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Interaction of [^{3}H]Bongkrekic Acid with the Mitochondrial Adenine Nucleotide Translocator†

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ABSTRACT: Chemical labeling by ^{3}H and biosynthetic labeling by ^{14}C of bongkrekic acid (BA) are described. In the rat liver cell, mitochondria are the only subcellular particles to bind [^{3}H]BA with high affinity. The high affinity sites for BA in mitochondria are located in the inner membrane. High affinity binding sites for BA are only displayed at pH below 7; they amount to 0.15–0.20 nmol/mg of protein in rat liver mitochondria and to 1.1–1.3 nmol/mg of protein in rat heart mitochondria. These values are similar to those found for the high affinity atractyloside binding sites and for the carboxyatractyloside binding sites. The kinetic parameters for BA binding to rat heart mitochondria at 20 °C are $K_d = 10\text{–}40 \times 10^{-9}$ M, $k_{+1} = 0.7 \times 10^5$ M^{-1} s^{-1}, $k_{-1} = 1.4 \times 10^{-3}$ M s^{-1}. Binding assays carried out with rat heart mitochondria, under equilibrium conditions, showed that the amount of BA bound to high affinity sites increases with temperature and reaches the maximum value of 1.1–1.3 nmol/mg of protein at 32–35 °C. At lower temperatures, and under equilibrium conditions, a significant fraction of high affinity sites remains masked and is not titrated by BA; these masked BA sites are revealed by addition of micromolar concentrations of ADP or by energization of the mitochondria. Carboxyatractyloside added to rat heart mitochondria preloaded with [^{3}H]BA is able to displace part of the bound [^{3}H]BA. Displacement of the bound BA is enhanced by simultaneous additions of carboxyatractyloside plus ADP, or by energization of the mitochondria. The synergistic effect of carboxyatractyloside and ADP on displacement of bound [^{3}H]BA is also observed in isolated inner membrane vesicles from rat liver mitochondria. When BA is preincubated with rat heart mitochondria before addition of [^{14}C]ADP for assay of ADP transport, the inhibition of ADP transport is a mixed-type inhibition. When BA is preincubated with the mitochondria together with a very small concentration of ADP (less than 0.5 μM), the inhibition of [^{14}C]ADP transport is markedly increased (up to ten times) and it becomes typically uncompetitive, which suggests the formation of a ternary complex, carrier–ADP–BA. The transition from a mixed-type inhibition, with high K_i value, to an uncompetitive type of inhibition, with low K_i value, upon addition of ADP, is explained by an ADP-induced conformational change of the ADP translocator.

Bongkrekic acid (BA)[1] is an inhibitor of ADP transport in mitochondria (Henderson and Lardy 1970; Henderson et al., 1970). Similarly to atractyloside and carboxyatractyloside which also inhibit ADP transport (for review, see Vignais et al., 1973b), BA prevents the oxidative phosphorylation of extramitochondrial ADP (Welling et al., 1960) but not the phosphorylation of intramitochondrial ADP (Kemp et al., 1970; Klingenberg et al., 1970). However, BA differs from atractyloside and carboxyatractyloside in virtue of some unique features. (1) Its inhibitory effect is not immediate (Henderson and Lardy, 1970); the lag time following addition of BA and the potency of inhibition depend on temperature and pH; in particular inhibition by BA is markedly increased at pH below 7 (Kemp et al., 1971). (2) Inhibitory efficiency of BA is enhanced by preincubation of mitochondria with micromolar amounts of ADP or ATP (Kemp et al., 1970, 1971). (3) BA decreases the amount of ADP or ATP required to elicit maximal contraction of mitochondria (Stoner and Sirak, 1973). (4) Addition of BA to heart mitochondria previously equilibrated with nonsaturating concentration of [^{14}C]ADP results in a significant increment of [^{14}C]ADP binding (Erdelt et al., 1972; Klingenberg and Buchholz, 1973).

The pH effect can readily be explained by assuming that BA interacts with the ADP translocator from the inside of the inner mitochondrial membrane and that it must be protonated to enter this membrane. Since the average pK of the carboxylic groups of BA is of the order of 5.5 (Lijmbach, 1969), the pH of the medium must be sufficiently low to allow a significant fraction of BA to be protonated. The other effects, especially the synergistic effect of ADP, are most probably inherent to the mechanism itself of ADP transport. Further insights into the mechanism of ADP transport can be obtained by binding studies using radioactively labeled BA. This paper reports data on the binding properties of [^{3}H]BA to mitochondria. These include the dependence of the binding parameters on the membrane environment of the ADP translocator and on the energy state of mitochondria. Parallel experiments have been

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[1] Abbreviations used are: BA, bongkrekic acid; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

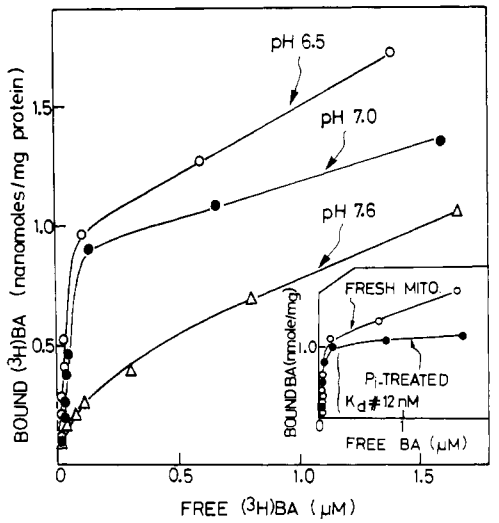

FIGURE 1: Effect of pH on binding of [^{3}H]BA to rat heart mitochondria. The medium used was the standard saline medium supplemented with 10 mM Tris. The pH was adjusted by addition of KOH or HCl. For other conditions, see Experimental Procedure (standard binding assay). Experiment described in the insert was carried out at pH 6.5.

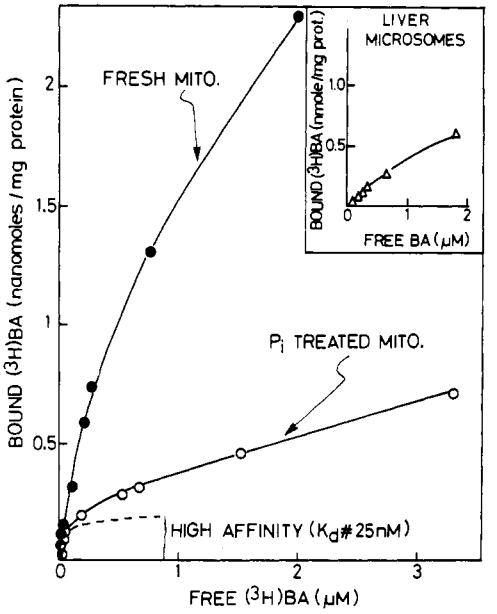

FIGURE 2: Effect of phosphate treatment on binding of [^{3}H]BA to rat liver mitochondria. Binding of [^{3}H]BA to rat liver microsomes. The medium was the standard saline medium, pH 6.5. For other conditions, see Experimental Procedure (standard binding assay).

carried out on the inhibitory effect of BA on ADP transport, in order to assess whether a correlation could be established between binding and inhibition.

Experimental Procedure

Preparation of BA. Strain NCIB 9450 of *Pseudomonas cocovenenans* was used for preparation of BA. The procedure followed for isolation of BA was essentially that described by Nugteren and Berends (1957), as modified by Lijmbach et al. (1970), except that the counter-current step was replaced by a DEAE-cellulose column chromatography. Ten grams of DEAE-cellulose (DE 52 Whatman) was equilibrated with 0.1 M NaCl, 20 mM Mops, pH 8; then 20 mg of BA in the same buffer was applied to the DEAE-cellulose column (10-cm high). After washing with 50 ml of the same buffer, the DEAE-cellulose was submitted to a linear gradient of NaCl in 20 mM Mops, pH 8. Fractions were monitored by silica gel thin-layer chromatography, using chloroform–methanol–acetic acid, 95:5:1 (v/v) solvent system. BA was recovered at 0.25 M NaCl. After acidification to pH 2–3 by 2 N HCl, BA was extracted with peroxide-free diethyl ether and concentrated under vacuum.

Biosynthetic Labeling of BA. In early studies on BA binding (Vignais et al., 1973b), BA was biosynthetically labeled by ^{14}C. The different metabolic sources of ^{14}C included glucose, glycerol, and methionine. They were mixed with a preculture of *Pseudomonas cocovenenans* grown for 16 h just before the addition of the defatted coconut pulp. The specific radioactivity of purified [^{14}C]BA was about 0.1 mCi per mmol.

Chemical Labeling of BA. Tritiated potassium hydroxide was prepared with tritiated water (5 Ci/ml) and potassium *tert*-butoxide (Aldrich) (Thomas and Biemann, 1965). Bongkrekic acid was dissolved in tritiated potassium hydroxide and incubated for 16 h in a small sealed tube at 100 °C. During the course of this reaction, an isomer of BA accumulated (Lauquin et al., 1976). [^{3}H]BA was extracted in ether after acidification of the incubation solution; it was further purified by silica gel thin-layer chromatography and DEAE-cellulose chromatography as described above. The specific radioactivity of [^{3}H]BA was 0.7–1.0 Ci per mmol. The incorporated ^{3}H was stable at pHs used for binding assays; [^{3}H]BA was diluted with unlabeled BA to bring the specific radioactivity to 30–60 dpm per pmol.

Subcellular Preparations. Rat heart mitochondria were prepared according to the method of Chance and Hagihara (1963) in 0.225 M mannitol, 0.075 M sucrose, 0.5 mM EDTA, 5 mM Tris buffer, pH 7.5. Rat liver mitochondria were isolated from 0.25 M sucrose homogenates by the Hogeboom procedure. Inner membrane plus matrix and outer membrane particles of rat liver mitochondria were obtained following the procedure of Parsons and Williams (1967) and inner membrane vesicles devoid of matrix were prepared according to a procedure developed in this laboratory (Colbeau et al., 1971). Rat liver microsomes corresponded to the fraction of homogenate sedimenting between 20 000 and 100 000*g*.

Transport and Binding Assays. The rate of [^{14}C]ADP transport was determined by the direct exchange procedure (Duée and Vignais, 1969), using a saline medium made of 110 mM KCl, 10 mM Mes, pH 6.5, 0.1 mM EDTA (standard saline medium). The reaction was started by addition of [^{14}C]ADP and terminated by addition of 5 μM carboxyatractyloside, followed by centrifugation (Vignais et al., 1973a). When BA was used as inhibitor, mitochondria were preincubated with BA for 3 min at 20 °C and then for 7 min at 0 °C before addition of [^{14}C]ADP.

Binding of [^{3}H]BA to mitochondria or mitochondrial membranes and to microsomes was assayed in the standard saline medium. The final protein concentration was 0.25 mg/ml. In standard binding assays, the incubation was carried out for 3 min at 20 °C and then 30 min at 0 °C in a series of centrifuge tubes with increasing concentration of [^{3}H]BA. Complete equilibrium between bound and free [^{3}H]BA was attained under these conditions. The total volume per tube was 5 ml. The reaction was terminated by high-speed centrifugation. The inside walls of the tubes were rinsed with the saline solution. The pellets were then solubilized in 1 ml of formamide at 180 °C and the radioactivity was determined by liquid scintillation. In some experiments, to allow a better determination of the high affinity binding, the mitochondria were preincubated for 10 min at 25 °C in a medium made of 50 mM

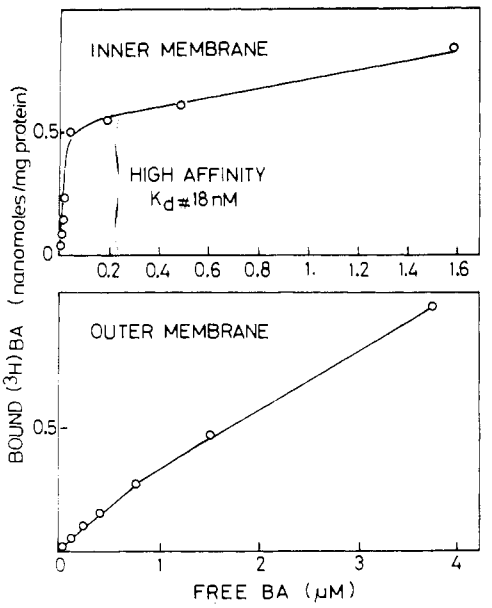

FIGURE 3: Binding of [^{3}H]BA to inner and outer membranes of rat liver mitochondria. Conditions as described in Experimental Procedure (standard binding assay, pH 6.5).

Table I: Distribution of Bound [^{3}H]BA in Fractions Obtained from Inner Membrane Plus Matrix Particles from Rat Liver Mitochondria.[a]

Fraction	Total Protein (mg)	Total Bound [^{3}H]BA (nmol)	Bound [^{3}H]BA (nmol/mg of protein)	Cyt. Ox.[b] Spec. Act.
Inner membrane + matrix	115	26	0.23	4.4
Inner membrane vesicles	58	16	0.28	5.3
Matrix	74	8	0.11	<0.1

[a] Rat liver mitochondria (200 mg of protein) were incubated in 200 ml of the standard EDTA–Mes–KCl medium, pH 6.5, with [^{3}H]bongkrekic acid (1 nmol/mg of protein) for 3 min at 20 °C and 30 min at 0 °C. Then the mitochondria were collected by centrifugation at 15 000*g* for 10 min, resuspended in 150 ml of 20 mM phosphate buffer, pH 7.5, and allowed to swell for 30 min at 2 °C. The matrix plus inner membrane fraction was collected by centrifugation at 1900*g* for 15 min. The crude inner membrane, the purified inner membrane, and the matrix fractions were prepared as described in Experimental Procedure. [b] Micromoles of cytochrome *c* oxidized per minute per milligram of protein.

sodium phosphate, pH 7.4, 50 mM sucrose, and 5 mM $MgCl_2$. After centrifugation, they were resuspended in 0.25 M sucrose and used for binding assays.

Binding of [^{14}C]ADP to rat heart mitochondria was carried out at nonsaturating concentrations of [^{14}C]ADP, using the same medium as for [^{3}H]BA binding. The specific radioactivity of [^{14}C]ADP was corrected for dilution by internal adenine nucleotides.

Results

Equilibrium Binding of [^{3}H]BA to Mitochondria. [^{3}H]BA binding curves obtained with rat heart and rat liver mitochondria under standard conditions (see Experimental Procedure) are shown in Figures 1 and 2. BA binding sites in fresh mitochondria were not saturable. However pretreatment of mitochondria with a phosphate medium decreased the low affinity binding without altering the number of high affinity sites; it was, therefore, used to ascertain the high affinity binding parameters. Mitochondria pretreated by phosphate clearly exhibited, at pH 6.5, a region of high affinity whose K_d value was 10–40 nM. Interestingly, *N*-ethylmaleimide, a penetrant SH reagent (Vignais and Vignais, 1973), also decreased the BA low affinity binding without changing the number of high affinity binding sites. By contrast, mersalyl, a nonpenetrant SH reagent, was without effect.

The number of BA high affinity sites titrated under standard conditions at pH 6.5 was of the order of 1 nmol/mg of protein in rat heart mitochondria and 0.15–0.20 nmol/mg of protein in rat liver mitochondria. There were no high affinity binding sites for BA in rat liver microsomes (Figure 1B).

The effect of temperature on BA high affinity binding has been tested on phosphate-pretreated mitochondria from rat heart. The period of incubation was extended to 1 h for each temperature tested between 0 and 40 °C to ensure complete equilibrium between bound and free BA. Increasing the temperature did not affect the K_d value corresponding to the high affinity binding, but it led to a significant increase in the number of high affinity sites for [^{3}H]BA, up to the maximum value of 1.2–1.3 nmol/mg of protein at 32–35 °C.

As shown in Figure 1, the BA high affinity binding capacity and affinity are markedly increased below pH 7. This is consistent with the fact that acidic pH enhances the inhibitory activity of BA on ADP transport (Kemp et al., 1970, 1971). At pH below 7, a fraction of BA molecules is protonated, as the p*K* of the carboxylic groups of BA is of the order of 5.5 (Lijmbach, 1969). BA in its protonated form is a penetrant reagent, as shown by the following experiment. Rat liver mitochondria were incubated with [^{3}H]BA at pH 6.5 and then sedimented by centrifugation and fractionated in outer membrane and inner membrane plus matrix particles. The inner membrane plus matrix particles were treated mechanically in the presence of 20 mM phosphate buffer, pH 7.2 (Colbeau et al., 1971), and the resulting matrix fluid and inner membrane vesicles were separated by sucrose gradient centrifugation. Data in Table I indicate that two-thirds of the [^{3}H]BA was present in the inner membrane and the remainder in the matrix fluid. A parallel experiment carried out with [^{35}S]carboxyatractyloside confirmed previous data showing the presence of negligible amounts of [^{35}S]carboxyatractyloside in the matrix space and indicating that carboxyatractyloside is a nonpenetrant inhibitor (Vignais et al., 1973a). The presence of [^{3}H]BA in the matrix space as opposed to the absence of carboxyatractyloside indicates that BA, at pH 6.5, is able to penetrate the inner mitochondrial membrane. Since acidic pH is required for the inhibitory effects of BA on ADP transport, it is inferred that the inhibitory effect of BA is linked to its penetrant character.

The first step in the fragmentation of [^{3}H]BA-loaded mitochondria into inner membrane plus matrix particles and outer membrane (see above) was the recovery of a supernatant fraction containing the molecular species of the intermembrane space. An abnormally high concentration of [^{3}H]BA was found in this fraction, suggesting that [^{3}H]BA either accumulates in the intermembrane space or is loosely bound to the sides of the outer and inner membranes facing the intermembrane space. It is possible that part of low affinity BA sites shown in binding curves, using whole mitochondria, is due to accumulation of BA in the intermembrane space.

Outer and inner membranes from rat liver mitochondria have been isolated to test their ability to bind [^{3}H]BA. Inner

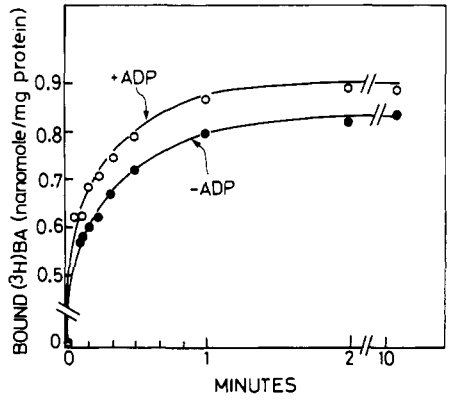

FIGURE 4: Kinetics of [^{3}H]BA binding to rat heart mitochondria. Mitochondria (1.25 mg of protein) were added at zero time to 5 ml of the standard saline medium, pH 6.5, containing 0.2 μM [^{3}H]BA. Incubations were carried out at 20 °C and stopped at a given time by quick filtration through Millipore filter fitted to a syringe. A similar assay was carried out with the same medium supplemented with 10 μM ADP. The free ligand was measured in the filtrate. Bound [^{3}H]BA was determined by subtracting free [^{3}H]BA from total [^{3}H]BA present in the sample. A control without mitochondria was carried out to assess the loss of [^{3}H]BA by unspecific binding to Millipore filter.

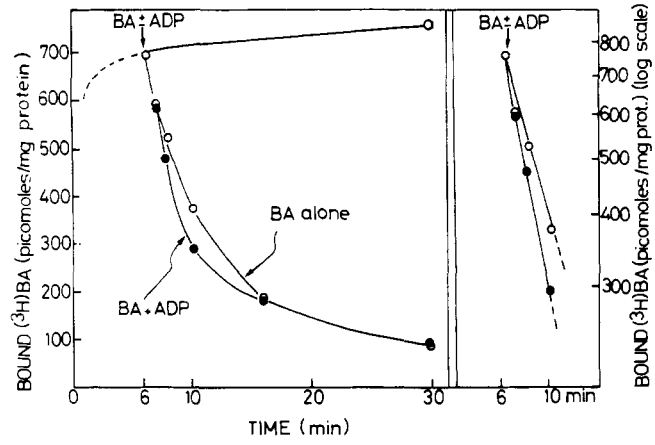

FIGURE 5: Kinetics of [^{3}H]BA dissociation from rat heart mitochondria and effect of ADP. Mitochondria (0.25 mg/ml) were suspended in the standard saline medium, pH 6.5, containing 0.18 μM [^{3}H]BA. After a 6-min incubation, a zero time control of 5 ml was sampled and filtered through Millipore filter, immediately before adding 4 μM nonradioactive BA without or with ADP. Aliquots of 5 ml were sampled at the indicated time and filtered. Temperature: 20 °C. The semilogarithmic plots, on the right side, allow direct calculation of the dissociation rate constants [(open circles) without ADP; (dark circles) with ADP].

membrane vesicles devoid of matrix were able to bind about 0.5 nmol of [^{3}H]BA per mg of protein with high affinity (K_d = 18 nM). In contrast, outer mitochondrial membrane was devoid of high affinity binding sites for [^{3}H]BA (Figure 3).

Kinetics of [^{3}H]BA Binding. Figure 4 illustrates the kinetics of binding of [^{3}H]BA to rat heart mitochondria at 20 °C. In this experiment [^{3}H]BA was added to a final concentration of 0.2 μM, corresponding to the high affinity portion of the binding curve. Seventy percent of the high affinity sites were saturated in less than 5 s, the plateau being reached in about 1 min. Experimental determination of the binding rate constant k_{+1} was hampered by lack of resolution in the binding reaction. ADP enhanced both the rate of BA binding and the number of BA binding sites in rat heart mitochondria, but not in rat liver mitochondria. This effect of ADP will be discussed further.

The rate of dissociation of bound [^{3}H]BA was determined by addition of a large excess of unlabeled BA to rat heart mitochondria previously equilibrated with [^{3}H]BA (Figure 5). The dissociation process was a first-order reaction as shown by the linear plot of the remaining bound [^{3}H]BA as a function of time. The dissociation rate constant k_{-1}, which was equal to 1.4×10^{-3} s^{-1} at 20 °C was increased to a value of 1.6×10^{-3} s^{-1} when ADP was added together with the unlabeled BA. From the experimental values of K_d and k_{-1} at 20 °C for rat heart mitochondria, it was possible to deduce a mean k_{+1} value of 0.7×10^{-5} M^{-1} s^{-1}, assuming that the binding of BA to the mitochondria is a second-order reaction.

Effect of ADP and Energization on Binding Affinity and Binding Capacity for [^{3}H]BA. The number of high affinity sites for [^{3}H]BA in rat heart mitochondria assayed under standard binding conditions (which allow complete equilibrium between free and bound BA) was increased by 20–30% upon addition of micromolar concentrations of ADP (Figure 6A). This suggests that added ADP is able to unmask a fraction of BA sites which were not accessible to [^{3}H]BA. The half-maximum effect was obtained with a concentration of ADP less than 0.5 μM. The ADP effect was shared by ATP, but not by other nucleotides such as GDP, CDP, and UDP. The enhancing effect of ADP was obtained only at pH below 7. It was also observed in phosphate-treated rat heart mitochondria but

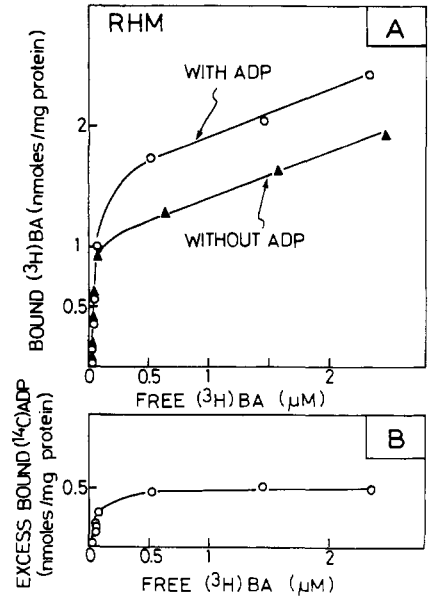

FIGURE 6: Enhancing effect of ADP on [^{3}H]BA binding capacity of rat heart mitochondria. Reciprocal enhancing effect of BA on [^{14}C]ADP binding. The standard incubation medium, pH 6.5, was supplemented with 5 μM [^{14}C]ADP and increasing concentrations of [^{3}H]BA. For other conditions, see Experimental Procedure (standard binding assay).

not in rat liver mitochondria.

The excess binding of BA, dependent on addition of ADP, has its reciprocal counterpart, namely the excess binding of ADP in the presence of BA (Figure 6B; see also Erdelt et al., 1972). When rat heart mitochondria were incubated with nonsaturating concentrations of [^{14}C]ADP, [^{14}C]ADP did not bind to the totality of the specific ADP sites; the subsequent addition of BA led to a substantial increase of [^{14}C]ADP binding. The amount of [^{14}C]ADP bound in excess due to the addition of [^{3}H]BA was of the same order as the amount of [^{3}H]BA bound in excess due to the addition of [^{14}C]ADP.

Energization resulted in the same effect on BA binding as addition of ADP. Energized mitochondria (preincubated with succinate and oligomycin) exhibited 20–30% more high affinity

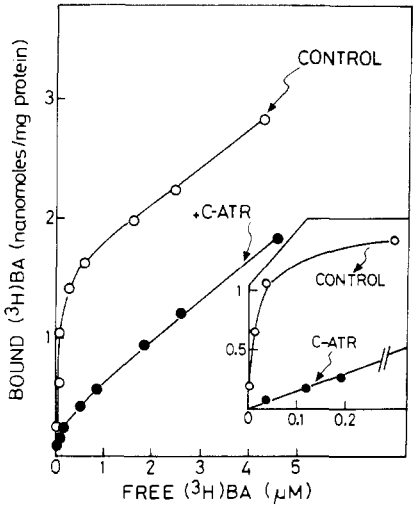

FIGURE 7: Competing effect of carboxyatractyloside on [^{3}H]BA binding to rat heart mitochondria. Carboxyatractyloside was added together with [^{3}H]BA to a final concentration of 5 μM. For other conditions, see Experimental Procedure (standard binding assay, pH 6.5).

Table II: Effect of the Energy State and BA on the Binding of [^{14}C]ADP to Rat Heart Mitochondria.[a]

Conditions	Bound [^{14}C]ADP (nmol/mg of protein)	Excess bound [^{14}C]ADP caused by BA Addition (nmol/mg of protein)
Control	3.36 ± 0.12[b] (6)[c]	
BA	3.80 ± 0.10 (6)	0.44
FCCP + oligomycin	4.80 ± 0.11 (4)	
FCCP + oligomycin + BA	5.03 ± 0.03 (4)	0.23
Succinate + oligomycin	2.82, 2.95 (2)	
Succinate + oligomycin + BA	3.10, 3.20 (2)	0.26

[a] Rat heart mitochondria (1 mg of protein) were preincubated in series of centrifuge tubes containing 5 ml of the standard saline medium. The preincubation was at 20 °C for 1 min with 1 μM FCCP plus 10 μg of oligomycin (deenergization) or 1 mM succinate plus 10 μg of oligomycin (energization). After a 1-min preincubation, [^{14}C]ADP was added to a final concentration of 5 μM and kept in contact with the mitochondria for 2 min before addition of BA to a final concentration of 3 μM. After a 2-min incubation with BA, the tubes were centrifuged and the radioactivity in the pellets was measured by scintillation. Temperature: 20 °C. [b] Standard deviation. [c] Number of determinations.

binding sites for [^{3}H]BA than nonenergized mitochondria (preincubated with FCCP plus oligomycin), when [^{3}H]BA binding was assayed under standard conditions (see Methods). Variations in [^{14}C]ADP binding found in energized and deenergized mitochondria were exactly opposite to variations in [^{3}H]BA binding. For example, energization decreased the amount of bound [^{14}C]ADP (Table II), whereas it increased the amount of bound [^{3}H]BA. The reverse holds for deenergized mitochondria. However, addition of BA to energized and deenergized mitochondria incubated with [^{14}C]ADP resulted in both cases in some increase in [^{14}C]ADP binding (Table II). These data indicate that BA controls the binding of ADP and that conversely ADP controls the binding of BA, but BA binding and ADP binding are not similarly controlled by the energy state of mitochondria. In this respect it is noteworthy that the energy state of mitochondria has no significant effect on the binding affinity and capacity of rat heart mitochondria for atractyloside (not shown).

Interaction between BA and Carboxyatractyloside for Binding. When carboxyatractyloside was added together with [^{3}H]BA to mitochondria, it decreased the high affinity binding capacity for [^{3}H]BA. Interaction for binding between carboxyatractyloside and BA was apparently noncompetitive (Figure 7).

Data on the release of bound [^{3}H]BA by a number of specific ligands of the ADP translocator are presented in Table III. The particles used were rat heart mitochondria and inner membrane vesicles from rat liver mitochondria. Bound [^{3}H]BA could be readily and nearly totally displaced by addition of unlabeled BA, which speaks in favor of specific BA binding sites (experiments 2 and 3). A small displacement of bound [^{3}H]BA was obtained by addition of carboxyatractyloside. Sonication of mitochondria during the incubation with carboxyatractyloside did not improve the release of bound [^{3}H]BA (experiment 1). Addition of ADP (experiments 1 and 3) or energization (experiment 2) markedly increased the displacement of bound [^{3}H]BA by carboxyatractyloside. It may be recalled that bound carboxyatractyloside in rat liver or rat heart mitochondria is removed very efficiently by bongkrekic acid plus ADP, but not by bongkrekic acid or ADP added separately (Vignais et al., 1973b).

Inhibitory Effects of BA on the ADP Transport. In their studies of the effect of BA on the initial rate of the dinitrophenol-induced hydrolysis of ATP, Kemp et al. (1971) showed that the inhibition by BA was competitive with respect to ATP when BA was added simultaneously with the uncoupler. When the mitochondria were preincubated with BA, the inhibition was of a mixed type (competitive and noncompetitive; Kemp et al., 1971). By direct analysis of the effect of preincubated BA on the rate of [^{14}C]ADP transport in rat heart mitochondria, we found a mixed-type inhibition (Figure 8), in agreement with Kemp et al. (1971); the K_i value for the inhibition of ADP transport by BA was of the order of 0.4 μM, which is at least ten times higher than the K_d value deduced from binding assays. Inhibition of ADP transport by BA was stronger in energized mitochondria than in deenergized mitochondria (not shown).

When heart mitochondria were preincubated with a micromolar concentration of ADP before addition of BA and then assayed for [^{14}C]ADP transport, the inhibition of ADP transport was increased up to ten times (Figure 9) and it was then uncompetitive (Figure 10). This is illustrated by the parallel lines obtained in a double reciprocal plot of the rate of [^{14}C]ADP transport at different fixed concentrations of BA (Figure 10). From the ordinate intercepts $[1 + (i/K_i)]/K_m$, a K_i value of 0.04–0.06 μM was calculated in close agreement with the K_d value found in binding assays. It can be concluded that preincubation of heart mitochondria with minute amounts of ADP modifies not only the type of inhibition caused by BA, but it also markedly decreases the K_i of the ADP translocator for BA to a value which is in the same range as the K_d value.

Discussion

We have prepared radioactively labeled BA to study its binding properties. Chemical labeling of BA by ^{3}H and biosynthetic labeling of BA by ^{14}C are described. However chemical tritiation resulted in a much higher specific activity than biosynthetic labeling by ^{14}C and, therefore, [^{3}H]BA was routinely used in the experiments described in this paper. Through the use of [^{3}H]BA, it has been possible to show that: (1) BA readily penetrates the inner mitochondrial membrane

Table III: Effect of Carboxyatractyloside and ADP on the Release of Bound [^{3}H]BA.[a]

Expt	[^{3}H]BA-Loaded Particles	Additions	Remaining Bound [^{3}H]-BA (nmol/mg of protein)	[^{3}H]BA Released (%)
1	Rat heart mitochondria	None (control)	0.92	
		Carboxyatractyloside	0.77	16
		ADP	0.91	<1
		Carboxyatractyloside + ADP	0.67	27
	Sonicated rat heart mitochondria[b]	None	0.78	
		Carboxyatractyloside	0.60	23
		ADP	0.78	0
		Carboxyatractyloside + ADP	0.29	63
2	Rat heart mitochondria	Succinate + oligomycin	1.06	
		Succinate + oligomycin + carboxyatractyloside	0.45	57
		FCCP + oligomycin	1.08	
		FCCP + oligomycin + carboxyatractyloside	0.85	20
		BA (unlabeled)	0.16	85
3	Inner membrane from rat liver mitochondria	None	0.63	
		Carboxyatractyloside	0.53	14
		ADP	0.61	<2
		Carboxyatractyloside + ADP	0.28	56
		BA (unlabeled)	0.05	92

[a] Rat heart mitochondria or inner mitochondrial vesicles from rat liver were loaded with [^{3}H]BA by incubation in the standard saline medium (1 mg of protein/ml) with [^{3}H]BA (1 nmol/mg of protein). After centrifugation, the pellet was resuspended in the standard saline medium at a concentration of 0.25 mg/ml. Five-milliliter aliquots of the particle suspension were distributed in centrifuge tubes and additions of different ligands in volume less than 50 μl were made to the following final concentrations: 10 μM carboxyatractyloside, 10 μM BA, 50 μM ADP, 1 mM succinate, 1 μM FCCP, 2 μg oligomycin/ml. After 3 min of incubation at 20 °C and 30 min at 2 °C, tubes were centrifuged and the radioactivity of the pellets was counted by scintillation. The values are corrected for the spontaneous release of [^{3}H]BA, which was less than 10%. [b] Sonication for 1 min was performed during incubation with the added ligands.

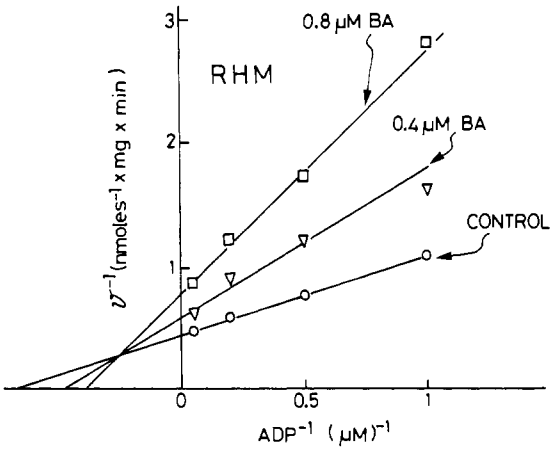

FIGURE 8: Kinetics of inhibition of ADP transport by BA preincubated with rat heart mitochondria. Mitochondria (1 mg of protein) in 3 ml of standard saline medium, pH 6.5, were preincubated in the presence of BA at the indicated concentration for 3 min at 20 °C and then 7 min at 0 °C. ADP transport was started by addition of different concentrations of ADP ranging from 1 to 20 μM. For other conditions, see Experimental Procedure. Temperature: 0 °C.

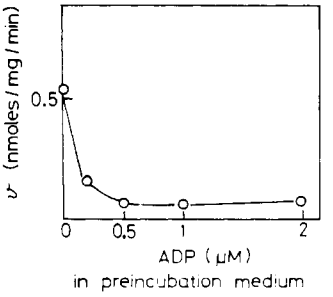

FIGURE 9: Potentiating effect of ADP added in the preincubation medium on the inhibitory effect of BA on ADP transport. Same conditions as in Figure 8, except that ADP was added to the preincubation medium at the indicated concentration. The final concentration of BA was 0.6 μM.

at acidic pH; (2) the maximum number of high affinity sites for BA is similar to that found for atractyloside and carboxyatractyloside, i.e., 1.2–1.3 nmol per mg of protein in rat heart mitochondria and 0.15–0.20 nmol per mg of protein in rat liver mitochondria. The penetrant character of BA at acidic pH is at variance with the nonpenetrant character of atractyloside (Vignais et al., 1971) and of carboxyatractyloside (Vignais et al., 1973a). Requirement of an acidic pH means that BA must be protonated to penetrate the inner mitochondrial membrane and to reach its specific binding site.

Binding assays under equilibrium conditions showed that the amount of BA bound to high affinity sites increases with temperature and reaches a maximum value at 32–35 °C. At lower temperatures, a smaller number of high affinity sites were revealed, although the binding assays were carried out under equilibrium conditions. For example, in the standard binding assay (3-min incubation at 20 °C, followed by 30 min at 0 °C), the amount of BA bound to high affinity sites was 20–30% less than that found at the optimal temperature of 32–35 °C. The effect of temperature on BA binding under equilibrium conditions can be explained by an increased solubility of BA in the lipid core of the mitochondrial membrane and a better accessibility of BA to the ADP translocator. One may imagine that clusters of lipids in the mitochondrial membrane prevent the accessibility of BA to the ADP translocator. Increasing the temperature would break the clusters, making the translocator more accessible to BA.

Preincubation of rat heart mitochondria with a micromolar

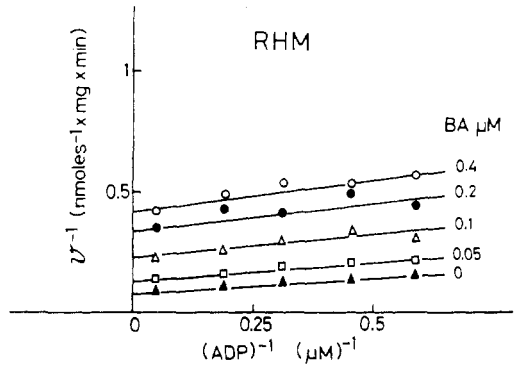

FIGURE 10: Kinetics of inhibition of ADP transport by BA preincubated with rat heart mitochondria in the presence of ADP. ADP was added in the preincubation medium to the final concentration of 0.5 μM. Other conditions are as in Figure 8.

amount of ADP leads not only to an increase of the number of high affinity sites for BA, but also to a decrease of the K_i value of the ADP transport for BA and to a change of the type of inhibition from a mixed type to an uncompetitive type. This unexpected transition is most likely the result of an ADP-induced conformational change of the ADP translocator, altering specifically the BA binding site. It is noteworthy that, without preincubation of mitochondria with ADP, the K_i value of ADP transport for BA is roughly ten times higher than the K_d value. Preincubation with ADP decreases the K_i to a value which is similar to the K_d, and inhibition of ADP transport by BA becomes coincident with BA binding. This observation suggests that the uncompetitive character of inhibition caused by BA corresponds to its true mechanism of action on ADP transport.

Models of transport across biological membranes fall in two general classes: the mobile carrier and the fixed pore (or channel) (for review, see Singer, 1974). To explain the extra ADP binding upon addition of BA, Klingenberg and Buchholz (1973) postulated that the ADP transport system is a mobile carrier, which is distributed on the inner or outer face of the inner mitochondrial membrane according to a pattern which depends on the environmental conditions. In the absence of ADP, the immobile empty carrier would accumulate on the outer face of the membrane. Upon addition of ADP, the mobile carrier-ADP complex would move to the inner face. BA, as a permeable ligand, would trap the ADP carrier on the inner face of the membrane and, thanks to its higher affinity, would displace the bound ADP into the matrix. The mobile carrier model led Klingenberg and Buchholz (1973) to speculate that BA and ADP bind to the same site on the carrier. This is in contradiction with data presented in this paper, which, on the contrary, suggest the existence of two separate binding sites for BA and ADP. These data are the following: (1) under appropriate conditions of incubation, BA behaves as an uncompetitive inhibitor of ADP transport; i.e., it binds to another site than ADP to make the ternary complex, carrier-ADP-BA (Figure 10); (2) energization of mitochondria results in an increase of high affinity BA binding sites and, on the contrary, in a decrease of ADP binding sites (Table II).

In the presence of traces of external ADP, bound BA is displaced by carboxyatractyloside and conversely bound carboxyatractyloside is displaced by BA (Vignais et al., 1973a). Direct interaction between BA and carboxyatractyloside is not likely to occur, as BA is a penetrant inhibitor and carboxyatractyloside, a nonpenetrant inhibitor. The interaction between the two inhibitors is probably indirect; this indirect interaction can be tentatively explained by assuming a channel model which is characterized by a binding asymmetry, the BA and carboxyatractyloside-binding sites being located on the inner and outer regions of the channel, respectively. Binding of carboxyatractyloside to the outer region of the channel would result, in the presence of external ADP, in a change of conformation propagated to the inner region of the channel where BA is bound; the affinity for BA would then be decreased, resulting in a release of bound BA. A similar explanation holds for the displacement of bound carboxyatractyloside by BA.

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