ATRACTYLOSIDE AND BONGKREKIC ACID SITES IN THE MITOCHONDRIAL ADP/ATP CARRIER PROTEIN

An appraisal of their unicity by chemical modifications

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1. Introduction

One of the peculiarities of the mitochondrial ADP/ATP carrier is its ability to bind, in an asymmetric manner, two specific natural inhibitors, ATR and BA, and their derivatives, CATR, Ac-CATR and IsoBA; the atractylosides bind to the ADP/ATP carrier from the outside of mitochondria, and the bongkrekic acids from the inside [1]. Immunological [2] and fluorescence [3] studies have provided evidence that the conformation assumed by the ADP/ATP carrier protein is different, depending upon whether the carrier binds the atractylosides (ATR conformation) or the bongkrekic acids (BA conformation). A minimal hypothesis formulated by our group [4,5] to explain the binding asymmetry of the ADP/ATP carrier protein with respect to the atractylosides and bongkrekic acids was that each family of inhibitors is recognized by a specific pre-existing sequence of amino acids belonging to the ATR and BA sites, although overlapping of the ATR and BA sites was not excluded. The fact that ATR and BA compete for binding was interpreted by indirect interaction between ATR and BA sites mediated by conformational changes. On the basis of current views on transport mechanism [6], the ADP/ATP carrier may be described as a dimer limiting a channel opened alternatively to the outside and the inside (outer and inner conformations), and through which the substrate is directed to either side [4]. As a consequence, inhibition of ADP/ATP transport by ATR and BA would be related to the immobilization of the carrier in the outer and inner conformations, respectively (fig.1). In the original version of his one-site mechanism, Klingenberg [7] postulated that all specific ligands (substrates and inhibitors) of the ADP/ATP carrier bind to a common area. Competition between ATR and BA for binding was explained to be the result of mutual exclusion for access to the same site. In the course of ADP/ATP transport, the

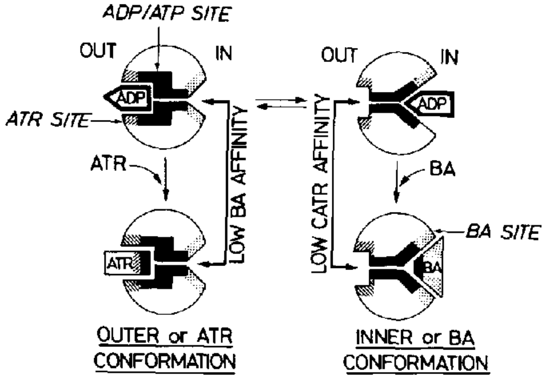

Fig.1. The double inhibitor site model: This model assumes 2 pre-existing specific binding sites for ATR and BA. The ADP/ATP site is capable of reorientation, i.e., it can be exposed to the outside and inside alternatively, and takes accordingly the outer and inner conformation. The outer conformation equivalent to the ATR conformation is stabilized by ATR or its derivatives. The inner conformation equivalent to the BA conformation is stabilized by BA or its derivatives. Note that the ATR, BA and ADP/ATP sites can overlap.

Abbreviations: MES, 2-(*N*-morpholino)-ethane sulfonic acid; MOPS, 3-(*N*-morpholino)-propane sulfonic acid; ATR, atractyloside; BA, bongkrekic acid; CATR, carboxyatractyloside; Ac-CATR, acetyl carboxyatractyloside; isoBA, isobongkrekic acid; NEM, *N*-ethylmaleiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; HNB, 2-hydroxy 5-nitrobenzylbromide

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carrier site was supposed to adopt two different conformational states, the *c*-state and the *m*-state, corresponding to whether the site is turned to the outside or to the inside respectively (which is the equivalent of the outer and inner conformations in the preceding formulation). The *c*-state would be recognized specifically by ATR, and the *m*-state by BA. In [8] it was admitted that beside the common binding area, called also binding center, molecules of ATR and BA should occupy additional areas, although no specific function was ascribed for these additional areas. In brief, Klingenberg et al. [7,8] and our group [4,5] agree on the two different extreme conformations assumed by the ADP/ATP carrier protein (ATR conformation or *c*-state, and BA conformation or *m*-state). The identity of the ATR and BA sites remains, however, a matter of debate (two pre-existing sites capable of interacting and changing their conformation [4,5] or same site with two possible conformations [7,8]).

Because the ADP/ATP carrier is unique among transport systems by its ability to be probed in two conformations (ATR and BA conformations) that probably correspond to two frozen states of the transport process, full elucidation of the topology of the ATR and BA sites is clearly desirable for better understanding of the functioning of this carrier. Along this line, selective chemical modifications of strategic amino acids in the ADP/ATP carrier have already provided interesting data [4,5,8]. The penetrant reagents used so far and their main targets include NEM (SH groups) [9], HNB (tryptophanyl residues) [10], phenylglyoxal and butanedione (arginyl residues [11, 12]). Inactivation of the binding properties of the ADP/ATP carrier by UV light was possibly due to modification of tryptophanyl residues [4]. NEM, HNB and UV light were found to alter primarily ATR binding. Phenylglyoxal and butanedione altered both ATR and BA binding, although ATR binding was inhibited 3–4-times more than BA binding. The potentiating or protective effects of a number of parameters on inactivation by the above reagents, reported in [5], were consistent with the dual-inhibitor site mechanism [4]. However, in [8] it was claimed that all the above modifications stabilized the *m*-state, thus preventing ATR binding, and they maintained that the single site model could accommodate all our results.

These experiments show that the single-site model [8] cannot explain all inactivation data; for example, stabilization of the *m*-state by preincubation of mitochondria with isoBA did not enhance inactivation of Ac-CATR binding by butanedione. The same observation holds for another reagent, EEDQ, highly specific for the activation and modification of carboxyl groups [13,14]. Further, the protection afforded by ADP on butanedione inactivation of both Ac-CATR and BA binding could not fit the one-site model. The data are better explained by the dual inhibitor-site model, with possible partial overlapping of the sites, as proposed in [4,5].

2. Materials and methods

2.1. *Materials*

[^{3}H]Atractyloside and [^{3}H]bongkrekic acid were prepared as in [15] and [16], respectively. Isobongkrekic acid was prepared as in [17], and [^{14}C]acetylcarboxyatractyloside as in [18], except that the purification procedure by TLC chromatography and DEAE-cellulose was replaced by a reverse-phase high-pressure liquid chromatography on a Waters apparatus equipped with a C_{18}-microbondapack column, with the mobile phase made of water, methanol, acetic acid and 1 M ammonium acetate (60/40/1/2, by vol.). Atractyloside was purchased from Sigma and ADP from PL Biochemicals.

2.2. *Subcellular preparations*

Rat heart mitochondria were isolated as in [19].

2.3. *Chemical modifications*

In the case of NEM modification, freshly prepared rat heart mitochondria were suspended at 1 mg/ml in saline buffer made of 120 mM KCl, 10 mM MOPS, 1 mM EDTA (pH 7.2) supplemented with 10 μM ADP. After 5 min preincubation at 25°C under aerobiosis, NEM was added to final conc. 200 μM. After different periods of reaction with NEM at 25°C, aliquot fractions were withdrawn and diluted 5-fold with 4 ml ice-cold standard medium made of 120 mM KCl, 10 mM MES, 1 mM EDTA (pH 6.5) supplemented with 1 mM cysteine to stop the NEM reaction. [^{14}C]-Ac-CATR binding was assayed by addition of a saturating concentration of [^{14}C]Ac-CATR (2 nmol/mg protein) and 10 μM ADP.

Treatment of mitochondria with EEDQ was carried out at 25°C in the above standard medium (pH 6.5) using 1 mg mitochondrial protein/ml. At zero time, EEDQ was added upon vigorous stirring. The reaction was stopped by a 5-fold dilution with the ice-cold standard medium, and 2 nmol [^{14}C]Ac-CATR/mg protein and 10 μM ADP were added for study of [^{14}C]Ac-CATR binding.

Butanedione inactivation was performed according to [20], in a medium made of 70 mM borate and 50 mM KCl (pH 8) at 25°C. Mitochondrial protein was 1 mg/ml. Butanedione was 5 mM for ATR binding inactivation, or 10 mM for BA binding inactivation. The reaction was stopped by cooling to 0°C and lowering the pH to 6.5 by a 5-fold dilution with 4 ml 50 mM KCl, 70 mM MES (pH 6.4). This latter medium was supplemented with 10 μM ADP and a saturating concentration of [^{14}C]Ac-CATR or [^{3}H]BA (2 nmol/mg protein) for binding assays.

The assays for [^{14}C]Ac-CATR or [^{3}H]BA binding were performed by incubation of the chemically modified mitochondria for 30 min at 0°C with the radioactive ligand, followed by centrifugation for 5 min at 22 000 × *g*. The pellets were digested with 1 ml 5% Triton X-100 and 0.5 M NaCl, and the radioactivity was counted by liquid scintillation. Binding controls, without chemical modifiers, were run to take into account the small spontaneous decrease in [^{14}C]Ac-CATR binding capacity, due to incubation of mitochondria at 25°C. In some experiments, unlabelled ATR or isoBA were added prior to the modification step. As noticed above, a small amount of ADP (10 μM final conc.) was systematically added with [^{14}C]Ac-CATR or [^{3}H]BA. At this low concentration ADP did not perturb Ac-CATR binding [18]; it even slightly enhanced BA binding [16]; furthermore, ADP allowed the efficient removal of bound isoBA by Ac-CATR and that of bound ATR by BA.

3. Results

3.1. *Effect of preincubation of mitochondria with isoBA on inhibition of Ac-CATR binding by NEM, butanedione and EEDQ*

NEM inhibits ATR binding [21] but not BA binding [16], and butanedione inhibits ATR binding 3–4-times faster than BA binding [5]. Like NEM, EEDQ inhibits ATR binding without modification of BA binding (fig.2). In all cases, the inhibition of ATR (and BA) binding caused by covalent binding of NEM [21], butanedione [5] and EEDQ (fig.2) is an all-or-none process, i.e., that the binding capacity of mitochondria for ATR (or BA) is decreased, whereas the affinity of the remaining sites for ATR or BA remains unaltered. This is typical of an inactivation process.

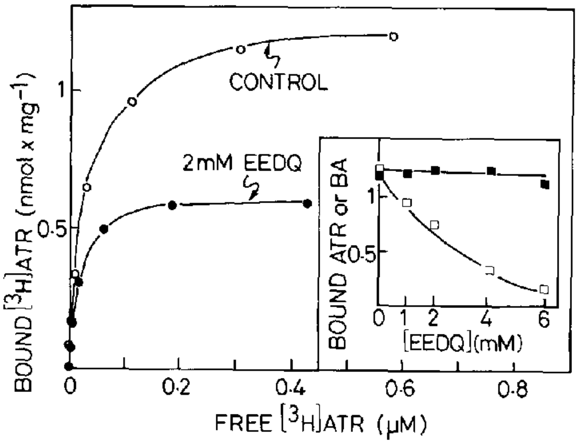

Fig.2. Effect of EEDQ on ATR and BA binding to heart mitochondria: The incubation conditions are in section 2. Mitochondria were treated by 2 mM EEDQ for 25 min at 25°C.

Inhibition of ATR binding by NEM requires preincubation of the mitochondria with traces of ADP or ATP [21]. This result was explained in [8] by an ADP-induced shift from the *c*-state (with high affinity for ATR) to the *m*-state (which is unable to bind ATR); it was further postulated [8] that SH-groups buried in the *c*-state are unmasked in the *m*-state, and can therefore be alkylated by NEM, resulting in a

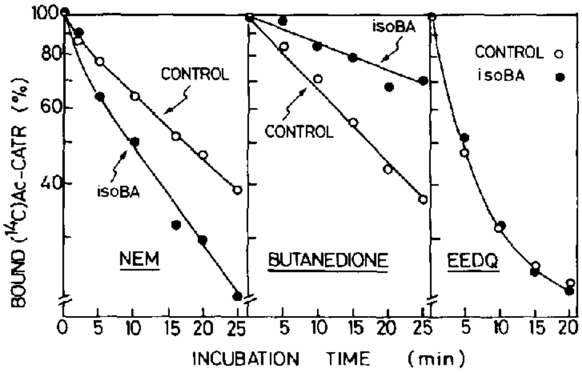

Fig.3. Effect of preincubation of heart mitochondria with isoBA on the inhibition of [^{14}C]Ac-CATR binding by NEM, butanedione and EEDQ. Unlabelled isoBA was added prior to the chemical modification, at 2 nmol/mg protein final conc. The incubation conditions are given in section 2. The time scale in abscissa corresponds to the incubation period with 200 μM NEM, 5 mM butanedione or 2 mM EEDQ. The amounts of bound [^{14}C]Ac-CATR in the absence of chemical modifiers (100% binding) were 0.95 and 1.15 nmol/mg protein depending on whether the mitochondria are preincubated with or without isoBA.

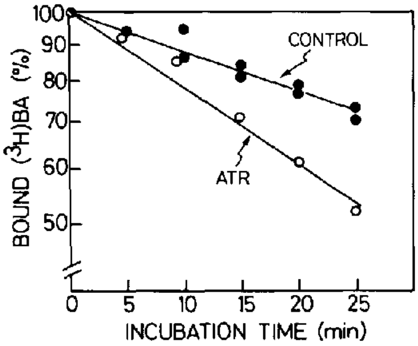

Fig.4. Sensitization of [^{3}H]BA binding to butanedione inhibition by preincubation with ATR. Heart mitochondria were suspended in borate buffer (pH 8.0) (cf. section 2). A fraction of the suspension was supplemented with unlabelled ATR 2 nmol/mg protein. At time zero, 10 mM butanedione was added, and at the times indicated on the abscissa, 1 ml aliquot of the suspension was withdrawn and diluted as in section 2 for assay of [^{3}H]BA binding. The amounts of bound [^{3}H]BA in the absence of chemical modifiers (100% binding) were 0.85 and 1.00 nmol/mg protein, with mitochondria preincubated with or without ATR, respectively.

frozen *m*-state favorable to the binding of BA, but not to that of ATR. The reasoning in [8] was extended to all the reagents examined so far, which in fact have been found to inhibit ATR binding more than BA binding (cf. section 1). If the above reasoning is valid, it follows that a shift from the *c*-state to the *m*-state, induced by addition of isoBA, might potentiate, like ADP does, the inhibitory effect of NEM, EEDQ and butanedione on ATR binding. In the experiment illustrated in fig.3, ATR was substituted for by the tighter ligand, Ac-CATR, and BA by the looser ligand, isoBA. These conditions facilitated the displacement of isoBA bound during the preincubation period by the further added [^{14}C]Ac-CATR. As shown in fig.3, in contrast with the prediction in [8], opposite effects of preincubation with isoBA on NEM and butanedione inhibition were obtained. IsoBA increased inhibition of [^{14}C]Ac-CATR binding brought about by NEM, and it protected [^{14}C]Ac-CATR binding against butanedione inhibition. It had no effect on inhibition of [^{14}C]Ac-CATR binding by EEDQ. In the case of butanedione, which inactivates both BA and ATR binding, the reverse experiment was carried out, which consisted in testing the binding of [^{3}H]BA to mitochondria after pretreatment by ATR and then inhibition by butanedione. The results showed that ATR preincubation potentiated inhibition of BA binding by butanedione (fig.4).

3.2. *Effect of ADP on inhibition of [^{14}C]Ac-CATR and [^{3}H]BA binding by butanedione*

A crucial point of the one-site mechanism is that, in the absence of external ADP, the ADP/ATP carrier site is turned to the outside (*c*-state) [8], and that a trace of external ADP induces the site-shift from the

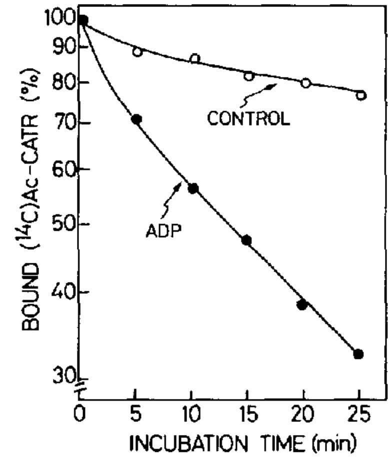

Fig.5. ADP-induced stimulation of NEM inhibition of [^{14}C]-Ac-CATR binding. Heart mitochondria were suspended in the MOPS buffer, with or without 10 µM ADP (cf. section 2). At zero time, 200 µM NEM was added. The assay was continued as in section 2. The values of bound [^{14}C]Ac-CATR corresponding to 100% binding is 1.10 nmol/mg protein.

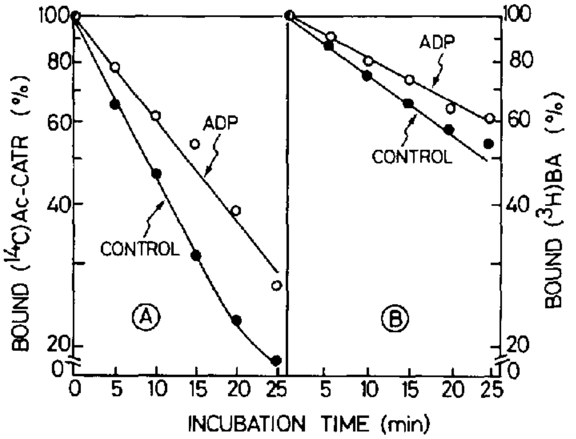

Fig.6. Effects of preincubation with ADP on butanedione inhibition of [^{14}C]Ac-CATR and [^{3}H]BA binding. Heart mitochondria were suspended in the presence or absence of 1 mM ADP in a borate medium (pH 8.0). The further steps (reaction with butanedione), followed by binding of [^{14}C]Ac-CATR or [^{3}H]BA were done as in section 2. The values of bound [^{14}C]Ac-CATR and [^{3}H]BA corresponding to 100% binding are 1.10 and 0.98 nmol/mg protein, respectively.

c- to the *m*-state. This postulated effect of ADP was supported by the finding that μM levels of externally added ADP enhanced the NEM inhibition of [^{3}H]ATR binding [21] and [^{14}C]Ac-CATR binding (fig.5). Just the opposite was found when butanedione was used as inactivating reagent instead of NEM. Micromolar levels of ADP had no enhancing effect of the kinetics of inhibition. Further, when added at mM levels, ADP protected both [^{14}C]Ac-CATR binding and [^{3}H]BA binding against butanedione inhibition (fig.6). It is therefore clear that the effect exerted by ADP on the inhibition of ATR or BA binding by chemical modifiers is more complex than claimed by [8]. It must be added that the protective effect of ADP against butanedione inhibition was shared also by ATP, but not by other nucleotides, which points to the specificity of the effect.

4. Discussion

A number of penetrant reagents have been used to study the response of ATR and BA binding to chemical modifications on specific amino acid residues of the ADP/ATP carrier [4,5,8,21]. In [8] chemical reagents like NEM, phenylglyoxal, butanedione and others, that inhibit more ATR binding than BA binding, were proposed to stabilize the ADP/ATP carrier in the *m*-state (cf. section 1); it follows that, once the carrier conformation is frozen in the *m*-state corresponding to the BA or inner conformation, it can still bind BA, but can no longer bind ATR. To test the above predictable effects of chemical reagents, we conducted a series of experiments in which the *m*-state (or inner conformation) was induced by addition of isoBA or ADP, and the *c*-state (or outer conformation) by addition of ATR. The data obtained for NEM inhibition were consistent with the idea that NEM reacts with SH group(s) at distance from the ATR (or BA) site, resulting in the stabilization of the *m*-state (or BA conformation). However, the data on EEDQ and butanedione inhibition clearly showed that the chemical impact of these reagents could not be interpreted as simply as for NEM. For example, contrary to expectation, addition of isoBA prior to modification of mitochondria by EEDQ did not increase EEDQ inhibition of Ac-CATR binding. Whereas NEM and EEDQ inhibit Ac-CATR binding, but not BA binding, arginine reagents like phenylglyoxal and butanedione were found to inhibit not only ATR binding, but also BA binding, a crucial fact which was overlooked in [8]. IsoBA preincubated with mitochondria protected inhibition of [^{14}C]Ac-CATR binding by butanedione. However, preincubation with ATR led to enhancement of the inhibition of BA binding by butanedione. The ATR conformation seems therefore more reactive than the BA conformation to butanedione; yet ATR protected its own binding site against butanedione. Although a first view, these data are difficult to reconcile, they can be rationalized on the following basis:

1. Butanedione would modify arginyl residue(s) at the ATR site; this is consistent with the rapid inhibition of ATR binding;
2. Butanedione would act on other arginine residue(s) at distance from the ATR and BA sites, resulting in the shift and stabilization of the carrier protein in the ATR conformation or *c*-state; this would explain the slow loss of BA binding.

The postulated rapid inhibition of ATR binding by butanedione modification of arginyl residue(s) at the ATR site is in accordance with the linear relationship previously found between the inhibition of ATR binding by phenylglyoxal and the binding of [^{14}C]-phenylglyoxal to arginyl residue(s) in the carrier protein [5], which suggests a direct titration of the ATR site.

Considering now the effects of ADP (or ATP) on inhibition of Ac-CATR and BA binding by NEM and butanedione, it is obvious that NEM and butanedione inhibitions respond to ADP in different ways and therefore involve different mechanisms:

(i) ADP exerts a protective effect against butanedione inhibition; on the contrary, ADP enhances NEM inhibition;

(ii) Butanedione inhibition is prevented by mM levels of ADP; enhancement of NEM inhibition requires only μM levels of ADP.

Whereas the enhancing effect of ADP on NEM inhibition of Ac-CATR binding (fig.5) may be interpreted by a shift of the substrate site from the *c*-state to the *m*-state [8], this interpretation is no longer tenable to explain the protection afforded by mM levels of ADP against butanedione inhibition of Ac-CATR binding and BA binding. A possible explanation of these data is that the substrate site of the carrier (to which ADP or ATP specifically binds) partially overlaps the BA and ATR sites. It is noteworthy that our inactivation data are not incompatible with a sequential mechanism of ADP/ATP transport involving a ternary complex consisting of the carrier and two nucleotides, one coming from the inside, the other from the outside [22,23]; this would lead to assume that each subunit of the dimer in the model of fig.1 contains a nucleotide binding site.

These data are consistent with the idea that the binding sites for ATR and BA differ by at least a few amino acid residues [5]; it remains quite possible that the ATR and BA sites share a common area which overlaps the ADP/ATP site. It must be stressed that the dual inhibitor site model does not preclude alternating reorientation of the substrate site, nor the conformational changes associated with the reorientation.

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