^{35}S-ATRACTYLOSIDE BINDING AFFINITY TO THE INNER MITOCHONDRIAL MEMBRANE

P.V.VIGNAIS, P.M.VIGNAIS and M.G.COLOMB
Laboratoire de Biochimie, Centre d'Etudes Nucléaires et Faculté de Médicine, 38-Grenoble, France

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Isolated inner mitochondrial membrane contains a small number of binding sites for atractyloside (of the order of 0.1 nmole/mg of protein) with very high binding affinity (half saturation at 0.014 μM atractyloside). The high affinity binding ability of the inner mitochondrial membrane is markedly decreased upon aging, acidification of the medium or addition of ADP, but remains unchanged in the presence of uncouplers such as FCCP. Added ADP causes a two-step transition from the high affinity binding to low affinity binding ($K_d > 0.50$ μM) and concomitantly a significant increase of the measured number of binding sites (about a doubling). The half maximum effect in the first step transition is given by 1 μM ADP. The use of ^{35}S-atractyloside as a probe of the inner mitochondrial membrane conformation specifically related to the adenine nucleotide translocation is discussed.

1. Introduction

When added to whole liver mitochondria ^{35}S-atractyloside preferentially binds to the inner membrane [1, 2]. In this paper, we report some typical properties concerning the binding of ^{35}S-atractyloside to isolated inner mitochondrial membrane. Attention has been focused on the loss of the high affinity binding of atractyloside upon addition of ADP, a remarkable characteristic which may be paralleled with the inhibition, by atractyloside of the ADP-translocation [3–6] which is apparently competitive [7–9].

2. Materials and methods

^{35}S-Atractyloside was extracted [10] from the rhizomes and roots of young plants of *Atractylis gummifera* which had been grown in the presence of ^{35}S-sulfate. The alcoholic extract [10] was purified by chromatography on alumina column and thin-layer silica gel as previously described [1] and then by electrophoresis on cellulose acetate (Sepharose III). The electrophoretic migration of ^{35}S-atractyloside (fig. 1) shows that, after a first purification run, the entire radioactivity is found in a single band which moves like authentic atractyloside and which, when tested by oxygraphy, contains all the biological activity.

Nucleotides were purchased from Sigma Chemical Company, St-Louis Mo. U.S.A., adenosine 5′-methylene diphosphonate (AOPCP) from Miles and Company, Inc., Elkhart, Indiana, U.S.A. and adenosine 5′-hypophosphate (AOPHP) was a generous gift of Professor J.P.Ebel from Strasbourg.

The isolation of the inner mitochondrial membrane from rat liver mitochondria according to Parsons et al. [11] and the enzymatic criteria used to assay the purity of membrane preparations were described in a previous paper [1].

The composition of the incubation medium used in experiments on binding of ^{35}S-atractyloside is detailed in the legends. After incubation at 2° for 45 min, the

Abbreviations:
AOPCP: Adenosine 5′-methylene diphosphonate
AOPHP: Adenosine 5′-hypophosphate
FCCP : Carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone
ANS : Anilino naphthalene sulfonate

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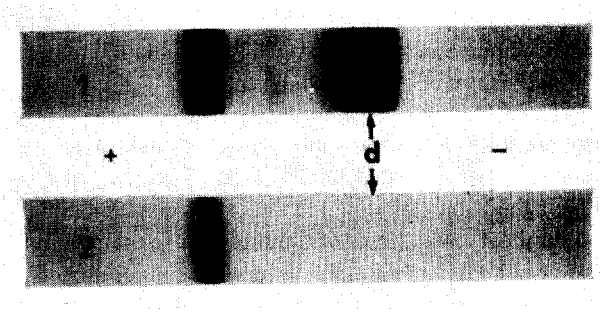

Fig. 1. Electrophoretic migration of ^{35}S-atractyloside. 1) Crude extract: 2) Pure ^{35}S-atractyloside. The run was performed on cellulose polyacetate strip (1" × 6¾", Sepraphore III) in 0.2 M tris-HCl, pH 8.5 (30 min, 4 mA per strip). Radioactivity was located by autoradiography.

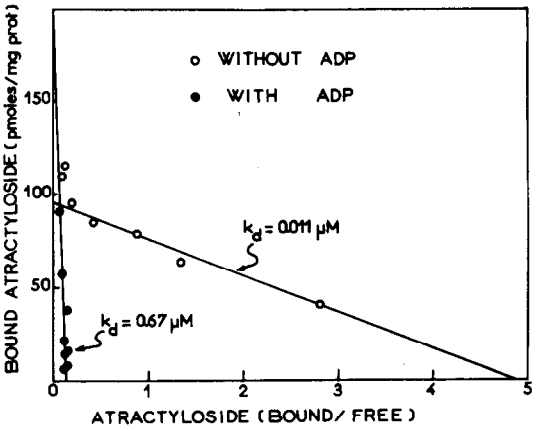

Fig. 2. Scatchard plot of the binding of ^{35}S-atractyloside to the inner mitochondrial membrane in the absence and the presence of ADP. Two parallel series of incubation were carried out. In both series, the inner membrane preparation was incubated for 45 min at 0° in 2.4 ml of 10 mM tris-sulfate, pH 7.5, 120 mM KCl, 6 mM $MgCl_2$ and ^{35}S-atractyloside (specific radioactivity 7 × 10^5 cpm/μmole) at various concentrations. ADP was omitted in one series of incubation tubes and present at 2.5 mM in the other. After incubation, the membranes were collected by centrifugation at 30,000 *g* for 20 min and the radioactivity of the pellet was measured as described in Methods.

mitochondrial membranes were collected by centrifugation. The walls of the tube 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 naphthalene per liter of dioxan) and counted in a Nuclear-Chicago scintillation counter.

3. Results

3.1. *Atractyloside binding*

The binding affinity of isolated inner mitochondrial membrane for ^{35}S-atractyloside has been estimated by the method of Scatchard [12] by plotting the amount of ^{35}S-atractyloside bound per mg of protein against the ratio of bound to free atractyloside (fig. 2).

In the absence of ADP, the experimental points can be joined by a straight line which leads to a calculated value of the dissociation constant (K_d) of 0.011 μM and a number (A) of binding sites of 95 pmoles per mg of protein. Average values of K_d and A in 12 experiments were 0.014 μM (σ = 0.004) and 90 pmoles/mg protein (σ = 20). In some experiments, the points determined at high atractyloside concentrations (fig. 2 open circles) begin to define a new line, the slope of which would represent low affinity binding. An uncertainty exists as to the location of the break point in the curve at which the weak binding sites would begin to be titrated, but the way in which the curve is drawn would tend to overestimate rather than underestimate the K_d values for high affinity.

Aging of the mitochondrial inner membrane results in the loss of the high affinity atractyloside binding sites (more than 25% after 16 hr of aging at 2°).

3.2. *ADP effect on atractyloside binding and specificity*

As shown in fig. 2, the addition of ADP (2.6 mM) results in a significant increase (about a doubling) of the detected number of atractyloside binding sites and concomitantly in a considerable decrease in the affinity of the inner membrane for atractyloside (K_d = 0.67 μM).

The loss of atractyloside affinity of the inner mitochondrial membrane upon ADP addition takes place in two steps which depend on the ADP concentration, the first one occurring at ADP concentrations lower

than 3 μM, the second one at ADP concentrations higher than 0.3 mM (fig. 3). The first step consists of a sharp but limited increase of both the K_d value and the amount of bound atractyloside. The K_d value which is of the order of 0.01 μM in the absence of ADP reached a value of 0.11 μM when the concentration of ADP was 3 μM; the half maximum effect was brought about at an ADP concentration of 1 μM, a value which is of the same order of magnitude as that found for the K_m relative to the ADP translocation in mitochondria [13, 14] or for the K_d relative to the ADP binding to mitochondrial membranes [2, 15, 16]. This decrease of affinity is parallel by an increase of the amount of bound atractyloside. This first step is followed by a plateau phase in which there is no further change in the atractyloside affinity when the ADP concentration is varied from 3 μM to 300 μM (insert of fig. 3). When the ADP concentration is raised above 300 μM (insert of fig. 3) the affinity binding for atractyloside decreases further but the number of binding sites for atractyloside apparently remains the same.

In the low concentration range, the effect of ADP on atractyloside binding is specific. It is not given by other nucleotides such as AMP, GDP, CDP or UDP. It is only partially given by analogues of ADP such as adenosine 5′-methylene diphosphonate (AOPCP) and adenosine 5′-phosphohypophosphate (AOPHP) which are exchanged to a limited extent with intramitochondrial adenine nucleotides (AOPCP ref. [14, 17]; AOPHP: unpublished results).

The effect of ADP is not likely related to the energy state of the inner mitochondrial membrane since it was found that FCCP (5 μM) does not alter the high affinity binding for atractyloside.

In short, two classes of binding sites for atractyloside can be described at the level of the inner mitochondrial membrane: high affinity binding sites demonstrated in the absence of added ADP and low affinity binding sites revealed by added ADP. The high affinity binding sites seem to be present only in the inner mitochondrial membrane; the low amount of such sites detected in preparations of outer mitochondrial membrane and of microsomal membranes (less than 10 pmoles per mg of protein) was likely due to pieces of inner mitochondrial membrane present as a contaminant.

3.3. *Ionic effect*

The atractyloside binding affinity is somewhat affected by the ionic strength of the medium (fig. 4). Thus, in order to minimize the small ionic effect brought about by added ADP, all the experiments described in this paper were made in saline media with ionic strength as high as 0.15. On the other hand,

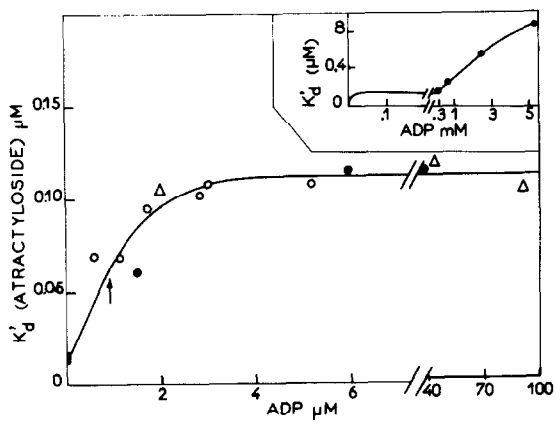

Fig. 3. Effect of different concentrations of ADP on the K_d value for atractyloside. Same conditions as in fig. 2: ○—○ 1.5 mg of protein; ●—● 1.6 mg of protein; △—△ 1.2 mg of protein. The variation in the whole range of ADP concentrations is given in the insert. The lower curve gives the first transition step to the plateau.

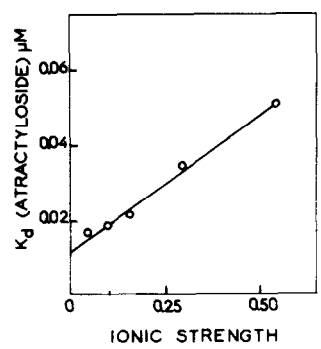

Fig. 4. Effect of the ionic strength on the binding affinity of atractyloside to inner mitochondrial membrane. The incubation medium (2.4 ml) used for each K_d determination, was made of 10 mM tris-sulfate, pH 7.5, 6 mM $MgCl_2$, ^{35}S-atractyloside and KCl. 1.8 mg of protein was used. Other conditions of incubation as in fig. 2.

the presence of Mg^{2+} was found necessary to maximize the change of affinity induced by ADP.

3.4. *pH Effect*

No alteration of the high affinity of the inner mitochondrial membrane for atractyloside is observed in the pH range of 5 to 9.8. However, below pH 5, the affinity for atractyloside abruptly decreases whereas the amount of bound atractyloside per mg of protein increases (fig. 5). This effect of pH may be related to changes in the ionization state of polar groups of either the membrane components specifically involved in atractyloside binding, or of the atractyloside molecule itself (for instance the protonation of the carboxylic group of the atractyligenin moeity which is the lipophilic part of atractyloside). In keeping with this idea, the loss of binding affinity at low pH values would correspond to a topological displacement of atractyloside to lipophilic binding sites (may be phospholipids) with low binding affinity.

3.5. *Reciprocal displacement of ADP and atractyloside*

The analysis of the atractyloside affinity to *whole mitochondria* by the Scatchard method allows the identification of two types of binding sites in the absence of added ADP: the first experimental points obtained at low atractyloside concentrations can be joined by a straight line (fig. 6 dotted line) which paradoxically indicates a low affinity (K_d > 0.5 μM) similar to the low affinity revealed by addition of ADP (fig. 6, solid circles) (K_d > 0.5 μM). This unexpected effect is likely due to the inhibition of the binding of ^{35}S-atractyloside by intramitochondrial ADP or ATP. After these endogenous nucleotides have been "titrated" by atractyloside, the high affinity type of binding (fig. 6 upper curve, K_d=0.013 μM) is immediately displayed. This would mean that bound endogenous ADP or ATP is displaced by atractyloside in the same way as bound atractyloside is displaced by ADP.

4. Discussion

Two possible explanations may be formulated to account for the above results: The first one that assumes a direct binding of atractyloside to the ADP translocase and a second based on interactions between atractyloside and components of the inner membrane which do not necessarily belong to the ADP translocase but which are structurally dependent of the functioning of this translocase.

According to the first hypothesis, the ADP trans-

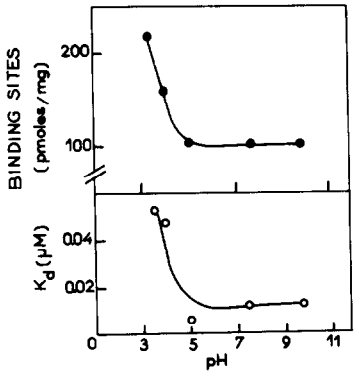

Fig. 5. Effect of the pH of the medium on the binding affinity and the number of binding sites of atractyloside to the inner mitochondrial membrane. The medium used for each K_d determination at a given pH, was made of 10 mM tris-sulfate buffer, 6 mM $MgCl_2$, 0.12 M KCl, 0.01 M glutamate and ^{35}S-atractyloside in a final volume of 2.4 ml. 1.7 mg of membrane protein was used.

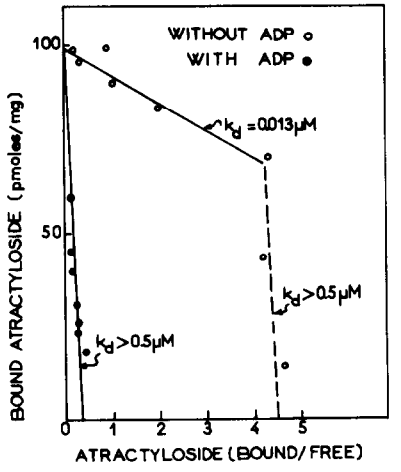

Fig. 6. Scatchard plot of the binding of ^{35}S-atractyloside to whole rat liver mitochondria. Same conditions as in fig. 2. The amount of mitochondria was 4.0 mg of protein.

locase units in the inner mitochondrial membrane would display two types of binding sites for atractyloside: one of high affinity detected in the absence of ADP, the other of low affinity revealed in the presence of ADP or at high concentrations of atractyloside. When added to the inner membrane, ADP would prevent the binding of atractyloside to the high affinity binding sites, possibly by allosteric interaction [9, 15], and this would then allow the disclosure of the remaining low affinity binding sites.

In the second alternative, micromolar concentrations of ADP, by inducing a conformation transition at a few membrane areas specifically related to the ADP translocation, would make accessible to the atractyloside those low affinity binding sites that are assumed to be hidden in the membrane core. In this case, ^{35}S-atractyloside could be considered as a probe of membrane conformation along with fluorescent indicators or spin labelled compounds. However, for the commonly used fluorescent probe ANS, the K_d values and the number of binding sites are considerably higher (about 1000 times) [18, 19] than those found here for atractyloside. In contrast with the specific and discrete changes of conformation detected by ^{35}S-atractyloside obviously only gross changes of conformation are revealed by ANS. Another difference between atractyloside and ANS bears on their respective response to uncouplers or inhibitors of oxidative phosphorylation in that the ANS fluorescence [18–20] but not the ^{35}S-atractyloside binding depends on the energized state of the mitochondrial membrane. In this latter respect, the behaviour of atractyloside is similar to that of aurovertin [21, 22].

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