DIFFERENTIAL INACTIVATION OF ATRACTYLOSIDE AND BONGKREKIC ACID BINDING SITES ON THE ADENINE NUCLEOTIDE CARRIER BY ULTRAVIOLET LIGHT

Its implication for the carrier mechanism

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1. Introduction

An intriguing feature of the mitochondrial ADP/ATP carrier is its binding asymmetry with respect to two specific inhibitors, atractyloside and bongkrekic acid. In whole mitochondria, atractyloside binds to the outer (cytosolic) face of the inner membrane and bongkrekic acid to the inner (matrix) face. Atractyloside inhibits the binding of bongkrekic acid and vice-versa. Two possible mechanisms can be formulated to explain these data.

1. The two inhibitors bind to the two opposite sites of the carrier protein which spans the membrane to form a channel; the atractyloside site faces the outside and the bongkrekic acid site faces the matrix space. The interaction between the two sites is indirect [1,2].
2. The two inhibitors bind to the same site of the carrier; this site is able to assume two different conformations depending on the orientation of the carrier in the membrane (atractyloside conformation when the carrier is opened to the outside, bongkrekic acid conformation when the carrier is opened to matrix space) [3,4].

The two postulated models could in principle be discriminated by specific inactivation of atractyloside and bongkrekic acid binding sites.

Data presented here show that atractyloside binding is much more sensitive to ultraviolet light irradiation than bongkrekic acid binding. This result points to the occurrence of different binding sites for atractyloside and bongkrekic acid and it therefore favours the channel model. Inactivation of atractyloside sites does not modify the K_d for atractyloside, indicating that the loss of atractyloside binding is an all or none event. In addition, either ADP or ATP specifically sensitizes the atractyloside binding site to inactivation by ultraviolet light, and ADP/ATP transport is inactivated by ultraviolet light to about the same extent as atractyloside binding. These latter data suggest that atractyloside and ADP/ATP sites are closely related.

Abbreviation: MES, 2(*N*-morpholino)ethane sulfonic acid

2. Materials and methods

[^{3}H]Atractyloside and [^{3}H]bongkrekic acid were prepared as in [5,6]. Most experiments were performed with either beef heart [7] or rat heart [8] mitochondria. Some of them were carried out with sonicated particles from beef heart mitochondria [9].

In binding experiments, mitochondria at 1 mg protein/ml were routinely preincubated for 15 min at 20°C in a hypotonic medium made of 0.05 M sucrose, 0.05 phosphate buffer (pH 7.4) and 5 mM $MgCl_2$ at 1 mg protein/ml. This pretreatment allowed a substantial decrease of the unspecific low affinity binding especially for bongkrekic acid without modifying the specific high affinity binding. The mitochondria were then sedimented by centrifugation and resuspended in 0.12 M KCl, 10 mM MES, 1 mM EDTA, final pH 6.5 at 5 mg/ml. The suspension (4 ml) was placed in a 5 cm diam. Petri dish on crushed ice at ~10 cm

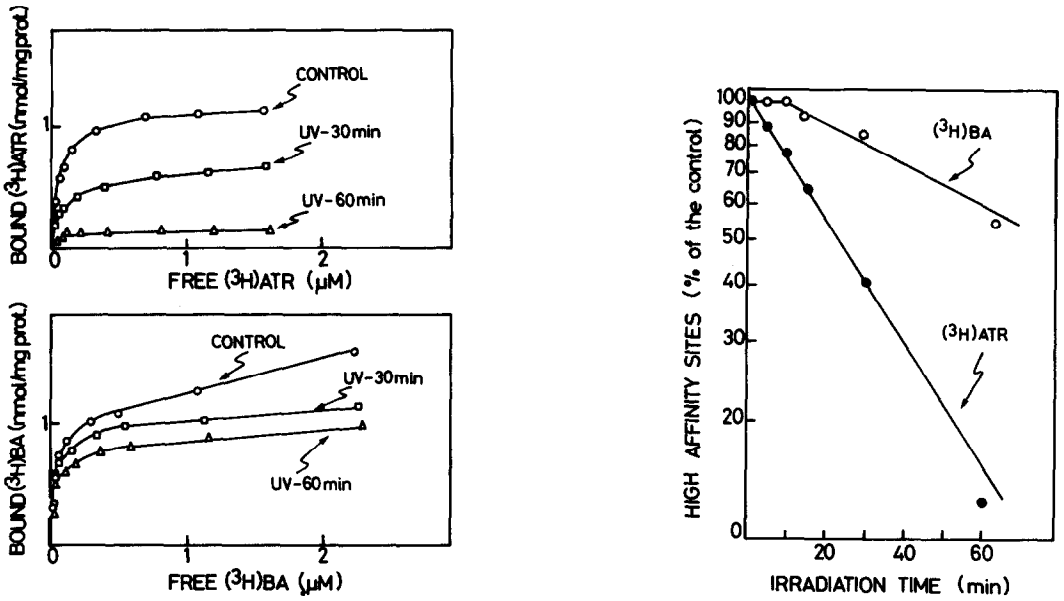

Fig.1. Effect of ultraviolet light on binding of [^{3}H]atractyloside and [^{3}H]bonkrekic acid. (A) The suspension of beef heart mitochondria (5 mg/ml) in 0.12 M KCl, 10 mM MES, 1 mM EDTA (pH 6.5) was irradiated by a 15 W Philips T-UV lamp for 30 and 60 min as in section 2. Aliquots corresponding to 1 mg protein were withdrawn and incubated in 5 ml of the same medium in the presence of increasing concentrations of [^{3}H]atractyloside or [^{3}H]bongkrekic acid for 40 min at 0°C. (B) The mitochondria were irradiated by ultraviolet light for different lengths of time up to 60 min and then incubated with 0.4 μM [^{3}H]atractyloside or [^{3}H]bongkrekic acid for 40 min at 0°C. At this concentration, only high affinity sites are titrated.

from a 15 W Philips T-UV germicidal lamp equipped with a reflector. The peak energy output of the lamp was at 254 nm. Aliquots of 0.2 ml (1 mg protein) were withdrawn after irradiation and incubated in 5 ml of the same saline medium in the presence of increasing concentrations of [^{3}H]atractyloside or [^{3}H]bongkrekic acid for 40 min at 0°C. After incubation the mitochondria were collected by centrifugation, and their radioactivity estimated by liquid scintillation following digestion of the pellet by formamide.

ADP transport was assayed by the direct exchange procedure at 0°C using [^{14}C]ADP as external adenine nucleotide [10]. Internal ATP, ADP and AMP were determined as in [11,12].

3. Results

3.1. *Effect of ultraviolet light on binding of [^{3}H]atractyloside and [^{3}H]bongkrekic acid to heart mitochondria*

Ultraviolet light is known to modify tryptophan, tyrosine, cysteine and methionine residues in proteins [13,14]. The following shows that the atractyloside binding site in the ADP/ATP carrier protein is particularly susceptible to ultraviolet light.

The binding curve of atractyloside and bongkrekic acid to heart mitochondria is characterized by a high affinity region with K_d values between 20 and 30 nM and a number of high affinity sites of about 1 nmol/mg protein (fig.1A). Irradiation of heart mitochondria by ultraviolet light decreased the number of high affinity sites for atractyloside. There was no significant modification of the K_d value indicating that ultraviolet light inactivates atractyloside binding to specific sites according to an all or none process. Inactivation of atractyloside sites by ultraviolet light approximated first order kinetics (fig.2).

Bongkrekic acid sites were much less sensitive to ultraviolet light than atractyloside sites (fig.1A). A kinetic study of inactivation of bongkrekic acid sites is illustrated in fig.1B. It shows a lag period of ~10 min followed by a first order inactivation kinetics. Inactiva-

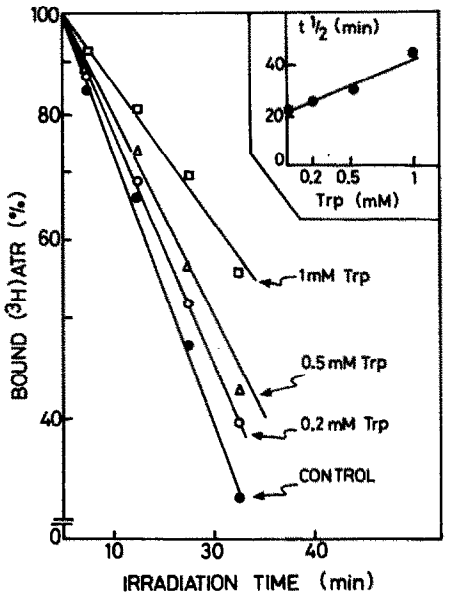

Fig.2. Effect of added tryptophan on ultraviolet light inactivation of atractyloside binding sites. Tryptophan was present during photoirradiation at concentrations of 0.2, 0.5 and 1 mM as indicated. Other conditions are as in fig.1.

tion of bongkrekic acid sites was 4 times slower than that of atractyloside sites. As for inactivation of atractyloside binding, there was no effect of ultraviolet light on the K_d value for bongkrekic acid. Latency of inactivation and slow rate of inactivation of bongkrekic acid sites were also observed with inside-out submitochondrial particles, where bongkrekic acid has direct access to the inverted surface of the mitochondrial membrane. The lag period for inactivation of bongkrekic acid sites may be explained by indirect effects; for example cumulative modifications of amino acid residues located at some distance from the bongkrekic acid site may be required to initiate a change in the conformation of the carrier protein, and this could result in a modified bongkrekic acid site unable to recognize its specific ligand. The typical differential effect of ultraviolet light on atractyloside and bongkrekic acid binding was also found in liver mitochondria. All subsequent assays were performed with heart mitochondria.

Binding assays were routinely carried out with air as a gas phase. Inactivation of atractyloside binding sites and, to a less degree, that of bongkrekic acid sites were slightly more rapid when the medium was gassed with O_2. Conversely inactivation was markedly decreased (~3 times), when air was substituted by argon (table 1). Kinetics of inactivation also depended on temperature and concentration of mitochondria during the irradiation step. Increasing the temperature increased the rate of inactivation. Decreasing the protein concentration significantly increased the sensitivity of atractyloside sites to inactivation. There was no significant effect of pH at pH 6–8.

The same differential inactivation effects on atractyloside and bongkrekic acid sites were obtained by including between the ultraviolet lamp and the suspension of mitochondria a Corning filter 9863 delivering a light band between 240 and 300 nm or by using a Xenon lamp equipped with an interference filter delivering a light band between 275 and 285 nm. This result rules out major participation of membrane lipids in the effects of ultraviolet light, since lipids are susceptible to damage at ultraviolet wavelengths shorter than 230 nm [15,16] and it strongly suggests that ultraviolet-sensitive amino acid residues at the atractyloside binding site are destroyed by ultraviolet light.

Inactivation of atractyloside binding by ultraviolet light irradiation was not modified by addition of 1 mM dithiothreitol, 1 mM 2-mercaptoethylamine, 10 mM methionine or 10 mM thioglycolic acid. The first two

Table 1
Effect of atractyloside, ADP and oxygen on the half-time of inactivation of atractyloside binding sites by ultraviolet light

Additions	Gas phase	% of change of the half-time of inactivation with respect to standard inactivation
0.4 μM atractyloside	Air	+85%
50 μM ADP	Air	−40%
None	O_2	−10%
None	Argon	+120%

The half-time of inactivation of atractyloside binding to beef heart mitochondria was determined in each case comparatively to a control inactivation carried out in the standard conditions of the experiment of fig.1B, as in section 2. When used, O_2 or argon were bubbled in the medium containing the mitochondria and the suspension was placed in closed quartz tubes which were irradiated by ultraviolet light. Irradiation in the presence of atractyloside or ADP was carried out in air

compounds were added to protect cysteine residues, the last one to protect methionine residues. It is therefore likely that these amino acids are not critical targets in ultraviolet light irradiation. On the contrary, tryptophan added prior to irradiation protected the atractyloside binding sites from inactivation, probably due to ultraviolet light absorbancy by the tryptophan solution acting as a filter. As shown in fig.2, the half-time of inactivation increased with the concentration of added tryptophan; it doubled when the tryptophan reached 1 mM.

3.2. *Effects of specific ligands of the ADP/ATP carrier on photoinactivation of atractyloside binding sites*

When added to mitochondria at a saturating concentration prior to ultraviolet light irradiation, atractyloside protected against inactivation of atractyloside binding sites (table 1). On the contrary, ADP or ATP at concentrations $\geqslant 20\ \mu M$ increased significantly the rate of inactivation of atractyloside binding sites (table 1). This unexpected effect was not given by other nucleotides. We checked that ultraviolet light did not induce covalent binding of ADP to the adenine nucleotide carrier protein; this was shown by photoirradiation of mitochondria in the presence of [^{14}C]-ADP and analysis of the radioactivity pattern after sodium dodecyl sulfate gel electrophoresis of the mitochondrial lysate. Most likely ADP or ATP, by binding to specific sites of the carrier, acted as photosensitizers catalysing light energy transfer to sensitive amino acid residues at the atractyloside binding site. This result suggests that the binding sites for ADP or ATP and atractyloside are closely related. It is noteworthy that, in the above assays involving added ligands, kinetics of inactivation remained first order.

Bongkrekic acid inhibits binding of atractyloside to mitochondria [1]. The remaining atractyloside binding in ultraviolet light-irradiated mitochondria was inhibited by bongkrekic acid to the same extent as atractyloside binding in control mitochondria. This observation is consistent with the view that ultraviolet inactivation is an all or none process.

3.3. *Effect of ultraviolet light on ADP transport*

Ultraviolet irradiation inactivated ADP transport. As inactivation of ADP transport was accompanied by a significant release of adenine nucleotides (cf. legend of table 2), appropriate corrections of rates of ADP transport had to be made. The experiments described in table 2 show that ultraviolet light irradiation decreased the rate of ADP transport to the same extent as the amount of bound atractyloside. The fraction of ADP transport that escaped ultraviolet light inactivation was fully sensitive to atractyloside or carboxyatractyloside.

Table 2
Comparison of the inhibitory effects of ultraviolet light on ADP transport and atractyloside binding

Irradiation	Rate of ADP transport (nmol.mg protein^{-1}.min^{-1})	Bound [^{3}H]atractyloside (nmol.mg protein^{-1}.min^{-1})
None	13.8	1.12
Ultraviolet light	8.9 (36%)	0.72 (35%)

Rat heart mitochondria at 5 mg/ml in 0.25 M sucrose, 10 mM MES (pH 6.5), 1 mM EDTA were irradiated by ultraviolet light for 10 min as in section 2. The suspension was divided in 3 fractions. One was used for measurement of ADP transport, the other for atractyloside binding, and the last for adenine nucleotide content (in the present experiment, 40% of the internal ADP + ATP were released upon ultraviolet light irradiation). ADP transport was started by adding 0.2 ml of the above suspension (1 mg protein) to 1 ml 0.10 M KCl, 20 mM MES and 1 mM EDTA (pH 6.5) and 50 μM [^{14}C]ADP. The incubation was at 0°C for various times from 15 s to 5 min and was terminated by addition of 5 μM carboxyatractyloside, followed by centrifugation at 20 000 × *g* for 10 min. The pellets were digested in 1 ml formamide at 180°C and their radioactivity was determined by liquid scintillation. The calculated rates of transport were corrected for loss of internal adenine nucleotides. [^{3}H]Atractyloside binding was assayed as described in the legend of fig.1 and in section 2. Numbers in brackets refer to percentage of inhibition of ADP transport and atractyloside binding

4. Discussion

The sensitivities of atractyloside and bongkrekic acid binding sites to ultraviolet light differ in two aspects.

1. The bongkrekic acid sites are inactivated much more slowly than the atractyloside sites.
2. The bongkrekic acid sites are inactivated after a

lag period whereas inactivation of atractyloside is immediate.

Furthermore the kinetics of inactivation of atractyloside sites is typically first order. The lag period characteristic of bongkrekic acid inactivation points to indirect effects of ultraviolet light on bongkrekic acid sites, that may be opposed to probable direct effects on atractyloside sites as suggested by first-order kinetics of inactivation. In other words, atractyloside binding requires ultraviolet-sensitive amino acid residues (possibly aromatic amino acids), whereas amino acids involved in the binding of bongkrekic acid are not sensitive to ultraviolet light. The lag period required for the inhibition of atractyloside binding by ultraviolet light can be explained by a secondary change of conformation of bongkrekic acid sites, caused by additive destructive events occurring at some distance from this sites. It can be concluded that the binding sites for atractyloside and bongkrekic acid are not similar.

Identical binding sites for the two inhibitors were postulated by [3,4] in the reorientating carrier model,

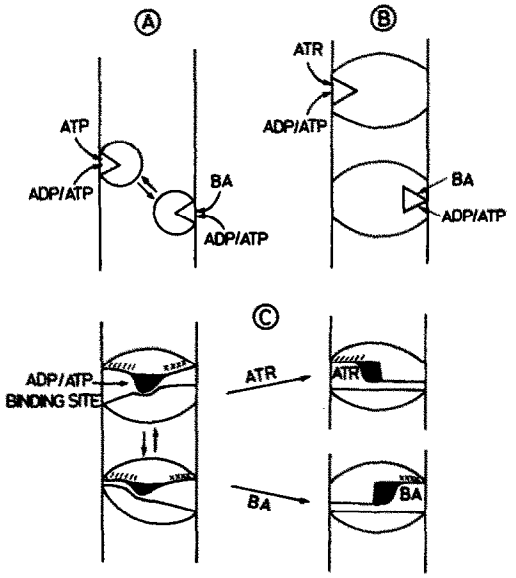

Fig.3. Single site and double site carrier models. (A) and (B) refer to the reorientating mechanism of ADP/ATP transport with a single site occupied either by atractyloside (ATR), bongkrekic acid (BA) or ADP/ATP. (C) is a double-site gated channel (one site for atractyloside (ATR) and another for bongkrekic acid (BA)). The ADP/ATP site is supposed to share a small common area with the atractyloside site.

presented either as a mobile carrier (fig.3A) [3] or as a fixed carrier with a 'switching site' (fig.3B) [4]. Inactivation studies presented here do not fit transport models, in which atractyloside and bongkrekic acid sites are identical. An amended version of the single site model, more compatible with the present data, could be proposed, which implies that the amino acid residues used for atractyloside binding are not the same as those involved in bongkrekic acid binding. Any speculation on conformational changes during the switching step is beyond the scope of the present discussion.

Differential photoinactivation of atractyloside and bongkrekic acid binding sites is more readily explained by a double site model. The double site model shown in fig.3C is an asymmetric channel with different preexisting sites for atractyloside and bongkrekic acid (fig.3C). It may be recalled that atractyloside is not a penetrant and attacks the carrier only from the outside; bongkrekic acid is a penetrant inhibitor, and it has to attack the carrier from the inside to inhibit ADP/ATP transport. Therefore, the atractyloside and bongkrekic acid sites of the channel are postulated to face the outside and the inside, respectively. This double site model does not eliminate the possibility that the recognition domains for atractyloside and bongkrekic acid share a small common area.

To be compatible with the exchange–diffusion mechanism that is characteristic of ADP/ATP transport, the channel must be opened alternatively to the inside and the outside. This may conceivably be accomplished by a gate that possesses the ADP/ATP site and that could be a specific domain of the channel protein (fig.3C). Since ADP/ATP transport is inactivated by ultraviolet light to about the same extent as atractyloside binding, it may be assumed that the ADP/ATP and atractyloside sites are closely related. Experiments are now in progress to characterize strategic amino acids in atractyloside, bongkrekic acid and ADP/ATP binding sites by means of selective chemical modifications.

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