-line drifts found in this way were indistinguishable in the samples shown here. Errors introduced by this method will largely cancel out when one compares the results of the two samples. Fig. 4 shows that the kinetics of the light-on reaction in weak light as well as in strong light were virtually the same in preilluminated and in control chromatophores. The extent of the light-on reaction (μmol H^+ per mg bacteriochlorophyll) may have been about 5–10 % less in the preilluminated sample, as compared to the control. The kinetics of the dark decay were the same in preilluminated and in control chromatophores.

We also measured light-induced proton uptake in the absence of PMS. This was done at pH 6.0, since, in agreement with earlier results [33, 34], the extent of the light-induced proton uptake in the absence of PMS was very small at pH 8.0. Again, the kinetics and the extent of the light-on reaction were very similar in preilluminated and in control chromatophores (Fig. 5). The kinetics of the dark decay could not be followed accurately at pH 6.0, due to the more extensive base-line drift in this case (see Methods).

It appears that chromatophores which were inactivated by 50–60 % in endogenous and PMS-stimulated photophosphorylation (Table I, Exp. 1) and in uncoupler-stimulated ATPase (Table II), had a practically unimpaired capacity for light-induced proton uptake in the presence (Fig. 4) and absence (Fig. 5) of PMS. This suggests that the photoinactivation of the coupling factor activities were not caused by an increase in proton permeability, nor by electron transfer inhibition. An increase in proton permeability should result in uncoupling of electron transfer, an acceleration of the dark decay of the light-induced proton uptake, and (especially at low light intensities when the rate of electron transport is limited) a decrease of the extent of the light-induced proton uptake. Electron transfer inhibition should result in an inhibition of the initial rate, and of the extent of the light-induced proton uptake; this inhibition should be the same in weak, and in strong light (cf. ref. 35). A close connection between proton uptake and light-induced electron transport has been shown to exist in chloroplasts [36, 37] and evidence that it also exists in chromatophores is accumula-

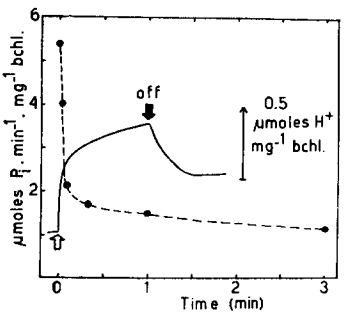

Fig. 6. ●– – –●, time course of the photoinactivation of the phosphorylation system. Chromatophores containing 48 μg bacteriochlorophyll were added to standard reaction medium minus ADP, while the light was on. The light energy was 54 mW. At the times indicated, ADP was added in the light and the maximum photophosphorylation rate was determined. —, kinetics of light-induced proton uptake in the absence of ADP. The light was switched on at $t = 0$. Chromatophores containing 50 μg bacteriochlorophyll were added to standard reaction medium without ADP, at 0.4 min prior to the switching on of the light. The light energy was 54 mW.

ting [38, 39]. Thus, these results indicate that the inactivation shown in Tables I and II was due to "energy transfer" inhibition. This conclusion is supported by the fact that in one batch of chromatophores, uncoupler-stimulated ATPase (Table II), and endogenous as well as PMS-stimulated photophosphorylation (Table I, Exp. 1) were all approximately equally inhibited.

The rate and extent of the light-induced proton uptake in the absence of PMS was exceptionally low in the samples shown in Fig. 5. As discussed above, this was probably due to loss of an endogenous electron transfer component, as a result of the extra washing which was required in this type of experiments.

In control experiments, the photoinactivation appeared to take place regardless of whether the preillumination took place in the electrode vessel or in an all-glass vessel. Moreover, in either case, inclusion of EDTA (0.1 mM) in the preillumination medium did not significantly influence the degree of photoinactivation. This indicates that heavy metal ions were not involved in the photoinactivation (in contrast to results with chloroplasts reported by Uribe [40]), and that the material of the electrode vessel (lucite and stainless steel) did not influence the results. An iron analysis with the *o*-phenanthrolin method [41] was carried out with reaction medium which had been treated in the electrode vessel as in normal experiments, except that no chromatophores had been added. No iron was detected, indicating that the concentration was less than 0.1 μM.

Fig. 6 shows the time course of the photoinactivation caused by preillumination without ADP. The larger part of the inhibition was obtained during the first 6 s of preillumination. A much longer time was required to obtain further inhibition. The time course of the photoinactivation on the one hand, and the kinetics of the light-induced proton uptake in the absence of ADP on the other hand, were similar. The proton uptake measured with the electrode technique, likewise exhibited biphasic kinetics, with a rapid phase which was essentially complete in 6 s.

Table III shows that preillumination in the presence of PMS, but without ADP, had no inhibitory effect if CCCP was present at a concentration (10 μM) sufficient

TABLE III

PROTECTIVE EFFECT OF CCCP ON THE PHOTOINACTIVATION OF THE PHOSPHORYLATION SYSTEM

Chromatophores containing 48 μg bacteriochlorophyll were added to standard reaction medium without ADP 0.4 min before the switching on of the light. After 6 min of preillumination, the light was turned off and the chromatophores were sedimented by centrifugation and resuspended into a minimal amount of fresh reaction medium without ADP and PMS. A control sample was treated similarly except that it was not preilluminated. CCCP (10 μM) was added either before the start of the preillumination (bottom row) or just before centrifugation (2 top rows). After this pretreatment, the maximum photophosphorylation rate was determined as described. Light energy, 45 mW. Bchl, bacteriochlorophyll.

Pretreatment	Maximum photophosphorylation rate (μM $P_i \cdot$ mg Bchl$^{-1} \cdot$ min^{-1})	Inhibition* (%)
Control+CCCP	3.9	0
Preilluminated−CCCP	1.0	75
Preilluminated+CCCP	3.6	8

* Relative to the activity of the control (top line).

TABLE IV

EFFECT OF THE PRESENCE OF PMS DURING PREILLUMINATION, ON THE PHOTOINACTIVATION OF THE PHOTOPHOSPHORYLATION SYSTEM

Standard reaction medium, except that PMS was present as indicated below. Each sample contained 54 μg bacteriochlorophyll (Bchl). Light energy, 54 mW. Pretreated samples (3 top lines): chromatophores were added to the reaction medium without ADP, 0.2 min before the switching on of the light. A preillumination period of 3 min was followed by a dark period of 3 min, in which ADP was added. Photophosphorylation was measured subsequently in the presence of PMS. PMS was added either along with ADP (2 top lines), or before the chromatophores were added (3rd line from top). The dark control (top line) was kept in the dark during the preillumination period, but was otherwise treated identically. Inhibitions are given relative to the dark control. Non-pretreated samples (bottom line): chromatophores were added to the reaction medium containing ADP, with or without PMS, at 0.2 min prior to the switching on of the light. N.D., not determined.

Chromatophores	Maximum photophosphorylation rate (μM $P_i \cdot$ mg Bchl$^{-1} \cdot$ min^{-1})		Inhibition (%)
	−PMS	+PMS	
Pretreated			
Dark−PMS	N.D.	5.6	0
Preillum.−PMS	N.D.	5.1.	9
Preillum.+PMS	N.D.	1.6	71
Non-pretreated	3.5	6.5	N.D.

to completely inhibit photophosphorylation (see Fig. 3 and Discussion). This indicated that the presence of PMS and absence of ADP, by themselves, were not sufficient for photoinactivation to occur. The photoinactivation apparently requires an energized membrane (light-induced proton uptake was also completely inhibited by 10 μM CCCP).

The bottom line of Table IV shows that in untreated chromatophores, the rate of endogenous photophosphorylation was about 40 % of the rate of PMS-stimulated photophosphorylation. A similar ratio was usually found; it can be ascribed to a higher degree of membrane energization, due to the enhanced rate of cyclic electron flow in the presence of PMS [42, 43].

The three top lines of Table IV show that preillumination in the absence of both PMS and ADP caused only 9 % inactivation of PMS-stimulated photophosphorylation. By contrast, the presence of PMS during preillumination resulted in an inactivation of 71 %. Whether this stimulation of the photoinactivation by PMS is due solely to the higher degree of membrane energization obtained during preillumination in the presence of PMS (see above) remains to be established.

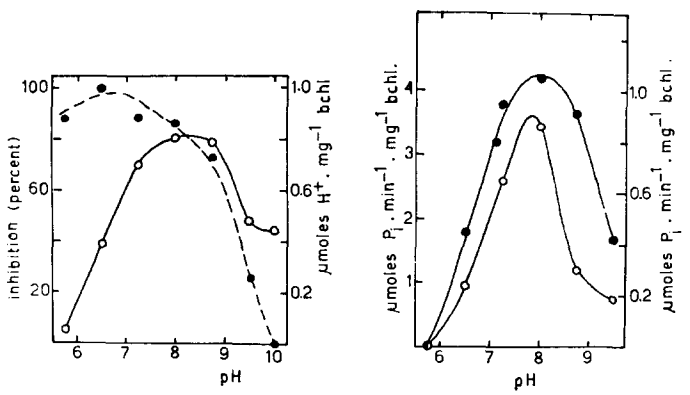

Fig. 7. The extent of the proton uptake during preillumination, and the inhibitory effect of preillumination, as a function of pH during the preillumination. Chromatophores containing 17–20 μg bacteriochlorophyll were added to standard reaction medium supplemented with 30 mM KCl, without ADP and at the indicated pH, at 0.8 min prior to the onset of light. After 4 min of preillumination, the light was turned off for 4–6 min, during which ADP was added; the pH was adjusted to 8.0 with 0.1 M NaOH or HCl prior to the measurement of photophosphorylation. Controls were treated similarly, except that they were kept in the dark during the preillumination period. The maximum photophosphorylation rate of the dark controls was 3.8–4.4 μmol Pi esterified per mg bacteriochlorophyll and per min. The light energy was 45 mW. ○ — ○ (left hand scale), percentage of inhibition of photophosphorylation, as obtained from the maximum photophosphorylation rate (at pH 8.0), of the preilluminated sample, relative to that of the control. ● - - - ●, maximum extent of the proton uptake observed during the preillumination (right hand scale).

Fig. 8. pH-dependence of endogenous and PMS-stimulated photophosphorylation. The final pH of standard reaction medium supplemented with 30 mM KCl, was as indicated. The light energy was 45 mW. Values for the molar ratio of $d[H^+]/d[P_i]$ at different pH values were taken from ref. 24. ○ — ○, left hand scale: PMS present. Chromatophores containing 48 μg bacteriochlorophyll were added to the reaction medium at 1.6 min prior to switching on of the light. ● — ●, right hand scale: PMS absent. Chromatophores containing 41–44 μg bactrriochlorophyll were added 1 min prior to switching on the light.

TABLE V

EFFECTS OF PHOTOPHOSPHORYLATION SUBSTRATES ON THE PHOTOINACTIVATION OF THE PHOSPHORYLATION SYSTEM

Chromatophores were preilluminated for 4 min in the presence of PMS and with substrates as indicated. After switching off the light, the lacking phosphorylation substrates were added and the chromatophores were sedimented by centrifugation and resuspended in approx. 0.1 ml of fresh reaction medium without ADP and PMS. Photophosphorylation was measured as usually. Inhibitions are given relative to the activity of the control after centrifugation (top line). Reaction medium was standard. Light energy, 45 mW. Bacteriochlorophyll (Bchl), 15–26 μg.

Substrates present during preillumination	Maximum photophosphorylation rate (μM Pi · mg Bchl^{-1} · min^{-1})		Inhibition (%)
	During preillumination	After preillumination and centrifugation	
ADP+P_i+Mg^{2+}	1.40	1.73	0
ADP+P_i	0	0.46	73
P_i+Mg^{2+}	0	0.47	73
ADP+Mg^{2+}	0	0.58	66

TABLE VI

EFFECT OF ATP DURING PREILLUMINATION, ON THE PHOTOINACTIVATION OF THE PHOTOPHOSPHORYLATION SYSTEM

Chromatophores containing 49 μg bacteriochlorophyll (Bchl) were added to the standard reaction medium while the light was on (54 mW). In the control sample (top line), ADP was present before addition of the chromatophores. In the preilluminated samples, photophosphorylation was started (and preillumination was terminated) by addition of ADP in the light, at 3 min after addition of the chromatophores. ATP was present as indicated at a final concentration of 2.2 mM. ADP, P_i and Mg were present at the usual concentrations.

Substrates present during		Maximum photophosphorylation rate (μM P_i · mg Bchl^{-1} · min^{-1})	Inhibition (%)
Preillumination	Photophosphorylation (initially)		
–	ADP+P_i+Mg^{2+}	5.4	0
P_i+Mg^{2+}	ADP+P_i+Mg^{2+}	1.1	80
ATP+P_i+Mg^{2+}	ATP+ADP+P_i+Mg^{2+}	0.6	89

* Relative to the activity of the control (top line).

In the experiments shown in Fig. 7, photophosphorylation at pH 8.0 was measured after a preillumination or dark incubation at the indicated pH values. The maximum photophosphorylation rate of the preilluminated sample was determined relative to that of the dark control sample. There was no correlation between the extent of proton uptake attained during the preillumination (solid circles) and the degree of photoinactivation caused by the preillumination (open circles). In the experiments shown, the extent of light-induced proton uptake decreased strongly at pH values above 8.7; no proton uptake was detectable at pH 10. However, the

photophosphorylation at pH 8.0 was found to be appreciably inhibited after preillumination at pH 10.0. If membrane energization in the absence of phosphorylation substrates is the cause of the inactivation, it must be assumed that a proton/cation exchange prevented a net proton uptake during preillumination at pH 10. Preillumination at pH 5.8 led to a considerable proton uptake, but it did not result in an appreciable inhibition of photophosphorylation.

Fig. 8 shows the pH-optimum curve for endogenous and PMS-stimulated photophosphorylation. Similar results were obtained earlier [44, 45]. The rate of PMS-stimulated photophosphorylation declined more steeply than that of endogenous photophosphorylation when the pH was raised to values above 8.0 (Fig. 8). This is probably related to an immediate, reversible inhibition of photophosphorylation caused by photoreduced PMS (see above). The pH-optimum curve for endogenous photophosphorylation was however remarkably similar to the pH-optimum curve for the photoinactivation of the phosphorylation system (Fig. 7).

As shown in Fig. 2, phosphorylation conditions offered a complete protection against the photoinactivation of photophosphorylation. This is also shown in the first line of Table V. It was of interest to see whether individual phosphorylation substrates would offer protection against the photoinactivation. Some results are shown in Table V. Similar degrees of inhibition were obtained in other experiments in which control chromatophores had an activity two to three times higher. The photoinactivation was virtually the same, no matter whether ADP, P_i or Mg^{2+} was omitted during preillumination in the presence of PMS.

The findings referred to in the second alinea of Results already suggested that ATP did not offer protection against the photoinactivation. This is also clear from results given in Table VI. Preillumination in the absence of ADP resulted in an even more severe inhibition when ATP was present during the preillumination. This difference was partly due to the fact that ATP at 2.2 mM gave a 15 % inhibition of photophosphorylation in untreated chromatophores (not shown; cf. ref. 46), presumably due to chelation of free Mg^{2+} [47]. Even so, it is clear that ATP did not offer any protection against the photoinactivation caused by preillumination in the absence of ADP.

DISCUSSION

Preillumination of chromatophores in the presence of PMS under non-phosphorylation conditions resulted in an irreversible inactivation of coupling factor activities (Tables I and II). The site of inactivation is presumably the coupling factor itself, in view of the unimpaired capability of the inactivated chromatophores for light-induced proton uptake (Figs. 4 and 5). Heavy metal ions do not seem to be involved in the photoinactivation.

The photoinactivation requires an energized membrane (Table III), and is greatly stimulated by PMS (Table IV). One possible explanation for this is that the photoinactivation occurs only if during preillumination in the absence of ADP, the membrane energization exceeds a certain value. PMS would be required only to raise the light-induced membrane energization above that value. This would be reminiscent of the *N*-ethylmaleimide photoinactivation in chloroplasts, which also occurs predominantly at a very high degree of membrane energization [48]. Alterna-

tively, PMS may participate more directly in the reaction(s) leading to photoinactivation.

The photoinactivation was virtually the same, no matter whether ADP, P_i or Mg^{2+} was omitted during preillumination in the presence of PMS (Table V), indicating that none of these compounds offered an appreciable protection against the photoinactivation. Likewise, ATP offered no protective effect (Table VI). These results suggest that the photoinactivation is not prevented by allosteric effects exerted by phosphorylation substrates or ATP on the coupling factor enzyme. This would be in contrast with previous results on photoinactivation of the coupling factor enzyme in chromatophores [20] and chloroplasts [13–17, 40, 49]. Whether such allosteric effects play a role in photoinactivations induced by shorter preillumination periods, e.g. 6 s (see Fig. 6), remains to be established.

In chloroplasts, the magnesium salt of ADP rather than the free acid probably serves as a substrate during photophosphorylation [47], and it was suggested that this may also be the case in *R. rubrum* chromatophores [47]. This would explain why the absence of either Mg^{2+} or ADP during preillumination resulted in an equally extensive photoinactivation (Table V), if it is assumed that the photoinactivation of the coupling factor enzyme occurs whenever photophosphorylation is inhibited for lack of substrates.

The similar pH-dependences of endogenous photophosphorylation (Fig. 8) and of the photoinactivation of the photophosphorylation system (Fig. 7), raise the the possibility that these two reactions proceed via one or more common intermediate states or compounds. These intermediates would lead to photophosphorylation or photoinactivation, depending on whether exogenous phosphorylation substrates are present or not. Accepting this as a working hypothesis, one can at present only speculate about the nature of these intermediates. A possible candidate might be an energy-dependent (Table III) conformational change which takes place predominantly at pH-values around 8.0 (Figs. 7 and 8). One could also think of light-induced, energy-dependent changes in the content of tightly-bound noucletides; such changes have been shown to occur in chloroplasts [50] and chromatophores [51–53]. No photoinactivation occurs under phosphorylation conditions (Fig. 2, Table V, first line). This may be because the turnover of the coupling factor enzyme prevents accumulation of the intermediate(s) required for photoinactivation.

By depletion and reconstitution experiments with untreated and photoinactivated chromatophores, it may be possible to determine whether the site of inactivation is in the F_1-component or in the membrane sector [2] of the coupling factor enzyme. Until we know this much, there is little use in speculating about the nature of the reactions leading to photoinactivation.

An investigation of this problem seems worthwhile, however, because it may yield some interesting data on the nature of the mechanism whereby membrane energization drives the photophosphorylation reaction.

In Fig. 3 (triangles, right hand scale), the photophosphorylation activity of normal chromatophores in the presence of PMS was titrated with CCCP (see legends to Fig. 3). The resultant curve shows negative values, indicating a high ATPase activity at high concentrations of CCCP. This was probably due to the fact that CCCP was added in the light in progressively increasing concentrations; when the light was shut off after addition of CCCP to 15 μM/l, the ATPase rate declined to a low value

(arrow) and subsequent illumination had no effect. The explanation is probably that prior to switching off the light, the coupling factor was in a high-energy state which was maintained by the light and by its own activity. After switching off of the light, the enzyme returned to a low-energy state, which could not be reversed by light alone. Similar explanations have been offered before [32, 54].

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