ARYL-AZIDO ATRACTYLOSIDES AS PHOTOAFFINITY LABELS FOR THE MITOCHONDRIAL ADENINE NUCLEOTIDE CARRIER

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1. Introduction

It is well known that atractyloside (ATR) and carboxyatractyloside (CATR) are potent and specific inhibitors of ADP transport in mitochondria [1,2]. Labeled ATR or CATR have been used for the purification of an ATR- or CATR-binding protein [3–5]. Isolation of the ATR– or CATR–protein complex was made possible by mild techniques of purification which do not dissociate the inhibitor from its binding site in the protein. These techniques include affinity chromatography [3] and hydroxyapatite chromatography [4]. In contrast strong detergents, such as sodium dodecylsulfate (SDS) resulted in the displacement of the bound inhibitor and therefore prevented the possibility to characterize the inhibitor–protein complex by SDS polyacrylamide gel electrophoresis (SPAGE). To obviate this difficulty, it was necessary to bind covalently labeled ATR or CATR to its specific binding protein. This paper describes the synthesis of a photoactive aryl-azido-ATR (N_4-azido-2-nitrophenyl-aminobutyryl-ATR or NAP_4-ATR) and its use to label covalently the ADP carrier in mitochondria. It is shown that NAP_4-ATR, in the dark, inhibits competitively ADP transport with the same efficiency as ATR and competes with ATR for binding to mitochondria. The nitrene derivative formed upon irradiation of NAP_4-ATR binds covalently to mitochondria. By this means, the ATR-binding protein has been characterized by SPAGE; its molecular weight is about 30 000.

2. Materials and methods

2.1. *NAP-aminopropionic acid and NAP-aminobutyric acid*

These compounds were synthetized by the method of Fleet et al. [7] applied to the 3-aminopropionic acid and the 4-aminobutyric acid. The starting material was the 4-fluoro-3-nitrophenylazide. The two NAP amino derivatives were crystallized from ethanol–water and characterized by their IR spectrum (2130 cm^{-1} band) and UV spectrum (λ_{max} 261 and 460 nm; ϵ_{mM} = 27 200 and 5900 respectively). Their purity was assessed by thin-layer chromatography in butanol satured with water. Attempt to synthetize the NAP-glycine derivative of ATR failed. [^{3}H] 4-aminobutyric acid (25 Ci/mmol) used for the synthesis of NAP-[^{3}H] aminobutyric acid was obtained from the 'Commissariat à l'Energie Atomique' (Saclay) and diluted with unlabeled 4- aminobutyric acid to about 5×10^8 dpm/μmol. The synthesis of NAP_4-ATR and NAP_3-ATR is described under Results. [^{3}H] ATR (3×10^7 dpm/μmol) was prepared according to Brandolin et al. [3] and used for the synthesis of NAP_3-[^{3}H] ATR and NAP_4-[^{3}H] ATR.

2.2. *Biological assays*

Mitochondria from rat heart were prepared by the method of Chance and Hagihara [8]. Except in experiments requiring photoactivation, binding and inhibition assays were carried out in a dark room, using a red safety lamp. Oxygen uptake was measured

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at 25°C with a Gilson oxygraph equipped with a Clark electrode. ADP transport was assayed as described in [9].

Binding of NAP_4-[^{3}H] ATR to rat heart mitochondria was performed as follows. Mitochondria (1 mg protein) in 1 ml of sucrose-mannitol medium (containing 0.225 M sucrose, 0.075 M mannitol, 5 mM Tris–HCl, 0.5 mM EDTA, pH 7.4) were incubated at 0°C for 45 min in centrifuge tubes with increasing concentrations of NAP_4-[^{3}H] ATR. After centrifugation the pellets were dissolved in formamide (1 ml) at 180°C, and radioactivity was measured by liquid scintillation counting.

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. Five ml of the mitochondrial suspension in sucrose-mannitol medium (1 mg protein/ml) was introduced 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.

2.3. *Gel electrophoresis*

SPAGE was carried out as described by Weber and Osborn [10], using a 10.3% acrylamide gel. Mitochondria (10 mg/ml) were lysed by addition of SDS and mercaptoethanol to a final concentration of 2% each. After heating for 10 min at 100°C, glycerol was added to a final concentration of 20% as well as traces of a tracking dye (Bromophenol Blue). An aliquot fraction of mitochondrial protein (100 to 150 μg) was loaded on each gel. Electrophoresis was carried out at 5 mA/gel for 22 hours. Staining with Coomassie Blue and destaining with methanol, acetic acid were performed as described in [10]. The gels were sliced with a Gilson slicer. Each slice (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 counting.

3. Results

3.1. *Synthesis of 6'[4N(4-azido-2-nitrophenyl)-amino] propionyl atractyloside, (NAP_3-ATR) and of 6'[4N(4-azido-2-nitrophenyl)-amino] butyryl atractyloside, (NAP_4-ATR)*

In the method which is described below, the primary alcohol function of the glucose moiety of ATR was linked to the carboxylic group of the propionyl or butyryl derivatives of the 4-fluoro-3-nitrophenyl azide in the presence of carbonyl diimidazole in dry dimethyl formamide. Carbonyl-diimidazole has been introduced by Gottikh et al. [11] for activation of carboxyl groups in esterification reactions. Routinely the synthesis was carried out as follows. The aminopropionyl (or aminobutyryl) derivative of the 4-fluoro-3-phenyl azide (50 μmol) was added to carbonyl-diimidazole (170 μmol) in 30 μl of dimethyl-formamide. The reaction mixture was stirred for 15 min at 25°C. Then atractyloside (10 μmol) in 1 ml of water was added and allowed to react at room temperature overnight. After evaporation under vacuum, the residue was washed three times with chloroform (5 ml) to eliminate the unreacted butyryl or propionyl derivatives, and then dissolved in 300 μl of methanol. Further purification was achieved by thin layer chromatography on silica gel plates with $CHCl_3$, methanol, acetic acid, water (55/20/2/1, v/v). The plates were sprayed with one percent vanillin in concentrated sulfuric acid to visualize the various products. Beside the unreacted ATR (R_f 0.28) two major orange colored bands (R_f 0.48 and 0.56) and a minor one (R_f 0.36) were obtained. Each was scraped off, eluted with methanol and characterized by IR spectrum (band at 2130 cm^{-1} characteristic of the azido group) and visible spectrum (peak at 460 nm, ϵ_{mM} 5900). Based on the absorbancy at 460 nm and on the specific radioactivity of [^{3}H] ATR, the molar ratio of NAP to ATR could be assessed. The faster moving band (R_f 0.56) was characterized by a molar ratio of NAP to ATR of 2, whereas the band with the R_f value of 0.48 had a ratio of NAP to ATR of 1. Only the latter compound exhibited a strong inhibitory effect of ADP transport. Because the primary alcohol group in the glucose moiety of ATR is highly reactive and can be esterified without loss of the binding and inhibitory properties of ATR [3], it is most likely that the compound with a NAP to ATR ratio of 1 corresponds to the monoester derivative of ATR at the C'6 position of the glucose residue of ATR. The monoester derivative obtained from the aminopropionyl precursor is designated NAP_3-ATR. The corresponding derivative obtained from the aminobutyryl precursor is designated NAP_4-ATR (fig.1).

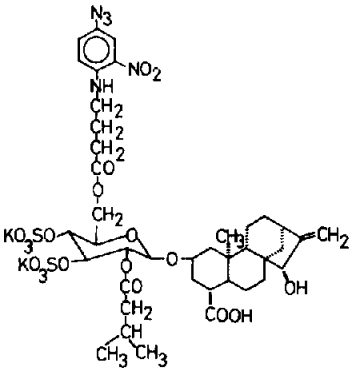

Fig.1. Structure of 4-azido-2-nitrophenyl-aminobutyryl actractyloside (NAP_4-ATR).

3.2. *Inhibition and binding experiments (dark reactions)*

NAP_4-ATR and NAP_3-ATR were both tested for their ability ot inhibit the ADP-stimulated respiration of mitochondria in the dark. Both were potent inhibitors. Half inhibition was obtained with 0.6 μM NAP_4-ATR and 0.2 μM NAP_3-ATR, which indicates that these compounds are as efficient as ATR. The inhibition was relieved by excess ADP or by uncoupler (fig.2). The primary effect of the inhibition was on ADP transport. Typical inhibition data of ADP transport by NAP_4-ATR are given in table 1. The diester derivatives of ATR were 10 times less inhibitory than the monoester derivatives.

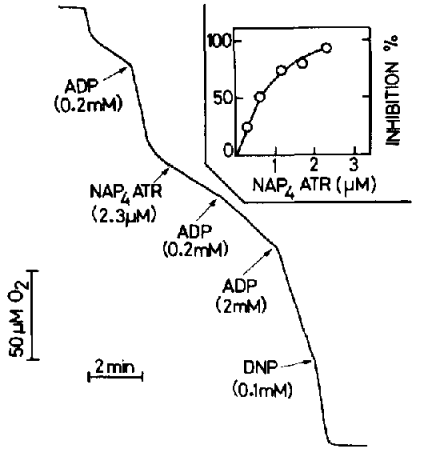

Fig.2. Inhibition of the ADP-stimulated respiration of rat liver mitochondria with NAP_4-ATR. The incubation medium contained 112 mM KCl, 16 mM phosphate, 6 mM $MgCl_2$, and 10 mM glutamate, pH 7.4 and 4 mg of mitochondrial protein. Final volume 2 ml. Other additions were as indicated. The incubation was carried out in the dark. The *insert* shows the effect of different concentrations of NAP_4-ATR on the ADP-stimulated respiration.

Table 1
Effect of NAP_4-ATR on ADP transport in rat heart mitochondria

NAP_4-ATR (μM)	Rate of ADP transport (nmol/mg protein)	Inhibition (%)
None	11.5	–
0.18	7.5	35
0.38	2.7	76
0.76	0.3	97

The mitochondria (0.5 mg protein) were preincubated at 0°C in 1.5 ml of a medium containing 112 mM KCl, 10 mM Tris, 1 mM EDTA, pH 7.4, with or without NAP_4-ATR. ADP transport was started by the addition of 50 μM [^{14}C] ADP, and stopped after 20 sec by carboxyatractyloside (cf. Materials and methods). The whole assay was carried in the dark.

NAP_4-[^{3}H] ATR bound in the dark to rat heart mitochondria with high affinity (number of high affinity binding sites: 1.2 nmol/mg protein). The K_d value (40 mM) was similar to that found for ATR. Similar data were obtained with NAP_3-

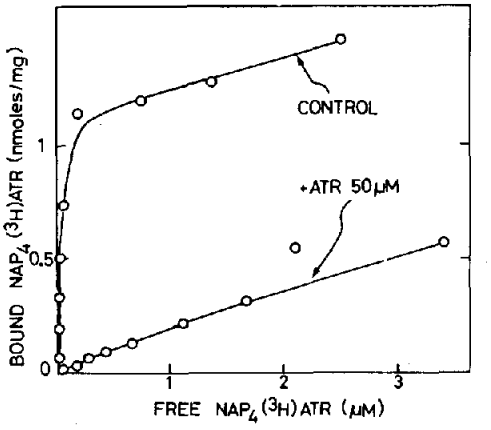

Fig.3. Competition between NAP_4-[^{3}H] ATR and unlabeled ATR for binding to rat heart mitochondria. Mitochondria (1 mg protein) were incubated in 1 ml of the sucrose-mannitol medium at 0°C for 45 min with increasing concentration of NAP_4-[^{3}H] ATR with or without ATR (50 μM). The whole incubation was carried out in the dark. For details, cf. Methods.

Table 2
Displacement of NAP_4-[^{3}H] ATR bound in the dark or in the light by unlabeled ATR

Conditions	Bound NAP_4-[^{3}H] ATR (nmol/mg protein)		Bound NAP_4-[^{3}H] ATR removable by ATR (nmol/mg protein)
	Without ATR	With ATR	
Dark	1.54	0.61	0.93
Light	1.86	1.52	0.34

Rat heart mitochondria (5 mg) in 5 ml of sucrose-mannitol medium were incubated with 2.6 μM NAP-[^{3}H] ATR for 30 min at 0°C in the dark. Then the suspension was divided in two fractions of 2.5 ml. One of them was let in the dark. The other one was photoirradiated as described in Materials and methods. From each suspension (dark and light), a fraction of 1 ml was taken out and made to react with 50 μM ATR for another 30 min at 0°C. After centrifugation, the radioactivity of the pellets was measured by scintillation counting (cf. Methods). The difference between the bound NAP_4-[^{3}H] ATR removable by ATR in the dark and that removable by ATR in the light is designated as 'covalently bound NAP_4-[^{3}H] ATR'.

[^{3}H] ATR. Unlabeled ATR and CATR were able to compete for the binding of NAP_4-[^{3}H] ATR or NAP_3-[^{3}H] ATR to mitochondria (fig.3). They were also able to displace bound NAP_4-[^{3}H] ATR and NAP_3-[^{3}H] ATR from their binding sites. These results indicate that NAP_3-ATR and NAP_4-ATR bind to the same site as ATR on the ADP carrier.

3.3. *Photolabeling experiments with NAP_4-[^{3}H] ATR*

When NAP_4-[^{3}H] ATR was incubated at a saturating concentration with rat heart mitochondria in the light, some radioactivity was covalently incorporated, as indicated by the fact that added ATR (50 μM) displaced only a small fraction of the bound radioactivity (table 2). In the experiment of table 2, calculation based on the amounts of bound NAP-[^{3}H] ATR removable by ATR in the dark and in the light respectively led to a value of about 0.6 nmol of NAP-[^{3}H] ATR convalently bound to the ADP carrier per mg of mitochondrial protein. The time course of the photolabeling of the ADP carrier in rat heart mitochondria by NAP_4-[^{3}H] ATR is shown in fig.4. Covalent binding of NAP_3-[^{3}H] ATR was less efficient than that of NAP_4-[^{3}H] ATR, possibly because the former label does not probably meet the steric requirement for covalent binding at the nitrene phenyl moiety of the molecule.

Rat heart mitochondria photoirradiated in the presence of NAP_4-[^{3}H] ATR were treated with SDS in the presence of mercaptoethanol and the protein extract was analyzed by SPAGE. The profile of radioactivity in the gel shows a major peak corresponding to a mol. wt. of 30 000 and a minor one with a mol. wt. of 42–45 000 (fig.5). Similar data were obtained with NAP_4-ATR labeled by ^{3}H on the butyryl arm. Sequential addition of ATR and NAP_4-[^{3}H] ATR, followed by photoirradiation resulted in a peak of radioactivity with a mol. wt. of 42–45 000 and in a complete disappearance of the 30 000 mol. wt. peak.

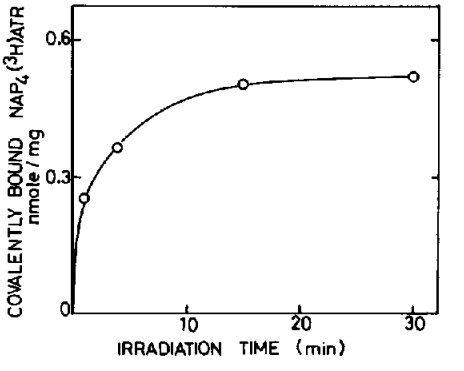

Fig.4. Kinetics of covalent binding of NAP_4-[^{3}H] ATR to rat heart mitochondria. The binding assay was carried out as described in table 2. The 'covalently bound NAP_4-[^{3}H] ATR' is defined in the legend of table 2.

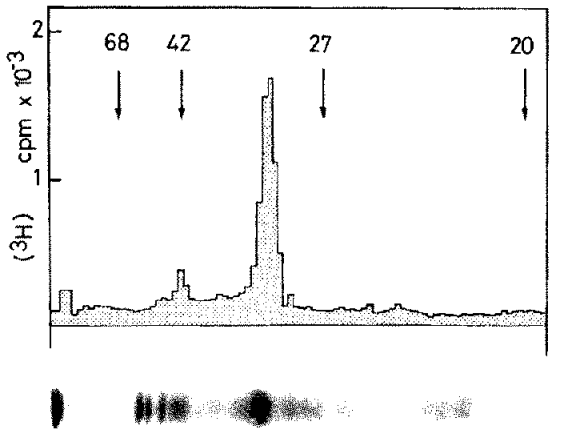

Fig.5. Separation of the NAP_4-[^{3}H] ATR binding protein by SPAGE. The figure shows the stained gel and the corresponding profile of radioactivity of a lysate from rat heart mitochondria photoirradiated with NAP_4-[^{3}H] ATR. Details are given in Methods and Results. Arrows correspond to the mol. wt. of Bovine serum albumin (68×10^3), egg albumin (42×10^3), triose phosphate isomerase (27×10^3), trypsin inhibitor (20×10^3).

An effect similar to that of ATR was obtained with bongkrekic acid or CATR. When the [^{3}H] NAP_4 residue (instead of NAP_4-[^{3}H] ATR) was incubated with mitochondria and then the mixture subjected to photoirradiation, a major radioactive peak corresponding to a mol. wt. of 42–45 000 could also be detected and a minor one with a mol. wt. of 56 000 as well.

The above photolabeling experiments were carried out with rat heart mitochondria. Similar results, with NAP_4-[^{3}H] ATR showing in particular a major peak of radioactivity corresponding to a mol. wt. of 30 000 were obtained with rat liver mitochondria.

4. Discussion

Photolabeling [6,12] has proved to be a useful method to label covalently receptors with specific ligands. The mitochondrial ADP carrier was a good candidate for photolabeling since it is specifically and efficiently inhibitied by ATR, a glucoside which can be linked to azido derivatives at the primary alcohol of the glucose moiety without modification of its inhibitory properties. The present paper reports the preparation of two aryl-azido derivatives of ATR (NAP_3-ATR and NAP_4-ATR) and the use of these derivatives for the separation of an ATR-binding protein by SPAGE after photoactivation. After photoactivation NAP_4-ATR has been found more reactive than NAP_3-ATR. When exposed to visible light, NAP_4-ATR irreversibly binds to mitochondria and inactivates ADP transport. NAP_4-[^{3}H] ATR binds covalently to a 30 000 mol. wt. protein as shown by SPAGE. That the 30 000 mol. wt. protein is specific for binding ATR is indicated by the fact that mitochondria preincubated with NAP_4-[^{3}H]-ATR in the dark and then treated with ATR (or CATR or bongkrekic acid) are no longer labeled by NAP_4-[^{3}H] ATR when they are photoirradiated. Instead, a protein with a mol. wt. of 42–45 000 becomes labeled.

It has been reported that photoactivated NAP which is the terminal residue of NAP_4-ATR binds covalently to two polypeptides of the inner mitochondrial membrane: subunit 1 of the mitochondrial ATPase (mol. wt. 56 000) and a protein with a mol. wt. of 31 000. The latter protein is believed to be a specific uncoupler binding protein [13]. NAP_4 used in our experiments differs from NAP by the aminobutyryl chain which is attached to the aryl residue. This may explain why the binding specificity of NAP is different from that of NAP_4.

Previous work concerning the purification of a [^{3}H] ATR-binding, protein from rat liver mitochondria by a mild emulphogen treatment followed by Sephadex chromatography led to the recovery of a radioactive peak corresponding to an apparent molecular weight of 60 000 [3]. This peak corresponded to the [^{3}H] ATR-binding protein included in micelles of detergent. The exact molecular weight of the embedded protein was difficult to determine by this method, because of the contribution of the micelles of detergent [14]. The data reported in the present paper indicate that the molecular weight of the ATR-binding protein in rat liver mitochondria is 30 000.

In conclusion, photolabeling experiments based on the use of an aryl-azido derivative of ATR show that both rat liver and rat heart mitochondria contain an ATR-binding protein with a mol. wt. of 30 000.

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