ON THE RELATION BETWEEN ADENINE NUCLEOTIDE CARRIER SITES AND ATRACTYLOSIDE BINDING IN MITOCHONDRIA

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1. Introduction

In previous studies the adenine nucleotide carrier sites were determined by the competition of adenine nucleotide (ANP) binding with atractyloside (ATR) using isotope labelled ANP [1, 2]. Further insight can be expected by studying the binding of ATR using isotope labelled ATR. The production of labelled ATR has been attempted for considerable time and by various methods in this laboratory. The most feasible method was the biosynthesis of ^{35}S-ATR as produced in young plants, a method also applied by Vignais and Vignais [3, 4]. Thus a compound with high specific ^{35}S activity can be isolated, which has very similar properties to authentic ATR and can be applied in binding studies for ATR.

In the present communication, the binding of ^{35}S-ATR is studied in the same membrane preparations (ANP-depleted mitochondria from beef heart and rat liver) as those used previously for the study of the 'specific' ANP binding. These studies were aimed at elucidating the interaction between the binding of ANP and ATR similar as applied for the determination of the 'specific' ANP binding sites [1].

2. Methods

Seedlings of *Atractylis gummifera* were grown on carrier free ^{35}S-sulfate solutions and harvested after several weeks. The rhizomes were homogenized and extracted with hot ethanol, fractionated on Al_2O_3 columns by elution with ethanol–H_2O gradient. The radioactive fractions containing ATR were further purified by thin layer chromatography using an elution system according to [5]. The purity of the ATR preparation was checked by polyacrylamide gel and cellulose acetate electrophoresis. The titer of the ATR solution was determined by comparing with authentic ATR the inhibition of the ANP exchange in rat liver mitochondria. For this purpose the ANP exchange was inhibited by increasing amounts of the ^{35}S-ATR containing extract and, in a parallel experiment, of an ATR solution of known concentration. Similar titers for the ^{35}S-ATR extracts were obtained by isotope dilution using the binding of ^{35}S-ATR to mitochondrial membranes. In contrast, calibration of the extracts by inhibition of the O_2 uptake of rat liver mitochondria gave considerably smaller titers. This latter method, also applied by Vignais and Vignais [4], is indirect and can be expected to contain more systematic errors than the inhibition of the ANP exchange.

During 12 months different preparations of ATR were obtained with a total radioactivity of about 3 μCi and specific activities of 2 to 10 μCi/μmole.

Abbreviations:
ANP: adenine nucleotide
ATR: atractyloside

3. Binding of ATR depending on its concentration

The binding of ATR to ANP depleted beef heart mitochondria depending on the amounts of ATR added is shown in fig. 1. The binding is followed with and without ADP in order to demonstrate a possible competition of ADP with an ATR binding. A clear saturation binding curve is obtained which, surprising-

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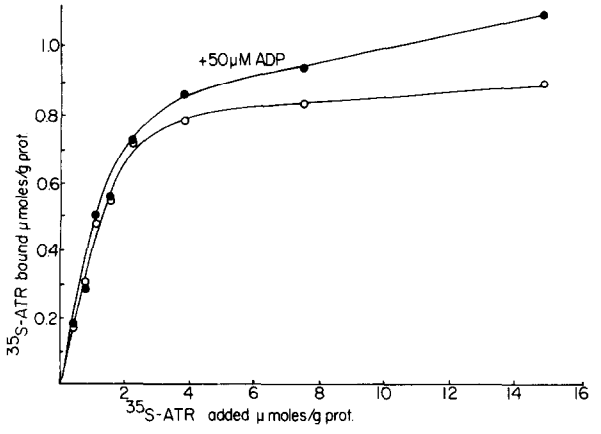

Fig. 1. The ^{35}S-ATR binding to membranes from beef heart mitochondria (BHM). Depleted BHM (1.06 mg protein/0.5 ml) incubated in standard incubation medium at 0°, pH 7.0. After addition of ^{35}S-ATR 2 min later centrifugation. The pellets are treated as described before [1] and the extractions counted for ^{35}S.

ly, is rather little influenced by ADP. In contrast to the expectations, at high ATR concentrations the maximum binding is even increased by ADP. A similar result has been observed also with liver mitochondria which will be shown below.

The binding parameters are analysed in a Scatchard plot shown in fig. 2. In view of the high affinity for ATR the amount of free ATR at low concentrations

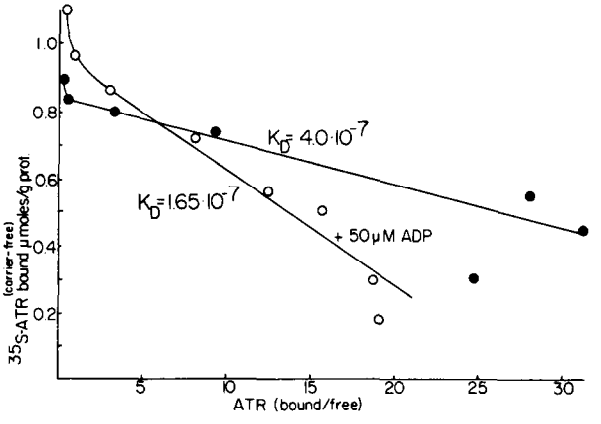

Fig. 2. Scatchard plot of ^{35}S-ATR binding to membranes from beef heart mitochondria. Data from fig. 1.

is very small and therefore the error of measurements is rather large as seen by the large scatter of points. Approximate linear curves are obtained except for the region of high concentration of ATR. Particularly with ADP this region of low affinity binding is increased. From the slope of the linear region the dissociation constants K_D are evaluated showing that under the influence of ADP the dissociation is increased about 2.5-fold in accordance with an expected competition of ADP with ATR. For the maximum number of binding sites only approximate values can be given in the range of 0.6 to 1.0 μmole/g protein.

In rat liver mitochondria (fig. 3) the binding of ATR both in undepleted and in depleted mitochondria is compared. In the undepleted mitochondria (fig. 3A) the binding curve of ATR has an opposite curvature to the depleted mitochondria, showing that the affinity apparently increases at higher concentration of ATR. In the presence of ADP the curvature is abolished and a single line is obtained which gives the same number of binding sites as without ADP (n = 0.12 μM/g protein). In effect, the affinity for ATR is decreased in the whole concentration range by ADP.

In the depleted rat liver mitochondria (fig. 3B) the affinity is generally lower for ATR than in the untreated mitochondria. The binding curves are partially non-linear, indicating high and low affinity sites. Under the influence of ADP the maximum number of binding sites apparently increases similar as in the beef heart mitochondria, altough a clear extrapolation is not possible. The affinity decreases under the influence of ADP with a $K_D = 7 \times 10^{-8}$ M to 6×10^{-7} M (+ADP).

Table 1
Binding parameters for ^{35}S-ATR in mitochondria.

n(μmole/g protein)	(+ADP)	K_D(×10^{-7} M)	(+ADP)
rat liver (100 μM)			
0.16	0.16	0.4	2.0
rat liver, depleted (200 μM)			
0.14	0.12	0.7	6.0
beef heart, depleted (50 μM)			
1.1	0.95	1.65	4.0

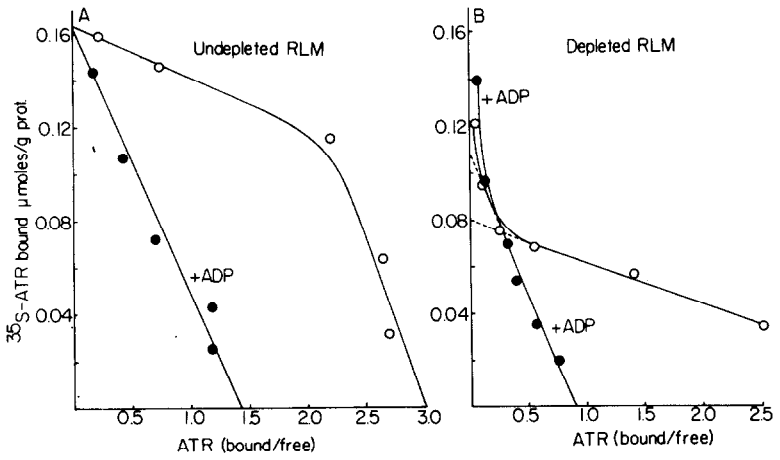

Fig. 3. ^{35}S-ATR binding in rat liver mitochondria (RLM) analysed in a Scatchard plot. (A) Binding in undepleted RLM (0.91 mg protein/0.5 ml). (B) Binding to RLM (20 mg protein/0.5 ml) depleted by treatment with 0.1 M phosphate buffer according to [1]. Incubation of mitochondria in standard medium. After addition of ^{35}S-ATR 2 min later centrifugation. Extraction of the pellets as described [1].

The results on the binding parameters are summarized in table 1 for the various types of mitochondrial membranes. Of particular interest is the comparison of these data with the ATR removable ANP binding in mitochondrial membranes. The dissociation constants K_D for the specific ANP binding are nearly 2 orders of magnitude higher than for the ATR binding. The higher affinity is in accordance with the very effective removal of bound ANP. A simple competition appears not to exist and these relations require further studies.

4. Stoichiometry of ATR binding and ANP removal

Of particular interest for the identification of binding sites for ATR and ANP at the ANP carrier is the stoichiometry of binding. As shown in table 1, the number of maximum binding sites for ATR, n_{ATR}, is about equal to that for ANP in liver mitochondria, n_{ADP} = 0.14 [1] as compared to n_{ATR} = 0.12 to 0.16 µmole/g protein, and in membranes from beef heart mitochondria the relation is n_{ADP} = 0.7 [1] to n_{ATR} = 0.9–1.1 µmole/g protein. In this case there is nearly 2 times more ATR than ADP bound.

The specific ANP binding at the carrier sites was defined by the portion of ANP removed by addition of ATR. An experiment directly examining this question is shown in fig. 4. Here simultaneously the binding of ^{3}H-ADP and ^{35}S-ATR is compared. The additions of increasing concentration of ^{35}S-ATR is performed at various concentrations of ^{3}H-ADP. Only at high concentrations of ^{3}H-ADP, when the ANP binding sites are nearly saturated, the maximum Δ^{3}H-ADP/ATR ratio can be expected since ATR binds to both unoccupied and ^{3}H-ADP occupied sites.

In accordance with the expectations, the ^{3}H-ADP removal reaches a maximum when ^{35}S-ATR binding also becomes saturated as seen at the cluster of points at the right end of the curves. This demonstrates that ^{3}H-ADP removal and ^{35}S-ATR binding are closely correlated. The slope gives the ratio of Δ^{3}H-ADP/ATR which increases, according to expectations, with the amount of ^{3}H-ADP added and the degree of occupation of the ANP binding sites. This experiment again demonstrates directly that more ADP is removed than ^{35}S-ATR bound.

4.1. *Binding of ATR to sonic particles*

It has earlier been found that in sonic particles ANP is still bound but that the binding is largely insensitive to ATR [1]. The ANP binding appears to be retained and it was therefore of great interest whether the binding of ATR by sonication is impaired. Further-

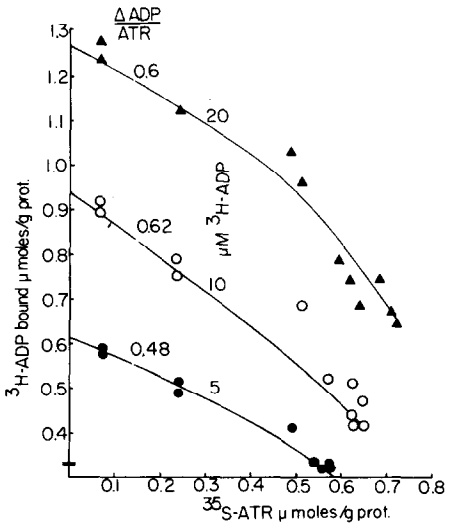

Fig. 4. Stoichiometry of ^{3}H-ADP removal and ATR binding. Depleted BHM (1.5 mg/0.5 ml) are incubated in standard medium as described. In each sample (0.5 ml) 0.04 μCi ^{3}H-ADP are added combined with cold ADP to 5, 10 and 20 μM.

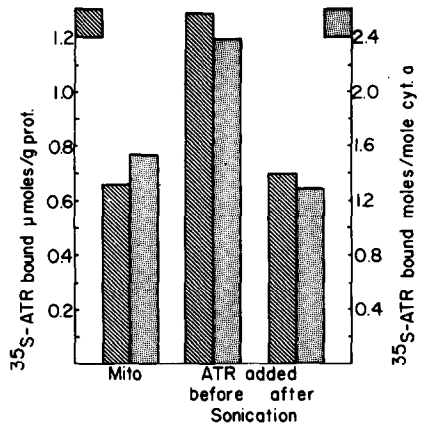

Fig. 5. Comparison of ^{35}S-ATR binding in mitochondria and sonic particles. Depleted BHM and sonic particles are incubated in standard medium under addition of 20 μM ATR. In one sample ATR was added after sonication. The sonic membranes were centrifuged at 100,000 *g* for 20 min. The pellet was resuspended in standard incubation medium for binding. In another sample ATR was added before sonic treatment. Centrifugation of the membranes as described above.

more it was asked whether the reversion of the inner membrane on sonication prevents ATR from reaching those sites which in the mitochondrial membranes are localized on the outer surface. For this purpose the binding of ATR was compared to mitochondrial membranes, to sonic particles and to sonic particles prepared from mitochondrial membranes already exposed to ^{35}S-ATR before sonication.

A typical experiment is shown in fig. 5 where the binding of ^{35}S-ATR to the three membrane preparations is compared and related to the cytochrome *a* content for eliminating the error of protein loss due to the sonication. The data show that the binding is retained in sonic particles to about the same extent as in the mitochondrial membranes. Considerably more ATR is, however, bound when added before sonication. Apparently in sonic particles as well as in mitochondrial membranes ATR has access only to 60% of the binding sites. The residual binding sites can be reached only when on sonication both faces of the membrane become accessible to ATR.

The results show that ATR has access only to outer sites of the ANP translocator in agreement with conclusions drawn from comparison of the inhibition by ATR of the ANP exchange and the ANP efflux of mitochondria [6, 7]. It has previously been assumed that there are binding sites localized on the inner surface of the membrane for ANP [1] and the present results extent this dual localization for the ATR binding sites. This would again indicate that the ANP carrier fits a dimer model with 2 binding sites at one cooperating carrier complex.

5. Conclusions

The data would indicate that only part of the binding sites for ATR are identical with the ANP sites: because of the stoichiometric excess of ATR bound to ANP removed and the persistence of ATR binding in sonic particles despite the insensitivity of ANP binding to ATR. Thus there could be effector sites for ATR similar as speculated by Winkler and Lehninger [8]. One should consider, however, that also only part of the ANP binding sites in intact mitochondria is ATR sensitive. This corresponds to the

reciprocal situation that not all ATR sites are accessible to ANP. This reasoning would permit that there is only one class of ANP binding sites in the membranes which are partially altered in the preparation. The main reason for this assumption are some similarities in the structure of ATR and ADP and furthermore that only ANP is the physiological substrate and effector for the ANP carrier, but not ATR.

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