REVISED DATA ON (^{35}S)-ATRACTYLOSIDE BINDING TO MITOCHONDRIAL MEMBRANES.

P.V. Vignais and P.M. Vignais

Biochimie, C.E.N.-G. et Faculté de Médecine, Cédex-85
38 - Grenoble, France.

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In contrast to a previous report from this laboratory (^{35}S)-atractyloside is shown to bind preferentially to the inner membrane of rat liver mitochondria.

In a recent communication (1), it was reported that atractyloside preferentially binds to the outer membrane of mitochondria. This conclusion was based on experiments carried out with preparations of (^{35}S)-atractyloside containing labeled contaminants and decomposition products. In order to discriminate between (^{35}S)-atractyloside and radioactive contaminants bound to the membranes, only the radioactivity displaced by ADP had been taken into account. However, further experiments have shown that highly labeled contaminants in the (^{35}S)-atractyloside preparations used could bind to the outer membrane of mitochondria and could be partially displaced by ADP after binding. This observation has prompted an extensive purification of (^{35}S)-atractyloside and its use in direct binding experiments. In contrast with what was previously reported, data presented in this paper show that atractyloside preferentially binds to the inner mitochondrial membrane.

Methods :

Mitochondria were isolated in 0.27 M sucrose buffered by 2 mM Tris, HCl, pH 7.4 from a 10% homogenate by differential centrifugation. After centrifugation into mitochondrial membranes according to Parsons et al. (2), the "inner membrane + matrix" fraction was rehomogenized

in 20 mM phosphate buffer, pH 7.4, in a tightly fitting Potter-Elvejhem type homogenizer in order to isolate the matrix proteins from the inner membrane. After centrifugation through the same 3 layer sucrose density gradients (23.2%, 37.7% and 51.3%, w/v) (2) in a SW-39 Spinco rotor at 39,000 r.p.m. for 45 min., the inner membrane fraction is collected at the 3rd interface (between 37.7 and 51.3%) while most of the matrix proteins remain on top of the gradient (1).

(^{35}S)-atractyloside was extracted (3) from the rhizomes and roots of the thistle Atractylis gummifera which had been grown in the presence of (^{35}S)-sulfate. The alcoholic extract (3) was purified by chromatography on a neutral alumina column. The radioactive peak eluted with aqueous ethanol (50%, v/v) was purified by thin layer chromatography on silica gel H using the following solvent : chloroform/methanol/acetic acid/water (55:25:8:4, v/v). The radioactive spot moving like authentic atractyloside was eluted with aqueous ethanol (85%, v/v) and was further purified by electrophoresis on cellulose acetate (sepraphore III) in 0.2 M Tris, HCl, pH 8.5. The estimation of the amount of atractyloside present in (^{35}S)-atractyloside preparations, was based upon the degree of inhibition of mitochondrial respiration after having calibrated the system with crystalline atractyloside.

The composition of the incubation medium used to study the binding of (^{35}S)-atractyloside is detailed in the legend of Table I. After incubation, whole mitochondria or mitochondrial membranes were collected by centrifugation. The walls of the tubes were carefully washed with distilled water and the pellet was dissolved in 1 ml of formamide at 180°. Aliquot fractions were transferred to 20 ml of phosphor solution (6 g. of 2,5-diphenyloxazole (PPO), 0.3 g. of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) and 100 g. of naphtalene per liter of dioxan) and counted in a Nuclear Chicago scintillation counter.

The degree of purification of the membrane fractions was assessed on the basis of the specific activity of enzyme markers : monoamine oxidase for outer membrane (4) measured at pH 7.5 and 25° according to Tabor et al. (5), and cytochrome oxidase for inner membrane measured at pH 7.4 and 25° according to Appelmans et al. (6). Triton X-100 was used

to lyse the membranes prior to enzymatic analysis. The protein content was assessed by the biuret method (7).

Results :

To study the distribution of atractyloside in mitochondrial compartments, (Table I), mitochondria were first incubated for 60 min.

Table I

Distribution of bound (^{35}S)-atractyloside in submitochondrial fractions.

Fraction	Total protein (mg)	Bound (^{35}S)-atractyloside picomoles /mg protein	nanomoles (total)	Cytochrome° oxidase	Monoamine°° oxidase
mitochondria	650	59	38.4	1.9	5.2
low speed pellet	390 (60)	66	25.7 (67)	2.5 (79)	3.2 (37)
high speed pellet	32 (5)	51	1.6 (4)	0.4 (1)	34.0 (32)
supernatant	140 (22)	40	5.6 (15)	<0.1	<0.1
inner memb. + matrix	250	82	20.5	2.7	1.8
inner memb.	74	120	8.9	5.1	1.8
outer memb.	10	21	0.2	0.3	101.0

° micromole cyt.c oxidized/min./mg protein.
°° nanomole benzaldehyde formed/min./mg protein.
in brackets : per cent of recovery (calculated from total activity).

Mitochondria (800 mg of protein in 15 ml of 0.27 M sucrose) were incubated with 10 mM glutamate, 120 mM KCl, 10 mM Tris, sulfate pH 7.5, 6 mM $MgCl_2$ and 54 nanomoles of (^{35}S)-atractyloside (52,000 c.p.m.) in a final volume of 180 ml. After 60 min. of incubation at 0° the mitochondria were collected by centrifugation at 20,000 g for 20 min. (650 mg of protein), resuspended in 280 ml of 20 mM phosphate buffer, pH 7.4 (from which solution aliquots were taken for analysis) and allowed to swell for 30 min. at 2° (2). Three sub-fractions were subsequently obtained : a crude inner membrane + matrix fraction (low speed pellet) collected by centrifugation at 1,900 g x 15 min., a crude outer membrane fraction (high speed pellet) spun down at 35,000 g x 20 min. and the remaining supernatant fluid. The two crude membrane fractions were further purified as described in Methods but relatively large losses made it impossible to draw a balance-sheet of the recovery.

at 0° with (^{35}S)-atractyloside and were then collected by centrifugation. The mitochondrial pellet was suspended in 20 mM phosphate buffer and fractionated as described by Parsons et al. (2). Although the amount of (^{35}S)-atractyloside bound per mg of protein in the high speed pellet (crude outer membrane fraction) is of the same order of magnitude as that of the low speed pellet (crude "inner membrane + matrix" fraction), the total amount of (^{35}S)-atractyloside found in the low speed pellet is much higher than in the high speed pellet. On the other hand, the supernatant fluid contains about 15 per cent of (^{35}S)-atractyloside present in the original mitochondrial suspension (°). Purified fractions of "inner membrane + matrix" and "inner membrane" contain, per mg of protein, a markedly higher amount of (^{35}S)-atractyloside than the purified outer membrane fraction (Table I).

In a previous report (1) where atractyloside binding was analyzed with partially purified (^{35}S)-atractyloside and evaluated according to the radioactivity displaced by ADP, the outer membrane had been found to bind more radioactivity per mg of protein than the inner membrane. This erroneous result can be paralleled with the observation (see Introduction) that the displacement by ADP of bound atractyloside cannot be used as a criteria to differentiate (^{35}S)-atractyloside amongst (^{35}S)-organic compounds in a partially purified extract of rhizomes of Atractylis gummifera .

A subsequent report will show the occurence of high affinity (K_d <0.06 μM) and low affinity (K_d >0.4 μM) atractyloside binding sites in mitochondrial membranes ; the low affinity binding sites are essentially present in the outer membrane whereas the high affinity atractyloside binding sites are mostly located in the inner membrane.

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(°) On the basis of 2 μl of water per mg of protein in the mitochondrial pellet (8) and of 10% of inter-mitochondria water, it can be calculated that the radioactivity due to extramitochondrial water would represent less than 3% of the radioactivity of the original mitochondrial suspension.

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