THE NEW ATRACTYLOSIDE TYPE COMPOUND AS A HIGH AFFINITY LIGAND TO THE ADENINE NUCLEOTIDE CARRIER

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1. Introduction

In a preceding communication [1] the isolation was reported of a new atractyloside-type compound ATR′ from roots of *Atractylis gummifera* as the ^{35}S-labelled compound. This compound was shown to be also an effective inhibitor of the adenine nucleotide translocation in mitochondria. In the present communication studies on the binding of [^{35}S]ATR′ to mitochondrial membranes are presented similar to the previously reported studies on the binding of [^{35}S]ATR and [^{35}S]CAT [2–6]. The investigation concerns in particular the comparison of binding of [^{35}S]ATR′ with that of ATR and CAT.

2. Results

A more detailed study of ATR′ binding was performed on beef heart mitochondria since the experimental conditions are more favorable here and previously most studies on the binding of [^{35}S]ATR and [^{35}S]CAT were performed with these mitochondria. Fig. 1 illustrates the ATR′ binding in dependence on the amount of ATR′ added. The maximum number of ATR′ bound is about 2 μmole/g protein. This value agrees approximately with those reported for ATR and CAT. The mass action plot derived from these experiments is linear over nearly the whole range. From the slope the dissociation constant, $K_d = 8 \times 10^{-9}$ is evaluated indicating a very high affinity of ATR′. It is approximately equal to that of CAT and one to two orders higher than that for ATR [2, 3].

For a more discriminating comparison of ATR′ with ATR and CAT the competition of the binding between ATR′ and ATR or CAT was studied. It was noted earlier that for the interaction between two ligands at the carrier the sequence of addition can be of great importance. Therefore, also in the competition studies more information is obtained when ATR′ is added either before, after, or simultaneously with ATR or CAT. In the experiment of fig. 2 the competition of ATR′ with ATR and CAT is shown on adding ATR or CAT simultaneously with ATR′. Surprisingly, no difference in the competition between ATR, CAT and ATR′ is noted although it might be expected that ATR is less effective than CAT in competing with the binding of ATR′ due to its lower affinity. When first ATR′ is added, the subsequent addition of ATR and also of CAT is rather ineffective in removing ATR′. In the reversed sequence of additions, ATR′ is prevented from binding completely by CAT and up to 85% by ATR.

These data appear to reflect a certain irreversibility of the binding common to all three ATR homologues. The dissociation rate of one ligand once bound appears to be so slow that it does not exchange with the added ligand during the incubation time of 2 to 3 min. Therefore, the incubation time was extended up to 60 min after it was found in further experiments (not shown) that at this time the equilibration with ATR′ appears to be nearly complete. The results of such competition experiments at prolonged incubation time are shown in fig. 3. The concentration range of

Abbreviations:

ATR, atractyloside; BA, bongkrekic acid; CAT, carboxyatractyloside.

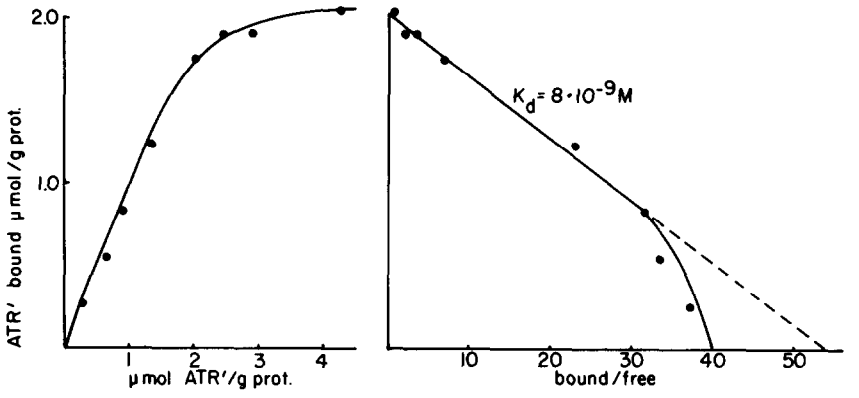

Fig. 1. [^{35}S]ATR' binding to beef heart mitochondria (BHM). Depleted BHM (0.04 mg protein/0.2 ml) were incubated in 0.25 M sucrose, 10 mM MOPS, 1 mM EDTA, pH 6.8 at 20°. [^{35}S]ATR' was added and mitochondria were centrifugated after 60 min. Sediments were extracted with 2% Lubrol. For further details see [7].

ATR and CAT has been decreased as compared to the experiments in fig. 2 in order to improve the resolution. Under these conditions, in fact the expected difference between ATR and CAT and the competition on simultaneous addition of the ligands is found. Increasing ATR up to a ratio of ATR/ATR' = 35, only about 30% of ATR' is removed, whereas at the same ratio CAT/ATR' the binding of ATR' has approached zero similar as in fig. 2. Obviously, as a result of the long incubation time, the binding of ATR' has increased and slowly reduced the initial competition by ATR. This occurs not only on simultaneous addition but even when ATR' is added 5 min after ATR (case ATR–ATR'). Thus, approximately the same level of ATR' binding (of about 60 to 70% of the level without ATR) is obtained independently of the sequence of ATR and ATR' addition, This indicates that equilibration between ATR' and ATR of the binding has been obtained during

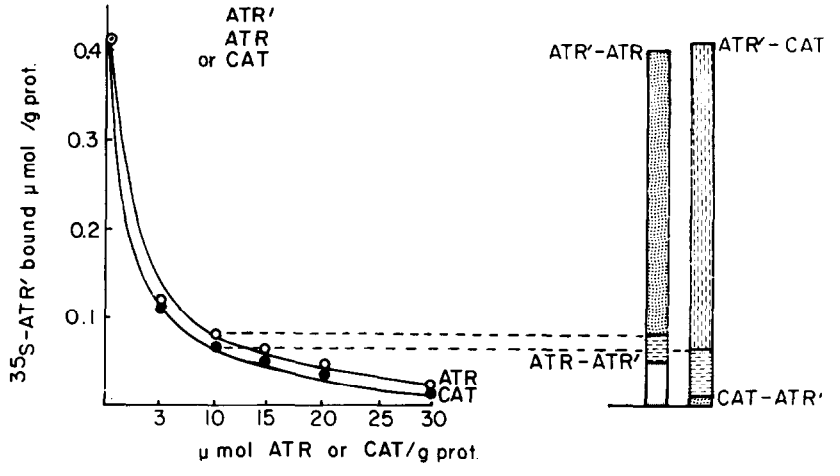

Fig. 2. Competition of [^{35}S]ATR' binding with ATR or CAT. Depleted BHM (0.11 mg protein/0.5 ml) were incubated in 0.25 M sucrose, 10 mM MOPS, 1 mM EDTA, pH 6.8 at 20°. ATR or CAT were added simultaneously with [^{35}S]ATR' (0.06 nmole) to BHM. After 2 min incubation, mitochondria were centrifugated and sediments extracted in 2% Lubrol. In addition the columns show the binding obtained by difference sequence of [^{35}S]ATR' and ATR or CAT. Binding sequences: ATR' plus CAT or ATR–2 min-centrifugation or ATR'–1 min–ATR or CAT–2 min-centrifugation.

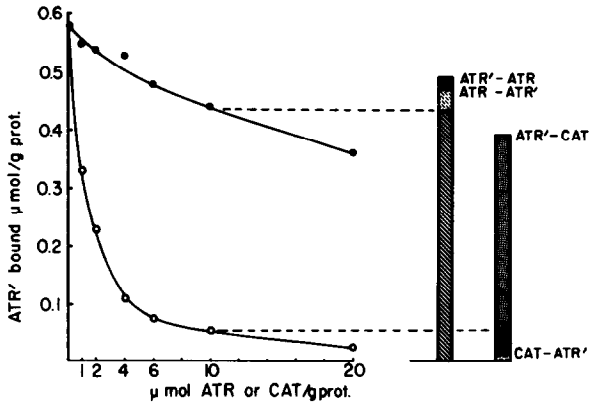

Fig. 3. Experiment as in fig. 5 (protein content 0.09 mg/0.5 ml), 60 min of incubation until centrifugation.

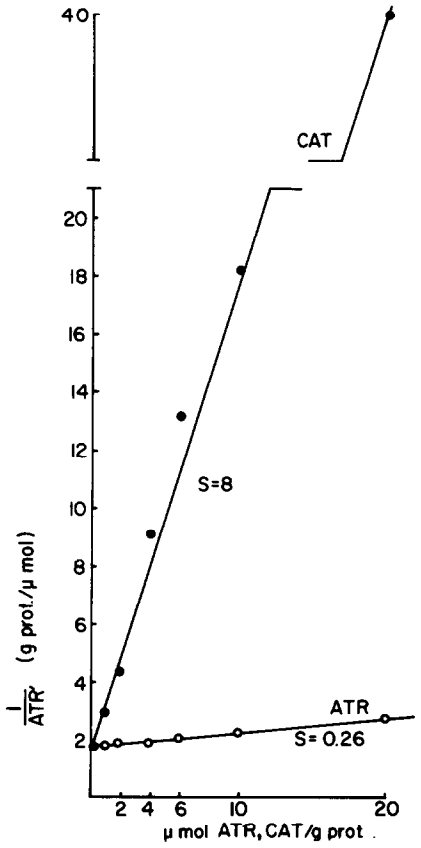

Fig. 4. Quantitative evaluation of the competition between ATR' and CAT or ATR. Reciprocal plot of data from fig. 3.

the long incubation time. In contrast, in competition with CAT large differences in the binding of ATR' remain between different addition sequences. Obviously, here no equilibration between both ligands has been reached even after 60 min.

The competition experiment is further graphically evaluated according to the following relation which holds for the competition between two ligands:

$$\frac{C_0}{CA'} = 1 + \frac{K_{A'}}{A'}\left(1+\frac{A}{K_A}\right) \qquad (1)$$

where C is the carrier, A' = ATR' and A = ATR or CAT, CA' = carrier–ATR' complex.

Since the free concentrations of ATR', ATR and CAT are not small as compared to their total concentration, the equation becomes complex and not explicitly solvable when A' and A are substituted by total concentration A'_0 and A_0. Nevertheless, the plot of $1/ATR'_{bound}$ against total amount of CAT and ATR added, gives a straight line (fig. 4) despite the non-linear relations inherent in equation (1). In fact the slopes of these lines fairly well represent the K_d's for CAT and ATR: the ratio of the slopes is 31, is approximate agreement with the ratios of K_d^{ATR}/K_d^{CAT} [4]. The results demonstrate that ATR' and CAT or ATR compete obviously for the same binding site at the carrier. The results further substantiate the functional similarity of ATR' to CAT.

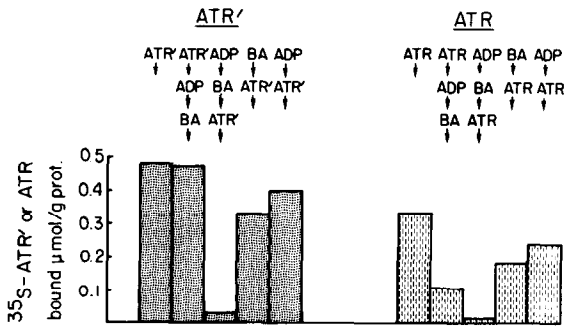

Fig. 5. Influence of bongkrekic acid on the binding of ATR'. Comparison of ATR' and ATR. Binding of [^{35}S]ATR' and [^{35}S]ATR according to different sequence of addition of ADP, BA and [^{35}S]ATR or [^{35}S]ATR'. Incubation medium as fig. 2. Protein content 0.11 mg/0.5 ml, 10 μM ADP, 10 μM BA, 1 nmole [^{35}S]ATR or [^{35}S]ATR'/mg protein. Total incubation time 8 min.

2.1. *The influence of bongkrekic acid on the binding of ATR'*

It was previously shown that bongkrekic acid (BA) is able to remove [^{35}S]ATR from the binding. Furthermore BA was unable to remove CAT, obviously due to the higher affinity of CAT as compared to ATR. Thus BA furnished a very important means of discriminating between [^{35}S]ATR and [^{35}S]CAT [4]. The application of this method is shown in the experiment in fig. 5 where the influence of BA on the binding of ATR' and ATR are compared. Here ATR' acts similar to CAT and different from ATR. When first ATR' is added and then BA (+ADP), no ATR' is removed. However, in the reversed sequence ADP–BA–ATR', ATR' is largely prevented from binding. BA alone also when added before ATR' cannot prevent the binding. The control experiment with ATR shows that here the sequence of addition is much less important. Even when ATR is added first, the subsequent additions of ADP and BA largely remove ATR. Thus also in this discriminating test ATR' is different from ATR and rather similar to CAT in that it resists removal on subsequent addition of BA. This is obviously the result of the high affinity of ATR' to the carrier. The strong difference between ATR and ATR' in the binding competition with BA serves as a qualitative assay for the discrimination between both compounds.

3. Discussion

The high affinity of ATR' (= epi-ATR) for the mitochondrial membranes is of great interest in particular in view of the following aspects. Originally, it might have been unexpected that the binding affinity of CAT is about 25-fold higher than of ATR, considering that the affinities of ATP and ADP differ only slightly and both CAT^{4-} and ATP^{4-} have four anionic charges at pH 7 as compared to three anionic charges of ATR^{3-} and ADP^{3-}. Thus the charge increase cannot explain the strong affinity increase of CAT as compared to ATR. The high affinity found for epi-ATR, which is close to that of CAT, may offer here an explanation. Both epi-ATR and CAT have in common the equatorial carboxyl-group which apparently is needed for high affinity binding. This shows that the configuration of the anionic charges is more important for the binding to the carrier than the number of charges. From this standpoint CAT and epi-ATR rather than ATR should be compared with ATP and ADP respectively.

Why does the equatorial carboxyl-group produce a higher affinity than the axial configuration? As one can deduce from space-filling models, the carboxylic group comes in the equatorial position somewhat closer to the two $-SO_3^-$ groups of the glucose moiety. On the other hand, the physiological ligands of the carrier, ADP or ATP, have their anionic charges in a very close neighborhood. Maximum binding affinity obviously is obtained when the anionic charges are not too far separated. The spatial arrangement of the charges of these ligands has been correlated in a previous discussion [3] with the conformation of the carrier and with the transition of the carrier from a mobile form on binding ADP or ATP, to an immobile form on binding ATR or CAT.

Acknowledgements

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