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PHOTOAFFINITY LABELING OF THE ADENINE NUCLEOTIDE CARRIER IN HEART AND YEAST MITOCHONDRIA BY AN ARYLAZIDO ADP ANALOG

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Summary

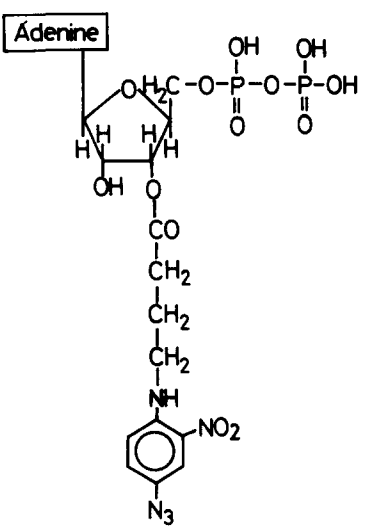

Fig. 1. Structure of *N*-4-azido-2-nitrophenylaminobutyryl-ADP.

1. Arylazido analogs of ADP and ATP (*N*-4-azido-2-nitrophenyl-aminobutyryl-ADP and *N*-4-azido-2-nitrophenylaminobutyryl-ATP) have been prepared in radioactive form and used in photolabeling experiments to identify the adenine nucleotide carrier in mitochondria and sonic submitochondrial particles.

2. When added in the dark to beef heart mitochondria, azidonitrophenyl-aminobutyryl-ADP binds to the adenine-nucleotide carrier. It is not transported across the membrane to the matrix space, but it inhibits ADP transport in mitochondria. The inhibition is of a mixed type with a K_i value of about 10 μM.

3. The nitrene derivative formed upon photoirradiation of tritiated azidonitrophenylaminobutyryl-ADP or -ATP binds to a polypeptide of apparent molecular weight 30 000 in beef heart mitochondria and 37 000 in *Saccharomyces cerevisiae* mitochondria. The photolabeling is prevented by preincubation of the mitochondria with atractyloside or carboxyatractyloside.

4. Photoirradiation of sonic submitochondrial particles from beef heart (inside-out particles) with tritiated azidonitrophenylaminobutyryl-ADP or -ATP results in the labeling of the 30 000-dalton polypeptide and also in the labeling of higher molecular weight peptides (50 000—55 000) probably belonging to F_1-ATPase. Addition of bongkrekic acid specifically decreases the photolabeling of the 30 000-dalton polypeptide.

5. An arylazido derivative of atractyloside (*N*-4-azido-2-nitrophenylaminobutyryl atractyloside) binds upon photoirradiation to the 30 000-dalton polypeptide in beef heart mitochondria and to the 37 000-dalton polypeptide in *S. cerevisiae* mitochondria.

6. Since the adenine nucleotide carrier is readily damaged by ultraviolet light, nitro-arylazido analogs of ADP and ATP or of atractyloside, which are photoactivated in visible light, were used in preference to other azido analogs, which require ultraviolet light for photoactivation.

7. Data presented in this paper support the view that the same mitochondrial protein belonging to the adenine nucleotide transport system is able to bind ADP (or ATP) and atractyloside.

Introduction

Photoaffinity labels are useful reagents to probe substrate or inhibitor sites in enzymes and membrane receptors [1]. Their molecule contains the ligand which interacts with the specific site in the enzyme or in the receptor and a photoactivable group which is able to bind covalently and unspecifically at or in the neighbourhood of the specific site upon photoirradiation. Azido derivatives are currently used as photoactivable groups. Upon photoirradiation, they yield a very reactive nitrene which is capable of insertion into carbon-hydrogen bonds in proteins. Because the adenine nucleotide carrier is very susceptible to photodecomposition by short wavelength irradiation (unpublished data), we have chosen to label the carrier protein by derivatives of ADP and of ATP, which are photoactivated at long wavelengths [2] rather than to use 8-azido-ADP and 8-azido-ATP which are photoactivated by ultraviolet irradiation [3—5].

In this paper, we show that *N*-4-azido-2-nitrophenylaminobutyryl-ADP (Fig. 1) and *N*-4-azido-2-nitrophenylaminobutyryl-ATP can bind covalently upon photoirradiation to the mitochondrial adenine nucleotide carrier. The binding specificity is assessed by the fact that atractyloside and bongkrekic acid, when preincubated with mitochondria, decrease the extent of the photolabeling. The protein which is photolabeled has a molecular weight of 30 000 in beef heart mitochondria and 37 000 in *Saccharomyces cerevisiae* mitochondria. Protein with molecular weights close to 30 000, characterized by high affinity for carboxyatractyloside or atractyloside, have been recently purified from

beef heart mitochondria [6] and from rat liver mitochondria [7—9]. An atractyloside binding protein with a molecular weight of 37 000 has also been purified from *S. cerevisiae* mitochondria (unpublished data). These proteins are most likely the same as those which bind the arylazido analogs of ADP and ATP.

Materials and Methods

1. Synthesis of tritiated N-4-azido-2-nitrophenylaminobutyryl-ADP and -ATP

Tritium-labeled 4-aminobutyric acid (25 Ci/mmol) was obtained from the Commissariat à l'Energie Atomique (Saclay, France) and diluted before use with unlabeled 4-aminobutyric acid to about $5 \cdot 10^{11}$ dpm/mmol. [^{3}H]azidonitrophenylaminobutyric acid was synthesized by the method of Fleet et al. [10]. Its purity was assessed by cellulose thin layer chromatography in butanol saturated with water. [^{3}H]Azidonitrophenylaminobutyryl-ADP was synthesized from [^{3}H]azidonitrophenylaminobutyric acid and ADP using the same method as that described by Jeng and Guillory [2,11] for the synthesis of unlabeled azidonitrophenylpropionyl-ATP. It was purified by cellulose thin layer chromatography in butanol/water/acetic acid (5 : 3 : 2 v/v) and identified with the orange band moving with an R_F of about 0.6. The material, after elution with water, was characterized by ultraviolet spectrum (peak at 260 nm) and visible spectrum (peak at 460 nm). The same procedure was used for the preparation of tritiated *N*-4-azido-2-nitrophenylaminobutyryl-ATP. Radiolabeling of the ADP and ATP analogs in the butyryl group was prefered to labeling of the adenine nucleotide moiety. This choice was dictated by the fact that after covalent binding of the photoaffinity reagent to mitochondria upon photoirradiation, the heat treatment which is used to digest the mitochondria by sodium dodecylsulfate prior to gel electrophoresis damages the nucleotide moiety, but not the azidonitrophenylaminobutyryl portion of the molecule.

Tritiated *N*-4-azido-2-nitrophenylaminobutyryl-atractyloside was prepared as described in [9].

2. Biological assays

Mitochondria from rat heart were prepared by the method of Tyler and Gonze [12], mitochondria from beef heart according to the method of Smith [13] and mitochondria from yeast according to the method of Balcavage and Mattoon [14]. The yeast strain used in this study was a diploid of *S. cerevisiae* JB 65 (P9/P9) obtained from Dr. J. Mattoon. Beef heart submitochondrial particles were prepared by sonication of beef heart mitochondria in the presence of ATP and $MgCl_2$ [15]. Adenine nucleotide transport was assayed as described in [16]. In photolabeling experiments, irradiation was carried out with an Osram lamp 250 W (Halogen Reflector lamp) equipped with a filter to cut off radiation below 300 nm. 5 ml of the mitochondrial suspension in sucrose/mannitol medium (0.5—1 mg protein/ml) was introduced together with the photoreagent in a 50 ml flask which was rotated horizontally at 200 rev./min in an ice bath. The mean distance between the lamp and the mitochondrial suspension was 10 cm.

3. Gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate was performed according to Weber and Osborn [17]. The cylindrical separating gels (length 12 cm, diameter 0.6 cm) contained 10.3% acrylamide. After photolabeling as described above, mitochondria were sedimented by centrifugation and resuspended in 0.225 M mannitol and 0.075 M sucrose to a final concentration of 10 mg/ml and then lysed by addition of sodium dodecyl sulfate and mercaptoethanol to a final concentration of 2% each. After heating at 100°C for 10 min, glycerol was added to a final concentration of 20% as well as traces of Bromophenol Blue as a tracking dye. An aliquot fraction of mitochondrial protein (100—150 μg) was loaded on each gel. Electrophoresis was carried out at 5 mA/gel for 16—18 h unless otherwise indicated. The gels were stained with 0.25% Coomassie Blue in acetic acid/methanol/water (1 : 4 : 5 v/v) and then destained in acetic acid/methanol/water (1 : 2 : 7 : v/v). After scanning with a Chromoscan gel scanner they were sliced with a Gilson slicer. Each slice (0.5 or 1 mm) was digested overnight by 1 ml of 10% H_2O_2 at 65°C. The radioactivity of each digest was measured by liquid scintillation. The following proteins were used as molecular weight standards : bovine serum albumin (68 000), ovalbumin (42 000), triose phosphate isomerase (27 500), trypsin inhibitor (20 000) and cytochrome *c* (12 400).

Results

Binding and transport of [^{3}H]azidonitrophenylaminobutyryl-ADP in rat heart mitochondria (dark reactions)

To measure the amount of [^{3}H]azidonitrophenylaminobutyryl-ADP bound to the adenine-nucleotide carrier after incubation with mitochondria in a dim light, we have used the same method [18,19] as that described for specific ADP or ATP binding, which was based on displacement of bound ADP or ATP by atractyloside, a specific and competitive inhibitor of adenine nucleotide transport. In the same experiment, we have measured the amount of arylazido-ADP analog transported into mitochondria via the adenine-nucleotide carrier. All incubations were performed at 2°C. The suspension of rat heart mitochondria was divided into three fractions. The first one was incubated with [^{3}H]azidonitrophenylaminobutyryl-ADP for 4 min. After centrifugation, the radioactivity of the pellet was measured by liquid scintillation (Pellet A). The second mitochondrial fraction was incubated as described for the first one with [^{3}H]-azidonitrophenylaminobutyryl-ADP, and at the end of the 4-min incubation period atractyloside was added at a sufficiently high concentration (40 μM), and left in contact for 2 min to remove the ADP analog bound to the adenine-nucleotide carrier. The mitochondria were then sedimented and the pellet was assayed for radioactivity (pellet B). The third mitochondrial fraction was preincubated with 40 μM atractyloside for 2 min prior to incubation with [^{3}H]-azidonitrophenylaminobutyryl-ADP, followed by centrifugation as described for the first fraction. The radioactivity found in the pellet (pellet C) was due to unspecific binding of the ADP analog.

The difference in radioactivity found in pellets A and B corresponded to [^{3}H]azidonitrophenylaminobutyryl-ADP specifically bound to the adenine

nucleotide carrier (bound radioactivity removable by atractyloside). The difference in radioactivity found in pellets B and C corresponded to [^{3}H]azidonitrophenylaminobutyryl-ADP transported into the matrix space of mitochondria by the adenine nucleotide carrier. From previous experiments [20], it is known that there is about 0.8—1.0 nmol of ADP/ATP carrier sites per mg of protein in rat heart mitochondria. Data in Table I show that [^{3}H]azidonitrophenylaminobutyryl-ADP binds to a significant extent to the adenine-nucleotide carrier in rat heart mitochondria (up to 0.31 nmol/mg protein) when added to a concentration of 8.6 μM. However it is transported across the mitochondrial membrane at a rate which is negligible.

Although azidonitrophenylaminobutyryl-ADP was not transported in rat heart mitochondria, it inhibited ADP transport in the dark. The inhibition was of a mixed type (competitive and non-competitive) with a K_i value of about 10 μM (Fig. 2), which is in the same range as the K_m for ADP.

Photolabeling of the adenine nucleotide carrier in beef heart mitochondria

The mitochondria were incubated under visible light with [^{3}H]azidonitrophenylaminobutyryl-ADP as described in the legend of Fig. 3. After centrifugation, the mitochondrial pellet was lysed with sodium dodecyl sulfate in the presence of mercaptoethanol, and the protein extract was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The distribution pattern of radioactivity showed only one radioactive peak corresponding to a molecular weight of about 30 000. When carboxyatractyloside or atractyloside was added to the mitochondria together with the tritiated ADP analog, the radioactive peak was markedly decreased. The same pattern was obtained when [^{3}H]azidonitrophenylaminobutyryl-ADP was replaced by the tritiated ATP analog. In a previous paper [9], it has been shown that a protein from heart mitochondria with a molecular weight of 30 000 is specifically labeled in the light with a photoactivable azido derivative of atractyloside. Collectively, this strongly suggests that the same 30 000-dalton protein in heart mitochondria carries sites for both ADP (or ATP) and atractyloside (or carboxyatractyloside).

TABLE I

CARRRIER BINDING AND CARRIER-MEDIATED TRANSPORT OF [^{3}H]AZIDONITROPHENYLAMINOBUTYRYL-ADP IN RAT HEART MITOCHONDRIA

Rat-heart mitochondria (0.5 mg protein) were added to 1 ml of a medium consisting of 100 mM KCl, 20 mM Tris · HCl buffer, pH 7.2 and [^{3}H]azidonitrophenylaminobutyryl-ADP (17 500 dpm/nmol) at the indicated concentrations. The incubation was carried out at 2°C in a dim light. When added atractyloside was present at a concentration of 40 μM. All other experimental conditions are given in the Results Section.

Added [^{3}H]azidonitrophenylaminobutyryl-ADP (μM)	Carrier-bound [^{3}H]azidonitrophenylaminobutyryl-ADP (nmol/mg protein)	Rate of transport of [^{3}H]azidonitrophenylaminobutyryl-ADP (nmol/mg protein/min)
1.8	0.12	<0.02
8.6	0.31	<0.02

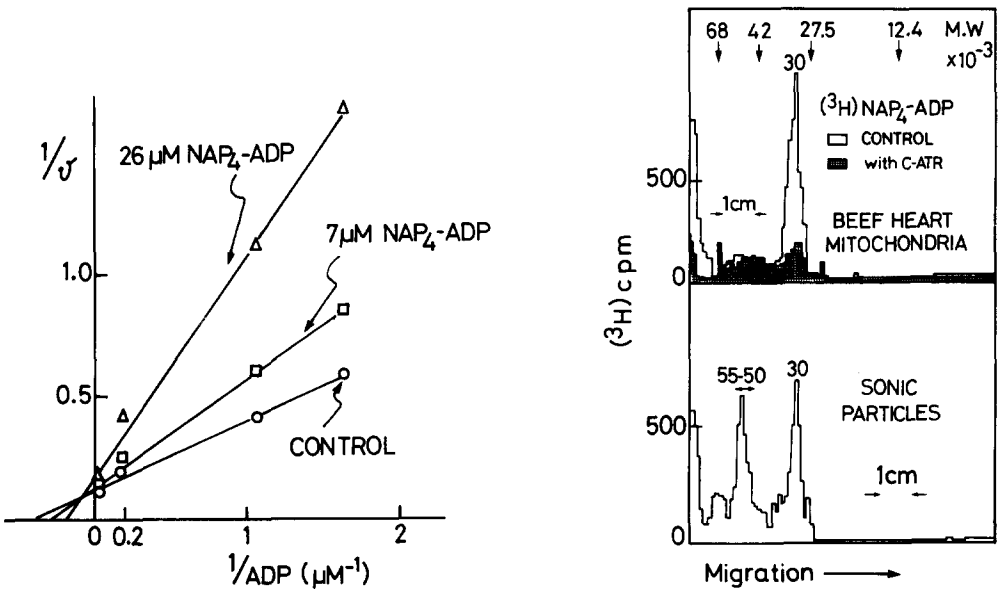

Fig. 2. Inhibition of ADP transport by azidonitrophenylaminobutyryl-ADP (NAP_4-ADP). The incubation medium (1 ml) contained 100 mM KCl, 20 mM Tris · HCl, pH 7.2, 2 mM EDTA and 0.4 mg of mitochondrial protein. Azidonitrophenylaminobutyryl-ADP (NAP_4-ADP) was added at the indicated concentrations. The incubation was carried out in the dark at 0°C. ADP transport was initiated by addition of [^{14}C]ADP (920 dpm/nmol) and stopped by addition of 10 μM carboxyatractyloside, followed by rapid centrifugation. The pellet was dissolved in formamide at 180°C and the radioactivity was assayed by scintillation counting. Unspecific binding of [^{14}C]ADP was assessed from the radioactivity incorporated in the presence of carboxyatractyloside (10 μM) added before [^{14}C]ADP. v is given in nmol/mg protein/min.

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of photolabeled proteins from heart mitochondria and sonic submitochondrial particles after irradiation with [^{3}H]azidonitrophenylaminobutyryl-ADP (NAP_4-ADP). Mitochondria from beef heart and the derived sonic submitochondrial particles were photoirradiated for 30 min at 0°C with 14 μM [^{3}H]azidonitrophenylaminobutyryl-ADP (NAP_4-ADP). When present, carboxyatractyloside was added at a final concentration of 20 μM prior to irradiation. Details are given in Materials and Methods. The indicated molecular weights correspond to standard reference proteins given in Materials and Methods.

Photolabeling of the adenine nucleotide carrier in sonic submitochondrial particles from beef heart

The polarity of submitochondrial particles prepared by sonication of heart mitochondria (sonic particles) is reversed with respect to that of whole mitochondria [21]. Beef heart sonic particles were incubated with [^{3}H]azidonitrophenylaminobutyryl-ADP in the light following the same procedure as that described for whole mitochondria (see above section). The analysis of the protein extract by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed, beside the 30 000-dalton protein, larger peptide(s) corresponding to a molecular weight of 50 000—55 000 (Fig. 3). These latter components are probably the larger subunits of the mitochondrial F_1-ATPase, as suggested by photolabeling of isolated F_1-ATPase [25]. The photolabeling of the 30 000-dalton protein was specifically lowered when the sonic particles were incubated first with bongkrekic acid, a selective inhibitor of ADP transport. The decrease was about 40—50% when bongkrekic acid was added at a final concen-

tration of 20 μM. This is in agreement with the fact that bongkrekic acid in sonic particles competes with ADP or ATP binding [15].

Photolabeling of the adenine nucleotide carrier in mitochondria from S. cerevisiae JB65

The same procedure as that described for heart mitochondria was used for labeling by photoaffinity the adenine nucleotide carrier in *S. cerevisiae* mitochondria with [^{3}H]azidonitrophenylaminobutyryl-ADP. A parallel experiment was performed with [^{3}H]azidonitrophenylaminobutyryl-atractyloside, a photoreagent which had been previously used to covalently label the adenine nucleotide carrier in heart mitochondria [9].

Preliminary experiments carried out with the atractyloside analog have shown that the labeled protein in *S. cerevisiae* mitochondria has a molecular weight significantly higher than that found in heart mitochondria (37 000 vs. 30 000). As the density of ADP/ATP carrier sites on yeast mitochondria [22] is less than in heart mitochondria (0.2—0.3 nmol/mg protein vs. 0.9—1.0 nmol/mg protein), we have initially preferred to use a partially purified extract of mitochondria enriched in ADP/ATP carrier protein to ascertain the molecular weight of the atractyloside-protein complex by gel electrophoresis. *S. cerevisiae* mitochondria were incubated with [^{3}H]azidonitrophenylaminobutyryl-atractyloside in the light. The mitochondrial pellet obtained after centrifugation was lysed with Triton X-100 and the lysate was applied to a small column of hydroxyapatite, following the procedure described by Riccio et al. for the isolation of the beef heart atractyloside-binding protein [6]. The fraction which passed directly through the hydroxyapatite column was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Coloration with Coomassie Blue revealed two proteins with molecular weights of 34 000 and

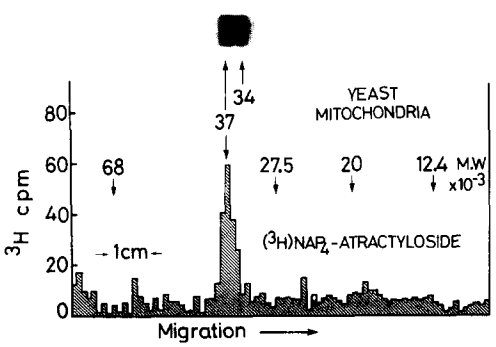

Fig. 4. Separation by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate of the azidonitrophenylaminobutyryl-atractyloside-binding protein in a partially purified extract of *S. cerevisiae* mitochondria. [^{3}H]Azidonitrophenylaminobutyryl-atractyloside (NAP_4-atractyloside) (550 000 dpm/nmol) at a final concentration of 0.5 μM was incubated in the light with *S. cerevisiae* mitochondria for 30 min at 0°C. After centrifugation the mitochondrial pellet was treated with Triton X-100 and the adenine-nucleotide carrier protein was purified by hydroxyapatite chromatography. The protein fraction which was not retained on hydroxyapatite was heated with sodium dodecyl sulfate and mercaptoethanol. Details are given in Materials and Methods and in Results. Arrows correspond to the molecular weight of reference proteins. The figure shows the stained gel and the corresponding profile of radioactivity. Of the two proteins revealed by Coomassie Blue staining, only the 37 000-dalton protein was radiolabeled.

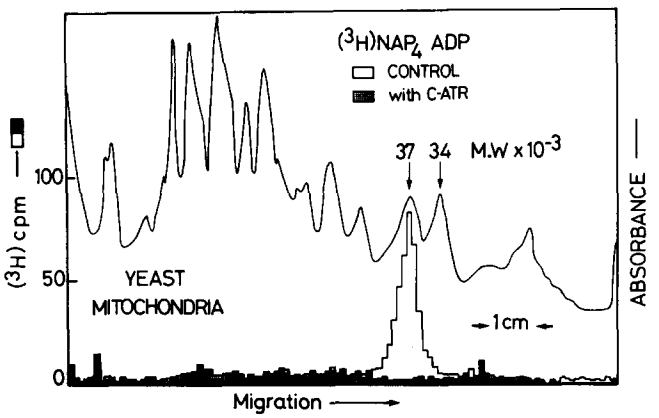

Fig. 5. Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate of a crude extract of yeast mitochondria after photolabeling with [^{3}H]azidonitrophenylaminobutyryl-ADP (NAP_4-ADP). *S. cerevisiae* mitochondria were photoirradiated with 2.5 μM [^{3}H]azidonitrophenylaminobutyryl-ADP (NAP_4-ADP) (550 000 dpm/nmol) for 30 min at 0°C. When present carboxyatractyloside was at the final concentration of 20 μM. Electrophoresis was carried out for 24 h for a better resolution of the 37 000-dalton protein. Details are given in Materials and Methods.

37 000. Only the 37 000-dalton protein was photolabeled by the atractyloside analog.

Incubation in the light of *S. cerevisiae* mitochondria with [^{3}H]azidonitrophenylaminobutyryl-ADP resulted in the labeling of a protein characterized by the same molecular weight (37 000) as the atractyloside-binding protein (Fig. 5). Photolabeling of the 37 000-dalton protein by the ADP analog was abolished by preincubation with carboxyatractyloside or atractyloside in similar fashion to the photolabeling of the 30 000-dalton protein in heart mitochondria (see above). The same results were obtained when [^{3}H]azidonitrophenylaminobutyryl-ADP was replaced by the ATP analog.

Discussion

Characterization of the adenine nucleotide carrier in mitochondria has been achieved either by labeling with a specific inhibitor (for example atractyloside or carboxyatractyloside) or with the substrates (ADP and ATP) (for review see ref. 23). In the latter case, the specific binding of ADP or ATP to the adenine nucleotide carrier was assessed by displacement of the bound nucleotide by atractyloside. Since 1974, a number of purification procedures of the adenine nucleotide carrier have been reported, based on differential adsorption to hydroxyapatite [6] or on the principle of affinity chromatography [7,8]. Purification was followed by the enrichment in bound [^{35}S]carboxyatractyloside or [^{3}H]atractyloside. However, although bound to the carrier with high affinity, the radiolabeled inhibitory ligand could be detached under drastic conditions, i.e. in sodium dodecyl sulfate gel electrophoresis. To obviate this difficulty, we synthesized a photolabeled derivative of [^{3}H]atractyloside to tag covalently [^{3}H]atractyloside to the carrier. By this means, we have been able to determine unambiguously by sodium dodecyl sulfate polyacrylamide gel electrophoresis of crude mitochondrial extracts the molecular weight of the atractyloside bind-

ing protein (probably the adenine nucleotide carrier). The molecular weight of the binding protein is 30 000 in beef heart and rat liver mitochondria [9] and 37 000 in *S. cerevisiae* mitochondria (this paper).

Photolabeling of the adenine nucleotide carrier in the mitochondrial membrane by arylazido derivatives of ADP and ATP prepared in radioactive form has also been achieved. When added to whole mitochondria, azidonitrophenylaminobutyryl-ADP does not enter the matrix space. It binds essentially to the outer face of the inner mitochondrial membrane and does not reach the inner face of this membrane where the nucleotide binding sites of F_1-ATPase are located. This holds also for the ATP analog. In contrast in submitochondrial particles obtained by sonication of beef heart mitochondria (inside-out particles), both ADP and ATP analogs bind not only to a 30 000-dalton protein, but also to other sites which most probably belong to F_1-ATPase. However the fact that the binding of the ADP and ATP analogs to the 30 000-dalton protein is specifically lowered by preincubation with bongkrekic acid is in agreement with the contention that this protein belongs to the adenine nucleotide carrier.

The choice of an azidonitrophenyl derivative of ADP or ATP rather that of 8-azido-ADP or ATP as photoaffinity label for the adenine nucleotide carrier was made following our observation that adenine nucleotide transport is markedly sensitive to ultraviolet irradiation. The nitro group shifts the absorption maximum into the visible region and allows photoactivation at wavelengths which do not damage the carrier protein. This is not the case for other mitochondrial proteins, like F_1-ATPase (unpublished data) which has been successfully photolabeled with 8-azido-ATP [5]. Another advantage of azidonitrophenylaminobutyryl-ADP over 8-azido-ADP is its higher affinity in the dark for the adenine nucleotide carrier; the K_i values are 10 μM (this paper) and 400 μM [4] respectively.

The binding specificity of azidonitrophenylaminobutyryl-ADP and of the ATP analog to the adenine nucleotide carrier in whole mitochondria is inferred from the following findings: (1) the ADP analog in the dark inhibits reversibly ADP transport; (2) when exposed to light, the ADP analog reacts irreversibly with the same site with which it was reversibly associated in the dark; (3) photolabeling of whole mitochondria by the ADP or ATP analogs is abolished by preincubation with appropriate concentrations of atractyloside or carboxyatractyloside.

The nitrene which is generated by photoirradiation of azidonitrophenylaminobutyryl-ADP and -ATP binds covalently to a mitochondrial protein characterized by a molecular weight of 30 000 in heart mitochondria and 37 000 in yeast mitochondria. The fact that the atractyloside-binding proteins from beef heart mitochondria and *S. cerevisiae* mitochondria have also molecular weights of 30 000 and 37 000 respectively supports the view that the same protein belonging to the adenine nucleotide transport system, is able to bind both ADP (or ATP) and atractyloside. Whether ADP (or ATP) and atractyloside bind to the same site or to distinct sites remains to be assessed.

Ruoho et al. [24] have emphasized a complexity in the use of photoaffinity labels in terms of labeling specificity. They suggest that in order for true photoaffinity labeling to occur, the rate of association of the activated photolabel to the receptor site must be significantly faster than the rate of dissociation; they

have proposed the use of scavengers of nitrene, possibly proteins, to check whether the labeling is a true photoaffinity labeling or a pseudoaffinity labeling. Photoaffinity experiments reported in this paper have been carried out with whole mitochondria or with submitochondrial particles. In all cases, only one discrete protein was covalently labeled in spite of the large number of membrane proteins which could potentially act as scavengers. The covalent labeling of the adenine nucleotide carrier in the mitochondrial membrane by photoactivated arylazido derivatives of ADP, ATP or atractyloside is therefore most likely a true photoaffinity labeling. This is probably related to the fact that the ADP, ATP or atractyloside moieties of the photolabels used have a high affinity for the adenine nucleotide carrier.

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