PARTIAL PURIFICATION OF AN ATRACTYLOSIDE-BINDING PROTEIN FROM MITOCHONDRIA

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1. Introduction

The ADP carrier in mitochondria is specifically inhibited by atractyloside (ATR) and carboxyatractyloside also called gummiferin, (CARB-ATR), (for review see [1]). When added to mitochondria, ATR and CARB-ATR bind with high affinity ($K_d < 30$ nM) to the inner mitochondrial membrane and the molar quantity of inhibitor bound is of the same order as that of ADP or ATP bound to the ADP carrier. The protein nature of the ATR and CARB-ATR receptor has been demonstrated by the decrease of binding affinity and capacity upon heat treatment or trypsin digestion or photoxidation [2]. These data taken together have been interpreted to indicate that ATR and CARB-ATR interact with a protein component of the mitochondrial ADP carrier. The high affinity and specificity of the binding of ATR and CARB-ATR suggested to us that these inhibitors could be used in radioactively labelled form to trace protein components of the ADP carrier and possibly the whole carrier system during its extraction from mitochondria.

This paper describes the purification from rat liver mitochondria of a protein, that binds ATR with high affinity. The purification procedure is based on affinity chromatography using an analogue of ATR, succinyl-atractyloside, linked to Sepharose through an 'arm' of diaminodipropylamine. The ATR binding protein could be specifically eluted by ATR or CARB-ATR. Chromatography of the protein on Sephadex revealed a major peak corresponding to a molecular weight of 60 000 and a low molecular weight component (15 000). A new method of preparation of [^{3}H]ATR is also described.

2. Materials and Methods

2.1. *Preparation of ^{35}S-labeled CARB-ATR*

^{35}S-labeled CARB-ATR was extracted from the rhizomes of the thistle *Atractylis gummifera* that had been grown in the presence of [^{35}S]sulfate, and was purified according to [2]. Its specific radioactivity was 1.2×10^6 dpm μmole.

2.2. *Preparation of [^{3}H]ATR*

The method used to introduce tritium into ATR was similar to that followed by Duffield et al. [3] to introduce deuterium into propionic acid starting from methylmalonic acid. CARB-ATR, which prossesses two carboxyl groups bound the C_4 atom of the diterpene cycle [2], can be decarboxylated to ATR by heating [4]. When this reaction is carried out in the presence of tritiated water, ^{3}H is incorporated into ATR on the C_4 atom of the diterpene, giving rise to [^{3}H]ATR. CARB-ATR (20 mg) was dissolved in 0.5 ml of tritiated water (90 mCi/mMole). The pH was adjusted to 2.0 by addition of N HCl, and 2 mg of the Pd-Al_2O_3 catalyst was added. The reaction was permitted to proceed at 150°C in a distillation apparatus to recover the excess of tritiated water. After the dry residue had been maintained for 3 hr at 150°C, 1 ml of a mixture of water methanol (1:1, v/v) was added and mixed with the residue. This solvent was then removed by distillation. This washing was repeated five times in order to remove all traces of tritiated water and of exchangeable ^{3}H. Between 70 and 90 per cent of CARB-ATR was transformed into [^{3}H]ATR by this procedure. [^{3}H]ATR was separated from the non-reacted CARB-ATR by chromatography on DEAE-

cellulose. The mixture of [^{3}H] ATR and CARB-ATR was dissolved in 5 ml of water, and the pH adjusted to 8.7 by addition of a few drops of 2 M Tris-base. The solution was poured on a column of DEAE-cellulose (0.8 meq/g) of 4 cm of diameter and 25 cm height, equilibrated with 8.8 mM triethylamine-NH_4 bicarbonate (TEAB buffer) pH 8.7. Then, the column was washed with 200 ml of the same buffer. Increasing the molarity of the TEAB buffer to 24.5 mM allowed the elution of [^{3}H] ATR while the CARB-ATR was retained on the column. The purity of the [^{3}H] ATR was verified by chromatography and by electrophoresis [2]; its specific radioactivity was approximately 60×10^6 dpm/μmole.

Another method of preparation of [^{3}H] ATR has also been used, based on the oxidation by chromic acid [5] or dimethylsulfoxide [6] of the primary alcohol group of the glucose moiety of ATR into an aldehyde group, followed by the re-reduction of the aldehyde into primary alcohol by [^{3}H] sodium borohydride. To a solution of ATR (40 mg) in dimethylsulfoxide (2 ml) were added 0.2 ml pyridine, 0.1 ml phosphoric acid and 180 mg *N-N* dicyclohexylcarbodiimide. The suspension was stirred for 4 hr at room temperature and then filtrated. The filtrate was chromatographed on Whatman 3 paper in butanol, acetic acid, water (4:1:5 v/v). The aldehyde derivative of ATR, which migrates at the same level as ATR, was recovered by elution with water. After concentration under vacuum, the residue was dissolved in 5 ml methanol; 5 mg boric acid and 4.5 potassium [^{3}H] borohydride (50 mCi/mmole) were added. After stirring for 2 hr, acetic acid was added dropwise and the solution was evaporated. The purification of [^{3}H] ATR was carried out as as described above. Although both tritiation methods, that involving the decarboxylation of CARB-ATR and that based on the replacement of H by ^{3}H in the primary alcohol group of the glucose moiety of ATR, resulted in [^{3}H] ATR with similar specific radioactivity, the former one gave a much higher yield.

2.3. *Synthesis and purification of succinyl atractyloside, (Succinyl-ATR)*

The succinylated derivative of ATR was obtained by esterification of the primary alcohol of the glucose moiety of ATR by a succinyl group of succinic anhydride. Although the molecular stoichiometry was 1 to 1, the reaction proceeded more rapidly with an excess of succinic anhydride. 100 mg of ATR and 100 mg of succinic anhydride were vigorously stirred overnight in 100 ml of freshly-distilled pyridine at 30°C; 100 ml methanol were added, and permitted to react for 2 hr at 20°C in order to transform the excess of succinic anhydride into methyl succinate. The methanol and pyridine were then removed by evaporation at 40°C under vacuum. After two to three washings with methanol followed by evaporation, the residue was dissolved in a few ml of water and the pH brought to 8.7 by addition of a few drops of N NaOH. Then the solution was diluted with water (about 100 ml) until the conductivity had a value not greater than $2 \times 10^{-3}\ \Omega^{-1} \cdot cm^{-1}$, prior to the next step of DEAE-cellulose chromatography. The DEAE-cellulose equilibrated with 8.8 mM TEAB buffer pH 8.7, was packed in a column of 4 cm × 30 cm and the mixture of succinyl–ATR and methyl succinate was applied on the top of the column. Methyl succinate was eluted with 400 ml of 1.75 mM TEAB buffer pH 8.7 and the non-reacted [^{3}H] ATR with 200 ml of 24.5 mM TEAB buffer. Increasing the concentration of the TEAB buffer to 140 mM allowed the elution of the succinyl–ATR in a volume of 50 to 60 ml. The purity of the succinyl–ATR was determined by thin-layer chromatography on silica gel using a solvent containing chloroform–methanol–acetic acid–water, (55:25:8:4), and staining with a vanillin reagent [7]. The solution of succinyl–ATR was concentrated under vacuo at 40°C and the residue taken in a few ml of water.

2.4. *Synthesis of succinyl-ATR amino Sepharose*

Succinyl–ATR was covalently linked by a diaminopropylamine 'arm' to Sepharose 4B (fig. 1). Diaminodipropyl Sepharose was prepared according to Cuatrecasas [8], after activation of the Sepharose by cyanogen bromide. 100 ml of packed Sepharose 4B, resuspended in 100 ml of water, were mixed with 15 g of cyanogen

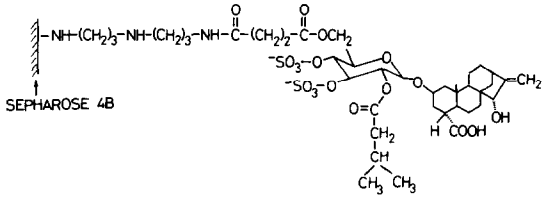

Fig. 1. Structure of succinyl–ATR-amino Sepharose.

bromide. The pH was brought to 11 by addition of NaOH and maintained at this value for 15 min at a temperature below 20° C. After cooling at 2° C, the activated Sepharose was quickly washed with 500 ml of 0.1 M sodium bicarbonate buffer pH 9.5, suspended in 100 ml of the same buffer with 200 nmoles of 3-3′ diaminodipropylamine, and left to react overnight at 4° C with stirring. 0.1 M glycine was then added to neutralize unreacted Sepharose groups, the pH being maintained at 9.5 [9]. The excess of glycine was removed by extensive washing on a Buchner funnel. The coupling of succinyl–ATR to diaminodipropyl Sepharose was essentially similar to that described by Cuatrecuasas for the preparation of an succinyl–estradiol Sepharose affinity column [8].

The amino–Sepharose was suspended in 100 ml of water and 300 mg of succinyl–ATR were added. The pH was brought to 4.7 by addition of 0.1 N HCl. 1 g of *N*-ethyl-*N*′-3-dimethylaminopropylcarbodiimide in 5 ml of water was slowly added and the coupling reaction was allowed to proceed overnight at room temperature. The resulting succinyl-ATR amino Sepharose was washed on a Buchner funnel 5 times with 200 ml of 1 mM phosphate buffer pH 7.6, 50 mM NaCl, 0.1% emulphogen and 10^{-3} mM dithiothreitol. When the substituted Sepharose was kept for periods of time longer than two to three days, 0.02% sodium azide was added to avoid bacterial contamination. In order to determine the binding capacity of the amino Sepharose for succinyl–ATR, a paraellel smallscale preparation was carried out with [^{3}H] succinyl-ATR. The binding capacity was found to be in the range of 0.15–0.30 μmole of succinyl–ATR per ml of Sepharose.

3. Results

Preliminary experiments, showed that when mitochondria were lysed by a non ionic detergent (Triton X-100 or emulphogen BC 720) they lost their ability to bind ATR or CARB-ATR. However, when ATR or CARB-ATR was allowed to bind to mitochondria first, and then detergent was added, the bound ATR or CARB-ATR was retained on macromolecular components that could be separated from remaining unbound inhibitor by gel chromatography. The bound inhibitor could be exchanged against added ATR or CARB-ATR, as shown by a double labeling experiment where mitochondria containing bound [^{3}H] ATR were lysed with emulphogen, and then subsequently incubated with ^{35}S-labeled ATR or ^{35}S-labeled CARB-ATR. After separation of the free and bound inhibitor by chromatography on a column of Biogel P4, it was found that the bound [^{3}H] ATR had been replaced by ^{35}S-labeled ATR or ^{35}S-labeled CARB-ATR. These displacement experiments provide the basis for the elution by [^{3}H] ATR or ^{35}S-labeled CARB-ATR as described below. Furthermore, the above data taken together indicate that the ATR-binding protein is altered markedly when it is extracted from mitochondria in the absence of ATR.

As shown in fig. 2 and 3, rat liver mitochondria bind succinyl [^{3}H] ATR with high-affinity (Kd ⩽ 50 nM). This bound succinyl [^{3}H] ATR can be displaced by ATR or CARB-ATR in less than 10 min at 2° C. It must be recalled that the K_d of the mitochondria for ATR and CARB-ATR are 20 to 30 nM and 5 to 10 nM respectively. It was therefore possible to recover the ATR or CARB-ATR-binding protein in radioactively labeled form by displacing it from the succinyl–ATR amino Sepharose with [^{3}H] ATR or ^{35}S-labeled CARB-ATR.

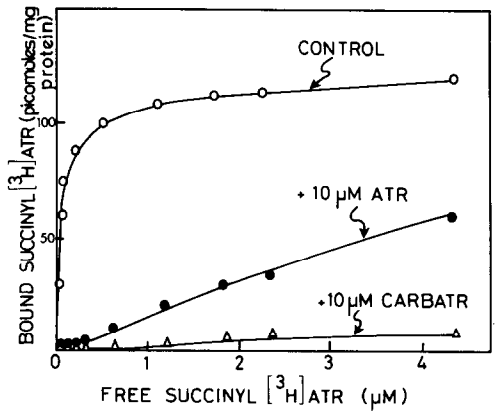

Fig. 2. Displacement of bound succinyl [^{3}H] ATR by ATR and CARB-ATR. Rat liver mitochondria (2.5 mg protein) were incubated in 5 ml of 0.12 M KCl, 10 mM Tris–HCl pH 7.3 and 0.1 mM EDTA for 30 min at 2° C with increasing concentrations of succinyl [^{3}H] ATR. Three parallel sets of incubation were carried out. After this first incubation period, unlabeled ATR was added to the tubes of one series to a final concentration of 10μM; unlabeled CARB-ATR was added to the other series to 10 μM; the third series was the control. After 15 min of incubation the tubes were centrifuged and the pellets were recovered for radioactivity counting.

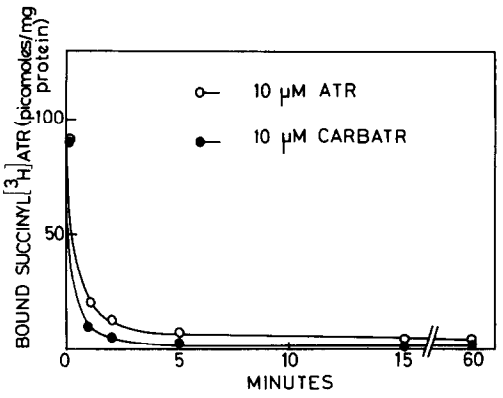

Fig. 3. Rate of displacement of bound succinyl [^{3}H] ATR by unlabeled ATR or CARB-ATR. Same conditions as in fig. 2. The concentration of succinyl [^{3}H] ATR used in the first incubation was 0.2 μM.

In a typical experiment, 1 g of rat liver mitochondria suspended in 30 ml of solution containing 0.25 M sucrose, 10 mM Tris–HCl pH 7.3 were incubated with 3 μm ATR for 30 min at 2°C. Then, 2 ml of a 20% emulphogen BC-720 solution in water was added (final concentration 1.5% and ratio of protein to emulphogen equal to 2.5), and left to stand for one hr at 5–6°C. The pH was maintained at 7.3–7.5. After lysis, the remaining mitochondrial fragments were removed by centrifugation at 145 000 g for 2 hr at 5°C. The supernatant was added to 100 ml of the succinyl–ATR-amino Sepharose in 100 ml of 1 mM phosphate buffer pH 7.4, 50 mM NaCl, 0.1 mM EDTA and 10^{-3} mM dithiothreitol and the suspension was gently stirred at 5–6°C overnight. After filtration on a Buchnerfunnel, the substituted Sepharose with bound protein was washed three times with 200 ml of 50 mM NaCl, 10 mM phosphate pH 7.4 and 0.1% emulphogen. Then [^{3}H] ATR was added to a final concentration of 50 μM and permitted to stand for 3 hr at 5–6°C. The resultant suspension was filtered on a Buchner funnel, and the supernatant fluid recovered. At this stage, the presence in the supernatant of protein with bound [^{3}H] ATR could be easily verified by gel filtration of an sample of the supernatant on a column of Biogel P4. As shown in table 1, when unlabeled ATR and CARB-ATR were added to the supernatant at a concentration roughly twice that of the free [^{3}H] ATR present in this supernatant, 6,9% and 80% of the bound [^{3}H] ATR was released as free [^{3}H] ATR, respectively. The released

Table 1
Displacement of bound [^{3}H] ATR by unlabeled ATR or CARB-ATR

Additions	Radioactivity present in the excluded fraction	Release of bound [^{3}H] ATR %
None (control	7636	
12.5 μM ATR	2360	69%
15.5 μM CARB-ATR	1551	80%

An aliquot of the eluate obtained by treatment of succinyl-ATR amino-Sepharose by [^{3}H] ATR (cf. Results) was diluted to bring the concentration of free [^{3}H] ATR to about 6 μM and then divided in three equal fractions. In one them, unlabeled ATR was added to a concentration of 12.5 μM; in the other one, unlabeled CARB-ATR was added to a concentration of 15.5 μM; the third one was the control. The volume of the fractions was adjusted to 0.5 ml and the three fractions were incubated at 5–6°C for 1 hr. The separation of high molecular weight components was carried out on Biogel P4 columns (1.5 cm X 45 cm) equilibrated with 1 mM phosphate pH 7.5, 50 mM NaCl, 0.1% emulphogen, 0.1 mM EDTA and 10^{-3} mM dithiothreitol. The values given in the table refer to the radioactivity of the high molecular weight components excluded from the Biogel.

[^{3}H] ATR was most likely replaced by the unlabeled ATR or CARB-ATR.

The supernatant fluid containing the bound [^{3}H] ATR was concentrated under vacuum at 25°C. The molecular weight of the protein containing high-affinity binding sites for ATR was estimated by chromatography on Sephadex in the presence of 0.1% emulphogen. At this stage, higher concentrations of emulphogen interfered with the binding of ATR. Chromatography on a G-100 Sephadex (fig. 4) allowed the separation of a main peak with a mol. wt. of 60 000 and a shoulder of about 15 000. When the 15 000 mol. wt. component was rechromatographed on Sephadex G-100, a peak corresponding to a mol. wt. of 60 000 was again eluted (insert of fig. 4) Occasionally, a minor peak at 30 000 appeared. Chromatography on G-150 Sephadex revealed the additional presence of a larger component with a mol. wt. of about 120 000. These data suggest that the ATR-binding protein has a tendancy to polymerize, as might be expected from its hydrophobic nature. However at this stage of our study, we have not been able to determine whether the 15 000 mol. wt. component is a subunit of the major 60 000 mol. wt. component.

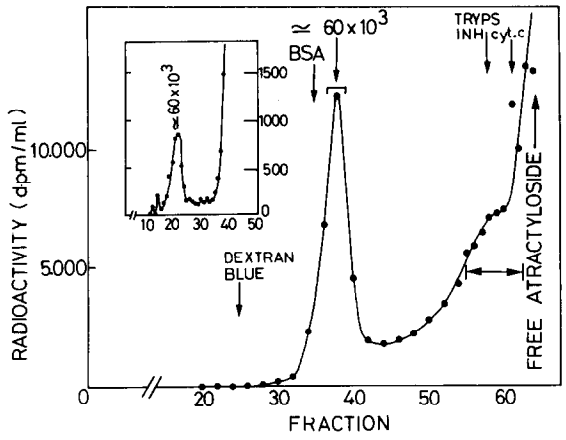

Fig. 4. Elution of an ATR-binding protein from a Sephadex G-100 column. The profile shows a main peak (mol. wt. 60 000) and a shoulder (15 000). The elution profile in the insert corresponds to a rechromatography of the 15 000 mol. wt. component.

^{35}S-labeled CARB-ATR was used in a similar manner as [^{3}H] ATR for displacement of protein bound to succinyl-ATR amino Sepharose. The high-affinity ^{35}S-labeled CARB-ATR binding protein also exhibited a major peak corresponding to a mol. wt. of 60 000.

The yield of the high-affinity ATR (or CARB-ATR)-binding protein, with a mol. wt. of 60 000, and its degree of purification, has been tentatively estimated, 1 g of rat liver mitochondria were found to yield 0.05–0.1 mg of the ATR-binding protein. The amount of bound [^{3}H] ATR varied between preparations from 5 to 10 nmoles per mg of the purified protein. Based on a the value of 0.14–0.20 nmoles of high-affinity binding sites for ATR or CARB-ATR per mg of mitochondrial protein [1], this represents about a fifty fold purification.

Acknowledgements

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