Synthesis of 6′-*O*-*p*-azidobenzoylatractyloside, a short arm photoactivable derivative of atractyloside

Studies of its binding and inhibitory properties

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1. INTRODUCTION

Photolabeling of the ADP/ATP carrier in the mitochondrial membrane by photoactivable derivatives of the substrate ADP and the inhibitor attractyloside (ATR) has found applications in the characterization of the carrier protein in mitochondria from different mammalian tissues and living species [1–5]. As ATR is a non-penetrant inhibitor of ADP/ATP transport, which binds to the carrier at the outer surface of the inner mitochondrial membrane, the following azido derivative of ATR, 6′-*O*-[4-{*N*-(4 azido-2-nitrophenyl) amino}butyryl]-ATR, was used in its radiolabeled form to map the atractyloside binding site of the carrier protein. However, because of the length of the chain bearing the azido group, it cannot be excluded that the photo-generated nitrene interacts not only with amino acid residues within the ATR site, but also with amino acids located at a distance from the ATR site. To obviate this difficulty, a short-arm photo-activable derivative of ATR, the 6′-*O*-*p*-azidobenzoyl-ATR, was synthesized (fig.1). The preparation and spectral properties of this new derivative are described here. Binding and inhibition studies showed that this derivative is recognized by the membrane-bound ADP/ATP carrier with virtually the same affinity and specificity as ATR itself, and is therefore of potential use in mapping studies.

Abbreviations: ATR, atractyloside; CATR, carboxyatractyloside; HPLC, high-pressure liquid chromatography; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; Mops, 3-*N*-morpholino propane sulfonic acid

2. MATERIALS AND METHODS

p-Amino[^{14}C]benzoic acid (49 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique (Saclay). *N,N*′-Carbonyldiimidazole was purchased from Merck, triethylamine from Fluka, and atractyloside from Sigma. [^{3}H]Atractyloside was prepared as described in [6]. High-pressure liquid chromatography (HPLC) was performed, utilizing a Waters liquid chromatograph equipped with two M 6000A solvent delivery systems and a U6K injector. The UV absorbance of the column

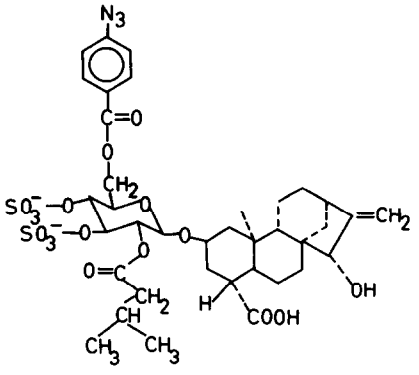

Fig.1 Structure of 6′-*O*-*p*-azidobenzoyl-ATR.

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effluent was monitored with a Shoeffel 770 spectrophotometer. The columns (300 × 7.8 mm) were filled with a reversed-phase support medium (C18 μBondapak from Waters); they were protected by a guard column (40 × 4.6 mm) dry-packed with a CO-pell ODS (30–38 μm) phase from Whatman.

Thin-layer chromatography (TLC) was done on silica gel-coated glass plates, using appropriate solvent systems. UV absorption spectra were recorded at room temperature with a Perkin-Elmer 557 spectrophotometer. Mass spectra were taken with a Kratos MS 50 apparatus. For silylation, samples (10–20 μg) were treated for 1 h at 100°C in closed vials with a mixture of pyridine, hexamethyldisilazane and trimethylchlorosilane (4:2:1, by vol.) [7]. The reagents were evaporated under vacuum at 20°C; the residue after solubilization in chloroform was introduced into the ion source, and vaporized at 200°C.

Reversible binding of the radiolabeled 6′-*O*-*p*-azidobenzoyl-ATR to mitochondria was done in the dark. Mitochondria (1 mg protein) in 1 ml standard saline medium made of 0.12 M KCl, 1 mM EDTA, 10 mM Mops (pH 6.8) were incubated at 0°C for 45 min in centrifuge tubes with increasing concentrations of the labeled compound. After centrifugation, the pellets were digested in 1 ml of 5% Triton X-100, 0.5 M NaCl and 10 mM Mops (pH 7.5) and radioactivity was measured by liquid scintillation counting. In photolabeling experiments, the suspension of mitochondria with the photolabeled derivative was irradiated with UV light delivered by a 15 W Philips UV germicidal lamp under an atmosphere of argon. It is known in fact that photoirradiation of mitochondria by UV light leads to some inactivation of the ADP/ATP carrier protein; this inactivation depends on O_2 and is considerably decreased in an atmosphere of argon. Moreover, the presence of the ATR derivative together with the mitochondria protects against inactivation [8]. Taking these facts into account, UV light irradiation was carried out as follows. 6′-*O*-*p*-Azido-[^{14}C]benzoyl-ATR was incubated with the mitochondria at a saturating concentration corresponding to 2 nmol/mg protein in the above standard saline medium. After 30 min in the dark, the suspension was poured into a 500 ml beaker to form a thin layer of 1–2 mm. The beaker was placed on crushed ice at 20 cm from the source of UV light, and the suspension of mitochondria was flushed with a stream of argon during photo-irradiation that lasted for ~ 10 min.

ADP/ATP transport in mitochondria was measured by the direct exchange procedure [9]. The incubation medium (1 ml) was made of 0.27 M mannitol, 1 mM EDTA and 10 mM Hepes (pH 6.8). The amount of mitochondria used corresponded to 1 mg protein. The reaction was started by addition of [^{14}C]ADP (5 × 10^6 dpm/μmol). The ATR derivative to be tested was preincubated with the mitochondria at 0°C for 15 min prior to addition of [^{14}C]ADP. The reaction was carried out at 0°C and terminated by addition of 20 μM CATR, followed by centrifugation. The pellets were digested, and the radioactivity measured by liquid scintillation counting as described for binding assays.

3. RESULTS AND DISCUSSION

3.1. *Synthesis and spectral properties of* 6′-O-p-*azido[^{14}C]benzoyl-ATR.*

p-Amino[^{14}C]benzoic acid (20 μmol, 10^8 dpm/μmol) was dissolved in 900 μl 4 N sulfuric acid, and cooled in an ice bath; 100 μl 0.3 M sodium nitrite were added dropwise, and the mixture was kept in the ice bath with mechanical stirring for 50 min; 2 ml diethylether were then added, followed by 300 μl 0.2 M sodium azide. The solution was stirred in the dark for another 30 min. On standing, the ether layer separated rapidly, and was pipetted out. The remaining aqueous solution was washed 4 times with 2 ml ether. The ether fractions were pooled, washed with 2 ml of an aqueous solution of NaCl, poured into a 100 ml flask, and the ether was evaporated under a stream of nitrogen [10]. The dry residue consisting of *p*-azido[^{14}C]benzoic acid was ready for coupling to ATR. The coupling step was carried out after activation of the carboxylic group of the *p*-azido[^{14}C]benzoic acid by *N,N′*-carbonyldiimidazole as in [11]. Carbonyldiimidazole (70 μmol) was dissolved in 330 μl dimethylformamide pre-dried over 3 Å molecular sieve and calcium hydride, and the solution was added to the *p*-azido-[^{14}C]benzoic acid in the flask. After stirring in the dark for 15 min at room temperature, a mixture of ATR (20 μmol) and triethylamine (20 μmol) in 1.2 ml water was added dropwise within 2 min, and stirring was continued for 30 min. A longer contact did not improve the yield of coupling. The mixture was then evaporated in vacuo below 40°C. The

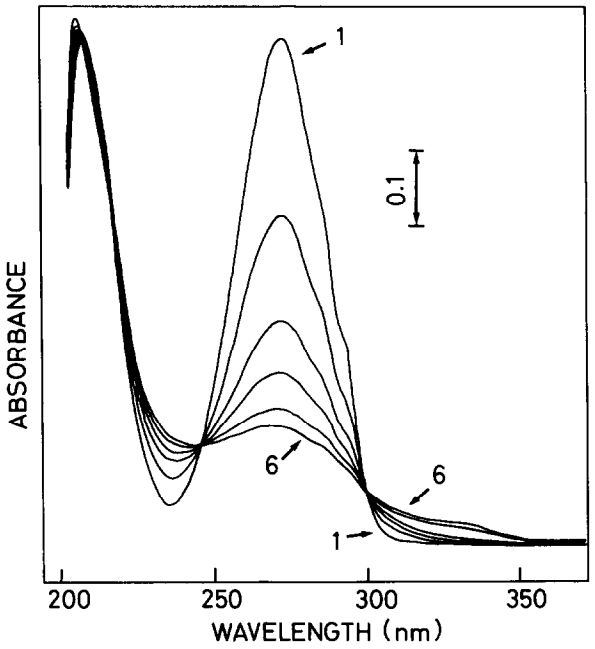

Fig.2 Absorption spectra and photolysis of 6′-*O*-*p*-azidobenzoyl-ATR. A solution of 45 μM 6′-*O*-*p*-azidobenzoyl-ATR in methanol in a 3 ml quartz cuvette of 1 cm pathway was subjected to successive photoirradiations delivered by a 15 W Philips UV germicidal lamp placed at 20 cm of the cuvette. Traces 1–6 correspond to spectra taken after 0, 10, 20, 30, 40 and 70 s irradiation, respectively.

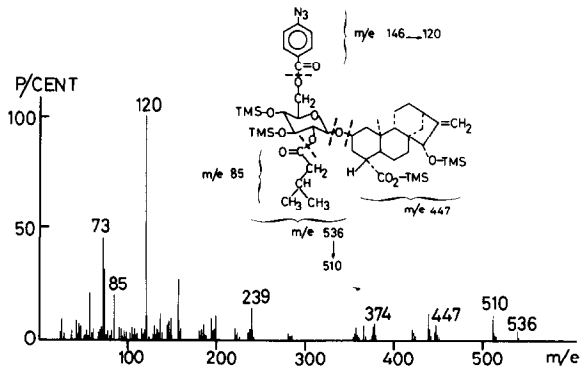

Fig.3 Characteristics of fragmentation products of silylated 6′-*O*-*p*-azidobenzoyl-ATR in mass spectrometry. Conditions are given in section 2.

gummy residue was dispersed in 1 ml distilled water and evaporated to dryness to remove the residual dimethylformamide. This procedure was repeated at least 3 times. The unreacted *p*-azido[^{14}C]benzoic acid was removed by several washings of the dry residue with chloroform. Finally, the residue was dissolved in 1 ml methanol, and the methanolic solution was subjected to HPLC. It was passed down a μBondapack C18 column (7.8 × 300 mm, 10 μm) with isocratic elution at 2 ml/min at room temperature. The solvent system used was: methanol/1 M NH_4-acetate/acetic acid/water (58/1/1/40). The effluents contained a main product that was characterized by a retention time of 25 min, and identified by mass spectrometry (see below) as the mono-*p*-azidobenzoyl ester of the glucose disulfate moiety of ATR (6′-*O*-*p*-azidobenzoyl-ATR). The unreacted *p*-azidobenzoic acid was eluted with a retention time of 15 min. The yield of its recovery with respect to the initial amount of added ATR was 25%. Its purity was assessed by TLC on silica gel with a solvent system made of $CHCl_3$–MeOH–CH_3COOH–H_2O (55:20:3:3, by vol.); its R_F-value was 0.34. Two other derivatives of ATR with retention times higher than 50 min on HPLC were detected in minute amounts. By analogy with the acetylated and long chain azido-ATR derivatives [2.12], the two minor ATR derivatives were probably the mono-*p*-azidobenzoyl ester of the diterpene moiety of ATR and the di-*p*-azidobenzoyl ester of ATR. Their R_F values on TLC, using the same solvent system as above were 0.20 and 0.42, respectively. Only 6′-*O*-*p*-azidobenzoyl-ATR exhibited the same biological properties as ATR, as shown thereafter.

The UV spectrum of 6′-*O*-*p*-azidobenzoyl-ATR in ethanol exhibited a maximum absorbancy at 272 nm (ε_{272} = 14 600 $M^{-1} \cdot cm^{-1}$) and shoulder at 290 nm (fig.2). Photoirradiation by UV light led to a decrease of the absorption peak at 272 nm, accompanied by a slight increase of the absorbance 320–340 nm. Data on the mass spectrum of silylated 6′-*O*-*p*-azidobenzoyl-ATR are presented in fig.3. The peaks corresponding to *m/e* 536 and 510 were ascribed to the disilylated glucose moiety of ATR combined with isovaleric acid and a residue of either *p*-azidobenzoic acid or *p*-aminobenzoic acid, respectively. These data are indicative of esterification of the primary alcohol of the glucose disulfate moiety of ATR by the carboxylic group of *p*-azido-

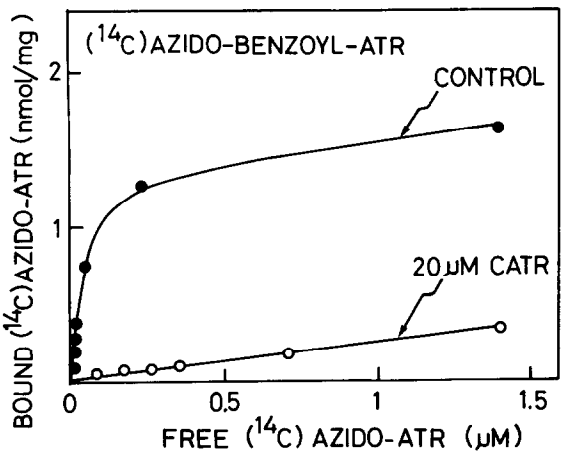

Fig.4 Binding of 6′-*O*-*p*-azido[^{14}C]benzoyl-ATR to rat heart mitochondria. Mitochondria (1 mg protein) were incubated in the dark in 1 ml standard saline medium (cf. section 2) for 30 min at 0°C with increasing concentrations of 6′-*O*-*p*-azido[^{14}C]benzoyl-ATR. Non-specific binding was assessed in the presence of 20 μM CATR. The pellets obtained after centrifugation were processed as in section 2.

benzoic acid. This ester linkage was completely hydrolyzed by treatment with 0.1 N NaOH at 45°C for 15 min. The aminobenzoyl residue revealed by mass spectrum was a decomposition product of *p*-azidobenzoic acid.

3.2. *Binding and inhibitory properties of* 6′-O-p-*azido[^{14}C]benzoyl-ATR*

Reversible binding of 6′-*O*-*p*-azido[^{14}C]benzoyl-ATR to the ADP/ATP carrier protein in the mitochondrial membrane was assayed with a suspension of rat heart mitochondria incubated with increasing concentrations of the azido derivative in the dark (fig.4). The properties of the high affinity binding sites for 6′-*O*-*p*-azidobenzoyl-ATR, number of sites close to 1 nmol/mg protein and K_d-value of ~30 nM, were virtually identical to those found for ATR binding. Preincubation of mitochondria with a large excess of unlabeled CATR prior to addition of 6′-*O*-*p*-azido[^{14}C]benzoyl-ATR resulted in abolition of the high affinity binding; only the low affinity binding remained unaltered. Similar binding date, showing high affinity binding, were obtained with rat liver mitochondria, except that the number of high affinity sites was only 0.2 nmol/mg protein in that case. These results are in accordance with previous work where it was shown that the biological potency of ATR was preserved in ATR derivatives, provided that the adduct was placed on the primary alcohol group of the glucose disulfate moiety of ATR [13].

Associated with the reversible binding of 6′-*O*-*p*-azidobenzoyl-ATR in the dark to mitochondria, there was a competive inhibition of ADP transport by the ATR derivative. This illustrated in fig.5 by the data of an ADP-transport experiment carried out with rat liver mitochondria; the K_i for 6′-*O*-*p*-azidobenzoyl-ATR was close to 100 nM, which is similar to the K_i for ATR [15]. These experiments clearly showed that 6′-*O*-*p*-azidobenzoyl-ATR-meets the criteria of affinity and specificity required for photoaffinity labeling.

Covalent photolabeling of the ADP/ATP carrier by 6′-*O*-*p*-azido[^{14}C]benzoyl-ATP required irradiation by UV light, in contrast to photolabeling with the orange-colored long chain azido ATR derivative, 6′-*O*-[4-{*N*(4 azido-2-nitrophenyl)amino}-butyryl]-ATR, that could be achieved under visible light [2]. An obvious advantage of using the short arm 6′-*O*-*p*-azidobenzoyl-ATR is that the active nitrene formed by photoactivation binds at or very close to the ATR site on the ADP/ATP carrier protein; this might not be the case for the long chain

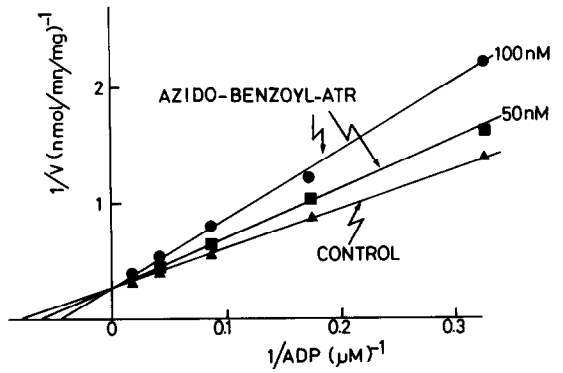

Fig.5. Kinetics of inhibition of ADP transport in rat liver mitochondria by 6′-*O*-*p*-azidobenzoyl ATR. Mitochondria (1 mg protein) in 1 ml mannitol medium were preincubated in the presence of 6′-*O*-*p*-azidobenzoyl-ATR at the indicated concentrations for 15 min at 0°C in the dark. ADP transport was started by addition of [^{14}C]ADP at 2.5– 40 μM. Other details are given in section 2.

azido-ATR derivative, in which the light-generated active nitrene may bind at some distance from the ATR site. The peculiar structural features of long and short chain azido-ATR derivatives might therefore be exploited to investigate the topography of the ADP/ATP carrier. The yield of covalent photolabeling was determined as follows. Heart mitochondria were photoirradiated in the presence of 6′-*O*-*p*-azido[^{14}C]benzoyl-ATR and the ADP/ATP carrier protein was prepared as in [14]. Extensive purification included a final washing of the protein by acetone and diethyl ether [4]. Based on the radioactivity bound to the precipitated protein, the percentage of covalently labeled carrier protein was estimated at ~ 10%.

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