Vol. 192, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1993 Pages 143-150

BIOCHEMICAL CHARACTERISATION OF THE ISOLATED Anc2 ADENINE NUCLEOTIDE CARRIER FROM *SACCHAROMYCES CEREVISIAE* MITOCHONDRIA

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Received February 23, 1993

Summary: The yeast mitochondrial adenine nucleotide carrier isoform encoded by the *ANC2* gene has been specifically expressed in a yeast strain disrupted for the two other genes, *ANC1* and *ANC3*. Isolation of the carrier in a functional form was achieved by utilisation of a mixture of two detergents, dodecylmaltoside and Emulphogen. The intrinsic fluorescence of the Anc2 protein was specifically and rapidly enhanced upon addition of the transportable nucleotides ADP and ATP. Fluorescence enhancement was prevented or reversed by the addition of a stoichiometric amount of CATR. Addition of CATR alone elicited a dose-dependent decrease of fluorescence. The *ANC2*-specific yeast strain offers the means to study a single ADP/ATP carrier, with a well-defined amino acid sequence, suitable for analysis of substrate- or inhibitor-induced conformational changes.

The adenine nucleotide carrier (ANC) is a nuclear encoded protein, located in the inner mitochondrial membrane, that catalyses the transmembrane exchange of ADP and ATP between the cytosolic and the matrix compartments. In yeast, three genes, *ANC1*, *ANC2* and *ANC3*, encoding isoforms of the adenine nucleotide carrier, have been isolated and characterized (1-3). Recently, a mutated form of the *ANC2* gene, containing a single base change, has been reported to be responsible for the *op1* mutation in yeast (3, 4 and unpublished data). The three genes exhibit a high degree of similarity; however, disruption of the genes *ANC1* and *ANC3* revealed that they are not essential for the growth of yeast on a non-fermentable carbon source, whereas disruption of the *ANC2* gene yielded a mutant unable to grow on glycerol (3, 4). Therefore, the

Abbreviations: CATR : carboxyatractyloside, DFP : diisopropyl fluorophosphate, LAPAO : 3-laurylamido-N,N'-dimethylaminopropylaminoxyde, Mops : 3-[N-morpholino]propanesulfonic acid, PMSF : phenylmethyl sulfonyl fluoride, CHAPS: 3-[3-(cholamidopropyl)-dimethylammonio]-1-propane sulfonate.

0006-291X/93 $4.00

ANC2 gene appears to encode the major isoform of the mitochondrial ADP/ATP carrier protein which is expressed in yeast grown under aerobic conditions.

In order to study the structure-function relationships of the Anc2 isoform of the yeast mitochondrial ADP/ATP carrier, we have constructed a *Saccharomyces cerevisiae* strain in which *ANC2* was the only active gene for the carrier. We report here a preliminary biochemical characterisation of the carrier isoform expressed in mitochondria of this strain. We investigated the interactions of the ADP/ATP carrier with specific ligands, either in the membrane-bound state or in the isolated state in the presence of detergent. In the latter case, studies were based on intrinsic fluorescence measurements since it has been established that the tryptophanyl fluorescence of the beef-heart ADP/ATP carrier is modified in response to conformational changes induced by ADP or ATP (5, 6).

MATERIAL AND METHODS

Nucleotides and carboxyatractyloside were purchased from Sigma and Calbiochem, respectively. Dodecylmaltoside was obtained from Boehringer. Emulphogen BC720 (GAF Corporation, New York) was purified following the procedure described by Ashani and Catravas (7). [^{3}H]atractyloside was synthesised as previously described (8). Protein concentration was determined with the BCA reagent kit from Pierce. DNA manipulations were performed by standard procedures (9, 10). Yeast cultures were grown at 28 °C in YP-Lac medium (2% Bacto-Peptone, 1% yeast extract, 2% lactate, 1% KH_2PO_4, pH 5.5). Mitochondria were prepared according to the method described by Daum et al. (11). Protoplasts were obtained by enzymatic treatment of cells with Zymolyase 20T (Calbiochem). They were disrupted in a medium consisting of 0.6 M mannitol, 0.1 mM EDTA, 10 mM Tris-Cl, 0.1% BSA (fatty acid free) and 1 mM PMSF, final pH 7.4. Mitochondria were collected by differential centrifugation and then washed and resuspended in the same medium but devoid of BSA and PMSF; they were stored in liquid nitrogen.

The ADP/ATP carrier protein from yeast mitochondria was isolated by chromatography on hydroxylapatite (BioRad) following the method described in (12) after modification. Yeast mitochondria were solubilized at a final concentration of 10 mg/mL in 0.5 M Na_2SO_4, 10 mM Tris-Cl, 1 mM EDTA, 1 mM PMSF, 1 mM DFP, 2% (w/v) dodecylmaltoside and 1.7% (v/v) purified Emulphogen BC720, final pH 7.4 for 10 min at 0 °C. The lysate was centrifuged at 20000 g for 10 min at 4 °C and the supernatant was applied to a hydroxylapatite column (2 ml of settled gel/mg total protein). The pass-through fraction was further purified by chromatography on Ultrogel AcA 202 gel equilibrated with 50 mM Mops, 0.1 mM EDTA and 0.2 % (v/v) Emulphogen BC720. The carrier protein fraction was eluted immediately after the void volume. For fluorescence assays, the AcA 202 protein was diluted four times with 136 mM glycerol, which resulted in a final protein concentration ranging between 0.03 and 0.05 mg/ml.

Fluorescence measurements were made at 10 °C in a high sensitivity fluorometer (Bio-Logic, Grenoble) under the experimental conditions previously described (5, 6). Briefly, the protein fluorescence was excited at 296 nm and the emitted fluorescence light was measured at a right angle through a 0.54 Corning filter coupled to an UV light filter, with a resulting band pass centered at 355 nm.

Binding assays of [^{3}H]atractyloside were carried out as follows. Mitochondria (0.1 ml, 10 mg/ml) were added to a standard medium (1 ml) consisting of 0.125 M KCl, 10 mM Tris-Cl, 1 mM EDTA, final pH 7.4 and containing increasing concentrations of tritiated atractyloside, up to 3 µM. After a 30 min incubation at 0 °C, mitochondria were sedimented and their radioactivity was determined by scintillation counting. Parallel assays were performed in the presence of 20 µM carboxyatractyloside to correct for the minor unspecific binding of atractyloside.

RESULTS

Construction of the *ANC2* strain 2N1-3

The yeast strain JL-1-3, kindly provided by J. Kolarov (13), is derived from W303-1B (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*); its three genes *ANC1*, *ANC2*,

ANC3 are interrupted by *LEU2*, *HIS3* and *URA3*, respectively. The *ANC2* gene obtained by complementation of the *op1* mutation (unpublished work) was reintroduced into JL-1-3 by homologous recombination of a *Pst*I-*Eco*RI DNA fragment (2.85 kb) using a non-selective transformation (14). Transformants able to grow on a non-fermentable carbon source (glycerol) were checked for their ability to grow on a medium lacking histidine, and then integration at the *ANC2* locus was verified by Southern blot analysis (figure 1). A His^- transformant with the expected genomic structure was saved and named 2N1-3.

Binding of atractyloside to Anc2 mitochondria

As shown on figure 2, binding of atractyloside to mitochondria isolated from the *ANC2* strain 2N1-3 increased as a function of the concentration of added atractyloside and reached a plateau. From the Scatchard plot of the binding data, the number of atractyloside binding sites was estimated at 0.5-0.6 nmol/mg of protein, the Kd value being 150-200 nM.

Substrate-induced fluorescence changes in the isolated Anc2 carrier

We have previously reported that the ADP/ATP carrier from beef heart mitochondria exhibited substrate-induced fluorescence changes due to modifications in the environment of tryptophanyl residues (5, 6). We therefore examined whether the ADP/ATP carrier from the yeast *ANC2* strain could also undergo conformational changes reflected by changes in its intrinsic fluorescence. In a preliminary study, a number of detergents were assayed for their

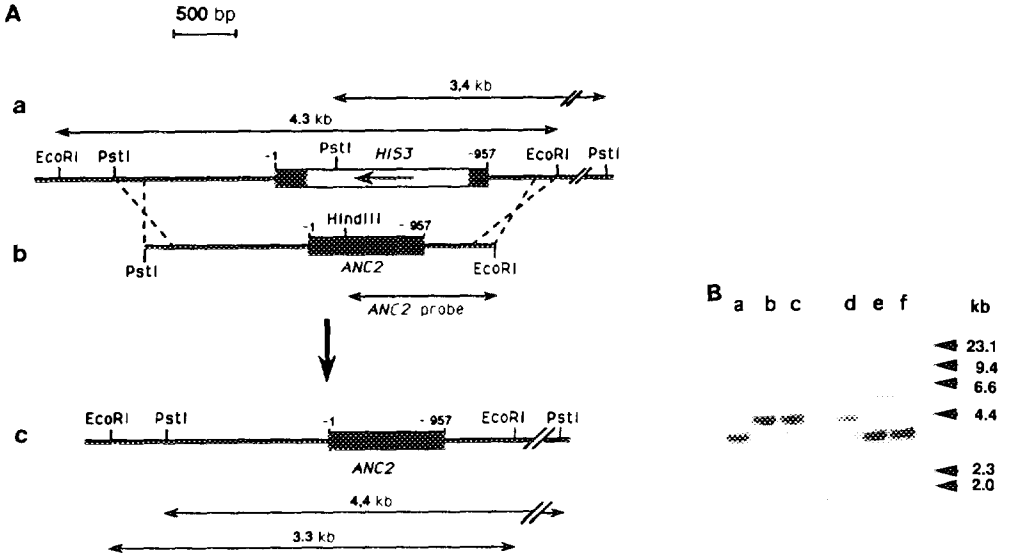

Figure 1. Construction of the *ANC2* strain 2N1-3.
A) Schematic representation of the homologous recombination at the *ANC2* locus. a) *HIS3*-disrupted *ANC2* locus on chromosome II of the yeast strain JL-1-3; b) *Pst*I-*Eco*RI DNA fragment bearing the wild-type *ANC2* allele; c) *ANC2* locus of the transformant 2N1-3. B) Southern blot hybridization analysis of yeast genomic DNA with the 1.22-kb *Hin*dIII-*Eco*RI *ANC2*-specific probe. Lanes a, d: JL-1-3 ; lanes b, e: wild type W303-1B; lanes c, f: transformant 2N1-3. *Pst*I digestion, lanes a, b, c; *Eco*RI digestion, lanes d, e, f.

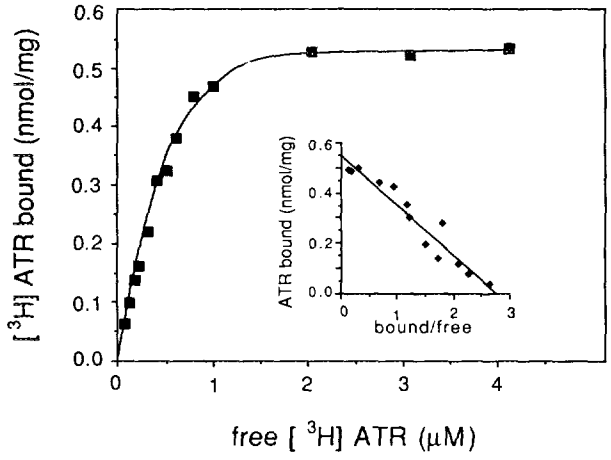

Figure 2. Binding of tritiated atractyloside to mitochondria from the *ANC2* strain. Mitochondria (0.1 ml, 10 mg/ml) were added to 1 ml of KCl standard medium containing increasing concentrations of [^{3}H]atractyloside and incubated for 30 min at 0 °C. After centrifugation, bound [^{3}H]atractyloside was determined by scintillation counting. When present, CATR was added to a final concentration of 20 μM. The inset corresponds to the Scatchard plot of the binding data.

ability to isolate the Anc2 carrier in a soluble and functional form. Two of them, namely dodecylmaltoside and Emulphogen, used in combination for the lysis of mitochondria were able to yield an ADP/ATP carrier preparation virtually devoid of porin, a protein which usually co-purifies with the ADP/ATP carrier. The ADP/ATP carrier was obtained in a reasonably functional state, with about 50% of the atractyloside binding sites being recovered. Attempts to purify the yeast Anc2 carrier in the presence of LAPAO or CHAPS, two detergents that have been successfully used to isolate the beef heart mitochondrial ADP/ATP carrier, led to inactivated carrier preparations.

Figure 3 shows the time-course of the ATP-induced change of fluorescence of the yeast ADP/ATP carrier, measured at 10°C. Addition of ATP at a sub-saturating concentration (5 μM) resulted in a very rapid, 4-5% enhancement of fluorescence. This high fluorescence state of the ADP/ATP carrier was stable for at least 1 minute at 10 °C. Upon addition of CATR, the fluorescence decreased rapidly to a level 3% lower than the initial level of fluorescence of the unliganded carrier. As illustrated on figure 3, the amplitude of the fluorescence signal increased with the concentration of added ATP to reach a plateau at about 8-10 μM ATP, the half-maximum effect being obtained with 0.6 μM ATP. ADP induced a fluorescence increase similar to that obtained with ATP, with a half-maximal effect at 2 μM, but other nucleotides such as AMP, UDP, UTP, GDP, GTP or 8Br-ADP used at a 50 μM final concentration were ineffective.

When added prior to ATP or ADP, CATR used at saturating concentrations was responsible for two distinct effects : (i) it induced a 3% decrease of the fluorescence of the carrier and (ii) it totally prevented the ATP (ADP)-induced fluorescence increase (figure 3). Careful analysis of the amplitude of the fluorescence changes indicated that the CATR-induced decrease of fluorescence and the ATP (ADP)-induced increase of fluorescence were independent

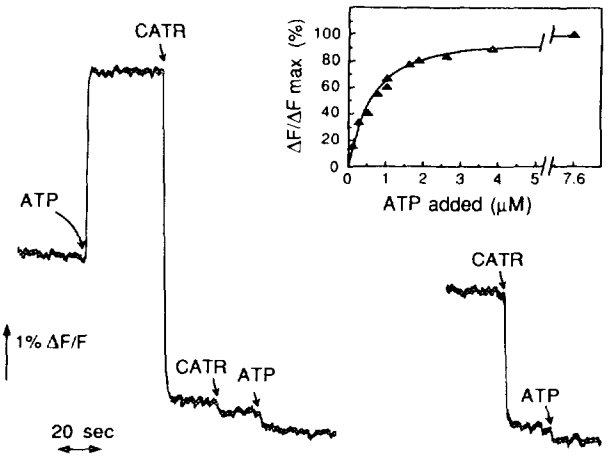

Figure 3. Time-course of ATP- and CATR-induced fluorescence changes of the isolated Anc2 carrier.
The assay was carried out at 10 °C. Fluorescence emission was recorded at 355 nm as described in "Material and Methods". The protein concentration was 0.040 mg/ml. ATP and CATR were added to a final concentration of 2.5 μM.
Inset : titration of the enhancing effect of ATP on the fluorescence of the isolated Anc2 carrier. The amplitude of the response was plotted as a function of the ATP concentration.

effects. They probably involve distinct tryptophanyl residues contributing to the fluorescence changes that were observed.

In an early study, it was reported that the beef heart carrier protein underwent a biphasic fluorescence change upon addition of ATP. When the beef heart carrier was prepared in the presence of dodecylmaltoside and Emulphogen, similarly to the yeast carrier, a monophasic ATP-induced fluorescent enhancement signal was observed, similar to that described above for the yeast Anc2 carrier (see figure 3). As in the case of the yeast carrier, the highly fluorescent level decreased rapidly to the initial level upon addition of CATR. When added first, CATR inhibited the ATP-induced fluorescence increase. However, in contrast to the CATR-induced fluorescence decrease observed with the yeast Anc2 carrier and to the effect observed previously with LAPAO as detergent, CATR elicited an increase of fluorescence of moderate amplitude with the beef heart carrier. Another unexpected result with the beef heart carrier was the reversion by ATP of the fluorescence signal induced by CATR.

Comparative titrations of the CATR binding sites present in the isolated Anc2 carrier

Titration of CATR binding sites can be performed by measuring either the extent of the CATR-dependent inhibition of the ATP-induced fluorescence increase or the CATR-induced fluorescence decrease in the presence of increasing concentrations of CATR. Both types of measurement were carried out with the Anc2 carrier and are illustrated in figure 4. The dose-inhibition curves were strictly linear, which is indicative of the very high affinity binding of CATR to the ADP/ATP carrier protein. The same end-points are found for both titrations, corresponding to 6-7 nmol of CATR sites/mg of protein. On the basis of the amount of CATR

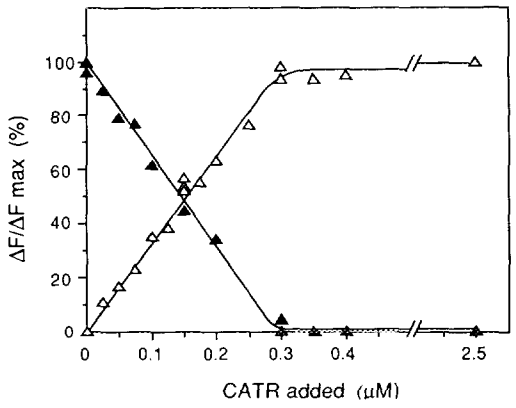

Figure 4. Fluorometric titrations of the CATR binding sites present on the Anc2 carrier protein.
The amplitude of the CATR-induced fluorescence decrease (▲) and the inhibition by CATR of the ATP-induced fluorescence increase (△), measured in separate assays on the same carrier preparation, were plotted as a function of CATR concentration. Protein concentration was 0.040 mg/ml. When used, ATP was added to a final concentration of 2.5 μM.

and atractyloside binding sites of the carrier determined either in its membrane-bound state or its isolated state, it can be calculated that the carrier preparation was enriched 10-12 times with about 50% recovery during the purification procedure.

DISCUSSION

In the yeast *S. cerevisiae*, the *ANC2* gene is the only one, out of three, that encodes a mitochondrial ADP/ATP carrier essential for the cells to grow under aerobic conditions on nonfermentable carbon sources. The prerequisite for the study of the structure-function relationships of the Anc2 carrier protein from yeast mitochondria was the construction of a strain that expresses exclusively this isoform of the carrier. This paper reports the preliminary characterisation of the mitochondrial Anc2 carrier from a triply disrupted strain *ΔANC1*, *ΔANC2*, *ΔANC3* in which the *ANC2* gene was reintroduced. The carrier protein content in mitochondria from the *ANC2* strain 2N1-3 was determined from the amount of bound atractyloside. The number of atractyloside binding sites amounted to 500-600 pmol/mg mitochondrial proteins, which indicated an efficient expression of the Anc2 carrier protein.

The Anc2 polypeptide contains three tryptophanyl groups (2) that are largely responsible for its intrinsic fluorescence. We have shown that the fluorescence of the Anc2 carrier could be significantly enhanced in the presence of ATP or ADP, a result that reflected conformational changes undergone by the protein upon binding of substrates. This effect was triggered by ADP or ATP used at low concentrations, half-maximum effects being produced in the presence of 0.6 μM ATP or 2 μM ADP. Non-transportable nucleotides were ineffective. When added prior to ATP, CATR, which is a very potent and specific inhibitor of ADP/ATP transport in mitochondria, inhibited the ATP-induced fluorescence change of the yeast carrier in a dose-dependent manner that allowed the titration of the CATR binding sites. These data led us to

conclude that the ATP (ADP)-induced fluorescence changes are typical of a functional carrier.

In our experimental conditions, the substrate-induced fluorescence changes of the yeast ADP/ATP carrier were kinetically monophasic, even when observed at 0 °C (not shown), whereas those elicited in the beef heart ADP/ATP carrier isolated in the presence of LAPAO were biphasic at 10 °C or lower (10). In the later case, it was postulated that the rapid phase was related to the binding of nucleotides and that the slow phase reflected a subsequent conformational change of the carrier, possibly occurring at the onset of the transport process. However, when the beef heart carrier was prepared in dodecylmaltoside and Emulphogen instead of LAPAO, the slow phase was abolished, resulting in fluorescence variations similar to those observed with the yeast ADP/ATP carrier protein. This result indicates that the nature of the detergent, which in some cases determines the conformation of the isolated ADP/ATP carrier (15), may also restrict the conformational changes, possibly because of different constraints imposed in detergent-carrier mixed micelles.

The transition of the ADP/ATP yeast carrier from a basal to a high fluorescence level upon addition of ADP or ATP is rapid. Within the limits of the time resolution of our experiments, it did not depend on temperature. Furthermore, the transition was triggered in a dose-dependent manner by the presence of ATP or ADP. From these observations, it may be suggested that the high-fluorescence conformational state of the carrier reflects the formation of the carrier-ATP (or -ADP) complex. Upon addition of CATR, which is able to displace the bound nucleotide, the yeast carrier is shifted to a fluorescence level lower than that of the unliganded state, corresponding probably to the formation of the inactivated CATR-carrier complex. This is supported by the fact that, in the absence of added nucleotide, CATR is able to induce a dose-dependent conformational change of the yeast ADP/ATP carrier that results in an intrinsic fluorescence decrease. To explain that the CATR-induced fluorescence signal adds to that of the CATR-induced reversal of the ATP (or ADP)-triggered fluorescence enhancement, one may suggest that distinct tryptophanyl residues are involved in response to the binding of CATR and ATP (ADP) to the carrier protein. The bovine and yeast ADP/ATP carrier contain five and three tryptophanyl residues, respectively; alignment of the amino acid sequences of both carriers shows that the locations of the three tryptophanyl groups present in the yeast carrier almost coincide with three tryptophanyl residues of the bovine carrier. The yeast W87 residue and its bovine analogue W109 belong to a highly conserved region while W126 and W235 and their bovine equivalents W109 and W113, respectively, are located in less conserved regions. These different local environments could explain the qualitative differences of fluorescence responses of the bovine and the Anc2 carriers upon interaction with CATR. However, one cannot exclude the possibility that discrete changes in the polypeptide chain arrangement occurring at distance from the tryptophanyl residues upon association of CATR could result in a different exposure of tryptophanyl group(s) or possibly of tyrosyl residues.

Acknowledgments: A. Le Saux was supported by a fellowship of the French Ministère de la Recherche et de l'Espace. This work was funded in part by research grants from the Centre National de la Recherche Scientifique, the University of Bordeaux II and the University Joseph Fourier, Faculté de Médecine de Grenoble.

REFERENCES

1. Adrian, G.S., McCammon, M.T., Montgomery, D.L. and Douglas, M.G. (1986) Mol. Cell. Biol. 6, 626-634.
2. Lawson, J.E. and Douglas, M.G. (1988) J. Biol. Chem. 263, 14812-14818.
3. Kolarov, J., Kolarova, N. and Nelson, N. (1990) J. Biol. Chem. 265, 12711-12716.
4. Lawson, J.E., Gawaz, M., Klingenberg, M. and Douglas, M.G. (1990) J. Biol. Chem. 265, 14195-14201.
5. Brandolin, G., Dupont, Y. and Vignais, P.V. (1981) Biochem. Biophys. Res. Comm. 98, 28-35.
6. Brandolin, G., Dupont, Y. and Vignais, P.V. (1985) Biochemistry 24, 1991-1997.
7. Ashani, Y. and Catravas, G.N. (1980) Anal. Biochem. 109, 55-62.
8. Brandolin, G., Meyer, C., Defaye, G., Vignais, P.M. and Vignais, P.V. (1974) FEBS Lett. 46, 149-153.
9. Maniatis, T., Fritsch, E.F. and Sambrook J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
10. Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Method in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
11. Daum, G., Bohni, P. and Schatz, G. (1982) J. Biol. Chem. 257, 13028-13033.
12. Krämer, R. and Klingenberg, M. (1977) Biochemistry 16, 4954-4961.
13. Drgon, T., Sabova, L., Nelson, N. and Kolarov, J. (1991) FEBS Lett. 289, 159-162.
14. Gietz, R.D. and Schiestl, R.H. (1991) Yeast 7, 253-263.
15. Block, M.R. and Vignais, P.V. (1986) Biochemistry 25, 374-379.