1992

Partial purification of a 32 kDa photoaffinity labeled benzyladenine-binding protein from mung bean mitochondrial membranes

Gayatri Shah
San Jose State University

Follow this and additional works at: https://scholarworks.sjsu.edu/etd_theses

Recommended Citation

Shah, Gayatri, "Partial purification of a 32 kDa photoaffinity labeled benzyladenine-binding protein from mung bean mitochondrial membranes" (1992). *Master's Theses*. 418.
DOI: <https://doi.org/10.31979/etd.uzkv-x3hj>
https://scholarworks.sjsu.edu/etd_theses/418

This Thesis is brought to you for free and open access by the Master's Theses and Graduate Research at SJSU ScholarWorks. It has been accepted for inclusion in Master's Theses by an authorized administrator of SJSU ScholarWorks. For more information, please contact scholarworks@sjsu.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 1350112

**Partial purification of a 32 kDa photoaffinity labeled
benzyladenine-binding protein from mung bean mitochondrial
membranes**

Shah, Gayatri Shirish, M.A.

San Jose State University, 1992

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

**PARTIAL PURIFICATION OF A 32 kDa PHOTOAFFINITY LABELED
BENZYLADENINE-BINDING PROTEIN FROM MUNG BEAN
MITOCHONDRIAL MEMBRANES**

A Thesis
Presented to
The Faculty of the Department of Biological Sciences
San Jose State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts

By
Gayatri Shah
August, 1992

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. A. Chris Brinegar

Dr. Roger G. Bringer

Dr. John Boothby

APPROVED FOR THE UNIVERSITY

Serena N. Stanford

ABSTRACT

PARTIAL PURIFICATION OF A 32 kDa PHOTOAFFINITY LABELED BENZYLADENINE-BINDING PROTEIN FROM MUNG BEAN MITOCHONDRIAL MEMBRANES

By Gayatri Shah

The photoaffinity labeled 32 kDa cytokinin-binding protein from mung bean mitochondria was partially purified. The 32 kDa protein was enriched by Triton X-100 washing of chloroform treated mitochondrial membranes, but contamination with the 57 kDa (F_1 ATPase) protein was a problem. Preliminary washing of mitochondrial membranes with 6 M urea followed by extraction with sodium dodecyl sulfate was an effective method of enrichment for the 32 kDa protein, suggesting that it is an integral membrane protein. Electrophoretic comparison with partially purified F_1 ATPase showed that the 32 kDa protein was not an F_1 ATPase subunit.

Results from mitochondrial suspensions incubated with tritiated 2-azido- N^6 -benzyladenine ($[^3H]AzBA$) for various times before UV irradiation showed no significant difference in the degree of labeling of the 92, 57, and 32 kDa proteins. Labeling at $-70^\circ C$ for 5 and 15 min also did not show any differences, however, the 32 kDa protein was more intensely labeled than the other proteins. Labeling mitochondria in the presence of Triton X-100, showed maximum labeling at 0.05%.

ACKNOWLEDGMENTS

I take this opportunity to thank Dr. Chris Brinegar for trusting and encouraging me with his patient guidance throughout this work. I am grateful to him for acting as my graduate advisor. I will always cherish the memories of working with him. Also my sincere thanks go to Dr. Roger Biringer and Dr. John Boothby for acting as my graduate committee members. Their helpful suggestions for improvements and corrections of this manuscript are gratefully acknowledged. Special thanks to Photography Department at the Instructional Research Center (San Jose State University, San Jose) for providing copies of photographs.

I would like to express my gratitude to my family. I thank Shirish and Palak for being patient and co-operative during the course of this work. A special thanks to my friend and colleague Sarasu for her friendship and support.

Gayatri Shah

July 20, 1992

TABLE OF CONTENTS

ABSTRACT	Page III
ACKNOWLEDGMENTS	IV
LIST OF FIGURES	VI
LIST OF ABBREVIATIONS	VII
INTRODUCTION	1
MATERIALS AND METHODS	7
RESULTS AND DISCUSSION	15
SUMMARY AND CONCLUSIONS	28
LITERATURE CITED	29

LIST OF FIGURES

Figure		Page
1	Respiratory chain of plant mitochondria.	2
2	The chemical structure of [³ H]-2-Azido-N ⁶ -benzyladenine. . .	5
3	Purified mitochondria after centrifugation through a percoll [®] gradient.	16
4	Thin layer chromatography of [³ H]-2-Azido-N ⁶ -benzyladenine. .	17
5	Labeling of mitochondria with [³ H]AzBA at -70 °C using 5 min and 15 min of UV illumination.	19
6	Effect of Triton X-100 concentration on mitochondrial labeling.	21
7	Effect of Triton X-100 concentration on the incorporation of [³ H]AzBA into TCA-precipitable mitochondrial protein.	22
8	Comparison of partially purified F ₁ ATPase with total mitochondrial proteins by SDS-PAGE.	23
9	Fractionation of mitochondria into soluble and membrane (peripheral and integral) proteins.	25
10	Partial purification of the 32 kDa Protein by Triton X-100 solubilization of chloroform disrupted mitochondrial membranes.	26
11	Partial purification of the 32 kDa protein by SDS solubilization of mitochondrial membranes washed with 6 M urea.	27

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
AzBA	2-azido-N ⁶ -benzyladenine
[¹⁴ C]AzBA	2-azido-N ⁶ -[¹⁴ C] benzyladenine
BA	Benzyladenine
BSA	Bovine serum albumin
HEPES	N-2-hydroethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
[³ H]AzBA	2-azido-N ⁶ - <i>m</i> -tritiobenzyladenine
kDa	Kilodaltons
MOPS	3-[N-morpholino]-propanesulfonic acid
Na ₂ EDTA	Disodium ethylenediaminetetraacetate
NADH	Nicotinamide adenine dinucleotide (reduced form)
PPO	2, 5-diphenyloxazole
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UV	Ultra-violet

INTRODUCTION

The cytochrome oxidase pathway in plant mitochondria consists of a respiratory chain which is responsible for electron transfer from NADH and succinate to O_2 . Both NADH and succinate are biosynthesized in the mitochondrial matrix and their initial oxidation takes place on the inner surface of the inner mitochondrial membrane. The plant respiratory chain consists of five complexes and has three oxidative phosphorylation sites (8) (Fig. 1). When electrons flow through this chain, protons are pumped across the inner mitochondrial membrane into the intermembrane space generating a proton gradient to facilitate ATP synthesis. The cytochrome oxidase pathway can be blocked by cyanide and is known as the cyanide-sensitive pathway. Cyanide inhibits the reduction of cytochrome oxidase (8).

Mitochondria in higher plants have another cyanide resistant pathway known as the alternative (or cyanide resistant) pathway which is thought to branch from the cytochrome oxidase pathway at ubiquinone. Since the alternative pathway branches from the cytochrome oxidase pathway after the first oxidative phosphorylation site, about one-third ATP is produced (6).

When electrons flow through the alternative pathway some physiological responses are evoked in certain plant tissues. The alternative pathway can play a major role in the respiration of imbibed seeds (19), and has also been implicated in many other physiological responses including pollination (6), germination (19), thermogenesis (6, 14), and ripening of fruits (6).

There is evidence that a class of plant hormones (or growth regulators) called cytokinins may somehow be involved in the regulation of alternative

Figure 1. Respiratory chain of plant mitochondria.

pathway respiration. Naturally occurring cytokinins, e. g., zeatin and isopentenyladenine, are purine derivatives and are known to play a regulatory role in the acceleration of cell division, formation of shoots, and metabolism of nucleic acids (23). Some commonly used synthetic cytokinins such as kinetin and benzyladenine (BA) are purine derivatives, but others such as diphenylurea are not (23).

Some cytokinins are effective inhibitors of the alternative pathway in some plant mitochondria when added exogenously (7, 16, 17, 18). The alternative pathway is immediately and completely inhibited by 1 mM BA (7), but lower concentrations of BA disengaged the alternative pathway without complete inhibition (20, 21). Miller (17) has shown that BA inhibits the electron transport chain at a site between NADH dehydrogenase and cytochrome b. A later study suggested that the inhibition site is located prior to the convergence of the NADH dehydrogenase and succinate dehydrogenase pathways (22). A study done with radiolabeled BA indicates that mitochondria have a strong affinity for BA (13).

Some natural cytokinins are usually effective in lower concentrations (≤ 1 μ M) than synthetic cytokinins (23), but Miller (16, 17, 18) discovered that naturally occurring zeatin is not effective in disengaging the alternative pathway. It has been suggested that derivatives like BA with its higher lipid solubility could enter the mitochondrial membrane and interrupt the alternative pathway (7). Zeatin has lower lipid solubility which may explain its ineffectiveness for alternative pathway inhibition. Therefore, lipophilic cytokinins may be more effective inhibitor of alternative pathway respiration (7, 8).

Theiler, *et al.* (25) and Keim and Fox (12) were the first to use azido photoaffinity derivatives of cytokinins to identify putative receptor proteins. These reagents, when irradiated with ultraviolet light, release N₂ from the azido group resulting in a highly reactive nitrene radical which will covalently bind to any proximate molecule and be useful in plant hormone receptor investigations. Brinegar, *et al.* (3) used 2-azido-N⁶-[¹⁴C]benzyladenine ([¹⁴C]AzBA) to label, isolate and characterize the binding site peptide from a wheat cytokinin-binding protein (CBF-1). Photoaffinity labeling has also been used by Hicks, *et al.* (10) to identify putative auxin receptors. Since very high specific activities of reagents are required to detect the low abundance of cytokinin-binding proteins, a tritiated form of AzBA was synthesized by Cooper, *et al.* (5) (Fig. 2). This isotopic form, 2-azido-N⁶-*m*-tritiobenzyladenine ([³H]AzBA) has a 1000-fold higher specific activity than the [¹⁴C] form.

Brinegar, *et al.* (4) and Blumenthal (2) found at least five polypeptides which interact with the cytokinin using [³H]AzBA-labeled proteins from isolated mung bean hypocotyl mitochondria. While the identity of two mung bean minor polypeptides remains unclear, the three major cytokinin-binding polypeptides are the α and β subunits of F₁ ATPase, and an unidentified 33 kDa polypeptide. F₁ ATPase complex purified from maize mitochondria consists of five subunits α , β , γ , δ , and ϵ with the molecular weight of 58 kDa, 56 kDa, 35 kDa, 22 kDa, and 8 kDa respectively (8). The unidentified 33 kDa may be the γ subunit of F₁ ATPase or perhaps an electron transport protein of the inner mitochondrial membrane. The molecular weight estimation for this protein has been revised recently from 33 kDa to 32 kDa (Shah and Brinegar, unpublished). Hereafter, it will be referred to as the "32 kDa protein."

Figure 2. The chemical structure of [3H]-2-Azido-N⁶-benzyladenine.

The goals of this study were 1) to vary the conditions of labeling to increase the specificity of interaction between [^3H]AzBA and mung bean mitochondrial proteins, and 2) to partially purify the 32 kDa cytokinin-binding protein by selective solubilization of mitochondrial membrane fractions.

MATERIALS AND METHODS

Mitochondria Isolation

Crude mitochondria from hypocotyls of mung bean (*Phaseolus aureus*), obtained from Carolina Biological Supply Co. (Gladstone, OR), were prepared by a modification of the method of Miller (17). Chemicals used for the isolation and purification were obtained from Sigma Chemical Co. (St. Louis, MO). All seedlings were grown in vermiculite in the dark at room temperature and used after 6 days. Hypocotyl tissue was crushed in a cold mortar with 2 mL of cold Buffer I per gram of tissue. Buffer I (adjusted to pH 7.5 with KOH) contained 20 mM N-2-hydroethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.5 M D-mannitol, 1 mM disodium ethylenediaminetetraacetate (Na₂EDTA), 10 mM potassium chloride, 10 mM dipotassium phosphate, 5 mM magnesium chloride, and 2 mg of Bovine Serum Albumin (BSA - fraction V) per mL. The ground material was filtered through 8 layers of wet cheesecloth, and centrifuged at 4 °C for 10 min at 1000 xg. The supernatant was recovered, centrifuged for 10 min at 10,000 xg to pellet the mitochondria, and resuspended in buffer I. After a 10 min centrifugation at 10,000 xg the final pellet was suspended in 1 mL of Buffer II (Buffer I minus BSA) per 30 g of initial hypocotyl tissue.

The crude mitochondrial suspension was purified by Percoll® (Pharmacia, Piscataway, NJ) gradient centrifugation, using a modification of the methods presented by Bergman, *et al.* (1) and Jackson, *et al.* (11). Briefly, a step gradient was prepared by layering 10 mL each of 60%, 27% and finally 17% (v/v) Percoll in a 50 mL centrifuge tube. All layers contained 0.5 M sucrose and 20 mM 3-[N-morpholino]-propanesulfonic acid (MOPS) , pH 7.5. Approximately

2 mL of the crude mitochondrial suspension were individually layered on top of the gradients, centrifuged at 10,000 xg for 30 min at 4 °C, and the mitochondrial band (at the 27%-60% Percoll interface) was collected with a Pasteur pipette. The mitochondria were washed with 30 mL of Buffer II and recentrifuged for 10 min at 10,000 xg. The resulting soft pellet contained the purified mitochondria. The pellet was gently resuspended in Buffer II until its volume was the same as that of the initial crude mitochondrial suspension. The temperature of all equipment and materials was kept at 4 °C during the preparation of mitochondria.

Synthesis of the Azido Photoaffinity Reagent

Synthesis of [³H]AzBA was performed by Geoff Cooper at the Plant Cell Research Institute, Dublin, CA. 2-Chloro-N⁶-*m*-iodobenzyladenine was prepared by the condensation of 2, 6-dichloropurine with *m*-iodobenzyladenine. The reagent was isotopically labeled by hydrodeiodination with tritium gas over a palladium catalyst to yield 2-chloro-N⁶-*m*-tritiobenzyladenine, as described by Cooper, *et al.* (5). Displacement of the chloride with hydrazine was followed by nitrosation (12) to produce 2-azido-N⁶-*m*-tritiobenzyladenine ([³H]AzBA) having a specific activity of 11.1 Ci/mmol. After HPLC purification, the reagent was dissolved in methanol to a concentration of 2.5 mM and stored at 4 °C in the dark.

Purity of the reagent was checked occasionally by thin layer chromatography on silica gel (E. Merck, Darmstadt, Germany) using 5% acetic acid in methylene chloride as the mobile phase. Fluorography was performed

using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) after spraying the plate with EN³HANCE (New England Nuclear, Boston, MA).

Incorporation of [³H]AzBA

After transferring 0.5 mL of suspended mitochondria to a tissue culture tube, 20 µL of [³H]AzBA were added dropwise with continuous swirling for a final concentration of 1 mM. After incubating on ice for 5 min, mitochondria were transferred to a 1 cm quartz cuvette. The cuvette was placed at a distance of 1 cm from a UV light source (Ultra-violet Products, Inc., San Gabriel, CA). The sample was illuminated with short wavelength UV light (500 µW/cm² at 254 nm) for 5 min. The mitochondria were then pelleted at 10,000 xg in a microcentrifuge at room temperature, washed once more with cold Buffer II, and the pellet was finally resuspended in 0.2 mL of cold Buffer II. Samples were analyzed by liquid scintillation counting, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

SDS-PAGE, Fluorography, and Scintillation Counting

SDS-PAGE was performed using a discontinuous buffer system (15). All reagents used were purchased from BioRad Laboratories (Richmond, CA). The running (12.5%) and stacking (4%) gels were prepared with a 37.5 : 1 weight ratio of acrylamide to bisacrylamide. Suspended mitochondrial samples were prepared by adding double strength sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 2% glycerol, 10% 2-mercaptoethanol and 0.01 mg/mL bromophenol blue). Pelleted mitochondrial samples were prepared in single strength sample buffer. Samples were heated for 3 min at 95 °C before loading on the gel.

Electrophoresis was performed for 50 to 60 min at 25 mA constant current on an HSI model SE 600 dual slab cell (Hoefer Scientific Instruments, San Francisco, CA). Gels were stained with 0.125% Coomassie Blue R-250 in 50% methanol and 10% acetic acid, and destained with 20% methanol and 10% acetic acid.

Gels with radiolabeled proteins were enhanced for fluorography with 20% 2,5-diphenyloxazole (PPO) in acetic acid as described by Skinner and Griswold (24) and dried at 60 °C for 1 hr on an HSI model SE 1160 slab gel dryer (Hoefer Scientific Instruments, San Francisco, CA). The dried gels were then exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70 °C for 4-5 weeks, and developed according to manufacturer's directions.

Labeled mitochondrial samples were precipitated for scintillation counting by adding an equal volume of cold 20% (w/v) trichloroacetic acid (TCA) for 15 min at 4 °C and then pelleted by centrifugation at 10,000 xg. The pellet was washed with 100 µL of cold 20% TCA. The washed pellet was dissolved in 100 µL of 1% SDS, and then transferred to scintillation vials along with 10 mL of Ready Safe® liquid scintillation cocktail (Beckman Instruments, Fullerton, CA). Vials were stored at 4 °C for 24 hr to avoid quenching and chemilluminescence. Counts were obtained on a model 1900CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT).

Variations in the Labeling Procedure

Incubation time prior to UV irradiation. Into six microcentrifuge tubes (each containing 0.2 mL of mitochondrial suspension) were added 8 µL of [³H]AzBA dropwise with constant swirling. The tubes were incubated on ice for

1, 7, 12, 20, 27, and 32 min, respectively. The samples were illuminated with UV light for 5 min, washed, and resuspended in Buffer II as described above.

Variation in labeling and irradiation time. Into 0.5 mL of mitochondrial suspension, 20 μ L of [3 H]AzBA were added and then incubated on ice for 5 min. One 250 μ L aliquot was transferred to a quartz cuvette and irradiated for 5 min at room temperature as described. Another 250 μ L aliquot was transferred to a well of a 96-well microtitre plate and allowed to freeze on crushed dry ice. The frozen sample was then illuminated from above with UV light for 5 min from a distance of 1 cm. After labeling, the mitochondria were washed and resuspended in Buffer II as noted above. In another experiment, two aliquots of mitochondria were frozen on dry ice as outlined above with one sample being irradiated for 5 min while the other was irradiated for 15 min.

Variation in Triton X-100 concentration. A mitochondrial suspension was divided into five microcentrifuge tubes (0.2 mL each). After centrifugation, the supernatants were removed and the pellets placed on ice. The pellets were resuspended in 0.2 mL of Buffer II containing 0.000%, 0.025%, 0.050%, 0.075%, and 0.100% Triton X-100. [3 H]AzBA was added to each sample to a final concentration of 1 mM and then labeled at room temperature as described above. The labeled mitochondrial pellets were resuspended in 0.2 mL Buffer II, and analyzed by liquid scintillation counting and SDS-PAGE/fluorography.

Partial Purification of F₁ ATPase

Mitochondria were lysed osmotically by adding 60 mL of water per 1.0 mL mitochondria preparation. The mitochondria were centrifuged at 27,000 xg for 30 min at 4 °C to recover the total membrane fraction, and then enriched in F₁

ATPase by the method of Hack and Leaver (9). Briefly, the pellet was resuspended in 2.0 mL cold 250 mM sucrose, 10 mM Tris-HCl buffer (pH 7.6). Chloroform (0.25 mL) was added, the suspension vortexed vigorously, and then microcentrifuged at room temperature for 30 sec. The supernatant was decanted and the proteins precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 350 mg/mL (65% saturation). The suspension was centrifuged as before at 10,000 xg for 15 min. The pellet contained the partially purified F_1 ATPase.

Partial Purification of the 32 kDa Protein

Several methods were investigated for the partial purification of the 32 kDa protein [the "33 kDa protein" described by Blumenthal (2) and Brinegar, *et al.* (4)] to remove peripheral proteins from integral membrane proteins.

Removal of peripheral membrane proteins by salt washing. Purified, labeled mitochondria were lysed with water, transferred into two L5-65 ultracentrifuge tubes and centrifuged at 100,000 xg for 90 min at 4 °C in a Beckman Type 65 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatants (soluble protein) and one pellet containing total membrane protein were stored at -20 °C. The pellet from the other tube was resuspended in 0.1 mL of 1 M Na_2CO_3 (pH 8.5) and centrifuged at 80,000 xg for 60 min at 4 °C. The pellet containing integral membrane proteins and the supernatant containing peripheral membrane proteins were stored at -20 °C.

Detergent solubilization from chloroform insoluble membrane material. Triton X-100 solubilized membrane protein was prepared as follows. Mitochondria (1.0 mL) were broken osmotically as before. After centrifuging at

10,000 xg for 30 min, the resulting membrane pellet was suspended in 1 mL of 250 mM Tris-HCl (pH 7.6), warmed to 20 °C and equally divided into two microcentrifuge tubes. Chloroform (0.25 mL) was added and after vigorous vortexing, the tubes were recentrifuged. The aqueous phase was discarded and the interface material was transferred to a microcentrifuge tube. An equal volume of 1% (v/v) Triton X-100, 50 mM Tris-HCl buffer (pH 8.0) was added, the tubes vigorously vortexed for 30 sec, and then microcentrifuged for 10 min. The supernatant (Triton-soluble interface protein) was transferred into another microcentrifuge tube and stored at -20 °C.

SDS solubilized membrane protein was prepared as follows. Mitochondria were prepared and chloroform extracted as above. The interface material obtained from the extraction was resuspended in 200 µL of 0.5 M NaCl, 50 mM Tris-HCl (pH 8.0) in an attempt to wash away more of the trapped peripheral proteins (e. g., F₁ ATPase). After microcentrifuging for 5 min, the supernatant was discarded, and the Triton-soluble interface protein was prepared as above. The pellet was resuspended in 200 µL of 0.1% SDS, 50 mM Tris-HCl (pH 7.0), microcentrifuged, and the supernatant was saved for SDS-PAGE. The pellet was resuspended in 200 µL of 1.0% SDS, 50 mM Tris-HCl (pH 7.0), microcentrifuged, and the supernatant was saved for SDS-PAGE. In a related experiment, mitochondria were prepared and chloroform extracted as noted above. In this case interface material was extracted with 0.1%, 0.2%, 0.5%, and 1.0% SDS. Supernatants from each extraction were frozen and saved for SDS-PAGE.

Removal of peripheral membrane protein by urea washing followed by detergent solubilization of integral membrane protein. Mitochondria were

osmotically burst using a large volume of water as before and centrifuged in five tubes at 27,000 $\times g$ for 30 min. One total mitochondria membrane pellet was stored at -20 °C for SDS-PAGE. The remaining four pellets were resuspended in 0.2 mL of 1, 2, 4, and 6 M urea solutions buffered in 50 mM Tris-HCl (pH 7.6). The suspensions were transferred to microcentrifuge tubes and centrifuged for 5 min. All four supernatants were saved for SDS-PAGE. The pellets were then resuspended in 0.2 mL of 1.0% SDS in 50 mM Tris-HCl (pH 7.0). After centrifuging, the supernatant was stored at -20 °C for SDS-PAGE and the pellets were discarded.

In a subsequent experiment, 250 μL of mitochondria were burst and centrifuged as described above. The pellet was then dissolved in 200 μL of 6 M buffered urea. It was resuspended and microcentrifuged for 5 min. This step was repeated three times and each time the supernatant was saved for SDS-PAGE. Finally, the pellet was resuspended in 0.2 mL of 1.0% SDS, 50 mM Tris-HCl (pH 7.0). After centrifuging, the SDS supernatant was saved and the pellet was discarded.

RESULTS AND DISCUSSION

Isolation of Mitochondria

Crude mitochondrial suspensions were purified by centrifugation through a Percoll® gradient (Fig. 3). The bottom band between the 27% and 60% layers consisted of pure mitochondria. Purified mitochondrial proteins were analyzed by SDS-PAGE, with the most prominent polypeptides appearing at 92, 57, 44, 39, 34, and 32 kDa (not shown).

Thin Layer Chromatography of [³H]AzBA

Prior to labeling the mitochondria, the purity of the photoaffinity reagent [³H]AzBA was checked by thin layer chromatography (TLC) on silica gel (Fig. 4). Fluorography of three batches of [³H]AzBA showed the same pattern in each. Some radiolabeled material remained adsorbed at the origin. The major migrating species ($R_f = 0.44$) was [³H]AzBA (5). Minor contaminants had R_f values of 0.92, 0.20, and 0.05.

Variations in the labeling procedure

When photoaffinity reagents are used to identify protein binding sites, it is important to check for specific and non-specific labeling. Specific labeling depends on both the nature of the receptor site and of the [³H]AzBA. Any random binding other than to the receptor is non-specific labeling. Various factors can contribute to specific and non-specific labeling, such as incubation time, labeling time and temperature, and the presence of detergents.

Figure 3. Purified mitochondria after centrifugation through a percoll® gradient. Band A consists of broken membrane material and non-mitochondria organelles. Band B is the purified mitochondria.

Figure 4. Thin layer chromatography of [^3H]-2-Azido- N^6 -benzyladenine. Sample of three different batches of [^3H]AzBA were run on a silica gel plate and the fluorogram was developed as described in Materials and Methods. See text for an explanation.

Incubation time prior to UV irradiation. Fluorography from SDS-PAGE of mitochondrial suspensions incubated with [³H]AzBA for various times before UV irradiation showed the three bands were labeled (92, 57, and 32 kDa) to the same degree (not shown). The TCA precipitated protein also showed no significant differences in incorporation of the label. Apparently, the AzBA binds to the target sites in ≤ 1 min. This is in agreement with Blumenthal (2) who showed that AzBA addition to respiring mitochondria inhibited oxygen uptake in a matter of seconds. Therefore, a 5 min incubation time prior to UV irradiation was chosen for all subsequent experiments.

Variation in labeling and irradiation time. At -70 °C the kinetic energy of AzBA is lower and reactions at this temperature should have reduced non specific labeling. Preparations labeled for 5 min at room temperature showed no significant difference from those labeled at -70 °C by fluorography and scintillation counting (not shown).

When the irradiation time was increased from 5 min to 15 min at -70 °C, the fluorogram again showed no significant difference in the labeling pattern (Fig. 5). However, the 32 kDa protein was more intensely labeled than the other proteins. In addition, some labeled material migrated with the dye front.

Variation in Triton X-100 concentration. Triton X-100 detergent (0.1%), when added to corn hypocotyl membranes before irradiation, has been shown to increase the specificity of labeling by a photoaffinity auxin derivative (10). Triton X-100 works by partial solubilization of the membrane, thereby making the proteins more accessible to the reagent. Therefore, various concentrations of Triton X-100 were added to the mitochondrial incubation medium prior to labeling with [³H]AzBA to determine the effect on labeling efficiency and

Figure 5. Labeling of mitochondria with [^3H]AzBA at -70°C using 5 min and 15 min of UV illumination. The fluorogram is of a 12.5% acrylamide SDS gel. Molecular weights of labeled proteins are expressed as kilodaltons. Lane 1, 5 min labeling. Lane 2, 15 min labeling.

specificity. Fluorography (Fig. 6) and scintillation counting (Fig. 7) showed that the highest degree of labeling was obtained with 0.05% Triton X-100. The 57 kDa and 32 kDa polypeptides were labeled to the greatest degree with the latter being the most intensely labeled. Scintillation counts showed a rise in labeling with increasing concentration of Triton X-100 up to 0.05%, but at 0.075% Triton X-100 labeling was diminished. At or above 0.075%, the membrane may have become too disrupted and altered the conformation and/or the binding abilities of target proteins.

Partial Purification of F₁ ATPase

The labeled proteins at 57 kDa were probably the α and β subunits of F₁ ATPase. The 32 kDa protein, however, could have been a separate protein or a part of the F₁ ATPase complex. When F₁ ATPase was partially purified from labeled mitochondria, the 57 kDa band was seen on the gel (Fig. 8), but no other major polypeptides of the enriched preparation migrated with an apparent molecular weight of 32 kDa. This result indicates that the 32 kDa protein is not a part of F₁ ATPase.

Partial Purification of the 32 kDa Protein

Since the 32 kDa protein was not a part of F₁ ATPase, our goal was to purify and identify it. Various strategies were investigated for purification of this protein from mitochondrial preparations. Classical fractionation of membrane proteins involves the removal of peripheral proteins from the integral membrane proteins by "washing" with sodium carbonate solutions. Sodium chloride can also be used (Dale Greenwalt, personal communication).

Figure 6. Effect of Triton X-100 concentration on mitochondrial labeling. The photograph is Coomassie Blue stained SDS-PAGE gel. Lanes 1-5 are mitochondrial suspensions treated with 0.000%, 0.025%, 0.050%, 0.075%, and 0.100% Triton X-100, respectively, before labeling with [^3H]AzBA. Lane 6 contains molecular weight standards (kDa). The bottom photograph is the fluorogram of the same gel.

Figure 7. Effect of Triton X-100 concentration on the incorporation of [³H]AzBA into TCA-precipitable mitochondrial protein.

Figure 8. Comparison of partially purified F₁ ATPase with total mitochondrial proteins by SDS-PAGE. Lane 1, Molecular weight standards (kDa). Lane 2, total mitochondria. Lane 3, Partially purified F₁ ATPase.

SDS-PAGE of samples derived from mitochondrial fractionation using 1 M Na_2CO_3 to wash off peripheral proteins showed F_1 ATPase as peripheral membrane protein. Some F_1 ATPase contamination was also seen in the integral membrane protein fraction (Fig. 9). Nearly all of the 32 kDa protein was found in the integral membrane protein fraction.

Interface material from mitochondrial suspensions which had been treated with chloroform and Triton X-100 to solubilize integral membrane proteins were mostly 32 kDa protein with some 57 kDa polypeptide contamination. These results were not reproducible. With the exception of first experiment (Fig. 10), there was always significant 57 kDa polypeptide present.

Purification of the 32 kDa protein using various concentration of SDS after chloroform extraction indicated that SDS at 0.1% was too low for stripping off the proteins whereas 1.0% SDS was too high, as it extracted both the 32 kDa protein and F_1 ATPase (data not shown). Intermediate concentrations of SDS were tried, but F_1 ATPase contamination was still problematic (data not shown).

The use of urea treatment for the purification of the 32 kDa protein from total mitochondrial protein showed that nearly all the F_1 ATPase was removed in the first wash of total mitochondrial membranes with 6 M urea (Fig. 11, lane 1), and that some of the 32 kDa protein was removed in all three washes (Fig. 11, lanes 1-3). Most of the 32 kDa protein, however, was recovered in an enriched form by SDS extraction of the remaining membranes (Fig. 11, lane 4).

Figure 9. Fractionation of mitochondria into soluble and membrane (peripheral and integral) proteins. Mitochondrial fractions were prepared by centrifugation and washing with 1 M Na_2CO_3 as described in Materials and Methods and analyzed by SDS-PAGE. Lane 1, Total membrane protein. Lane 2, Soluble protein. Lane 3, Peripheral membrane protein (1 M wash), Lane 4, Integral membrane protein. Lane 5, total mitochondria. Lane 6, Molecular weight standards (kDa).

Figure 10. Partial purification of the 32 kDa Protein by Triton X-100 solubilization of chloroform disrupted mitochondrial membranes. Mitochondrial membranes were treated with chloroform, centrifuged, and the insoluble material at the interface of the aqueous and organic phases was extracted with 1.0% Triton X-100 and analyzed by SDS-PAGE. Lane 1, Total mitochondria. Lane 2, Total membrane. Lane 3, Triton-soluble interface. Lane 4, Triton-insoluble interface. Lane 5, Molecular weight standards (kDa).

Figure 11. Partial purification of the 32 kDa protein by SDS solubilization of mitochondrial membranes washed with 6 M urea. Lane 1, Proteins solubilized by the first urea wash. Lane 2, Proteins solubilized by the second urea wash. Lane 3, Proteins solubilized by the third urea wash. Lane 4, Proteins solubilized by SDS extraction of urea washed membranes. Lane 5, Total mitochondrial membranes. Lane 6, Molecular weight standards (kDa).

SUMMARY AND CONCLUSIONS

Purified mitochondria were labeled with [^3H]AzBA photoaffinity reagent. Two intensely labeled bands observed were of 57 kDa and 32 kDa molecular weight. The 57 kDa band appears to be part of the F_1 ATPase complex, but the identity of the 32 kDa protein remains unclear (2). Isolation and identification of the 32 kDa protein was the goal of this study.

Pure mitochondria were isolated from hypocotyls of mung beans and labeled with [^3H]AzBA with variations in labeling procedure. Triton X-100 (0.05%), BA photolabeled (5 min, 20 °C) mitochondria were fractionated to determine the location of the 32 kDa cytokinin-binding protein and its relationship to F_1 ATPase. Results showed that the 32 kDa protein was not a part of F_1 ATPase, and is an integral membrane protein.

The 32 kDa protein was enriched using urea and SDS as detergents. The results show that 6 M urea is sufficient to wash off most of the F_1 ATPase and not the 32 kDa protein.

The 32 kDa protein may be part of electron transport chain, since it is an integral membrane protein, and is photolabeled with BA, a known inhibitor of the electron transport chain. Once identified, future studies should be directed at how BA affects the 32 kDa protein at the molecular level and how it regulates alternative respiration.

LITERATURE CITED

1. **Bergman A, Gardestrom P, Ericson I** (1980) Method to obtain a chlorophyll-free preparation of intact mitochondria from spinach leaves. *Plant Physiol* **66**: 442-445
2. **Blumenthal S** (1990) Photoaffinity labeling of benzyladenine-binding proteins in plant mitochondria. MS Thesis, San Jose State University, San Jose
3. **Brinegar AC, Cooper G, Steven A, Hauer CR, Shabanowitz J, Hunt DF, Fox JE** (1988) Characterization of a benzyladenine binding-site peptide isolated from a wheat cytokinin-binding protein: Sequence analysis and identification of a single affinity-labeled histidine residue by mass spectrometry. *Proc Natl Acad Sci USA* **85**: 5927-5931
4. **Brinegar AC, Blumenthal S, Cooper G** (1991) Photoaffinity labeling of mung bean mitochondrial proteins using [³H]-2-azido-N⁶-benzylaminopurine. *In* Proceedings of the international Symposium on Physiology and Biochemistry of Cytokinins in plants, Liblice, Czechoslovakia, edited by M. Kaminek, D. W. S. Mok, and E. Zazimalova. pp 1-7
5. **Cooper G, Bourell J, Kaminek M, Fox JE** (1988) Methods for synthesis of 2-azido-N⁶-*m*-tritiobenzylaminopurine, a photoaffinity labeled for cytokinin-binding proteins in plants. *J Labelled Comp Radiopharm* **25**: 957-962
6. **Day DA, Arron GP, Laties GG** (1980) Nature and control of respiratory pathways in plants: The interaction of cyanide-resistant respiration with the cyanide sensitive pathway. *In* DD Devices, ed, The Biochemistry of Plants, Vol 2. Academic Press, NY, pp 197-241
7. **Dizengremel P, Chauveau M, Roussaux J** (1982) Inhibition by adenine derivatives of the cyanide-insensitive electron transport pathway of plant mitochondria. *Plant Physiol* **70**: 585-589
8. **Douce R** (1985) *In* Mitochondria in Higher Plants: Structure, Function, and Biogenesis. Academic Press, NY

9. **Hack E and Leaver CJ** (1983) The α -subunit of the maize F_1 -ATPase is synthesised in the mitochondrion. *The EMBO Journal* **10**: 1783-1789
10. **Hicks GR, Rayle DL, Lomax TL** (1989) The diageotropica mutant of tomato lacks high specific activity auxin binding sites. *Science* **245**: 52-54
11. **Jackson C, Dench JE, Hall DO, Moore AL** (1979) Separation of mitochondria from contaminating subcellular structures utilizing silica gel gradient centrifugation. *Plant Physiol* **64**: 150-153
12. **Keim P, Fox JE** (1980) Interaction of a radiolabeled cytokinin photoaffinity probe with a receptor protein. *Biochem Biophys Res Commun* **96**: 1325-133
13. **Keim P, Erion J, Fox JE** (1981) The current status of cytokinin-binding moieties. *In* F Guern, C Peaud-Lenoel, eds, *Metabolism and Molecular Activities of Cytokinins*. Springer-Verlag, NY, pp 179-190
14. **Knuston RN** (1974) Heat production and temperature regulation in Eastern skunk cabbage. *Science* **186**: 746-747
15. **Laemmli UK** (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680-685
16. **Miller CO** (1979) Cytokinin inhibition of respiration by cells and mitochondria of soybean, *Glycine max* (L.) Merrill. *Planta* **146**: 503-511
17. **Miller CO** (1980) Cytokinin inhibition of respiration in mitochondria from six plant species. *Proc Natl Acad Sci USA* **77**: 4731-4735
18. **Miller CO** (1982) Cytokinin modification of mitochondrial function. *Plant Physiol* **69**: 1274-1277
19. **Morohashi Y, Matsushima H** (1983) Appearance and disappearance of cyanide-resistant respiration in *Vigna mungo* cotyledons during and following germination of axis. *Plant Physiol* **73**: 82-86
20. **Musgrave ME, Siedow JN** (1985) A relationship between cyanide-resistant respiration and plant responses to cytokinins. *Physiol Plant* **64**: 161-166

21. **Musgrave ME, Miller CO, Siedow JN** (1987) Do some plant responses to cytokinins involve the cyanide-resistant respiratory pathway? *Planta* **172**: 330-335
22. **Ralph RK, Wojcik SJ** (1982) Cytokinin effects on mitochondria and calcium metabolism. *Plant Sci Lett* **25**: 321-328
23. **Salisbury FB, Ross CW** (1978) *In* Plant Physiology, Ed 2. Waddsworth Publishing Company, Inc., CA, pp 182-184
24. **Skinner MK, Griswold MD** (1983) Fluorographic detection of radioactivity in polyacrylamide gels with 2, 5-diphenyloxazole in acetic acid and its comparison with existing procedures. *Biochem J* **209**: 281-284
25. **Theiler JB, Leonard NJ, Schmitz RY, Skoog F** (1976) Photoaffinity-labeled cytokinins: Synthesis and biological activity. *Plant Physiol* **58**: 803-805