Journal of Bioenergetics and Biomembranes, Vol. 35, No. 3, June 2003 (© 2003)

Four Mutations in Transmembrane Domains of the Mitochondrial ADP/ATP Carrier Increase Resistance to Bongkrekic Acid[1]

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Received January 13, 2003; accepted March 25, 2003

Two distinct conformations of the mitochondrial ADP/ATP carrier involved in the adenine nucleotide transport are called BA and CATR conformations, as they were distinguished by binding of specific inhibitors bongkrekic acid (BA) and carboxyatractyloside (CATR), respectively. To find out which amino acids are implicated in the transition between these two conformations, which occurs during transport, mutants of the *Saccharomyces cerevisiae* ADP/ATP carrier Anc2p responsible for resistance of yeast cells to BA were identified and characterized after in vivo chemical or UV mutagenesis. Only four different mutations could be identified in spite of a large number of mutants analyzed. They are located in the Anc2p transmembrane segments I (G30S), II (Y97C), III (L142S), and VI (G298S), and are independently enabling growth of cells in the presence of BA. The variant and wild-type Anc2p were produced practically to the same level in mitochondria, as evidenced by immunochemical analysis and by atractyloside binding experiments. ADP/ATP exchange mediated by Anc2p variants in isolated mitochondria was more efficient than that of the wild-type Anc2p in the presence of BA, confirming that BA resistance of the mutant cells was linked to the functional properties of the modified ADP/ATP carrier. These results suggest that resistance to BA is caused by alternate conformation of Anc2p due to appearance of Ser or Cys at specific positions. Different interactions of these residues with other amino acids and/or BA could prevent formation of stable inactive Anc2p • BA complex.

KEY WORDS: ADP/ATP carrier; bongkrekic acid resistant mutants; yeast mitochondria.

INTRODUCTION

The adenine nucleotide carrier (Ancp) of the inner mitochondrial membrane exchanges ADP and ATP between cytoplasm and mitochondrial matrix. It is thus a key energetic link between these two compartments of an eukaryotic cell. Three nuclear genes are encoding Ancp in yeast *Saccharomyces cerevisiae* (Adrian *et al.*, 1986; Kolarov *et al.*, 1990; Lawson and Douglas, 1988) but only *ANC2* gene encoding Anc2p protein is required for growth on nonfermentable carbon sources (O'Malley *et al.*, 1982) and can also support growth under anaerobic conditions, despite absence of the hypoxic isoform *ANC3* (Drgon *et al.*, 1991).

Ancp is a typical member of the mitochondrial carrier family (MCF) (Walker and Runswick, 1993). It has 318 amino acids arranged in three sequence-related domains,

Key to Abbreviations: ATR, atractyloside; Ap_5A, P_1P_5-di(adenosine-5′)-pentaphosphate; BA, bongkrekic acid; BA^R, BA resistant; CATR, carboxyatractyloside; EMS, ethylmethanesulfonate; E.U., enzymatic units (μmol/min); isoBA, isobongkrekic acid; TMS, transmembrane segment; WT, wild-type.

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0145-479X/03/0600-0243/0

each with about 100 amino acids. Native Anc2p has six transmembrane segments (TMS) with N- and C-terminal regions exposed to the intermembrane space and the functional unit of Ancp is at least a dimer (for reviews, see Brandolin *et al.*, 1993a; Klingenberg, 1993). Dimeric organization of Anc2p was recently corroborated by characterization of covalent tandem Anc2p dimers (Hatanaka *et al.*, 1999; Trézéguet *et al.*, 2000).

Mitochondrial ADP/ATP transport is specifically inhibited by two classes of natural high-affinity inhibitors. The first class is represented by bongkrekic acids (BA and its isomer isoBA). The second class comprises atractyloside (ATR) and carboxyatractyloside (CATR). Lipophilic BA is an uncompetitive inhibitor which binds to the Ancp sites accessible from the matrix side while nonpenetrant inhibitors ATR (competitive) and CATR (noncompetitive) interact with Ancp sites exposed to the cytosol. These inhibitors recognize two preexisting carrier conformations in equilibrium in the inner mitochondrial membrane, referred as the BA and CATR conformations, and form stable complexes with Ancp. In the absence of inhibitors, rapid reversible conversion from one conformation to the other is triggered only by substrates ADP or ATP and therefore is probably involved in the transport process. Ancp • BA or Ancp • CATR complexes have different chemical and immunochemical properties; this has been fully documented for bovine and yeast Ancp thanks to the utilization of photoactivatable analogues of ADP and ATR, chemical modifiers, antipeptide antibodies, proteases, and measurement of intrinsic fluorescence changes upon binding of substrates and inhibitors (for review, see Brandolin *et al.*, 1993a).

To understand the precise mechanism of the ADP/ATP translocation, it is necessary to identify Ancp amino acids involved in interactions with BA or CATR and/or in the conformational changes induced by inhibitors and relevant to transport. BA in contrast to CATR and ATR penetrates through cell membrane in protonated form at acidic pH. This fact made possible search for Anc2p mutants resistant to BA in vivo. In this study, a yeast strain sensitive to BA was mutagenized with UV light or by chemical agent and mutants resistant to BA (BA^R) supporting growth on nonfermentable carbon source were screened and analyzed. Four single Anc2p mutations conferring BA^R phenotype to cells were thus discovered and strains expressing individual Anc2p mutants from *ANC2* chromosomal locus were created. We have analyzed the cellular properties of mutant strains, characteristics of Anc2p BA^R mutants in mitochondria and their ability to maintain ADP/ATP transport in the absence or presence of specific inhibitors, BA, CATR, or ATR.

MATERIALS AND METHODS

Chemicals

Nucleotides were purchased from Sigma, CATR and P_1P_5-di(adenosine-5′)-pentaphosphate (Ap_5A) from Calbiochem. [^{3}H]ATR was synthesized as described in the literature (Brandolin *et al.*, 1974). BA and isoBA were prepared according to Lauquin *et al.* (1976) and Lauquin and Vignais (1976). Hexokinase/glucose-6-phosphate dehydrogenase enzyme mix was obtained from Roche Diagnostics GmbH. Protein concentration was determined with the BCA (bicinchoninic acid) reagent kit from Sigma.

Strains and Media

The *Escherichia coli* strain XL1-Blue (*recA1 endA1 gyrA96 (Nal^R) thi hsdR17 (r_K^- m_K^+) supE44 relA1 lac$^-$ F′ [Tn10 (tet^R) proAB$^+$ lacI^q lacZΔ M15])* was used for plasmid amplification. It was grown on modified Luria both (1% Bactotryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl (pH 7.5)) plus 100 μg ampicillin/mL when necessary. Yeast strains used in this study were: JL1-3-*ANC2 (Matα leu2-3,112 his3-11,15 ade2-1 trp1-1 ura3-1 can1-100 anc1::LEU2 anc3::URA3)* which refers to the strain 2N1-3 (Brandolin *et al.*, 1993b); JL1-3-*ANC2-W87Y-W235Y* (parental strain) refers to JL1-3-*ANC2-W126* (Le Saux *et al.*, 1996); JL1-3Δ2 *(Matα leu2-3,112 his3-11,15 ade2-1 trp1-1 ura3-1 can1-100 anc1::LEU2 Δanc2::HIS3 anc3::URA3)* (De Marcos Lousa *et al.*, 2002). Yeast cells were grown at 28°C on rich media YPD, YPGal, YPG, or YPL (1% yeast extract (Difco), 2% Bactopeptone (Difco) supplemented with 2% glucose, 2% galactose, 3% glycerol, or 2% lactate plus 1% KH_2PO_4, pH 5.5, respectively) or on synthetic complete medium lacking tryptophan or other amino acids (0.67% Bacto yeast nitrogen base, 2% glucose supplemented with uracil, adenine, and all amino acids but tryptophan or other amino acids) (Sherman *et al.*, 1983). Sensitivity to BA was examined on YPGCE plates (YPG medium with 50 mM sodium citrate, 2% ethanol (pH 4.0) with increasing concentrations of BA (range of BA concentration was from 0 to 15 μM). Anaerobic environment was generated using sachets (GENbox anaer) from bioMérieux, and controlled with indicator strip. Media for anaerobic growth were supplemented with 12 μg/mL ergosterol and 0.2% Tween 80. Ethidium bromide (EtBr) was added to YPD plates to a final concentration of 20 μg/mL when necessary.

Mutagenesis

JL1-3-*ANC2-W87Y-W235Y* was mutagenized by EMS (ethylmethane sulfonate) or by UV light as described (Lawrence, 1991). Treated cells were plated (10^6 cells/plate) and BA^R mutants were selected on YPGCE plates with different concentrations of BA (0.1, 0.5, 0.75, or 1.0 μM) or isoBA (5, 10, 20, or 30 μM) during incubation at 28°C in the dark.

Identification of Mutations and Construction of Strains

Mutant cells more resistant to BA than parental strain were analyzed with the following approach. *ANC2* genomic alleles were cloned by plasmid gap repair (Orr-Weaver *et al.*, 1981, 1983). *Eco*RI-*Apa*I fragment of centromeric plasmid pRS*ANC2*-fus-5′-3′ (Trézéguet *et al.*, 2000), which contained 5′ and 3′ noncoding regions but not *ANC2* ORF was used for this purpose. Reparation of plasmids was verified by Southern blot analyses of total yeast DNA digested with *Kpn*I which linearized the plasmid not within *ANC2* ORF. *Eco*RI-*Bam*HI DNA fragment corresponding to *ANC2* ORF (Trézéguet *et al.*, 2000) was used as radioactive probe. Plasmids were then isolated from yeast and transformed into *E. coli* and *ANC2* ORF were sequenced to identify new mutations, in addition to the two *ANC2* mutations of the parental strain, which could cause BA^R phenotype.

The four single mutations identified in the parental strain were independently created de novo in wild-type *ANC2* (WT*ANC2*) sequence by method described in the literature (Deng and Nickoloff, 1992) to ascertain they conferred BA resistance. Centromeric plasmid pMD101 (Le Saux *et al.*, 1996) bearing WT*ANC2* gene was mutagenized by Chameleon^{TM} double-stranded site-directed mutagenesis kit (Amersham Pharmacia Biotech, APBiotech) according to supplier instructions. The mutagenic primers were $5'_{76}$GATTTCTTAATGaGTGGTGTCAGTGCCG$_{103}3'$ (amino acid mutation G30 → S), $5'_{279}$CGTTATCCGTTgTTTCCCCACTC$_{301}3'$ (Y97 → C), $5'_{412}$GGTGCCTTGTCATcACTATTTGTTTA$_{437}3'$ (L142 → S), $5'_{499}$GCTCGTCAATTCAcGGGTTTGATCGA$_{524}3'$ (N171 → T), $5'_{881}$GAGGTGTCGCAaGTGCTGGTGTTAT$_{905}3'$ (G298 → S). The following oligonucleotides were used to generate two additional mutants: $5'_{279}$CGTTATCCGTTcTTTCCCCACTC$_{301}3'$ (Y97 → S, in the WT context), $5'_{412}$GGTGCCTTGTCAactCTATTTGTTTA$_{437}3'$ (L142 → T, in the parental context). Mutagenized plasmids were sequenced and the *ANC2* genes with one of the four BA^R individual mutations were reintroduced at *ANC2* chromosomal locus by homologus recombination in vivo after nonselective transformation (Brandolin *et al.*, 1993b). Correct integration was verified by Southern blot analysis and genomic sequences of *ANC2*-BA^R mutant strains were confirmed by sequencing of PCR products containing the whole *ANC2* ORF.

Transformation

E. coli strain was transformed according to standard method (Morisson, 1977). Yeast strain were transformed with LiCl treatment protocol (Gietz *et al.*, 1992).

DNA Analyses

Isolation of recombinant plasmids was performed as described (Holmes and Quigley, 1981). Yeast genomic DNA isolation, DNA electrophoresis, manipulation of DNA fragments, and Southern blotting were performed using standard procedures (Sherman *et al.*, 1983; Sambrook *et al.*, 1986). Sequencing was carried out with automated sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems) by Sanger method (Sanger *et al.*, 1977) with fluorescent dideoxynucleotides.

Southern Blot Analyses

Yeast genomic DNA was digested with *Eco*RI and membrane with separated DNA fragments was hybridized with radioactive specific oligonucleotide (5′-ACCAGCACtTGCGACAC-3′) 100% complementary to the region with identified nucleotide mutation G_{892} → A according to (Meinkoth and Wahl, 1984). Mutated nucleotide is shown lowercase letter. Washing at different temperatures allowed to discriminate between DNA samples with perfect match and those with mismatches.

Isolation of Mitochondria, Cytochrome Spectra and [^{3}H]ATR Binding Assays

Mitochondria were isolated from cells grown in YPL medium at 28°C and harvested during exponential growth phase according to Daum *et al.* (1982) with minor modifications (Le Saux *et al.*, 1996). Cytochrome spectra

were obtained as described in Le Saux *et al.* (1996), with mitochondria diluted at 2 mg/mL in 0.12 M KCl, 10 mM MOPS, 1 mM EDTA (pH 6.8). Specific binding of [^{3}H]ATR to Anc2p in isolated mitochondria was determined as a function of increasing concentration of added ATR (Brandolin *et al.*, 1993b), up to 6 μM final concentration. Anc2p content was calculated knowing that one ATR molecule binds per one Anc2p dimer (Block *et al.*, 1986).

Immunodetection

Mitochondrial proteins (10 μg) were separated by SDS-PAGE (12.5% polyacrylamide) (Laemmli, 1970) and transferred to a PVDF membrane according to Towbin *et al.* (1979). Two parallel gels were run and one stained with Coomassie Brilliant Blue to control protein amounts used for immunodetection. Anc2p was detected with rabbit polyclonal antibodies (1/2000 dilution) raised against a 14-residue synthetic peptide (YDQLQMILFGKKFK) (Brandolin *et al.*, 1989) corresponding to the C-terminal part of Anc2p. The secondary antibodies were coupled to horseradish peroxidase. The immune complexes were detected with enhanced chemiluminiscence (ECL) kit from APBiotech.

Measurement of ADP/ATP Transport in Isolated Mitochondria

Method for measurement of ADP/ATP transport was adapted from Passarella *et al.* (1988) and is described in De Marcos Lousa *et al.* (2002). Freshly isolated mitochondria (0.5 mg) were suspended and incubated at 22°C in 1 mL of 0.6 M mannitol, 0.1 mM EGTA, 2 mM $MgCl_2$, 10 mM KP_i, 5 mM α-ketoglutarate, 0.01 mM Ap_5A, 10 mM Tris-HCl (pH 7.4) in the presence of an ATP detecting system (2.5 mM glucose, hexokinase (1.7 enzymatic units, (E.U.)), glucose-6-phosphate dehydrogenase (0.85 E.U.), 0.2 mM $NADP^+$). Externally added ADP started exchange reaction with intramitochondrial ATP mediated by ADP/ATP carrier. NADPH formation ($\varepsilon_{340} = 6200\,M^{-1} \times cm^{-1}$), which is proportional to ATP efflux was monitored continously for 3 min at 340 nm. The rate of absorbance increase was obtained from linear part of the curve and used to calculate amount of exchanged ADP (nmol/min $\times$ mg protein). Mitochondrial adenylate kinase was inhibited with specific inhibitor Ap_5A (Barile *et al.*, 1994; Lienhard *et al.*, 1973). In some experiments mitochondria were preincubated for 3 min with BA, CATR, or ATR to study the effect of these inhibitors. Transport was initiated by addition of 100 μM total ADP when BA or CATR were used and 10 μM total ADP when ATR was used. To ensure efficient penetration of BA through inner mitochondrial membrane and its maximal inhibition effect (Lauquin and Vignais, 1976), the reaction medium was at pH 6.5 instead of pH 7.4. To determine K_M values, concentrations of free ADP were calculated using the program WinMAXC v2.05 created by Chris Patton (www.stanford.edu/%7Ecpatton/maxc.html).

RESULTS

Induction and Identification of Anc2p BA^R Mutations

To find out which amino acids of Anc2p are important for BA sensitivity, we have induced Anc2p mutants in vivo by chemical or UV mutagenesis. To increase probability to obtain BA^R mutants, we have used the most sensitive strain to BA from our yeast collection, JL1-3-*ANC2-W87Y-W235Y* (Le Saux *et al.*, 1996). This strain supported growth on YPGCE medium supplemented with BA concentration below 75 nM (data not shown) while WT*ANC2* strain could grow on medium with higher BA concentration (250 nM). This parental strain was constructed earlier to study the contribution of different Anc2p Trp residues to conformational changes induced by binding of substrates and inhibitors. It contained only one Trp residue, W126, while W87 and W235 were mutated into Tyr. These mutations did not prevent growth of parental strain on nonfermentable carbon sources and ADP/ATP exchange activity of Anc2p was preserved (Le Saux *et al.*, 1996). Mutants were selected by growing cells on plates with glycerol and ethanol, in the presence of BA. Under these conditions, cell growth needed a functional Anc2p maintaining mitochondrial ADP/ATP transport.

EMS mutagenesis of JL1-3-*ANC2-W87Y-W235Y* led to isolation of around 100 clones resistant to BA to various degrees. *ANC2* ORFs from three arbitrary chosen mutants growing on YPGCE medium with 1 μM BA were cloned by plasmid gap repair technique and sequenced. Anc2p mutation G298S (nucleotide change $G_{892} \rightarrow A$ in *ANC2* gene) was identified in two clones while the other mutant contained only parental *ANC2* mutations W87Y and W235Y, which means that its BA^R phenotype was not linked to mutations in *ANC2*. As we were interested preferentially in modifications of *ANC2* gene, these types of mutants were not further examined. Mutated *ANC2* gene was integrated into the *ANC2* locus of JL1-3Δ2 by homologous recombination (Brandolin *et al.*, 1993b) and integration was verified by Southern blot and PCR. We could thus ascertain that BA^R phenotype was indeed linked to the additional *anc2* mutation.

Interestingly, mutation G298S (nucleotide change $G_{892} \rightarrow A$) led to the loss of the unique *Alw*NI restriction site (5′-CAG↓NNN↑CTG-3′) in *ANC2* gene (5′$_{890}$CAGGTGCTG$_{898}$3′). All of the mutants obtained after EMS mutagenesis were thus screened for the presence of *Alw*NI site (see Materials and Methods). Surprisingly, half of them had lost this site (data not shown). As mutations in other nucleotides of *Alw*NI sequence, different from $G_{892} \rightarrow A$, may also abolish recognition by *Alw*NI, hybridization with specific oligonucleotide complementary to sequence with $G_{892} \rightarrow A$ mutation was performed (see Materials and Methods). Hybridization revealed that all of the mutants that had lost *Alw*NI site contained only the $G_{892} \rightarrow A$ nucleotide mutation (data not shown). High frequency of $G_{892} \rightarrow A$ transition raised the question whether amino acid G298 located in the transmembrane segment (TMS) VI (Fig. 1) plays a key role in resistance to BA, or whether frequent appearance of this mutation was due to properties of chosen mutagen. EMS induces, with more than 96% probability, transitions of G·C pairs to A·T (Kohalmi and Kuntz, 1988).

Finally, after screening the other EMS-induced BA^R mutants, we identified a different mutation in *ANC2*, which was also a transition of guanine to adenine, $G_{88} \rightarrow A$ substituting also a glycine for a serine (G30S) in TMS I.

Facing the high frequency of $G \rightarrow A$ occurrence, we then used UV mutagenesis because it has the advantage of producing all types of base substitutions, mostly at sites of adjacent pyrimidines, and particularly at 5′-TT-3′ sites (Lee *et al.*, 1988). Two series of UV mutagenesis gave rise to 72 independent clones resistant to BA to various degrees (see Materials and Methods). They were first screened for absence of *Alw*NI restriction site at the *ANC2* locus, which could indicate appearance of G298S mutation. Southern blot analysis performed like for mutants obtained by EMS unveiled that only one mutant had lost *Alw*NI site and indeed, sequencing identified the presence of the $G_{892} \rightarrow A$ mutation (data not shown). Thus, while only one G298S mutant out of 72 was generated by UV mutagenesis, as many as 50% of EMS induced mutants had this mutation. Therefore, it seems that high frequency

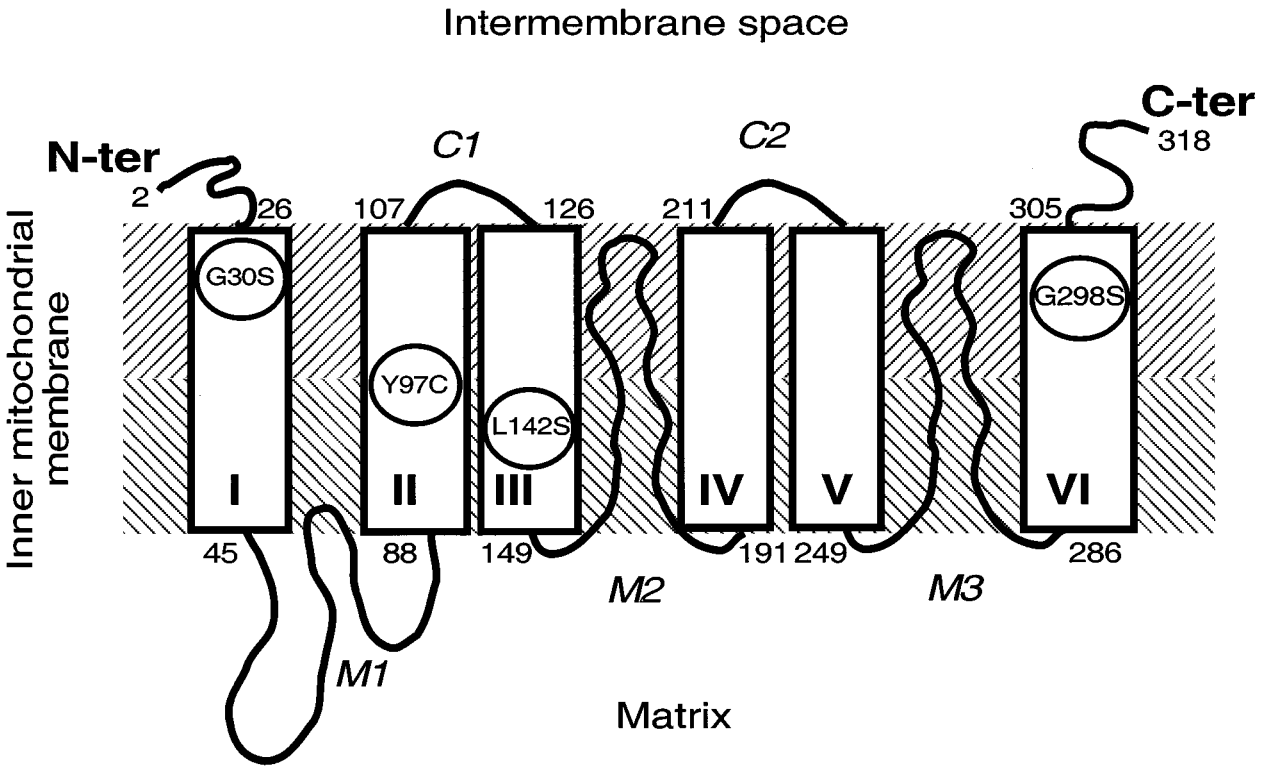

Fig. 1. Anticipated topological model of the Anc2p from *S. cerevisiae*. Anc2p (318 amino acids) contains six α-helical transmembrane segments (TMS, I–VI) connected by two cytosolic (C1, C2) and three matrix (M1, M2, M3) loops. Small numbers denote amino acid positions in the primary structure at the beginning and at the end of each putative TMS. Mature Anc2p starts with an *N*-acetylated N-terminal Ser and is only 317 residues long (Dianoux *et al.*, 2000). Mutations of four amino acids highlighted in circles are independently responsible for BA^R phenotype. One-letter amino acid code marks WT amino acids (before numbers) and mutated amino acids (after numbers).

of appearance of G298S mutation after EMS mutagenesis was primarily due to the EMS specific reactivity. From the pool of mutants with unmodified *Alw*NI sequence in *ANC2*, two further BA^R mutants (Fig. 1) have been identified. Mutation Y97C in TMS II was a result of the base transition $A_{290} \rightarrow G$, and mutation L142S in TMS III was due to the nucleotide transition $T_{425} \rightarrow C$. Overall, four independent BA^R mutations were identified in the parental strain, all corresponding to amino acids located in transmembrane segments.

To summarize, out of the 100 EMS-induced BA^R mutants obtained, 50 had the same G298S mutation and three had the G30S mutation. The other ones were not mutated within the *ANC2* ORF. Out of 67 UV-induced BA^R mutants obtained, 22 were analyzed: 6 presented the L142S mutation, 2 had the Y97C mutation and one was of the G298S type. The latter were not mutated within the *ANC2* ORF. In spite of the large number of analyzed mutants, only four different types of *ANC2* mutants could be obtained, whose levels of resistance were significant but not tremendously high (see below).

BA Resistance of Mutants Obtained by Site-Directed Mutagenesis

To get further insights into the role of the Anc2p mutated amino acids, we created by site-directed mutagenesis the Anc2p mutants, Y97S, and L142T. Y97S variant was as resistant as Y97C mutant, as judged from growth on plates with BA (data not shown). L142T replacement induced a BA^R phenotype almost as strong as the one induced by L142S (data not shown). Therefore, appearance of Ser or Cys and their ability to establish hydrogen bound via their electronegative atoms (O or S) in their side chains could be involved in the mechanism of resistance to BA.

BA Resistance Associated to G30S, Y97C, L142S, or G298S, Is Independent of the Mutations W87Y and W235Y

We have introduced the four BA^R mutations separately into the *WTANC2* gene because it was essential to evaluate whether BA^R phenotype resulted or not from a cumulative effect of each newly identified mutation with the two parental Anc2p mutations (W87Y and W235Y). *ANC2* was mutated by site-directed mutagenesis of plasmid pMD101, and the mutant genes were integrated at the *ANC2* locus of JL1-3Δ2, by homologous recombination. Integration of mutated *ANC2* genes was verified (i) by growth arrest of the strains on a medium devoid of histidine, since *HIS3* used for inactivation of endogenous *ANC2* was removed by integration of mutated *ANC2*; (ii) by Southern blot experiment; and (iii) by sequencing of PCR products obtained from genomic DNA corresponding to full ORF of *ANC2* (data not shown). Single Anc2p BA^R mutations examined in this work were thus expressed from the *ANC2* chromosomal locus containing WT promoter and terminator in a strain lacking the other two yeast Ancp isoforms. The nomenclature of the strains reflects introduced mutations in *ANC2* gene (Table I).

The degrees of resistance to BA were determined after growth on YPGCE plates supplemented with increasing amounts of inhibitor (Table II). They correspond to the highest BA concentrations allowing growth on YPGCE. We could thus evidence that BA^R phenotype was connected only to each one of the four identified single amino acid mutations of Anc2p (G30S, Y97C, L142S, and G298S), independently of the two mutations W87Y and W235Y originally present in the parental strain. Resistance to BA was even increased since the single mutants could grow in the presence of BA concentrations twice as high as those allowing growth of the original mutants (data not shown). This is in line with the high sensitivity of the parental strain in comparison to JL1-3-*ANC2*. These

Table I. Nomenclature of the Strains Harboring a WT or Mutated *ANC2* Gene

Strain	Codon changes in *ANC2* ORF	Short name	Source
JL1-3-*ANC2*[a]	—	WT*ANC2*	Brandolin *et al.* (1993)
JL1-3Δ2	ORF deleted	JL1-3Δ2	De Marcos Lousa *et al.* (2002)
JL1-3-*ANC2-W87Y-W235Y*[b]	TGG → TAC	Parental strain	Le Saux *et al.* (1996)
JL1-3-*ANC2-G30S*	GGT → AGT	*G30S*	This study
JL1-3-*ANC2-Y97C*	TAT → TGT	*Y97C*	This study
JL1-3-*ANC2-L142S*	TTA → TCA	*L142S*	This study
JL1-3-*ANC2-G298S*	GGT → AGT	*G298S*	This study

[a] Refers to the strain 2N1-3. Genes *ANC1* and *ANC3* are disrupted.

[b] Parental strain sensitive to BA refers to the JL1-3-*ANC2-W126* where two out of three Trp were replaced with Tyr. After mutagenesis BA^R mutants were selected and identified in this strain.

Table II. Growth Characteristics of WT*ANC2* and BA^R Mutant Strains

Strain	Doubling time (h)[a]	Saturation ($OD_{600\,nm}$)	Resistance to BA[b] (μM)		Temperature sensitivity[c]
			3 days	10 days	
WT*ANC2*[a]	3.8	9.7	0.125	0.25	−
G30S	4.2	9.5	0.5	3.0	+
Y97C	4.7	9.0	1.0	9.0	+
L142S	4.2	9.6	1.0	9.0	−
G298S	4.1	9.1	0.5	3.0	+

[a] Cells were grown in YPL under aeration with shaking at 28°C. Inocula were prepared from cells grown overnight in YPL.

[b] Cells were grown at 28°C on YPGCE plates containing increasing concentrations of BA. Numbers denote highest concentration of BA at which growth of the cells was observed after 3 and 10 days.

[c] A strain was considered temperature-sensitive when it grew slower than WT*ANC2* at 37°C on YPD, YPG, or YPL plates (see Fig. 2).

results also indicate that it was not necessary to operate with the wild-type strain, to select for highly BA resistant mutants, allowing the use of much less BA. Furthermore, the BA^R strains manifested significantly increased resistance to BA when compared to JL1-3-*ANC2*. The degree of resistance was around four times (*G30S*, *G298S*), and eight times higher (*Y97C*, *L142S*) when evaluated after 3-day growth and the difference from the WT*ANC2* strain was even more increased after 10-day growth (Table II). However, in spite of our efforts (two types of mutagenesis and analyses of a large number of mutant cells), it has not been possible to isolate mutants highly resistant to BA (more than 10-fold more the WT*ANC2* resistance level).

Growth properties were then examined at 28°C in liquid rich medium containing lactate, a nonfermentable carbon source (Table II). Mutations of Anc2p caused minor increase in doubling times, the most noticeable being for the strain *Y97C*. OD_{600} values at the growth plateau of stationary phase were in the range 9.0–9.6, which correspond to minor decrease of biomass yield as compared to the wild-type strain (Table II). These data indicate that mitochondrial metabolism in mutant strains could reach almost the same efficiency as in WT*ANC2* with respect to Anc2p function.

Growth of strains with mutated Anc2p was evaluated on solid media with different carbon sources and growth conditions (Fig. 2). All mutant strains were growing similarly to WT*ANC2* when using glucose or nonfermentable carbon sources (glycerol or lactate) at 28°C. All Anc2p mutants were also enabling growth in respiratory deficient ρ^- and ρ^o strains induced by EtBr, and they supported growth under anaerobic conditions as well as WT*ANC2*. However, growth differences between mutants were observed at 37°C on rich media (summarized in Table II). *G30S*, and even more *G298S* and *Y97C*, displayed temperature sensitive growth on glycerol medium. Only *L142S* was growing similarly to WT*ANC2* (Fig. 2).

Cytochrome and Anc2p Contents in Mitochondria Isolated From Wild-Type and Mutant Cells

Components of respiratory chain were assessed by measuring difference cytochrome spectra. As can be seen in Fig. 3, cytochromes *b*, *c*, c_1, and aa_3 were present in similar amounts in mitochondria with BA^R Anc2p mutants and in mitochondria with wild-type Anc2p (WTAnc2p). Therefore, the small increases of doubling times of BA^R strains were not attributable to diminished mitochondria biogenesis during growth on respiratory carbon source.

The amount of Anc2p in mitochondria from mutant strains was evaluated first by immunodecoration (see Materials and Methods). ADP/ATP carrier was detected as a single band of about 34 kDa with almost uniform intensity in all samples (Fig. 4). Specific antibodies against Anc2p did not interact with other mitochondrial protein than Anc2p, as was indicated by the absence of signal in Western blot analysis of mitochondria from strain JL1-3Δ2 lacking all of the ADP/ATP carrier isoforms (data not shown). It can be concluded that mitochondrial import of mutated Anc2p has been efficient for all mutants and that BA^R phenotype of mutant cells did not correlate with highly variable amounts of mutated Anc2p in mitochondria.

Anc2p amount was then quantified by [^{3}H]ATR binding experiments. ATR is a nonpenetrant inhibitor that recognizes regions of Anc2p exposed to the outside of the mitochondrial inner membrance (MIM) when the carrier is in the CATR conformation. Determination of ATR binding parameters was used for quantitating functional ADP/ATP carrier inserted into MIM, assuming that mutation of

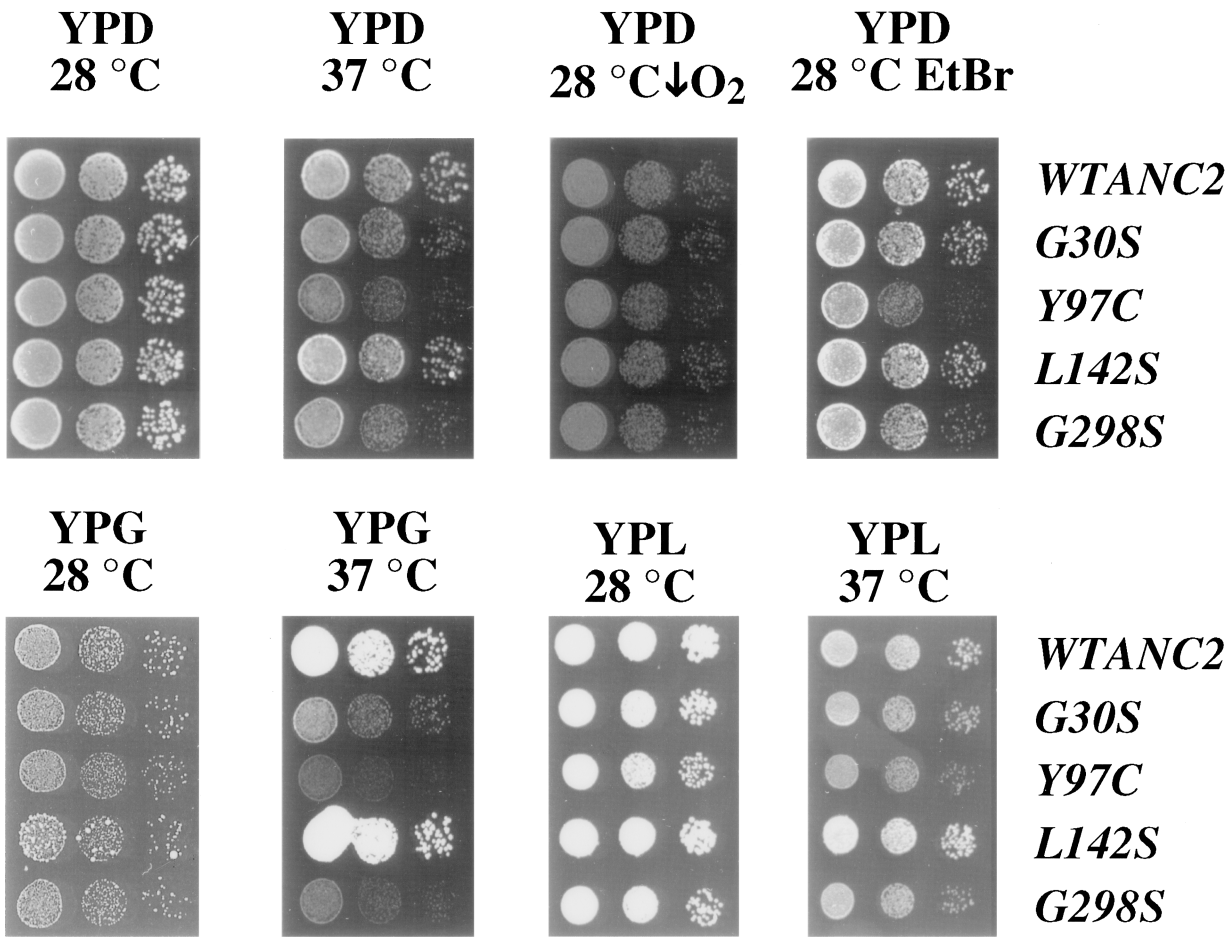

Fig. 2. Growth properties of strains with BA^R Anc2p mutations. Cells were spotted on plates with rich media containing either 2% glucose (YPD), 3% glycerol (YPG), or 2% lactate (YPL). Each lane contains spots with initially 10^4, 10^3, and 10^2 cells. Cells were cultivated at indicated temperatures for 2 days on YPD plates or 4 days on YPG and YPL plates. Symbols: ↓O2 (anaerobic cultivation); EtBr (plate supplemented with 20 μg/mL ethidium bromide).

Anc2p has not substantially changed its sensitivity to the inhibitor. Specific binding of [^{3}H]ATR to Anc2p increased in relation to the amount of added inhibitor and reached a plateau (see Methods). Maximal number of binding sites for ATR (B_{max}) and K_d^{ATR} values were calculated from curves fitted to experimental data. Binding parameters of mutant and WTAnc2p in intact mitochondria are summarized in Table III. Contents of BA^R Anc2p variants were in the range of 746–1058 pmol Anc2p/mg protein, which corresponded to −15% to +21% as compared to the wild type Anc2p content. Thus, increased BA resistance did not involve dramatic changes of Anc2p content in MIM. Affinity for ATR was moderately decreased for G30S, which therefore behaved similarly to WTAnc2p for ATR binding. The K_d^{ATR} values for Y97C and G298S were half that of WTAnc2p. As they were roughly in the range of values determined previously (100–210 nM) for WTAnc2p (Fiore *et al.*, 2000; Le Saux *et al.*, 1996), we can postulate that the affinity of ATR for these variants was not substantially modified. To contrast with these results, L142S had a dramatically reduced affinity for ATR (K_d^{ATR} is 15 times higher than that of WTAnc2p) that could account for a modified structure of Anc2p. However, *L142S* growth was not impaired at 37°C on nonfermentable carbon source (Fig. 2, Table II).

Adenine Nucleotide Exchange Activity of Anc2p BA^R Mutants

Transport properties of Anc2p mutants were measured under the conditions prevailing in mitochondria during growth on respiratory substrates, when cytosolic ADP is exchanged for intramitochondrially synthesized ATP in a $\Delta\Psi$ dependent manner. Freshly isolated mitochondria

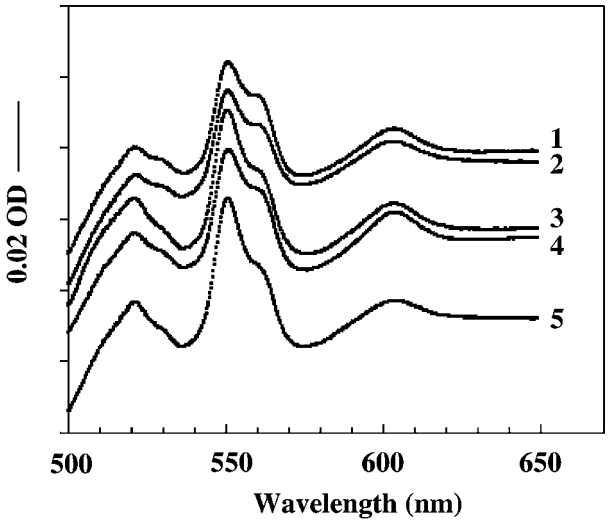

Fig. 3. Difference cytochrome spectra of mitochondria with BA^R Anc2p mutants. Mitochondria were isolated from WT or mutant cells grown in YPL medium which were harvested during exponential growth phase. The bar represents an absorption difference of 0.02 OD. (1) WTAnc2p; (2) G30S; (3) Y97C; (4) L142S; (5) G298S.

were incubated in the presence of respiratory substrate and phosphate to maintain active respiratory chain and ATP synthase. Upon addition of ADP, the amount of ATP exported by Anc2p from mitochondria was continuously measured (see Materials and Methods). In control experiment, mitochondria from JL1-3Δ2 were isolated

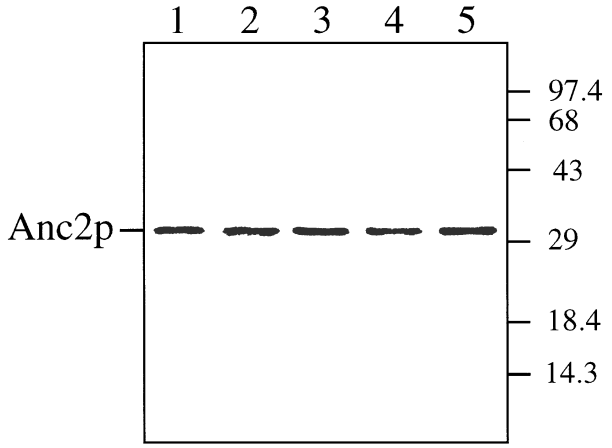

Fig. 4. Immunodetection of the WT and mutated Anc2p. Mitochondria were isolated from indicated strains grown in YPL. Mitochondrial proteins (10 μg) were separated by electrophoresis in SDS-polyacrylamide gel and transferred onto PVDF membrane. Anc2p was detected with rabbit polyclonal antibodies raised against 14 last amino acids of Anc2p C-terminal peptide. The second antibodies were coupled to horseradish peroxidase. The immune complexes were detected with ECL kit (APBiotech). (1) WTAnc2p; (2) G30S; (3) Y97C; (4) L142S; (5) G298S.

Table III. Specific Binding of [^{3}H]ATR to Mitochondria Isolated from WT and Mutant Strains

Carrier	K_d^{ATR} (nM)	B_{max} (pmol/mg protein)	Anc2p content (pmol/mg protein)[a]
WTAnc2p	220 ± 32	437 ± 63	874 ± 126
G30S	279 ± 9	414 ± 4	828 ± 8
Y97C	121 ± 10	476 ± 38	952 ± 72
L142S	3230 ± 100	373 ± 46	746 ± 92
G298S	112 ± 21	529 ± 18	1058 ± 36

Note. B_{max} refers to the maximal number of ATR binding sites.
[a] The Anc2p content was calculated knowing that 1 mol of ATR binds per 1 mol of Anc2p dimer.

from cells grown in YPGal and assayed for ADP/ATP transport. As expected, exchange of adenine nucleotides could not be detected (data not shown). This confirmed that transport observed in mitochondria from strains expressing WT and BA^R Anc2p variants was mediated only by the ADP/ATP carrier. K_M^{ADP} was 1.61 ± 0.21 μM for WTAnc2p and in the same range for G30S and Y97C (Table IV). It was reduced by 40% for G298S but increased by 100% for L142S. V_{max}^{ADP} values as well as the turnover number of the ADP/ATP exchange were increased for all mutants as compared to WTAnc2p (23–42% for the turnover number). These variations in K_M^{ADP}, V_{max}^{ADP}, and turnover number did not seem to significantly influence the cell physiology of BA^R mutants, as judged from similar growth properties of the mutant and wild-type strains at 28°C (Fig. 2, Table II).

Effect of Inhibitors BA, CATR, and ATR on the Transport Activity of Anc2p BA^R Mutants

Mitochondrial ADP/ATP transport was measured in the presence of BA to correlate BA resistance of cells

Table IV. Biochemical Characterization of ADP/ATP Transport in Isolated Mitochondria Mediated by WT or Mutated Anc2p Carrier

Carrier	K_M for ADP[a] (μM)	V_{max}[a] (nmol ADP/min × mg protein)	Turnover number[b] (min^{-1})
WTAnc2p	1.61 ± 0.21	69.6 ± 1.6	160
G30S	1.49 ± 0.03	89.1 ± 10.8	216
Y97C	1.76 ± 0.01	94.6 ± 16.9	198
L142S	2.74 ± 0.09	82.8 ± 11.2	222
G298S	1.00 ± 0.04	120.1 ± 2.8	228

[a] K_M and V_{max} values were calculated from kinetic data by using Michaelis–Menten equation. They represent concentration of free ADP as this is the form transported by Anc2p.
[b] Turnover number was calculated with dimeric Anc2p content obtained from [^{3}H]ATR binding to mitochondria.

to Anc2p variant properties. Mitochondria were preincubated with variable BA concentrations before ADP/ATP transport was initiated. All mutants displayed higher resistance to BA than WTAnc2p but the shapes of the inhibion curves were different (Fig. 5A). Substantial ADP/ATP transport activity was preserved in mutant mitochondria at concentrations of BA causing almost complete inactivation of WTAnc2p. For example, 97% of WTAnc2p transport were inhibited by 300 nM BA, but G30S, Y97C, and L142S retained 30% of activity and G298S, 60%, under the same conditions. To inhibit 90% of ADP/ATP transport by the variants, 400 nM BA was necessary for G30S and L142S, and more than 700 nM for Y97C. Half-maximal inhibition (IC_{50}^{BA}) of WTAnc2p transport was achieved with 145 nM BA. IC_{50}^{BA} was increased by about 50% for Y97C and L142S, 70% for G30S, and 110% for G298S (Table V).

Some of the BA^R mutations modified the K_d^{ATR} value of WTAnc2p (Table III). We therefore examined the influence of ATR on ADP/ATP transport. ATR inhibited transport activities of G30S, Y97C, and G298S more efficiently than it inhibited WTAnc2p (Fig. 5C), although the K_d^{ATR} values of these carriers were not very different (Table III). L142S behaved differently since its transport activity was more resistant to ATR than that of WTAnc2p. These results corroborate ATR binding experiments that had already evidenced that this mutant had strikingly increased K_d^{ATR} (Table III). IC_{50}^{ATR} values for mutants G30S, Y97C, and G298S was about 60% of the value determined for WTAnc2p (Table V). On the contrary, the L142S IC_{50}^{ATR} value was increased by around 33%. Thus, Ser at position 142 instead of leucine in TMS III modified interaction of Anc2p with BA as well as with ATR.

CATR is a specific noncompetitive inhibitor that stabilizes Ancp in the CATR conformation involved in nucleotide transport (Vignais *et al.*, 1985). As can be seen in Fig. 5(B), only one BA^R mutant, G30S, exhibited significantly increased resistance to CATR, the other mutants being only slightly less sensitive to CATR than WTAnc2p. The effects of CATR were restricted to a smaller range of concentration of inhibitor than in the case of BA. IC_{50}^{CATR} values were roughly similar for Y97C, L142S, G298S, and for WTAnc2p while IC_{50}^{CATR} value for G30S was increased by 40% (Table V).

DISCUSSION

Identification and Characterization of Anc2p BA^R Mutations

Four independent Anc2p mutations G30S, Y97C, L142S, and G298S were identified after in vivo mutagenesis. Those mutated residues are located in TMS I, II, III, and VI, respectively, and are conserved in all known Ancp sequences (Fiore *et al.*, 1998; Nelson *et al.*, 1998). The only exceptions are in *Sc*Anc1p, where an alanine instead of a glycine is present in the position corresponding to G298 of ScAnc2p, and in *Pj*Anc1p and *Pj*Anc2p, where a phenylalanine instead of a leucine is present in the position corresponding to L142 (accession numbers CAB88027 and CAB88028, respectively) (Bof *et al.*, 1999; Fiore *et al.*, 1998; Nebohácová *et al.*, 1999). One-hundred and twenty-nine sequences of MCF members were aligned by Nelson (1996) and it appeared that G30 (TMS 1) is conserved in most of the carrier sequences, Y97 is specifically present in all Ancp and phosphate carrier sequences, while L142 and G298 are mostly Ancp-specific.

Only G30 has been previously described as important for yeast Anc2p activity since G30C was an active second site revertant of the inactive R254I mutant, among 11 different revertants restoring $glycerol^+$ phenotype of the recipient strain (Nelson and Douglas, 1993). Surprisingly, G30V was found as the unique revertant of the inactive double mutant R96T and D149G (Nelson, 1996).

Properties of BA^R Anc2p Mutants in Isolated Mitochondria

Western blot analysis of mitochondrial protein and binding of [^{3}H]ATR established that the BA resistance was not correlated with highly modified amounts of Anc2p variants in mitochondria. Growth phenotypes of mutant strains in lactate (Table II) suggested that, in spite of BA resistance, ADP/ATP transport properties should not be impaired. Those were determined with isolated mitochondria, as measurements with functional organelles are much more approaching conditions in the living cells than in vitro reconstitution experiments with liposomes. As a matter of fact, K_M^{ADP}, V_{max}^{ADP}, and turnover number values of Anc2p variants indicated that their ADP/ATP transport capabilities were not impaired. Thus, G30, Y97, L142, and G298 are unlikely to be involved in the nucleotide binding site or translocation pathway. We note these amino acids are not located in the nucleotide binding regions S183-R191 (M2 loop) and I311-K318 (C-terminal part) of yeast Anc2p, determined by photolabeling with 2-azido-3′-*O*-naphtoyl-[β-^{32}P]-ADP (Dianoux *et al.*, 2000). Furthermore, none of the BA^R mutations involved amino acids located in the matrix loops M1, M2, or M3, which are all considered to be actively and cooperatively involved in the adenine nucleotide transport (Majima *et al.*, 1994).

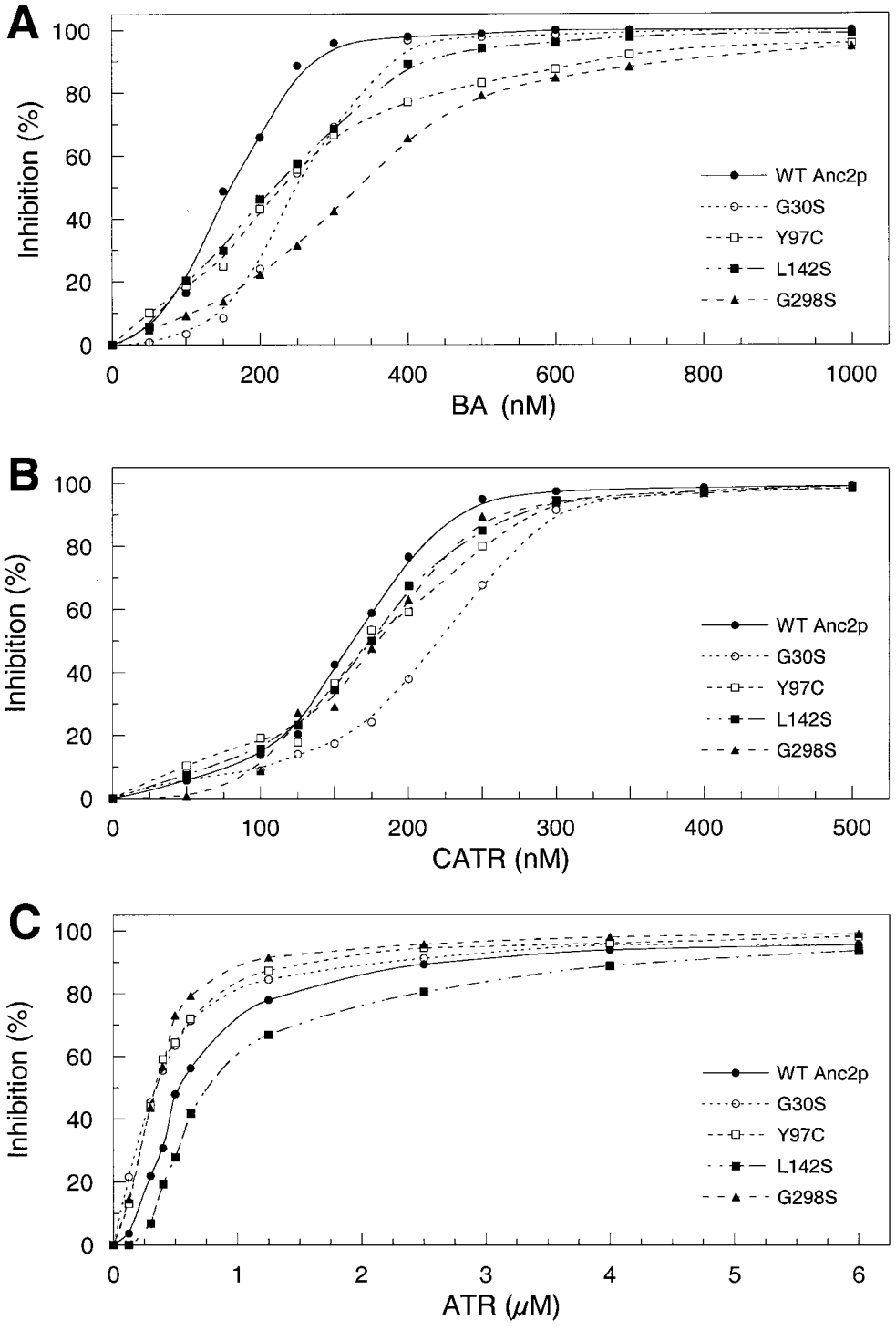

Fig. 5. Inhibition of mitochondrial ADP/ATP transport with specific inhibitors of Anc2p. Mitochondria were preincubated with increasing amount of inhibitor for 3 min. Transport was initiated by addition of ADP and continuously measured for 3 min (see Materials and Methods). (A) Inhibition effect of bongkrekic acid, (B) carboxyatractyloside, (C) atractyloside. The inhibition values in percent (%) are equal to 100 $(V_o - V_i)/V_o$, where V_o and V_i are velocities of ADP/ATP transport (nmol ADP/min × mg protein) in the absence and presence of particular concentration of inhibitor. Data points are represented as means from at least two independent experiments performed with different mitochondrial preparations.

Table V. Sensitivity of the Mitochondrial ADP/ATP Transport to the Specific Anc2p Inhibitors

Carrier	IC_{50}^{BA}[a] (nM)	IC_{50}^{CATR} (nM)	IC_{50}^{ATR} (nM)
WTAnc2p	145 ± 12	164 ± 15	577 ± 12
G30S	246 ± 37	227 ± 23	353 ± 35
Y97C	223 ± 1	172 ± 19	327 ± 27
L142S	215 ± 26	176 ± 14	768 ± 44
G298S	307 ± 7	173 ± 17	357 ± 5

[a] IC_{50} values (inhibition concentration yielding half-maximal inhibition) were obtained from data obtained in Fig. 5. Values are represented as mean ± SE and were determined from at least two independent experiments performed with different mitochondrial preparations.

Effect of BA, CATR, and ATR on the Function of BA^R Anc2p Variants

Growth of mutant strains on plates containing respiratory substrates and BA should be associated with preserved transport capacity of Anc2p variants in the presence of BA. Indeed, we have shown that all BA^R mutants have higher effectiveness of nucleotide transport than WTAnc2p in the presence of BA. For example, IC_{50}^{BA} for G298S, which was the most resistant BA^R mutant, was increased more than two times as compared to WTAnc2p. This is consistent with the higher BA resistance of the mutant cells as compared to WT cells. For a given BA concentration, ADP/ATP transport was completely inhibited or below the threshold value in WT cells but its level was still sufficient to support growth of the BA^R mutants on nonfermentable carbon sources.

Nevertheless, we can note that none of the mutants have lost the ability to bind BA. As conformation changes are involved in BA binding and in nucleotide transport, one can hypothesize that mutants that lose the ability to interact with BA would be theoretically more resistant but would probably also become inactive for transport by their inability to operate the required conformational changes to transport. This can explain why, in spite of our extensive search for BA^R mutants, hyperresistant mutants were not obtained.

It is currently postulated that ATR and BA binding sites are distinct, but they may partially overlap (Block *et al.*, 1979, 1981). Moreover, modification of amino acid residues outside ATR or BA binding sites could obviously provoke topological changes of Anc2p, influencing indirectly other essential residues involved in binding and/or effect of inhibitors. The region C159-M200 of beef heart Ancp has been identified as the ATR binding region by photolabeling with radioactive azido derivatives of ATR (Boulay *et al.*, 1983). It corresponds to yeast Anc2p V176-L217 region comprising M2 loop, TMS IV, and C2 loop. None of the BA^R mutations were located in this region. However, ATR binding properties were modified for some BA^R variants. L142S had significantly reduced affinity for ATR but its ADP/ATP transport activity was still inhibited by ATR and the IC_{50}^{ATR} value did not correlate well with the K_d^{ATR} value. This unexpected result suggests that ATR inhibition is favored under the experimental conditions used in the nucleotide transport assay. On the contrary, for the other mutants Y97C and G298S, there was a good correlation between the K_d^{ATR} and the IC_{50}^{ATR}, both parameters indicating a higher sensitivity to ATR as compared to the wild-type carrier. Thus, BA^R mutations could also influence interaction of Anc2p with ATR and as a consequence ATR and BA binding sites could partially overlap functionally, if not physically.

Recent experiments with yeast Anc2p allocate binding of CATR to the cytosolic loop C1 and to the C-terminal region, which is orientated towards the intermembrane space (Hatanaka *et al.*, 2001). Since CATR inhibition of the ADP/ATP transport was similar for three BA^R mutants and for WTAnc2p ,we can propose that C1 and Cter regions are probably not involved in the conformational changes induced by BA binding. This is in line with our previous results indicating that W87 and W126 but not W235 were able to report conformational changes induced by BA binding (Roux *et al.*, 1996).

The Role of Particular Amino Acids in the Mechanism of Resistance to BA

Not taking into account our results, the simplest explanation to account for BA resistance is that BA cannot bind to Anc2p mutants. The ability of BA^R mutants in parental strain context to bind [^{3}H]BA has been examined but the result were not conclusive (data not shown). Because of the amphiphilic nature of this inhibitor, BA binding analyses are complicated because it binds preferentially to Ancp but it is also unspecifically bound to mitochondrial membranes or could accumulate in matrix, especially at high concentrations (Klingenberg *et al.*, 1983). Since BA inhibited ADP/ATP transport, we can state that it still binds to mutant Anc2p but may not be able, or very poorly, to induce the conformational changes leading to the stable inactive Anc2p·BA complex. Indeed, it was reported that some single mutants, R96A, R204L, or D249S, inactive for the nucleotide transport, were also losing their ability to bind BA (Müller *et al.*, 1996, 1997).

BA^R mutants induced in vivo always resulted from one base substitution, which may explain preferential appearance of Ser over Thr. Indeed, to obtain Thr mutants in positions 30, 97, 142, or 298, two base replacements

per codon would have been necessary. In line with this the L142T variant was almost as resistant as the L142S one.

Formation of disulfide bridge could be connected with resistance to BA in the Y97C variant. Involvement of Cys residue of the M1 loop of the Anc2p in the BA^R phenotype could be hypothesized on the basis of Cys labeling experiments reported by Hatanaka *et al.* (2001). However, since Y97S is as resistant as Y97C, we can conclude that Cys is not required at this position for the induction of a BA^R phenotype and this is a further evidence that resistance of Y97C mutant could not be explained by a putative disulfide bridge of C97 with C73 or C97 in another monomer unit or with other Anc2p Cys.

Analyses of properties of BA^R mutants obtained either by in vivo or by in vitro mutagenesis stress importance of particular amino acids with OH group (G30S, Y97S, L142S, L142T, G298S) or SH group (Y97C) for resistance to BA. We assume that BA^R phenotype results from appearance of these amino acids at specific positions in TMS and is linked to their ability to create hydrogen bonds either with other Anc2p residues, within the monomer, or between two monomers. Mutants thus can adopt variant conformations which still allow ADP/ATP transport but counteract the inhibitory effect of BA. Presumably the transition of Anc2p • ATP • BA transient ternary complex (Roux *et al.*, 1996) to inactive Anc2p·BA complex is prevented. Additional substitutions of crucial residues with different amino acids and subsequent analyses will provide further insight into mechanism of resistance of Anc2p mutants to BA.

ACKNOWLEDGMENTS

This work was supported by the University of Bordeaux2, the Centre National de la Recherche Scientifique, the Région Rhône-Alpes, the Région Aquitaine and the Commissariat à l'Energie Atomique. IZ and CS have been respectively supported by a Marie Curie Fellowship (HPMF-CT-1999-00122) and Grant No. BIO4-CT-972119 from the European Community.

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