ON THE INHIBITION OF THE ADENINE NUCLEOTIDE TRANSLOCATION BY BONGKREKIC ACID

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Summary

Bongkrekic acid (BA) is shown to inhibit the adenine nucleotide (AdN) exchange in rat liver mitochondria. BA inhibits only above 20°, once applied at this temperature also at 0°. Quantitative evaluation of the inhibition of the translocation rate gives the number of inhibitor sites N_I = 0.2 to 0.3 µmoles/g protein and dissociation constant $K_I = 2 \times 10^{-8}$M. Similar results are obtained for inhibition of respiration. It is shown that, in accordance with the inhibition of translocation, BA inhibits phosphorylation only of exogenous but not of endogenous AdN, similar to atractyloside (AT).

It was reported some time ago by Welling, Cohen and Berends (1) that bongkrekic acid (BA) has an oligomycin-type effect on respiration and phosphorylation of rat liver mitochondria. Recently, Henderson and Lardy (2) observed that BA inhibits the adenine nucleotide (AdN) "binding" to mitochondria, which has been shown to reflect largely the AdN exchange (3), in a manner similar to atractyloside (AT). In view of our longstanding interest in the specific AdN exchange, the use of BA for studies of the mechanism of translocation appeared to us of great interest. Therefore the influence of BA* on the AdN exchange was studied by the procedures used for elucidating the AdN exchange, with particular attention to a quantitative evaluation of the inhibitory parameters.

Effect of Bongkrekic Acid on the Adenine Nucleotide Exchange

An unforeseen problem in the application of BA on the AdN exchange was the finding that BA requires increased time, and temperatures above 20° for its effectiveness. In experiments on respiration and oxidative phosphorylation reported so far (1), BA

* Bongkrekic acid was kindly supplied to us by Prof.W.Berends, Biochem.Lab. of the Technological University, Delft.

has been applied at 30°. For resolving the kinetics of the AdN translocation, usually lower temperatures have to be applied (3,4). However, when BA was applied at this lower temperature in initial experiments, no inhibition of translocation was found. This is demonstrated in Table 1, where the rate of AdN translocation is measured at 0° under the influence of BA and AT. When BA is added at 0°, even when present for more than 15 min, the translocation rate is not inhibited. After preincubation of the mitochondria with BA for 1 min at 25°, the rate of translocation is inhibited by 80%. AT inhibits the translocation by 70% also when added at 0°. Further results on this remarkable temperature influence will be published separately.

Table 1

Temperature Dependence of Bongkrekic Acid Application on the AdN Exchange

Temperature of addition	Translocation rate (at 0°) μmoles/min/g prot.
Bongkrekic Acid	
-	4.6
0°	7.0
25°	1.20
Atractyloside	
0°	1.32

AdN translocation rate measured by the "back exchange and inhibitor stop" method (8). Rat liver mitochondria with endogenous ^{14}C-prelabelled AdN are exposed to unlabelled ADP (0.2 mM) for 20 sec at 0°. Exchange is stopped by addition of AT. Evaluation according to assumption of first rate kinetics of exchange (see ref.4).

For measuring the effect of BA on the AdN translocation the procedure applied in the following experiments was to add BA to the mitochondria at 0°, warm the mitochondria up to 25° for 1 min and then perform the translocation again at 0°. In experiments performed according to this procedure, the inhibition of the AdN exchange by BA was measured as a function of the concentration of BA. As shown in Fig.1, the concentration of BA required for half-

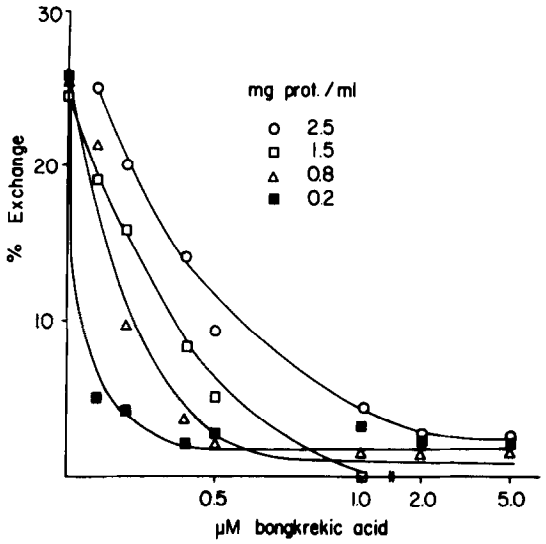

Fig.1. Influence of BA on the AdN exchange in liver mitochondria. The AdN exchange was measured by the method of the back exchange (see ref.8), i.e. endogenous AdN are equilibrated before exchange with ^{14}C-ADP and washed. Mitochondria at the various protein concentrations indicated were added to 1 mM sucrose-EDTA solution at 0°. Then BA at the respective concentrations was added. The vessels were then warmed up rapidly to 25°, kept for 1 minute at this temperature, and rapidly cooled down to 0°. For starting the exchange, 0.2 mM unlabelled ADP were added by a special device to 8 samples simultaneously. The exchange was stopped by addition of 50 μM AT. Then the vessels were centrifuged for sedimentation of the mitochondria in a microcentrifuge. Aliquots of the supernatant were counted for ^{14}C-ADP released by the exchange with added ADP. Appropriate controls for the release of nucleotides without exchange ("leakage") and for the AT-insensitive exchange were accounted for in the calculations of % exchange. 100% exchange is defined as the amount of AdN exchange after 20 minutes without BA.

maximum inhibition of % exchange increases with the amount of mitochondrial protein. Approximately full inhibition is reached in the range of 0.5 to 1 μM BA.

For a quantitative evaluation the % exchange was converted to translocation rates (V_T) on the basis of the first order reaction kinetics of the exchange and the size of the endogenous exchangeable pool of AdN. The degree of inhibition, $i = 1 - V_T/V_{To}$, serves to evaluate the number of inhibition sites N_I and the dissociation constant for the inhibitor K_I according to eq.1 (cf.ref.5):

$$\frac{I_o}{i} = N_I + \frac{K_I}{1-i}$$

i = degree of inhibition
I_o = total amount of inhibitor
K_I = dissociation constant of inhibitor
N_I = binding sites for inhibitor

According to eq.1, straight lines are to be expected in a plot of I_o/i versus 1/1-i, and therefore straight lines are drawn interpolating between the scattered points. The intercept with the ordinate gives the number of inhibiting binding sites for BA. For three concentrations of protein, a range of 0.22 to 0.3 µmoles binding sites/g protein was evaluated. The slope of the curve should correspond to the inhibition constant K_I. It varies between various concentrations in contrast to expectations, possibly due to some problem in the application of BA; K_I= 0.02 to 0.08 µM.

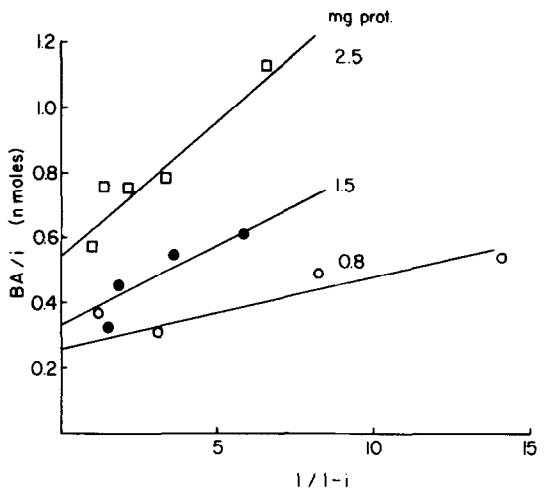

Fig.2. Graphical evaluation of the number of inhibition sites for the inhibition of the AdN translocation by BA. The data of Fig.1 were transformed from % exchange into (absolute) translocation rate V_T (µmoles/g protein/min), assuming first order kinetics of the exchange according to the procedure established previously (4). The translocation rate was used to calculate the binding of BA to the mitochondria according to eq.1. The degree of inhibition is $i = V_T(\text{inhibited})/V_{To}$.

Effect of Bongkrekic Acid on Respiration

On the basis of these results and those of Henderson and Lardy (2) the effects of BA on respiration, as shown first by Welling et al. (1), can be attributed to an inhibition of the AdN translocation. A comparison of the titre of BA and AT required for inhibiting respiration, as shown in Fig.3, demonstrates that BA apparently has a "higher affinity" for the inhibition than does AT. The titre for the BA inhibition can be evaluated as 0.3 µmoles/g protein in good agreement with the "titration" of the AdN translocation, as shown in Fig.2. AT is needed at higher con-

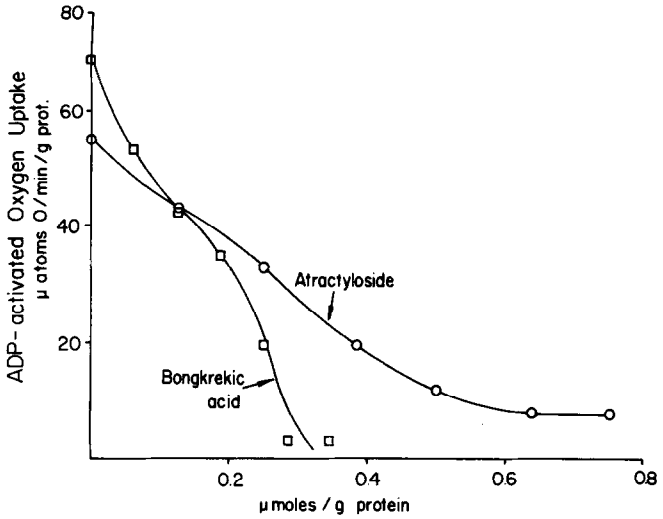

Fig.3. Dependence of the active state respiration on the concentration of BA and AT. Liver mitochondria incubated in the presence of 5 mM Pi, pH 7.2, air saturated. Substrates: 2 mM succinate, 2 mM glutamate. Active respiration was started by addition of 1 mM ADP. The respiratory rates refer to the ADP-stimulated respiration only. Amperometric recording of oxygen consumption using a differentiator.

centrations, apparently due to the higher dissociation constant of the AT binding site. These results agree with those of Welling et al. (1) who observed that BA inhibits respiration by 70% in the range of 10^{-7} to 10^{-9} Moles/mg protein.

Effect of Bongkrekic Acid on the Phosphorylation of Exogenous and Endogenous Adenine Nucleotides

Fig.4 shows the kinetics of phosphorylation of intramitochondrial AdN and added ADP under the influence of various inhibitors. The mitochondria are preincubated under nitrogen for conversion of endogenous ATP to ADP in a special apparatus for rapid sampling, (6). With a programmed device oxygen is injected at t=0 and samples are withdrawn according to the preprogrammed time sequence. At t=60 sec ADP is added. The control experiment on the addition of inhibitors shows that the phosphorylation of endogenous AdN is completed in about 5 sec. On addition of excess ADP, exogenous ATP is formed in a linear time dependence. In the presence of oligomycin, the phosphorylation both of endogenous and exogenous ATP is inhibited. In the presence of AT only the formation of exogenous ATP is inhibited, whereas endogenous phosphorylation is

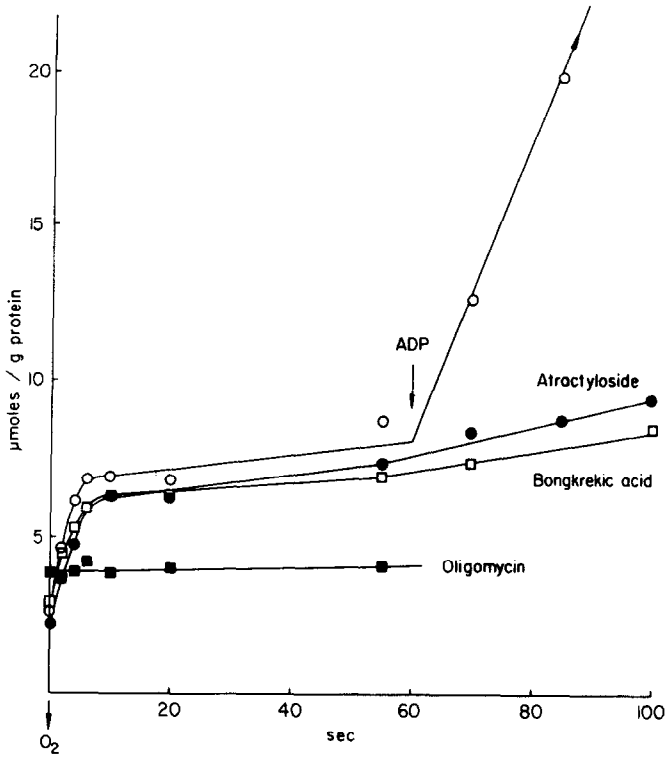

Fig.4. Effect of BA (10 µM) on the phosphorylation of endogenous and exogenous ADP. Comparison with the effects of oligomycin (15 µM) and AT (40 µM). Results obtained by a programmed sampling apparatus. Phosphorylation started by addition of oxygen at t=0. Mitochondria are preincubated anaerobically for 5 min. At t=-1 min ^{32}P is added. Rat liver mitochondria, 4.7 mg prot/ml, temperature 10°, 1 mM Pi, 5 mM succinate, 0.7 mM ADP. For further details of experiment see ref.6.

largely unaffected, in confirmation of previous studies (6). BA has a similar effect to AT; the inhibition of phosphorylation of exogenous ADP may be even stronger.

The differentiation in these experiments of the effect of oligomycin and AT shows clearly that BA acts similarly to AT. BA does not interfere with the phosphorylation reaction as does oligomycin but interferes with the phosphorylation of exogenous ADP only. This has been shown to involve the AdN translocation step.

Discussion

The present studies show that BA inhibits the AdN exchange in mitochondria. This is in agreement with the finding that BA blocks the "binding" of AdN to undepleted mitochondria (2), which

has been shown to reflect the AdN exchange (3). Thus BA can be regarded as an inhibitor of AdN translocation, similar to AT. In the presence of BA as well as AT, the phosphorylation of the intramitochondrial AdN remains uninhibited whereas the phosphorylation of exogenous AdN is fully blocked. BA ($K_I \leq 2 \times 10^{-8}$M) is at least as effective in inhibiting the AdN exchange as AT (at 400 µM ADP, $K_I \approx 2 \times 10^{-8}$M) (7). The very low K_I of BA is difficult to determine accurately and thus may actually still be lower.

The number of inhibition sites of BA (N_I) agrees fairly well when measured by the translocation of AdN or by inhibition of respiration (N_I = 0.2 to 0.3 µmoles/g protein). This value is somewhat higher than the number of specific binding sites of the AdN carrier as measured by specific ADP binding (0.18 µmoles/g protein). The present results permit the conclusion, with the above qualifications, that the number of binding sites for BA and for AdN are identical and therefore indicate that BA binds directly to the AdN carriers.

The concentration dependence of the inhibition of the AdN translocation by BA is linear, similar to that of AT (7). This does not support the contention of an "allosteric" mechanism of BA inhibition, as concluded from its influence on the respiration (3). In such an indirect assay a nonlinarity might be expected if the translocation is not rate-limiting, as actually shown also in Fig.3. The evaluation of the translocation rates from the exchange measurements corresponds most closely to measuring an enzyme activity and permits the quantitative evaluation of the influence of an inhibitor.

It might be surprising that substances of so different a structure as BA and AT both inhibit the same translocase. There is one analogy in the structures of ADP and both inhibitors: all have three anionic groups which have been postulated to be a minimum requirement for the translocase, since AMP is excluded (8). However, as shown in the subsequent publication (9), the mechanism of inhibition by AT and BA is basically different: BA prevents dissociation of ADP from the carrier, AT removes it.

Acknowledgements

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