(^{35}S)-ATRACTYLOSIDE* BINDING TO MITOCHONDRIAL MEMBRANES

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Although the inner mitochondrial membrane has a much higher affinity for ADP than the outer membrane, (^{35}S)-atractyloside was found to be specifically displaced by ADP from the outer membrane to which it binds selectively.

The evidence for the localization of the adenine nucleotide translocation system in the inner membrane of mitochondria has been put forward on the basis of its selective permeability toward ADP and ATP(1). Since atractyloside competitively inhibits the ADP-stimulated respiration (2-5) and other ADP- or ATP-dependent reactions in mitochondria (cf 4 for review), the inner mitochondrial membrane has been tentatively identified as the atractyloside barrier in mitochondria. However, although it has been shown by indirect inhibitory studies (6) that mitochondria bind atractyloside, no affinity measurements so far have been reported.

In the present study use was made of atractyloside labeled with ^{35}S in its sulfate moiety. In this preliminary report it will be shown that (^{35}S)-atractyloside selectively binds to the outer mitochondrial membrane and that bound atractyloside is specifically displaced by ADP. Data presented here also show that the inner mitochondrial membrane displays a much higher affinity for ADP than the outer membrane, which contrasts with the selective binding of atractyloside to the outer membrane.

Methods

Mitochondria isolated in 0.27 M sucrose buffered by 2 mM Tris, HCl, pH 7.4, from a 10 % homogenate by differential centrifugation were fractionated after phosphate induced swelling as described by Parsons et al. (7). A supplementary step was added to obtain an inner membrane fraction devoid of matrix : the inner membrane + matrix fraction recovered at the bottom of the 3 layer sucrose density gradient of the Parsons's procedure (7) (23.2 %, 37.7%,

* or atractylate

and 51.3 % w/v) was suspended once more in 20 mM phosphate buffer, pH 7.4 (final conc. ca 8 mg/ml) and rehomogenized in a tightly fitting Potter-Elvejhem type homogenizer and centrifuged through the same 3 layer sucrose density gradient with 0.5 ml of suspension (about 4 mg prot.) in a SW-39 Spinco Rotor at 39,000 r.p.m. for 45 min. Most of the matrix protein remains on top of the gradient and the inner membrane which gathers at the 3rd interface (between 37.7 % and 51.3 %) appears, under the electron microscope, mainly as large empty vesicles devoid of matrix (Vignais et al. in preparation).

(^{35}S)-atractyloside was isolated by the method of Wunschendorff and Braudel (8) from the rhizomes and roots of young plants of Atractylis Gummifera that had been grown in the presence of (^{35}S)-sulfate. The crude alcoholic extract was further purified by repeated column chromatography on a neutral alumina column with aqueous ethanol (50 % v/v) as eluant. This allows for the separation of atractyloside from pigments and from residual (^{35}S)-sulfate still present in the extract. The peak containing (^{35}S)-atractyloside was monitored by its radioactivity content and atractyloside was qualitatively characterized by its chromatographic behaviour on paper or on a thin layer of silicic acid (9). The amount of atractyloside was estimated polarographically from the inhibition, brought about by the extract, of mitochondrial respiration after calibrating the system with crystalline atractyloside.

The composition of the incubation medium used in experiments on binding of (^{35}S)-atractyloside or (^{14}C)-ADP is detailed in the legends. The length of incubation and the temperature chosen allow complete equilibrium between the bound and the free labeled compounds. After incubation, whole mitochondria or mitochondrial membranes were collected by centrifugation. The pellets were washed with a 0.9 % KCl solution buffered by 10 mM Tris, sulfate, pH 7.4, and then dissolved in 1 ml 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. In all the experiments bearing on the (^{35}S)-atractyloside binding, only that part of radioactivity removable by added ADP (ADP-sensitive bound atractyloside) was taken into account since (^{35}S)-atractyloside preparations contained labeled contaminants and decomposition products whose binding to mitochondrial membranes could be differentiated from that of atractyloside by

ADP insensitivity. For such a purpose two parallel series of incubations were carried out, the same incubation medium was used in both series except that ADP was added in one and omitted in the other. The amount of the ADP-sensitive bound atractyloside was calculated by substracting the amount of radioactivity bound in the presence of ADP from the amount of radioactivity bound in the absence of ADP.

AMP, ADP and ATP were assayed in neutralized perchloric extracts of mitochondrial membrane preparations according to Adam (10). Cytochrome oxidase activity was determined at pH 7.4 and 25° acc. to Appelmans et al.(11) and monoamine oxidase activity at pH 7.5 and 25° acc. to Tabor et al.(12). Triton X-100 was used to lyse the membranes prior to enzymatic analysis. The protein content was assessed by the biuret method (13).

Results

The capacity of isolated rat liver mitochondria to bind atractyloside was estimated by plotting the ADP-sensitive bound atractyloside (cf Methods) versus the ratio of ADP-sensitive bound atractyloside to free atractyloside (Scatchard plot). In the experiment represented in Fig.1 the maximum amount of the ADP-sensitive bound atractyloside (A) was 21 picomoles/mg protein and the concentration of atractyloside required for half maximal binding (K_d) was 0.14 $\mu\underline{M}$. In two other experiments, the values of A were found to be 24 and 20 picomoles/mg protein and those of K_d 0.16 $\mu\underline{M}$ and 0.17 $\mu\underline{M}$ respectively. The K_d values are of the same order of magnitude as the K_i value (0.1 μM) previously reported (5) for the atractyloside inhibition of the ADP-stimulated respiration in rat liver mitochondria. Not only ADP but also adenylylmethylene diphosphonate (AOPCP), the methylene analog of ADP, which is translocated into mitochondria as well as ADP (14) can displace bound (^{35}S)-atractyloside. UDP, CDP, GDP are virtually inactive.

Bruni et al. had reported earlier (6) that the amount of atractyloside which remains bound to rat liver mitochondria is 150 picomoles/mg protein, a value 6 to 7 times higher than those reported in the present paper. However the experimental procedure used by Bruni et al. (6) would be expected to lead to an overestimation of the number of binding sites. It consisted of a first incubation of fresh mitochondria with a known amount of atractyloside, then isolation of the mitochondria by centrifugation and estimation of the inhibitory capacity of the supernatant fluid on a new batch of fresh mitochondria. Besides

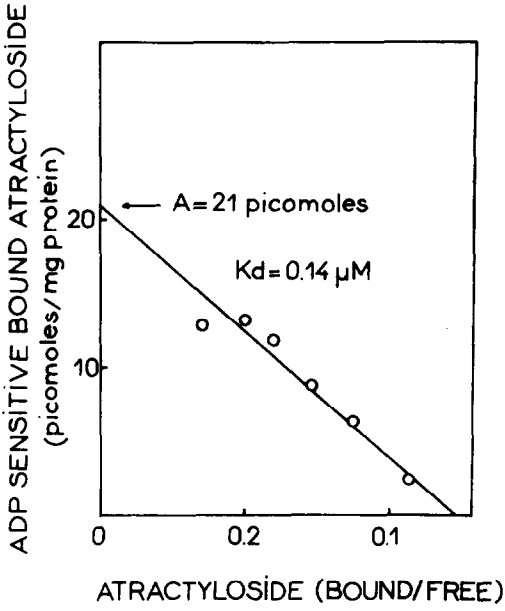

Fig. 1 : Scatchard plot of the binding of (^{35}S)-atractyloside to whole rat liver mitochondria. Two parallel series of incubation were carried out at 20° for 3 min. In both cases the incubation was started by addition of 0.2 ml of mitochondria in 0.27 M sucrose to 2.3 ml of the following medium : 10 mM glutamate, 6 mM $MgCl_2$, 120 mM Tris, sulfate, pH 7.4, and (^{35}S)-atractyloside (50,000 c.p.m.) - the initial atractyloside concentration ranging from 0.024 to 0.31 μM -. 5 mM ADP was present in one series of incubation tubes and omitted in the other. After 3 min. of incubation at 20°, the mitochondria were collected by centrifugation at 20,000 g for 20 min. at 2° and the ADP-sensitive bound atractyloside was determined as described in Methods.

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the unbound atractyloside the supernatant fluid might have contained an appreciable amount of ADP released, even at 0° (15), by mitochondria, which could have antagonized the inhibitory action of atractyloside on the second batch of mitochondria leading to an underestimation of the amount of atractyloside actually present.

The distribution of the ADP-sensitive bound atractyloside in mitochondrial membranes was determined on preparations of inner and outer membranes obtained from mitochondria previously loaded with (^{35}S)-atractyloside by short incubation with (^{35}S)-atractyloside both in the absence and in the presence of ADP. As shown in Table I, the outer membrane fraction displays a much higher affinity for atractyloside than the inner membrane fraction.

Table I

DISTRIBUTION OF THE ADP-SENSITIVE ATRACTYLOSIDE BINDING SITES IN MITOCHONDRIAL MEMBRANES.

Fractions	ADP-sensitive bound (^{35}S)-atractyloside*	Monoamine Oxidase**	Cytochrome Oxidase***
Mitochondria	5.8	4.7	1.25
Outer Membrane	17.0	140 ± 7	0.14 ± 0.02
Inner Membrane + Matrix	0.3	3.5 ± 0.1	2.50 ± 0.10
Inner Membrane	1.0	3.2 ± 0.7	3.60 ± 0.10

* picomoles/mg protein ** nanomoles benzaldehyde formed/mg protein
*** micromoles cytochrome c oxidized/mg protein

Two parallel series of incubations were carried out as described in Fig.1. In each of them mitochondria (1200 mg of protein) were incubated with 10 mM glutamate, 6 mM $MgCl_2$, 120 mM KCl, 10 mM Tris, sulfate, pH 7.4 and 2 µM (^{35}S)-atractyloside (1.1 x 10^6 c.p.m.) ; 2 mM ADP was present in one incubation medium and omitted in the other. The final volume was 60 ml. After 3 min. of incubation at 20° the mitochondria were collected by centrifugation at 20,000 g for 20 min. at 2°. Membrane fractions were isolated as described in Methods and their purity assessed on the basis of the specific activity of enzyme markers (monoamine oxidase for the outer membrane (16) and cytochrome oxidase for the inner membrane or inner membrane + matrix). The activities given are the mean of the values obtained for both series.

Allman et al. have said (17) that the atractyloside barrier is the outer mitochondrial membrane. Since they used GTP-AMP phosphotransferase as a marker of the outer membrane whereas it was found by other workers in the inner membrane (18)(19) the coincidence of the findings is probably fortuitous.

Contrary to atractyloside, (^{14}C)-ADP binds preferentially to the inner mitochondrial membrane. The maximal amount of (^{14}C)-ADP bound per mg of inner membrane protein as determined by Scatchard plots in Fig.2 (left side) is about 1 nanomole. Based on the protein recovery in fractionation experiments (unpubl. results) it can roughly be estimated that the inner membrane protein represents from 25 to 30 per cent of the total mitochondrial protein and calculated that at least 0.25 nanomoles of ADP can be bound per mg of total mitochondrial protein, an amount which is roughly 10 times higher than that of bound atractyloside. On the other hand, the K_d value for the binding of

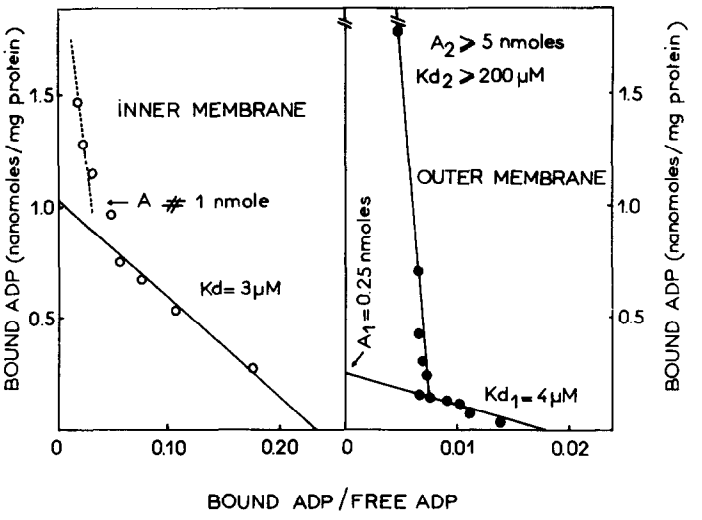

Fig. 2 : Scatchard plot of the binding of (^{14}C)-ADP to inner or outer membrane of rat liver mitochondria. Inner membrane (3 mg protein) and outer membrane (1.4 mg protein) were incubated for 30 min. at 0° in 4.8 ml of 10 m$\underline{M}$ Tris, HCl, pH 7.6, 120 m$\underline{M}$ KCl, 1 m$\underline{M}$ EDTA and (^{14}C)-ADP (2.2 x 10^5 c.p.m.) - the ADP concentrations used with inner membranes ranged from 1.0 to 51.8 μ$\underline{M}$ and those used with outer membranes from 1.0 to 117 μ$\underline{M}$ -. After incubation, the suspensions were centrifuged at 100,000 g for 30 min. at 0°, the pellets washed and their radioactivity determined as described in Methods.

ADP to the inner membrane as calculated from the slope of the Scatchard curve (full line, left side of Fig. 2) is 3.0 μ$\underline{M}$. In three other experiments the K_d values were 3.2, 3.0 and 2.1 μ$\underline{M}$. These K_d values are somewhat higher than that found by Winkler and Lehninger : 1.1 ± 0.2 μ$\underline{M}$ in a lubrol membrane preparation of rat liver mitochondria which was composed of both inner and outer membranes (20). Nevertheless they are in good agreement with the K_m for the ADP translocation in intact rat liver mitochondria : 4 μ$\underline{M}$ (21), 5.6 μ$\underline{M}$ (22)

The two slopes obtained for the plot of the binding of (^{14}C)-ADP to the outer membrane fraction (Fig.2, right) is likely to be due to the presence, in this fraction, of a small number of high affinity ADP-binding sites (0.25 nanomoles/mg protein) (K_d = 4.0 μ$\underline{M}$) and of a relatively large number of low affinity ADP-binding sites (≥ 5 nanomoles/mg protein) ($K_d \geq 200$ μ$\underline{M}$) ; the former which probably belongs to the inner membrane may partly reflect the contamination, by inner membrane, of the outer membrane fraction. It is interesting to note that the inner membrane devoid of matrix and the outer

membrane, even after extensive washing, still retain a significant amount of adenine nucleotides (from 2 to 3 nanomoles of AMP + ADP + ATP / mg protein). Preliminary experiments carried out with inner membranes previously loaded with (^{14}C)-ADP showed that the bound radioactivity is released upon addition of cold ADP which suggests, in agreement with data obtained by Winkler and Lehninger for lubrol membranes (20) that, at least in the case of the inner membrane, binding of the added ADP occurs by means of exchange with the adenine nucleotides already present there. The ADP binding to isolated mitochondrial membranes is only partially atractyloside sensitive (P. Vignais and O. Bârzu, in preparation) since atractyloside inhibition requires free Mg ions for maximal efficiency (23) ; however the medium used for ADP binding was supplemented with EDTA in order to avoid any conversion of the added ADP into ATP and AMP by enzymatic transphosphorylation.

As stated in the Methods, the experimental conditions used to study the binding of atractyloside or of (^{14}C)-ADP, although somewhat different, were chosen to allow maximal efficiency of binding at equilibrium to whole mitochondria or to mitochondrial membranes. With these restrictions in mind, the above results strongly suggest that the high affinity binding sites for ADP are located in the inner mitochondrial membrane. These results are in agreement with the proposal by Klingenberg and Pfaff (1) that the inner mitochondrial membrane is exclusively responsible for the adenine nucleotide translocation because of its selective permeability towards ADP and ATP. On the other hand, since the atractyloside binding sites are mostly located in the outer mitochondrial membrane and are therefore topologically distinct from the ADP binding sites, it is postulated that the atractyloside sensitivity of the ADP translocation in whole mitochondria results from indirect interactions between the atractyloside binding sites in the outer membrane and the ADP binding sites in the inner membrane. Another argument in favour of indirect interactions is that atractyligenin, the active moiety of atractyloside (5), competitively inhibits the ADP translocation although its structure is totally different from that of ADP. Indirect interactions between the atractyloside- and the ADP-binding sites may bring about a reversible change of the site-conformation as it was proposed in the concept of allosterism applied to regulatory enzymes (24) or to excitable membranes (25). Such interactions must require sites of contact with physical continuity between the inner and the outer membrane such as those described

by Hackenbrock in isolated rat liver mitochondria (26). Based on the protein content of a liver mitochondrion (1.1×10^{-13} g.) (27) and on the data reported here, a value of 1200 to 1400 atractyloside binding sites per mitochondrion can be calculated. If all the sites of contact between mitochondrial membranes (about 115 per mitochondrion with a diameter of 200 Å according to Hackenbrock (26)) are involved in the ADP translocation, ten molecules of atractyloside would be sufficient to saturate each area of outer membrane at the level of one site of contact, a value which is in reasonnable agreement with the size of these sites. Since the ratio of bound ADP to bound atractyloside in mitochondria is of the order of 10, it is inferred that each atractyloside binding site controls about 10 ADP binding sites. Consequently, if the binding of one molecule of atractyloside to the outer membrane initiates, in the inner membrane, a conformation change of one ADP binding site into an inhibited form, this change in turn must be propagated in a cooperative manner to the neighboring ADP-binding sites of the inner membrane. The nature of the primary effect of atractyloside on the outer membrane and the nature of the interactions remain an open question.

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