INTERACTIONS BETWEEN ADP AND ATRACTYLOSIDE ON THE MITOCHONDRIAL ADENINE NUCLEOTIDE CARRIER

P. V. VIGNAIS and P. M. VIGNAIS
Biochimie, Centre d'Etudes Nucléaires et Faculté de Médecine, Cédex 85, 38-Grenoble, France

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1. Introduction

The mechanism of the adenine nucleotide translocation in mitochondrial membranes can be approached by studying:

1) The binding of ADP and ATP which are the specific substrates for the adenine nucleotide carrier [1–4].

2) The binding of atractyloside which competitively inhibits the ADP-stimulated respiration [5–8] by inhibiting the adenine nucleotide translocation [1].

The study of ^{14}C-ADP or ^{14}C-ATP binding to mitochondrial membranes was worked out first by Winkler and Lehninger [9], then by Weidemann, Erdelt and Klingenberg [10–12]. For our part, we focused our attention on the second type of approach by using ^{35}S-atractyloside and inner membranes of rat liver mitochondria largely depleted of matrix. Some of our results have already been presented in preliminary communications [13–15].

Because of misunderstandings which have arisen over the interpretation of our data on ^{35}S-atractyloside binding (Weidemann, Erdelt and Klingenberg [12]), it seems desirable to elaborate some results previously published in a rather condensed way, and to describe some of the experimental conditions which are critical for the assessment and understanding of the processes involved.

2. Comments on the preparation of ^{35}S - atractyloside

The method of obtaining ^{35}S-atractyloside will be described in detail and discussed on a technical basis in a more comprehensive article to be published later. We shall summarize here briefly only some critical points. The labelling of atractyloside is obtained by growing the thistle *Atractylis gummifera* in the presence of ^{35}S-sulfate. Along with atractyloside, many pigments which move close to atractyloside in column or paper chromatography and which are able to bind to mitochondrial membranes become labelled. In the first preparations of ^{35}S-atractyloside, some unlabelled atractyloside had been added as carrier in the last steps of purification in order to check the coincidence of radioactivity with the coloration of the spot after migration in different solvent systems. This explains why the specific radioactivity of some of the batches used was not very high. At the beginning of these studies, the test used as purity control of the ^{35}S-atractyloside preparation was chromatography on silica gel plate [16] : at least 80 μg of atractyloside are necessary to obtain a good purple coloration after spraying *p*-dimethylaminobenzaldehyde; more is required if traces of contaminants are to be detected. When spraying *p*-dimethylaminobenzaldehyde, it did not appear that repeated runs on silica gel plates would alter the molecule of atractyloside. However, when ^{35}S-atractyloside was rechromatographed a second time on silica gel plate, although a single colored spot appeared after spraying *p*-dimethylaminobenzaldehyde, it was nevertheless possible to detect, by autoradiography of the plate, the presence of labelled compounds moving more slowly than atractyloside (when present in amounts large enough these compounds are revealed by the spray or by charring). It was therefore suspected that either the radioactive preparation was not stable or that radio-

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active contaminants could move together with atractyloside during migration. This is why the binding of ^{35}S-atractyloside was assessed by an indirect test: the measure of bound radioactivity which could be displaced by ADP. This approach was based on the observation that the ADP translocation in mitochondria is competitively inhibited by atractyloside and on the postulate that atractyloside bound to the adenine nucleotide carrier in mitochondrial membranes can be displaced by added ADP. At that time, we were far from suspecting that addition of ADP could, in some conditions (see below), increase the binding of atractyloside on the high affinity binding sites present in the inner mitochondrial membrane.

Subsequently a new separation procedure was set up (electrophoresis on cellulose polyacetate strip) [13, 15] which allowed quicker purity controls and necessitated much smaller amounts of atractyloside (5μg). By using this electrophoresis test we were able to see: 1) that ^{35}S-atractyloside is stable, 2) that the silica gel chromatographic step itself could bring about decomposition of ^{35}S-atractyloside. At the same time, binding studies had progressed and indicated the necessity of using very small concentrations of ^{35}S-atractyloside in order to be able to detect the high affinity binding sites. When using ^{35}S-atractyloside in direct binding experiments it was possible to demonstrate that atractyloside binds preferentially to the inner mitochondrial membrane [13]; namely, as is discussed below, the high affinity atractyloside binding sites are only found in the inner membrane, while only low affinity binding sites are found in the outer membrane [14, 15].

During these last three years, five different crops of radioactive *Atractylis gummifera* yielded ^{35}S-atractyloside preparations which were subsequently used for about 3 to 5 months. The specific radioactivity of ^{35}S-atractyloside used in the binding experiments ranged from 0.7×10^6 to 1×10^7 dpm/μmole.

3. Assessment of ADP effect

Two types of atractyloside binding sites in rat liver mitochondria have been differentiated on the basis of their affinity for atractyloside [14, 15], namely high affinity binding sites with a dissociation constant for atractyloside of the order of 0.01 μM and low affinity binding sites with a dissociation constant for atractyloside higher than 0.3 μM. High affinity binding sites were not found in the outer mitochondrial membrane [14, 15].

The high affinity for atractyloside displayed by the inner mitochondrial membrane is markedly decreased upon addition of micromolar concentrations of ADP (fig. 1 and [15]). Furthermore, whereas at low atractyloside concentrations ADP (or ATP) competes with atractyloside for binding to mitochondrial membranes, it paradoxically *increases the number of atractyloside binding sites at higher atractyloside concentrations* (figs. 1 and 2 and [15]). This paradoxical behaviour of ADP on atractyloside binding, if not carefully assessed in terms of binding affinity, may lead to erroneous conclusions on the properties of atractyloside binding.

The addition of increasing concentrations of ADP to mitochondrial membranes in equilibrium with ^{35}S-atractyloside led to a "titration" curve for atractyloside binding [15] with three characteristic portions which are the following: up to 3 μM ADP the apparent dissociation constant for atractyloside binding to inner mitochondrial membrane is increased by a factor of ten (0.01 μM in the absence of ADP, 0.1 μM at 3 μM ADP) and there is a parallel increase of the number of binding sites. Increasing the ADP concentration from 3 μM up to 300 μM does not increase the K_d value for atractyloside binding. Above 300 μM, the binding affinity for atractyloside is further decreased but the number of binding sites for atractyloside remains constant [15].

The effect of three different concentrations of ADP: 2 μM, 40 μM and 5.3 mM, representative of the three characteristic portions of the above mentioned "titration" curve, is illustrated in figs. 1 and 2. Above a critical concentration of total atractyloside (corresponding in figs. 1 and 2 to a ratio of bound to free atractyloside lower than 0.9 and 0.4, respectively), the amount of bound atractyloside is paradoxically higher (about twice) in the presence than in the absence of ADP. In these experiments, the final concentrations of atractyloside varied from 0.03 to 0.58 μM. This fact is to be correlated to the observation made by Weidemann, Erdelt and Klingenberg [12] of an "unexplained but reproducible small increase in the adenine nucleotide binding by addition of atractyloside at concentrations lower than 1 μM".

Whereas this phenomenon (increase of the atrac-

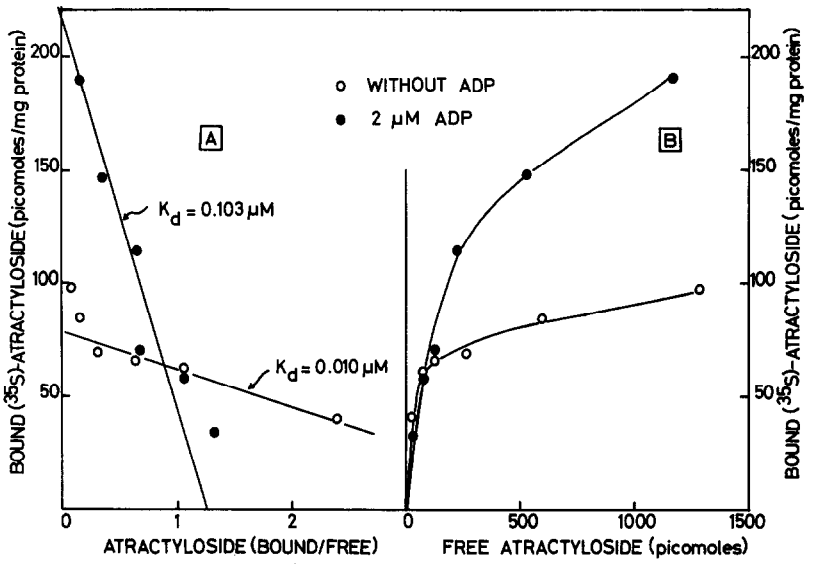

Fig. 1 : Binding of ^{35}S-atractyloside to the inner mitochondrial membrane in the absence and in the presence of 2 μM ADP. Two parallel series of incubation were carried out. In each series the inner membrane preparation [13, 18] was incubated for 45 min at 2° in 2.4 ml of 10 mM tris-sulfate pH 7.5, 120 mM KCl, 6 mM $MgCl_2$ and ^{35}S-atractyloside (7×10^5 dpm/μmole) at concentrations between 0.03 μM and 0.58 μM. Incubation was started by adding to each tube 0.1 ml of the mitochondrial inner membrane preparation (1.2 mg or protein) and ended by high speed centrifugation (30,000 *g*, 20 min). The pellets were dissolved in 1 ml of formamide at 180° and their radioactivity measured in a scintillation counter. The given ADP concentration is that found at the end of the incubation as estimated in the supernatant fluid by enzymatic assay [19].

A) Scatchard plot [20] of bound atractyloside against bound/free atractyloside.

B) Direct plot of bound atractyloside as a function of free atractyloside.

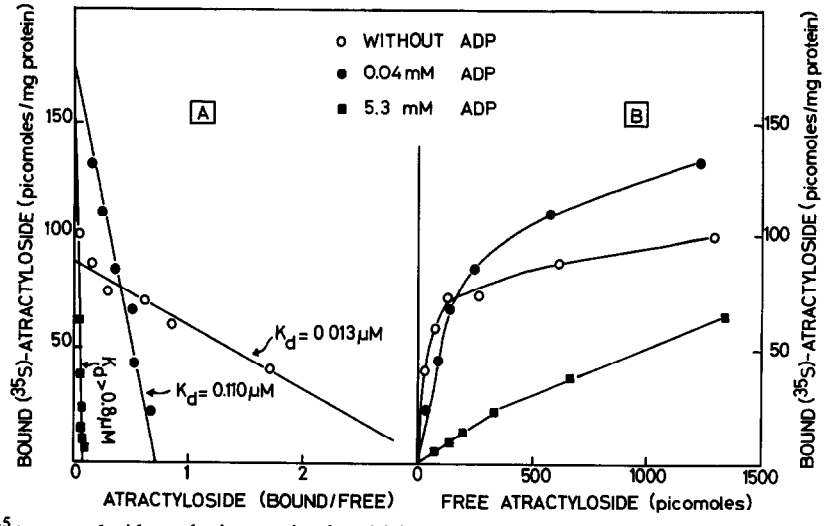

Fig. 2 : Binding of ^{35}S-atractyloside to the inner mitochondrial membrane in the absence and in the presence of 0.04 μM and 5.3 mM ADP. Same conditions as in fig. 1.

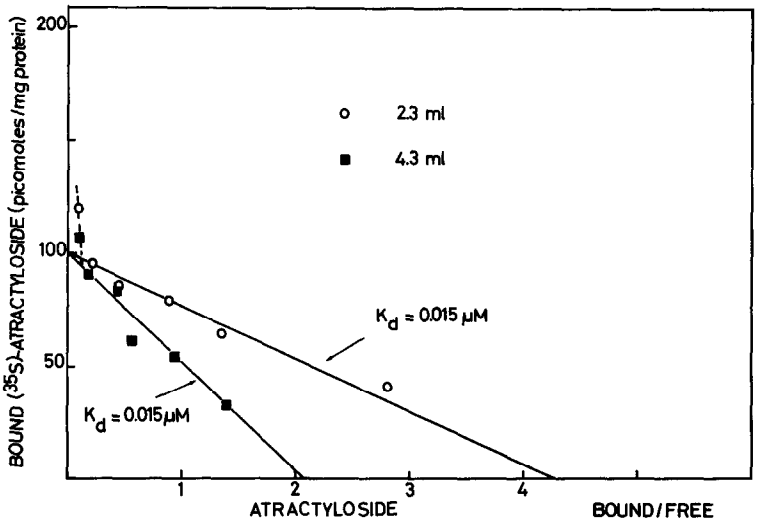

Fig. 3: Evaluation of the binding of ^{35}S-atractyloside to inner mitochondrial membrane in function of protein concentration. Same conditions as in fig. 1 except that the final volumes were 2.3 ml (o) and 4.3 ml (■).

tyloside binding by addition of micromolar concentrations of ADP) may reveal some aspects of the transport mechanism of ADP and ATP in mitochondrial membranes, it clearly shows that the use of ADP to remove bound atractyloside is restricted to a critical range of atractyloside concentration and that the ADP removable binding of atractyloside cannot be used without restriction as a criteria of the atractyloside binding. The stimulating effect of ADP on the atractyloside binding at critical concentrations may be relevant to the observed failure of ADP to remove ^{35}S-atractyloside from the inner mitochondrial membrane as reported in our first publication on atractyloside binding [17], an observation which led us to an unfortunate interpretation on the distribution of atractyloside binding sites between mitochondrial membranes.

4. Effect of protein concentration on the atractyloside binding affinity

The use of high concentration of membrane protein may result in unspecific effects for ligand binding. Being aware of this possibility, we kept the concentration of membrane protein at low values (lower than 0.7 mg/ml) to measure the atractyloside binding affinity [14, 15].

The following experiment clearly shows that in this range of protein concentration, the concentration of membrane protein may be largely varied without any change of the binding affinity for atractyloside. Two parallel series of incubation of inner membrane particles with ^{35}S-atractyloside were carried out. Each tube in each series contained increasing amounts of ^{35}S-atractyloside and the same amount of membrane protein. In one series, the volume of the incubation medium was about twice that of the second, and consequently, the protein concentration half (0.28 mg/ml and 0.52 mg/ml respectively) (fig. 3). In spite of the difference in protein concentration, the number of binding sites and the value of the dissociation constant are similar for both series of incubation (100 pmoles/mg protein and 0.015 μM respectively). Earlier experiments aimed at determining the permeability of mitochondria to atractyloside and the distribution of the atractyloside binding sites in whole mitochondria, by direct binding of atractyloside [13] required higher protein concentrations (800 mg of mitochondrial protein in 180 ml) in order to maintain, as much as possible, the integrity of the mitochondrial structure during the one hour incubation of this experiment. However, this experiment on direct binding of atractyloside [13]

did not involve the use of added ADP nor the displacement of atractyloside by added ADP. The comment by Weidemann et al. [12] that "no firm conclusions on the atractyloside binding can be drawn in view of the greatly diminished ability of low concentrations of atractyloside to displace nucleotides at protein concentrations over 2mg/ml" is not justified since 1) it does not apply to the relevant experiment, and 2) it does not discriminate between the data on the atractyloside binding distribution [13] and the data on the atractyloside binding affinity [14, 15].

Much effort is being directed towards obtaining labelled atractyloside of higher specific radioactivity. Labelled atractyloside is evidently an efficient tool to investigate the mechanism of adenine nucleotide translocation in the mitochondrial membrane. In connection with this approach it remains to be determined whether atractyloside binds only to the ADP binding sites or, besides, to specific sites controlling the adenine nucleotide carrier.

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