OBSERVATIONS ON THE CONTROL OF CHLOROPLAST STRUCTURE BY ADENOSINE TRIPHOSPHATE

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Membranes of mitochondria (1-2), chloroplasts (3-4), and photosynthetic bacteria (5), manifest mechanochemical changes that are coupled to energy transfer reactions. These changes in membrane structure or membrane deformations, conveniently measured by the recording of a physical parameter such as light-scattering, can be closely correlated to conditions favorable for oxidative or photophosphorylation. Since all of the reactants required for phosphorylation coupled to electron transport in both photosynthetic and non-photosynthetic systems are required for the change in structure, and since inhibitors of these functions abolish the change, it has been suggested (1-2, 4-5) that the structural parameter is under the control of energy-containing intermediates.

Recent experiments with mitochondria (6) have demonstrated that swelling induced by electron transport is oligomycin sensitive, whereas reversal of swelling by ADP (under conditions of oxidative phosphorylation) is specifically blocked by oligomycin. These observations implicate the ATPase site in the control of mechanochemical changes. Ohnishi and Ohnishi (7) have isolated a protein from mitochondria which undergoes conformational changes with ATP (light-scattering, viscosity) and manifests ATPase activity. It is an attractive hypothesis that in mitochondria, a contractile-like substance may be involved in the changes in macromolecular structure coupled to energy transduction. In view of this, it seemed worthwhile to examine the role of ATP and ATPase activity in the control of chloroplast structure in more detail. In brief, effects of ATP on structural changes in spinach chloroplasts have been demonstrated. Furthermore, a protein fraction has been isolated from chloroplast membranes which undergoes light-scattering changes with ATP and which hydrolyzes this substance.

Methods. Spinach chloroplasts were prepared in Tris-(hydroxy methyl) amino methane buffer (0.1 M, pH 8.0) - NaCl (0.35 M) according to Whatley

and Arnon (8). The exact conditions for study are given in the individual experiments. Light-scattering changes in chloroplasts (and in the chloroplast extract described below) were measured at 90° in a Brice-Phoenix Light-Scattering apparatus modified for recording. Incident and scattered light were filtered at 546 mμ. The scattering was adjusted to read 100% on the chart paper by using the minimum intensity of 546 mμ light and the instrument at maximum gain. Light in this region is near the minimum of the photochemical action spectrum. Increases and decreases in the scattered light intensity in response to actinic red light are expressed as % changes of the initial scattering level. The temperature of the system was accurately controlled during periods of illumination at 25 ± 0.1°C by circulating liquid around the jacketed 1 x 1 cm cuvette.

Preparation of protein extract from chloroplasts: Spinach leaves (2 kg) with stems removed were homogenized in a Waring Blender at 5°C, and chloroplasts prepared in the usual manner, but in Tris (0.1 M, pH 8.0) - sucrose (0.5 M). To free the chloroplasts of isolation medium, the last centrifugation step was made at high speed (9,000 g, 15 min). The pellet was resuspended in about 25 ml of KCl (0.6 M) - imidazole (0.1 M, pH 7.9), and then stirred at 0°C for two hours. The suspension was then centrifuged (90,000 g, 90 min. at 5°C). The supernatant fluid located between the pellet and a surface lipid layer was carefully removed, and diluted with 5 volumes of cold distilled water and the pH adjusted to 6.2 with 1 N HCl. The precipitate was collected by centrifugation (10,000 g, 15 min). The supernatant was discarded, and the residue redissolved in 5 ml of KCl (0.6 M) - imidazole (0.1 M, pH 7.9) by stirring at 0°C for 60 minutes. That portion of the precipitate which did not redissolve was centrifuged out (10,000 g, 15 min). The supernatant solution was again diluted with 5 volumes of cold distilled water to low ionic strength (KCl - 0.1 M), and the pH was adjusted to 6.3. ATPase and ITPase activities were estimated by an ammonium molybdate - stannous chloride procedure (cf. 9). Light-scattering tests were carried out as described above except for red light illumination (which was not required). Viscosity measurements were made at 25° ± 0.1°C with a Ubbeholde type viscometer.

Experimental. Earlier studies (3-5) established that light-scattering increases in chloroplasts occur under conditions of cyclic and non-cyclic photophosphorylation. In some cases however, scattering changes were lost even when chloroplasts were incubated in the presence of complete requirements for photophosphorylation. These "aged" chloroplasts developed a requirement for ATP to manifest scattering changes. Figure 1 dramatically demonstrates this ATP requirement. Illumination of chloroplasts under conditions for cyclic photophosphorylation led to an increase in

scattering of 2% upon illumination with red light. This small scattering increase was reversed after extinguishing actinic light. The addition of 3.3 mM ATP in the "dark" did not change the scattering level. However, when the red light was restored, a rapid and extensive increase in scattering ensued, reaching a steady state at a level 85% higher than the initial scattering intensity. This increased scattering state could be fully reversed by extinguishing the actinic light. A second light and dark period led to a similar cycle of scattering increase and decrease. The large reversible scattering responses, characteristic of "fresh" chloroplasts, had been restored by ATP. A similar restoration of the response can be obtained with ITP.

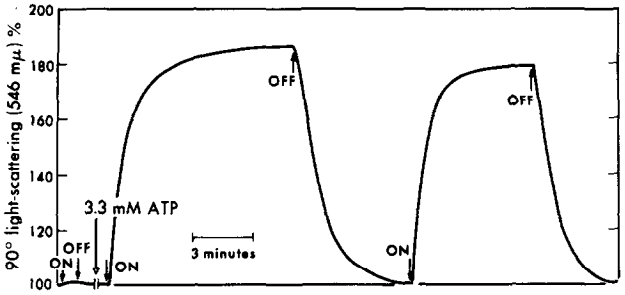

Figure 1. Restoration of reversible scattering changes in aged chloroplasts by ATP. The reaction system contained Tris (0.02 M, pH 8.0), NaCl (0.035 M), $MgCl_2$ (0.005 M), phosphate (0.004 M, pH 8.0), ADP (1 mM), ascorbate (2.5 mM), 2,6-dichlorophenol indophenol (30 μM), phenazine methosulfate (20 μM), and chloroplasts (approximately 10 μg chlorophyll/ml).

This remarkable restoration of the scattering response induced by red light in the presence of ATP suggested that this action of ATP might bear some relation to the existence of a light-induced ATPase in chloroplasts reported by Avron (10) and Petrack and Lipmann (11). Petrack and Lipmann demonstrated that light-induced ATPase of spinach chloroplasts was maximally activated in the presence of cysteine (0.08 M). Accordingly the action of ATP and also of ITP on the scattering responses in fresh chloroplasts was tested in the presence and absence of cysteine. Parallel determinations were made of nucleoside triphosphatase activity. The results (Table I) confirm the existence of the light activated ATPase observed by Petrack and Lipmann, and also show photohydrolysis of ITP. Under conditions where nucleoside triphosphatase activity is maximum (i.e. in the presence of cysteine), it may be seen that the scattering

TABLE I

Correlation of Photohydrolysis of ATP and ITP by Spinach Chloroplasts With Changes in Chloroplast Structure

The reaction mixture contained Tris buffer (0.02 M, pH 8.0), NaCl (0.035 M), $MgCl_2$ (0.005 M), ascorbate (0.83 mM), phenazine methosulfate (20 μM), and chloroplasts (12 μg chlorophyll/ml). The concentration of the nucleoside triphosphates were 2 mM, and that of cysteine, 0.083 M, where they appear in the table. The chloroplast preparation used in this experiment was prepared in the usual manner in Tris (0.1 M, pH 8.0) - NaCl (0.35 M), and then washed two additional times by centrifugation in a low salt medium, Tris buffer (0.005 M, pH 8.0) - NaCl (0.035 M). This procedure removes the soluble protein of chloroplasts. Illumination in the scattering experiment was with red light as described in methods. The extent of the % scattering increase is given by the (+) values and the extent of the decay in scattering after removal of actinic light by the (-) values. In parallel experiments made on nucleoside triphosphatase activity, illumination was made with an unfiltered Tungsten light source. The chlorophyll concentration in the photohydrolysis experiment was 200 μg chlorophyll/ml; other conditions were the same as in the scattering experiment.

	% Scattering Change		μ moles phosphate formed/mg chlorophyll/15 min.	
Condition	Light	Dark	Light	Dark
ATP	+22	-18	0.37	0.38
ITP	+21	-21	0.10	0.22
cysteine + ATP	+69	-9	2.73	0.39
cysteine + ITP	+83	-21	1.11	0.12

increases induced by red light under conditions of cyclic photophosphorylation are considerably larger as compared with those in the absence of cysteine. The scattering changes observed in the absence of cysteine when the nucleoside triphosphatase activity is lower, are largely reversed in the dark in the same fashion as illustrated in figure 1. However, in the presence of cysteine, the scattering increase is only slightly reversed when the red light is turned off. Addition of one of a number of substances which inhibit light-induced ATPase or ITPase activity such

as NH_4Cl (1 mM) or ADP (1 mM) bring about a full reversal of the scattering response (not shown in the Table). These findings strongly suggest an involvement of light-induced nucleoside triphosphatase activity in the control of structural changes geared to photosynthetic electron transport.

A similarity between these results and the properties of the contractile protein of muscle was at once apparent. Accordingly chloroplasts were extracted under the same conditions employed for the extraction of actomyosin from mammalian muscle (methods section). The results in Table II show that this protein fraction extracted from chloroplast membranes manifests ATPase and ITPase activity. The same fraction also shows decreases in light-scattering on addition of these nucleotides. Nucleoside triphosphatase activity is greater at pH 5.5 than at higher pH's. Likewise, the ability of these nucleotides to cause a decrease in scattering is also greater at the lower pH. The nucleoside triphosphatase activity at high ionic strength was lower than at low ionic strengths (pH 5.5). Similarly, the light-scattering responses observed in the preparations at high ionic strength were much smaller than those observed at low ionic strengths. Parallel experiments on the viscosity of the protein extract in the presence and absence of the nucleotides were made. These results did not show a decrease in viscosity with added nucleotides, which would be expected if this preparation behaved like the contractile proteins of mammalian muscle and mitochondria.

Although it may be too early to conclude that chloroplasts contain a contractile protein, the conclusion that ATPase (or ITPase) activity is somehow involved in light-induced changes of chloroplast structure seems inescapable. This conclusion is based upon the following evidence: a) Both ATP and ITP restore to aged chloroplasts the ability to manifest structural changes under conditions of photosynthetic electron transport. b) Under conditions where ATPase and ITPase activities are maximally activated in fresh chloroplasts (in the presence of cysteine), scattering increases with red light are larger and no longer reversible when the red light is extinguished. Reversal of the scattering changes may then be completed by addition of known inhibitors of chloroplast nucleoside triphosphatase. c) A protein fraction isolated from chloroplast membranes under the same conditions as employed for extraction of contractile proteins from muscle shows ATPase and ITPase activity and decreases in scattering with ATP and ITP, which suggest the existence of conformational changes. These results are indicative of a primary role for ATP, the product of photophosphorylation, in the control of chloroplast structure.

TABLE II

Light-Scattering Changes and Nucleoside Triphosphatase Activity of a Protein Extract of Chloroplast Membranes

Light-scattering changes and nucleoside triphosphatase activity were determined as described in the methods. The experiments at low ionic strengths were carried out in 0.04 M KCl - 0.12 M imidazole, and those at high ionic strengths in 0.6 M KCl - 0.12 M imidazole at the pH indicated. ATP and ITP concentrations were 4 mM.

	pH 5.5		pH 6.3		pH 7.5	
	ATP	ITP	ATP	ITP	ATP	ITP
Low Ionic Strength						
% Light-scattering decrease	16.0	16.0	8.0	6.5	7.0	6.5
Nucleoside triphosphatase*	2.10	1.70	1.50	1.40	0.25	0.21
High Ionic Strength						
% Light-Scattering decrease	2.0	3.0	--	--	--	--
Nucleoside triphosphatase*	0.32	0.28	--	--	--	--

* μmoles phosphate formed/mg protein/30 minutes at 25°C.

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