Peisach, J. (1975) *Ann. N.Y. Acad. Sci. 244*, 187–203.
Rubinow, S. C., & Kassner, R. J. (1984) *Biochemistry 23*, 2590–2595.
Taniguchi, S., & Kamen, M. D. (1963) *Biochim. Biophys. Acta 74*, 438–455.
Valentine, J. S., Sheridan, R. P., Allen, L. C., & Kahn, P. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1009–1013.
Weber, P. C. (1982) *Biochemistry 21*, 5116–5119.
Weber, P. C., Howard, A., Huu Xuong, N., & Salemme, F. R. (1981) *J. Mol. Biol. 153*, 399–424.
Wilhelm, E., Battino, R., & Wilcock, R. J. (1977) *Chem. Rev. 77*, 219–262.
Wyman, J. (1948) *Adv. Protein Chem. 4*, 407–531.
Wyman, J. (1965) *J. Mol. Biol. 11*, 631.

Substrate-Induced Modifications of the Intrinsic Fluorescence of the Isolated Adenine Nucleotide Carrier Protein: Demonstration of Distinct Conformational States

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ABSTRACT: The effects of ATP or ADP and the specific inhibitors carboxyatractyloside (CATR) and bongkrekic acid (BA) on the conformation of the isolated adenine nucleotide (AdN) carrier protein were studied by fluorescence spectroscopy. The addition of ATP to the AdN carrier resulted in a rapid fluorescence increase of the tryptophanyl residue(s) at 355 nm, which leveled up in less than 1 s at 22 °C. Among the natural nucleotides, only ATP and ADP were effective. At 10 °C or below, the kinetics of the fluorescence increase induced by ATP were biphasic, consisting of a rapid phase of less than 1 s, followed by a slower phase that lasted for a few seconds and had virtually the same amplitude as the rapid one. Both phases were abolished when CATR was added prior to ATP or fully reversed when CATR was added after the fluorescence response to ATP had been elicited. The number of CATR binding sites present on the carrier protein was determined by CATR specific inhibition of the ATP-induced increase in intrinsic fluorescence. The calculated number of CATR sites was equal to that found by another method based on the use of the same preparation of AdN carrier loaded with fluorescent nucleotide naphthoyl-ATP and on the CATR-induced release of the bound naphthoyl-ATP, demonstrating the reliability of the intrinsic fluorescence assay. Addition of BA prior to or together with ATP nearly doubled the amplitude of the ATP-induced fluorescence signal. At 10 °C or below, the fluorescence response to ATP in the presence of BA could also be decomposed into rapid and slow phases. The amplitude of the rapid phase was not modified in the presence of BA, but in contrast to the fluorescence signal induced by ATP alone, the amplitude of the slow phase was about 3 times higher than that of the rapid phase. The same results were obtained when ATP was replaced by ADP. In the absence of ATP, CATR was found to induce per se a modification of the intrinsic fluorescence that differed from that induced by ATP by its excitation and emission spectra. These results are discussed on the basis of a minimal model where the AdN carrier is supposed to exist in two native conformations, the CATR and BA conformations that are trapped and stabilized by CATR and BA, respectively; interconversion between the two conformations is triggered by ATP or ADP. However, on the basis of several observations that point to a tetrameric organization of the AdN carrier protein, it is equally possible that the transition between the CATR and BA conformations is multiphasic and may proceed with sequential modification of each subunit of the tetramer.

The mitochondrial AdN carrier isolated in detergent and purified by hydroxylapatite chromatography is able to respond specifically to the addition of ADP or ATP by modification of its intrinsic fluorescence (Brandolin et al., 1981). The nucleotide-induced fluorescence signal was most likely due to changes in the environment of the tryptophanyl residue(s) caused by conformational changes resulting from the binding of nucleotides to the carrier protein. Carboxyatractyloside (CATR),[1] an inhibitor of AdN transport that reacts with the carrier on the outer face of the inner mitochondrial membrane, prevented the fluorescence increase when added prior to ADP or ATP and decreased it when added after the fluorescence rise had been initiated. On the other hand, bongkrekic acid (BA), another inhibitor that attacks the carrier from the inside

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[1] Abbreviations: AdN carrier, adenine nucleotide carrier; CATR, carboxyatractyloside; BA, bongkrekic acid; LAPAO, 3-lauramido-*N*,*N*-dimethylpropylamine oxide; N-ATP, naphthoyladenosine 5′-triphosphate; FTP, formycin triphosphate; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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of the inner mitochondrial membrane, had just the opposite effect. These observations were interesting enough to warrant further examination of the intrinsic fluorescence properties of the purified AdN carrier protein. Actually, the AdN carrier obtained by hydroxylapatite chromatography and used in earlier experiments (Brandolin et al., 1981) contained contaminants, such as calcium, phosphate ions, and micromolar amounts of nucleotides, that prevented an accurate determination of the kinetics of the changes in intrinsic fluorescence. We have further purified the AdN carrier protein to eliminate these contaminants. The new preparation of AdN carrier allowed faster responses of the intrinsic fluorescence changes upon addition of specific ligands. In the work reported here, the kinetics of the ADP- or ATP-induced fluorescent signal are shown to be biphasic. The effects of the concentrations of substrate and inhibitory ligands on this biphasic signal are reported and discussed in terms of two conformations of the carrier, a conformation trapped by CATR and the other by BA, the transition between the two conformations being triggered by trace amounts of ATP or ADP.

Experimental Procedures

Nucleotides and CATR were purchased from Boehringer, hydroxylapatite was from Bio-Rad, and AcA 202 gel was from LKB. Bongkrekic acid was prepared as previously described (Lauquin & Vignais, 1976). The AdN carrier protein was extracted from beef heart mitochondria by purified LAPAO (Brandolin et al., 1980); it was isolated from the mitochondrial lysate by chromatography on hydroxylapatite (Krämer & Klingenberg, 1977) and further purified by chromatography on AcA 202 gel equilibrated with 50 mM Mops, 0.1 mM EDTA, and 0.5% (w/v) LAPAO at a final pH of 7.0. The carrier protein fraction was eluted just after the void volume. This step resulted in nearly complete removal of small molecular weight contaminants present in the hydroxylapatite preparation, namely, 2–4 mM P_i, 0.5–1 mM Ca^{2+}, 5 μM AMP, and less than 1 μM ADP and ATP.

Fluorescence was measured with a high-sensitivity fluorometer (Bio-Logic, Grenoble) equipped with a 150-W xenon lamp. The 1 × 1 cm fluorescence quartz cuvette was inserted in a temperature-controlled cell holder, thermostated at the required temperature with a circulating bath. The medium (final volume 2 mL) consisted of 80 mM glycerol, 20 mM Mops, 0.4 mM EDTA, and 0.5% LAPAO (w/v), pH 7.0. The purified carrier preparation was diluted with 136 mM glycerol and introduced in the cuvette to give a final protein concentration ranging between 0.04 and 0.20 mg/mL and a final glycerol concentration of 80 mM. The content of the cuvette was continuously stirred. Reagents were introduced by means of Hamilton automatic syringes in 2–10-μL aliquots. The fluorescence intensity was corrected for dilution and absorbance effects. The intrinsic fluorescence of the carrier protein was excited at 296 nm except when otherwise indicated. The emitted light was measured at right angle through a 0-54 Corning filter coupled to an ultraviolet light filter (Corning 7-54). The center of the resulting band-pass was at 355 nm with a peak transmission of 75% and a width of 60 nm at half-maximum of the peak. When the extrinsic fluorophore 3′-*O*-naphthoyl-ATP (N-ATP) was used, its fluorescence was excited at 312 nm, and the emitted light was measured through a K1 Balzers filter; the fluorescence peak was centered at 410 nm with 70% transmission at half-maximum of the peak, the band width being 50 nm. Other emission wavelengths were selected by means of interferential Baltzers filters B315, B320, B329, B336, and B383 coupled to a 305 UV light filter (Schott) (resulting band-pass centered at 315, 320, 329, 336, and 383 nm, respectively) or of a 0-52 Corning filter coupled to a 7-54 Corning filter to give a band-pass centered at 370 nm.

Results

Methodological Aspects Related to Fluorescence Properties of Isolated AdN Carrier Protein in Detergent. In a previous study, the AdN carrier protein in the detergent LAPAO was directly recovered from hydroxylapatite chromatography with a degree of purity of 70%–75% on the basis of the relative protein content (Brandolin et al., 1981). In the present study, the pass-through fraction recovered by passage of a LAPAO extract of beef heart mitochondria on hydroxylapatite was further purified by chromatography on an AcA 202 gel. The AcA 202 purified fraction was freed to P_i and Ca ions initially present at millimolar concentrations and of micromolar amounts of adenine nucleotides; it responded to ADP or ATP faster than the hydroxylapatite preparation. In particular, the plateau of the signal resulting from addition of ADP or ATP at 20 °C was attained in less than 1 s instead of 4–5 s. Furthermore, while in the hydroxylapatite preparation the fluorescence signal induced by ADP or ATP could not be reversed by CATR if added more than a few seconds later than ADP (or ATP), with the AcA 202 purified fraction it was possible to reverse the fluorescence signal by adding CATR even 5 min later than ADP (or ATP). Besides, the sensitivity to low concentrations of ADP or ATP was increased; for example, addition of 0.1 μM ADP or ATP to the AcA 202 preparation elicitated a full amplitude signal whereas the half-maximum response of the carrier protein obtained from hydroxylapatite required 5 μM ADP or ATP. It was previously shown that Mg ions by complexing to ADP or ATP decreased the rate of the fluorescence signal induced by addition of ADP or ATP to the AdN carrier (Brandolin et al., 1981); this led us to conclude that free ADP and free ATP are the true substrates for the AdN carrier. Likewise, the presence of Ca ions in hydroxylapatite preparation of the AdN carrier may explain the apparent lower efficiency of ADP or ATP for promoting changes in the intrinsic fluorescence of the AdN carrier. The screening of a large number of natural nucleotides with the AcA 202 preparation confirmed the specificity of the fluorescence response to ATP or ADP (Brandolin et al., 1981).

The emission spectrum of the carrier protein, when excited at 296 nm, had its λ_{max} at 330 nm. Upon addition of ATP, the emission spectrum of the carrier protein was shifted to the red (peak at 340 nm); it intersected the control emission spectrum at 345 nm. The fluorescent signal centered at 355 nm was therefore increased (inset of Figure 1). On the other hand, no effect of ATP on the excitation spectrum after corrections was observed. In the experiments described hereafter and performed with the purified carrier in LAPAO, the emitted light was selected by using a band-pass centered at 355 nm. The emission at 355 nm was chosen preferentially to that at 330 nm or at lower wavelengths to avoid a too large background due to light scattering. The same responses were produced by ADP or ATP; only the effects elicitated by ATP are reported here.

Temperature Effects on Rise of Fluorescent Signal Induced by ATP and Its Reversal by CATR. At 22 °C, addition of a saturating concentration of ATP resulted in an immediate rise of the fluorescence of the carrier protein; the plateau was attained in less than 1 s (Figure 1). Decreasing the temperature from 22 to 10 °C allowed resolution of the fluorescence increase in two phases of similar amplitude, a rapid one that lasted for less than 1 s and a slow one that lasted for 20 s. At

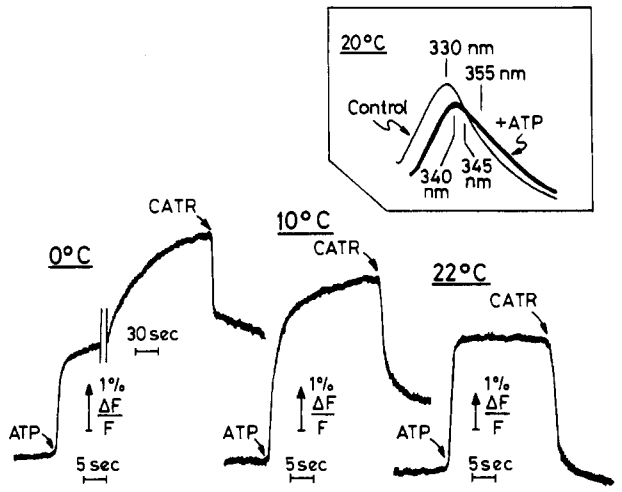

FIGURE 1: Temperature dependence of the kinetics of the ATP-induced fluorescence change of the AdN carrier in LAPAO solution. Conditions and medium are given under Experimental Procedures. The protein concentration was 0.2 mg/mL. ATP and CATR were used at the final concentrations of 10 and 5 μM, respectively. The different plateaus of fluorescence at 0, 10, and 22 °C reflect the dependence of the yield of fluorescence on the temperature. Excitation was at 296 nm. The emitted light wavelength was centered at 355 nm. (Inset) Fluorescence emission spectra of the AdN carrier protein in the absence or presence of 10 μM ATP (excitation at 296 nm). Note that at 355 nm, which corresponds to the center of the band-pass of the emitted light used for kinetic studies, addition of ATP results in an increase of the emitted fluorescence.

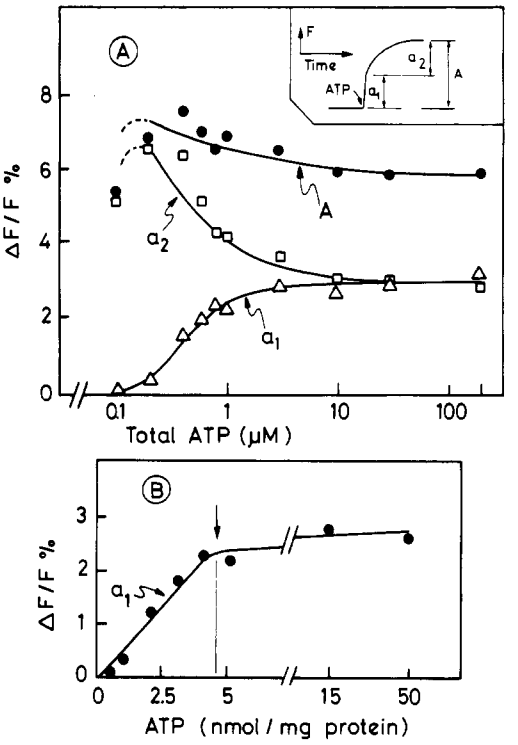

FIGURE 2: Effect of ATP concentration on the fast and slow phase of the ATP-induced fluorescence enhancement of the AdN carrier. Conditions are given under Experimental Procedures. (A) The protein concentration was 0.2 mg/mL. Measurements were performed at 10 °C, with an emitted light centered at 355 nm. The log of the ATP concentration is plotted on the abscissa. a_1 and a_2 correspond to the amplitudes of the rapid and slow steps of fluorescence increase, respectively. (B) Same fluorescence data as in (A) for the rapid phase of fluorescence increase, except that ATP concentration is plotted in normal scale. The end point of the titration was given either as concentration of ATP, $\simeq$1 μM (A), or number of added nanomoles of ATP per milligram of protein, $\simeq$5.

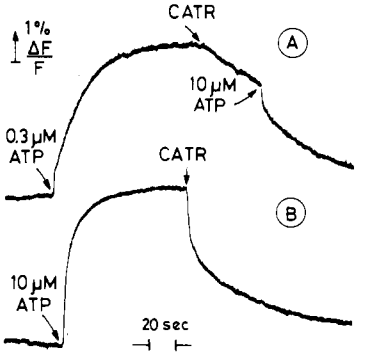

FIGURE 3: Comparative responses of the AdN carrier fluorescence to a substoichiometric concentration of ATP and an excess concentration of ATP with respect to the high-affinity nucleotide binding sites of the carrier protein. The preparation used and the conditions of incubation were the same as in Figure 2. (A) ATP was used at the final concentration of 0.3 μM, i.e., a concentration 3 times lower than that required to saturate the high-affinity nucleotide binding sites (cf. Figure 2). (B) ATP was used at the saturating concentration of 10 μM (cf. Figure 2).

0 °C, it took 2 s for the rapid phase to reach completion and several minutes for the final plateau to be attained.

At 22 °C, CATR reversed quickly (less than 1 s) and completely the fluorescence signal induced by ATP. At 10 °C, reversal by CATR occurred in two phases of equal amplitude, a rapid one and a slow one that lasted for less than 1 s and several seconds, respectively. At 0 °C, only the rapid phase was quickly reversed, full reversal of the slow phase requiring more than 5 min.

Effects of ATP Concentration on Rapid and Slow Phases of Fluorescence Increase. The experiments described hereafter were carried out at 10 °C. The amplitude of the overall fluorescence signal, consisting of the sum of the rapid and slow phases, did not markedly vary when the ATP concentration was increased from 0.1 to 200 μM. However, at [ATP] <1 μM, the amplitude of the rapid phase was less than that of the slow phase; at [ATP] >1 μM, the amplitudes of the rapid and slow phases were virtually equal.

The fluorescence level of the rapid phase increased linearly with the ATP concentration up to 1 μM ATP (trace a_1, Figure 2A) whereas that of the slow phase decreased (trace a_2, Figure 2A). This points to a titrating effect of ATP (Figure 2B) and to the presence of high-affinity ATP binding sites on the carrier protein. In the experiment of Figure 2, the end point of the titration corresponded to about 5 nmol/mg of protein. This value is probably underestimated due to the probable denaturation of a fraction of the carrier protein preparation during the extraction and purification steps and the subsequent loss of the binding capacity of the carrier.

When the amount of added ATP was less than that of the ATP binding sites in the carrier preparation, full fluorescent enhancement was still observed. This is typically shown in Figure 3A where ATP was used at the final concentration of 0.3 μM, i.e., a concentration 3–4 times less than that required to saturate the high-affinity ATP binding sites of the carrier; the fluorescence rise although much slower (Figure 3A) than that observed with 10 μM ATP (Figure 3B) increased regularly to reach a plateau whose amplitude was virtually equal to that attained with 10 μM ATP. Thus, a substoichiometric concentration of ATP with respect to that of the ATP binding sites in the carrier induced a full-amplitude fluorescence signal. Addition of CATR at a saturating concentration (5 μM) after the fluorescence plateau has been attained resulted in a very slow fluorescence decrease that was markedly accelerated by addition of 10 μM ATP (Figure 3A).

Comparative Titrations by CATR of Changes in Intrinsic

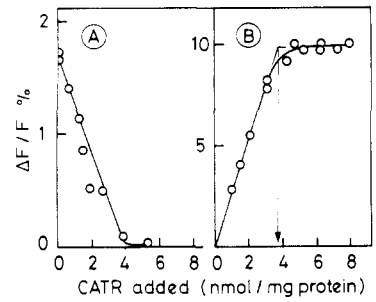

FIGURE 4: Comparison of two types of CATR titrations of the AdN carrier based on CATR inhibition of the ATP-induced increase in intrinsic fluorescence and on the CATR-induced release of bound N-ATP assessed by increase in intrinsic fluorescence. Same conditions as in Figure 2. The same carrier preparation at a concentration of 0.04 mg of protein/mL was used for the two titrations. The temperature was 0 °C. (A) Intrinsic fluorescence. ATP was used at the final concentration of 10 μM. CATR was added 20 s prior to addition of ATP. The amplitudes of the fluorescence responses at different concentrations of CATR were recorded and plotted against the CATR concentrations. (B) Extrinsic fluorescence. Conditions are given under Experimental Procedures. At difference with (A), CATR was added after N-ATP had bound to the carrier protein. CATR binding resulted in release of bound N-ATP; this was reflected by a rise in fluorescence that was proportional to the CATR concentration.

Fluorescence Induced by ATP and of the Extrinsic Fluorescence of Naphthoyl-ATP Bound to the AdN Carrier Protein. CATR, when added to the carrier protein prior to ATP, inhibited the ATP-induced modification of the fluorescence emission spectrum. A kinetic analysis showed that the rapid and slow fluorescence responses to the added ATP were inhibited by CATR to the same extent (not shown). As already mentioned, only the rapid response is exhibited at 0 °C.

Naphthoyl-ATP (N-ATP), a fluorescent ATP analogue that binds to, but is not transported by, the membrane-bound AdN carrier (Block et al., 1982), was previously used to titrate the CATR binding sites of the isolated carrier protein (Dupont et al., 1982). The CATR-induced release of bound N-ATP was accompanied by an increased fluorescence of the probe. The effects of increasing concentrations of CATR on the rapid ATP-induced changes of intrinsic fluorescence assayed at 0 °C and on the extrinsic fluorescence of bound N-ATP were compared. The end points of the two titrations were the same, corresponding to an amount of CATR of 4 nmol/mg of protein (Figure 4). This reinforces the idea of specific CATR binding sites on the isolated carrier protein; it demonstrates the reliability of the intrinsic fluorescence signals.

CATR-Induced Fluorescence Changes in Carrier Protein in the Absence of Nucleotide. CATR is able to reverse the ATP-induced increase in intrinsic fluorescence measured at 355 nm (Figure 1). Control assays showed that a small but reproducible increase in intrinsic fluorescence could be detected upon addition of CATR alone. It was therefore decided to explore in more detail the optimal conditions of elicitation of the CATR fluorescent signal. The assays were carried out at 0 °C to compare the CATR-induced fluorescence change with the rapid phase of the ATP-induced fluorescence change; it must be recalled that the rapid phase only is detectable at 0 °C. The excitation wavelength that allowed optimal detection of the CATR-specific signal was at 275–281 nm; for an optimal ATP-induced signal, a quite different excitation wavelength at 296–305 nm had to be used (Figure 5). With an excitation wavelength centered at 281 nm, addition of CATR resulted in a maximal fluorescence increase in the region of 310–330 nm, whereas addition of ATP had no significant effect (Figure 6A). On the other hand, with an excitation wavelength at 296 nm, the fluorescence increases

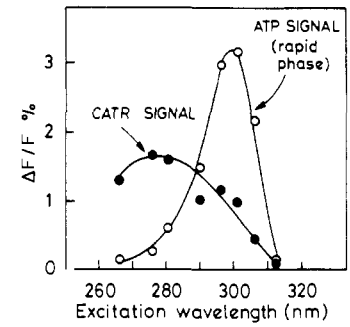

FIGURE 5: Effect of excitation wavelength on relative increase of emitted light by the AdN carrier at 355 nm. The protein concentration was 0.2 mg/mL and the temperature 0 °C. CATR and ATP were used at the final concentration of 2.5 μM. In the case of the CATR-induced fluorescence, the total extent of the fluorescence signal at 355 nm was taken into account. In the case of the ATP-induced fluorescence, only the signal at 355 nm corresponding to the rapid phase was taken into account.

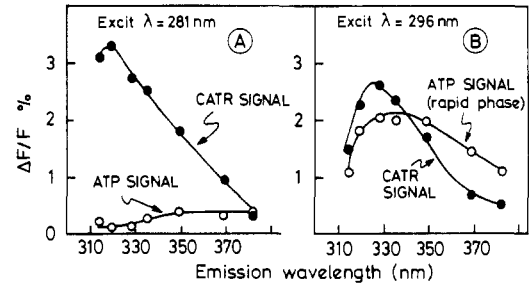

FIGURE 6: ATP- or CATR-induced fluorescence changes of the AdN carrier measured at different wavelengths for excitation light wavelengths of 281 (A) and 296 nm (B). The protein concentration was 0.2 mg/mL and the temperature was 0 °C. CATR and ATP were used at the final concentrations of 2.5 μM. The emission wavelengths were selected with appropriate filters (see Experimental Procedures).

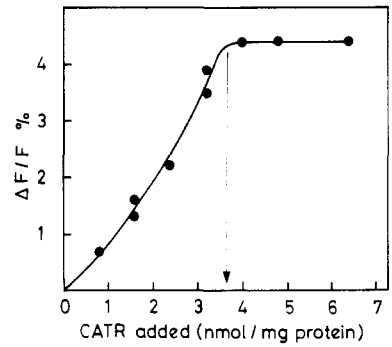

FIGURE 7: Titration of CATR binding sites of AdN carrier in the absence of added nucleotides. Same conditions as in Figure 2. The protein concentration was 0.05 mg/mL and the temperature 0 °C. The excitation wavelength was at 280 nm, and the emitted light was recorded at 329 nm. Increasing concentrations of CATR resulted in an increase of fluorescence at 329 nm.

caused by CATR and ATP were maximal at 325 and 330–350 nm, respectively (Figure 6B).

With the band-pass at 355 nm used in routine experiments to assay the ATP-induced increase in fluorescence, the amplitude of the CATR-induced signal at 0 °C was more than half that of the rapid signal induced by ATP. However, at 25 °C the relative contribution of the CATR signal was much less (only 5% of the signal caused by ATP). Because of its predominance over other signals at 0 °C, the CATR-induced signal was measured at 0 °C with excitation and emission lights centered at 281 and 329 nm, respectively, i.e., under conditions where the ATP-induced signal is negligible. As illustrated in Figure 7, the increase of intrinsic fluorescence

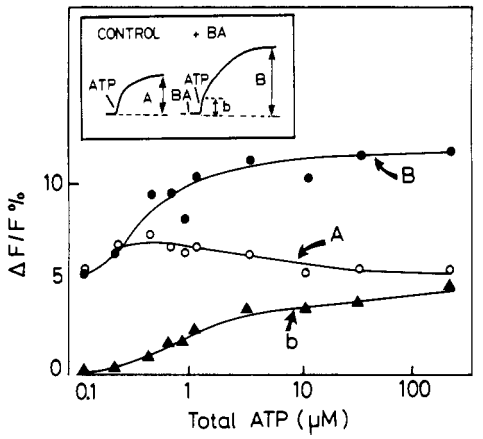

FIGURE 8: Synergistic effect of BA and ATP on the intrinsic fluorescence of the AdN carrier; effect of ATP concentration on the fluorescence increase at 355 nm at a fixed concentration of BA. Same conditions as in Figure 2 (high-affinity ATP binding sites: 5 nmol/mg of protein). The temperature was 22 °C. BA was used at the final concentration of 8.5 μM and added 30 s prior to ATP.

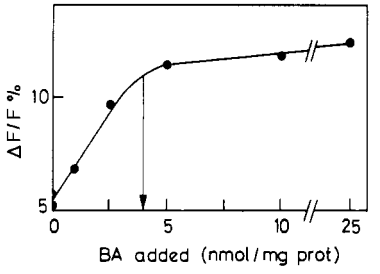

FIGURE 9: Titration of high-affinity BA binding sites in the AdN carrier. Same conditions as in Figure 8. The total amplitude of the signals (rapid and slow phases) at 2.5 μM ATP was measured as a function of the concentration of added BA.

depending on CATR alone was typically saturable. The CATR titration curve showed an end point at 4 nmol of CATR/mg of protein, a value similar to that found by the other two approaches mentioned above (Figure 4), namely, the CATR inhibition of the intrinsic fluorescence enhancement induced by ATP and the chase of bound N-ATP caused by CATR. However, instead of the rectilinear CATR titrations illustrated on Figure 4, the CATR titration of Figure 7 exhibited a curvilinear shape suggesting multiple CATR binding sites on the carrier protein.

BA-Dependent Increase of Fluorescence Signal Induced by ATP and Its Reversal by CATR. The amplitude of the total fluorescence response to ATP (including the rapid and slow phases) was approximately doubled when BA was added prior to or together with ATP. BA alone induced no measurable fluorescence perturbation. As with ATP, the response to ATP plus BA was composed of two steps, a rapid one terminating in less than 1 s at 20 °C and a slow one that reached a plateau after a few seconds (Figure 8, inset). The amplitude of the fluorescence signal corresponding to the rapid step was the same in the absence and presence of BA. It was the fluorescence signal related to the slow phase that was increased upon addition of BA; it was virtually tripled (Figure 8, inset). The saturation plateau for both the rapid and the slow phases was attained for an amount of ATP of 4 nmol/mg of protein.

The total fluorescence response (rapid plus slow phases) upon addition of increasing amounts of BA at fixed ATP concentration (2.5 μM) is illustrated in Figure 9. The maximal fluorescence response was obtained at 5 nmol of BA/mg of protein, a value similar to the amount of CATR required to titrate the CATR binding sites (Figure 4). The

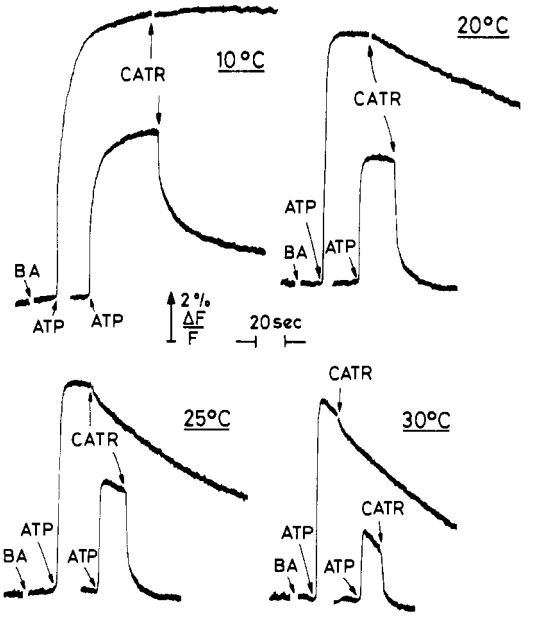

FIGURE 10: Reversal by CATR of the increase in intrinsic fluorescence brought about by addition of ATP or ATP plus BA. Same conditions as in Figure 8. ATP was added at the final concentration of 10 μM, BA at 8.5 μM, and CATR at 2.5 μM. The temperatures used were 10, 20, 25, and 30 °C. For comparison, control assays were performed in the absence of BA.

fluorescence signal that was induced by ATP plus BA could be readily reversed by CATR provided the temperature was higher than 10 °C (Figure 10). The temperature affected differently the reversal of the fluorescence signal induced by ATP alone or by ATP plus BA; at 20 °C the ATP-induced signal was readily reversed by CATR, whereas the reversal of the signal induced by ATP plus BA required a temperature higher than 20 °C.

DISCUSSION

Reliability of Titration by CATR or BA of the ATP- (or ADP-) Induced Fluorescence Changes in Isolated AdN Carrier Protein. This paper reports on the modification of the intrinsic fluorescence of the purified AdN carrier protein brought about by ATP. It is important to state again that ADP has the same effect as ATP. The emission fluorescence spectrum of the isolated AdN carrier, obtained with an excitation wavelength of 296 nm, was characterized by a peak centered at 330 nm, which is typical of the tryptophan fluorescence. There are five tryptophanyl residues per carrier unit of M_r 32 000 (Aquila et al., 1982). The red shift of the emission spectrum upon addition of ATP (or ADP) indicates that binding of ATP (or ADP) to the AdN carrier protein intiates conformational changes in the protein that modify the environment of the tryptophanyl residue(s). It is not known, however, which tryptophanyl residue(s) is (are) involved in the fluorescence changes.

The CATR titration data in Figure 4 based on CATR inhibition of the ATP- (or ADP-) induced fluorescence changes indicated 5 nmol of CATR binding sites/mg of protein. With the same carrier protein preparation loaded with N-ATP, titration of the release of bound N-ATP by CATR gave the same number of CATR binding sites. Moreover, on the basis of the finding that CATR added to the isolated carrier protein is capable of modifying the fluorescence emission at 329 nm with an excitation wavelength at 280 nm (Figure 7), a third type of titration of the CATR binding sites was performed; the number of CATR binding sites determined by this third method was exactly the same as that found by the two pre-

ceding ones. The similar results obtained by three different methods of titration justify the confidence that one may have in the significance of the fluorescence changes brought about by addition of CATR to the carrier protein.

On the other hand, the number of high-affinity BA binding sites found in the presence of ATP (or ADP), namely, 5 nmol of sites/mg of protein, was identical with the number of CATR binding sites. Furthermore, the fluorescence changes induced by BA in the presence of ATP or ADP were reversed by CATR. These data taken together point to the reliability of the titration assay for specific BA binding sites.

Molecular Significance of ATP- or ADP-Induced Conformational Changes Monitored by Fluorescence Spectroscopy in Isolated AdN Carrier Protein. There are several lines of evidence indicating that the nucleotide-induced conformational changes of the isolated AdN carrier, as reflected by fluorescence changes, are similar to those occurring in the membrane-bound carrier during AdN transport: (1) Among natural nucleotides, only ADP and ATP are transported by mitochondria (Duée & Vignais, 1969) and are able to induce fluorescence changes in the isolated AdN carrier. (2) ADP/ATP transport in mitochondria is a two-step process; the first step corresponds to the binding of ADP or ATP to the membrane-bound carrier, the second step being the translocation of ADP or ATP across the membrane (Schlimme, 1980). Of the two steps, the second one that involves conformational changes of the carrier protein is probably the temperature-sensitive component of the transport process. Likewise, it is possible to distinguish two phases in the ATP-(ADP-) induced fluorescence changes in the isolated carrier by varying the temperature: an initial rapid phase independent of temperature, at least to the limit of the kinetic resolution of our assays, and a slower phase that becomes faster as temperature is increased. The first phase reflects ATP (or ADP) binding whereas the second one is probably related to slow conformational changes of the ATP (ADP)–carrier complex that mimic the vectorial events in the membrane-bound carrier. It is suggested that in the course of ADP or ATP binding discrete conformational changes are initiated at the binding site or at close proximity that act obligatorily as a triggering signal to allow the full development of the subsequent change of conformation characteristic of the slow phase of fluorescence increase. (3) ADP/ATP transport in mitochondria proceeds by exchange diffusion; i.e., AdN are exchanged across the inner mitochondrial membrane with an obligatory 1:1 stoichiometry. The mechanistic implication of this process is that the nucleotide-loaded carrier should be the only form that is able to undergo the conformational changes responsible for AdN transport; should conformational changes occur in a free unoccupied carrier, this would result in uncoupling of the AdN exchange. The peculiar dependence of the fluorescence change of the isolated carrier on ATP concentration illustrated in Figure 3 confirms clearly this view. To explain that the same plateau of fluorescence is attained with a substoichiometric concentration of ATP or with an excess of ATP, it must be assumed that the stable conformation of the AdN carrier with low fluorescence is switched, upon binding of ATP, to another conformation with high fluorescence that is stable even after departure of ATP. The return to the low fluorescence conformation is possible provided that this conformation is trapped by CATR; the return is accelerated by high concentrations of ATP. In other words, the low and high fluorescence conformations are stable, and the transition from one conformation to the other one requires ATP (or ADP).

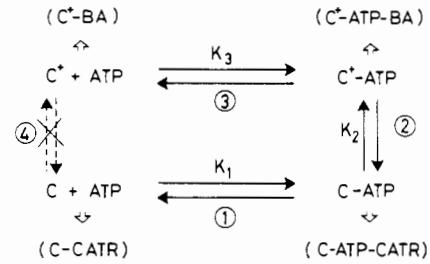

FIGURE 11: Scheme illustrating the obligatory binding of ATP to the carrier for the transition from the basal state C to the activated state C^+. BA is supposed to react with the C^+ state and CATR with the C state. Although the formation of the ternary complexes C^+–BA–ATP and C–CATR–ATP is plausible, it is not supported by direct evidence. The direct transition from C to C^+ is forbidden. For details, see text.

A Mechanistic Model To Interpret the Triggering Effect of ATP or ADP on the Transition between the CATR and BA Conformations. That the AdN carrier in mitochondria can assume two distinct conformations specifically recognized by CATR and BA is supported by experimental data based on immunological and chemical approaches (Buchanan et al., 1976; Block et al., 1981, 1983). Experiments relying on the reversible binding of N-ADP, a fluorescent and nontransportable analogue of ADP, to the membrane-bound AdN carrier have shown that the CATR and BA conformations are native conformations that exist prior to the addition of CATR and BA and that the transition between the two conformations is triggered by trace amounts of ATP or ADP (Block et al., 1983). These data, and more particularly the triggering effect of ADP or ATP, are corroborated and extended by the results of the present investigation. Before discussing these results in terms of a mechanistic model, it is worth recalling a few salient features. (1) Two distinct levels of intrinsic fluorescence are observed depending on the supplementation of the AdN carrier with either CATR or BA plus ATP (or ADP). (2) The fluorescence level attained in the presence of BA and ATP (or ADP) can be reversed by CATR. (3) At temperatures of 0 or 10 °C, the time course of the fluorescence increase and that of the reversal by CATR are biphasic and depend on ATP concentration, which suggest that the above increase and quenching of fluorescence reflect similar pathways of reactions. It is likely, as discussed above, that the rapid rise in fluorescence corresponds to the binding of ATP to the carrier, and the reverse process to the release of ATP, whereas the slow phase reflects conformational changes.

These data taken together and other results reported in this paper can be most simply rationalized by the scheme illustrated in Figure 11. This scheme is based essentially on the assumption that the carrier exists in two conformational states only, C and C^+ corresponding to a low and high fluorescence level, respectively. The transition from the C state to the C^+ state cannot occur spontaneously (forbidden step 4 in Figure 11). For this transition to occur, the carrier must bind ATP (step 1). The formation of the C–ATP complex is reflected by the rapid fluorescence increase at 355 nm observed under our experimental conditions. The C–ATP complex undergoes a conformational transition to give the C^+–ATP complex (step 2); this corresponds to a slower increase in fluorescence at 355 nm clearly revealed at or below 10 °C. The C^+–ATP complex can dissociate reversibly to give C^+ and ATP (step 3). Under steady-state conditions in the presence of ATP (or ADP), the respective concentrations of C, C–ATP, C^+–ATP, and C^+ obviously depend on the values of the equilibrium constants for the different reactions, i.e., K_1, K_2, and K_3. To explain the further fluorescence increase in the presence of BA, it suffices

to postulate that BA binds either to C^+ or to the C^+–ATP complex, to give the C^+–BA or C^+–ATP–BA complexes, respectively. The function of BA is therefore to displace the equilibrium, which results in decrease of free C and accumulation of the different forms of C^+. It is important to recall that ATP or ADP, even in trace amounts, is required for the effect of BA to occur, which reinforces the idea of the obligatory transition C–ATP $\rightleftharpoons$ C^+–ATP for the binding of BA. Conversely, CATR is postulated to react with either C or C–ATP. When added to the high-fluorescence state of the carrier (C^+) in the presence of ATP (or ADP), CATR displaces the equilibrium toward the low-fluorescence state, C or C–CATR. An alternative but less likely explanation is that CATR can react with C^+–ATP and substitutes to ATP in C^+–ATP to give C^+–CATR, resulting in an abrupt decrease in fluorescence; the transition from the C^+–CATR complex to the C–CATR complex would account for the slow fluorescence decrease.

The major feature of the above scheme is that the transition between the two conformations is triggered by trace amounts of ATP or ADP, as it occurs in the membrane-bound AdN carrier (Block et al., 1983). It is probable that the effect of ATP and ADP on the intrinsic fluorescence of the carrier is shared by other transportable nucleotides. In fact, it was previously reported that formycin triphosphate, a transportable ATP analogue that binds to the isolated carrier is displaced by BA (Brandolin et al., 1982) in contrast to N-ATP, an analogue that binds to the carrier but is not transported (Dupont et al., 1982).

On the basis of modifications of its fluorescence properties upon addition of CATR in the absence of nucleotides, the isolated carrier was found to bind CATR. This result affords direct evidence for a native conformation of the isolated carrier able to recognize and to bind CATR. Block et al. (1983), using a different approach, reached a similar conclusion with the membrane-bound carrier of heart mitochondria.

An examination of the increments of fluorescence levels upon addition of ATP (ADP) shows that the rapid and slow phases that characterize the ATP-induced transition have an equal amplitude. On the other hand, in the presence of BA plus ATP (or ADP), the slow phase is virtually tripled whereas the rapid one remains constant. It is conceivable that these different fluorescence amplitudes may depend on the values of K_1, K_2, and K_3 and on the trapping of C^+ by BA as discussed above. However, an alternative explanation is that discrete molecular events occur in the course of the transition from the CATR conformation to the BA conformation. In this context, it may be recalled that by means of titration with fluorescent nucleotides, four nucleotide binding sites have been demonstrated in the isolated carrier (Dupont et al., 1982; Brandolin et al., 1982). It is plausible that the four nucleotide sites are distributed in distinct subunits of a tetramer. The tetramer would exist in two conformations, α_4 and β_4, the transition between α_4 and β_4 occurring in a sequential manner at the level of each subunit; each conformational change would correspond to one-fourth of the total fluorescence signal. Although appealing, the tetrameric hypothesis is apparently in conflict with structural studies showing that the CATR– and BA–carrier complexes behave as dimers (Krämer & Klingenberg, 1977; Block et al., 1982a). However, recent binding studies on the isolated AdN carrier (Dupont et al., 1982; Brandolin et al., 1982) and on the membrane-bound carrier (Block & Vignais, 1984) tend to support the tetramer hypothesis. The tetrameric organization of the AdN carrier is also consistent with the combined observations that there is more than one CATR binding site per transport unit (Figure 7) and that CATR binds to one subunit only of the carrier dimer (Krämer & Klingenberg, 1977; Block et al., 1981).

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Registry No. ATP, 56-65-5; ADP, 58-64-0; CATR, 33286-30-5; BA, 11076-19-0.

REFERENCES

Aquila, H., Misra, D., Eulitz, M., & Klingenberg, M. (1982) *Hoppe Seyler's Z. Physiol. Chem. 363*, 345–349.

Block, M. R., & Vignais, P. V. (1984) *Biochim. Biophys. Acta 767*, 369–376.

Block, M. R., Lauquin, G. J.-M., & Vignais, P. V. (1981) *FEBS Lett. 131*, 213–218.

Block, M. R., Zaccaï, G., Lauquin, G. J.-M., & Vignais, P. V. (1982a) *Biochem. Biophys. Res. Commun. 109*, 471–477.

Block, M. R., Lauquin, G. J.-M., & Vignais, P. V. (1982b) *Biochemistry 21*, 5451–5457.

Block, M. R., Lauquin, G. J.-M., & Vignais, P. V. (1983) *Biochemistry 22*, 2202–2208.

Brandolin, G., Doussière, J., Gulik, A., Gulik-Krzywicki, T., Lauquin, G. J.-M., & Vignais, P. V. (1980) *Biochim. Biophys. Acta 592*, 592–614.

Brandolin, G., Dupont, Y., & Vignais, P. V. (1981) *Biochem. Biophys. Res. Commun. 98*, 28–35.

Brandolin, G., Dupont, Y., & Vignais, P. V. (1982) *Biochemistry 21*, 6348–6353.

Buchanan, B. B., Eiermann, W., Riccio, P., Aquila, H., & Klingenberg, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2280–2284.

Duée, E., & Vignais, P. V. (1969) *J. Biol. Chem. 244*, 3920–3931.

Dupont, Y., Brandolin, G., & Vignais, P. V. (1982) *Biochemistry 21*, 6343–6347.

Krämer, R., & Klingenberg, M. (1977) *Biochemistry 16*, 4954–4961.

Lauquin, G. J.-M., & Vignais, P. V. (1976) *Biochemistry 15*, 2316–2322.

Schlimme, E., Boos, K. S., & de Groot, E. J. (1980) *Biochemistry 19*, 5569–5574.