LIGHT INACTIVATION OF PHOTOPHOSPHORYLATION BY SWISS-CHARD CHLOROPLASTS

MORDHAY AVRON

Biochemistry Section, Weizmann Institute of Science, Rehovoth (Israel)

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SUMMARY

1. Photophosphorylation by swiss-chard chloroplasts was rapidly inactivated by preincubation of the reaction mixture in light. 50% inactivation was observed after 90 sec exposure to 160,000 lux.

2. The presence of phenazine methosulfate during the light preincubation period largely prevented the inactivation.

3. The inactivation was observed under a nitrogen atmosphere, when the preincubation was carried out under red light, with chloroplasts or chloroplast fragments, but not in the dark.

4. The quantitative requirements for light and phenazine-methosulfate during the preincubation and the following reaction periods were found similar to those of the photophosphorylative reaction proper.

5. No bleaching of chlorophyll could be observed during the light inactivation period. Addition of non-light-pretreated chloroplasts to pretreated ones did not restore their photophosphorylative abilities.

6. It is suggested that the light inactivation observed was the result of a destructive reaction of the normally produced early products in photophosphorylation.

INTRODUCTION

Light dependent phosphorylation was shown to be catalysed by swiss-chard chloroplasts at an extremely rapid rate[1]. Under optimal conditions rates of 2000 μmoles of ATP synthesized/mg chlorophyll/h could be demonstrated. It was noted, however, that the attainment of such rates was dependent, among others, on the presence of phenazine methosulfate as cofactor, the use of very high light intensities, and a very short reaction time (2 min or less). The unusually high activity of phenazine methosulfate, as compared with other cofactors, has been emphasized previously[2]. It is thought that this high activity might be related to the observations reported herewith.

The investigation of the light inactivation of photophosphorylation was prompted by the rapid decrease of photophosphorylative rate with time noted under the above mentioned optimal conditions[1]. Although it is still not possible to prevent

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Tris, tris-(hydroxymethyl)aminomethane; PMS, phenazine methosulfate.

this decrease, it is hoped that the following might offer some clues for future investigations.

MATERIALS AND METHODS

For details of methods see ref. 1.

Preincubation experiments were run by exposing identical volumes of reaction mixtures to light for a specified time in the absence of one or more specified components (ADP was absent in all treatments); equal total volumes of materials were added in the dark as fast as possible, the flask contents were mixed, and returned to the light for a second specified time period. ATP production proceeded, therefore, only during the second exposure to light, since ADP was always absent during the initial exposure. It should be noted that by the second exposure to light all reaction mixtures had received the same identical components. The difference between treatments was limited, therefore, to changes as to which components were added before and which after the initial incubation in light.

Red light was produced by passing the white light through two layers of red cellophane paper. The paper had about 90% transmittance in the 650–700 mμ region, and less than 1% transmittance in the 400–570 mμ region. Unless otherwise indicated, white light was always used.

RESULTS

The decrease in the photophosphorylative activity of swiss-chard chloroplasts by pretreatment in light is shown in Fig. 1. It is evident that the high light intensity

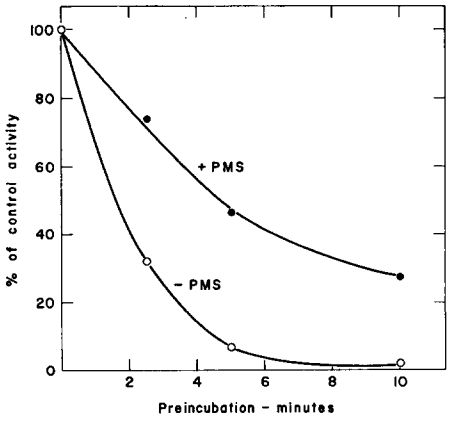

Fig. 1. Effect of preincubating chloroplasts in light for increasing periods in the presence or absence of PMS on their photophosphorylative abilities. Reaction mixture contained during the preincubation period the following components, in μmoles: Tris, pH 7.8, 45; NaCl, 60; $MgCl_2$, 12; sodium ascorbate, 30; phosphate, pH 7.8, 12 (containing $2–5 \cdot 10^5$ counts/min of ^{32}P), PMS (only in + PMS samples), 0.09, and once-washed chloroplasts containing 18 μg chlorophyll, in a total volume of 2.80 ml. After preincubation in light of 160,000 lux for the specified times, 4 μmoles of ADP (and 0.09 μmoles of PMS in the samples which did not contain PMS during preincubation) were added in a total volume of 0.20 ml. The contents were mixed and exposed to 160,000 lux of light for 2 min. Trichloroacetic acid was added to a final concentration of 2%, the reaction mixtures centrifuged, and the supernatant analysed for its [^{32}P]ATP content as already described[1]. Temperature, 17°, Gas phase:air. 100 on the ordinate corresponds to an activity of 1200 μmoles ATP formed/mg chlorophyll/h.

caused rapid inactivation. 50 % inactivation, as compared to non-pretreated controls, was attained after only 90 sec of light pretreatment. Virtually total inactivation required only about 8 min of preincubation in the light.

Early in the studies of the phenomenon it became clear that PMS, the cofactor of the reaction, could largely prevent the inactivation process. Fig. 1 illustrates the inactivation observed in the presence and absence of PMS during the preincubation period. In the presence of PMS, 50 % inactivation was attained only after about 5 min of light pretreatment. The extent of inactivation, and of its prevention induced by the presence of PMS during preincubation, has been variable, but both effects have always been large and clear cut. In some preparations, the presence of PMS during the preincubation period brought about virtually complete protection from light inactivation. The data of Fig. 1 show a typical, rather than an extreme case.

Several other materials were tested as to their ability to show the same protective effect as PMS. The other cofactors tried, flavin mononucleotide, and menadione, showed small to no protective action. Ascorbic acid had some protective effect, but never as large as the one exhibited by PMS.

Expts. 1 and 3 in Table I illustrate that the results were totally dependent on light, in the presence of PMS. Some inactivation occurred, however, if PMS was absent during a dark preincubation. The latter was always small in relation to the inactivation caused by preincubation in the light (Expt. 3 in Table I). Although

TABLE I

EFFECT OF LIGHT AND GAS PHASE ON PHOTOINACTIVATION

Conditions as described under Fig. 1, except for variation, as indicated: Expt. 1, once washed chloroplasts containing 31 μg chlorophyll per flask. Control value: 530 μmoles ATP formed/mg chlorophyll/h. Expt. 2, darkness was obtained by completely covering reaction flasks with aluminum foil. Once washed chloroplasts containing 20 μg chlorophyll per flask. Control value: 600 μmoles ATP formed/mg chlorophyll/h. Gas phase was changed to nitrogen by bubbling prepurified nitrogen through a gas disperser into alkaline pyrogallol, then through water and then through the flasks. The nitrogen was passed for 5 min before the light was turned on, and continuously throughout the reaction period. Expt. 3, once washed chloroplasts containing 17 μg chlorophyll per flask. Control value, 1015 μmoles ATP formed/mg chlorophyll/h. Darkness attained as in Expt. 1. Expt. 4, once washed chloroplasts containing 35 μg chlorophyll per flask. Control value: 700 μmoles ATP formed/mg chlorophyll/h. Red light was produced as described under METHODS.

Expt. No.	*Conditions*						*% of control*
	During preincubation			*During reaction*		*Throughout*	
	Time (min)	*Illumination (lux)*	*Absent*	*Time (min)*	*Illumination (lux)*	*gas phase*	
1	20	Dark	ADP	5	45,000	Air	97
1	20	Dark	ADP + PMS	5	45,000	Air	87
2	20	45,000	ADP	5	45,000	Air	38
2	20	45,000	ADP + PMS	5	45,000	Air	5
2	20	45,000	ADP	5	45,000	Nitrogen	53
2	20	45,000	ADP + PMS	5	45,000	Nitrogen	41
3	5	Dark	ADP	5	160,000	Air	100
3	5	Dark	ADP + PMS	5	160,000	Air	72
3	5	160,000	ADP	5	160,000	Air	45
3	5	160,000	ADP + PMS	5	160,000	Air	15
4	5	160,000	ADP	5	160,000	Air	57
4	5	160,000	ADP + PMS	5	160,000	Air	18
4	5	Red, 160,000	ADP	5	Red, 160,000	Air	70
4	5	Red, 160,000	ADP + PMS	5	Red, 160,000	Air	44

slightly depressed, the same general phenomena appeared when the reaction was run anaerobically, under a nitrogen atmosphere (Expt. 2 in Table I), or in red light in place of white light (Expt. 4 in Table I). It cannot be attributed, therefore, solely to photooxidation by free oxygen, or to action of light on pigments which do not absorb in the red region of the spectrum.

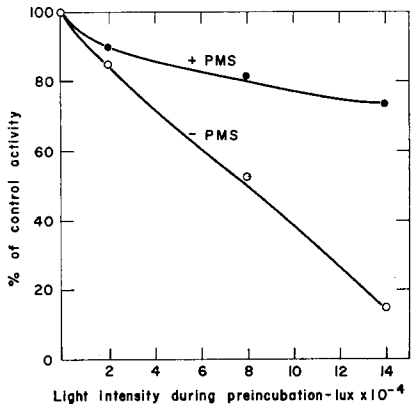

Fig. 2. Effect of light intensity during the preincubation period. Conditions as described under Fig. 1, except for the following: the light intensity during the preincubation period was varied as indicated. The light remained at 160,000 lux in all cases during the second exposure to light. Red light used throughout (see METHODS). Preincubation for 10 min. Reaction (second exposure) for 5 min. Once washed chloroplasts containing 36 μg chlorophyll per flask. 100 on the ordinate corresponds to an activity of 1050 μmoles ATP formed/mg chlorophyll/h.

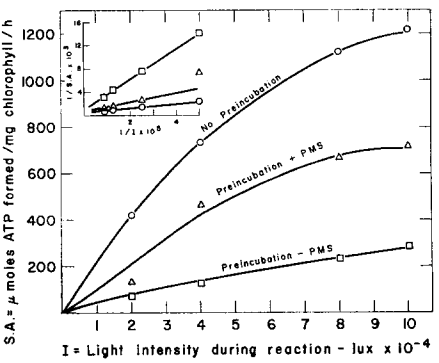

Fig. 3. Effect of light intensity during the reaction, after a preincubation period. Conditions as described under Fig. 1, except for the following: the light intensity was varied during the reaction (second exposure) as indicated. The light remained at 160,000 lux during the preincubation period. Preincubation for 5 min. Reaction for 5 min. Once-washed chloroplasts containing 32 μg chlorophyll per flask.

The same results were obtained when chloroplast fragments were used in place of whole chloroplasts, and when the chloroplasts were isolated from spinach, in place of swiss-chard.

The light-intensity requirements during the preincubation period are shown in Fig. 2. It can be seen that high light intensities were required for maximal inactivation. As shown previously[1], similar light intensities were necessary for maximal photophosphorylation in these chloroplasts. The high light intensity required was, however, in contrast with the light requirements for photoinactivation of the enzyme photosynthetic pyridine–nucleotide reductase. In the latter case saturation was reached at low light intensities[3].

The requirement for light after the preincubation period is recorded in Fig. 3. It is evident that the rates of the reaction were markedly decreased by the light pretreatment, but the amount of light necessary for maximal activity during the reaction has not changed markedly. This is interpreted to indicate that the main factor damaged during the light-preincubation period is closely associated with the initial light absorption reaction and not with later steps. Plotting the same data in the manner suggested by LUMRY *et al.*[4], as shown in the inset of Fig. 3, reemphasizes

this conclusion. A change in slope in such a plot indicates interference with the light reaction, while a change in intercept indicates interference with a dark reaction.

If, as indicated above, the damage is caused mostly within the initial reactions, associated with light absorption, the effect should be relatively independent of temperature. Table II describes an experiment in which the preincubation was carried out at two different temperatures while the reaction was at the same temperature. When the chloroplasts were pretreated at 2°, in the absence of ADP and PMS, the inhibitory action was decreased as compared with pretreatment at 17°, but was nevertheless unquestionably present. When PMS was present during the preincubation period the damage was largely prevented at 17°, but not at all at 2°. It seems, therefore, that light inactivation was indeed mostly a photoreaction not involving chemical or enzymic reactions. The prevention of inactivation by PMS, on the other hand, must have involved such chemical or enzymic reactions.

TABLE II

EFFECT OF TEMPERATURE DURING THE PREILLUMINATION PERIOD

Conditions as described under Fig. 1, except for the following variations: All four flasks were exposed to 2° for 10 min, but only those marked 2° were illuminated at 160,000 lux (the other two being completely covered with aluminum foil). The temperature of the bath was now raised to 17° in the dark, the flasks marked 2° covered completely with aluminum foil, and the flasks marked 17° exposed to 160,000 lux for 10 min. ADP, or ADP + PMS, as indicated, was now added, the contents mixed, and all flasks exposed to 160,000 lux for a reaction period of 5 min. Once washed chloroplasts containing 32 μg chlorophyll per flask. Control value: 1500 μmoles ATP formed/mg chlorophyll/h.

Temperature during preillumination	*Absent during preincubation*	*% of control*
17°	ADP	75
17°	ADP + PMS	9
2°	ADP	30
2°	ADP + PMS	41

The effect of several concentrations of PMS in the prevention of photoinactivation is compared in Fig. 4 with the effect of the same concentrations on the photophosphorylative reaction proper. It can be seen that increasing concentrations of PMS reacted in a very similar fashion in the two functions, especially in the low concentration range. This would seem to indicate that PMS is reacting in both cases with the same intermediate within the chloroplasts, and that the two actions observed are essentially two different manifestations of the same basic interaction.

The effect of high intensity light in bleaching chlorophyll is well known[5]. However, the chlorophyll content of a reaction mixture, before and after a light incubation period which caused 76 % inactivation of photophosphorylation, showed no decrease whatsoever. Chlorophyll content was measured by the method of ARNON[6]. The light effect which was responsible for the inactivation of photophosphorylation was therefore not identical with that causing the bleaching reaction. It could, of course, be an initial phase in the bleaching process.

The possibility that light inactivated a component which could be added back, has also been tested. The results presented in Table III, indicate that the addition of non-pretreated chloroplasts, to pretreated (*i.e.* photoinactivated) ones, did not

increase the photophosphorylative ability of the light pretreated chloroplasts. The amount of ATP formed in the reaction in which both types of chloroplasts were present (3rd line) was approximately equal to the sum of that formed by each type of chloroplasts by itself (1st and 2nd line). It did not approach the amount of ATP formed by the same amount of non-preincubated chloroplasts (4th line). The component which was inactivated could not, therefore, be restored by the addition of non-preincubated chloroplasts.

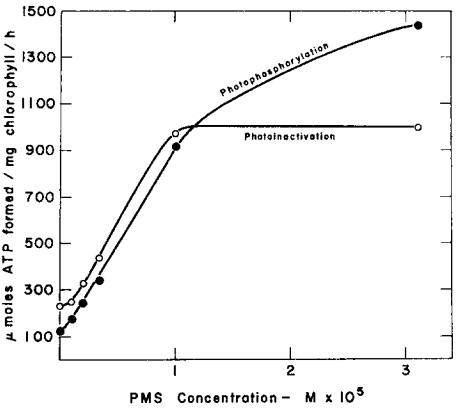

Fig. 4. Effect of PMS concentration on photoinactivation and photophosphorylation. In the photoinactivation experiments conditions were as described under Fig. 1, except for the PMS concentration during the preincubation period, which was varied as described. After the preincubation, additional PMS was added so that all flasks contained the identical optimal amount of PMS (0.09 µmoles per flask) during the following reaction period. Preincubation for 5 min. Reaction for 2 min. In the photophosphorylation experiments the [^{32}P]ATP formed after 2 min of reaction with the concentration of PMS indicated was measured. No preincubation was done in this case. Light, 160,000 lux. Once washed chloroplasts containing 25 µg chlorophyll per flask.

In other experiments, the addition of an extract from swiss-chard leaves which had photosynthetic pyridine-nucleotide reductase[3] activity, was also ineffective in reversing the light inactivation.

TABLE III

LACK OF REVERSAL OF PHOTOINACTIVATION BY ADDITIONAL CHLOROPLASTS

Conditions as described under Fig. 1, except for the indicated variations. Once washed chloroplasts containing 27 µg chlorophyll/0.1 ml in the 5-min preincubation experiment. Chloroplast fragments containing 22 µg chlorophyll/0.1 ml in the 10 min preincubation experiment. Preincubation at 160,000 lux for the times indicated. Reaction at the same light intensity for 5 min.

Added before preincubation	*Added after preincubation*	*µmoles ATP formed*	
		5 min preincubation	*10 min preincubation*
0.1 ml chloroplasts	ADP + PMS	0.77	0.37
none	ADP + PMS + 0.1 ml chloroplasts	2.61	2.07
0.1 ml chloroplasts	ADP + PMS + 0.1 ml chloroplasts	3.54	2.74
none	ADP + PMS + 0.2 ml chloroplasts	4.22	4.22

DISCUSSION

The observations reported herewith emphasize the double role played by light in photophosphorylation. It is, of course, a necessary component of the reaction, but at the same time it causes a rapid and as yet irreversible inactivation of the chloroplasts. The two effects can be measured independent of each other, as was done throughout this presentation. It could be shown, however, that the same inactivation occurred during the progress of photophosphorylation. This can be clearly seen in

Fig. 5, where four simultaneous reactions were run, each receiving its ADP at a different time. When the rate of the reactions which received their ADP at later than zero-time was replotted on the control curve, it was evident that they conformed well with its shape. It is thus apparent that the inactivation was not dependent upon whether or not phosphorylation proceeded simultaneously with it.

The light inactivation reaction explains the necessity of running the reaction for short periods of time in order to realize the very high rates of photophosphoryla-

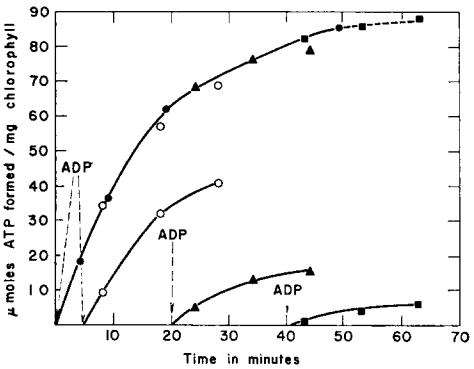

Fig. 5. Comparison of photoinactivation during photophosphorylation and after light-preincubation. Conditions as described under Fig. 1, except for ADP addition at the times marked. Light, 100,000 lux. Chloroplast fragments containing 15 μg chlorophyll per flask.

tion previously reported[1]. At the high intensities of light necessary to attain those rates, inactivation by light must have been extremely rapid, causing a sharp decline in the rate as time progressed.

The light inactivation of photophosphorylation studied may well be the initial reaction damaged during the "induction phase" in the experiments reported by KANDLER AND SIRONVAL[5,7]. The earliest effect which they could detect was a decrease of the photosynthetic rate after 10 min illumination of *chlorella* cells, at 100,000 lux. They also observed a large decrease in the phosphorylative rate of whole chlorella cells after preincubation in the light for 2.5 h (shorter preincubation times were not reported). The latter decrease was not related to a decrease in the oxygen absorption capacity of the cells and it was therefore suggested that "oxidative phosphorylation is very likely a main site of action of photooxidation during the induction phase". In view of the results presented here, it would seem more likely to regard photophosphorylation, rather than oxidative phosphorylation, as the phosphorylative step damaged early in the photoinactivation process. The low rate of phosphorylation which remained in their experiments, after 2.5 h of illumination at 100,000 lux, could indeed be due to oxidative-phosphorylation which was not damaged by the preillumination period.

The ability of PMS to partially prevent the inactivation might well be related to its outstanding ability as a cofactor in photophosphorylation. The similarity in its concentration requirements in both processes (Fig. 4) indicates that it might interact with the same components in the chloroplasts in performing both functions. The ability of PMS to prevent the slow inactivation of the chloroplasts in the dark (Table I), is also of interest in view of the difficulties encountered in maintaining chloroplasts of high phosphorylative capacity for long periods of time.

In this connection, it is of interest to compare the action of PMS with that of carotenoids in photosynthetic bacteria. FULLER AND ANDERSON[8] observed a large

photoinactivation of the photophosphorylative reaction in chromatophores prepared from carotenoidless bacteria. The normal bacteria were not nearly as sensitive. It would seem, therefore, that the carotenoids serve a function similar to that exhibited by PMS in this communication. Although added carotene did not have any protective effect similar to PMS in our chloroplast system, it could, of course, have such a protective function *in vivo*. In that case, the light inactivation studied here will essentially be similar in mechanism to that studied in photosynthetic bacteria[8,9].

The characteristics of the photo-inactivation reaction suggest that the light is acting through the chlorophyll molecule in a manner similar to its effect in promoting photophosphorylation. Such a conclusion is in agreement with the observations that (a) red light inactivated the chloroplasts, (b) inactivation was present under nitrogen, as was photophosphorylation, and (c) the light intensity requirements for the inactivation reaction were similar to those necessary for photophosphorylation.

The data at hand do not permit one to more than speculate on the mechanism through which light depresses the ability of chloroplasts to phosphorylate. It is, however, reasonable to assume that some of the reducing and/or oxidizing agents generated very early in the photoreaction of the chlorophyll molecule, are here acting in a destructive manner on the chlorophyll molecule itself, or on some components closely associated with it. Such an hypothesis would agree with the ability of phenazine methosulfate to prevent the inactivation, since it is most likely capable of reacting with these reducing or oxidizing agents.

Finally, it should be pointed out, that light inactivated chloroplasts could serve as a very convenient source of the enzymic complex of chloroplasts devoid of its ability to catalyse light dependent reactions. Such preparations may prove useful in the further study of the properties of the non-light-dependent enzymic processes of chloroplasts.

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