1990

Photoaffinity labeling of benzyladenine-binding proteins in plant mitochondria

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DOI: <https://doi.org/10.31979/etd.xpz2-n8pn>
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**PHOTOAFFINITY LABELING OF BENZYLADENINE-BINDING
PROTEINS IN PLANT MITOCHONDRIA**

**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
Sonal Blumenthal
December, 1990**

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ABSTRACT

PHOTOAFFINITY LABELING OF BENZYLADENINE-BINDING PROTEINS IN PLANT MITOCHONDRIA

By Sonal Blumenthal

The photoaffinity label [^3H]-2-azido- N^6 -benzyladenine ([^3H]AzBA) was used to identify putative benzyladenine-binding proteins in mung bean (*Phaseolus aureus*) mitochondria. Obtaining evidence of cytokinin-binding proteins is the first step in elucidating the molecular mechanism of cytokinin action in the inhibition of alternative respiration in plant mitochondria. With increasing ultraviolet-irradiation time or [^3H]AzBA concentration, there was greater mitochondrial labeling. To optimize binding conditions, an irradiation time of 3 min and a concentration of 50 μM [^3H]AzBA were chosen. Fluorography of the mitochondrial proteins separated by SDS-PAGE showed there to be several polypeptides that were prominently labeled by the reagent. The most intensely labeled polypeptides were estimated to be 33, 34, 45, 57, and 70 kDa. The 34 and 57 kDa polypeptides are suspected of being ATP synthase subunits, while the identity of the 33, 45, and 70 kDa polypeptides remain unclear. In the presence of increasing concentrations of ADP or benzyladenine, labeling of all mitochondrial proteins was greatly reduced. Preliminary evidence indicated that approximately 89% of the incorporated radioactivity was from membrane proteins. Of this fraction, the labeling of integral proteins was nearly twice that of peripheral proteins.

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Chris Brinegar, who has not only been my advisor, but has also been a good friend. His friendship and confidence in me are deeply appreciated. Under Dr. Brinegar's guidance, I found the strength to "hang in there" and endure the joys and pains of research. Soon, I will embark on a new journey working towards my doctoral degree, but I will always cherish the memories of working with Chris.

Many thanks to Drs. Roger Biringer and David Bruck for their hours of advice and participation on my thesis committee. Their suggestions were extremely helpful...even the red ink! Special thanks to Drs. John Boothby, Steve White, and Bob Cluss for their much appreciated research advice and assistance. Special thanks also to the staffs of the Biology Department Office, Biology Service Center, Botany Preparation Room, Photography Department at the Instructional Research Center, and Microbiology Service Center for making life a little easier during the investigation and preparation of this thesis.

I would like to express my gratitude to my family and friends who have helped me more than they know. Jeff, your endless patience, love, and encouragement mean a lot to me. Its comforting to know I could count on you to understand and listen to my SJSU tales. Now we have five more years to go at UT Austin! Mom, Dad, Lopa, and Arati, the emotional support you

have given me over the years will also be remembered. To my friends at SJSU who have come and gone over the years, thanks for taking the "hum" out of school and making it lots of fun!

Financial support for the synthesis of the photoaffinity derivative described in this thesis was provided by the Plant Cell Research Institute. Special thanks to Geoffry Cooper formerly at PCRI for synthesizing the derivative.

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INTRODUCTION

The cytochrome pathway of the mitochondrial electron transport chain is the main route by which electrons flow. This route, also known as the cyanide-sensitive pathway, can be blocked by cyanide which inhibits the reduction of cytochrome oxidase. In addition to the main respiratory pathway, the mitochondria of many higher plants also possess an alternative, cyanide-resistant pathway. This second route cannot be blocked or inhibited by cyanide and is thought to branch off from the ubiquinone region prior to cytochrome b (Appendix A). The cytochrome pathway has three oxidative phosphorylation sites which use the transport of electrons to pump protons across the inner membrane, generating a proton gradient to drive ATP synthesis. Both the alternative pathway and the cytochrome pathway allow transport of electrons to oxygen; however, the alternative route produces mainly heat and only one-third as much ATP, because divergence from the main pathway occurs after the first oxidative phosphorylation site. If electron transport through the alternative pathway is fast enough (high respiration rate), both heat and ATP are formed at rapid rates, but this formation is at considerable expense of food reserves (3, 8, 28).

In some plant tissue, the flow of electrons through the alternative pathway can be considerable and has been shown to evoke a number of physiological responses. Respiration inhibitors such as cyanide, or growth

regulators such as some cytokinins have been shown to break dormancy in *Lactuca sativa* (lettuce) seeds in combination with other factors (3). Studies with imbibed seeds have shown that, in early stages of germination, the alternative pathway can become a significant part of total respiration although its importance is not yet known (22, 29, 33). During the opening of the inflorescence (the collection of flowers sharing a common stalk) in many of the Araceae (*e.g.*, arum lilies), the alternative pathway facilitates pollination by causing volatilization of several insect attractants (8). Thermogenesis (heat production by the alternative pathway during high respiration) permits the inflorescence of eastern skunk cabbage to survive the cold (8, 15). Finally, the alternative pathway may also be responsible for the respiratory climacteric rise in some ripening fruits (*e.g.*, avocado and banana) (8). Climacteric fruits are those fruits that ripen as respiration rate increases sharply. A comprehensive review of both the cytochrome and alternative pathways can be found in Day (8).

"Cytokinin" is a general term used for plant hormones or growth regulators that are involved in the stimulation of cell division and shoot formation, nucleic acid metabolism, and root-shoot interactions (*e.g.*, delaying senescence and promoting lateral bud outgrowth). Natural cytokinins are all chemically related to the purine adenine, whereas synthetic cytokinins may or may not be purine derivatives. Zeatin and isopentenyladenine are examples of naturally occurring cytokinins found in various plant parts. Of

the many synthetic cytokinins available, kinetin and benzyladenine (BA) are those most commonly used by physiologists (28). Both kinetin and BA are purine derivatives. A comprehensive review of cytokinins may be found in Moore (21).

In early studies, Tetley and Thimann (31) showed that in senescing oat leaf tissue, the respiration affected by cytokinin treatment was resistant to cyanide. Later, Miller (18, 19, 20) and Dizengremel, *et al.* (9) showed that, in mitochondria isolated from a variety of plant sources, some cytokinins are effective inhibitors of the alternative pathway. They reported that 1 mM BA resulted in immediate and complete inhibition of the alternative pathway. Musgrave and Siedow (23, 24), however, found that in intact tissues, lower concentrations (20 to 40 μ M BA) disengaged the alternative pathway without completely inhibiting it. Miller (19) showed that cytokinins, such as BA, inhibit electron transport at a site between NADH dehydrogenase and cytochrome b (Appendix A). Ralph and Wojcik (26) suggested that this site is prior to the convergence of the NADH dehydrogenase and succinate dehydrogenase pathways. Miller (18, 19, 20), however, discovered that the natural cytokinin zeatin is ineffective in disengaging the alternative pathway. For BA to have substantial effects on mitochondrial respiration unusually high, nonphysiological concentrations were required for a compound serving as a hormone (19). Natural hormones are usually effective at internal concentrations of 1 μ M or less (28).

Dizengremel, *et al.* (9) proposed that adenine derivatives with high lipid solubility (such as BA) could enter the mitochondrial membrane and interact with a hypothetical receptor to interrupt the alternative pathway. From their studies with tritiated BA, Keim, *et al.* (14) presented evidence that mitochondria do have a strong affinity for BA. However, unlike BA, zeatin has a low degree of lipid solubility, which could account for its ineffectiveness in inhibiting the alternative pathway. It is possible that a lipophilic group is essential in positioning BA to produce an inhibitory effect (9, 20). For a cell to use zeatin, a special mechanism may be needed (20).

Photoaffinity labeling has been used to identify receptor proteins. Azido photoaffinity derivatives of cytokinins were first introduced by Theiler, *et al.* (32) and Keim and Fox (13). When these reagents are irradiated with ultraviolet light, N₂ is released from the azido group resulting in a highly reactive nitrene radical, which can covalently bond with any molecule in close proximity. To label, isolate, and sequence the binding site of an abundant wheat embryo cytokinin-binding protein (CBF-1), Brinegar, *et al.* (5) used 2-azido-N⁶- [14C]benzyladenine ([14C]AzBA). This study showed the utility of using a photoaffinity labeled derivative to detect cytokinin binding. Hicks, *et al.* (11) also successfully used azido-photoaffinity labeling to identify putative auxin receptors. Because detection of low abundance cytokinin-binding proteins requires higher specific activity of the label, a tritiated form of AzBA was synthesized by Cooper, *et al.* (7). The new isotopic

form, 2-azido-N⁶-*m*-tritiobenzyladenine ([³H]AzBA) has a 1000-fold greater specific activity than the ¹⁴C form (Appendix B).

The goal of this study was to use photoaffinity labeling for to identify putative BA-binding proteins in mung bean mitochondria exhibiting relatively high rates of alternative respiration. As of yet, no experimental connection has been made between a cytokinin-binding protein in mitochondria and a known molecular or cellular response to cytokinin. Obtaining evidence of putative cytokinin-binding proteins would be the first step in elucidating the molecular mechanism of cytokinin action in mitochondria.

MATERIALS AND METHODS

Preparation of Mitochondria

All chemicals used for the isolation and purification of mitochondria were obtained from Sigma Chemical Company (St. Louis, MO). Crude mitochondria from hypocotyls of mung bean (*Phaseolus aureus*), obtained from the Carolina Biological Supply Co. (Gladstone, OR), were prepared by a modification of the method of Miller (19). All seedlings were grown in vermiculite in the dark at 28°C and used after 6 d. Hypocotyl tissue was crushed in a cold mortar with 2 mL of grinding medium per g of tissue. The ground material was filtered through 8 layers of wet cheesecloth. The filtrate was centrifuged at 4°C for 10 min at 1000xg, and the resulting supernatant was centrifuged for 10 min at 10,000xg. The 10,000xg pellet was resuspended in washing medium and centrifuged again for 10 min at 10,000xg. The grinding and washing medium (adjusted to pH 7.5 with HCl) contained 20 mM Hepes (N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.5 M D-mannitol, 1 mM Na₂EDTA (disodium ethylenediaminetetraacetate), 10 mM potassium chloride, 10 mM dipotassium phosphate, 5 mM magnesium chloride, and 2 mg of BSA (bovine serum albumin - fraction V) per mL. The final suspension medium contained the same reagents as the grinding and washing medium with the omission of BSA. The pellet was then

resuspended so that, for 12 g of initial hypocotyl tissue, 1 mL of final suspension medium was used. Hereafter, the final suspension medium will be referred to simply as "medium."

The crude mitochondrial suspension obtained was purified by centrifugation through a Percoll^R gradient. The following purification procedure was modified from Bergman, *et al.* (2) and Jackson, *et al.* (12). The step gradient was prepared by first layering 10 mL 60%, 10 mL 27%, and finally 10 mL 17% (v/v) Percoll in a 50 mL centrifuge tube. All layers contained 0.20 M sucrose and 10 mM Mops [3-(N-morpholino)-propanesulfonic acid]. The final pH was adjusted to 7.2 with HCl. About 4 mL of the crude mitochondrial suspension were layered on top of the gradient and centrifuged at 10,000xg for 30 min at 4°C. After centrifugation, the mitochondrial band at the interface between the 27% and 60% Percoll layers was collected with a Pasteur pipette. The mitochondria were washed with 50 mL of medium and recentrifuged for 10 min at 10,000xg. The resulting soft pellet contained the purified mitochondria. The pellet was gently resuspended with medium until its volume equalled that of the initial crude mitochondrial suspension. The temperature of all equipment and materials was kept at 4°C during the preparation of the mitochondria.

The integrity of the purified mitochondria was checked by transmission electron microscopy (25), cytochrome c oxidase assay (33), and measurement of ADP/O ratios (19).

Oxygen Uptake Measurements

Oxygen consumption was determined polarographically at room temperature by means of a model 53 biological O₂ monitor and electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). Crude and purified mitochondria were checked separately for membrane integrity by means of ADP/O ratios. Air-saturated medium (2.175 mL) and the crude or purified mitochondrial suspension (0.725 mL) were added to the mixing chamber. After establishing a steady O₂ consumption rate in the presence of 15 mM malate, 0.3 mM ATP, 0.2 mM thiamine pyrophosphate, and 1 mM pyruvic acid, ADP was added to achieve a final concentration of 0.5 mM, and the rate of oxygen uptake was measured. ADP/O ratios were calculated as described by Miller (19). This ratio is defined as μmol of ADP added divided by the μmol of atomic oxygen utilized during state-3 respiration (*i.e.*, respiration in the presence of ADP). The magnitude of the ADP/O ratio depends in part on the origin of electron pairs transported in the mitochondrial membranes. Since the electrons originated from malate, ADP/O ratios will ideally approach 3.0 (the number of ADP-phosphorylation sites through which the electrons derived from malate pass).

Alternative respiration was measured in the presence of 1 mM SHAM (salicylhydroxamic acid prepared in 50% DMSO), an inhibitor of the alternative pathway, to the aforementioned mitochondrial suspension and

reagents during state-3 respiration. Also, state-3 rates were measured in the presence of 0.5 mM benzyladenine (BA) or 0.5 mM unlabeled 2-azido-N⁶-benzyladenine (AzBA), both in DMSO, to compare their effect on oxygen consumption. The final volume in the mixing chamber after all additions was 3 ml.

Synthesis of the Azido Photoaffinity Reagent

2-Chloro-N⁶-*m*-iodobenzyladenine was formed from 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.* (7). Displacement of the chloride with hydrazine was followed by nitrosation (13) 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.

Treatment of Mitochondria with [³H]AzBA

Aliquots (0.5 mL) of the purified mitochondrial suspension were preincubated on ice for 5 min with or without ADP or BA for 5 min. [³H]AzBA was added to the aliquots and incubated on ice for 5 min. Each aliquot was then transferred to a quartz cuvette and exposed to 254 nm

ultraviolet light in a model UVGL-58 mineral light lamp (Ultra-Violet Products, Inc., San Gabriel, CA) at a distance of 1 cm from the surface of the cuvette. After labeling, 0.4 mL of the mitochondrial suspension was removed, washed twice with medium by centrifugation at 10,000xg, and resuspended in 0.2 mL of medium. The suspension was then divided for protein quantitation, scintillation counting, and electrophoresis. All samples were kept in suspended or pelleted form and stored at -20°C until needed.

Protein Quantitation

The protein concentration of each of the samples was determined by the method of Lowry (17) and/or a modification of the method of Bradford (4). Using the modified Bradford method, 500 μ L of the Bradford reagent was added to 100 μ L of the sample (or diluted sample). The samples were assayed relative to BSA standards. Color development occurred within 5 min. All samples (0.2 mL) were read at 595 nm with a model EL 310 microtiter reader (Bio-Tek Instruments, Inc., Burlington, VT). Crude and purified mitochondria contained a protein concentration of approximately 0.3 to 0.4 mg/mL and 0.05 to 0.15 mg/mL, respectively. These values are comparable to those of Miller (20).

Analysis of Mitochondrial Proteins by SDS-PAGE, Fluorography, and Scanning Laser Densitometry

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system of Laemmli (16). All reagents were of electrophoresis grade and purchased from Bio-Rad Laboratories (Richmond, CA). Molecular weight markers from Bio-Rad included the following proteins: phosphorylase B (97,400 kDa), bovine serum albumin (66,200 kDa), ovalbumin (42,699 kDa), carbonic anhydrase (31,000 kDa), soybean trypsin inhibitor (21,500 kDa), and lysozyme (14,400 kDa). The labeled mitochondrial samples in medium were dissolved in an equal volume of double-strength SDS sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.125 M Tris-Cl, pH 6.8). The pelleted labeled mitochondrial samples were dissolved in single-strength SDS sample buffer. All samples were heated for 3 min at 95°C prior to loading on the gel. To each lane were added 10 to 20 µg of the sample proteins, or 2 µL of the marker proteins (diluted ten-fold with single-strength SDS sample buffer).

The stacking (4%) and separating (7.5 to 15% gradient) slab gels were prepared with a 36.5 : 1 weight ratio of acrylamide to bisacrylamide. Electrophoresis was for 2 to 3 h at 20 mA constant current per slab (16 x 18 x 0.075 cm) on an HSI model SE600 dual slab cell (Hoefer Scientific Instruments, San Francisco, CA). Gels were fixed and stained with 0.125% Coomassie

Brilliant Blue R-250 in 50% methanol, 10% acetic acid and destained with 50% methanol, 10% acetic acid followed by 7% acetic acid, 5% methanol. Gels with [³H]AzBA-labeled proteins were enhanced for fluorography with 20% PPO (2, 5-diphenyloxazole) in acetic acid, as described in Skinner and Griswold (29), and dried at 65°C for 1 hr on an HSI model SE 1160 slab dryer. The dried gels were then exposed to Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY) at -80°C for 1 month. Fluorograms were developed according to the manufacturer's directions and quantified by means of an UltroScan XL scanning laser densitometer (LKB Instruments, Inc., Piscataway, NJ).

Analysis of Intact Mitochondria and Proteins by Scintillation Counting

The proteins in each mitochondrial sample were precipitated by the addition of an equal volume of cold 20% (w/v) trichloroacetic acid (TCA) for 15 min at 4°C. The proteins were pelleted at 10,000xg and washed once with 100 µL cold 20% (w/v) TCA. The proteins were again pelleted, dissolved in 100 µL 1% SDS, and transferred to a scintillation vial along with 10 mL of Ready Safe™ scintillation cocktail (Beckman Instruments, Inc., Fullerton, CA). For each sample, the microcentrifuge tube originally containing the proteins was rinsed with scintillation cocktail from the vial and then the proteins were returned back to the vial. For intact mitochondria, the samples were each transferred directly to scintillation vials with 10 mL of Ready Safe scintillation cocktail.

The vials for all samples were stored at 4°C for at least 24 h to avoid chemiluminescence and quenching. Counts were determined by means of a model 1900CA Tri-Carb liquid scintillation analyzer (Packard Instrument Co., Meriden, CT).

Fractionation of Labeled Mitochondria

The purified mitochondrial suspension was treated with 50 μM [^3H]AzBA and 3 min UV light as previously described, except that the volume used for each aliquot was increased to 2 mL. After completion of the labeling and washing steps, the sample to be fractionated was resuspended in 0.4 mL dH_2O , whereas the control sample was resuspended in 0.2 mL dH_2O . Both samples were incubated on ice for 10 min with occasional vortexing. The control sample was temporarily stored at 4°C.

The mitochondrial sample to be fractionated was transferred to two L5-65 ultracentrifuge tubes, each containing 0.2 mL. The sample was ultracentrifuged at 100,000 $\times g$ for 90 min at 4°C in a Beckman type 65 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant from both tubes were pooled and dried with a model 10-145 MR-BA lyophilizer (Virtis Co., Inc., Gardiner, NY). After lyophilization, the dried supernatant (soluble fraction) was dissolved in 0.2 mL dH_2O . The pellet (total membrane fraction) from one of the two tubes was resuspended in 0.2 mL dH_2O . Both the soluble

and total membrane fractions were temporarily stored at 4°C with the control sample.

The pellet from the remaining tube was resuspended in 0.2 mL 0.1 M Na_2CO_3 for 30 min at 4°C with occasional vortexing. The resuspended pellet was then ultracentrifuged at 80,000xg for 60 min at 4°C. The supernatant (peripheral membrane protein fraction) was removed and temporarily stored at 4°C with the other fractions. The pellet (integral membrane protein fraction) was resuspended in 0.2 mL dH_2O and 0.2 mL cold 50% DMSO.

All samples were divided for protein quantitation, scintillation counting, and electrophoresis as previously described and stored at -20°C until needed.

RESULTS

Membrane Integrity of Purified Mitochondria

Purified mitochondria from mung bean hypocotyl tissue were examined for membrane integrity and for purity by transmission electron microscopy. Presented in Fig. 1 are intact mitochondria with an inner convoluted membrane enclosing the matrix and a smooth outer membrane. Although the majority of the mitochondria were intact, occasional damaged or envelope-free mitochondria were also observed. Cytochrome c oxidase assay indicated that the mitochondria were routinely 70 to 80 % intact. Oxygen uptake measurements gave ADP/O values of 1.8 to 2.5 when malate was used as the substrate. These values, which compared favorably with literature values (19), indicated that there was good coupling between electron transport and ATP synthesis. Good coupling is present only in intact mitochondrial membranes. In the presence of malate and ADP, alternative respiration was typically 15 to 20% of the total respiration as measured by SHAM inhibition of the cyanide-resistant pathway (Fig. 2). The effects of BA and AzBA on total respiration were compared by measuring the inhibition of state-3 respiration in the presence of malate and ADP (Fig. 3). The DMSO solvent had little or no influence as shown by the lack of change in rate when 40 μ l DMSO was added. BA immediately inhibited O_2 uptake by 89.5%,

followed by a lesser inhibition of 52.5%. AzBA caused a single inhibition of 46.9%. Although the kinetics of respiration were somewhat different, the final inhibition caused by BA and AzBA was approximately the same.

Effect of Irradiation Time on Labeling of Mitochondrial Proteins with [³H]AzBA

With increasing ultraviolet-irradiation time up to 2.5 min, there was greater labeling of mitochondria and their TCA-precipitable proteins with [³H]AzBA (Fig. 4). Labeling of mitochondrial proteins was negligible in the absence of ultraviolet (UV) light, whereas that of intact mitochondria was significant. At all times, the labeling of intact mitochondria with [³H]AzBA was greater than that of mitochondrial proteins. This disparity could have been due to reaction of the reagent with nonprotein molecules or to the sequestering of the compound within the mitochondria. The SDS-PAGE-derived fluorogram (Fig. 5) shows that covalent labeling of proteins occurred only in mitochondria that were irradiated, as mitochondria exposed to 0 min UV light produced no polypeptide labeling. SDS treatment before electrophoresis apparently stripped away any noncovalently bound [³H]AzBA from mitochondrial proteins. When mitochondria were exposed to 2.5 or 5 min UV light, similar polypeptide labeling patterns resulted. To optimize binding while limiting unnecessary mitochondrial damage, an irradiation time of 3 min was chosen for all subsequent experiments.

Figure 1. Transmission electron micrograph of Percoll-purified mung bean hypocotyl mitochondria. The mitochondria were fixed with glutaraldehyde and osmium tetroxide and stained with uranyl acetate and lead citrate, according to the method of Neuburger (25). Bar = 0.5 μ m. x57,000.

Figure 2. Influence of SHAM on state-3 respiration of mitochondria when added after ADP. The final concentration of SHAM was 1 mM.

Figure 3. Influence of DMSO, BA, and AzBA on state-3 respiration of mitochondria when added after ADP. The final concentration of BA or AzBA was 0.5 mM. The amount of DMSO added was 40 μ L.

Figure 4. Effect of UV-irradiation time on the labeling of intact mitochondria and their TCA-precipitable proteins with 50 μM $[^3\text{H}]\text{AzBA}$. The data points represent the average of two 25 μL replicates.

Figure 5. Effect of UV-irradiation time on the photoaffinity labeling of mitochondrial polypeptides. Lanes A, B, and C: Fluorogram of SDS-PAGE-separated polypeptides from mitochondria treated with 0, 2.5, and 5 min UV light, respectively, after incubation in 50 μ M [3 H]AzBA. Estimated molecular weights (in kilodaltons) of the most intensely labeled polypeptides are indicated to the right of lane C.

Labeling of Mitochondrial Proteins with [³H]AzBA

Mitochondrial uptake of the photoaffinity reagent increased rapidly with increasing [³H]AzBA concentration, up to approximately 150 μ M, above which incorporation of the radioactivity increased gradually (Fig. 6). The methanol in which the reagent was dissolved did not significantly affect the uptake of [³H]AzBA (data not shown). Differences in uptake of the reagent between intact mitochondria and their TCA-precipitable proteins could be due to the same reasons mentioned in the previous section. The Coomassie-stained gel and fluorogram in Fig. 7 show that at 50 μ M [³H]AzBA several polypeptides were prominently labeled. The most intensely labeled polypeptides were estimated to be 33, 34, 45, 57, and 70 kilodaltons (kDa). Although not apparent from Fig. 7, the 57 kDa band was a doublet of polypeptides (see Fig. 14). The 70 kDa band constituted a very small percentage of the total protein, as shown by the Coomassie-stained gel. However, as shown on the fluorogram, this small percentage of the total protein had a greater affinity for [³H]AzBA and shows a greater difference in relative labeling compared to the other polypeptides. Because levels of [³H]AzBA greater than 50 μ M gave polypeptide patterns similar to that above (data not shown), this concentration was used in all subsequent experiments. Resting mitochondria were used for all labeling experiments. In actively respiring mitochondria, it is possible that the labeling pattern would be different than the one presented in Fig. 7.

Figure 6. Incorporation of [³H]AzBA into intact mitochondria and its TCA-precipitable proteins. Irradiation time was 3 min. The data points represent the average of two 25 μL replicates.

Figure 7. SDS-PAGE and fluorography of photoaffinity labeled mitochondrial polypeptides. Lane A: molecular weight markers; lane B: Coomassie-stained mitochondrial polypeptides labeled with 50 μ M [3 H]AzBA and irradiated for 3 min; lane C: fluorogram of lane B. Molecular weight markers (in kDa) are indicated to the left of lane A. Estimated molecular weights of the most intensely labeled polypeptides are indicated between lanes B and C.

Effect of ADP on the Labeling of Mitochondrial Proteins with [³H]AzBA

The 57 kDa doublet and 34 kDa polypeptide shown on the fluorogram (Fig. 7, lane C) have molecular weights similar to the three largest subunits of ATP synthase (Fig. 8) (10). Because ATP synthase protudes from the inner membrane into the matrix, and is the most abundant mitochondrial protein, its easy accessibility makes it possible that it was labeled nonspecifically by [³H]AzBA. Alternatively, it is possible that the binding was specific since [³H]AzBA, like natural cytokinins, has an adenine ring that is structurally similar to ADP. If [³H]AzBA bonded to the ATP synthase nucleotide binding site, then the addition of ADP should prevent the 34 and 57 kDa polypeptides from being labeled. The objective was to allow labeling of all BA-binding polypeptides but not the ATP synthase polypeptides.

As the concentration of ADP was increased, the intensity of labeling of all the mitochondrial polypeptides decreased (Fig. 9). This result indicated that while ADP did displace [³H]AzBA, its blockage was not specific to the suspected ATP synthase polypeptides. A densitometer scan (Fig. 10) of the fluorogram also confirmed this nonspecificity in ADP's blocking ability. The measured radioactive counts show that the labeling of mitochondrial proteins by [³H]AzBA was greatly reduced with the addition of ADP (Fig. 11). Above 0.8 mM ADP, incorporation of [³H]AzBA leveled off to approximately 44% of the incorporation measured in the absence of ADP. Higher ADP

concentrations, even as high as 10 mM, did not completely inhibit labeling (Fig. 12).

Figure 8. Molecular weights and hypothesized arrangement of ATP synthase subunits from maize mitochondria, as reported in Douce (10).

Figure 9. Effect of ADP concentration on the photoaffinity labeling of mitochondrial polypeptides. Lanes A through E: fluorogram of polypeptides from mitochondria treated with 0.0, 0.09, 0.3, 0.6, and 0.9 mM ADP, respectively, prior to being labeled with 50 μ M [3 H]AzBA. Irradiation time was 3 min. Estimated molecular weights (in kDa) of the most intensely labeled polypeptides are indicated to the left of lane A.

Figure 10. Densitometer scan of the fluorogram from Fig. 9. Relative absorbance (in arbitrary units) of the 33, 57, and 70 kDa bands are plotted as a function ADP concentration. Relative absorbance less than 0.3 is background.

Figure 11. Effect of ADP (0 to 1 mM) on the labeling of TCA-precipitable mitochondrial proteins with 50 μM $[^3\text{H}]\text{AzBA}$. Irradiation time was 3 min. The data points represent the average of two 25 μL replicates.

Figure 12. Effect of ADP (0 to 10 mM) on the labeling of TCA-precipitable mitochondrial proteins with 50 μM $[^3\text{H}]\text{AzBA}$. Irradiation time was 3 min. The data points represent the average of two 25 μL replicates.

Effect of BA on the Labeling of Mitochondrial Proteins with [³H]AzBA

A competition binding experiment was conducted with [³H]AzBA and BA. The incorporated radioactivity (Fig. 13) indicates that the labeling of mitochondrial proteins with [³H]AzBA decreased significantly with the addition of BA up to approximately 0.8 mM. Thereafter, the incorporation of [³H]AzBA leveled off to approximately 34% of the incorporation measured in the absence of BA. The DMSO in which the BA was dissolved did not significantly affect the uptake of [³H]AzBA. However, the use of BA concentrations greater than 2 mM produced unusually high radioactive incorporation beyond that of the control (data not shown), perhaps due to the coprecipitation of the hydrophobic [³H]AzBA and BA in the protein precipitate after TCA treatment. The SDS-PAGE- derived fluorogram in Fig. 14 shows that the intensity of labeling of all mitochondrial polypeptides decreased as the concentration of BA was increased up to 0.9 mM. It should be noted that the 70 kDa polypeptide is not as highly labeled in this experiment, even in the control (no BA) sample.

Figure 13. Effect of BA on the labeling of TCA-precipitable mitochondrial proteins with 50 μM $[^3\text{H}]\text{AzBA}$. Irradiation time was 3 min. The data points represent the average of two 25 μL replicates.

Figure 14. Effect of BA concentration on the photoaffinity labeling of mitochondrial polypeptides. Lanes A through E: fluorogram of polypeptides from mitochondria treated with 0.0, 0.09, 0.3, 0.6, and 0.9 mM BA, respectively, prior to labeling with 50 μ M [3 H]AzBA. Irradiation time was 3 min. Estimated molecular weights (kDa) of the most intensely labeled polypeptides are indicated to the left of lane A.

Fractionation of Labeled Mitochondria

Measurements of radioactivity of fractionated mitochondria labeled with [^3H]AzBA (Table 1) show that membrane proteins account for approximately 89% of the total label associated with mitochondrial proteins, while the soluble proteins represent only approximately 8%. Of the membrane proteins, labeling of integral proteins was nearly twice that of peripheral proteins. To determine in which mitochondrial fraction the previously observed labeled polypeptides were present, the fractionated samples were electrophoresed. Although cross contamination occurred between fractions, there was evidence in the Coomassie-stained gel (not shown) that the 70 kDa polypeptide was from the soluble fraction, the 57 kDa polypeptide was from the peripheral fraction, and the 33 kDa polypeptide was from the integral fraction. It was unclear from the gel from which fraction the 45 and 34 kDa polypeptides originated.

Table 1. Labeling of whole and fractionated mitochondria with 50 μ M [3 H]AzBA.

	<u>Radioactivity (cpm)</u>	<u>% Total Protein</u>	<u>% Membrane Protein</u>
Total Mitochondrial Proteins	85517	100	----
1. Soluble Proteins	6617	8	----
2. Membrane Proteins	76083	89	100
a. Integral Proteins	48025	56	63
b. Peripheral Proteins	25525	30	34

Irradiation time was 3 min. Each value represents the average of two 25 μ L replicates normalized to the same protein concentration.

DISCUSSION

Several mitochondrial polypeptides were labeled with $[^3\text{H}]\text{AzBA}$ in this study. The most intensely labeled polypeptides were estimated to be 33, 34, 45, 57, and 70 kDa. Based on their molecular weights and abundance, the 34 and 57 kDa polypeptides are suspected of being ATP synthase subunits. The identity of the 33, 45, and 70 kDa polypeptides remains unclear. Because both ADP and BA can compete out $[^3\text{H}]\text{AzBA}$ and are structurally similar (adenine ring), specificity of labeling was brought into question.

Competition binding studies by Keim and Fox (1980) revealed that the affinities of AzBA ($K_d = 6.8 \times 10^{-7} \text{ M}$) and BA ($K_d = 6.7 \times 10^{-7} \text{ M}$) for the wheat embryo cytokinin-binding protein (CBF-1) were similar. Their observations served as the promise for the present study -- that the affinities of $[^3\text{H}]\text{AzBA}$ and BA for mitochondrial cytokinin-binding proteins are similar. Oxygen consumption measurements indicated that while the kinetics of respiration inhibition were somewhat different in response to BA and AzBA, the final inhibition caused by the two reagents was nearly the same. The difference in kinetics might have been due to a membrane effect, as BA is slightly more hydrophobic than AzBA. The result might be a faster passage of BA into or through mitochondrial membranes and, therefore, a quicker and more pronounced initial inhibition of respiration.

Nevertheless, which if any mitochondrial proteins were being labeled by [³H]AzBA as a result of its similarity to BA could not be determined with assurance in this study.

When using photoaffinity reagents to identify putative receptor proteins, it is important to differentiate between specific and nonspecific labeling and to optimize binding conditions. The photoaffinity labeling method depends upon the generation of a highly reactive species by irradiation of a chemical precursor. Ideally, a very reactive reagent is generated only at the receptor site of interest, and it reacts indiscriminately with any chemical group within that site that is in close proximity. In the case of [³H]AzBA, any labeling that results from an interaction between the receptor site and either the whole benzyladenine molecule or the adenine ring would be considered specific. Specific labeling is dependent on the unique chemical characteristics of both the receptor site and [³H]AzBA. Nonspecific labeling is any random binding that occurs outside the receptor site. This could be with the same macromolecule or with different "irrelevant" macromolecules (proteins or nonproteins) in more complex systems, such as mitochondria (1).

Various factors contribute to specific and nonspecific labeling. While prolonged irradiation time increases labeling, it can also result in undesirable effects such as slow nonspecific labeling by irradiation products and by destruction or alteration of the receptor site (1). A minimal irradiation time

of 3 min was chosen, because it gave easily detectable labeling while limiting mitochondrial damage. Following irradiation, the amount of noncovalently bonded label remaining in intact mitochondria was dependent on the efficiency and number of wash steps. Covalently bonded label was measured by precipitation of the mitochondrial proteins in trichloroacetic acid followed by washing of the precipitate. However, because the noncovalently bound photoaffinity reagent could have still been physically entrapped by the protein precipitate by coprecipitation, care was taken in interpreting radioactive counts.

The temperature of the incubation medium strongly influences binding kinetics. The temperature at which irradiation of azides, including [^3H]AzBA, is carried out can dramatically affect the reactivity of the nitrene that is formed (1). Since K_d decreases, and thus affinity increases, at low temperature, both the degree and specificity of labeling was improved by cooling the mitochondrial sample. In the present study, all labeling was done on ice.

The concentration of [^3H]AzBA used during photoaffinity labeling is also important. Because the reagent must be added as a methanol solution, an excessive amount of the reagent was avoided to reduce nonspecific labeling from denaturation of the mitochondria. Even at low organic solvent concentrations where denaturation is undetectable, subtle changes can be induced in biological samples (1). An [^3H]AzBA concentration of 50 μM was

used, because it labeled mitochondrial proteins sufficiently for gel electrophoresis and scintillation counting without causing mitochondrial damage.

The use of a lipophilic photoaffinity reagent, such as [^3H]AzBA, might lead to higher nonspecific labeling in mitochondria and other complex membrane systems than in water-soluble systems due to concentrating effects within the protein-rich inner membrane. Since ATP synthase protrudes from the inner membrane into the matrix, and is the most abundant mitochondrial protein, it could be an inviting target for nonspecific interactions. It is also possible that binding to the ATP synthase site is specific to adenine-type ligands, such as [^3H]AzBA and natural cytokinins. The displacement of most of the labeling of the putative receptor proteins argues in favor of significant specific labeling although not necessarily "cytokinin-specific" labeling.

In addition to the ATP synthase subunits, other subunits of components of the electron transport chain are known with molecular weights similar to the 45 and 70 kDa polypeptides labeled by [^3H]AzBA. Succinate: coenzyme Q reductase (complex II) has a subunit at 70kDa, and both coenzyme QH₂: cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) have subunits at approximately 45 kDa (27). In maize mitochondria (*c.f.*, 10), an abundant 30 kDa polypeptide appears in the total mitochondrial protein fraction, but not in the purified ATP synthase F₁

component (10). On SDS-PAGE gels, this polypeptide band is similar in intensity to the 33 kDa mung bean polypeptide on the Coomassie-stained gel in this investigation. Identification of proteins based on molecular weight is tenuous but can be helpful in guiding future experiments to determine the identity of the [^3H]AzBA-labeled polypeptides.

Fractionation of mitochondria into simpler systems (*e.g.*, inner membranes, electron transport complexes, and ATP synthase) would help to better localize and identify BA binding proteins and also reduce nonspecific labeling. By comparing the polypeptide banding patterns of electrophoretic gels from these simpler systems with the bands described here, the fraction containing the labeled proteins could be determined. Labeling purified ATP synthase with [^3H]AzBA would help test whether the 34 and 57 kDa polypeptides are ATP synthase subunits as hypothesized. To ascertain whether the 34 or 57 kDa polypeptides were labeled specifically or nonspecifically, the affinities between [^3H]AzBA and these polypeptides and other proteins of interest could be compared. It should be noted that even low affinity labeling of abundant ATP synthase subunits could result in intense bands on the fluorogram. Conversely, a small amount of a specifically labeled, high affinity BA-binding protein would yield a less intense band. Labeling purified inner membranes and electron transport complexes would also be useful in determining whether the 33 kDa polypeptide is an integral membrane protein and whether the 70 kDa

polypeptide has a higher affinity for [^3H]AzBA than the other polypeptides. Labeling simpler systems may also give more information as to what fraction the 45 kDa polypeptide belongs.

The presence of the detergent, Triton X-100 (0.1%), in the mitochondrial incubation medium during irradiation has been shown to increase the intensity of polypeptide labeling by a photoaffinity auxin derivative (11). Triton X-100 works by either stripping away peripheral proteins or slightly solubilizing membranes, thereby making target proteins more accessible to the reagent. Triton X-100 could be tried with the present system or simpler systems (*e.g.*, inner mitochondrial membranes), as my data indicated significant labeling of integral proteins. Cooling samples below freezing with liquid nitrogen during irradiation might reduce nonspecific labeling (11).

The data presented in this study have not conclusively identified a BA-binding protein (or proteins) specifically involved in regulating the alternative pathway, but some conclusions can be drawn nonetheless. If BA, like [^3H]AzBA, interacts with several mitochondrial proteins, then O_2 consumption and electron transport inhibition data should not be explained by a single site of action for BA. If BA interacts with ATP synthase, as is suspected of [^3H]AzBA, then competition between ADP and BA for the catalytic site could create what might be an "uncoupling" effect due to ATP synthase inhibition. O_2 consumption would in turn be reduced and would

reduce O₂ consumption measurements when using BA as an inhibitor of the alternative pathway. It has been speculated by others that BA indirectly inhibits electron transport through an uncoupling effect (26). is an idea that has been proposed before (26). Although these mechanisms may be involved, Siedow and Musgrave (*c.f.* 24) insist that for a cytokinin (BA) response to occur, the alternative pathway must function. They found that a loss of responsiveness to exogenous cytokinin was due to the genetic loss of the alternative pathway. This brings us back to an earlier question posed by Musgrave, et al. : "Do some plant responses to cytokinins involve the cyanide-resistant respiratory pathway?" (24). As nature would have it, instead of answering this question, more questions have arisen.

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Appendix A. The pathways for utilization of reducing power and electrons in plant mitochondria. NADH-DH, NADH dehydrogenase; SDH (FAD), flavoprotein-linked succinate dehydrogenase; sites I - III, sites of proton extrusion where ATP (circled) is produced; cyt, cytochrome. (Source: Bewley JD, Black M (1985) *In SEEDS - Physiology of Development and Germination*. Plenum Press, NY)

Appendix B. [³H] -2-Azido-N⁶-benzyladenine