RETENTION OF ENERGY TRANSFER IN GLYCERINATED CHLOROPLASTS

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Chloroplasts (1) and mitochondria (2) undergo structural changes that are coupled to energy-dependent reactions. These changes are closely correlated with conditions of either ATP synthesis or hydrolysis and can be conveniently measured by some physical parameter such as light scattering or absorbancy (3). Furthermore, the addition of ATP plus a divalent cation such as Mg^{++} to chloroplasts incubated either in the dark (4) or light results in a rapid shrinkage especially under conditions where ATP hydrolysis has been light triggered (3). These observations led to the hypothesis that some type of contractile protein was involved in these mechanochemical changes. Although Packer and Marchant (3) and Ohnishi (5) have reported the isolation of a protein from chloroplasts which undergoes conformational changes with ATP, the correlation between the system described in chloroplasts and bona fide contractile systems is still uncertain.

In this study we extend the correlation between chloroplast structural changes and muscle contraction by the preparation of chloroplast "models" which are analogous to the muscle models of Szent Györgyi (6). This approach was suggested by the recent reports of Nakazawa (7) and Kazakova (8), who have established that mitochondria retain certain responses to ATP following glycerol treatment.

Methods

Spinach chloroplasts were prepared in a medium of NaCl (0.35 M), P_i (40 mM) and EDTA (2 mM) at pH 7.5. Nine volumes of a glycerol solution which contained 50% glycerol, KCl (0.125 M) and Tris-HCl buffer (20 mM, pH 7.5) were added to the thick chloroplast suspension recovered in the centrifuge tube. This mixture was stored at -15°C for 3-10 days. Such treatment would be expected (6) to gradually remove most of the water soluble proteins leaving any contractile apparatus intact. Before use, the glycerinated chloroplasts were washed twice by centrifugation

and resuspended in NaCl (0.35 M), EDTA (2 mM) and Tris-HCl (40 mM, pH 7.5). In certain control experiments, fresh chloroplasts prepared as described above minus glycerol treatment were employed. As a further control, chloroplasts were also prepared and stored at -15°C in KCl (0.125 M), Tris-HCl (20 mM, pH 7.5). Controls were given the same washing and subsequent resuspension as the glycerinated chloroplasts.

The light-scattering measurements were taken in a Brice-Phoenix light-scattering apparatus (1). Changes in the scattered light intensity in response to induction with actinic red light are expressed as percentages of the initial scattering level. The hydrolysis of ATP was found by measuring the change in phosphate concentration by means of a phosphomolybdic acid method (3). To establish conditions for light-triggered ATPase, the chloroplasts in the complete system were pre-illuminated for 5 minutes. After the pre-illumination period, ATP was immediately added and its hydrolysis was allowed to proceed in the dark for 15 minutes (3). Photophosphorylation was also investigated by the phosphomolybdic acid method. However, the chloroplasts were continuously illuminated in a system containing ADP and P_i. Chloroplast swelling was determined by recording decreases in the absorbancy at 540 mμ during a 30-minute interval (9). NADP reduction was determined by measuring absorbancy increases at 340 mμ in the presence of added spinach chloroplast ferredoxin.

Experimental

Our investigation of glycerinated chloroplasts has followed two approaches: first, to establish an analogy to the muscle models; and second, to examine the effects of glycerol treatment on the retention and loss of some aspects of electron and energy transfer.

Effect of Mg^{++} + ATP. We have found that chloroplasts which have been treated with glycerol contract upon the addition of Mg^{++} and ATP. Figure 1 shows the rate of contraction when measured by increases in the absorbance at 540 mμ as a function of time. The time course of the reaction was about twelve minutes. In comparison, no optical density changes were observed with the frozen, unglycerinated chloroplasts which were used as a control for non-specific effects, or in untreated controls.

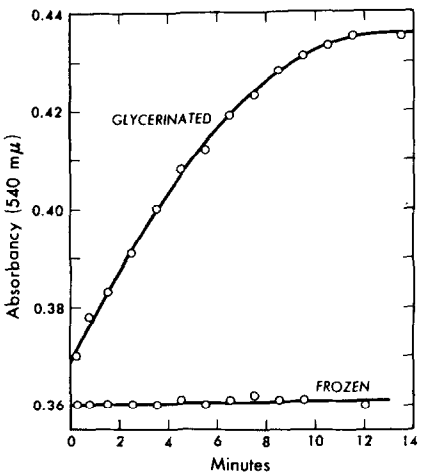

Figure 1. Evidence for contraction of glycerinated chloroplasts by ATP. Conditions: KCl (0.125 M), Tris buffer (20 mM, pH 8), $MgCl_2$ (5 mM), and chloroplasts added in an amount to give an initial absorbancy of about 0.35. ATP (5 mM) was added at zero-time. 23°C.

Retention of Light-Dependent Properties. Glycerinated chloroplasts also retain some of their typical responses to light. Table I shows that glycerinated chloroplasts manifest the light-triggered hydrolysis of ATP. The retention of this activity as compared with that of fresh chloroplasts was about 45%, while chloroplasts which were frozen without the glycerol treatment showed no response to light. Furthermore, the degree to which retention of light-triggered ATP hydrolysis depends upon various cofactors such as Thiol, Mg^{++}, and phenazine methosulfate (PMS) is the same as for fresh chloroplasts. Light-triggered ATPase in fresh chloroplasts generally disappears about three hours following isolation of the chloroplasts, while the preparations shown in Table I had been stored for four, five, and seven days respectively. However, we did not find any retention of photophosphorylation in glycerinated chloroplasts using our method of study. Perhaps glycerol treatment removes the soluble "extractable factor" found by Avron, et al., (10) to be a requirement for photophosphorylation. Glycerol treatment has been found to remove about 85% of the total chloroplast protein; the mg chlorophyll/mg protein ratio of glycerol-extracted chloroplasts was 0.24.

Since the action of ATP hydrolysis in fresh chloroplasts is closely related to the occurrence of structural changes, we investigated whether glycerinated chloroplasts are still capable of manifesting light-scattering responses. Upon illumination with

TABLE I

Retention of ATP Hydrolysis and Light-Induced Structural Changes

in Glycerinated Chloroplasts

ATP Hydrolysis. The reaction mixture contained NaCl (35 mM), Tris buffer (20 mM, pH 8), PMS (20 μM), $MgCl_2$ (5 mM), cysteine (50 mM), chloroplasts (50 μg chlorophyll per ml) and ATP (3 mM).
NADP Reduction. The reaction mixture contained NaCl (35 mM), Tris buffer (20 mM, pH 8), NADP(0.3 mM), chloroplasts (15 μg chlorophyll/ml), and ferredoxin (30 μg/ml).
Light-Scattering. The reaction mixture contained NaCl (35 mM), Tris buffer (20 mM, pH 8), PMS (20 μM), $MgCl_2$ (5 mM), inorganic phosphate (40 mM), and chloroplasts (10 μg chlorophyll per ml).
Swelling. The reaction mixture contained NaCl (350 mM), Tris buffer (20 mM, pH 8) and PMS (20 μM).
Osmotic Swelling. The reaction mixture contained 35 or 350 mM NaCl
In the table, days refer to length of storage of frozen and glycerinated chloroplasts at -15°C. Other details in text.

	ATP Hydrolysis μ moles P_i/mg chl/hr						NADP Reduction μ moles/mg chl/hr		
	pre-illumination			dark			light		
Days:	4	5	7	4	5	7	4	5	7
Fresh Chloroplasts	35	26	36	7	6	12	68	80	69
Glycerinated Chloroplasts	16	14	17	5	4	4	4	8	2
Frozen Chloroplasts	1	1	2	1	1	2	2	0	0

	Light-Scattering % Increase Light-Induced			Swelling % Decrease in $A_{540\ m\mu}$ Light minus Dark			Osmotic Swelling % Increase in $A_{540\ m}$ 35-350 mM NaCl		
Days:	4	5	7	4	5	7	4	5	7
Fresh Chloroplasts	81	60	83	43	50	64	110	98	110
Gycerinated Chloroplasts	50	52	56	1	2	5	29	32	29
Frozen Chloroplasts	0	0	0	4	7	6	27	24	27

actinic light, about 70% of this activity was retained as compared to fresh chloroplasts. These responses were observed in a cyclic

electron transfer system mediated by PMS. The characteristics of the scattering curve are the same as for fresh chloroplasts. The scattering increases are reversed by darkness or by the addition of NH_4Cl. Thus, this process, as in fresh chloroplasts, is energy dependent. It was found that while glycerinated chloroplasts could support energy-dependent process by a cyclic electron transfer system, they had lost their ability to reduce NADP in a non-cyclic system (Table I). Ferredoxin and NADP reductase are known to be readily extractable enzymes in chloroplasts (11). The possibility that small amounts of glycerol might inhibit light-scattering responses supported by non-cyclic electron flow was tested with fresh chloroplasts using a system containing added NADP, ferredoxin, and various concentrations of glycerol. No inhibition was found until the glycerol concentration exceeded 20%.

Preparations of glycerinated chloroplasts (such as in Table I) have in general not been found to retain light-induced swelling. This action of light on chloroplast volume is not energy dependent (9). In several instances, however, some retention of light-induced swelling by glycerinated chloroplasts was observed. Chloroplasts treated with glycerol also lack osmotic properties as determined by a test for absorbancy change at 540 mμ (9) over a ten-fold range of NaCl concentration between 35-350 mM.

Discussion

This study has shown that chloroplasts which have been treated with glycerol and stored at -15°C retain many, but not all of the characteristics manifested by fresh chloroplasts. Our results agree in general with those found by Nakazawa (7) for glycerinated mitochondria since glycerinated chloroplasts contract upon the addition of Mg^{++} and ATP. The report by Kazakova (8) of swelling in glycerinated mitochondria with Mg^{++} and ATP is unexplained. Nakazawa's observation (7) of retention of succinate oxidation in glycerinated mitochondria (supplemented with cytochrome c) also correlates with our finding of evidence for retention of cyclic electron transfer in glycerinated chloroplasts. Zelitch and Barber (12) have found that certain oxidative activities of chloroplasts may be inhibited in the presence of glycerol, a result possibly also originating from extraction of soluble chloroplast components. Our results suggest the possibility of obtaining a simpler system to study certain characteristics and reactions associated with the insoluble part of the membrane system. The retention of energy transfer in glycerol-treated chloroplasts should facilitate the study

of energy coupling between soluble synthetic reactions and the membrane system.

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