

Modulation of Oat Mitochondrial ATPase Activity by Ca^{2+} and Phytochrome

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ABSTRACT

The activity of a Mg^{2+} -dependent ATPase present in highly purified preparations of *Avena* mitochondria was photoreversibly modulated by red/far-red light treatments. These results were obtained either with mitochondria isolated from plants irradiated with white light prior to the extraction or with mitochondria isolated from unirradiated plants only when purified phytochrome was exogenously added to the reaction mixture. Red light, which converts phytochrome to the far red-absorbing form (Pfr) depressed the ATPase activity, and far-red light reversed this effect. Addition of exogenous CaCl_2 also depressed the ATPase activity, and the kinetics of inhibition were similar to the kinetics of the Pfr effects on the ATPase. The calcium chelator, ethyleneglycol-bis-(β -amino-ethyl ether)- N,N' -tetraacetic acid, blocked the effects of both CaCl_2 and Pfr on the ATPase. These results are consistent with the interpretation that Pfr promotes a release of Ca^{2+} from the mitochondrial matrix, thereby inducing an increase in the concentration of intermembranal and extra-mitochondrial Ca^{2+} .

transduction process has received continued support. Certain responses elicited by phytochrome, including fern spore germination (34) and the modulation of the mitochondrial enzyme NADH dehydrogenase (4, 5), can be replicated by manipulation of the external Ca^{2+} concentration. Furthermore, phytochrome-controlled movement of Ca^{2+} has been shown to occur in *Mougeotia* (9), *Avena* protoplasts (14), and extracted plant mitochondria (29).

These works, though few in number, strongly suggest that phytochrome, through modulation of cation fluxes across membranes, is capable of altering enzymic activity responsible for initiating developmental changes in the plant. Since it has been reported that phytochrome can modulate metabolite permeability (15) and the activity of two enzymes in mitochondria (4, 24), we felt it was possible that the activity of other mitochondrial enzymes might also be influenced by phytochrome. Calcium has been reported to alter mitochondrial ATPase activity in corn (20) and pea (12). This report presents the findings from an examination of the effects of Ca^{2+} and phytochrome on the mitochondrial ATPase of oats.

MATERIALS AND METHODS

Based on the knowledge that certain phytochrome-mediated responses alter membrane potentials in less than 2 s, it has been suggested that one of the first events in this transduction process could be the interaction of phytochrome with biological membranes (25). Pelletability and binding studies have demonstrated that upon photoactivation, phytochrome associates tightly with membranes (3, 33). This association is believed to alter the permeability of the membrane thereby modifying the transmembrane potential, as work with *Phaseolus*, *Avena*, and *Nitella* (18, 27, 36) suggests. Often coincident with transmembrane potential alterations is the migration of ions down their electrochemical gradients. Several studies have shown a correlation between activation of phytochrome and the movement of a cation (2, 18, 29). The significance of this ion flux is that it provides a possible mechanism by which a secondary messenger may become involved in relaying the phytochrome signal into morphogenetic responses.

Haupt and Weisenseel (17) proposed the existence of such a transduction system in which Ca^{2+} concentration changes in the cytosol, modulated by phytochrome, could initiate significant alterations in enzymic activity. Their reasoning was based partially on the knowledge that Ca^{2+} played an important role in many plant cellular activities and had also been shown to function as a secondary messenger in animal systems. This hypothesis that Ca^{2+} may act as a secondary messenger in the phytochrome-

Isolation of Mitochondria. Mitochondria were isolated from 4-d-old dark-grown oat seedlings (*Avena sativa* L. cv Garry). The plants were cooled (4°C) for 30 min prior to extraction and then harvested near the node under a green safe-light. The cut seedlings were then exposed to white light (15 min). All subsequent manipulations were carried out under a green safe-light at 3 to 4°C. The extraction procedure utilized was that of Day and Hanson (7) with slight modifications. Briefly, the harvested tissue was added to the extraction buffer (0.4 M sucrose, 10 mM KCl, 0.1% BSA, 5.0 mM EGTA, 9.0 mM NaHSO_3 , and 10 mM Hepes, final pH 7.6) in a ratio of 1:2 (w/v). This tissue was ground with a Moulinex homogenizer and the brei was filtered through two layers of nylon cloth (20 μm mesh). The filtered extract was centrifuged at 1,000g for 10 min and the resulting supernatant was centrifuged at 10,000g for 10 min. The pellet obtained was resuspended in wash solution (0.4 M sucrose, 10 mM Hepes, pH 7.6) and centrifuged at 2,000g for 10 min. The supernatant was layered over a 0.6 M sucrose pad (pH 7.6) and centrifuged at 11,000g for 20 min. The purified mitochondria were suspended in storage buffer (0.25 M sucrose, 0.1% BSA, 10 mM Hepes, final pH 7.2). Mitochondria thus isolated are referred to as 'preirradiated'. For some extractions, preisolation white light irradiation of the seedlings was omitted. Mitochondria obtained from these plants are designated 'dark' mitochondria. Submitochondrial particles were obtained by the procedure of Passam and Palmer (26).

Phytochrome Purification. The exogenous phytochrome utilized in these experiments was purified from 4-d-old etiolated *Avena* seedlings as described previously (30) with the following modifications. Following elution from the Brushite chromatog-

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raphy column, the phytochrome was concentrated with an unbuffered ammonium sulfate solution. The resulting phytochrome pellet was resuspended in a 7.0 mM K-phosphate buffer containing 1.0 mM EDTA, 10 mM NaHSO₃, and 1.0 mM PMSF³ (pH 7.8) and desalted through a Sephadex G-50 column. The phytochrome was then loaded onto a DEAE-Bio-Gel column and eluted with a gradient from 7.0 to 200 mM K-phosphate with 1.0 mM EDTA. The phytochrome fractions were then loaded on a hydroxyapatite column. Following elution from this column, the phytochrome was again concentrated by precipitation with ammonium sulfate. The pellet was solubilized with 0.1 M sodium phosphate and 1.0 mM EDTA buffer (pH 7.8) and separated on a Bio-Gel A-1.5m molecular sieve column.

The purity and mol wt of the purified phytochrome was estimated by SDS-polyacrylamide gel analysis as described in Georgevich *et al.* (11). Prior to its use with the mitochondrial preparations, the phytochrome was equilibrated in 10 mM Hepes, 5.0 mM KCl (pH 7.2) on a Bio-Gel P6 molecular sieve column. A spectral scan was then done, and only phytochrome which retained its characteristic spectral properties was utilized.

Irradiations. Illuminations with actinic R or FR light were done with a Ratiospect apparatus. The actinic R light source produced an irradiance of approximately 1.7 $\mu\text{W cm}^{-2}$. The irradiance from the FR light source was about 2.7 $\mu\text{W cm}^{-2}$. The interference filters had 15 nm band widths and a λ_{max} of 665 nm (R filter) or 722 nm (FR filter). In the photoreversibility experiments, where it was desirable to insure total photoconversion of the phytochrome in 4 min or less, an additional light source was utilized. This second light source consisted of a Sylvania Day 52 500-w bulb mounted in a lantern slide projector. The light beam was passed through either an interference filter identical to that in the Ratiospect to produce the actinic R light or through a CBS 750 FR filter (Carolina Biological Supply), which eliminates virtually all light below 700 nm. The irradiance achieved from this R light source was 13 $\mu\text{W cm}^{-2}$ and from the FR light source was 7.5 $\mu\text{W cm}^{-2}$. When both light sources were used, the samples to be irradiated were placed between the sources. All irradiance values were obtained with an International Light Spectroradiometer.

When seedlings were irradiated with white light, the source was overhead 40-w Westinghouse cool-white fluorescent bulbs, which had an irradiance of 13 $\mu\text{W cm}^{-2}$.

Estimation of Mitochondrial Integrity and Purity. The purified mitochondria were determined to be functionally normal and intact by electron microscopy and biochemical tests described by Douce *et al.* (8). For morphometric analysis, a pellet of purified mitochondria was fixed with 1% glutaraldehyde. The material was then washed, postfixed in OsO₄, exposed to a 1% tannic acid mordant, dehydrated in an ethanol-acetone series, and embedded in low-viscosity embedding medium. Sections were taken through the entire pellet and eleven representative micrographs ($\times 4,600$) were used to determine the per cent relative volume of the cellular components present using the coherent multipurpose test system as described by Weibel (35). In accord with the test, enough points on each micrograph were counted to generate an SE of 5% or less for components comprising more than 2% relative volume.

Nucleoside Triphosphatase Activity Assays. A dilute aliquot of mitochondria or submitochondrial particles in storage buffer was added to 1 ml of the assay medium (5.0 mM MgCl₂, 50 μM CaCl₂, 30 mM Hepes, pH 6.5). The reaction was begun with the addition of ATP or other nucleotides, as indicated, to a final concentration of 3.0 mM. Reactions were allowed to continue for 15 min at 26°C. The addition of an equal volume of ice-cold

10% TCA stopped the reaction. After centrifugation at 5,000 rpm (rotor 221, International Clinical Centrifuge) for 5 min, the released phosphate in the supernatant was estimated by the method of Sanui (31). The absorbance of phosphomolybdate released in the butyl acetate phase was measured at 310 nm. The amount of Pi liberated was calculated from a standard curve. Each experiment was conducted with duplicate samples and with zero time and buffer controls (minus mitochondria). The Δ Pi values were calculated from the number attained from subtracting the buffer control value from the average of the readings obtained from a given treatment at 15 min. Any variations in this basic format for assaying the ATPase activity are given in the appropriate figure and table legends. Disodium salts of ATP, GTP, ITP, and UTP were obtained from Sigma Chemical Company. In one set of experiments in which the effect of Na⁺ on the mitochondrial ATPase was tested, Tris-ATP was utilized.

In the absence of EGTA, there was a background level of 1 μM Ca²⁺ in the standard ATPase reaction solution as determined by atomic absorption.

Replication of Results. All results presented on the characterization of the ATPase have been repeated at least three times, using different mitochondrial preparations, with qualitatively similar results. Each figure and table illustrates the results obtained from one representative experiment unless otherwise stated. Each data point in both the figures and tables is the average of two measurements from replicate samples of the same treatment minus the buffer control value. In no case was there greater than a 4% difference between replicates from any one experiment, although there was greater variation in the rate of ATP hydrolysis from one mitochondrial preparation to the next.

Protein Assay. Protein was assayed by the method of Lowry *et al.* (22) after solubilization of the mitochondria with 5% SDS. As a standard, BSA in 5% SDS was used.

RESULTS

Mitochondrial Preparation. The modified protocol of Day and Hanson (7) yielded mitochondria which were intact and physiologically active. The degree of outer membrane intactness was determined by the procedure of Douce *et al.* (8). Utilizing this procedure, preparations were found to be consistently at least 85% intact. The mitochondria were tightly coupled as evidenced by ADP/O ratios of 1.5 to 1.7 when succinate was employed as a substrate. To ascertain the purity of the mitochondrial preparation, a morphometric study as outlined by Weibel (35) was conducted. The data from this analysis of a standard preparation are shown in Table I. The results indicate that in spite of the manipulations carried out to fix and embed the pellet, 80% of the total area occupied by membranes in the micrographs examined was recognizable mitochondria. Microbodies, etioplasts, or fragments of etioplasts constituted 6.0% of the final preparation. The remaining 13.5% of the area was composed of micro-

Table I. Morphometric Analysis of Purified Mitochondrial Pellet

Cellular Components	Relative Volume Occupied		
	Total pellet		Range (High-Low) ^a
	Mean value	SD	
	%		%
Mitochondria	80	4.7	88.6-73.3
Etioplasts and microbodies	6.0	3.7	12.2-1.8
Other ^b	13.5	7.0	17.2-3.5

^a Represents the highest and lowest value obtained from samples through one entire pellet.

^b Refers to microsomes and membrane material that could not be positively identified.

³ Abbreviations: PMSF, phenylmethylsulfonyl fluoride; R, red light; FR, far-red light.

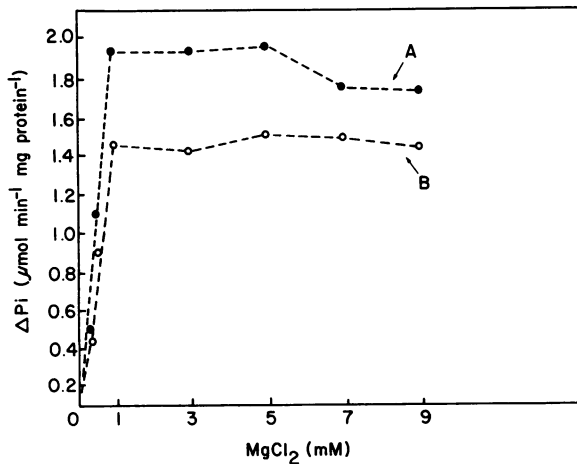


FIG. 1. Effect of Mg^{2+} on the ATPase activity in oat mitochondria. In A, only various concentrations of Mg^{2+} were present while in B, $50 \mu M$ Ca^{2+} was also present.

some and undefined membrane material, some of which may have originated from broken mitochondria.

Evaluation of Isolated Phytochrome. When electrophoresed on a calibrated 7.5% SDS-polyacrylamide gel, most of the phytochrome isolated by the above procedure produced a single peptide band which had an apparent mol wt of 118,000. The stained gel also showed a minor peptide fragment with an apparent mol wt of 60,000. Both fragments were identified as phytochrome by Western blot analysis using monoclonal antibody against phytochrome (Serlin, Silberman, and Roux, unpublished). Spectrally, the phytochrome in the Pr form showed an absorption maximum at 667 nm and in the Pfr form at 726 to 727 nm. The $\Delta A_{667\text{ nm}}$ to $\Delta A_{727\text{ nm}}$ ratio of this phytochrome was 1.0 and the ratio of the *A* at 727 versus 667 nm when the phytochrome was in the Pfr form was 1.2. The specific absorbance ratio ($A_{667\text{ nm}}/A_{280\text{ nm}}$) for the phytochrome was 0.71.

Mitochondrial ATPase. The ATPase from oat mitochondria showed a marked need for Mg^{2+} . As the concentration of Mg^{2+} was increased from a concentration of 0 to 1.0 mM, there was noticeable stimulation of the ATPase (Fig. 1). In the range of 1.0 to 5.0 mM Mg^{2+} , there was little overall change in the rate of ATP hydrolysis, but above 5.0 mM, there appeared to be a gradual decline in the rate (Fig. 1, A). The monovalent cations K^+ and Na^+ , unlike Mg^{2+} , did not enhance the level of the ATPase activity present in the mitochondria (data not shown).

Figure 2 illustrates the influence of pH on the rate of ATP hydrolysis. Two pH optima, 6.5 and 8.5, were observed for the oat mitochondrial ATPase. With the rise in the pH from 6.5 to 8.5, the mitochondrial suspension changed from turbid to translucent. Previous workers who have recorded the same phenomenon attributed the change to an alteration in membrane permeability (19, 21), so pH 6.5 was thereafter employed.

In three experiments done, oligomycin ($0.2 \mu g/ml$) caused 42%, 40%, and 37% reduction in the activity of the ATPase. Increasing the concentration of oligomycin further caused only slightly more reduction in the activity of the ATPase. This partial inhibition of the ATPase activity by oligomycin is in agreement with other published reports on oligomycin's effects on certain plant mitochondria (13, 23).

The respiratory uncoupler 2,4-dinitrophenol produced an increase in the activity of the ATPase. At $100 \mu M$, 2,4-dinitrophenol caused a 46% enhancement in the rate of ATP hydrolysis over that attained in the control (data not shown).

The ATPase in intact mitochondria showed a specificity for ATP as a substrate. The enzyme portion remaining in the sub-mitochondrial particles, the F_0 -ATPase complex, however, was

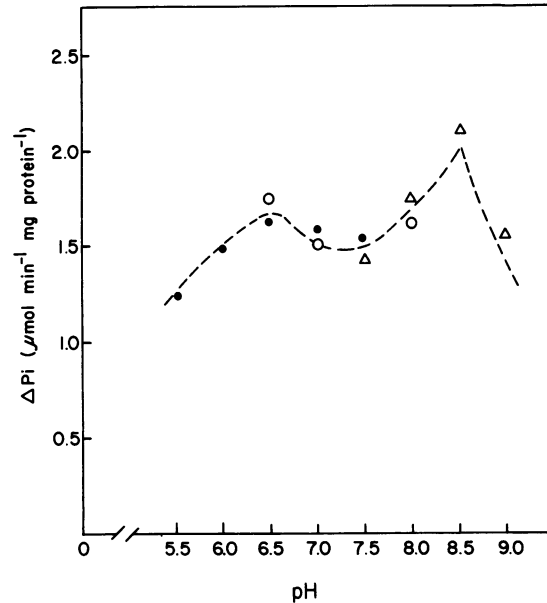


FIG. 2. Effect of pH on ATPase activity in the presence of 5.0 mM $MgCl_2$ and $50 \mu M$ $CaCl_2$. Different buffers were utilized around their pK_a values to maintain specific pH values. (●), Pipes buffer present; (○), Hepes buffer present; (Δ), Bicine buffer present. None of the buffers chelate either Mg^{2+} or Ca^{2+} . The concentration of each buffer is 30 mM.

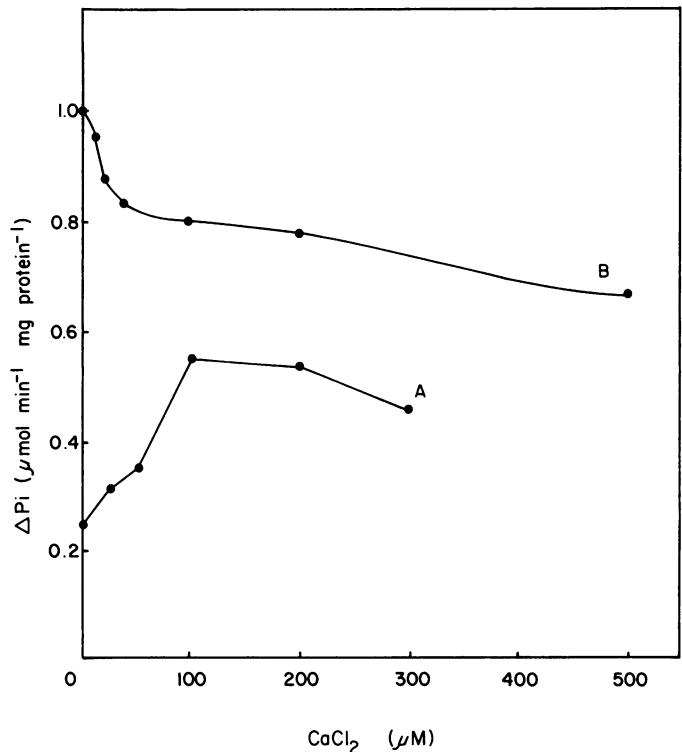


FIG. 3. Effect of various concentrations of Ca^{2+} on the ATPase activity in oat mitochondria. In A, Ca^{2+} was the only divalent cation present. In B, $5.0 \text{ mM } Mg^{2+}$ was present.

able to utilize UTP, GTP, and ITP also. In fact, the enzyme exhibited a greater rate of hydrolysis for these nucleotides than for ATP. In the case of ITP, the rate was twice that attained when ATP was supplied (data not shown).

In the absence of Mg^{2+} , the rate of hydrolysis of ATP by intact mitochondria increased as the level of Ca^{2+} was raised above the background concentration of $1 \mu M$ until a concentration of

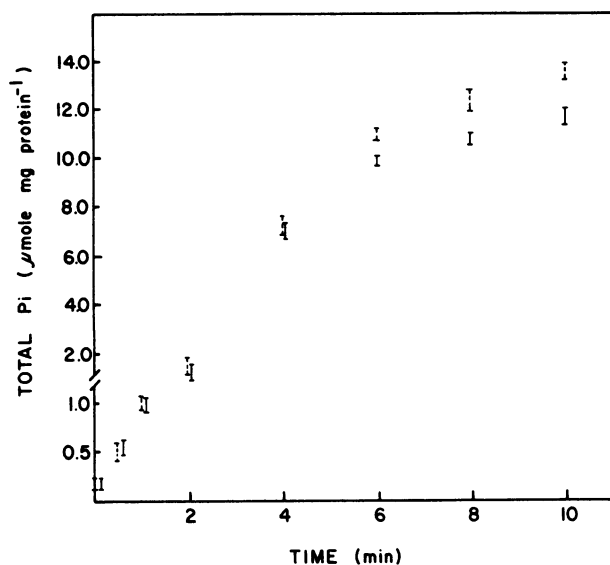


FIG. 4. Kinetics of the effect of Ca^{2+} on the Mg^{2+} -stimulated ATPase activity in oat mitochondria. Each data point represents the high and low value recorded at each time interval. Broken bars represent points obtained when only 5 mM Mg^{2+} was present; solid bars represent values obtained when 50 μM Ca^{2+} was also present.

approximately 100 μM was reached (Fig. 3). At concentrations above 100 μM Ca^{2+} , the rate of hydrolysis of the ATPase declined (Fig. 3). The maximum rate of ATPase activity obtained when Ca^{2+} was added was considerably less than that exhibited by the enzyme when Mg^{2+} was the only divalent cation present; approximately 9 to 16% of that observed with just Mg^{2+} . When the Mg^{2+} concentration was set at 5.0 mM, the addition of increasing amounts of Ca^{2+} caused a continued reduction in the level of ATPase activity (Fig. 3). This depression in the activity was observed from the zero Ca^{2+} value obtained by adding 1 mM EGTA to the reaction solution to the highest concentration tested, 500 μM Ca^{2+} (Fig. 3). The levels of activities attained when Ca^{2+} was held constant (50 μM) and the concentration of Mg^{2+} was varied were lower than those achieved when only equal concentrations of Mg^{2+} were used (Fig. 1, B). The kinetics of Ca^{2+} (50 μM) effects on the Mg^{2+} -dependent ATPase are presented in Figure 4. There was a lag period of between 4 and 6 min before Ca^{2+} caused a noticeable effect on the ATPase. Experiments carried out with EGTA verified this observation (Table II). When EGTA was added to a reaction mixture that contained Mg^{2+} and Ca^{2+} 6 min after the reaction was initiated, it did not negate the Ca^{2+} -induced reduction in the level of activity. The presence of Ca^{2+} in the solution for 6 min prior to

EGTA addition was enough to modify the activity of the ATPase. However, when EGTA was present at the beginning of the incubation period, the level of activity attained was raised approximately to the level achieved when no Ca^{2+} was present (Table II). As could be predicted from Figure 3, the higher values reached when EGTA was present is a consequence of the chelation of all Ca^{2+} , including the background 1 μM amount, present in the reaction solution.

Phytochrome Effects. In the initial experiments to investigate phytochrome effects on the mitochondrial ATPase activity, the effects of R only and FR only were tested, using both preirradiated (Fig. 5) and dark (Fig. 6) mitochondria. When preirradiated mitochondria were used, the per cent decrease in ATPase activity induced by R was 3 to 5 times the small per cent decrease induced by FR (Fig. 5). No exogenous phytochrome was needed for this response. When dark mitochondria were tested, R and FR had essentially no effect in the absence of exogenous phytochrome (Fig. 6A), but in its presence, R again decreased the ATPase activity 3 to 5 times more than the small decrease induced by FR (Fig. 6B). In each of Figures 5 and 6, the results of two separate experiments are shown, indicating the reproducibility of the relative effects of R and FR on the ATPase activity.

Photoreversibility experiments were conducted to determine whether it was possible to reverse the effect of R treatment with FR. In both the R followed by FR treatment and in the reverse treatment, the mitochondria were exposed to a total of 4 min of light. The results from one of these experiments are shown in Figure 7. Consistent with data previously presented, the control nonirradiated sample exhibited the highest rate of ATPase activity, designated here 100% activity. Further, the per cent activity attained by the mitochondrial ATPase following either the R or FR irradiation was comparable to that shown in Figure 5. In the photoreversibility tests, FR significantly reversed the effects of R, and R significantly reversed the effects of FR on the ATPase activity (Fig. 7).

Time courses of the rate of ATP hydrolysis for nonirradiated and irradiated mitochondria are shown in Figure 8. In each instance, the only divalent cation present in the assay medium was 5.0 mM Mg^{2+} , and the irradiations were 3 min. A distinction in the rate of ATP hydrolysis between the control nonirradiated sample and the red-irradiated sample was discernable prior to the 5 min time point. The red-irradiated sample which had 1.0 mM EGTA added 5 min after the irradiation had begun, exhibited a rate of hydrolysis comparable to that of the red-irradiated sample lacking EGTA. Conversely, if the EGTA was present in the mitochondrial suspension prior to the irradiation, the rate of ATP hydrolysis followed that recorded from the nonirradiated control sample.

DISCUSSION

Vierstra and Quail (32) found that photoconversion of phytochrome to the Pfr form prior to extracting it from etiolated oats

Table II. Effect of EGTA on Ca^{2+} -Dependent ATPase

Values given are the mean \pm SD of data normalized from two experiments. Final concentration of substances added were: Mg^{2+} , 5.0 mM; Ca^{2+} , 50 μM ; EGTA, 1 mM.

Time	Divalent Cation and Chelator Addition			
	Mg^{2+}	$\text{Mg}^{2+} + \text{Ca}^{2+} + \text{EGTA}^*$ (* added at start)	$\text{Mg}^{2+} + \text{Ca}^{2+}$	$\text{Mg}^{2+} + \text{Ca}^{2+} + \text{EGTA}^*$ (* added after 6 min)
min	$\mu\text{mol Pi mg}^{-1} \text{ protein}$			
0	0.119 \pm 0.006	0.126 \pm 0.005	0.113 \pm 0.004	0.121 \pm 0.004
5	6.09 \pm 0.03	6.42 \pm 0.14	5.99 \pm 0.02	
9	11.16 \pm 0.05 ^a	11.68 \pm 0.09 ^c	9.47 \pm 0.03 ^b	9.49 \pm 0.08 ^b
12	14.76 \pm 0.09 ^a	15.60 \pm 0.09 ^c	12.60 \pm 0.09 ^b	12.65 \pm 0.02 ^b
15	18.81 \pm 0.03 ^a	19.01 \pm 0.01 ^c	15.90 \pm 0.11 ^b	15.68 \pm 0.04 ^b

^{a-c} For each time point at 9 min and thereafter, the values with different superscripts differ significantly at the 95% confidence level as determined by nonpaired *t* tests.

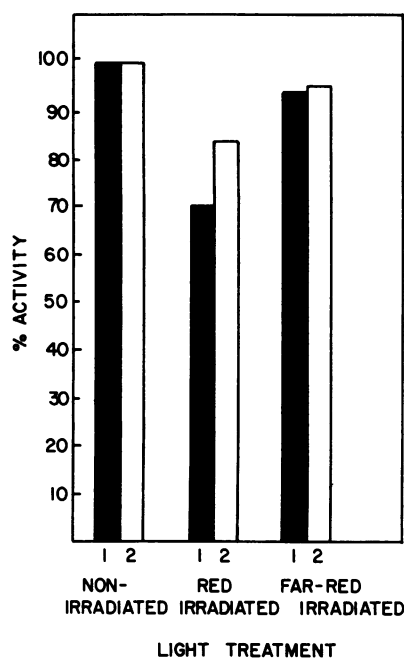


FIG. 5. Effect of R and FR light on the ATPase activity of preirradiated mitochondria. The only divalent cation added to the assay medium was 5.0 mM Mg^{2+} . Two distinct experiments are shown (1 and 2).

helped maintain it as a larger and presumably more native 124,000 D form. It is possible, then, that the endogenously bound phytochrome associated with preirradiated mitochondria in this study was the 124,000 D form. The exogenous phytochrome was comprised mainly of 118,000 D phytochrome. But because two of its spectral properties (λ_{max} of Pfr = 727 nm; $A_{727\text{ nm}}/A_{667\text{ nm}}$ of Pfr = 1.2) were intermediate between those characteristic of 124,000 D phytochrome and those characteristic of 118,000 D phytochrome (30), it is possible that some 124,000 D phytochrome was present in the population of exogenous phytochrome molecules used. This study, then, does not rigorously determine whether the peptide removed in the degradation of 124,000 D phytochrome to 118,000 D phytochrome is required for the ATPase effects shown here.

The apparent physical disruption of oat mitochondria by pH 8.5 conditions made this pH unacceptable for the physiological studies with phytochrome. But by choosing the pH 6.5 optimum (Fig. 2), we risked obscuring the identity of the ATPase being assayed, since the ATPase of plasma membrane-enriched membranes also has a pH 6.5 optimum (10). Although we cannot eliminate entirely the possibility of some contribution to the results by the plasma-membrane ATPases, it is clear that they could only be a minor contaminant at best for the following reasons. (a) Potential plasma membrane vesicles represent at most 10% of the membranes in the preparation. (b) The Mg^{2+} -dependent ATPase in this study was stimulated by 2,4-dinitrophenol and inhibited by oligomycin, characteristic of mitochondrial but not plasma membrane ATPases. (c) Sonication of the mitochondria to produce submitochondrial particles altered the substrate specificity of the ATPase of this study which would be expected for the F_0F_1 -type ATPase, but not for a plasma membrane ATPase. (d) The ATPase under study was not stimulated by K^+ while the plasma membrane ATPase is. Furthermore, work by Gallagher and Leonard (10) on the mitochondrial and plasma membrane ATPases from corn roots suggests that, at pH 6.4, contamination by plasma membrane ATPase activity in the mitochondrial fraction is observable only after K^+ is added to the reaction medium.

The finding that actinic R and FR photoreversibly modulates

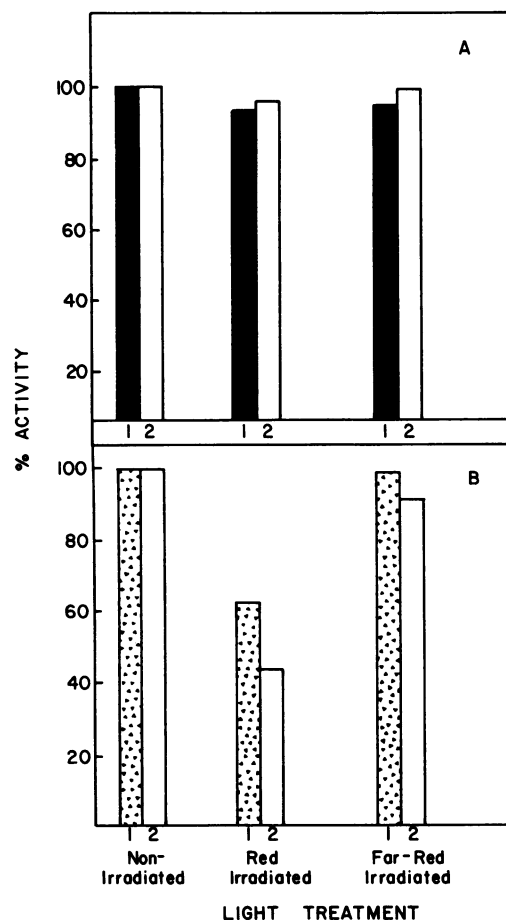


FIG. 6. Effect of R and FR light on the ATPase activity of dark mitochondria. In A, the ATPase activity of the dark mitochondria is shown. B shows the activity after exogenous phytochrome (10 $\mu\text{g}/\text{mg}$ mitochondrial protein) was added to the mitochondria. In both instances, the only divalent cation added was Mg^{2+} (5.0 mM). In A and in B, two distinct experiments are shown. In each case, similarly shaded columns represent data from the same experiment.

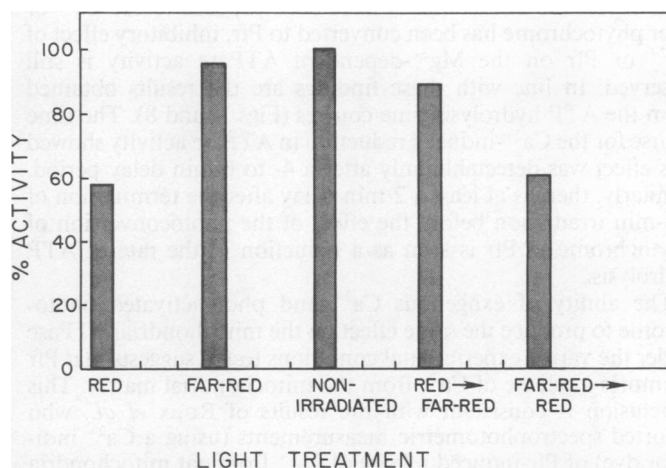


FIG. 7. Photoreversible effects of phytochrome on mitochondrial ATPase activity. Preirradiated mitochondria were used in these experiments. Each mitochondrial sample was irradiated for a total of 4 min. When both R and FR irradiations were given, they were of equal duration. See "Materials and Methods" for a description of the light sources. Five mM Mg^{2+} was the only divalent cation present in the assay medium.

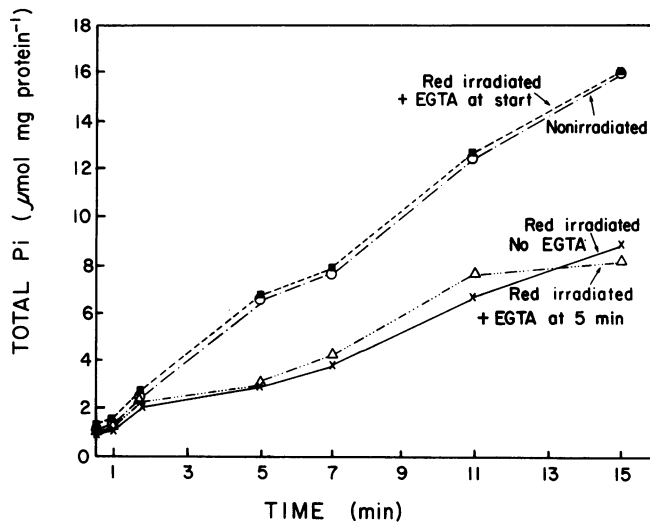


FIG. 8. Time courses of EGTA and Pfr effects on mitochondrial ATPase activity. In all cases, the only divalent cation added to the assay medium was 5.0 mM Mg^{2+} and, when present, 1.0 mM EGTA was used. Preirradiated mitochondria were employed. The R irradiations were given during the first 3 min of the time courses shown.

the rate of ATPase activity in preirradiated mitochondria strongly suggests that this is a phytochrome-controlled response (Fig. 7). The results using dark mitochondria, in which the addition of exogenous phytochrome was required to impart R-sensitivity to the ATPase activity also supports the same conclusion. These results also suggest that preirradiated mitochondria have more photoresponsive phytochrome associated with them than do the dark mitochondria.

The hypothesis that phytochrome can act through its regulation of a secondary messenger, Ca^{2+} (17, 29), is supported by this study. When examined under comparable conditions, phytochrome in its Pfr form and externally added Ca^{2+} induced a similar response in the activity of the ATPase, *i.e.* inhibition. The effect of both Ca^{2+} and Pfr, respectively, on the Mg^{2+} -dependent ATPase can be negated if EGTA is added to the mitochondrial suspension prior to the introduction of these agents to the reaction. Conversely, if the Ca^{2+} chelator is added 6 min after the mitochondria have been exposed to the Ca^{2+} or after phytochrome has been converted to Pfr, inhibitory effect of Ca^{2+} or Pfr on the Mg^{2+} -dependent ATPase activity is still observed. In line with these findings are the results obtained from the ATP hydrolysis time courses (Figs. 4 and 8). The time course for the Ca^{2+} -induced reduction in ATPase activity showed this effect was detectable only after a 4- to 6-min delay period. Similarly, there is at least a 2-min delay after the termination of a 3-min irradiation before the effect of the photoconversion of phytochrome to Pfr is seen as a reduction in the rate of ATP hydrolysis.

The ability of exogenous Ca^{2+} and photoactivated phytochrome to produce the same effect on the mitochondrial ATPase under the varied experimental conditions tested suggests that Pfr promotes a release of Ca^{2+} from the mitochondrial matrix. This conclusion is consistent with the results of Roux *et al.*, who reported spectrophotometric measurements (using a Ca^{2+} indicator dye) of Pfr-induced release of Ca^{2+} from oat mitochondria (29).

Work carried out on another mitochondrial enzyme also supports this inferred direction of Ca^{2+} movement. The mitochondrial enzyme, NADH dehydrogenase which is located on the outer surface of the inner membrane, also responds similarly to exogenous Ca^{2+} and to Pfr but in this case the response is stimulation. Cedel and Roux (3) have shown that the activity of

this enzyme is higher after the light induced conversion of Pr to Pfr. The results of Robinson and Wellburn (28) also suggest that Pfr promotes NADH dehydrogenase activity. They demonstrated that R increased and FR decreased NADH-dependent ATP formation (presumably by oxidation of NADH [16]) in oat mitochondria. Evidence that an addition of extramitochondrial Ca^{2+} also increases the activity of this enzyme comes from the work of Cowley and Palmer (5). A plausible inference from these results is that the R-induced enhancement of NADH dehydrogenase activity was mediated by an R-induced release of matrix Ca^{2+} .

Exactly how this redistribution of Ca^{2+} brings about a reduction in the activity of the ATPase is still unclear. Under the experimental conditions utilized, the Ca^{2+} -stimulated mitochondrial phospholipase which could through its activity change the permeability of the membranes, was not activated (Serlin and Roux, unpublished data). It is possible that the rise in extramitochondrial Ca^{2+} causes a change in the fluidity of the membrane through a rearrangement of certain phospholipids (6). Such a rearrangement could effect either the activity of the enzyme directly by changing the spatial orientation of the enzyme within the membrane or indirectly by modifying some integral regulatory site, *i.e.* a substrate transferase.

The data reported here are in agreement with previous studies (9, 14, 29) which illustrated Pfr-induced Ca^{2+} movement across plant membranes. A predicted consequence of this movement would be an increased Ca^{2+} concentration in the cytosol and an activation of Ca^{2+} and calmodulin-dependent enzymes. Two calmodulin-dependent enzymes in plants appear also to be regulated by phytochrome (1, 29). These results and the data presented here indicate that the possible mediation of phytochrome responses by Ca^{2+} and Ca^{2+} -dependent regulator proteins warrants further study.

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