Conformational Changes of the Yeast Mitochondrial Adenosine Diphosphate/Adenosine Triphosphate Carrier Studied through Its Intrinsic Fluorescence. 1. Tryptophanyl Residues of the Carrier Can Be Mutated without Impairing Protein Activity[†]

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ABSTRACT: During the transport process the mitochondrial adenine nucleotide carrier (Ancp) undergoes conformational changes which result in modifications of the intrinsic fluorescence of the carrier. To further study these changes by a fluorometric approach, the three tryptophanyl residues (Trp87, Trp126, and Trp235) of the *Saccharomyces cerevisiae* Anc2p were individually mutated to their tyrosine counterparts. The resulting mutated genes (two-Trp, one-Trp or Trp-less variants) were integrated at the *ANC2* locus. A prerequisite for such studies is that all the engineered carrier molecules are still able to catalyze ADP/ATP exchange. The cellular characteristics of the strains expressing the mutated Anc2p and the biochemical properties of the variant Anc2p in mitochondria were examined. Although Trp87 is absolutely conserved in all 30 available Ancp sequences, none of the tryptophanyl residues is essential to the carrier protein folding and the transport activity. The mutated and wild-type Anc2p were expressed to the same level, as evidenced by both ligand binding and immunochemical analyses. When isolated in the presence of detergent, all the variant Anc2p preparations contained ergosterol in similar amounts (9 mol/mol of 35 kDa Anc2p) but no specific interaction was revealed. Our results show that the tryptophan-mutated Anc2p are suitable for fluorescence studies, which are reported in the accompanying paper by Roux et al. [(1996) *Biochemistry 35*, 16125−16131].

The adenine nucleotide carrier (Ancp)[1] is a nuclear encoded protein of about 300 amino acids located in the inner mitochondrial membrane that catalyzes the transmembrane exchange of ADP and ATP between cytosol and mitochondria. It exists in two distinct conformations that can bind atractyloside (ATR) and carboxyatractyloside (CATR) or bongkrekic acid (BA), respectively. These conformations, referred to as CATR and BA conformations, are characterized by different reactivities of well-defined regions of the protein to chemical, enzymatic, and immunochemical reagents [for review, see Vignais et al. (1985) and Brandolin et al. (1993a)]. Conformational changes of the beef heart Ancp have been thoroughly studied by measurement of intrinsic fluorescence changes. The tryptophanyl fluorescence of the beef heart Ancp is modified in response to conformational changes induced by ATP (or ADP), CATR, and BA (Brandolin et al., 1981, 1985). The Anc1p isoform, which is predominant in beef heart mitochondria, has five tryptophanyl residues, and the intrinsic fluorescence changes result from modifications in the environment of one or more of the five tryptophanyl residues.

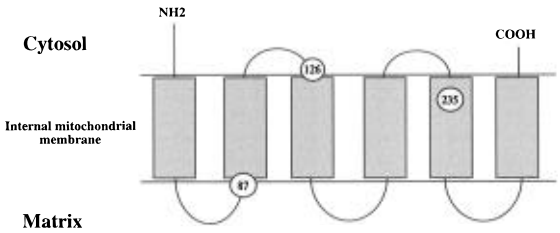

FIGURE 1: Schematic representation of the secondary structure of the Anc2p from *S. cerevisiae.* This representation was based on the Kyte and Doolittle algorithm and on biochemical studies of the carrier topology.

Saccharomyces cerevisiae is a convenient organism since its genes can be easily mutated or replaced. A *S. cerevisiae* strain was constructed in which the only functional adenine nucleotide carrier gene was *ANC2* (Brandolin et al., 1993b). The corresponding encoded protein Anc2p contains three tryptophanyl residues, located at positions 87, 126, and 235 (Figure 1) (Lawson & Douglas, 1988; Brandolin et al., 1993a), that are largely responsible for its intrinsic fluorescence. The intrinsic fluorescence of the isolated Anc2p in detergent solution was enhanced in the presence of ATP or

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[1] Abbreviations: Ancp, adenine nucleotide carrier; ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; N-ATP, 3′-*O*-naphthoyl adenosine triphosphate.

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ADP and decreased in the presence of CATR, a result that reflected conformational changes of the carrier upon binding of specific ligands (Brandolin et al., 1993b). Nontransportable nucleotides were ineffective. Examination of the amino acid sequence revealed the presence of three repeats of about a hundred amino acids each (Saraste & Walker, 1982) with one tryptophanyl residue per repeat in the case of yeast Anc2p. To determine the Anc2p region(s) involved in the conformational changes, we replaced each of the tryptophanyl residues by tyrosyl residues (W → Y) in order to quench selectively part of the intrinsic fluorescence signal. We describe in this paper the construction and the characterization of a number of mutated Anc2p proteins which will be used for structural studies [accompanying paper by Roux et al., 1996].

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media. *Escherichia coli* strains were as follows: TG1: Δ*(lac−pro) supE thi hsd*5 F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]. HB101: Δ*(gpt−proA)62 leuB6 supE44 ara-14 galK2 lacY1* Δ*(mcrC−mrr) rpsL20* (Str^r) *xyl-5 mtl-1 recA13* (Boyer & Roulland-Dussoix, 1969). XL1-Blue: *recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_K^- m_K^+)-*supE44 relA1 lac*$^-$ F′ [Tn10 (tet^r) *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]. Bacterial strains were grown on modified Luria broth [1% bactotryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl, pH 7.5 (Miller, 1972)] plus 100 μg of ampicillin/mL when necessary. The following *S. cerevisiae* strains were used in this study: W303-1B (*MAT*α *leu2-3,112 his3-11 ade2-1 trp1-1 ura3-1 can1-100*) and its derivative, kindly provided by J. Kolarov, JL1-3 (*anc1::LEU2 anc2::HIS3 anc3::URA3*) (Drgon et al., 1991); and JL1-3-*ANC2* (*MAT*α *leu2-3,112 his3-11 ade2-1 trp1-1 ura3-1 can1-100 anc1::LEU2 anc3::URA3*), which refers to the 2N1-3 strain described in Brandolin et al. (1993b).

Yeast cells were grown at 28 °C on YPD, YPG, or YPLact [1% yeast extract (Difco) and 2% bactopeptone (Difco) supplemented with 2% glucose, 3% glycerol, or 2% lactate plus 1% KH_2PO_4, pH 5.5, respectively) and synthetic complete media deprived of histidine or tryptophan (0.67% bacto yeast nitrogen base and 2% dextrose supplemented with uracil, adenine, and all amino acids but tryptophan or histidine) (Sherman et al., 1974). Sensitivity to BA was examined on a YPG medium buffered at pH 4.0 with 50 mM sodium citrate and supplemented with 2% ethanol. The vectors used in this study were phagemid pRS314 (TRP1/CEN6/ARSH4) (Sikorski & Hieter, 1989) and phage M13mp18.

Chemicals. Nucleotides and ergosterol were purchased from Sigma and carboxyatractyloside from Calbiochem. [^{3}H]-ATR was synthesized as previously described (Brandolin et al., 1974). [^{14}C]N-ATP was synthesized and purified as previously described in Block et al. (1982). [2,8-^{3}H]ATP was purchased from Amersham Corp. Protein concentration was determined with the BCA (bicinchoninic acid) reagent kit from Pierce.

Cloning of the ANC2 Gene. The *ANC2* DNA fragment was cloned from a genomic library of the S288C *S. cerevisiae* strain into the vector YEp24 (Carlson & Botstein, 1982). The 2.85-kb *Pst*I-*Eco*RI *ANC2* fragment was isolated from a clone able to complement the *op1* mutation (unpublished results) that prevented growth on a nonfermentable carbon source (Kovac et al., 1967) and its nucleotide sequence was determined (GenBank/EMBL Data Bank, accession number X74427). A *Pst*I−*Sal*I or *Pst*I−*Eco*RI fragment containing the open reading frame of *ANC2* (954 bp), 1350 bp from the 5′-flanking DNA, and 540 bp from the 3′-flanking DNA was introduced into the phagemid pRS314 or the phage M13mp18 at the *Pst*I−*Sal*I or *Pst*I−*Eco*RI polylinker sites, respectively. The resulting vectors were pMD101 (Figure 2; M. Donzeau, DEA Bordeaux, personal communication,1990) and M13mp18*ANC2*, respectively.

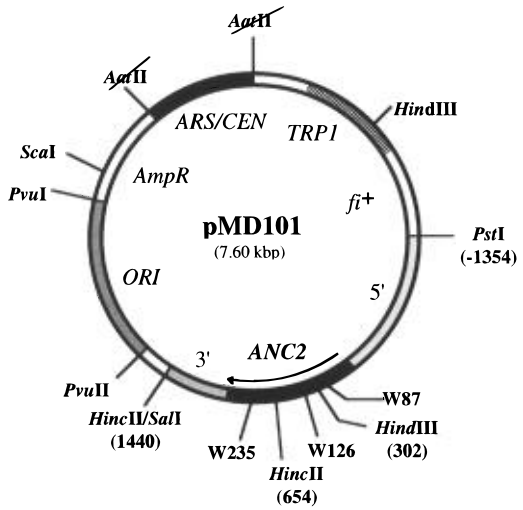

FIGURE 2: Graphic map of phagemid pMD101. A 2.8-kb fragment containing the *ANC2* open reading frame (954 bp), 1350 bp from the 5′ noncoding region, and 540 bp from the 3′ noncoding region was cloned into the *Pst*I−*Sal*I sites of pRS314.

Construction of the Mutated ANC2 Genes. Oligonucleotide site-directed mutagenesis was used to replace the *ANC2* tryptophan codons by tyrosine codons. The W87Y and W235Y mutations were generated by the method of Taylor et al. (1985), using M13mp18*ANC2* as a template. The W126Y mutation was generated by the method described by Vandeyar et al. (1988), using pMD101 as a template. The mutagenic primers were 5′$_{250}$ATCTCATTCTacAGAGG-TAAC$_{271}$3′ (mutation W87Y), 5′$_{367}$TACGCCAAATacTTTGC-CGGT$_{388}$3′ (mutation W126Y), and 5′$_{694}$TTGTTGGGT-TacGTTGTTACT$_{715}$3′ (mutation W235Y). Numbering of nucleotides is that expected if position +1 corresponds to the A of the ATG initiation codon. The mutated genes *ANC2-W87Y* and *ANC2-W235Y* were cloned into phagemid pRS314 on a *Pst*I−*Sal*I fragment as described for the pMD101 construction. The resultant plasmids were pMD*ANC2-W87Y* and pMD*ANC2-W235Y*, respectively. pMD101 was used as a template to generate the *ANC2-W126Y* mutated gene and the recombinant plasmid was pMD*ANC2-W126Y*.

To construct *ANC2* genes with two or three tryptophan codon substitutions, the single mutations were combined, using natural restriction sites present on phagemid pRS314 and on *ANC2* between codons 87 and 126 or 235. To isolate the *ANC2-W87Y-W126Y* mutant, the phagemids pMD*ANC2-W87Y* and pMD*ANC2-W126Y* were digested by *Hin*dIII. After purification, the 2.58-kb DNA fragment containing the W87Y mutation was ligated with the 5.06-kb DNA fragment containing the W126Y mutation. The recombinant phagemid was pMD*ANC2-W87Y-W126Y*. The pMD*ANC2-W87Y-W235Y* phagemid was constructed in a similar way but using pMD*ANC2-W87Y* and pMD*ANC2-W235Y*. To obtain the

ANC2-W126Y-W235Y mutant, another strategy was used. Two *Hin*cII sites are present in the *ANC2* DNA fragment cloned into pRS314. The first one (GTTGAT) is located between codons 126 and 235 (position 654) and the second one (GTCGAC) is in the 3′ flanking region (position 1440) (Figure 2). This latter was methylated with *Taq*I methylase on pMD*ANC2-W126Y* and on pMD*ANC2-W235Y*. These plasmids were then restricted with *Pst*I and *Hin*cII and the resulting 1.99-kb DNA fragment containing the W126Y mutation and the 5.65-kb DNA fragment containing the W235Y mutation were purified and ligated together. The new phagemid was pMD*ANC2-W126Y-W235Y*. The pMD*ANC2*-3Y phagemid harboring the *ANC2* gene containing no tryptophan codon was constructed in a similar way, using pMD*ANC2-W126Y-W235Y* and pMD*ANC2-W87Y*. The desired nucleotide substitutions were confirmed by nucleotide sequencing of the whole mutated *ANC2* open reading frame by the dideoxy chain termination method of Sanger et al. (1977).

Transformation. *E. coli* strains were transformed according to standard methods (Morrison et al., 1977; Cohen et al. 1972). Yeast strains were transformed according to the LiCl treatment procedure of Gietz et al. (1991, 1992).

DNA Analyses. Isolation of recombinant phagemids or phages were performed as described by Holmes et al. (1981) and Sanger et al. (1980), respectively. Yeast genomic DNA, gel electrophoresis, and manipulation of DNA fragments were performed by standard procedures (Sambrook et al., 1989; Sherman et al., 1974).

Preparation of Mitochondria and Mitochondrial Respiration. Yeast cells were grown in YPLact medium at 28 °C and harvested when the OD_{600nm} of the culture reached a value of 3 (log phase) by a 20-min centrifugation at 750*g* and washed in cold water. Mitochondria were isolated by enzymatic digestion of the cell wall by Zymolyase 20T (Calbiochem) (Daum et al., 1982). Oxygen consumption was measured with a Clark electrode at 30 °C. Isolated mitochondria (0.3−1 mg of protein) were suspended in 5 mM KCl, 0.6 M mannitol, 10 mM morpholinopropanesulfonic acid (Mops), 10 mM potassium phosphate, 4 mM $MgCl_2$, and 0.2% bovine serum albumin, pH 6.8. The respiratory substrate was 0.5 mM NADH; respiration was stimulated by addition of 0.17 mM ADP.

Determination of the Cytochrome Content. The cytochrome content of isolated mitochondria was measured by recording the reduced minus oxidized visible spectrum. Mitochondria were diluted at 2−3 mg of protein/mL in 0.6 M mannitol, 10 mM Tris-HCl, and 0.1 mM EGTA, pH 7.4. The oxidized state was obtained after addition of potassium ferricyanide, and the reduced state, after addition of sodium dithionite. The millimolar extinction coefficient used was 24 for cytochrome aa_3 (λ = 605−630 nm) (Van Gelder & Muijsers, 1967).

Immunodetection. Proteins were subjected to electrophoresis on a SDS−10% polyacrylamide gel with a 5% stacking gel (Laemmli, 1970) and then transferred to nitrocellulose filter. Anc2p was detected using polyclonal antibodies raised against a 14-residue peptide corresponding to the C-terminal sequence (Y-D-Q-L-Q-M-I-L-F-G-K-K-F-K) of Anc2p. The peptide and the corresponding antibodies were obtained as described in Brandolin et al. (1989). The immune complexes were detected according to the manufacturer's indications (Promega).

[^{3}H]ATR or [^{14}C]N-ATP Binding Assays. [^{3}H]ATR or [^{14}C]N-ATP binding assays on isolated mitochondria were carried out as described by Brandolin et al. (1993b). Briefly, mitochondria were diluted (0.5−1 mg of protein/mL) in a standard medium (1 mL) consisting of 0.12 M KCl, 10 mM Mops, and 1 mM EDTA, pH 6.8, and [^{3}H]ATR or [^{14}C]N-ATP were added at increasing concentrations up to 3 or 19 μM, respectively. After a 45-min incubation at 0 °C, the mitochondria were sedimented and radioactivity of the pellet was determined by scintillation counting (Ready Value, Beckman). Parallel assays were performed in the presence of 20 μM CATR to correct for minor nonspecific binding of [^{3}H]ATR or [^{14}C]N-ATP.

Assay of Adenine Nucleotide Exchange. The adenine nucleotide translocation was measured by a slightly modified back-exchange procedure (Lauquin & Vignais, 1973). Freshly isolated and well-coupled mitochondria were first suspended in 0.6 M mannitol, 0.2% bovine serum albumin, 10 mM Mops, and 0.1 mM EDTA, pH 6.8 (15 mg of protein/mL) and then incubated in the presence of 15 μCi of [2,8-^{3}H]-ATP (40 Ci/mmol) for 45 min at 0 °C. They were washed twice with the suspension medium to remove free [2,8-^{3}H]-ATP and finally resuspended in the same medium (about 30 mg of protein/mL). For translocation experiments, mitochondria were resuspended in 0.6 M mannitol, 10 mM Mops, and 2 mM EDTA, pH 6.8, at 0.5 mg of protein/mL. The exchange was initiated by addition of unlabeled ATP (up to 50 μM) and stopped after 10 s by addition of CATR (10 μM final). After centrifugation for 5 min at 16000*g*, the release of [2,8-^{3}H]ATP was estimated by scintillation counting of the radioactivity present in the supernatant.

Time-resolved measurements of ATP transport were carried out at 22 °C (Brandolin et al., 1990) using the rapid filtration system described by Dupont (1984). Briefly, the substrate to be transported was delivered to particles adsorbed on a nitrocellulose filter. The filter was perfused for a preset period of time with a solution containing the substrate at a given concentration and at a flow rate high enough to keep it constant. For each transport measurement, mitochondria (1 mg of protein) preloaded with [2,8-^{3}H]ATP were immobilized on the nitrocellulose filter. The filter was perfused with 50 μM ATP in 125 mM KCl, 10 mM sodium phosphate, 5 mM $MgCl_2$ and 10 mM sodium succinate, pH 7.3, for a preset period of time ranging from 10 ms to 10 s. The filter was then removed and dissolved in scintillation fluid and the bound radioactivity was measured. The nonspecifically bound radioactivity was assessed from assays performed with mitochondria preincubated with 10 μM CATR for 1 min.

Determination of Mitochondrial ATP and ADP. After mitochondria were loaded with [2,8-^{3}H]ATP, ATP, and ADP were extracted by perchloric acid. Aliquots of 300 μL of mitochondrial proteins (30 mg of protein/mL) were treated with 30 μL of 70% $HClO_4$ for 15 min at 0 °C. The denatured proteins were sedimented by centrifugation at 16000*g* for 5 min. The supernatant was neutralized with 2 M KOH and 0.3 M Mops. After 1 h at 0 °C, the potassium perchlorate was removed by centrifugation and the supernatant was filtered on a cellulose triacetate filter (MWCO 12 000). ADP and ATP in the perchlorate extract were assayed using a Beckman HPLC Gold System. A 20-μL sample was applied on a 3-μm reverse-phase column (RP-18 Velosep type; 100 × 3.2 mm) according to Stocchi et al. (1987) with some modifications. The mobile phase used consisted in two

eluants, namely, 8 mM tetrabutylammonium hydrogen sulfate in 0.1 M potassium phosphate, pH 6.0 (buffer A), and 30% (v/v) methanol in buffer A (buffer B). The elution conditions were as follows: 5 min with 100% buffer A at 0.5 mL/min, 15 min with buffer B increasing linearly from 0% to 50% at 1 mL/min, 5 min with buffer B increasing from 50% to 100% at 1 mL/min. Buffer B was held at 100% for 4 min and then decreased to 0% and to a flow rate of 0.5 mL/min in 3 min. The adenine nucleotides were detected at $\lambda = 254$ nm. Identification and quantification of ADP and ATP were made by reference to standard solutions of known concentrations.

Isolation of Anc2p. Anc2p and all tryptophanyl variant carriers were isolated by first solubilizing membranes (10 mg of protein/mL) in a mixture of 2% (w/v) dodecyl maltoside (Sigma) and 2% (w/v) Emulphogen BC720 (GAF Corp., New York). They were purified by chromatography on hydroxyapatite (HTP), followed by gel filtration on Ultrogel AcA 202 (Sepracore) as described in Brandolin et al. (1993b). The various Anc2p were eluted in the passthrough fractions. Their purity was estimated to be 80−90% from Coomassie Blue staining after SDS−PAGE. About 50% of the CATR binding sites were recovered. These parameters were the same for the wild-type and the mutated Anc2p.

Extraction of Ergosterol. The AcA 202 column eluate, containing Anc2p isolated from 300 mg of mitochondrial proteins, was extracted with 5 volumes of chloroform/methanol (2/1 v/v). After phase separation, the organic phase was filtered through a sintered glass funnel and dried over solid $CaCl_2$ prior to being evaporated to dryness at 40 °C in a rotary evaporator. The extract was solubilized with 5 mL of dichloromethane and analyzed by ascending thin-layer chromatography on a silica gel-coated glass plate (Merck) containing a fluorescent indicator. The chromatogram was developed in chloroform. Ergosterol was visualized under UV light as a single spot with an R_f of 0.2. High-performance liquid chromatography (HPLC) was performed with a Kontron system equipped with two 420 pumps, using a 300- × 6-mm silica column (microporasil, Waters). UV absorbance of the effluent was monitored continuously with a 420 spectrophotometer detector at $\lambda = 270$ nm. The column was eluted for 20 min at a flow rate of 1 mL/min with a linear gradient of methanol increasing from 0% to 50% in dichloromethane. Under these conditions, ergosterol was eluted with a retention time of 7.7 min.

RESULTS

Anc2p Tryptophanyl Residues Are Not Essential for Cell Growth and Adenine Nucleotide Exchange Activity. The three mutated *ANC2* genes in which the tryptophan codons were singly substituted by tyrosine codons were generated by site-directed mutagenesis. The mutated genes in which two or three tyrosine codons were substituted for tryptophan codons were constructed by recombination of plasmids as described in Experimental Procedures. Mutated genes were named *ANC2* followed by the position of the remaining tryptophan codon(s) (for example, *ANC2-W87-W235*) or *ANC2*-ΔW for the gene devoid of any tryptophan codons (Table 1).

To ensure that the mutated *ANC2* genes all encoded an active carrier, these genes were cloned on the yeast centromeric phagemid pRS314, and then used to transform the JL1-3 strain, which is unable to grow on a nonfermentable carbon source. Irrespective of the mutated *ANC2* genes, the transformants were all able to grow on glycerol, indicating that each of them encoded an active Anc2p. As a control experiment, phagemid pRS314 was used to transform JL1-3 and none of the transformants was able to grow on glycerol (data not shown). To facilitate biochemical analyses, the mutated *ANC2* genes were integrated into JL1-3 genomic DNA by homologous recombination (Brandolin et al., 1993b). The yeast strain nomenclature followed the mutated *ANC2* gene nomenclature (Table 1).

Table 1: Nomenclature of the JL1-3 Strains Harboring a Wild-Type or a Mutated *ANC2* Gene

strain	position of the remaining Trp codon	position of the Trp → Tyr codon substitution
JL1-3-*ANC2*	87, 126, 235	
JL1-3-*ANC2-W87-W126*	87, 126	235
JL1-3-*ANC2-W87-W235*	87, 235	126
JL1-3-*ANC2-W126-W235*	126, 235	87
JL1-3-*ANC2-W87*	87	126, 235
JL1-3-*ANC2-W126*	126	87, 235
JL1-3-*ANC2-W235*	235	87, 126
JL1-3-*ANC2*-Δ*W*		87, 126, 235

The different strains expressing a wild-type or a mutated *ANC2* gene were grown at 28 °C using lactate as carbon source. As shown in Table 2, they were all able to grow under these conditions. The OD_{600nm} values of the cultures at the growth plateau were in the range of 10−12 for all the strains (Table 2), indicating that there was no significant difference in the growth yields. However, two different growth behaviors were noticed. The strains expressing a carrier with two or three W → Y substitutions had longer lag phases and growth doubling times than the strain harboring the wild-type *ANC2* gene; they reached a plateau after 100 h of growth instead of 50 h. This was also the case for the mutated strain JL1-3-*ANC2-W87-W235*, but in this case the plateau was reached after 75 h. The other strains had the same growth behavior as JL1-3-*ANC2*.

Using glucose as a fermentable carbon source, all the strains harboring a wild-type or a mutated *ANC2* gene were able to grow at the same rate at 20, 28, or 37 °C. However, at 37 °C, when the cells were grown on the nonfermentable carbon sources glycerol or lactate, the strains with a longer lag phase at 28 °C did not grow (Table 2).

BA is a highly specific inhibitor of Ancp. Unlike ATR, it can penetrate the yeast cell membrane at slightly acidic pH. Resistance of JL1-3-*ANC2* strains to BA was examined, a strain being characterized as sensitive when it could not grow at 28 °C on a specific medium containing 5 μM BA (Table 2) (Experimental Procedures). The JL1-3-*ANC2* strain was able to grow on such a medium as well as the strains expressing the monosubstituted genes *ANC2-W87-W126* and *ANC2-W126-W235*. All the other strains were sensitive to BA. Thus, a higher sensitivity to BA is accompanied by a higher temperature sensitivity at 37 °C for growth of the cells on a nonfermentable carbon source (Table 2).

This first set of experiments led to the conclusion that none of the three tryptophanyl residues of Anc2p was essential to ensure a functional Ancp since all the mutated strains were able to grow on a nonfermentable carbon source. However, the detection of differences in growth properties of cells from

Table 2: Growth Characteristics of Wild-Type and Mutated Strains

strain	lag phase (h)[a]	doubling time (h)	saturation (OD_{600nm})	sensitivity to BA[b]	temperature sensitivity[c]
JL1-3-*ANC2*	*d*	2.5	12	−	−
JL1-3-*ANC2-W87-W126*	*d*	2.5	12	−	−
JL1-3-*ANC2-W87-W235*	10	8.3	12	+	+
JL1-3-*ANC2-W126-W235*	*d*	2.5	12	−	−
JL1-3-*ANC2-W87*	10	8.3	11.5	+	+
JL1-3-*ANC2-W126*	10	8.0	11.5	+	+
JL1-3-*ANC2-W235*	10	8.3	10	+	+
JL1-3-*ANC2-*Δ*W*	10	8.0	10.5	+	+

[a] Cells were diluted from overnight cultures and grown in YPLact under aeration at 28 °C. Growth was monitored at $\lambda = 600$ nm. For JL1-3-*ANC2*, JL1-3-*ANC2-W126-W235*, and JL1-3-*ANC2-W87-W126*, the plateau phase was reached after 50 h. For JL1-3-*ANC2-W87-W235*, it was reached after 75 h, and for the other strains, after 100 h. [b] Sensitivity to BA was estimated from growth inhibition on a specific medium buffered at pH 4.5 and containing 5 μM BA. [c] A strain was temperature-sensitive when it could not grow at 37 °C on glycerol or lactate. [d] Lag phase < 0.5 h.

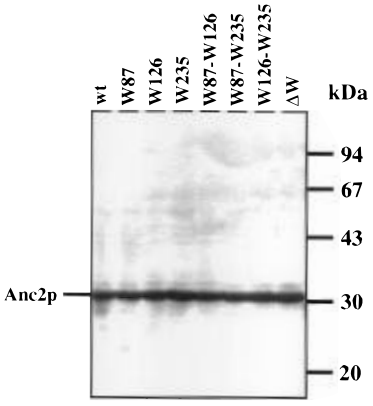

FIGURE 3: Western blot analyses of the wild-type (wt) and the mutated Anc2p. The primary antibody was a rabbit polyclonal antibody directed against a C-terminal peptide of Anc2p (the 14 last amino acids). Dilution of the serum was 1:4000. Secondary antibodies were anti-rabbit IgG coupled to alkaline phosphatase (1:5000). The immunocomplexes were detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

some of the mutated strains led us to hypothesize that some of the tryptophanyl residues might marginally be involved in the correct folding or in transport activity of the carrier protein, resulting in high transport efficiency.

The Mitochondrial Content of Anc2p Is Not Modified by the Trp → Tyr Substitutions. The amount of mutated Anc2p in mitochondria of transformed yeast strains was analyzed by an immunochemical approach and also by binding of a specific inhibitor and a nontransportable ATP analogue.

For immunochemical analyses, mitochondrial proteins were separated by SDS−PAGE, and Anc2p was detected with an antibody raised against the C-terminal peptide of wild-type Anc2p (Experimental Procedures). As shown in Figure 3, for each sample, only one band with an apparent equivalent intensity was detected at a position corresponding to a mass of about 35 kDa, indicating that, whatever the W → Y substitution, equal amounts of Anc2p are present in mitochondria.

Binding experiments were carried out with atractylate (ATR), a highly specific inhibitor of Anc2p, and 3′-*O*-naphthoyl-ATP (N-ATP), a nontransportable fluorescent analogue of ATP. Specific binding of [^{3}H]ATR or [^{14}C]N-ATP to isolated mitochondria was determined as a function of the added ligand concentration after correction for nonspecific binding (see Experimental Procedures). Results are given in Table 3, and binding curves of [^{3}H]ATR and of [^{14}C]N-ATP to mitochondria from JL1-3-*ANC2*-ΔW are shown in Figures 4 and 5, respectively. As previously reported with mitochondria from the strain JL1-3-*ANC2* (Brandolin et al., 1993b), binding of [^{3}H]ATR increased as a function of added labeled ligand and reached a plateau. From the Scatchard plot, a number of binding sites of 0.59−0.65 nmol/mg of protein was calculated for the mutated strains, which is not significantly different from the amount determined for the wild-type Anc2p (Table 3). As shown in Figure 5, the amount of bound [^{14}C]N-ATP increased with the amount of added ligand to reach a plateau. The number of [^{14}C]N-ATP binding sites was 0.43 nmol/mg of protein for the control Anc2p and was in the range of 0.46−0.64 nmol/mg of protein for the variant Anc2p (Table 3). Taken together, the immunochemical and binding data indicated that the mutated Anc2p and the wild-type Anc2p were present in similar amounts in the inner mitochondrial membrane.

In order to correct against variable levels of membrane protein contaminants between the mitochondria preparations from the mutants, the numbers of binding sites for [^{3}H]ATR and [^{14}C]N-ATP were normalized to the amount of cytochrome aa_3. The calculated ratio values were similar (2.0−4.3) for the wild-type and various mutant membranes.

The [^{3}H]ATR K_d values of the mutated Anc2p (0.17−0.25 μM) were virtually the same as that of the wild-type Anc2p (0.21 μM) (Table 3). Likewise, the K_d value for [^{14}C]N-ATP for the wild-type Anc2p (0.96 μM) was similar to the K_d values found for the mutated carriers (0.40−1.07 μM) (Table 3). We determined the specifically bound [^{14}C]N-ATP after correction for the nonspecifically bound [^{14}C]N-ATP that was estimated, from parallel assays, as bound radioactivity not removed by CATR. The greater variation in K_d values for [^{14}C]N-ATP compared to those for [^{3}H]ATR suggests that the specifically bound [^{14}C]N-ATP chased by CATR was not totally released into the aqueous medium. In addition, [^{14}C]N-ATP binds somewhat to the membrane because of the hydrophobic character of its naphthoyl moiety. It may also bind to other proteins endowed with good affinity for ATP. These considerations led us to conclude that the W → Y substitutions alter neither the [^{14}C]N-ATP binding nor the functioning of Anc2p.

Respiratory Properties of the Mutated Strains. Although the growth rate of cells was altered by some of the W → Y substitutions introduced in Anc2p, the mitochondrial respiratory control was not significantly modified, with values ranging from 2.3 to 3.1 (Table 4). Mitochondrial respiration was measured as described in Experimental Procedures. These results confirm that the Anc2p in all the mutated

Table 3: [^{3}H]ATR and [^{14}C]N-ATP Binding to Isolated Mitochondria from Wild-Type and Mutated Strains

	[^{3}H]ATR			[^{14}C]N-ATP		
strain	(nmol/mg of protein)	mol/mol of cyt aa_3	K_d (μM)	nmol/mg of protein	mol/mol of cyt aa_3	K_d (μM)
JL1-3-*ANC2*	0.64	3.7	0.21	0.43	2.5	0.96
JL1-3-*ANC2-W87-W126*	0.60	3.6	0.25	0.50	3.0	0.49
JL1-3-*ANC2-W87-W235*	0.64	2.5	0.18	0.64	2.5	1.07
JL1-3-*ANC2-W126-W235*	0.65	4.3	0.18	0.46	3.1	1.04
JL1-3-*ANC2-W87*	0.61	3.4	0.20	0.56	3.2	0.52
JL1-3-*ANC2-W126*	0.60	3.0	0.21	0.60	3.0	0.40
JL1-3-*ANC2-W235*	0.64	4.2	0.17	0.63	4.1	0.65
JL1-3-*ANC2-*Δ*W*	0.59	2.0	0.16	0.64	2.2	0.48

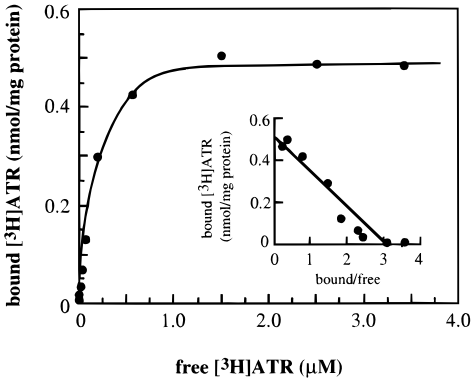

FIGURE 4: Binding of tritiated atractyloside to mitochondria from the JL1-3-*ANC2*-ΔW strain. Mitochondria (1 mg of protein/mL) were incubated in 1 mL of a standard medium (0.12 M KCl, 10 mM Mops, and 1 mM EDTA, pH 6.8) containing increasing concentrations of [^{3}H]ATR for 45 min at 0 °C. After centrifugation, bound [^{3}H]ATR was estimated by scintillation counting of the radioactivity associated with the pellet. The inset corresponds to the Scatchard plot of the binding data.

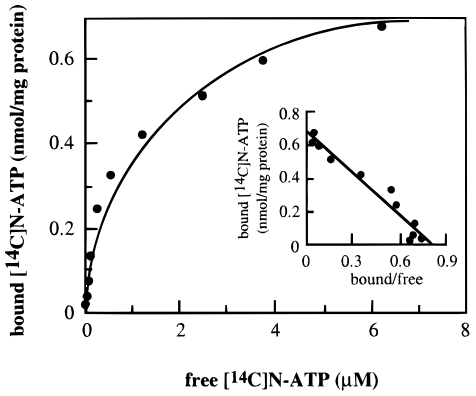

FIGURE 5: Binding of [^{14}C]N-ATP to mitochondria from the JL1-3-*ANC2*-Δ*W* strain. Mitochondria (0.6 mg of protein/mL) were incubated in 1 mL of a standard medium (0.12 M KCl, 10 mM Mops, and 1 mM EDTA, pH 6.8) containing increasing amounts of [^{14}C]N-ATP for 45 min at 0 °C. After centrifugation, bound [^{14}C]N-ATP was estimated by scintillation counting of radioactivity associated with the pellet. The inset corresponds to the Scatchard plot of the binding data.

strains was functional, being able to respond to added ADP.

Nucleotide Exchange Activity of the Mutated Carriers. The nucleotide exchange activity of control and mutated Anc2p was performed at 0 °C using freshly isolated and well-coupled mitochondria (see Experimental Procedures). The exchange activity was expressed in terms of percent of exchanged [2,8-^{3}H]ATP. The apparent K_m for ATP (Figure 6 and Table 4) was 2.8 ± 0.4 μM for the control Anc2p (four determinations on different preparations of mitochondria) and in the range of 2−3.9 μM for the mutated Anc2p.

Table 4: Biochemical Characterization of Mitochondria from the Strains Expressing Wild-Type or Mutated Anc2p Carrier

strain	respiratory control[a]	apparent K_m (μM)[a]	k_{cat}[b] (min^{-1})	[ATP + ADP] (nmol/mg of protein)
JL1-3-*ANC2*	2.3	2.8	438	1.6
JL1-3-*ANC2-W87-W126*	2.6	3.3	216	1.1
JL1-3-*ANC2-W87-W235*	2.3	3.5	318	2.3
JL1-3-*ANC2-W126-W235*	2.3	3.5	456	1.8
JL1-3-*ANC2-W87*	3.0	3.3	264	3.3
JL1-3-*ANC2-W126*	2.8	3.9	408	3.2
JL1-3-*ANC2-W235*	3.1	2.9	474	2.5
JL1-3-*ANC2-*Δ*W*	2.5	2.0	252	2.3

[a] The values correspond to the mean of at least two experiments. [b] k_{cat} was determined in an independent set of experiments. The standard error value for the control experiment is 10−15%.

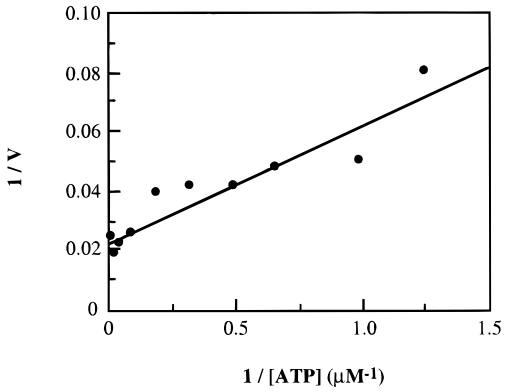

FIGURE 6: ADP/ATP exchange activity. The exchange was initiated by addition of increasing concentrations of unlabeled ATP to mitochondria from the JL1-3-*ANC2*-ΔW strain (0.5 mg of protein) loaded with [2,8-^{3}H]ATP. After 10 s, the exchange was stopped by addition of CATR (10 μM final). After centrifugation, radioactivity associated with the supernatant was estimated by scintillation counting. *V* stands for percent exchange of [2,8-^{3}H]ATP.

The differences in K_m values were essentially attributable to experimental determinations and it can therefore be concluded that the nucleotide exchange activity of the Anc2p is not modified by the W → Y substitutions.

The ATP + ADP content of the mitochondria was determined after incubation with [2,8-^{3}H]ATP (see Experimental Procedures). The exchangeable adenine nucleotide pool was not significantly affected by the mutations introduced in the Anc2p protein (Table 4). Thus, a mutated Anc2p was not responsible for nucleotide leakage which could account for a slower growth rate of some of the mutated strains.

The turnover of the ADP/ATP exchange measured by the rapid filtration technique (Experimental Procedures) was in the range of 400−480 min^{-1} for mitochondria isolated from

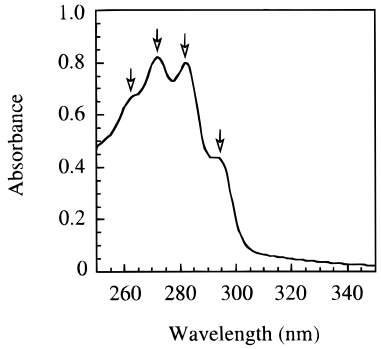

FIGURE 7: Absorption spectrum of wild-type Anc2p isolated in detergent. Protein concentration was 0.25 mg/mL. The main peaks indicated by arrows correspond to $\lambda = 263$, 272, 282, and 293.5 nm.

JL1-3-*ANC2*, JL1-3-*ANC2-W126*, JL1-3-*ANC2-W235*, and JL1-3-*ANC2-W126-W235* and in the range of 210−310 min^{-1} for JL1-3-*ANC2-W87*, JL1-3-*ANC2-W87-W126*, JL1-3-*ANC2-W87-W235*, and JL1-3-*ANC2*-ΔW (Table 4). Comparison of Tables 1 and 4 shows that there was no correlation between a slower growth rate at 28 °C on a nonfermentable carbon source and a decreased activity of the ADP/ATP exchange.

Absorption Spectra of Anc2p Isolated in the Presence of Detergent. Anc2p in detergent solution exhibited an unexpectedly high absorbance at $\lambda = 280$ nm relative to the protein content. As shown in Figure 7, the absorption spectrum of purified Anc2p with the presence of four main peaks at $\lambda = 263$, 272, 282, and 293.5 nm was not typical of a protein. It was due to a contaminant that could be extracted from the carrier solution by chloroform/methanol and was unambiguously identified as ergosterol on the basis of its spectroscopic characteristics and its chromatographic behavior. The R_f value determined by thin-layer chromatography and the retention time measured by HPLC analysis were identical to those of ergosterol from Sigma Chemical Co. (see Experimental Procedures). Identification of the isolated compound with ergosterol was confirmed by mass spectrometry analysis that yielded an M^+ value of 396 (data not shown). All the Anc2p variant preparations used in this study contained similar amounts of ergosterol, estimated at 0.1 mg/mg of protein from absorbance at $\lambda = 282$ nm (extinction coefficient of 11.9×10^3 M^{-1} cm^{-1}). This corresponded roughly to 9 mol of ergosterol/mol of 35-kDa Anc2p.

DISCUSSION

In previous work aimed at exploring conformational changes of the isolated beef heart ADP/ATP carrier, the intrinsic fluorescence approach proved to be quite valuable (Brandolin et al., 1981, 1985). This approach revealed the ADP(ATP)-induced transition of the beef heart carrier between two conformations which could be stabilized by CATR and BA, respectively. However, due to the presence of different isoforms of the ADP/ATP carrier, the fluorescence signals provided only a global assessment of the conformational changes of the bulk of the carrier isoforms. That is why it was decided to turn toward the yeast *S. cerevisiae* and to use an engineered strain in which only one isoform of the ADP/ATP carrier, the Anc2p isoform, was expressed. Similar to the beef ADP/ATP carrier, the isolated Anc2p, which contains three tryptophanyl residues at positions 87, 126, and 235, undergoes substrate- and inhibitor-specific conformational changes (Brandolin et al., 1993b). Using the yeast strain expressing only Anc2p, we engineered carriers in which the tryptophanyl residues were replaced sequentially by tyrosyl residues. Obviously, any conclusion about the contribution of tryptophanyl residues in the fluorescence changes of the carrier requires that the mutated carriers are functional. This in turn implies that tryptophanyl residues of Anc2p are not essential to its functioning, as it is demonstrated in the present report. There are at least two similar examples in the literature. The tryptophanyl residues of the lactate dehydrogenase from *Bacillus stearothermophilus* were replaced by tyrosyl residues without impairing protein activity (Waldman et al., 1987, 1988). Likewise, the six tryptophanyl residues of the *E. coli lac* permease were replaced individually by phenylalanine without altering the permease activity (Menezes et al., 1990).

Tryptophanyl residues of Anc2p were replaced by tyrosine, an aromatic amino acid which is not fluorescent under the conditions of tryptophan fluorescence measurements. The choice of tyrosine over phenylalanine was dictated in part by the ability of tyrosine and tryptophan, unlike phenylalanine, to establish hydrogen bonding with the adjacent residues by the OH of the phenol group and the NH of the indole ring, respectively.

The mutated *ANC2* genes were cloned into a vector and expressed in JL1-3, a yeast strain in which all the *ANC* genes had been interrupted (Drgon et al., 1991). All the mutated *ANC2* genes could restore growth of this strain on a nonfermentable carbon source, indicating that the tryptophanyl residues were not essential. We then replaced the *anc2::HIS3* allele of the JL1-3 strain by the wild-type *ANC2* gene (Brandolin et al., 1993b) or the mutated *ANC2* genes. Indeed, Nelson et al. (1993) reported the isolation of false revertants of inactive *ANC2* genes which arose from recombination between chromosomal and plasmid-borne *ANC* genes. They used the strain JL1-2, which still expressed the *ANC3* allele after disruption of *ANC1* and *ANC2*. The *ANC3* gene was identified through a study of an *op1* revertant arising from recombination between the *ANC2* and an *ANC* gene located on the same chromosome and termed *ANC3* (Kolarov et al., 1990).

In the present work, although all mutated strains were able to grow on a nonfermentable carbon source, differences in cell growth rate on lactate were observed. The strains expressing doubly or triply substituted *ANC2* genes or the mutated *ANC2-W126Y* gene exhibited longer growth doubling times and lag phases, and were also temperature- and BA-sensitive. In principle, these alterations might originate from modifications in the folding or the stability of the carrier protein. Results of specific binding and immunochemical studies led us to conclude that the modified behavior of the mutated strains was not due to a decrease in the amount of the mutated Anc2p incorporated into the inner mitochondrial membrane. Furthermore, the respiration control of the mutated strains was the same as that of the wild-type strain.

Were the intrinsic properties of the carriers modified by the mutations? We avoided reconstitution experiments of ADP/ATP transport since this approach makes it difficult to quantify estimation of carrier catalytic parameters. Studies of the ADP/ATP exchange with mitochondria appeared to be more relevant. Examination of the turnover number and

K_m for ATP did not reveal significant differences for the mutated Anc2p as compared to the wild-type Anc2p. The same holds for K_d values for ATR and N-ATP. Different cellular properties of the mutated strains as compared to the wild type do not correlate with modified characteristics of the isolated carriers, at least for the parameters we examined.

The isolated Anc2p was characterized by an absorption spectrum atypical for a protein, with unusually high absorption peaks in the 260−300-nm region. The compound responsible was identified as ergosterol. Ergosterol coeluted with Anc2p through the chromatographic purification steps. Using the fluorometric titration of CATR binding sites [see accompanying paper by Roux et al. (1996)] as reference for the determination of Anc2p, it was calculated that the molar ratio of ergosterol to Anc2p monomer amounted up to 9. This value, which corresponds roughly to 0.1 mg of ergosterol/mg of protein, was 10 times higher than the ratio of ergosterol to yeast mitochondrial proteins, which was equivalent to 0.01 mg/mg of protein (Daum et al., 1982). These results point to the propensity of ergosterol to be extracted from mitochondria by detergent treatment and to be eluted in the passthrough fraction during HTP chromatography. Specific interactions of the beef heart and the yeast ADP/ATP carrier with cardiolipin have been reported to occur (Brandolin et al., 1980; Hoffmann et al., 1994), but at present there is no evidence of specific association of ergosterol with the yeast carrier, either isolated or in the membrane-bound state.

Assuming that sequence homologies result in folding similarities, it has been postulated that all members of the mitochondrial carrier family (MCF) are folded in a similar way (Walker, 1992) with six putative transmembrane segments (Figure 1). A cytosolic orientation of the N- and C-termini was described for the phosphate carrier (Capobianco et al., 1991), the citrate carrier (Capobianco et al., 1995), and the uncoupling protein (Eckerskorn & Klingenberg, 1987; Klingenberg & Appel, 1989; Miroux et al., 1993), for example. A cytosolic orientation of the bovine Ancp N-terminus was evidenced from immunochemical experiments but no firm conclusion could be drawn for the C-terminus using the same approach (Brandolin et al., 1989). An even number of transmembrane segment was recently proposed from experiments where a covalent dimer of the Anc2p was engineered and proved to be functional *in vivo* (V. Trézéguet, unpublished results). Altogether these data led to the topology model depicted in Figure 1. As can be seen, Trp87 is close to an essential residue, Arg96, whose mutation to histidine is responsible for the op1 phenotype (Kolarov et al., 1990; M. Donzeau, DEA Bordeaux, personal communication, 1990). Comparison of the amino acid sequences of 30 Ancp deduced from the known *ANC* genes (Brandolin et al., 1993a; Le Saux, 1995) showed that Trp87 is strictly conserved. This might suggest that Trp87 is involved in either the activity or the folding of Anc2p. However, Trp87 could be replaced by tyrosine without notable modifications of Anc2p properties. The other two tryptophan residues of Anc2p, Trp126 and Trp235, are conserved at an equivalent position only in the plant and fungus Ancp. In mammalian Ancp, tyrosine or phenylalanine occurs at a position equivalent to Trp126 and a glutamine is present at a position equivalent to the yeast Trp235. A tryptophanyl residue is located two residues upstream in the former case and four residues upstream in the latter case. Trp87 and Trp126 are located at the borders of the second and the third putative transmembrane segments, respectively (Figure 1). Their environment is rather hydrophilic and their exposure makes them readily accessible to chemical modifying reagents. Trp 235 is in a more hydrophobic environment since it appears to be located deeper inside the fifth putative transmembrane segment (Figure 1).

As a conclusion, the various mutant forms of Anc2p which were generated and biochemically characterized in this paper appear to be suitable for the tryptophan fluorescence spectroscopy studies described in the accompanying paper (Roux et al., 1996).

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