Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 28-35

SUBSTRATE-INDUCED FLUORESCENCE CHANGES OF THE ISOLATED ADP/ATP CARRIER PROTEIN IN SOLUTION

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Received October 24,1980

ABSTRACT : Fluorescence studies were carried out on a purified preparation of the ADP/ATP carrier protein solubilized in 3-laurylamido-N-N-dimethylpropyl-aminoxide. The intrinsic fluorescence of the protein was modified upon addition of ADP and ATP and specific inhibitory ligands (carboxyatractyloside and bongkrekic acid). The fluorescence was transitorily enhanced by micromolar concentrations of ADP or ATP. The rise in fluorescence lasted for 10 sec at 25°C. It was suppressed by carboxyatractyloside and on the contrary enhanced by bongkrekic acid. These data were interpreted as reflecting conformational changes probably related to the functioning of the ADP/ATP carrier. Mg^{++} inhibited the ADP- or ATP-induced rise in fluorescence, indicating that the free forms (and not the Mg^{++} complexed forms) of ADP and ATP are the true substrates for the ADP/ATP carrier.

INTRODUCTION

Earlier investigations of the interaction of mitochondria with ADP or ATP revealed that the mitochondrial ADP/ATP carrier undergoes, upon binding of external ADP or ATP, conformational changes, illustrated for example by unmasking of -SH groups (1) (2) (3). Because substrate-induced conformational changes are probably inherent to the basic mechanism of ADP/ATP transport, a direct study of this process with the isolated carrier protein was undertaken.

The ADP/ATP carrier protein contains 3 to 4 tryptophanyl residues (4) which are responsible for the large intrinsic fluorescence exhibited by the protein. Here we report preliminary studies on changes of the intrinsic fluorescence of the ADP/ATP carrier protein induced by ADP or ATP. That the observed fluorescence changes are related to the mechanism of ADP/ATP trans-

0006-291X/81/010028-08$01.00/0

port is supported by the fact that they are sensitive to specific inhibitors, like carboxyatractyloside (CATR) and bongkrekic acid (BA).

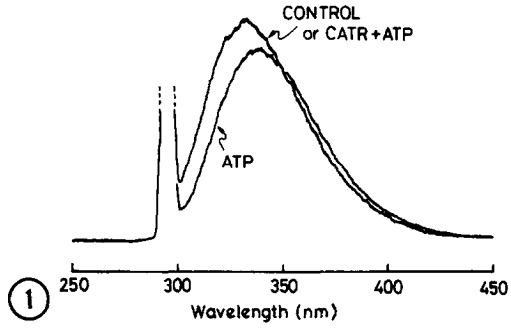
MATERIALS AND METHODS

Nucleotides and carboxyatractyloside were purchased from Boehringer and α-β-methylene adenosine diphosphate (AOPCP) from Miles. Bongkrekic acid was prepared as in (5). Laurylamido-N-N-dimethylpropylaminoxide was synthetized as described in (6). The ADP/ATP carrier protein from beef heart mitochondria was prepared by chromatography on hydroxyapatite (Biorad) as described in (7), using pure laurylamido-N-N-dimethylpropylaminoxide as detergent instead of Aminoxid WS35. The pass-through fraction, which contained the protein, was diluted twice with 136 mM glycerol to give a final protein concentration ranging between 0.15 and 0.25 mg/ml ; the final pH was 6.5. This fraction was used for fluorescence measurements. All fluorescence measurements were made at 25°C. Fluorescence emission spectra were recorded on a Perkin-Elmer MPF 2A fluorimeter using a 1 x 1cm fluorescence cuvette in 2 ml final volume (Fig. 1). Kinetics of fluorescence changes (Fig. 2-6) were measured with a DURRUM D117 fluorimeter as described in a recent paper (8). The protein fluorescence was excited at 295nm ; the emitted light was collected at 90° through quartz lenses and filtered with a 0-54 Corning filter coupled to an ultraviolet light filter. The resulting band-pass was centered at 355 nm with a peak transmission of 75 % and a width of 60 nm at half maximum of the peak. With this set-up and efficient stirring there was virtually no photolysis within the time range of the experiments.

RESULTS

The emission spectrum of the purified ADP/ATP carrier protein in laurylamido-N-N-dimethylpropylaminoxide (LAPAO) was characteristic of tryptophan fluorescence with a broad peak at 330 nm (Fig. 1). The fluorescence data which are presented hereafter can be classified into two categories on the basis of the time parameter ; accordingly we shall refer to long term effects (more than 20-30 sec) and short term effects (less than 20 sec). For example, the modified spectrum of the carrier protein recorded 5 min after addition of ATP (Fig.1) corresponds to long term effects (ADP had the same effect). Long term spectral modifications due to ADP or ATP consisted mostly in a red shift, resulting in fluorescence quenching for wavelengths lower than 345 nm and enhancement of fluorescence above 345 nm. No reversal was observed when CATR was added after ADP or ATP. The ADP(ATP)-induced spectral modification was prevented by incubation with CATR prior to addition of ADP (or ATP).

In the following experiments the time course of the change in fluorescence emission, using a filter with a pass-band centered at 355 nm



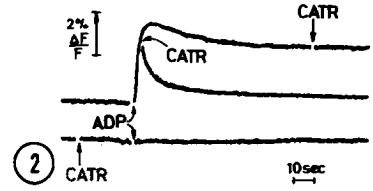


Figure 1. Fluorescence emission spectra of the ADP/ATP carrier protein.

The carrier protein was prepared, as described in Materials and Methods and used at a concentration of 0.22 mg/ml, final pH 6.5. Excitation was at 295 nm using a 2 nm slit width. The emission slit width was 5 nm. ATP and CATR were used at the final concentrations of 50 µM and 10 µM respectively. Other conditions are as described in Materials and Methods.

Figure 2. Time course of the ADP-induced fluorescence change of the ADP/ATP carrier protein.

Emitted light was recorded at 355 nm. Other conditions are described in Materials and Methods. The protein concentration was 0.125 mg/ml. ADP and CATR were added to give a final concentration of 100 µM and 5 µM respectively.

(cf Methods) was followed over periods of time ranging from less than 1 sec to 5 min. Addition of 100 µM ADP (or ATP), a saturating concentration, resulted in a 4 to 6 % enhancement of fluorescence (Fig. 2) ; the time to reach the maximum was about 10 sec. This was followed by a slow fluorescence decrease down to a final level which was 2 to 3 % higher than the initial one and corresponded in fact to the fluorescence state of the ATP-treated carrier described in Figure 1. The half maximum effect on the initial rise in fluorescence was given by 5µM ADP. No effect was detected with the following nucleotides used at a final concentration of 50 µM : CDP, UDP, GDP, IDP, CTP, UTP, GTP, ITP, AMP and AOPCP. There was no pH dependence between pH 6.5 and 7.5.

When CATR was added before the rise in fluorescence reached its maximum, a complete reversal of the enhanced fluorescence was observed (Fig. 2). When added after the fluorescence decline, CATR was no longer active. CATR alone did not induce any fluorescence modification, but it totally prevented the ADP(ATP)-induced fluorescence increase. The dose-inhibition curve characteristic of CATR inhibition was strictly linear (Fig. 3). As the concentration in carrier protein (5 µM) is higher than the

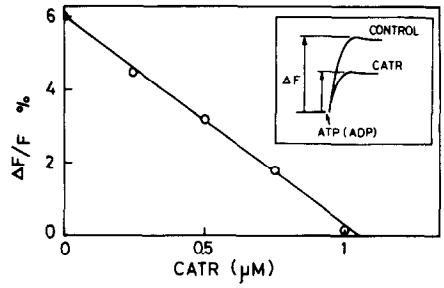


Figure 3. Inhibition by CATR of the ATP-induced fluorescence change of the ADP/ATP carrier protein.

CATR was added at different concentrations prior to addition of ATP (50 μM final concentration). Protein concentration : 0.15 mg/ml. The amplitude of the fast fluorescence change (Inset) was plotted as a function of CATR concentration.

apparent dissociation constant of the inhibitor (310 nM) (7), the concentration in CATR sites can be virtually equated to the CATR concentration yielding full inhibition. In the presence experiment, this value was 1.1 μM i.e. about one fourth of the carrier concentration, assuming a purity of 70-80 % (7) and a M.W. of 30,000 for the carrier protein (or one half of the carrier concentration assuming that the native form of the carrier is a 60,000 M.W. dimer which would be fully inhibited by one molecule of carboxyatractyloside). The binding of CATR to only part of the ADP/ ATP carrier protein molecules may be explained by inactivation of a fraction of the carrier protein during the preparation.

BA, another specific inhibitor of ADP/ATP transport, had just the opposite effect of that of CATR on the ADP/ATP-induced enhancement of fluorescence (Fig. 4). Addition of 5 μM BA resulted in a 60 % increase of the extent of the fluorescence rise obtained by addition of a saturating concentration of ATP (50 μM) (Fig. 4). The same effect was obtained with ADP. The overstimulated fluorescence resulting from the addition of both ATP (or ADP) and BA was slowly reversed by CATR to the basal level (Fig. 4). The $K_{1/2}$ for the effect of BA was 1.5-2.0 μM in the presence of 10 μM ATP (Fig. 5). On the other hand, the concentration of ATP (or ADP) required to obtain a given fluorescence response was markedly decreased by BA (more than twice) with respect to the control. BA alone had no effect.

It has been postulated that the true substrates for the mitochondrial ADP/ATP transport are the free forms of ADP and ATP (9) (10). This view is strongly supported by the fact that Mg^{++} inhibited the ADP(ATP)-induced rise in fluorescence of the isolated carrier protein (Fig. 6). The inhibitory

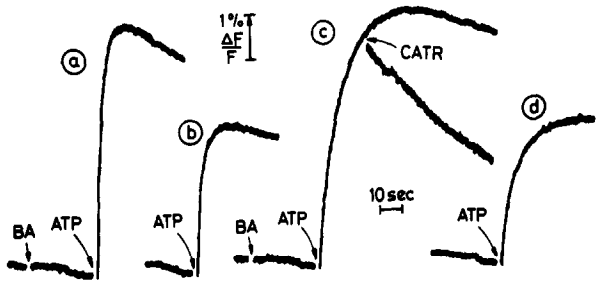


Figure 4. Effect of BA on the ATP-induced fluorescence change of the ADP/ATP carrier protein.

Same conditions as in Figure 2. The following concentrations were used : a- and b- 50 μM ATP, 5 μM BA ; c- and d- 10 μM ATP, 5 μM BA, 2 μM CATR.

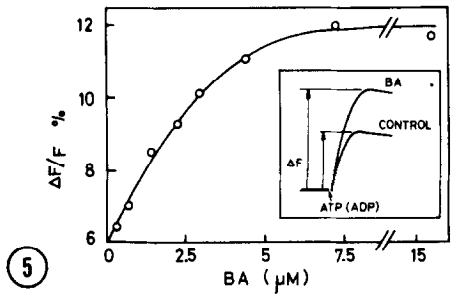
effect was more severe with ATP than with ADP, which suggested that Mg^{++} did not interact directly with the protein, but interfered essentially by chelating ATP or ADP. Since the apparent affinity of Mg^{++} for ATP is about 10 times higher than for ADP at neutral pH (11), it is expected that free ATP is more severely depleted than free ADP upon addition of Mg^{++}.

DISCUSSION

This work represents the first attempt to investigate substrate-induced conformational changes of the ADP/ATP carrier protein by study of its intrinsic fluorescence. That the ADP(ATP)-induced modifications in fluorescence intensity reflect conformational changes related to the functioning of the carrier is supported by two lines of evidence : 1- The fluorescence changes are sensitive to specific inhibitors of ADP/ATP transport, for example CATR and BA. 2- They are specific for ADP and ATP like mitochondrial ADP/ATP transport ; 3- The half maximum effect is obtained with ATP and ADP concentrations of 5 and 25 μM, which are close to the K_M values for ADP/ATP transport in mitochondria (3).

The present fluorescence data show that ADP, ATP, CATR and BA interact with the isolated carrier protein. Interaction with those ligands does not require the carrier to be incorporated into phospholipid vesicles.

The slow decline in fluorescence that follows the rapid ADP- or ATP-induced rise of fluorescence probably reflects an inactivation of the ADP/ATP carrier, since at that time neither CATR, nor ADP or ATP longer affects the fluorescence intensity of the protein. Inactivation by long term contact with ADP or ATP is corroborated by the marked red shift in the fluorescence



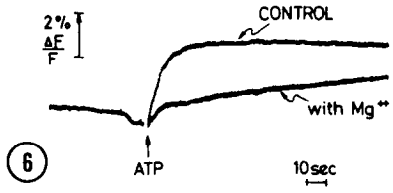


Figure 5. Titration of the enhancing effect of BA on the ATP-induced fluorescence change of the ADP/ATP carrier protein.

BA was added at different concentrations prior to addition of 10 μM ATP. Protein concentration was 0.15 mg/ml. The amplitude of fast response (Inset) was plotted as a function of BA concentration.

Figure 6. Effect of Mg^{++} on the ATP-induced enhancement of fluorescence of the ADP/ATP carrier protein.

Same conditions as in Figure 2. The following concentrations were used : ATP 50 μM, $MgSO_4$ 2 mM, protein concentration 0.12 mg/ml.

emission spectrum of the carrier protein. Similar red shift occurs in protein denatured by heat or urea (12).

The different fluorescence states of the isolated ADP/ATP carrier protein revealed by this study are interpreted on the basis of different conformational states (Fig. 7). In the absence of nucleotide, the carrier is in a low fluorescence state (E) that is supposed to correspond to a basal conformation of the carrier. In the presence of ADP or ATP, there is a transitory increase in fluorescence to a new state (E^+), that is supposed to correspond to an activated conformation of the carrier. This fast transition is followed by a slow irreversible decline to an inactivated form (E' fluorescence state). CATR prevents the ADP(ATP)-induced enhancement of fluorescence, most likely by binding firmly to the carrier in its basal conformation ; the carrier-CATR complex is supposed to correspond to the fluorescence state E_o. On the other hand, CATR is able to reverse the fluorescence state E^+ to give back the basal fluorescence state E ; it is noteworthy that full reversal is possible only when CATR is added during or shortly after the fluorescence rise that follows addition of ADP or ATP. An attractive hypothesis is that the basal conformation of the carrier (E fluorescence state) and its activated conformation (E^+ fluorescence state) are in equilibrium, and that CATR by binding to the basal conformation shifts the equilibrium towards this conformation. However, a direct binding of CATR to the activated conformation of the carrier is not excluded.

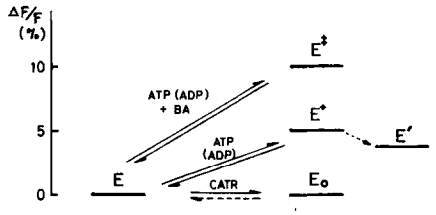


Figure 7. Fluorescence states of the ADP/ATP carrier protein at 355 nm.

The scheme does not imply that the E^+ state corresponds to an ADP or ATP bound form of the carrier, nor that the $E^‡$ state corresponds to an ADP(ATP)-BA bound form of the carrier.

Another point of interest concerns the question of whether ADP and ATP are transported by the carrier in their free or Mg^{2+}-complexed forms. Although data in literature make it clear that the free forms of ADP and ATP are substrates (9) (10), it has not been demonstrated that they are the true substrates. In fact the role played by Mg^{++} in a number of experiments dealing with mitochondria or reconstituted transport systems was difficult to assess, due to the interaction of Mg^{++} with phospholipids. The present data give the first direct evidence that the true substrates of the ADP/ATP carrier are the free forms of ADP and ATP.

A third highly fluorescent state $E^‡$ is introduced to take into account the further enhancement of fluorescence of the carrier in the presence of both BA and ADP(ATP). CATR is able to reverse the $E^‡$ fluorescence state to the E state ; as shown above, CATR is also able to reverse the E^+ fluorescence state to the E state. This effect of CATR probably reflects an equilibrium between the three states E, E^+ and $E^‡$. One may wonder whether the population of carrier units in the E^+ state could not be a mixture of two extreme conformations corresponding to the basal state E and the highly fluorescent state $E^‡$ respectively. The effect of BA would be to shift the equilibrium to the $E^‡$ state. Another possibility is that the E^+ and $E^‡$ states correspond to different well-defined conformations of the carrier. In this case, the conformation of the $E^‡$ state could be either a binary (carrier-BA), or ternary (carrier-BA-ADP(ATP)) complex.

ACKNOWLEDGEMENTS

The authors wish to thank Sylvain Blanquet for its interest and participation in the initial stage of this work. The investigation was supported by research grants from the "Délégation à la Recherche Scientifique et Technique" and the "Fondation pour la Recherche Médicale".

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