# EFFECT OF BONGKREKIC ACID ON THE ADENINE NUCLEOTIDE CARRIER IN MITOCHONDRIA: TIGHTENING OF ADENINE NUCLEOTIDE BINDING AND DIFFERENTIATION BETWEEN INNER AND OUTER SITES

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## Summary

The dissociation constants for binding of ADP at the adenine nucleotide (AdN) translocation site in heart mitochondria are decreased approximately 100-fold by bongkrekic acid (BA), indicating a strong increase in the affinity of the carrier for AdN. Accordingly, the rate of dissociation of AdN from the carrier sites is greatly decreased and becomes rate-limiting in the AdN translocation. The application of BA before or after equilibration of the endogenous pool with $^{14}C$-ADP also permits evaluation of the proportion of carrier sites (25%) accessible only to the endogenous pool.

Recently bongkrekic acid (BA) has been shown to inhibit adenine nucleotide (AdN) translocation across the inner mitochondrial membrane, in a manner similar to atractyloside (AT) (1,2). In the present paper, the effect of BA on the specific AdN binding to the carrier sites is examined. Previously (3,4) a specific binding of AdN to the carrier sites has been demonstrated and the dissociation constants, number of binding sites, etc. have been elucidated. The carrier-bound AdN is removed by AT, which successfully competes for a common binding site because of its higher affinity. As will be reported here, BA has the opposite effect: it fixes AdN to the carrier by increasing the affinity 100-fold, so that dissociation of AdN from the carrier becomes very slow. Furthermore, by virtue of this effect, BA application serves to elucidate separate inner and outer carrier binding sites.

## Methods

Beef heart mitochondria (5) stored at $-40^{\circ}$ retain the capacity for AdN exchange but contain less exchangeable AdN than fresh mitochondria (4). Such a "depleted" preparation is suitable for the differentiation of binding and exchange according to the principles discussed elsewhere (4). To make similar distinctions with BA, the experimental design consisted of the following sequences of

additions: (i) $^{14}$C-ADP alone, (ii) AT before $^{14}$C-ADP, (iii) BA before $^{14}$C-ADP, (iv) AT after $^{14}$C-ADP and (v) BA after $^{14}$C-ADP. With the exception of samples (ii) and (iii), where no AdN exchange took place, the total exchange time allowed was 2 min. The samples contained the following additions in 1.0 ml : beef heart mitochondria in 0.25 M sucrose (0.5 - 2.0 mg); 20 mM TRIS-maleate buffer, pH 7.0, 2 mM TRIS-EDTA, and 1 mM 3'-AMP (to inhibit adenylate kinase). BA and AT were added to give a final concentration of 10 μM. The $^{14}$C-ADP (4 x $10^4$ d.p.m.) contained sufficient unlabelled ADP to give final concentrations of 0.1 - 50 μM. To facilitate the application of BA (1) the experiments were conducted at 25°. The mitochondrial pellets were assayed for radioactivity following sedimentation, rinsing and deproteinization with $HClO_4$. The nucleotides remaining in the supernatant were separated into AMP, ADP and ATP by small column chromatography (6) to assess the interconversions of $^{14}$C-ADP. A more detailed treatment of the methods used is given elsewhere (4).

## Results

The effect of BA on the binding of AdN to the carrier sites in depleted mitochondria is shown in Fig.1. This experiment is conducted according to the procedures established in previous detailed studies (3,4) where AT was used to discriminate between the specifically bound, unspecifically bound and exchanged portions, as in the left column of Fig.1. $^{14}$C-ADP was added far below the range of saturation of the carrier sites (1 μM). AT and BA had opposite effects on the bound $^{14}$C ADP: where AT removed up to 25% of the label, the addition of BA increased $^{14}$C-ADP uptake by nearly 40%. The effect of BA cannot be explained by an increased exchange, since the $^{14}$C-ADP is already equilibrated with the small inner pool of AdN in the depleted mitochondria before BA addition. The increase is rather to be attributed to increased binding at the carrier. The total AdN bound in the presence of BA added after AdN is therefore the sum of AdN removable by AT (a) and extra AdN bound after addition of BA (d), i.e. $x = a + d$.

When AT and BA were added before AdN, less $^{14}$C-ADP was bound than in their absence. The decrease due to AT includes both the portion bound to the carrier sites (a) and the exchanged portion (b). BA added before AdN should inhibit incorporation of $^{14}$C-ADP into the exchangeable pool, yet after pretreatment with BA considerably more $^{14}$C-ADP was bound than with AT. The difference in

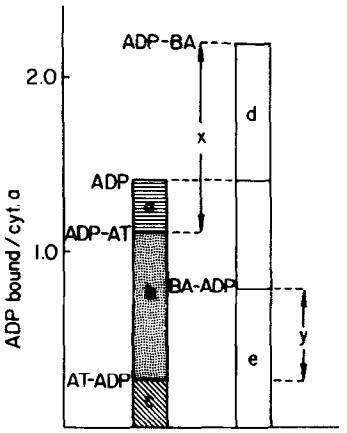


Fig.1. Effect of adding bongkrekic acid (BA) and atractyloside (AT) before and after $^{14}$C-ADP to beef heart mitochondria.

The sequence of addition of $^{14}$C-ADP (1 μM) and inhibitors, and the corresponding level of $^{14}$C-ADP uptake, are indicated by the solid horizontal lines in the columns. The shaded areas in the left-hand column represent those portions of the total $^{14}$C-ADP uptake defined by AT: a = AT-removable binding; b = AdN exchange; c = AT-insensitive binding. The other lettered areas are defined as follows: d = extra AdN uptake (in excess of control) when BA is added after $^{14}$C-ADP; e = total AdN uptake when BA is added before $^{14}$C-ADP. For the definition of x and y see the text. In calculating the $^{14}$C-ADP uptake, the specific activity was corrected for dilution by the endogenous AdN pool in samples ADP, ADP-AT and ADP-BA, and was uncorrected, because of inhibition of exchange, in samples AT-ADP and BA-ADP.

the amount of $^{14}$C-ADP bound with BA (e) and with AT (c) can therefore be taken as the binding to the translocation sites, i.e. y = e - c. Thus, also, after preincubation with BA, considerably more $^{14}$C-ADP is bound to the carrier sites. It is important to note, however, that the portion y is smaller than the portion x when BA is added after $^{14}$C-ADP.

This method of evaluating the portions which are specifically bound to the carrier when $^{14}$C-ADP is added before (x) and after (y) BA was applied over a wide range of ADP concentrations (0.1-50 μM), as shown in Fig.2. For comparison, the binding of $^{14}$C-ADP in the absence of BA was measured (as the portion discharged by AT) and found to reach saturation at 30 μM ADP. With BA added either before or after $^{14}$C-ADP more than 70% of the sites were saturated at 1 μM. The loss of some binding capacity above 10 μM ADP is an experimental artefact due to the change in this range to a higher protein concentration and a possible masking of some of the binding sites by protein-protein interaction.

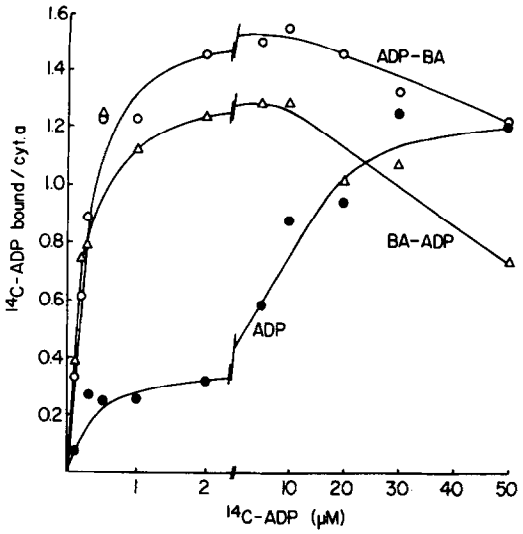


Fig.2. Concentration dependence of $^{14}$C-ADP binding to translocation sites in beef heart mitochondria and the influence of bongkrekic acid.
The experimental design and evaluation corresponds to that given in Fig.1. To obtain sufficient counts at each $^{14}$C-ADP concentration, the protein concentration was varied as follows: 0.1 - 1 μM, 0.4 mg/ml; 2 - 10 μM, 1 mg/ml; 20 - 50 μM, 1.8 mg/ml. The binding plotted is that defined from Fig.1: ●, no BA added = a; Δ, BA added before ADP = y; ○, BA added after ADP = x.

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Three conclusions may be drawn from these data:

1. The AdN binding sites have a greatly enhanced affinity for ADP under the influence of BA.
2. Significantly more sites can be occupied by $^{14}$C-ADP when BA is added after the endogenous pool has been equilibrated with $^{14}$C-ADP.
3. The total number of sites occupied by $^{14}$C-ADP when BA is added after ADP is the same as the number of sites occupied at much higher ADP concentrations in the absence of BA.

These studies on the concentration dependence of the binding were graphically evaluated according to the previously applied procedures for determining the number of binding sites and dissociation constants (3,4). The average data from two identical experiments are given in Table 1. AdN binding to the carrier sites in heart mitochondria has been analyzed, in the absence of BA, to show two binding sites of unequal affinity ($K_D$= 0.3 μM, $K_D$'= 6.6 μM) (4). In the presence of BA only one type of site is detectable, but the affinity of ADP is then 10-200 times higher ($K_D$= 0.02 to

TABLE 1

Parameters of AdN Binding to Translocation Sites under the Influence of Bongkrekic Acid

| Parameters measured | Experimental Conditions | | |
|---|---|---|---|
| | Control | BA added after ADP | BA added before ADP |
| Total sites/cyt.a ($C_o$) | 1.31 | 1.32 | 1.02 |
| Types of sites (%) | | | |
| High affinity ($K_D$<1μM) | 33 | 100 | 100 |
| Low affinity | 67 | 0 | 0 |
| Dissociation constants (μM) | | | |
| $K_D$ | 0.32 | 0.053 | 0.018 |
| $K_D'$ | 6.57 | | |

$0.05 \times 10^{-6}$M). If BA is added after $^{14}$C-ADP, the same number of sites ($C_o$= 1.32) can be occupied as at saturation in the absence of BA ($C_o$= 1.31). If BA is added before $^{14}$C-ADP, 25% of the sites are no longer accessible ($C_o$= 1.02). This corresponds approximately to the proportion of higher affinity sites previously identified in the absence of BA. It is an open question whether the sites not accessible to $^{14}$C-ADP after pretreatment with BA are identical with the high affinity sites.

The strong decrease in the dissociation constant should be reflected also in a decrease in the dissociation rate. This can best be measured by following the release of bound $^{14}$C-ADP by an exchange against an excess of unlabelled ADP. As shown in Fig.3, in the absence of BA bound $^{14}$C-ADP is exchanged with unlabelled ADP in less than 30 sec to the new equilibrium position. In the presence of BA the equilibration is considerably slower and not yet fully established in 15 min. This demonstrates that under the influence of BA ADP is extremely tightly fixed to the carrier site, an effect which strongly decreases the dissociation rate. Such a decrease should lead to a strong inhibition of the overall process of AdN translocation, which includes this dissociation step. Thus the effect of BA in inhibiting the AdN translocation is satisfactorily explained by the binding studies.

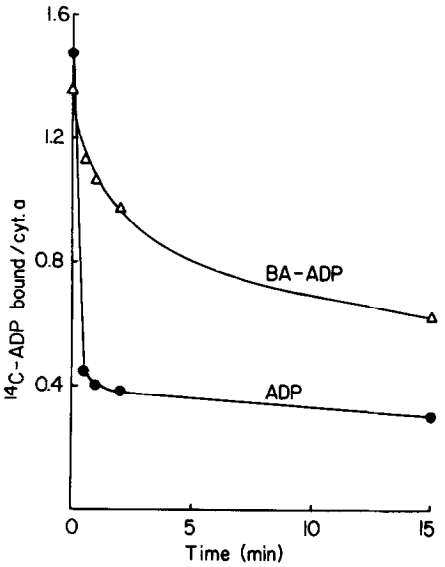


Fig. 3. Time course of equilibration of $^{14}$C-ADP (1 μM) with an excess of unlabelled ADP (10 μM) in mitochondria pretreated with bongkrekic acid (10 μM).
Mitochondrial samples (1.0 ml), either untreated or pretreated with BA, were prelabelled with 1 μM $^{14}$C-ADP. 10 nmoles unlabelled ADP was added after 2 min (0 time) and the time course of the equilibration followed for a further 15 min. The data are expressed as moles of original $^{14}$C-ADP remaining in the mitochondrial pellet/mole of cytochrome a. ● , no BA added; Δ , pretreated with BA.

## Discussion

An interpretation of the discriminating effect of BA based on the mobile carrier is illustrated in Fig. 4 and is in agreement with the interpretation that the two portions of the carrier sites correspond to an inner and outer location.

In the largely depleted mitochondria a small residual pool of unlabelled AdN is in equilibrium with carriers at the inner side of the membrane. The inner pool can be fully equilibrated with added $^{14}$C-ADP and in this case the inner and outer binding sites are equally labelled. If the affinity is then raised by addition of BA all the carrier sites, independently of their "position" (i.e. inside or outside), should be fully saturated with $^{14}$C-ADP. The only additional assumption for this explanation is that BA is permeable through the membrane, which might be expected in view of its lipid nature (7).

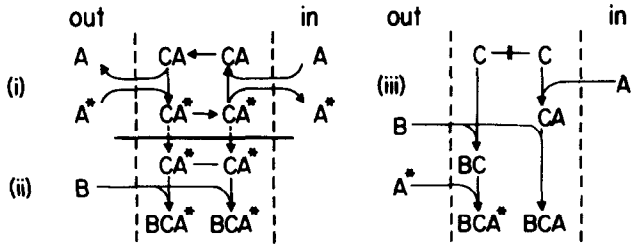


Fig.4. Scheme illustrating the distinction between "inner" and "outer" binding sites for AdN on the basis of a mobile carrier model. For clarity the following symbols are used: C = carrier, A = AdN, B = bongkrekic acid. The labelled nucleotides are signified with an asterisk. The following sequence is shown: (i) equilibration of inner and outer pools with A*; (ii) addition of B after equilibration with A*; (iii) addition of B before addition of A*.

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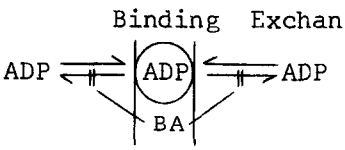
When BA is added before $^{14}$C-ADP, unlabelled endogenous AdN becomes fixed to the inner sites, while the outer sites remain unoccupied. $^{14}$C-ADP now added is fixed only to the unoccupied outer sites. The inner sites remain unlabelled since under the influence of BA endogenous unlabelled AdN does not exchange with $^{14}$C-ADP. Therefore the number of sites which cannot be labelled when BA is added before $^{14}$C-ADP gives an indication of the proportion of the sites located at the inner surface. In this way the discriminating application of BA permits one to gain insight into the mechanism of the AdN carrier.

In view of these results, the term "binding", widely used (2,8) for the total incorporation of labelled AdN into mitochondria, may be misleading and should therefore be defined more precisely. This "binding" actually includes the exchange with endogenous AdN and binding to the translocation sites:




AdN are "bound", in the proper sense, only to the AdN carrier, as was recently demonstrated (3,4). This binding is increased by BA, whereas the exchange is inhibited. The decreased "binding" observed by Henderson & Lardy (2) reflects therefore the sum of an increased binding to the carrier and an overwhelmingly larger decrease of the exchange.

It might be thought to be consistent with common usage to call the increased AdN binding in the presence of BA an "allosteric effect". However, it is not seen that the introduction of this term in the present context would serve to clarify the mechanism.

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