Conformational Changes of the Yeast Mitochondrial Adenosine Diphosphate/Adenosine Triphosphate Carrier Studied through Its Intrinsic Fluorescence. 2. Assignment of Tryptophanyl Residues of the Carrier to the Responses to Specific Ligands[†]

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ABSTRACT: Tryptophanyl substitution of the *Saccharomyces cerevisiae* adenine nucleotide carrier (Anc2p isoform) was not deleterious for the transport activity or the folding of the carrier [preceding paper by Le Saux et al. (1996) *Biochemistry 35*, 16116−16124]. Conformational changes of the isolated wild-type and Trp-substituted Anc2p variants, induced upon binding of specific substrates [adenosine triphosphate (ATP) or diphosphate (ADP)] or inhibitors [carboxyatractyloside (CATR) or bongkrekic acid (BA)], were studied by measurement of intrinsic fluorescence. Titration of CATR and BA binding sites ended in the same number of sites, namely, 6−7 nmol/mg of wild-type and variant Anc2p. Isolated Anc2p in detergent presented similar emission spectra, suggesting that all tryptophanyl residues were in environments of similar hydrophobicity. Trp87 and Trp126 contributed largely and to a similar extent to the fluorescence enhancement observed in response to ATP binding, while Trp235 contributed negatively and to a small extent to the fluorescence change. Both Trp126 and Trp235, and to a lower extent Trp87, participate in the CATR-induced fluorescence decrease of Anc2p. Responses to BA binding were observed only in the presence of ATP; they consisted of a further fluorescence increase of the Anc2p·ATP complex, which was mainly due to Trp87 and Trp126, Trp235 being much less responsive. The different fluorescence responses of the three Trp residues of Anc2p variants to ATP, CATR, and BA are in agreement with distinct binding sites for these ligands and distinct conformations of the carrier protein recognizing specifically CATR or BA. A mechanistic model is proposed to interpret the transitions between the different conformational states of Anc2p.

Upon addition of specific substrates and inhibitors, the beef heart mitochondrial ADP/ATP carrier protein (Ancp)[1] undergoes conformational changes that result in masking/unmasking of SH groups (Leblanc & Clauser, 1972; Vignais & Vignais, 1972; Boulay et al., 1983), tyrosyl residues (Brdiczka & Schumacher, 1976), or antigenic determinants (Buchanan et al., 1976; Brandolin et al., 1985). Substrate- and inhibitor-dependent conformational changes have been observed not only with the membrane-bound ADP/ATP carrier but also with the isolated carrier. A convenient approach to probe these conformational changes has been the fluorescence spectroscopy of endogenous tryptophanyl residues. This technique has been applied to the Ancp isolated from beef heart mitochondria (Brandolin et al., 1981, 1985; Block & Vignais, 1986) and from *Saccharomyces cerevisiae* mitochondria (Brandolin et al., 1993a). In both cases, Ancp was found to adopt two different conformations recognized specifically by carboxyatractyloside (CATR) and by bongkrekic acid (BA), respectively. The transition between the two conformations was very slow in the absence of transportable nucleotides but was markedly accelerated in the presence of the two substrates ATP or ADP. These results suggested that the nucleotide-induced conformational changes of the isolated Ancp are similar to those occurring in the membrane-bound carrier during ADP/ATP transport.

The Anc2p isoform of the ADP/ATP carrier in the yeast *S. cerevisiae* contains three tryptophanyl residues located at positions 87, 126, and 235 in the polypeptide chain. To assess the specific contribution of each Trp residue to the fluorescence changes of the Anc2p isoform, we have replaced each of the three Trp residues or pairs of them by Tyr residues, using site-directed mutagenesis. As reported in the preceding paper (Le Saux et al., 1996), seven tryptophan-substituted variants of Anc2p have been engineered. These transformed carrier proteins were expressed to virtually the same level and their ADP/ATP transport activity in mito-

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

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chondria was not significantly different from that of the wild-type Anc2p. In the present paper, we describe the fluorescence changes of the Anc2p variants initiated by ATP (or ADP), CATR, and BA. We report that Trp87 and Trp126 contribute predominantly to the enhancement of fluorescence of Anc2p elicited upon ATP (or ADP) binding, whereas Trp235 exerts a negative contribution. CATR induced a quenching of the fluorescence of the three tryptophanyl residues of the Anc2p, with Trp235 and Trp126 being mainly affected. In addition, we have found that the BA-dependent conformational change of the Anc2p is linked to the formation of a transient ternary Anc2p·ATP·BA complex.

EXPERIMENTAL PROCEDURES

Chemicals. Nucleotides, dodecyl maltoside, and carboxyatractyloside were purchased from Sigma. Hydroxyapatite and Ultrogel AcA 202 gel were purchased from Bio-Rad and Sepracore, respectively. Emulphogen BC720 (GAF Corp., New York) was purified following the procedure described by Ashami and Catravas (1980). Bongkrekic acid was prepared as previously described (Lauquin & Vignais, 1976). 3′-*O*-Naphthoyl-ATP (N-ATP) was synthesized as previously described by Block et al. (1982). Protein concentration was determined with the BCA (bicinchoninic acid) reagent kit from Pierce.

Strains and Media. *Saccharomyces cerevisiae* yeast strains used in this study were those described in the preceding paper (Le Saux et al., 1996), i.e., JL1-3-*ANC2* containing either the wild-type or the tryptophanyl-mutated *ANC2* genes. It is recalled that the mutated genes are designed by *ANC2* followed by the remaining tryptophan codon(s) or by *ANC2*-Δ*W* for the gene devoid of tryptophan codons.

Yeast strains were grown aerobically at 28 °C in YPLact medium (2% bactopeptone (Difco), 1% yeast extract, 2% lactate, and 1% KH_2PO_4, pH 5.5).

Preparation of Mitochondria. Cells were harvested in the logarithmic phase and mitochondria were isolated from yeast homogenates according to the method described by Daum et al. (1982).

Isolation of Anc2p. Anc2p and all tryptophanyl variant carriers were isolated as described in the accompanying paper by Le Saux et al. (1996).

Time Course of Fluorescence Changes. Fluorescence was measured with a high-sensitivity fluorometer (Bio-Logic, Grenoble, France) equipped with a 150-W Xenon (Hg) lamp. The emission slit width was fixed at 8 nm and the emitted light was measured at a right angle. Samples were diluted with 136 mM glycerol to obtain a final protein concentration ranging from 0.03 to 0.07 mg/mL. Samples (2 mL) were then placed in a 1- × 1-cm fluorescence quartz cuvette that was inserted in a temperature-controlled cell holder. The contents of the cuvette were continuously stirred. Reagents were introduced by means of Hamilton syringes in 2−20-μL aliquots. The fluorescence intensity was corrected for dilution and absorption effects.

The tryptophanyl fluorescence of the protein was excited at $\lambda = 297$ nm with a slit width of 5 nm and experiments were performed at 10 °C, except when specified. The emitted light was measured through a 0−54 Corning filter coupled to an ultraviolet light filter (Corning 7−54). The center of the resulting band-pass was at $\lambda = 345$ nm with a peak transmission of 75% and a width of 60 nm at half-maximum of the peak. Ergosterol present in the purified fractions of Anc2p resulted in an inner filter effect which might impede comparison of the fluorescence signals of Anc2p isolated from independent mitochondrial preparations [see the accompanying paper by Le Saux et al. (1996)]. To circumvent this technical difficulty, bovine serum albumin was used as a fluorescence standard to calibrate the fluorometer. The amplifier unit was set to provide a fluorescence signal of 400 mV upon addition of bovine serum albumin (20 μg/mL) to the Anc2p fraction in the assay cuvette. Thus, amplitudes of fluorescence changes could be compared for Anc2p variants that retained the same number of tryptophanyl residues. Using the high-sensitivity fluorometer, relative fluorescence changes as low as 0.3% could be measured reproducibly and taken as significant.

Binding of N-ATP (1 μM final concentration) was assayed by fluorometry. The excitation wavelength was set at $\lambda = 312$ nm with a slit width of 8 nm and the emitted light was measured through a K40 Corning filter with a band-pass at $\lambda = 410$ nm, a transmission of 75%, and a bandwidth of 50 nm at half-maximum of the peak. Displacement of bound N-ATP by CATR, BA, or ATP resulted in a fluorescence increase.

Fluorescence Emission Spectra. Fluorescence emission spectra were recorded at 10 °C using a Fluoromax spectrofluorometer (Spex) equipped with a photon-counting system. Tryptophan fluorescence was selected by excitation at $\lambda = 297$ nm with a slit width of 2 nm. Reagents were added in 5-μL aliquots with Hamilton syringes. Emission spectra were collected between $\lambda = 310$ and 500 nm at a scanning rate of 60 nm/min with a slit width of 6 nm. Each fluorescence spectrum was corrected for background fluorescence. Data were collected and analyzed using Grams/386 software (Galactic).

RESULTS

In all the following experiments, wild-type and Trp-substituted variants of Anc2p were assayed after purification in the presence of detergent.

Titration of CATR and BA Binding Sites. In the preceding paper (Le Saux et al., 1996) we have shown that membrane-bound Anc2p variants with various tryptophan substitutions are still competent in the specific recognition of ligands, including N-ATP, a nontransportable fluorescent analogue of ATP that binds with high affinity to the beef heart ADP/ATP carrier (Block et al., 1982; Dupont et al., 1982; Block & Vignais, 1986). In the present study, N-ATP has been used as a fluorescent probe to explore the binding properties of the isolated Anc2p variants in the presence of a fixed concentration of N-ATP (1 μM). Addition of CATR, BA and ATP induced a strong fluorescence increase at $\lambda = 400$ nm, reflecting the release of bound N-ATP.

The amplitude of the CATR-dependent fluorescence changes of Anc2p varied linearly with the concentration of added CATR until a plateau was attained, allowing end-point titrations of CATR binding sites. By this approach, the number of CATR binding sites amounted to 6−7 nmol/mg of protein, corresponding to 1 mol of CATR bound/2 carrier dimers. In the case of the beef heart ADP/ATP carrier, 1 carrier dimer binds 1 mol of CATR (Klingenberg et al., 1978; Block et al., 1986). Assuming that the native yeast and heart Anc2p behave similarly with respect to CATR binding, one may conclude that, in our extraction and purification condi-

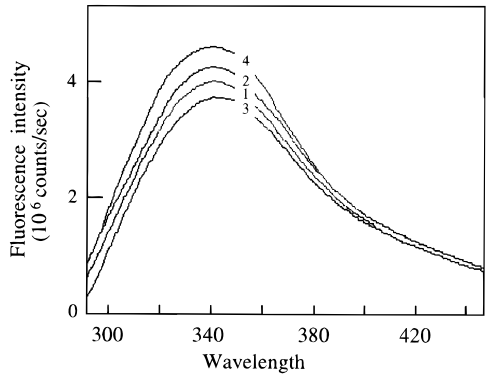

FIGURE 1: Fluorescence emission spectra of wild-type Anc2p isolated in dodecyl maltoside and Emulphogen (1) in the absence of any added ligand or supplemented with (2) ATP, (3) CATR, or (4) ATP plus BA. Spectra were recorded with isolated Anc2p as described in Experimental Procedures. Fluorescence was excited at $\lambda = 297$ nm. ATP, CATR, and BA were used at 5 μM final concentration. Protein concentration was 0.07 mg/mL.

tions, about half of the isolated yeast Anc2p remains competent in binding. The loss of binding sites is most likely attributable to a deleterious effect of the detergent used for solubilization of the carrier. Yet, in a number of preliminary assays, the combination of Emulphogen and dodecyl maltoside proved to be less deleterious for the yeast carrier than other detergents such as dodecylamido-*N*,*N*-dimethylpropylaminoxide or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate that have been used for extraction of the beef heart carrier (Brandolin et al., 1985; Block & Vignais, 1986).

Similar titration assays were carried out with BA. However, as previously shown for the isolated beef heart ADP/ATP carrier (Dupont et al., 1982), full displacement of Anc2p-bound N-ATP by BA required the presence of micromolar amounts of ATP. An equal number of CATR and BA binding sites were found for all tryptophan-substituted Anc2p variants including the tryptophan-less Anc2p (Anc2pΔW). These results demonstrated that the tryptophanyl residues in Anc2p were not necessary for the binding of inhibitors and substrate analogues and thus could be replaced by Tyr residues.

CATR or BA plus ATP was found to displace bound N-ATP more efficiently than ATP did. The half-maximum effect for release of bound N-ATP requires 0.5−0.6 mM ATP, whatever the type of Anc2p used, i.e., wild type or variants. This result confirms that the tryptophan substitutions in Anc2p had no effect on ATP recognition.

Effects of ATP, CATR, and BA on the Emission Fluorescence Spectra. When excited at $\lambda = 297$ nm in order to select for tryptophan fluorescence, the emission spectrum of the isolated Anc2p containing one, two, or three tryptophanyl residues exhibited a maximum at $\lambda = 340$ nm (Figure 1). In the preceding paper (Le Saux et al., 1996) it was shown that the purified Anc2p contained ergosterol. The absence of any significant contribution of ergosterol to the Anc2p fluorescence was demonstrated in control experiments in which no fluorescence change was observed when the carrier in detergent solution was supplemented with ergosterol added at concentrations up to 120 μM. This concentration is about 10 times higher than those usually found in Anc2p preparations.

As shown in Figure 1, the tryptophanyl fluorescence emission spectrum of the wild-type Anc2p was significantly

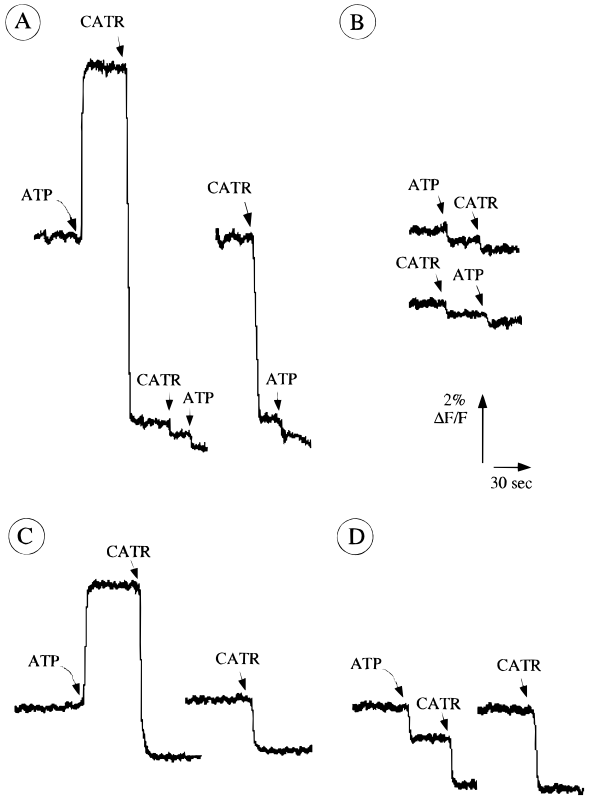

FIGURE 2: Time course of the fluorescence variations of isolated (A) wild-type Anc2p and (B) Anc2pΔW, (C) Anc2pW126, and (D) Anc2pW87-W235 variants. Fluorescence changes were observed after addition of ATP or CATR (5 μM final concentration). Conditions were as described in Experimental Procedures.

modified upon addition of specific ligands, but there was no shift of the maximum emission wavelength. ADP or ATP used at saturating concentration (5 μM) induced an enhancement of Anc2p fluorescence. This effect was specific since other nucleoside di- or triphosphates, even used at concentrations as high as 50 μM, were ineffective (data not shown). When CATR was added alone, or added either prior to or after ATP, the fluorescence emission of Anc2p was decreased and the low level of fluorescence that was attained reflected the formation of a stable carrier·CATR complex (Figure 1).

The effect of BA on the fluorescence spectrum of the isolated wild-type Anc2p was measured after correction for the inner filter effect due to BA itself. In the absence of ATP or ADP, BA did not modify the Anc2p fluorescence. In contrast, when added after ATP, BA induced a further enhancement of fluorescence (Figure 1).

Specific effects of ATP, CATR, and BA on tryptophanyl emission spectra have been analyzed for the singly and doubly substituted forms of Anc2p. For all modified Anc2p, the maximum emission wavelength was centered at $\lambda = 340$ nm as for the wild-type Anc2p.

Time Course of ATP- and CATR-Induced Fluorescence Changes. The effect of Trp substitution on the time course of Anc2p fluorescence changes is illustrated in Figure 2. Experiments were performed with a high-sensitivity fluorometer with the excitation light set at $\lambda = 297$ nm, and the changes in the emitted light were continuously monitored at $\lambda = 345$ nm. Direct comparison of wild-type and variant Anc2p responses to ATP, CATR, and BA was made possible because the position of the maximum emission wavelength in all these conditions was the same whatever the added ligand.

Table 1: Fluorescence Responses of Wild-Type and Tryptophan-Substituted Variants of Anc2p to the Binding of Specific Ligands[a]

protein	ligand[a]			
	ATP	CATR	BA + ATP	BA
Anc2p	5.0	−5.0	10.0	5.0
Anc2pW87-W126	6.0	−1.5	9.9	3.9
Anc2pW87-W235	−1.0	−2.0	−0.7	0.3
Anc2pW126-W235	0.5	−2.5	2.8	2.3
Anc2pW87	2.6	−0.9	4.0	1.4
Anc2pW126	3.0	−1.5	5.5	2.5
Anc2pW235	−0.6	−1.6	−0.3	0.3
Anc2pΔW	*b*	*b*	*b*	*b*

[a] Results are expressed in percent relative fluorescence changes. [b] Undetectable effect.

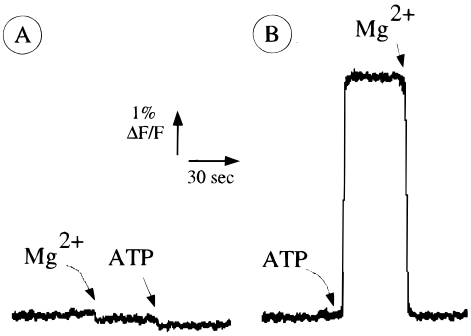

FIGURE 3: Effect of Mg^{2+} on ATP-induced fluorescence change of Anc2pW87-W126 variant. $MgCl_2$ (1 mM) was added (A) before or (B) after ATP (5 μM). Conditions were as described in Experimental Procedures.

As previously shown (Brandolin et al., 1993a), addition of ATP (or ADP) at saturating concentration (5 μM) to wild-type Anc2p in detergent resulted in a rapid and monophasic rise in fluorescence amounting to a 5% relative fluorescence change (Figure 2A). Other nucleotides were ineffective. Kinetics of the ATP- (or ADP-) induced effect were not significantly modified when the temperature was lowered to −5 °C, using a medium supplemented with glycerol and whatever the nucleotide concentrations used (data not shown). The high fluorescence level elicited by ATP (or ADP) was stable for at least 1 min. It could be reversed by CATR to a lower level ($\Delta F/F = -5\%$) than that determined prior to ATP addition, reflecting probably the formation of a carrier·CATR complex (Figure 2A). It was also reached when CATR was added prior to ATP. In this case, the response to ATP added after CATR was totally prevented, thus demonstrating the specificity of its effect. Similar experiments were performed with the Trp-substituted Anc2p variants. Demonstration that the observed fluorescence signals were specific and due exclusively to tryptophanyl residues was obtained from control experiments performed with Trp-less Anc2p (Figure 2B). As could be expected, neither ATP nor CATR induced any fluorescence change of the Anc2pΔW variant, despite the fact that this variant displayed CATR, BA, and ATP binding properties similar to those of the other Trp-substituted variants [see accompanying paper by Le Saux et al. (1996)].

Fluorescence Responses to ATP Binding. The stepwise replacement of the three tryptophanyl residues of Anc2p by tyrosyl residues resulted in modification of either the amplitude or the direction of the variation (increase or decrease) of the ATP-induced fluorescence changes. This is illustrated in Figure 2, panels C and D, for the variants Anc2pW126 and Anc2pW87-W235, respectively. In the latter case, the ATP-induced fluorescence change corresponded to a fluorescence quenching instead of an enhancement as observed with the wild-type Anc2p. The fluorescence responses of the seven Anc2p variants used in this study to added ATP are listed in Table 1. The amplitudes ranged from a 6% relative fluorescence increase for Anc2pW87-W126 to a 1% relative fluorescence decrease for Anc2pW87-W235. The ATP-induced fluorescence responses could be studied in detail only for Anc2pW87, Anc2pW126, and Anc2pW87-W126 since for the other variants the amplitudes were too small to be measured accurately (Table 1). The fluorescence changes increased with the concentration of added ATP, reaching a plateau at approximately 10 μM ATP. Half-maximum effect was obtained with 0.5−0.6 μM ATP, irrespective of the tryptophanyl residues remaining in the protein.

In a previous work, the beef heart Ancp was shown to recognize only the free forms of ATP and ADP (Brandolin et al., 1981). The same conclusion holds for the *S. cerevisiae* Anc2p as illustrated by the inhibitory effect of Mg^{2+} on the ATP-induced fluorescence increase of Anc2pW87-W126 (Figure 3A). The effect of Mg^{2+} was smaller when ADP was used instead of ATP, indicating that Mg^{2+} acted by complexing the nucleotides and not through inactivation of the carrier. Similar results were obtained for the other Anc2p variants and for the wild-type Anc2p (data not shown). When $MgCl_2$ was added after the ATP-induced rise of fluorescence, it completely reversed the signal back to the initial level (Figure 3B), suggesting that the fluorescence level reached in the presence of ATP corresponded to the formation of a stable Anc2p·ATP complex.

The 2.6% and 3% relative fluorescence increases observed with the doubly substituted variants Anc2pW87 and Anc2pW126, respectively, indicated that Trp87 and Trp126 are efficient reporters of ATP binding. Together, they are probably responsible for most of the fluorescence response of Anc2p elicited by ATP. In fact, Trp235 exhibited only a small fluorescence response to ATP binding, as illustrated by the limited quenching (−0.6% $\Delta F/F$) observed for Anc2pW235 (Table 1). This effect was reproducible and could not be attributed to an inner filter effect of ATP, which was negligible in our experimental conditions. Furthermore, addition of ATP to Anc2p containing both Trp87 and Trp126 but not Trp235 resulted in a larger fluorescence increase, whereas this effect was considerably attenuated in the monosubstituted variants Anc2pW87-W235 and Anc2pW126-W235 (Table 1). Thus, Trp235 exerts a negative effect on the ATP-induced fluorescence increase of Trp87 and Trp126, this effect being lower for Trp126.

Fluorescence Responses to CATR Binding. The fact that the wild-type Anc2p and its variants isolated in detergent could bind CATR in the absence of ADP or ATP indicated that these proteins were obtained in the CATR conformation. The formation of the Anc2p·CATR complex resulted in the quenching of the carrier fluorescence (Table 1).

The different effects of CATR on the intrinsic fluorescence of the wild-type Anc2p were also observed with the Anc2p variants: (1) when added to the carrier prior to ATP (or ADP), CATR decreased the protein fluorescence and prevented the ATP- (ADP-) induced fluorescence changes, and (2) when added after ATP (or ADP), CATR was able to reverse the ATP- (or ADP-) induced fluorescence signal, an

effect which cumulated with the CATR-induced fluorescence decrease (Figure 2). The CATR binding sites of the Anc2p variants were titrated by measuring either the CATR-induced fluorescence decrease or the CATR-dependent inhibition of the ATP- (ADP-) induced fluorescence changes (Brandolin et al., 1993a). The amount of saturable CATR binding sites measured for the wild-type Anc2p and the related variants (6−7 nmol of CATR/mg of protein) was in the same range as the amount measured from the N-ATP displacement experiments, which is in agreement with the view that the CATR-induced tryptophan fluorescence changes corresponded to the formation of an Anc2p·CATR complex. Titration of the CATR binding sites of the variants Anc2pW87-W235 and Anc2pW235 could not be carried out because the substrate- or inhibitor-induced fluorescence changes were too small.

Stepwise substitution of tryptophanyl residues of Anc2p resulted in CATR-induced quenching of fluorescence of lower amplitude than that observed for the wild-type carrier (Table 1). By use of singly and doubly substituted variants, it was concluded that Trp87 is less efficient than Trp126 and Trp235 as a reporter of conformational changes resulting from CATR binding. Table 1 shows, for example, that the CATR-induced fluorescence quenching of Anc2pW126-W235, which amounts to 2.5% $\Delta F/F$, is decreased to 2% upon the removal of Trp126 and the simultaneous introduction of Trp87 and to 1.5% upon the removal of Trp235 and the simultaneous introduction of Trp87.

Time Course of the ATP(+BA)-Induced Fluorescence Increase. Addition of BA to wild-type Anc2p in detergent induced no measurable effect on the intrinsic fluorescence. However, when BA was added together with ATP, the fluorescence increased by 10%, thus virtually doubling the ATP-induced fluorescence signal (Table 1). This indicated that neither ATP nor ADP remained bound to the carrier during the purification procedure. Upon addition of increasing amounts of BA, a saturation plateau of fluorescence was attained, which is presumed to correspond to the formation of an Anc2p·BA complex. Lowering the temperature to 10 °C allowed the time course of the ATP(+BA)-induced fluorescence to be resolved in a rapid phase of less than 1 s, followed by a slow one that lasted for 20−30 s. The amplitude of the rapid phase was virtually the same in the presence or absence of BA but increased with ATP concentration, thus corresponding probably to the formation of an Anc2p·ATP complex (Figure 4). The final amplitude of the total fluorescence signal (rapid plus slow phases) increased with the concentration of BA but was not dependent on the concentration of added ATP, even when substoichiometric amounts of ATP were used. This indicated that the Anc2p·BA complex probably does not retain bound ATP but that ATP, even in substoichiometric amounts, is necessary for the formation of the Anc2p·BA complex. Increasing the ATP concentration, but not the BA concentration, resulted in acceleration of the slow phase. For example, the half-time of the slow phase decreased from 129 s with 0.25 μM ATP to 9 s in the presence of 5 μM ATP (Figure 4). In contrast, kinetics of the slow phase were not affected by BA concentration. Contrasting with its ability to reverse the ATP-induced fluorescence enhancement of Anc2p, Mg^{2+} had no effect on the highly fluorescent state of the carrier reached in the presence of ATP and BA (data not shown).

Increasing the BA concentration in the presence of a saturating concentration of ATP resulted in an increased amplitude of the slow phase, the amplitude of the rapid phase remaining constant and amounting to a 5% relative fluorescence change for the wild-type Anc2p. At a saturating concentration of BA, the fluorescence response reached a plateau which corresponded to 10% $\Delta F/F$. From the end point of titration, an amount of 6−7 nmol of BA bound/mg of protein could be calculated (Figure 4C), a value identical to the amount of CATR binding sites.

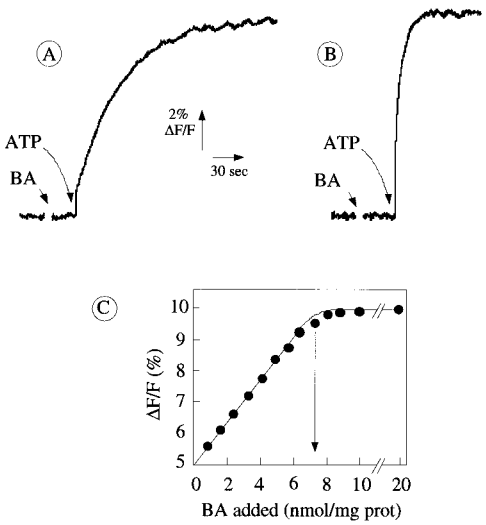

FIGURE 4: Effect of ATP concentration on the time course of BA-induced fluorescence change on isolated wild-type Anc2p. BA was used at the concentration of 5 μM, with (A) 0.25 μM ATP or (B) 5 μM ATP. (C) Titration of high-affinity BA binding sites. The total amplitude of the signals (rapid and slow phases) in the presence of 5 μM ATP was measured as a function of added BA concentration. Conditions were as described in Experimental Procedures.

The magnitude of the fluorescence response of Anc2p variants in the presence of BA(+ATP) depended on the tryptophanyl residues remaining in the protein (Table 1). As a general rule, the final fluorescence level reached in the presence of BA(+ATP) was higher than in the presence of ATP alone. The relative fluorescence change was −0.7% for the variant Anc2pW87-W235 and 10% for the wild-type Anc2p. No significant change in the amount of BA binding sites could be evidenced in the tryptophan variants of Anc2p.

The fluorescence changes elicited by BA itself on the Anc2p variants were determined from the BA(+ATP)-induced fluorescence signals after subtraction of the ATP-induced effect. Results summarized in Table 1 show that, in the case of doubly substituted variants, Trp87 and Trp126 are significantly more responsive to BA. The ability of both Trp87 and Trp126 to report the BA-induced conformation change was confirmed with the variant Anc2pW87-W126. This variant presented a higher fluorescence change than that exhibited by Anc2pW87 and Anc2pW126. For the variants Anc2pW87-W235 and Anc2pW126-W235, which both retain Trp235, the relative fluorescence changes were reduced to 0.3% and 2.3%, respectively. As already shown, Trp235 provided a negative effect on the BA-induced fluorescence change of Trp87, resulting possibly in a negative contribution to the total fluorescence change of Anc2p in the presence of BA(+ATP).

DISCUSSION

This paper reports on the effect of the substrates, ADP and ATP, and the inhibitors, CATR and BA, on the steady-state intrinsic fluorescence of the purified wild-type and tryptophan-substituted Anc2p from *S. cerevisiae*. The Anc2p

isoform of the ADP/ATP carrier is essential for *S. cerevisiae* to grow on nonfermentable carbon sources. It was isolated in detergent from a yeast strain expressing only this isoform (Brandolin et al., 1993a). The Anc2p contains three tryptophanyl residues per carrier unit of 35 kDa (Lawson & Douglas, 1988). In order to assess the contribution of each tryptophanyl residue to the substrate- and inhibitor-induced fluorescence changes, each tryptophanyl residue or pair of them was replaced by tyrosyl residues. In the accompanying paper (Le Saux et al., 1996), it was shown that all mutated forms of Anc2p are efficiently expressed and that they display functional characteristics similar to those found for the wild-type carrier. Control experiments carried out with the Trp-depleted Anc2p, Anc2pΔW, revealed that it was still functional, making it clear that the Trp substitution was not a lethal process. Indeed, this condition had to be fulfilled for subsequent studies dealing with the fluorescence of Trp residues as reporters of conformational changes.

Characterization of Anc2p Preparations. The fluorescence emission spectrum of the isolated Anc2p in detergent, using an excitation wavelength of $\lambda = 297$ nm, was characterized by an emission maximum at $\lambda = 340$ nm which was shifted neither in singly nor in doubly substituted variants of Anc2p, indicating that Trp87, Trp126, and Trp235 of Anc2p had environments of similar hydrophobicity. The three tryptophanyl residues are located in a region of the amino acid sequences highly conserved between the different ADP/ATP carriers, as deduced from the sequence analyses of the *ANC* genes (Brandolin et al., 1993b; Le Saux, 1995). According to the classification proposed by Burstein et al. (1973), the tryptophanyl residues of the Anc2p, with emission maximum at $\lambda = 340$ nm, would belong to the spectral class II. Consequently, they are thought not to be buried in apolar regions but located in a rather hydrophilic environment.

Conformational transitions of the carrier induced by specific ligands, such as ATP, CATR, and BA, were reflected by modifications of the fluorescence intensity but without any detectable shift of the emission maximum. This indicates that upon binding of substrates or inhibitors, only subtle modifications occurred in the local environment of tryptophanyl residues of Anc2p with respect to their relative hydrophobicity.

Conformational States of Anc2p As Characterized by Intrinsic Fluorescence. When isolated in the presence of dodecyl maltoside and Emulphogen, Anc2p adopts the CATR conformation (Brandolin et al., 1993a). This is reflected by a CATR-induced fluorescence decrease that was used to titrate the CATR binding sites. At a saturating concentration of CATR, a low fluorescence level was reached, which corresponded to all carrier molecules trapped under the form of an inhibited Anc2p·CATR complex. Isolated Anc2p could also bind specifically ATP and ADP, and this resulted in tryptophan fluorescence changes. Since these changes were specific for ADP and ATP, they were assumed to reflect conformational changes of Anc2p occurring in the course of ADP/ATP transport. The amplitude and the direction of fluorescence changes depended on the remaining tryptophanyl residues, but tryptophan substitutions did not alter significantly ATP recognition by the carrier. This is consistent with the fact that mutated Anc2p retained full transport activity in mitochondria [accompanying paper by Le Saux et al. (1996)].

The model depicted in Figure 5A is proposed to interpret the transition between the different conformational states of

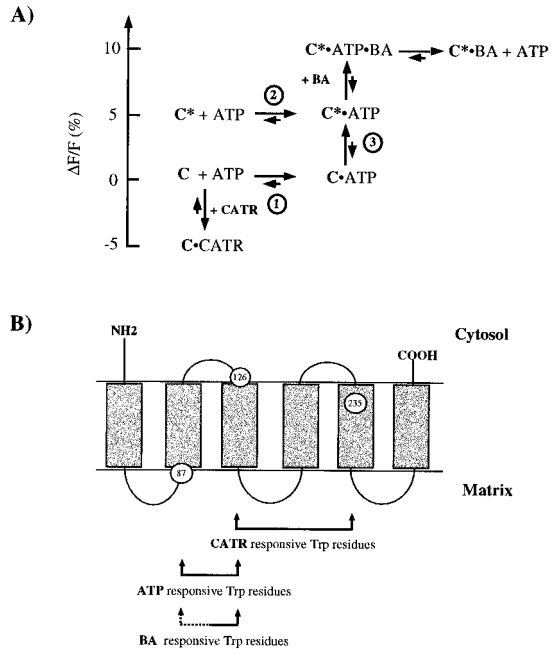

FIGURE 5: (A) Mechanistic model illustrating the transitions between conformational states of isolated Anc2p (indicated as C). (B) Contribution of Trp residues to the probing of substrate- or inhibitor-induced conformational changes of Anc2p. Transmembrane topography of Anc2p is from accompanying paper by Le Saux et al. (1996). Dotted line indicates that Trp87 responds to a lower extent than Trp126 to BA binding.

Anc2p. Similar transitions occur for all Anc2p variants, although resulting in fluorescence changes of different amplitudes. The isolated Anc2p is in the CATR conformation, indicated as C in Figure 5A, and binds added CATR to form the C·CATR complex. Unlike the beef carrier (Brandolin et al., 1985), the yeast Anc2p exhibits distinct fluorescence levels between the basal C conformation and the conformation of the C·CATR complex. The transition of the unliganded carrier in the C conformation to a conformation C*, able to bind BA, cannot occur spontaneously. It is triggered only in the presence of the transportable nucleotides ATP or ADP. Binding of ATP results in the formation of the C·ATP complex, which undergoes a rapid conformational transition toward the more stable C*·ATP complex. In our experimental conditions, the rate of the ATP-induced fluorescence changes was independent of the ATP concentration, even at low temperatures, suggesting that the C* and C*·ATP species are largely predominant. Reversal of the ATP-induced effect elicited by addition of CATR is due to the binding of CATR to high-affinity sites, resulting in displacement of equilibrium (1) or (2) or (3) to form the low-fluorescence Anc2p·CATR complex.

BA-induced fluorescence changes required addition of ATP. However, ATP does not remain bound to the C*·BA complex, as demonstrated by the lack of effect of Mg^{2+} on the high fluorescence level. The fact that the effect of BA is elicited by added ATP, i.e., is favored by the formation of C·ATP and C*·ATP complexes according to the equilibria (1) and (3), implies that BA binds to the C*·ATP complex. Thus, the C*·BA complex is formed from the transient ternary C*·ATP·BA complex.

Contribution of Individual Tryptophanyl Residues to the Fluorescence Change of Anc2p. The 5% relative fluorescence enhancement which accompanies the binding of ATP to the wild-type Anc2p arises mainly from Trp87 and Trp126 which contribute to the fluorescence change to a similar

extent. The ATP- (ADP-) induced fluorescence enhancement of Anc2p may result from either (1) an increased hydrophobicity of the local environment of both Trp87 and Trp126, although their spectral maxima were not shifted [such an explanation was proposed by Ellis et al. (1991) in studies on chloramphenicol acetyltransferase] or (2) the suppression of tryptophanyl residue quenching due to interactions with other side-chain residues or (3) molecular events taking place at the ATP binding site of Anc2p which could also include displacement of solvent molecules upon ATP binding and/or interaction of the adenine ring of ATP with the indole rings of Trp87 and Trp126. This latter hypothesis would be consistent with the very rapid kinetics of ATP-induced fluorescence change.

In contrast to Trp87 and Trp126, Trp235 was shown to be less responsive to ATP binding. Surprisingly, this low fluorescence response to added ATP was still observed when Trp235 was present together with either Trp87 or Trp126 (Table 1), despite the fact that there was no modification of ATP binding (Le Saux et al., 1996). Energy-transfer processes that would occur between either Trp87 or Trp126 and Trp235 can be ruled out since there is no overlap of the fluorescence emission peak, centered at $\lambda = 340$ nm, with the absorption maximum at $\lambda = 297$ nm for these three tryptophanyl residues. The negative contribution of Trp235 to the fluorescence changes elicited by ATP may be due to a restricted flexibility of the polypeptide chain of the carrier brought about by Trp235, impairing the ATP-induced modification of environment in the vicinities of Trp87 and Trp126. This effect might also take place in the wild-type Anc2p, thus limiting the amplitude of the ATP-induced fluorescence signal.

The use of singly and doubly substituted variants of Anc2p showed that each tryptophanyl residue could report CATR binding to Anc2p by a fluorescence decrease, the amplitude of which was the same for Trp235 and Trp126 and significantly smaller for Trp87. Although the tryptophanyl residues are scattered along the polypeptide chain, it cannot be excluded that they all belong to the CATR binding site and that their fluorescence is quenched by direct interaction with CATR. The tryptophan local environment may also be modified following the CATR-induced conformational transition propagated along the Anc2p polypeptide chain. Fluorescence quenching would result from either the contact of tryptophanyl residues with charged residues or hydrophilic regions of the protein or the formation of charge-transfer complexes (Lakowicz, 1983). Differences in the fluorescence responses of Trp87 and Trp126 to the binding of CATR and ATP imply that these two ligands affect the conformation of Anc2p in a different manner, in agreement with the proposal of distinct binding sites for ATP and for CATR (Vignais et al., 1985).

Addition of BA and ATP to the Anc2p variants resulted in a fluorescence increase of the Anc2p·ATP complex (Table 1). The effect of BA itself was in proportion with that of ATP. The fluorescence signal was predominantly due to Trp126 and slightly less to Trp87; Trp235 was much less responsive. Similarly to the enhancing effect of ATP on the fluorescence of Anc2p, the effect of BA might result either from an increased hydrophobicity of the environment of tryptophanyl residues or from decreased interaction of these residues with quenching residues. Such effects would arise from direct contact of the tryptophanyl residues with BA or by modification of their exposure to polar/nonpolar regions during conformational modifications of the polypeptide chain of the carrier. It should be noted that BA elicited a fluorescence enhancement even in the variants Anc2pW87-W235 and Anc2pW235, which responded to ATP by a fluorescence decrease. Thus, the binding of ATP and BA + ATP are sensed in a different manner by Anc2p. This conclusion is in agreement with the finding that Trp235 prevented the effect elicited by BA + ATP in the variant Anc2pW87-W235 but not in the variant Anc2pW126-W235.

In summary, the present study shows that the three tryptophanyl residues of the yeast Anc2p are suitable intrinsic reporters of structural changes occurring in the carrier. Each tryptophanyl residue is able to sense specifically the conformational transitions elicited by ATP, CATR, and BA (Figure 5B). These results provide a basis for further studies using the time-resolved fluorescence and anisotropy decays.

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