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6'-O-DANSYL-γ-AMINOBUTYRYL ATRACTYLOSIDE, A FLUORESCENT PROBE OF THE ADP/ATP CARRIER: EXPLORATION OF CONFORMATIONAL CHANGES OF THE MEMBRANE-BOUND ADP/ATP CARRIER ELICITED BY SUBSTRATES AND INHIBITORS

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Summary. A fluorescent atractyloside analogue, the 6'-O-dansyl-γ-aminobutyryl atractyloside (DGA), has been used to probe the binding of the inhibitors carboxyatractyloside (CATR) and bongkrekic acid (BA) and nucleotide substrates to the membrane-bound ADP/ATP carrier protein in beef heart mitochondria. Binding and release of DGA were followed by fluorescence responses. Specifically bound DGA was fully released by CATR alone, or by BA in the presence of micromolar amounts of ADP. In the absence of the inhibitors, ADP increased the rate of the specific binding of DGA. The effect of ADP was shared by transportable nucleotides. Non transportable nucleotides were ineffective. These data are consistent with the previously described CATR and BA conformations of the ADP/ATP carrier that are able to bind CATR and BA respectively, the transition between the two conformations being accelerated by micromolar concentrations of transportable nucleotides.

The tryptophanyl residues of the isolated ADP/ATP carrier (1) and the naphtoyl derivatives of ADP or ATP (2) have been previously used as fluorescent probes to study conformational changes of the carrier protein in response to specific ligands. Two conformers of the ADP/ATP carrier were demonstrated, which were characterized by their ability to bind carboxyatractyloside (CATR conformation) and bongkrekic acid (BA conformation). The synthesis of a number of fluorescent derivatives of atractyloside (ATR), including 6'-O-dansyl-γ-aminobutyryl ATR (DGA), was recently described (3) ; their affinities and specificities for the ADP/ATP carrier are virtually the same as those of the original ATR ; they therefore mimick ATR, with the advantage that their free or bound states can be readily followed by fluorescence changes. In this work, we have investigated the binding of DGA to beef heart mitochondria and the release of bound DGA upon addition of ADP or ATP and the specific inhibitors, CATR and BA.

Abbreviations: ATR : atractyloside ; CATR : carboxyatractyloside ; BA : bongkrekic acid ; DGA : 6'-O-dansyl-γ-aminobutyrylatractyloside.

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EXPERIMENTAL PROCEDURES

Materials. Formycin triphosphate was purchased from Calbiochem. Other nucleotides and carboxyatractyloside were purchased from Boehringer. Bongkrekic acid was prepared as described in a previous report (4). 6'-O-dansyl-γ-aminobutyryl atractyloside was synthesized as described in (3). Bovine heart mitochondria were prepared as in (5) and stored in liquid nitrogen.

Fluorescence Techniques. DGA fluorescence was measured in a 1x1 cm fluorescence cuvette with continuous stirring in a 2 ml final volume. Reagents were injected with Hamilton syringes in small volumes (2 to 5 µl). DGA was excited at 350 nm with a 150W Xe lamp using a Zeiss M4QII monochromator. The emitted light was measured at right angle through a K50 Balzers filter at 500 nm.

RESULTS

Specific binding of DGA assessed by fluorescence changes

As shown in Fig. 1A, the addition of DGA to beef heart mitochondria at 25°C resulted in a biphasic increase of fluorescence, starting with a rapid rise in fluorescence at 520 nm, followed by a slower rise that was completed in one min. Addition of CATR at a saturating concentration after the plateau of fluorescence had been attained led to partial fluorescence quenching (Fig. 1A). When the order of addition of DGA and CATR was reversed, i.e. CATR was added first, followed by DGA, an abrupt, but limited increase in fluorescence was observed ; the final plateau of fluorescence (Fig. 1B) was the same as that obtained when CATR was added after DGA (Fig. 1A). Based on the specific interaction of CATR with the ADP/ATP carrier, it is inferred that the extent of binding of DGA to the ADP/ATP carrier (specific DGA binding) is reflected by the size of the fluorescence quenching caused by addition of CATR.

When the temperature was decreased from 25 to 0°C, both the second phase of the biphasic increase in fluorescence that followed addition of

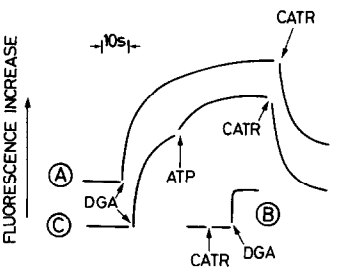

Figure 1. Fluorescence changes induced by addition of ATP and CATR to the carrier protein-DGA complex. The DGA fluorescence was measured at 500 nm with an excitation wavelength of 350 nm. The medium contained 0.12 M KCl, 1 mM EDTA, 10 mM MES, pH 6.5. The final concentration of beef heart mitochondria was 0.07 mg/ml and that of DGA, 0.13 µM. ATP and CATR were added at the final concentration of 1 µM. The temperature was 25°C.

DGA, and the response to CATR were considerably slowed down (not shown). However, the first phase was not modified, indicating that the specific binding of DGA is related to the slow fluorescent component and is characterized by a high temperature-dependence. The rapid fluorescence increase is probably due to the partition of DGA, an amphiphilic molecule, between the lipid core of the mitochondrial membrane and the incubation medium. There was no effect of pH, between pH 6 and 8, on the fluorescence signal.

The specificity of the CATR-induced quenching is testified by the titration data illustrated in Fig. 2. The number of CATR binding sites was assessed by measuring the relative fluorescence quenching resulting from addition of increasing concentrations of CATR for a saturating concentration of DGA. The titration curve was linear and the end point corresponded to 1.4 nmol CATR added/mg protein ; a similar value for the density of CATR binding sites in beef heart mitochondria was been found when the titration was carried out with radiolabeled CATR (6).

A titration of the DGA specific binding sites, based on the removal of the specifically bound DGA by CATR and the concomitant fluorescence change, was performed, using an excess amount of mitochondria (0.12 mg/ml) (Fig. 3). The binding curve was biphasic with an extended rectilinear region for low concentrations of added DGA. By extrapolation to the plateau, the number of titrated DGA binding sites was 1.4 nmol /mg protein, i.e. the same number as that found for the CATR binding sites (Fig.2).

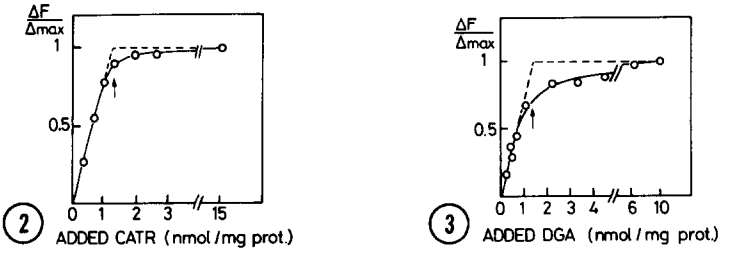

Figure 2. Titration of CATR binding sites by release of bound DGA. Titration of CATR binding sites was performed by measuring the amplitude of the relative decrease in relative fluorescence, $\Delta F/\Delta F_{Max}$, caused by the release of bound DGA upon addition of increasing concentrations of CATR (cf. Fig. 1). ΔF_{Max} was the maximal fluorescence decrease obtained when the carrier protein was incubated with a saturating concentration of CATR (1 μM). Experimental conditions were as in Fig. 1. Beef heart mitochondria were used at the final concentration of 0.07 mg/mL and DGA at the saturating concentration of 0.4 μM.

Figure 3. Titration of the specific DGA sites on the ADP/ATP carrier of beef heart mitochondria. The fluorescence of the specifically bound DGA was measured by total displacement by a large excess of CATR (1 μM), the displacement corresponding to a decrease in fluorescence intensity. The concentration of beef heart mitochondria was 0.12 mg/ml. The medium and the fluorescence conditions were the same as those described in Fig. 1.

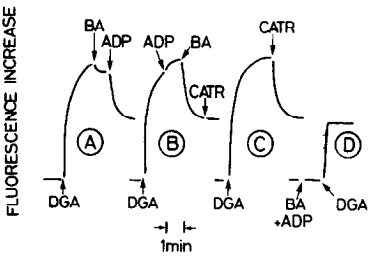

Figure 4. Fluorescence changes induced by addition of BA, ADP and CATR to the carrier protein-DGA complex. Conditions were the same as those described in Fig. 1. DGA was added at the final concentration of 0.13 µM. Additions were as follows BA 2.5 µM, ADP 25 µM, and CATR 2 µM.

Compared effect of ADP, CATR and BA on the specific DGA binding

The effect of ATP on the fluorescence rise following the addition of DGA to mitochondria is illustrated in Fig. 1C. When added at a micromolar concentration during the course of the slow rise, ATP (or ADP) accelerated the fluorescence increase, but the final level of the fluorescence plateau was not changed, nor the extent of the reversal by CATR (compare to Fig. 1A). Transportable nucleotides, like formycin triphosphate (7) and 5'-adenylyl imidodiphosphate (8), exhibited the same effect as ADP or ATP. Non-transportable nucleotides were ineffective.

At variance with the extensive quenching effect of CATR, the fluorescence quenching elicited by a saturating concentration of BA (2.5 µM) was very limited (Fig. 4A). ADP added either prior to or after BA amplified the BA-induced fluorescence quenching to virtually the same extent as that obtained with CATR (Fig. 4A, B and C). There was no additive effect of BA (plus ADP) and CATR on fluorescence quenching. Finally, as for CATR, the quenching effect of BA plus ADP appeared to affect the slow phase of fluorescence increase elicited by DGA. In fact, when BA plus ADP were added first, only the slow fluorescence response to the addition of DGA was inhibited (Fig. 4D). The eliciting effect of ADP added together with BA on the fluorescence quenching of DGA was shared by transportable nucleotides like ATP, formycin triphosphate and adenylylimidodiphosphate, but not by the non-transportable nucleotides AMP (9), naphthoyl-ADP (10) and 8Br-ATP (11).

DISCUSSION

In the present study, ADP, ATP and other transportable nucleotides have been shown 1. to accelerate the specific binding of DGA to the ADP/ATP carrier to a saturation value, 2. to promote the rapid release

of bound DGA in the presence of BA. On the other hand, CATR is able to remove bound DGA in the absence of added nucleotide. These results fit in with the existence of two conformations of the ADP/ATP carrier, the CATR conformation and the BA conformation, and the facilitation of the transition between the two conformations induced by transportable nucleotides (see Introduction). In brief, it is postulated that, in the mitochondrial membrane, the ADP/ATP carrier units are present either in the CATR conformation or in the BA conformation. Upon addition of DGA, the carrier units on the CATR conformation are immediately trapped by DGA,an analogue of ATR ; the subsequent slow release is due to the spontaneous transition of the BA conformation to the CATR conformation. The DGA-binding acceleration caused by ADP (or ATP) is explained by the ADP- or ATP-promoted facilitation of the transition from the BA conformation to the CATR conformation. The rapid release of bound DGA by CATR is due to the favorable competition of CATR, a quasi-irreversible inhibitor, over DGA for binding to the ADP/ATP carrier. When DGA binding has reached a plateau, all ADP/ATP carriers are presumably in the CATR conformation. Addition of BA alone at this point is poorly effective in the release of bound DGA ; however, if ADP (or ATP) is added, even in trace amounts, BA becomes effective in the full release of bound DGA, which means that the carrier conformation has now shifted from the CATR conformation (bound DGA) to the BA conformation (bound BA and released DGA). The postulated species and reactions are summarized in Fig. 5.

CATR, ATR and DGA are non-permeant reagents, in contrast to BA which penetrates the lipid core of the mitochondrial membrane (at acid pH) and attacks the carrier from the inside ; it is therefore inferred that the carrier in the CATR conformation has its CATR binding site exposed to the outside, whereas in the BA conformation, it has its BA binding site exposed to the inside.

Recent experiments have demonstrated the specific and high affinity binding of DGA to the isolated ADP/ATP carrier protein in detergent. In

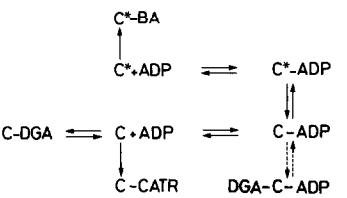

Figure 5. Scheme depicting the ADP (or ATP)-dependent transition between the CATR conformation (C) and the BA conformation (C^*). The CATR conformation, C, reacts reversibly with DGA. The concentrations of C, C^*, C-ADP and C^*-ADP depend on the values of the equilibrium constants for the different reactions. The formation of a transient ternary complex DGA-C-ADP cannot be excluded. For other details, see Discussion.

these experiments, the changes in the DGA emission fluorescence could be followed after excitation not only at 350 nm, but also at 295 nm, which points to an energy transfer mechanism between tryptophanyl group(s) in the isolated carrier protein and bound DGA. The nature of this transfer is presently under investigation.

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REFERENCES

1. Brandolin, G., Dupont, Y. and Vignais, P.V. (1985) Biochemistry **24**, 1991-1997.

2. Block, M.R., Lauquin, G.J.M. and Vignais, P.V. (1983) Biochemistry **22**, 2202-2208.

3. Boulay, F., Brandolin, G., Lauquin, G.J.M. and Vignais, P.V. (1983) Anal. Biochem. **128**, 323-330.

4. Lauquin, G.J.M. and Vignais, P.V. (1976) Biochemistry 15, 2316-2322.

5. Smith, A.L. (1967) Methods Enzymol. 10, 81-86.

6. Block, M.R., Pougeois, R. and Vignais, P.V. (1980) FEBS Lett. 117, 335-340.

7. Schlimme, E., Boos, K.S. and de Groot, E.J. (1980) Biochemistry 19, 5569-5574.

8. Yount, R.G. (1975) Adv. Enzymol. **43**, 1-56.

9. Duée, E.D. and Vignais, P.V. (1969) J. Biol. Chem. **244**, 3920-3931.

10. Block, M.R., Lauquin, G.J.M. and Vignais, P.V. (1982) Biochemistry 21, 5451-5457.

11. Schlimme, E. and Stahl, K.W. (1974) Hoppe-Seyler's Z. Physiol. Chem. **335**, 1139-1142.