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LIGHT-INDUCED ELECTRON TRANSFER IN *CHROMATIUM* STRAIN D

III. PHOTOPHOSPHORYLATION BY *CHROMATIUM* CHROMATOPHORES

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SUMMARY

Photophosphorylation at relatively high rates occurs in light-particle preparations described previously as derived from *Chromatium* chromatophores. It is shown that the optimal conditions for photophosphorylation correlate with changes in absorbance established in previous researches as the result of ambient redox potentials between 50 and 100 mV. Effects of various dyes, phenazine methosulfate, oxygen and ascorbate are presented and discussed. Attempts to demonstrate by direct measurement the effective redox potentials for photophosphorylation are described. The evidence accumulated favors occurrence of a cyclic phosphorylation mechanism associated with one of the electron transport pathways (the 'P-890 pathway') postulated in a model presented in a previous paper.

INTRODUCTION

In previous papers[1,2], we have described the preparation and characterization of *Chromatium* chromatophores ('light particles') and absorbance changes induced in them by actinic radiation. In a continuation of these researches, as described in this paper, we have demonstrated, that these particles are capable of a physiological function—photophosphorylation—which can be correlated with light-induced absorbance changes.

Although there have been extensive studies of photophosphorylation in the Athiorhodacea[3,4], the Thiorhodacea have received less attention[5,6]. Further, the existing studies of *Chromatium* have not been carried to the point of correlation between light-induced absorbance changes and a characterized phosphorylating system.

The present study describes results bearing on such correlations as exhibited under the previously defined 'optimum conditions'[2] for the light reaction, relative to light intensity, bacteriochlorophyll (BChl) concentration, pH and buffers.

Abbreviation: BChl, bacteriochlorophyll.

METHODS

Chromatium light particles were prepared as previously described[1] and stored under Ar at 4° in the dark prior to use. The apparatus used for the determination of light-induced absorbance changes has been described[2].

ATP formation was measured using a reaction system[7] consisting of hexokinase (Sigma Chemical Co., St. Louis, Mo.) and glucose *plus* glucose-6-phosphate dehydrogenase (Sigma) and $NADP^+$ (Sigma), in which $NADP^+$ is produced in amounts equivalent to the amount of ATP generated photosynthetically. The components of the reaction mixture are indicated in Table I.

Assays were performed in the anaerobic cuvette system described previously[2]. At least 12 ml of reaction components were required. The reaction mixture was deaerated by bubbling with oxygen-free N_2 (ref. 2). The particles were added to a final concentration of 30–40 μM in BChl, a portion of this suspension was placed in the reference cuvette, and the suspension was illuminated with $2.2 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1} of 888 mμ light (a Schott–Jena interference filter was used). The conditions described have been previously determined as 'optimum' (see ref. 2) and were used in all experiments described, unless otherwise noted. The reduction of $NADP^+$ was followed by observing the increase in $A_{340\,m\mu}$ as a function of time, using a Cary 14R recording spectrophotometer.

The amount of ATP formed was calculated from the maximum rate of reduction of $NADP^+$, determined from the increase in $A_{340\,m\mu}$/h using $\Delta\varepsilon_{mM} = 6.2$, and then multiplying this value by the appropriate factor to give the final result in units—mμmoles ATP formed per h per μmole BChl.

This method of measuring photophosphorylation had the merit of permitting dynamic measurements of the reaction and further allowed experiments to be conducted under the same conditions used for previous studies[2] of light-induced changes of chromatophore components.

RESULTS

Fig. 1A shows a typical measurement of photophosphorylation. The sample was monitored at 340 mμ and the recorder started just prior to illumination. After a lag time of several minutes, the increase in absorbance became constant and continued in a linear fashion indefinitely (1 h was the longest time employed with no variation in rate). Upon cessation of illumination, the reaction continued at a steadily decelerating rate, ending in about 2 min. The lag period was inherent in the assay system. No phosphorylation could be detected until a sufficient pool of ATP had been generated to support the reduction of the pyridine nucleotide. The existence of the lag period was not relevant for present purposes because we were interested only in comparative steady-state levels rather than absolute rates.

This method enabled direct observation of light-induced phosphorylation without necessity for correction due to dark reactions during the course of the experiment. The only problem encountered was a slight variation in the level of phosphorylation from day to day. For this reason, each set of experiments, including the control utilizing the complete reaction mixture, was performed in duplicate on the same day.

Comparison of particles

Table I*A* shows a comparison of levels of phosphorylation in the classical[1], 'heavy' and 'light' particles[1]. Also presented are the standard deviations for four determinations on each fraction.

The light particles show a greater than 2-fold increase in photophosphorylation over that seen in the classical chromatophores and heavy particles. The standard deviations indicate excellent reproducibility in all fractions. The levels found compare

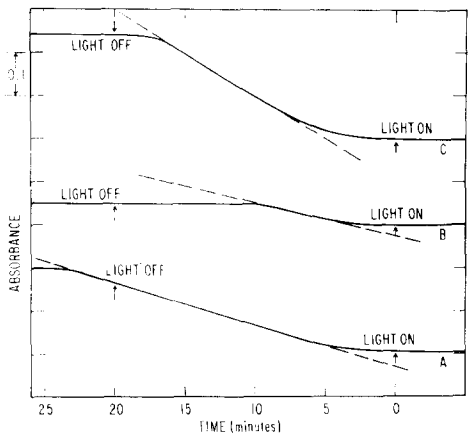

Fig. 1. Measurement of photophosphorylation. Reaction mixture as described (Table I), BChl concentration 35 μM, $I = 2.2 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 888 mμ. A. Complete, anaerobic conditions. B. Complete, *plus* 3 μM methylene blue, anaerobic conditions. C. Complete, aerobic conditions.

TABLE I

PHOSPHORYLATION BY *Chromatium* CHROMATOPHORES

The components of the reaction mixture included (mM): $MgCl_2$ 2, K_2HPO_4 30, ADP 0.5, glucose 20, $NADP^+$ 0.5 and the 'coupling enzymes' (U/ml) hexokinase 0.7 and glucose-6-phosphate dehydrogenase 0.5. All components were suspended in 0.1 M glycylglycine–10% sucrose (pH 7.5) at 22° ±2°. The BChl concentration was 35 μM and the intensity of 888 mμ actinic light was $2.2 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}.

Assay system	*mμmoles ATP per h per μmole BChl*		
	Classical chromatophores	*Heavy particles*	*Light particles*
A. Photophosphorylation			
Complete	1410 ($\sigma = 120$)	1170 ($\sigma = 110$)	3470 ($\sigma = 82$)
Minus $NADP^+$ and coupling enzymes	0	0	0
Minus coupling enzymes	350	0	0
B. Dark controls			
Dark, no $NADP^+$ no coupling enzyme	0	0	0
Dark, no coupling enzyme	1110	1170	0
Dark, 2 min after addition of coupling enzymes	5000*	2000*	100*
Dark, complete (myokinase)	2340	900	350

* These values were not determined from the rate of ATP formation, but represent the amount of ATP detected in the first 2 min after addition of the coupling enzymes.

favorably with those reported previously. ANDERSON AND FULLER[6] obtained levels 5–10 times as great; however, they routinely added a supernatant factor and used saturating light intensity. The levels reported here are 5–10 times higher than those reported by NEWTON AND KAMEN[5].

With the methods used, the only controls required were the light-dependent controls (reaction mixtures without $NADP^+$ or without coupling enzymes). These are also presented in Table I*A*. The apparent photoreduction of $NADP^+$ by the classical chromatophores, as shown, was only observed once out of five determinations, so it was not certain that the classical chromatophores were capable of this function. All other attempts to demonstrate a photoreduction of $NADP^+$ under a variety of conditions failed with all of the particle fractions.

Although the dark reactions were compensated for during the measurement of light-induced phosphorylation, it was of interest to measure their magnitude. These measurements were made by placing water in the reference cuvette and following the change at 340 mμ in the dark. Table I*B* shows the result of these experiments (average values for four determinations with each assay system). The occurrence of the dark reduction of $NADP^+$ without coupling enzymes, while not understood, indicates the presence of a reasonably large pool of endogenous reductant capable of reducing $NADP^+$.

The endogenous level of ATP *plus* glucose 6-phosphate was determined by following the increase in absorbance immediately after the addition of the coupling enzymes (hexokinase and glucose 6-phosphate). The levels observed were difficult to quantitate because of myokinase activity during the time the endogenous ATP was being measured. In general, we observed a rapid increase in absorbance on the addition of coupling enzymes and then a leveling off to a steady increase (myokinase) after 2 min. These results indicate that there was a large amount of ATP present (Table I*B*) in the particles. The light particles showed a marked removal of a large portion of this endogenous ATP.

The myokinase levels observed indicated that the light particles had been greatly purified relative to the myokinase, and that the large ratio of photophosphorylation to myokinase activity in the light particles probably accounted for the greater reproducibility in the levels of phosphorylation by the light particles.

Light intensity

In contrast to the studies[2] on light-induced cytochrome and chlorophyll changes, $2.2 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1} was not the saturation light intensity for photophosphorylation and gave only approximately half the maximal rate observed at light-saturation with $8.2 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}.

Light-induced absorbance changes under photophosphorylating conditions

It has been previously shown that a deaerated suspension of light particles gives light-induced absorbance changes which are quite similar to those observed when a redox potential of ca. 200 mV is imposed on the particles[2]. As shown in Fig. 2A, the light reaction in the presence of ADP, P_i and $MgCl_2$ gives changes identical to those which occur with an imposed redox potential between 100 and 50 mV. In this region, five of a possible six components identified[2] in the cytochrome region undergo light-induced changes, including both C-552 and C-555. Evidently, in the presence of added

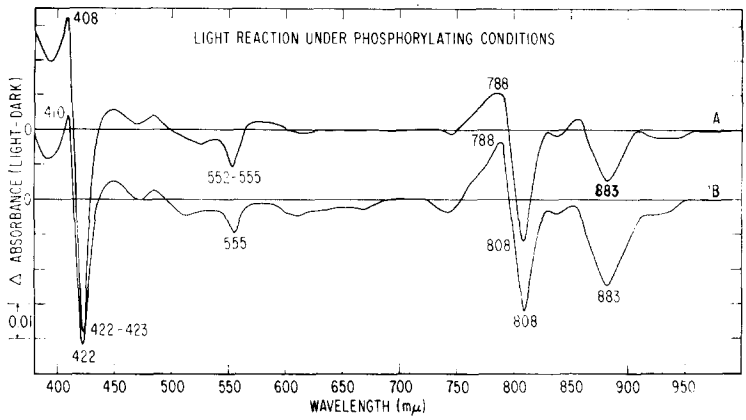

Fig. 2. A. Light-induced absorbance changes under photophosphorylating conditions. Complete reaction mixture (Table I). B. Same, only aerobic conditions. BChl concentration 35 μM, $I = 2.2 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 888 mμ.

ADP the light particles react as if the redox potential of the electron carriers became poised at a value effective in the so-called 'transition region'[2].

Fig. 2B shows the light reaction in the presence of ADP, P_i, $MgCl_2$ and molecular oxygen. Under these conditions, the changes induced by light are identical to those observed under an imposed redox potential of 200 mV. When light particles are diluted to a BChl concentration of 35 μM and allowed to stand in air for 12 h at 0°, there is a 70–90 % decrease in the magnitude of the light reaction of cytochromes and BChl (ref. 2). When ADP and P_i is added to light particles and these are then treated as described, there is only a 50 % decrease in the magnitude of the light-induced absorbance changes.

These results indicate that, besides effecting the poising of the redox potential of the electron carriers (100 to 50 mV), the presence of ADP and P_i can counteract to some extent the inactivation of the light reaction by molecular oxygen, perhaps by facilitating the action of the endogenous reductant[2] on the oxidation state of the electron carriers.

Redox range of photophosphorylation

Attempts to poise the potential of the particles in the transition region of the light-induced absorbance changes with redox buffers were hampered by the lack of redox buffers in this range which did not involve the use of strong chelating agents (Fe-EDTA, Fe-oxalate, *etc.*). These buffers would be expected to interfere by chelation of Mg^{2+}, thus inhibiting both hexokinase and photophosphorylation. This was verified by the observation that addition of redox buffers such as Fe-EDTA and Fe-oxalate inhibited photophosphorylation at potentials in the range 200 to 0 mV.

In an attempt to measure the potential during photophosphorylation, minimal amounts of redox buffers with midpoint potentials in the redox region 125 to 0 mV were used.

Methylene blue ($E_0' = 11$ mV)[8] was added to the light particles and amounts of ATP formed were measured. Upon illumination, the potential rose rapidly and then slowly drifted downward (Table II).

TABLE II

EFFECT OF METHYLENE BLUE ON PHOTOPHOSPHORYLATION

Assay mix	*mμmoles ATP per h per μmole BChl*	*E_h during photo-phosphorylation (mV)*	*Duration of photo-phosphorylation (min)*
Control	1270	—	>60
Plus 10 μM methylene blue	1370	52 to 30	10
Plus 3 μM methylene blue	1240	44 to 24	10
Plus 1 μM methylene blue	1360	29 to 10	15

Methylene blue did not affect the level of photophosphorylation; however, the reaction was terminated after a short time (see Fig. 1B). The potentials observed were consistent with the lower portion of the transition region of the light-induced absorbance changes.

Brilliant cresyl blue ($E_0' = 40$ mV)[8] was used in the same fashion as methylene blue. The results were much like those for methylene blue, with the measured redox potential 10 to 25 mV.

The light particles were treated with phenazine methosulfate ($E_0' = 80$ mV)[8]. Results are given in Table III.

TABLE III

EFFECT OF PHENAZINE METHOSULFATE ON PHOTOPHOSPHORYLATION

Assay mix	*mμmoles ATP per h per μmole BChl*	*E_h during photo-phosphorylation (mV)*	*Duration of photo-phosphorylation (min)*
Control	1570	—	>60
Plus 500 μM phenazine methosulfate	0	110	0
Plus 100 μM phenazine methosulfate	1980	78 to 58	20
Plus 50 μM phenazine methosulfate	1740	80 to 52	20
Plus 10 μM phenazine methosulfate	0	32	0

The results with phenazine methosulfate are different from those cited above in that either too much or too little reagent inhibited photophosphorylation. Apparently, at high concentrations of phenazine methosulfate the imposed potential was too high to allow photophosphorylation and at lower concentrations the potential was too low. This interpretation would suggest a poor coupling between the redox electrodes and phenazine methosulfate, because the measured potential is in a range where photophosphorylation has been observed. Alternatively, at high phenazine methosulfate concentrations, the ATP-generating site could be bypassed.

Effect of ascorbate

Particular attention has been paid to the effect of ascorbate ($E_0' = 58$ mV)[8] because of its widespread use as an activator of photophosphorylation[3,5]. Table IV gives results obtained.

TABLE IV

EFFECT OF ASCORBATE ON PHOTOPHOSPHORYLATION

Assay mix	*mμmoles ATP per h per μmole BChl*	*E_h during photo-phosphorylation (mV)*	*Duration of photo-phosphorylation (min)*
Control	1570	—	>60
Plus 30 mM ascorbate	0	15	—
Plus 3 mM ascorbate	0	26	—
Plus 300 μM ascorbate	2840	48	>60
Plus 30 μM ascorbate	2500	68	>60
Plus 10 μM ascorbate	1890	60	>60

The results suggest that too high an ascorbate concentration inhibits photophosphorylation. This may be a kinetic effect—that is, C-555 is reduced by ascorbate more rapidly than it can be oxidized by light. It has been shown that in the presence of 3 mM ascorbate, C-555 does not participate in the steady-state light-induced absorbance changes.

At 300 μM ascorbate it has been shown that the light reaction corresponds to the transition region (both C-555 and C-552 being light-oxidized)[2]. Concentrations of 30 and 10 μM ascorbate result in the light-oxidation of only C-555.

These results indicate that photophosphorylation is stimulated in the redox region, 150 to 50 mV by ascorbate, and the light-oxidation of C-552 is not required for photophosphorylation. These interpretations are not consistent with the redox potentials measured; however, ascorbate does not couple efficiently with the electrodes without added mediators[8]. This complication permits estimates of the optimum redox potentials for photophosphorylation, but not determination of the precise values.

The stimulation of photophosphorylation and lack of effect on the lifetime of photophosphorylation by ascorbate indicate that this reagent exerts no detrimental effects when present at the concentrations used.

Effect of oxygen

To study the effect of oxygen on photophosphorylation by the light particles, N_2 was replaced with air and the suspensions aerated by bubbling for 5 min prior to illumination (Table V).

TABLE V

EFFECT OF OXYGEN ON PHOTOPHOSPHORYLATION

Assay mix	*μmoles ATP per h per μmole BChl*	*Duration of photo-phosphorylation (min)*
Control	2980	>60
Plus oxygen	5900	20
Incubated in presence of oxygen for 12 h	930	10

There was a striking activation of photophosphorylation in the presence of oxygen; however, the production of ATP stopped after 20 min (see Fig. 1C). It can also be seen from Table V that incubation of the particles in the presence of oxygen resulted eventually in an inhibition of photophosphorylation. As previously noted, the light reaction was not initially modified in the presence of oxygen and ADP, but after long incubation the light reaction was inhibited.

DISCUSSION

Our studies of photophosphorylation demonstrate that the light particles are fully capable of carrying on photophosphorylation at levels 2–3 times those observed with the classical chromatophores, most likely because of the absence of components which lower the level of phosphorylation by interaction with electron transport components as well as removal of non-photophosphorylating BChl-containing fractions.

Measurements incidental to the assay for photophosphorylation which include determinations of myokinase and endogenous ATP also demonstrate that the light particles have been greatly purified relative to the classical chromatophores and heavy particles. Further, the results show that the removal of the bulk of reductases[2] and other soluble enzyme systems, such as myokinase, does not affect photophosphorylation markedly.

Electron transport associated with photophosphorylation must be of cyclic nature because we added no electron donors or acceptors. The continued production of ATP with time indicates that the electron carriers associated with photophosphorylation are being alternately oxidized and reduced.

The light-induced absorbance changes under photophosphorylating conditions indicate that the light reaction is in the transition region, that is, both the P890 and P905 reaction centers[2] are interacting with light upon illumination. The addition of various redox agents (phenazine methosulphate, methylene blue, cresyl blue, and ascorbate) which can couple with both the particle-bound proteins and the redox electrodes, indicate that phosphorylation takes place in the redox region 25 to 150 mV. The optimum level of photophosphorylation is obtained when the apparent redox potential of the system is between 50 and 100 mV.

Photophosphorylation by the light particles in the presence of molecular oxygen is stimulated initially to high levels, however, this response is short-lived. The light reaction in air in the presence of ADP and P_i is similar to that observed when the particles are poised anaerobically at approx. 200 mV (ref. 2). This implies that C-552 is not involved in this photophosphorylation. These results may indicate the occurrence of a non-cyclic phosphorylation with oxygen as the terminal electron acceptor. From the final level of ATP formed in the presence of oxygen, we deduce that approx. 90 times as much ATP is formed as there is reduced C-555 originally present. This may be understood on the basis of a pool of reductant able to reduce the cytochrome available. This suggestion is consistent with the existence of the previously discussed endogenous reductant[2], which was found to be present to the extent of 20–100 equiv/mole BChl or approx. 1700–8000 equiv/mole of C-555.

The mechanism of control exerted by ADP on the redox level of the electron carriers and the ability of ADP to inhibit oxygen inactivation is not understood. A

similar control has been observed in mitochondria[9]; this process, termed 'reverse-electron transport' is dependent on the ATP/ADP ratio. CHANCE *et al.*[10] have suggested that this process is operative in the photosynthetic bacteria.

At redox potentials below 25 mV, no evidence of phosphorylation was obtained. In this redox region, C-552 is the only cytochrome identified as reactive in the light-induced absorbance changes in the cytochrome α region.

No evidence of photophosphorylation is obtained above a measured potential 100 mV, although the form and magnitude of the light reaction in the presence of 3, 10 and 30 μM ascorbate[2] would suggest that the potential is 100 to 200 mV under these conditions.

We conclude that cyclic photophosphorylation takes place and is associated with the P890 reaction center. The light-oxidation of C-552 that is observed when photophosphorylation is taking place apparently is not associated either with the cyclic electron transport system or with the generation of ATP.

When photophosphorylation is occurring, it is always associated with a light reaction similar to that observed when the redox potential of the environment of the light particles is poised at 25 to 150 mV. Thus, photophosphorylation is associated with a steady state in which the components involved are mainly oxidized.

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REFERENCES

1 M. A. CUSANOVICH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 153 (1968) 376.
2 M. A. CUSANOVICH, R. G. BARTSCH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 153 (1968) 397.
3 T. HORIO AND J. YAMASHITA, in H. GEST, A. SAN PIETRO AND L. P. VERNON, *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio, 1963, p. 275.
4 T. HORIO AND M. D. KAMEN, *Biochemistry*, 1 (1962) 144.
5 J. W. NEWTON AND M. D. KAMEN, *Biochim. Biophys. Acta*, 25 (1957) 474.
6 I. C. ANDERSON AND R. C. FULLER, *Arch. Biochem. Biophys.*, 76 (1958) 168.
7 O. H. LOWRY, J. V. PASSONEAU, F. X. HASSELBERGER AND D. W. SCHULZ, *J. Biol. Chem.*, 239 (1964) 18.
8 W. M. CLARK, *Oxidation–Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, Md., 1st ed., 1960.
9 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 238 (1963) 432.
10 B. CHANCE, M. NISHIMURA, S. B. ROY AND H. SCHLEYER, *Biochem. Z.*, 338 (1963) 654.