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CHARACTERIZATION BY PHOTOAFFINITY LABELING OF THE ADENINE NUCLEOTIDE CARRIER IN PLANT MITOCHONDRIA

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Summary

1. The covalent photolabeling of the adenine nucleotide carrier in potato mitochondria and bean mitochondria by photoactivable derivatives of atractyloside and ADP is described.

2. In the absence of photoirradiation, *N*-4-azido-2-nitrophenylaminobutyryl atractyloside (arylazido atractyloside) was found to compete with atractyloside and ADP for binding to potato and bean mitochondria. Bound arylazido atractyloside was displaced by atractyloside and ADP.

3. Upon incubation with potato or bean mitochondria and photoirradiation, [^{3}H]arylazido atractyloside was found to bind covalently to a mitochondrial polypeptide of apparent molecular weight 35 000. Photolabeling by [^{3}H]-arylazido atractyloside was prevented by preincubation of mitochondria with carboxyatractyloside or ADP.

4. Photoirradiation with ^{3}H-labeled *N*-4-azido-2-nitrophenylaminobutyryl ADP ([^{3}H]arylazide ADP) also resulted in the covalent photolabeling of the 35 000 dalton polypeptide. Photolabeling by [^{3}H]arylazido ADP was prevented by preincubation with carboxyatractyloside.

5. The 35 000 dalton polypeptide covalently labeled by [^{3}H]arylazido-atractyloside was isolated by hydroxyapatite gel chromatography and its homogeneity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Abbreviations: Arylazido atractyloside, *N*-4-azido-2-nitrophenylaminobutyryl atractyloside; Arylazido ADP: *N*-4-azido-2-nitrophenylaminobutyryl ADP.

6. The molecular weight of the adenine nucleotide carrier protein of potato and bean mitochondria (35 000) is close to that of *Saccharomyces cerevisiae* mitochondria (molecular weight 37 000) and significantly higher than that of mammalian mitochondria (molecular weight 30 000).

7. The photolabeling data presented in this paper indicate that in spite of the fact that potato mitochondria bind atractyloside rather loosely, they possess a specific recognition site like bean mitochondria which bind atractyloside much more firmly. The same polypeptide, namely the adenine nucleotide carrier protein, is able to bind both ADP and atractyloside.

Introduction

The adenine nucleotide carrier in plant mitochondria differs from that in mammalian mitochondria by its low sensitivity to atractyloside, a well-known competitive inhibitor of ADP/ATP transport in heart or liver mitochondria [1]. For example, it has been reported that oxidative phosphorylation of external ADP in Jerusalem artichoke mitochondria is insensitive to atractyloside [2,3] and that in cauliflower [4,5], corn [4,6], and potato [7] mitochondria, it is sensitive to only high concentrations of atractyloside. We showed previously that the binding affinity of potato mitochondria for atractyloside is ten times lower than that of liver or heart mitochondria [7]. The bound atractyloside was displaced by ADP and by bongkrekic acid, another specific inhibitor of the adenine nucleotide carrier. On this basis, it was tentatively concluded that potato mitochondria possess specific binding sites for atractyloside. In the case of bean mitochondria however, the inhibition by atractyloside is comparable to that found in mammalian mitochondria [8], and the affinity for atractyloside is similar to that of mammalian mitochondria.

Photoactivable arylazido derivatives of atractyloside and ADP have been previously used to label covalently the adenine nucleotide carrier in heart, liver and yeast mitochondria [9,10]. These photoactivable derivatives yield upon photoirradiation highly reactive nitrenes [11]. On the other hand, they are able to recognize specifically the adenine nucleotide carrier protein in the mitochondrial membrane by their specific moiety, namely atractyloside and ADP. There are therefore appropriate tools to solve the problem of whether plant mitochondria possess an adenine nucleotide carrier with a specific atractyloside binding site. The present paper describes the characterization of the adenine nucleotide carrier in potato mitochondria by photoaffinity labeling, using photoactivable derivatives of atractyloside and ADP. Because of the unambiguous high affinity of bean mitochondria for atractyloside, those mitochondria were taken as reference in photolabeling assays. A single peptide of 35—36 000 daltons was photolabeled both in potato and bean mitochondria.

Materials and Methods

Mitochondria from potato tubers were isolated as described by Douce et al. [12], except that the last two washing steps were made in the absence of serum albumin. Serido beans (*Vigna sinensis* (L) Savi cv Serido) seeds were surface-

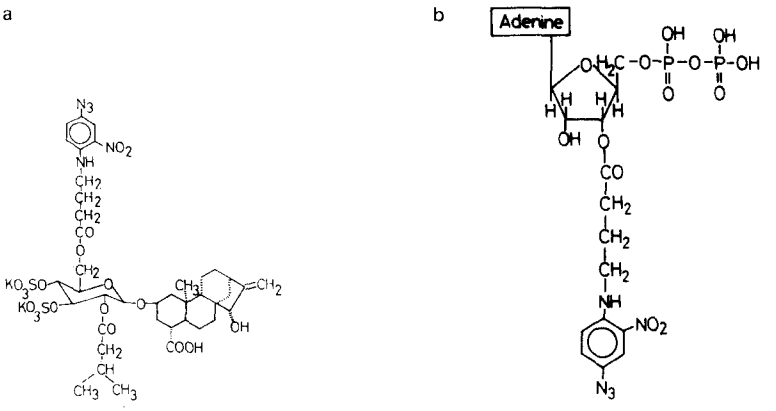

Fig. 1. (a) Structure of *N*-4-azido-2-nitrophenylaminobutyryl atractyloside (arylazido atractyloside). (b) Structure of *N*-4-azido-2-nitrophenylaminobutyryl ADP (arylazido ADP).

sterilized with 0.5% NaOCl and germinated at 28°C, in vermiculite moistened with distilled water. Mitochondria were isolated from 6-day-old, dark-grown bean hypocotyls by the method of Ikuma [13] as modified by Silva Lima and Pinheiro [14]; here again, serum albumin was omitted in the last two washing steps. Before use, the respiration of mitochondria were assayed for respiratory control and P/O ratios. Respiratory control ratios were between 3 and 6, using succinate as substrate (see previous data in Refs. 7 and 8).

Tritiated *N*-4-azido-2-nitrophenylaminobutyryl atractyloside ([^{3}H]arylazido atractyloside) and tritiated *N*-4-azido-2-nitrophenylaminobutyryl ADP ([^{3}H]-arylazido ADP) (Fig. 1) were synthesized as described in [9] and [10], respectively. Their specific radioactivity was approximately $8 \cdot 10^5$ dpm/nmol.

Binding assays 'in the dark' were carried out in series of tubes containing 5 ml of an incubation medium consisting of 0.12 M KCl, 10 mM morpholinopropane sulfonic buffer, pH 7.2, 0.1 M EDTA and increasing concentrations of [^{3}H]arylazido atractyloside. The incubation was started by addition of mitochondria (about 1 mg). After an incubation of 30 min at 0°C, the mitochondria were collected by centrifugation, and their radioactivity estimated by liquid scintillation following digestion of the pellet by 1 ml formamide at 180°C. The incubation time was sufficient to allow equilibrium between bound and free ligand. Parallel binding assays were conducted under similar conditions with [^{3}H]atractyloside.

For photolabeling assays, mitochondria were suspended in the same medium as that used for binding assays with [^{3}H]arylazido atractyloside and [^{3}H]arylazido ADP (see legends of Figs. 2 and 3) and let to stand for 30 min at 0°C. Then irradiation was carried out with an Osram lamp 250 W (halogen reflector lamp) equipped with a filter to eliminate radiations below 300 nm. (ultraviolet light decreases atractyloside binding [15]). 5 ml of the mitochondrial suspension (3 mg/ml) with the photoactivable reagent were introduced in a 50 ml round flask which was rotated horizontally at 200 rev./min in an ice bath, at a distance of 10 cm from the lamp. Photoirradiation was carried out for a 30 min period. After photolabeling, mitochondria were sedimented by centrifugation

and resuspended in the same medium (final concentration 10 mg protein/ml) without the photoactivable reagent. They were then lysed using sodium dodecyl sulfate and mercaptoethanol, each at a final concentration of 2%. After heating at 100°C for 2 min, glycerol was added at a final concentration of 20%, together with traces of bromophenol blue as a tracking dye. Electrophoresis in 10% (w/v) polyacrylamide gels was conducted as described by Weber and Osborn [16], using as buffer 100 mM sodium phosphate (pH 7.4) containing 0.1% sodium dodecyl sulfate. After staining and destaining the gels were sliced. Each slice (1 mm) was digested overnight with 1 ml of 10% H_2O_2 at 65°C and the radioactivity of each digest measured by liquid scintillation. The following non-radioactive proteins were used as molecular weight standards: bovine serum albumin (68 000), ovalbumin (42 000), triosephosphate isomerase (27 500) and cytochrome *c* (12 300).

Results

Reversible binding of [^{3}H]arylazido atractyloside in the dark

Binding assays employing [^{3}H]arylazido atractyloside were carried out in the dark to avoid photoactivation. The binding curves were analyzed by a graphical treatment according to Rosenthal [17]. The number of high affinity sites amounted to about 0.45 nmol/mg protein for potato mitochondria and 0.16 nmol/mg protein for bean mitochondria (Table I). The K_d values were 0.20 μM and 0.06 μM, respectively. These data are close to those obtained with [^{3}H]atractyloside (see Table I) although the binding affinity was higher with the photoactivable derivatives, possibly due to a more appropriate positioning of the ligand. Addition of 50 μM unlabeled atractyloside together with [^{3}H]-arylazido atractyloside resulted in a marked decrease in bound radioactivity, indicating the same binding site for atractyloside and arylazido atractyloside (Table II). Addition of 200 μM ADP also decreased the amount of bound [^{3}H]-arylazido atractyloside, which is consistent with a competition between arylazido atractyloside and ADP for the adenine nucleotide carrier (Table II).

A complementary experiment was performed to verify whether atractyloside or ADP are able to displace [^{3}H]arylazido atractyloside previously bound to the mitochondria. The mitochondria were preincubated in the dark with [^{3}H]-arylazido atractyloside, sedimented by centrifugation and resuspended. The

TABLE I

BINDING PARAMETERS FOR THE ATRACTYLOSIDE HIGH AFFINITY SITES OF POTATO MITOCHONDRIA AND BEAN MITOCHONDRIA

Conditions are described in Materials and Methods. Results are the mean average of at least two assays. *n*, the amount of bound [^{3}H]arylazido atractyloside and [^{3}H]atractyloside.

Mitochondrial species	[^{3}H]Arylazido atractyloside		[^{3}H]Atractyloside	
	K_d (μM)	*n* (nmol/mg protein)	K_d (μM)	*n* (nmol/mg protein)
Potato	0.20	0.45	0.40	0.50
Bean	0.06	0.16	0.08	0.18

TABLE II

COMPETITION FOR BINDING BETWEEN [^{3}H]ARYLAZIDO ATRACTYLOSIDE AND ATRACTYLOSIDE OR ADP

Conditions are described in Materials and Methods. Incubation was carried out in the presence of 0.3 μM [^{3}H]arylazido atractyloside and, either 50 μM atractyloside or 200 μM ADP.

Addition	Bound [^{3}H]arylazido atractyloside	
	Potato mitochondria (nmol/mg protein)	Bean mitochondria (nmol/mg protein)
None	0.40	0.15
Atractyloside	0.08	0.02
ADP	0.20	0.04

labeled mitochondria were then incubated with atractyloside or ADP. In both cases, as expected, the bound [^{3}H]arylazido actractyloside was released to the medium (data not shown).

Photolabeling by [^{3}H]arylazido atractyloside and [^{3}H]arylazido ADP, and characterization of the labeled protein in mitochondrial extract by SDS-polyacrylamide gel electrophoresis

Photoirradiation of potato mitochondria and bean mitochondria in the presence of [^{3}H]arylazido atractyloside was carried out in order to study the component which binds the ligand. The profile of radioactivity after gel electrophoresis of the lysate showed a single peak corresponding to a molecular weight of 35—36 000. Addition of ADP prior to that of [^{3}H]arylazido atractyloside prevented the binding of [^{3}H]arylazido atractyloside (Fig. 2). A similar

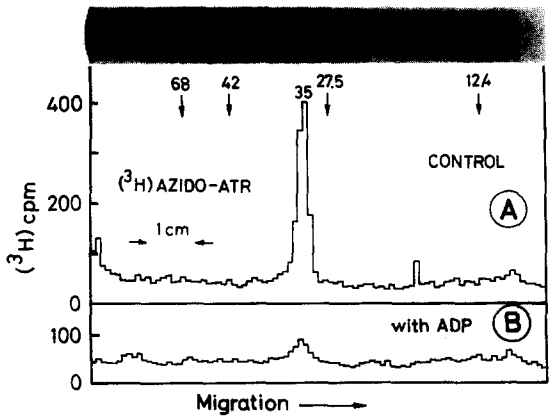

Fig. 2. Sodium dodecyl sulfate gel electrophoresis of potato mitochondria covalently labeled by [^{3}H]-arylazido atractyloside. Conditions are described in Materials and Methods. The concentration of [^{3}H]-arylazido atractyloside was 5 μM. The control photolabeling assay is given in (A). The assay including preincubation with ADP is given in (B). ADP was added at a final concentration of 200 μM, and preincubated with the mitochondria for 5 min at 0°C prior to the addition of [^{3}H]arylazido atractyloside. The indicated molecular weights correspond to standard reference proteins given in Materials and Methods.

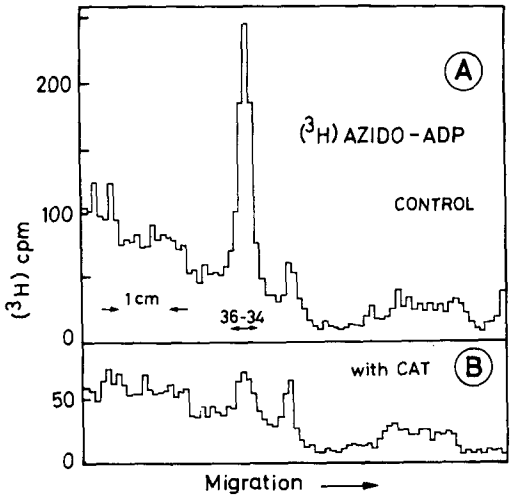

Fig. 3. Sodium dodecyl sulfate gel electrophoresis of potato mitochondria covalently labeled by [^{3}H]-arylazido ADP. Conditions are described in Materials and Methods. (A) Control assay. (B) Assay including preincubation with carboxyatractyloside (CAT). The concentration of [^{3}H]arylazido ADP was 3 μM. Carboxyatractyloside was added at a final concentration of 50 μM where indicated, and preincubated with the mitochondria for 5 min at 0°C prior to the addition of [^{3}H]arylazido ADP.

profile of radioactivity with a peak at 35–36 000 was obtained with a lysate of bean mitochondria incubated with [^{3}H]arylazido atractyloside.

The same procedure as that used for [^{3}H]arylazido atractyloside was employed to label the adenine nucleotide carrier by [^{3}H]arylazido ADP covalently. The radioactivity profile after gel electrophoresis showed one major peak with the same molecular weight as the [^{3}H]arylazido atractyloside binding proteins (Fig. 3). This peak disappeared when the mitochondria were treated by carboxyatractyloside prior to addition of [^{3}H]arylazido ADP, which further indicates that the same protein, namely the adenine nucleotide carrier protein, is able to bind ADP, atractyloside and carboxyatractyloside.

Photoactivation experiments, with [^{3}H]arylazido ADP, similar to those described above were performed with submitochondrial particles obtained by sonication of potato mitochondria. In that case, photoirradiation resulted in the photolabeling of not only the adenine nucleotide carrier protein but also of other protein(s) whose migration in sodium dodecyl sulfate gel electrophoresis corresponds to a molecular weight of 50–55 000. By analogy to a similar situation in beef heart submitochondrial particles [10] the 50–55 000 dalton peptides can be assigned to the two largest subunits of the mitochondrial F_1-ATPase. The major peak of radioactivity at 35–36 000 found with intact mitochondria photolabeled with [^{3}H]arylazido ADP indicates that this reagent is non-penetrant. Thus, in intact mitochondria it has access to the adenine nucleotide carrier, but not to the F_1-ATPase which is located in the inner face of the inner mitochondrial membrane.

Purification of the carrier protein photolabeled with [^{3}H]arylazido atractyloside or [^{3}H]arylazido ADP by hydroxyapatite chromatography

The photolabeled adenine nucleotide carrier protein was purified by

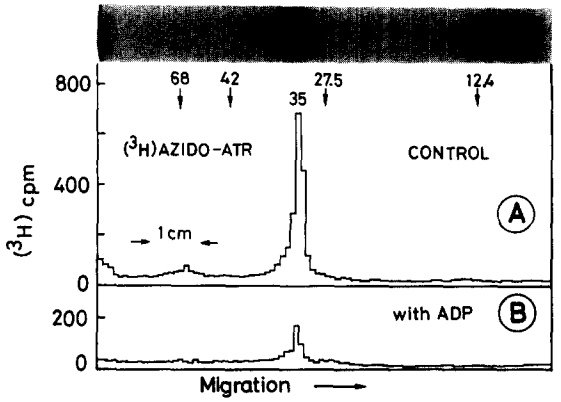

Fig. 4. Sodium dodecyl sulfate gel electrophoresis of the purified ADP-ATP carrier protein from potato mitochondria. (A) The purified carrier protein was obtained by hydroxyapatite chromatography of a Triton extract of potato mitochondria pretreated with [^{3}H]arylazido atractyloside as follows. Potato mitochondria (35 mg in 5 ml) were photolabeled with 5 μM [^{3}H]arylazido atractyloside. The pellet collected by centrifugation was resuspended in 5 ml of 0.5 M NaCl, 10 mM morpholinopropane sulfonic acid buffer, pH 7.2, 1 mM EDTA and 5% Triton X-100. After centrifugation to eliminate the remaining fragments of mitochondria, 1.5 ml of the supernatant were charged to the column of hydroxyapatite (40 mm × 15 mm). The pass-through fraction was used for SDS-polyacrylamide gel electrophoresis. (B) A parallel purification was run on a Triton extract of mitochondria, for which photolabeling by [^{3}H]-arylazido atractyloside had been preceeded by a 5 min incubation with 200 μM ADP.

hydroxyapatite chromatography as follows. After photoirradiation with [^{3}H]-arylazido atractyloside, the mitochondria were lyzed with Triton X-100 and the lysate submitted to a chromatography on hydroxyapatite [18]. The fraction not bound by the column was analyzed by SDS-polyacrylamide gel electrophoresis. It showed essentially one peak of radioactivity with a molecular weight of 35—36 000 coincident with the major protein band in the gel (Fig. 4). The same procedure applied for the purification of the carrier protein photolabeled with [^{3}H]arylazido ADP also yielded a 35—36 000 dalton peptide covalently labeled with [^{3}H]arylazido ADP.

Discussion

Photoactive arylazido derivatives of atractyloside have been introduced to label covalently and characterize the adenine nucleotide carrier in mammalian and yeast mitochondria [9,10]. These derivatives are orange colored and can be photoactivated under visible light to generate a reactive nitrene. They were preferred to other azido reagents which require ultraviolet light for photoactivation because the atractyloside site in mammalian mitochondria is very sensitive to ultraviolet light [15]. The present work extends previous studies on mammalian and yeast mitochondria to plant mitochondria. It was actually initiated to answer the question whether some plant mitochondria which have a low affinity for atractyloside possess nevertheless a specific recognition site for atractyloside. Data presented in this paper show unambiguously that potato mitochondria, in spite of low affinity for atractyloside, are able to recognize specifically and to bind covalently upon photoirradiation the corresponding

photoactivable derivative.By comparison, bean mitochondria which bind atractyloside with high affinity, also bind covalently arylazido atractyloside upon photoirradiation. In both cases the carrier protein was identified among mitochondrial proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a single peptide with a molecular weight of 35—36 000. A protein with the same molecular weight was found to be able to bind covalently arylazido ADP. In both cases, preincubation with a large excess of carboxyatractyloside or ADP before the photoirradiation prevented the photolabeling. These results fully corroborate other photolabeling data obtained with mammalian and yeast mitochondria, showing that the atractyloside and ADP sites belong to a same protein.

Another comment concerns the molecular weight of the carrier protein in potato and bean mitochondria (35—36 000); it is close to that found for the ADP-ATP carrier protein from *Saccharomyces cerevisiae* mitochondria, 37 000 [10,19], but significantly higher than that of the carrier protein from heart or liver mitochondria, 30 000 [9,18,20].

Acknowledgements

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References

1 Vignais, P.V. (1976) Biochim. Biophys. Acta 456, 1—38
2 Passam, H.C., Souverijn, J.H.M. and Kemp, A. (1973) Biochim. Biophys. Acta 305, 88—94
3 Passam, H.C. and Coleman, J.O.D. (1975) J. Exp. Bot. 26, 536—543
4 Jung, D.W. and Hanson, J.B. (1973) Biochim. Biophys. Acta 325, 189—192
5 Janovitz, A., Chavez, E. and Klapp, M. (1976) Arch. Biochem. Biophys. 173, 264—268
6 Earnshaw, M.J. and Hughes, E.A. (1976) Plant Sci. Lett. 6, 343—348
7 Vignais, P.V., Douce, R., Lauquin, G.J.M. and Vignais, P.M. (1976) Biochim. Biophys. Acta 440, 688—696
8 Silva Lima, M., Denslow, N.D. and Fernandes de Melo, D. (1977) Physiol. Plant 41, 193—196
9 Lauquin, G.J.M., Brandolin, G. and Vignais, P.V. (1976) FEBS Lett. 67, 306—311
10 Lauquin, G.J.M., Brandolin, G., Lunardi, J. and Vignais, P.V. (1978) Biochim. Biophys. Acta 501, 10—19
11 Fleet, G.W.J., Knowles, J.R. and Porter, R.R. (1969) Nature 224, 511—512
12 Douce, R., Christensen, E.L. and Bonner, W.D., Jr. (1972) Biochim. Biophys. Acta 275, 148—160
13 Ikuma, H. (1970) Plant Physiol. 45, 773—778
14 Silva Lima, M.G. and Pinheiro, P.A. (1975) Biochimie (Paris) 57, 1401—1403
15 Lauquin, G.J.M., Villiers, C., Michejda, J., Brandolin, G., Boulay, F., Césarini, R. and Vignais, P.V. (1978) in Proton and Calcium Pumps (G.F. Azzone et al., eds.), pp. 251—269, Elsevier/North-Holland Biomedical Press
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Rosenthal, H.E. (1967) Anal. Biochem. 20, 525—532
18 Riccio, P., Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 133—138
19 Boulay, F., Brandolin, G., Lauquin, G.J.M., Jollès, J., Jollès, P. and Vignais, P.V. (1979) FEBS Lett. 98, 161—164
20 Bojanovski, D., Schlimme, E., Wang, C.S. and Alaupovic, P. (1976) Eur. J. Biochem. 71, 539—548