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Light-induced import of the chromoprotein, phytochrome, into mitochondria

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Mitochondria extracted from plants that were irradiated with actinic light in vivo have associated with them the chromoprotein, phytochrome. This phytochrome retains its native subunit size of 124 kDa after proteolytic treatment of the mitochondria with trypsin and chymotrypsin. This result suggests that phytochrome is not exposed on the outer surface of the outer mitochondrial membrane. Phytochrome, so protected, is not found to be associated with mitochondria derived from unirradiated plants. The possibility that the photoactivation of phytochrome induces a conformational change in its structure which facilitates its transport into the mitochondrion is discussed.

Introduction

The plant chromoprotein, phytochrome, has been shown to play a prominent role in triggering a number of photomorphogenic changes during various stages in the life of a plant. The phytochrome-mediated events are initiated through the photoconversion of the pigment from its physiologically inactive, red-light absorbing form (Pr), which has a cytosolic locale [1], to its physiologically active far-red-light absorbing form (Pfr). It has been proposed that, following its photoactivation, phytochrome associates with various membrane systems and therein modulates physiological responses.

A number of in vitro studies have demonstrated an association of phytochrome with purified membrane components, such as etioplasts [2], plasma membrane [3] and mitochondria [4]. Some concern has been expressed that this binding phenomenon may be an artifact, arising during the organelle extraction procedure. Countering this interpretation are data which show that the binding induces functional membrane changes which are likely to have physiological significance [5–8]. Moreover, studies in which phytochrome was exogenously added to isolated plasma membrane vesicles and mitochondria have shown that there are distinct specific and nonspecific binding sites for phytochrome on these membranes [3,4]. No specific binding sites for phytochrome have been found on mammalian membranes [4]. It is still unclear, though, how phytochrome interacts spatially with these membrane structures following its photoactivation.

The present study was undertaken to clarify how the photoactivated form of phytochrome (Pfr) is positioned in biological membranes. So that the results could be related to earlier functional studies, we chose a well-defined experimental system in which phytochrome-regulated enzymic activity had clearly been demonstrated: highly purified mitochondria from etiolated oat seedlings [6–8].

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Of equal interest was evaluating the size of the endogenously bound phytochrome molecule. Recent work has demonstrated that the native subunit size of the soluble phytochrome in etiolated oat seedlings was 124 kDa [9] rather than the 118 kDa molecule that had been extensively characterized previously [10]. Yet, the 118 kDa species, when added exogenously, induced photoreversible changes in both biological [8] and artificial membranes [11]. In this communication, we demonstrate that the subunit size of the phytochrome endogenously bound to oat mitochondria is 124 kDa. It also appears that the binding of phytochrome to a biomembrane does not irreversibly alter the reactivity of the molecule with monoclonal antibodies.

Throughout this paper, the term 'endogenously bound phytochrome' will be used to refer to that endogenous phytochrome which remains associated with the purified mitochondria at the end of the organelle purification.

Methods

Isolation of mitochondria. Mitochondria with endogenously bound phytochrome were isolated from 3.5-day-old etiolated seedlings of *Avena sativa* cv. Garry (Agway) which were irradiated with 15 min of either white light or red light immediately prior to extraction [8]. The only modification in the procedure was the addition of aprotinin (10^4 kallikrein inactivating units/l extraction buffer) to the extraction medium. 'Dark' mitochondria were extracted by the same procedure except that the oat seedlings used were never exposed to any light. Transfer of the seedlings to the cold room, chilling for 30 min, the harvesting of the oats and the grinding of the seedlings in the extraction buffer were all carried out in complete darkness. All subsequent steps in the mitochondrial isolation procedure were performed under a green safe light. Purity and integrity of the isolated mitochondria were assayed as described previously [8].

Enzyme digestion. To digest away any proteins bound to the external surface of the outer mitochondrial membrane, the purified mitochondria were resuspended in a solution consisting of 10 mM Hepes, 0.25 M sucrose and trypsin and chymotrypsin (each at a final concentration of 120 μg/ml) (pH 8.0). After incubating the mixture for 30 min at 4°C, the reaction was terminated by adding an equal volume of a solution consisting of 10 mM Hepes, 0.25 M sucrose, 1 mM *N*-α-*p*-tosyl-L-lysinechloromethyl ketone (TLCK) (final concentration) and 50 kallikrein-inactivating units aprotinin (pH 7.2). The mitochondria were re-isolated by centrifuging the mixture for 10 min at $10\,000 \times g$. As a control, some of the purified mitochondria were never exposed to the enzyme solution but were placed directly into the TLCK/aprotinin solution. The activity of both the trypsin and chymotrypsin (both three-times crystallized) were tested by the standard procedures using chromogenic agents. To ensure that the aprotinin added to the initial extraction medium was not present at the end of the mitochondrial isolation to inactivate the proteinases, enzymic activity was also monitored in the presence of purified mitochondria.

Lysis of mitochondria and iodination procedure. Mitochondria pelleted out of the TLCK/aprotinin mixture were resuspended in a minimum volume (usually 500 μl) of lysis buffer (RIPA buffer) which contained 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.2% (w/v) SDS, 150 mM NaCl, and 50 mM Tris (pH 7.4). The suspension was vortexed for 1 min and then allowed to incubate for 1 h at room temperature.

The chloramine-T method of iodination was utilized to label the resultant available proteins released by the lysis procedure. To 250 μl of the lysis mixture 1 mCi of I^{125} (Amersham, 100 mCi/ml) was added. Next, 20 μl from a freshly prepared stock of chloramine T (25 mg/10 ml of a 0.05 M sodium phosphate solution, pH 7.5) was mixed in and the reaction was allowed to proceed for 3–4 min. To stop the reaction, 100 μl of a freshly prepared sodium metabisulphite solution (1.2 mg/ml of a 0.05 M sodium phosphate solution, pH 7.5) was added. Isolated 124 kDa phytochrome was iodinated by the same procedure. A modified procedure of Litts et al. [12] was utilized to isolate the phytochrome [13].

Immunoprecipitation. Monoclonal antibodies against oat phytochrome were produced and evaluated as described previously [14]. From the available clone lines, D49 and D26 were utilized. They were used because of their excellent reactiv-

ity to various fragments of trypsin-digested phytochrome and their high titer for phytochrome [14]. These two monoclonals were used both in tandem and separately. Antibodies (1–3 μg/mg of mitochondrial protein) were added to the iodinated protein solution and mixed overnight with continuous rotation at 4°C. From a 10% (v/v) solution of Protein A-Sepharose (Sigma, P-3391) suspended in the lysis buffer, 35 μl were added to the antibody/protein solution. This mixture was incubated at room temperature for 1 h with constant rotation thus allowing the protein A to bind to the monoclonal antibodies. The formed complexes were pelleted by centrifugation in a Beckman Microfuge (model B) for 3 min. The resultant pellets were resuspended in 1.0 ml of the lysis buffer, allowed to mix for 5 min and then re-pelleted. This washing procedure was carried out 5 times. The final pellet was then suspended in a SDS-polyacrylamide gel electrophoresis sample buffer containing mercaptoethanol (Hoeffer Scientific, for composition), boiled for 3–4 min and loaded onto a gel.

Gel electrophoresis, fluorography and quantification of mitochondria-associated phytochrome. To produce fluorograms, samples were electrophoretically separated on 5% or 7.5% SDS-polyacrylamide mini-gels (Idea Scientific Mini-Slab apparatus). Following the method of Bonner and Laskey [15], the mini-gels were impregnated with diphenyl oxazole, dried and then exposed to Kodak XAR-5 X-ray film to produce the fluorograms.

To ascertain the percentage of phytochrome associated with the mitochondria, following proteinase treatment, relative to the percentage present prior to the digestion, two aliquots from control and from proteinase-treated samples, respectively, were taken after resuspension of the immunoprecipitates. Samples of equal protein content were removed from each of the aliquots and counted in a Beckman series 4000 gamma counter.

Results

The mitochondrial isolation method used yielded mitochondria that were intact and physiologically active as shown previously [8]. An assessment of outer membrane intactness revealed that the preparations were more than 85% intact. The purity of the preparations was determined by morphometric analysis [8]. The results demonstrated that at least 80% of the total pellet membrane volume was occupied by recognizable intact mitochondria.

Analysis of the phytochrome endogenously bound to the purified mitochondria showed that its subunit size was 124 kDa (Fig. 1, compare lane A with lane F). When mitochondria containing

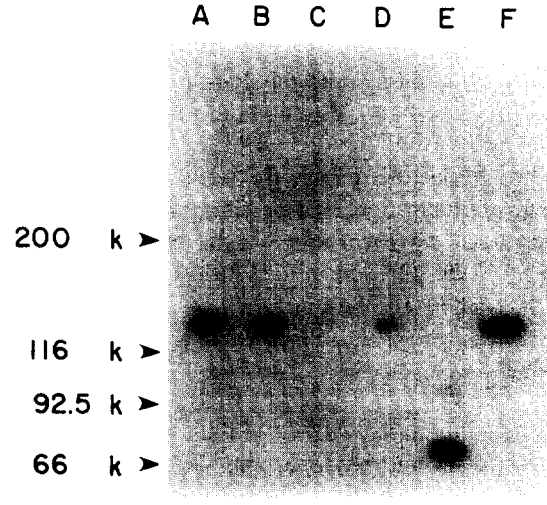

Fig. 1. Fluorogram of ^{125}I-labeled phytochrome recovered by immunoprecipitation from untreated or proteinase-treated mitochondria. All mitochondria were extracted from irradiated tissue (see Methods).
Lane A, phytochrome recovered from mitochondria not exposed to proteinases.
Lane B, phytochrome recovered from mitochondria treated with proteinase mixture.
Lane C, standard of purified phytochrome (1 ng of protein loaded).
Lane D, phytochrome recovered from mitochondria treated with proteinase mixture (50% less loaded).
Lane E, peptides of labeled phytochrome recovered by immunoprecipitation from the digestion of 0.1 mg Pr for 30 min at 4°C with 12 μg each of trypsin and chymotrypsin, in 0.1 ml of buffer (see Methods). 400 ng loaded.
Lane F, standard of purified phytochrome (150 ng of phytochrome loaded).
The resuspended immunoprecipitates for the samples presented in lanes A, B, and D contained equivalent concentrations of Protein A-Sepharose. Equivalent volumes of these resuspensions were loaded in lanes A and B; 50% less was loaded in lane D. Arrows indicate the migration of the standard molecular weight proteins: 200000 (myosin), 116000 (β-galactosidase), 92500 (phosphorylase D) and 66000 (bovine serum albumin). A 5% SDS-polyacrylamide gel used.

endogenously bound phytochrome were subjected to a trypsin-chymotrypsin digestion, the phytochrome recovered from these mitochondria was also the 124 kDa species (compare Fig. 1, lanes B and F). When the same manipulations were carried out on 'dark' mitochondria, no phytochrome was recovered (Fig. 2, lane D). In a second sample of 'dark' mitochondrial, 124 kDa phytochrome ($A_{667}/A_{278} = 0.63$) at a concentration of 38.8 μg/mg of mitochondrial protein was added. The phytochrome was permitted to incubate with the 'dark' mitochondria for 15 min and then the mitochondria were pelleted. They were then resuspended in the trypsin/chymotrypsin enzyme mixture and thereafter handled as described above. No phytochrome was detectable in the mitochondria following procedure (lane E in Fig. 2). Further, polypeptide fragments produced by proteinase digestion of exogenous phytochrome were recognized by the antibodies used (Fig. 1, lane E and Ref. 14).

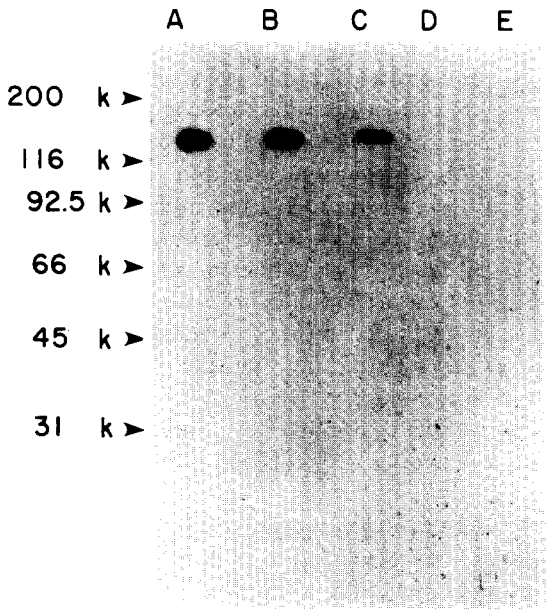

Fig. 2. Fluorogram of ^{125}I-labeled phytochrome immunoprecipitated from mitochondria that were extracted from irradiated or unirradiated tissue. Mitochondrial samples designated 'dark' were extracted from unirradiated tissue; all other samples were extracted from irradiated tissue.
Lane A, phytochrome recovered from proteinase-treated mitochondria.
Lane B, standard of purified phytochrome (200 ng of protein loaded).
Lane C, phytochrome recovered from proteinase-treated mitochondria (20% less loaded).
Lane D, phytochrome recovered from 'dark' mitochondria.
Lane E, phytochrome recovered from proteinase-treated 'dark' mitochondria which had been incubated with exogenously added phytochrome in its inactive Pr form.
The resuspended immunoprecipitates for the samples represented in lanes A, C, D and E contained equivalent concentrations of Protein A-Sepharose. Equivalent volumes of these resuspensions were loaded in lanes A, D, and E; 20% less was loaded on lane C. Arrows indicate the migration of the standard molecular weight proteins: 200000 (myosin), 116000 (β-galactosidase), 92000 (phosphorylase D), 66000 (bovine serum albumin), 45000 (ovalbumin) and 31000 (carbonic anhydrase). A 7.5% SDS-polyacrylamide gel was used.

To test whether the proteinases were functional in the presence of the purified mitochondria, we examined their effectiveness in digesting α-*N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester mixed with mitochondria. The results indicated the proteinases were fully active in the presence of the mitochondria.

The analysis of the amount of phytochrome remaining with the mitochondria following proteinase treatment relative to the amount present in the undigested mitochondria is shown in Table I. Between 70 and 76% of the total amount of phytochrome endogenously associated with the mitochondria through the in vivo irradiation remains with the mitochondria following the trypsin/chymotrypsin digestion.

TABLE I

IMMUNOPRECIPITATED PHYTOCHROME FROM UNTREATED AND PROTEINASE-TREATED MITOCHONDRIA

Samples I and II were from the same preparation of mitochondria, but were iodinated (^{125}I) and subjected to immunoprecipitation as separate samples (see Methods). All cpm values were normalized for equivalent amounts of mitochondrial protein and are average values derived from duplicate aliquots counted.

Sample	Immunoprecipitated phytochrome (cpm)		Average B/A (%)
	untreated (A)	proteinase treated (B)	
I	102660	77850	73.5
II	101840	72570	

Discussion

Based on the evidence presented in Figs. 1 and 2, it is clear that the species of phytochrome endogenously bound to mitochondria has a monomer size of 124 kDa. Previous work has shown that this phytochrome induces photoreversible function changes in purified oat mitochondria [6–8]. This association of phytochrome with mitochondria required the photoconversion of the chromoprotein to Pfr in vivo, for without the in vivo actinic irradiation there was no phytochrome detectable in the mitochondrial preparation. Neither do 'dark' mitochondria show any functional responses to red- and far-red-light irradiations [7,8], consistent with the interpretation that they have extremely little or no phytochrome.

The proteolytic digestion of mitochondria by trypsin and chymotrypsin is routinely employed to remove any proteins projecting out from the cytoplasmic surface of their outer membranes [17]. Recent evidence suggests that these proteinases may digest not only pea chloroplast outer membrane proteins, but also a few select inner membrane proteins [17]. Nonetheless, a significant fraction of the phytochrome associated with oat mitochondria is not susceptible to trypsin or chymotrypsin digestion (Table I), for its subunit molecular mass remained at 124 kDa after proteolysis of the mitochondria (Fig. 1).

This result indicates that a large portion of the total pool of phytochrome associated with the mitochondria is localized interior to the surface of the outer membrane. Past evidence has demonstrated that at least some of the mitochondria associated phytochrome can also be immunocytochemically localized on the outer membrane [7]. Taken together, these findings suggest that at any given time following photoactivation, there are two distinct pools of phytochrome associated with the mitochondria, one of which is positioned so that it is inaccessible to the proteinase action. At this point, the data do not resolve the questions of whether this proteinase-inaccessible phytochrome is deeply embedded in the outer membrane, whether a portion of it extends into the intermembranal space or whether the entire protein has been transported into the intermembrane or inner membrane regions.

Saunders et al. [18] demonstrated that after the photoconversion of phytochrome to the Pfr form, monoclonal antibodies against phytochrome were unable to detect the protein within intact frozen tissue sections. Since their antibody did recognize Pfr, Saunders et al. suggest that after activation phytochrome may become inaccessible to the antibody through a change in the molecular environment of the protein. Their results are consistent with the proposal that Pfr has a membrane-embedded locale.

The proteinase-accessible phytochrome represents about 25% of the total mitochondrial-associated phytochrome pool. This phytochrome may be in the same population that can be immunocytochemically localized on the outer membrane [7], and it may be only temporarily positioned at an external receptor site while enroute to an inner locale. This exposed phytochrome appears to be extensively degraded by the proteinases, since no digestion fragments of phytochrome were recovered by immunoprecipitation (Fig. 1), although the monoclonal antibodies employed recognize tryptic fragments of phytochrome as small as approx. 11 kDa [14].

An internal locale for phytochrome would be consistent with the location of the mitochondrial activities it modulates when it is photoactivated. These activities are all considered to be inner-membrane functions: NADH dehydrogenase activity [6], release of Ca^{2+}, probably from matrix stores [7], and ATPase activity [8]. If some portion of phytochrome was localized proximal to the inner membrane, its regulation of inner-membrane functions would be more direct.

An internal locale for phytochrome in mitochondria would also raise the important question of the mechanism of phytochrome insertion. Current evidence suggests that the positioning of intra-organellar proteins can occur through any one of a number of different mechanisms (Refs. 19 and 20 for reviews). One of these mechanisms depends on the recognition between an insertion sequence on a portion of the protein and a membrane-bound receptor. In several cases, proteins have been shown to undergo conformational changes, exposing internal insertion sequences, prior to their migration into specific membranes [19,20]. The insertion of phytochrome into

mitochondria requires a light-triggered conformational change. If phytochrome has an insertion sequence, then it must be exposed only upon photoactivation of the molecule. Evidence that there may be a region of phytochrome which becomes newly exposed upon photoconversion from Pr to Pfr comes from work by Hahn et al. [21]. They report that the Pfr chromophore region of the molecule is more susceptible to differential oxidation than the Pr chromophore region. Their results are consistent with the interpretation that there is a change in the environment around the chromophore after the photoconversion.

It remains to be seen whether there is a region in the primary sequence of phytochrome which has any of the features of an insertion sequence. It is clear, though, that the two most stable conformations of phytochrome have radically different membrane-associative properties. Because the sequence and other structural features of this protein are rapidly being clarified, it will be an excellent subject for studying how the structure of a protein governs its insertion into biomembranes.

Acknowledgements

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