Houk, T. W., Jr., & Ue, K. (1974) *Anal. Biochem. 62*, 66-74.
Kasai, M., Asakura, S., & Oosawa, F. (1962) *Biochim. Biophys. Acta 57*, 22-31.
Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33-38.
MacLean-Fletcher, S., & Pollard, T. D. (1980) *Biochem. Biophys. Res. Commun. 96*, 18-27.
Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10-21.
Oosawa, F., & Asakura, S. (1975) *Thermodynamics of the Polymerization of Protein*, Academic Press, New York.
Pardee, J. D., & Spudich, J. A. (1982) *J. Cell Biol. 93*, 648-654.
Pollard, T. D., & Mooseker, M. S. (1981) *J. Cell Biol. 88*, 654-659.
Rich, S. A., & Estes, J. E. (1976) *J. Mol. Biol. 104*, 777-792.
Rouayrenc, J., & Travers, F. (1981) *Eur. J. Biochem. 116*, 73-77.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866-4871.
Tait, J. F., & Frieden, C. (1982) *Biochemistry 21*, 3666-3674.
Wang, Y.-L., & Taylor, D. L. (1981) *Cell (Cambridge, Mass.) 27*, 429-436.
Wegner, A., & Engel, J. (1975) *Biophys. Chem. 3*, 215-225.
Wegner, A., & Savko, P. (1982) *Biochemistry 21*, 1909-1913.

Use of 3′-*O*-Naphthoyladenosine 5′-Diphosphate To Probe Distinct Conformational States of Membrane-Bound Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier†

Marc R. Block,* Guy J.-M. Lauquin, and Pierre V. Vignais

ABSTRACT: The fluorescent analogue of ADP, 3′-*O*-naphthoyladenosine 5′-diphosphate (N-ADP), which binds to the mitochondrial ADP/ATP carrier but is not transported [Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1982) *Biochemistry 21*, 5451-5457], has been used to probe the interactions with the ADP/ATP carrier of two specific inhibitors, carboxyatractyloside (CATR) and bongkrekic acid (BA), both in heart mitochondria and in inside-out submitochondrial particles obtained by sonication of mitochondria (sonic particles). The peculiarity of these inhibitors is that they are mutually exclusive for binding; moreover, in mitochondria, CATR attacks the carrier from the outside, and BA from the inside, while in sonic particles the reverse is true. In the present work, the mitochondria and sonic particles were loaded with N-ADP, and the amount of carrier-bound N-ADP released upon addition of CATR and BA was monitored fluorometrically. The kinetics of N-ADP release could be easily resolved by lowering the temperature. In mitochondria, the release was clearly biphasic at 10 °C; the first phase induced by CATR corresponded to the release of 40-70% of the carrier-bound N-ADP and lasted for less than 0.5 s; it ended abruptly and was followed by a very slow release of the rest of the carrier-bound N-ADP, which required more than 20 min for completion. Addition of BA at the onset of the slow phase dramatically accelerated the release of N-ADP; acceleration also occurred upon addition of micromolar concentrations of ADP or any transportable nucleotide. Reversing the sequence of additions, i.e., starting by the addition of BA, led to similar results, namely, a two-step release of the bound N-ADP consisting of a rapid phase of partial release of bound N-ADP followed by a slow one that was accelerated by CATR or ADP. In the case of sonic particles loaded with N-ADP, BA was able to induce the extensive release of the carrier-bound N-ADP at 10 °C, either in the absence or presence of ADP. On the other hand, CATR was inefficient in releasing bound N-ADP at 10 °C, unless ADP or another transportable nucleotide was added. These data provide the first direct experimental evidence in favor of an ADP/ATP carrier model in which the CATR or BA conformations exist prior to the addition of CATR or BA. Any given ADP/ATP carrier unit in the mitochondrial membrane is suggested to exist either in the CATR conformation or in the BA conformation. Bound N-ADP would be released upon binding either of CATR to those carrier units in the CATR conformation or of BA to those in the BA conformation. In heart mitochondria, between 40 and 70% of the carrier units would be in the CATR conformation, depending on the nature of the preparation, the remainder being in the BA conformation. In sonic particles, however, most of the carrier units would be in the BA conformation. The transition between the CATR and BA conformations is very slow at 10 °C; it is increased by raising the temperature or by adding micromolar concentrations of ADP or any transportable nucleotide, suggesting that it is an intrinsic event in ADP/ATP transport.

Previous studies from this laboratory (Block et al., 1982) have led to the characterization of a new fluorescent ligand of the ADP/ATP carrier, namely, 3′-*O*-naphthoyl-1-adenosine 5′-diphosphate (N-ADP).[1] This fluorescent analogue of ADP was able to bind to the ADP/ATP carrier in mitochondria and inside-out submitochondrial particles (sonic particles) but was not transported, in accordance with the well-known specificity of the carrier protein for ADP and ATP (Duée & Vignais, 1969; Boos & Schlimme, 1979). However, N-ADP was found to inhibit ADP transport. The binding of N-ADP to the membrane-bound ADP/ATP carrier and, conversely, its release were associated with opposing changes in the fluorescence intensity, namely, a fluorescence decrease in the case of N-

† From the Laboratoire de Biochimie (CNRS/ERA 903 et INSERM U.191), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires and Faculté de Médecine de Grenoble, 85X, 38041 Grenoble Cedex, France. *Received October 20, 1982.* This work was supported in part by a grant from the Fondation pour la Recherche Médicale.

[1] Abbreviations: sonic particles, inside-out particles prepared by ultrasonic irradiation of beef heart mitochondria; CATR, carboxyatractyloside; BA, bongkrekic acid; AMPPNP, 5′-adenylyl imidodiphosphate; N-ADP, 3′-*O*-naphthoyl-1-adenosine 5′-diphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

0006-2960/83/0422-2202$01.50/0

ADP binding and a fluorescence increase in the case of N-ADP release. The amount of carrier-bound N-ADP in mitochondria previously loaded with N-ADP was assessed as the N-ADP released upon addition of the specific inhibitory ligands carboxyatractyloside (CATR) or bongkrekic acid (BA). The same assay was applied to sonic particles preloaded with N-ADP. In this case, however, because of lack of accessibility by CATR (Shertzer & Racker, 1974; Lauquin et al., 1977a), only BA was used as a releasing agent. All these assays had been carried out at 30 °C; at this temperature, the release of bound N-ADP was achieved in less than 2 min, and the same fluorescence responses were observed with CATR or BA (Block et al., 1982). Interestingly, when the temperature was decreased to 10 °C, the kinetics of release of N-ADP upon addition of CATR to mitochondria could be resolved in two steps, a rapid one occurring in less than 0.5 s followed by a slow one that lasted for about 20 min. The slow phase was considerably accelerated by raising the temperature or by addition of ADP or bongkrekic acid. Manipulating the temperature of the medium thus opened the way to further studies on the susceptibility of the ADP/ATP carrier protein to CATR and BA, using the release of N-ADP to probe the binding of the two inhibitors. The data presented in this paper can be readily explained by postulating a transition between two conformational states recognizing CATR and BA, respectively; this is consistent with previous results based on immunochemical and spectroscopic experiments (Buchanan et al., 1976; Brandolin et al., 1981) showing that the ADP/ATP carrier protein can assume two distinct conformations corresponding to the CATR–carrier and BA–carrier complexes, respectively. These data also show that the CATR and BA conformations exist in the absence of added CATR or ATR.

Experimental Procedures

Materials. [^{14}C]ADP was purchased from New England Nuclear, ADP from P-L Biochemicals, 1-naphthoic acid from Fluka, and carboxyatractyloside (CATR) from Boehringer Mannheim. Synthesis of N-ADP included an activation step of naphthoic acid by a carboxyl reagent, carbonyldiimidazole (Gottikh et al., 1970), as previously described (Block et al., 1982).

Biological Preparations. Beef heart mitochondria were prepared according to Smith (1967) and were stored frozen in liquid nitrogen. Rat heart mitochondria were prepared according to Chance & Hagihara (1963) and were used in the following hours. Sonic particles were routinely obtained by sonication of frozen beef heart mitochondria as described by Beyer (1967) with few modifications (Lauquin et al., 1977a).

Fluorescence Assays. Fluorescence assays were performed with a Perkin-Elmer MPF 2A fluorometer equipped with a stirring device. The fluorescence was excited at 310 nm (2-nm band-pass), and emission was measured at 395 nm with a 6-nm band-pass. The cuvette holder was thermostated at 10 °C unless indicated. Routinely, 0.5–2 mg of membrane protein were suspended in a medium composed of 120 mM KCl, 10 mM Mes, pH 6.5, and 1 mM EDTA (standard saline medium); the final volume was 2.5 mL. The suspension was supplemented with N-ADP and allowed to incubate for a few minutes to allow stabilization of the fluorescence. The carrier-bound N-ADP was assessed by release upon addition of CATR and BA. Release of N-ADP was reflected by an increase of the fluorescence intensity (Block et al., 1982).

Titration of Specifically Bound [^{14}C]N-ADP. Beef heart mitochondria (0.5 mg in 1 mL of standard saline medium, pH 6.5) were preincubated with increasing concentrations of [^{14}C]N-ADP for 20 min at 0 °C in three parallel series of tubes. The tubes of the first series were incubated for another 5 min without any further addition and then centrifuged; the pellet-bound radioactivity reflected both specifically and unspecifically bound [^{14}C]N-ADP. The tubes of the second series were supplemented with 5 nmol of CATR/mg of protein, and after 1 min of contact (a period of time more than sufficient for CATR binding), they were centrifuged. The tubes of the third series were supplemented with both CATR and BA (5 nmol of each inhibitor/mg of protein); after a 5-min period to allow BA to enter the matrix space of the mitochondria and to bind to its specific site on the ADP/ATP carrier, the tubes were centrifuged. All centrifugations were performed in an Eppendorf 3200 centrifuge. The pellets were digested with 1 mL of 5% Triton X-100 and 0.5 M KCl, and radioactivity was assayed by liquid scintillation. The differences of radioactivity between the pellets of series 1 and 2 corresponded to the carrier-bound [^{14}C]N-ADP quickly removable by CATR; those between the tubes of series 1 and 3 corresponded to the [^{14}C]N-ADP removable by CATR plus BA, i.e., to the totality of the carrier-bound [^{14}C]N-ADP.

Results

Temperature Effect on Removal of Bound N-ADP by CATR and BA. As previously reported (Block et al., 1982), all the specifically bound N-ADP is removed in less than 2 min, upon addition of saturating concentrations of CATR or BA to N-ADP-loaded heart mitochondria at 30 °C. The same effect is observed when BA is added to sonic particles preloaded with N-ADP. Once the maximal level of release was attained with one of the two inhibitors, for example, CATR, there was no further effect of the other inhibitor, i.e., BA.

Decreasing the temperature from 30 to 10 °C allowed the resolution of the kinetics of N-ADP release from heart mitochondria in two steps, as illustrated by fluorescence measurements (Figure 1, trace A). At 10 °C upon addition of CATR at saturating concentration, there was a first phase of rapid release corresponding to a fraction of the carrier-bound N-ADP (35% in the present experiment). This phase lasted for less than 0.5 s and could not be analyzed by conventional techniques; it was followed by a second phase of very slow release of the residual bound N-ADP, the maximal fluorescence response being attained within 20 min (not shown). Increasing the temperature from 10 to 20 and 30 °C accelerated the slow phase but did not change the fraction of the quickly released N-ADP, which ranged from 35 to 40% in all cases. The Arrhenius plot relative to the rate of the slow phase was linear between 10 and 25 °C. The value of the energy of activation in this range of temperature was about 40 kJ/mol. The fact that the maximal fluorescence response to inhibitors was lower at 30 (trace C) than at 10 °C (trace A) is due to the decrease of the quantum yield when temperature is increased, as measured with free N-ADP (not shown).

The second phase of release of bound N-ADP (slow phase) was dramatically accelerated by addition of BA, the plateau phase of the fluorescence increase being then attained within 0.5 s (Figure 1, trace A). Reversing the order of addition of the inhibitors, i.e., BA added first followed by CATR, resulted in a similar pattern of release (Figure 2), i.e., part of the bound N-ADP was quickly released upon addition of BA, the remaining bound N-ADP being removed by CATR.

The extent of the rapid phase of N-ADP release upon addition of CATR or BA depended on the nature of the mitochondrial preparation. With freshly prepared rat heart mitochondria, up to 80% of the bound N-ADP was readily removed by CATR and the remaining fraction by BA (Figure 2, trace A); with frozen and thawed beef heart mitochondria,

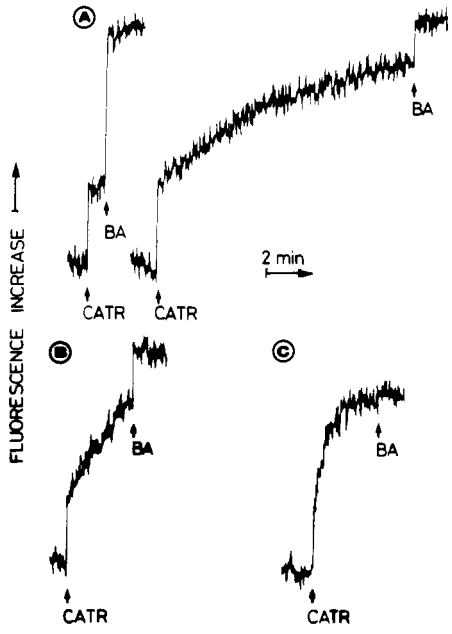

FIGURE 1: Effect of temperature on biphasic release of bound N-ADP induced by CATR or BA, as monitored by spectrofluorometry. Beef heart mitochondria (2 mg of protein) were suspended in 0.12 M KCl, 10 mM Mes, pH 6.5, 1 mM EDTA (standard saline medium), and 5 μM N-ADP in a final volume of 2.5 mL and introduced in the cuvette of the spectrofluorometer (cf. Experimental Procedures). After a period of 3 min to allow stabilization of the fluorescence base line, CATR (10 nmol) or BA (10 nmol) was added as indicated. The total increase in fluorescence emission, which reflects the release of the carrier-bound N-ADP, was about 5% of the initial fluorescence. The temperature was set at 10 °C in experiment A, 20 °C in experiment B, and 30 °C in experiment C.

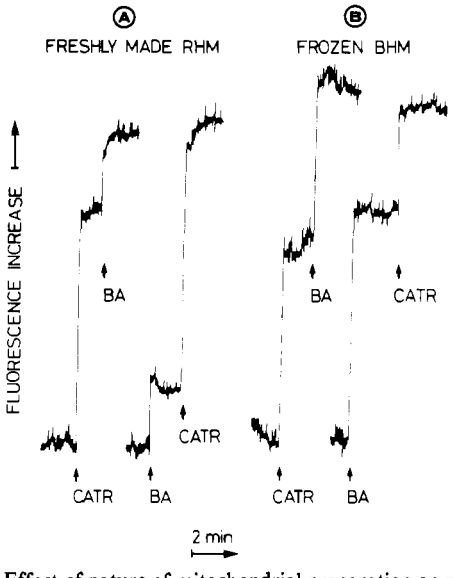

FIGURE 2: Effect of nature of mitochondrial preparation on percentage of N-ADP released upon addition of CATR or BA. The conditions were as described in the legend of Figure 1. RHM refers to rat heart mitochondria (A) and BHM to beef heart mitochondria (B). The temperature was 10 °C.

about half of the bound N-ADP was removed by CATR and the rest by BA (Figure 2, trace B). The large difference in the amount of released N-ADP in freshly made rat heart mitochondria and frozen beef heart mitochondria was not due to the animal species but to freeze thawing; in fact, a similar difference was observed between freshly made and frozen rat heart mitochondria. The energy state of the mitochondria was apparently not the primary factor in the control of the release of bound N-ADP by CATR, since there was no significant

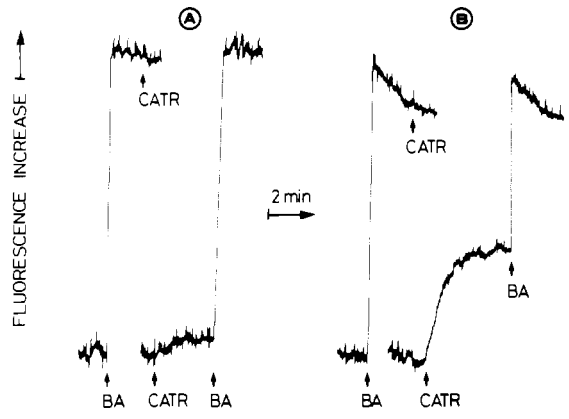

FIGURE 3: Temperature dependency of release of bound N-ADP in sonic particles upon addition of CATR or BA. Sonic particles (1 mg of protein) were suspended in the standard saline medium, pH 6.5, with 5 μM N-ADP. The final volume was 2.5 mL. Assays were carried out at 10 (A) and 30 °C (B) as described in the legend of Figure 1. CATR and BA were added as indicated.

difference in the extent and rate of N-ADP release when mitochondria were incubated in the presence of succinate plus oligomycin for energization or FCCP for deenergization (not shown). It was checked that N-ADP was not an uncoupler at the concentrations used.

Sonic particles loaded with N-ADP were much more responsive at 10 °C than mitochondria to a first addition of BA; indeed, the release of N-ADP was extensive and rapid (<0.5 s) (Figure 3, trace A). On the other hand, CATR at 10 °C was ineffective. A simple explanation is that the CATR site is located on the inner side of the inverted membrane in sonic particles and is therefore not directly accessible to the externally added CATR (Vignais, 1976). CATR addition at 30 °C, however, resulted in a significant (40%) release of bound N-ADP (Figure 3, trace B). This most likely reflected a fraction of leaky particles permeant to CATR [see Lauquin et al. (1977b)]. Permeation of CATR also occurred at 10 °C; but at this temperature, the releasing effect of CATR on bound N-ADP required a catalytic amount of added ADP (see below).

Quantitative Assessment of Release of N-ADP by CATR and BA. In the experiment illustrated in Figure 4A, aliquot fractions of the beef heart mitochondrial suspension were loaded with different concentrations of N-ADP, and the carrier-bound N-ADP was released at 10 °C by saturating concentrations of either CATR alone or both CATR and BA. Both CATR and CATR plus BA curves showed saturation; they differ essentially in that the maximal amount of N-ADP released was, as expected, higher in the presence of CATR plus BA than in the presence of CATR alone. On the basis of the same principle, a more accurate titration was carried out with [^{14}C]N-ADP (Figure 4B). The amounts of [^{14}C]N-ADP quickly displaced by CATR alone or by CATR plus BA were plotted against free [^{14}C]N-ADP. Both titration curves were of the Michaelis type, in good accordance with fluorescence data. The Scatchard plot of the radioactivity data revealed identical K_d values of about 2.5 μM for [^{14}C]N-ADP and a total number of unmasked sites of 0.7 nmol/mg of protein upon addition of CATR alone and 1.3 nmol/mg of protein upon addition of CATR plus BA. At 30 °C, the total number of specific N-ADP sites, equated to the N-ADP released upon addition of CATR, was 1.4–1.6 nmol/mg of protein with a K_d value of 3 μM.

Titration of CATR and BA Binding Sites by Release of Bound N-ADP. Two titrations were carried out at 10 and 30

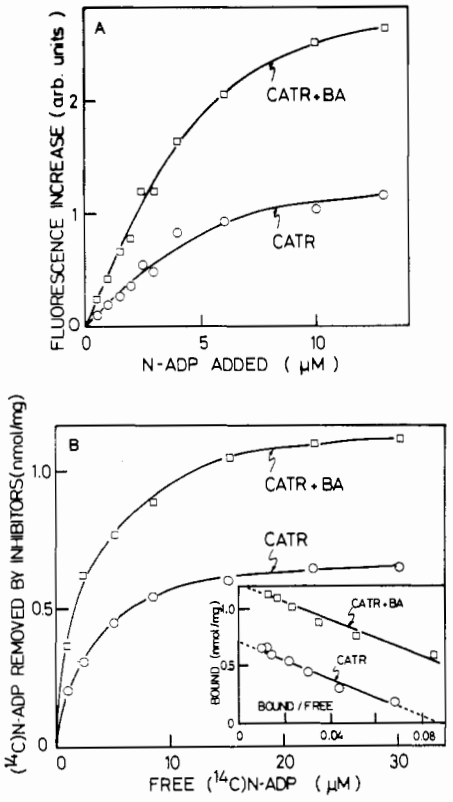

FIGURE 4: Titration of carrier-bound N-ADP by CATR or by CATR plus BA. (A) Fluorescence monitoring. Beef heart mitochondria (2 mg of protein) were suspended in 2.5 mL of the standard saline medium, pH 6.5, at 10 °C. Increasing amounts of N-ADP were added, and after 3-min equilibration, the fluorescence response to addition of CATR and BA (10 nmol/each) was recorded. Other conditions are as on the legend of Figure 1. (B) Radioactivity assay. Experimental conditions are described under Experimental Procedures. (○) corresponds to the bound [^{14}C]N-ADP displaced by CATR and (□) to the bound [^{14}C]N-ADP displaceable by CATR plus BA. The Scatchard plot of the data is given in the inset.

°C (Figure 5) by use of the fluorescence assay described in Figure 1. Beef heart mitochondria were first loaded with N-ADP. The rapid increase in the fluorescence following the addition of CATR and BA was recorded and plotted as a function of the added inhibitors. At 30 °C, the end point of the titration was achieved with 1.3 nmol of CATR/mg of protein, i.e., virtually the same amount of CATR as that required to saturate the CATR binding sites in heart mitochondria (Block et al., 1980). This could be expected because of the very high affinity of CATR for the ADP/ATP carrier (K_d < 10 nM) (Vignais et al., 1973). When a similar titration of N-ADP release by CATR was performed at 10 °C, the end point corresponded to about 0.8 nmol of CATR/mg of protein. The residual carrier-bound N-ADP was released by BA, and the minimal amount of BA needed for release of this residual N-ADP ranged between 0.4 and 0.5 nmol/mg of protein. Clearly at 10 °C, the extensive release of bound N-ADP required both CATR and BA added at subsaturating concentrations. The sum of these subsaturating concentrations of CATR and BA was roughly equal to the saturating concentration of CATR at 30 °C, i.e., 1.3 nmol/mg of protein.

Effect of Externally Added ADP on Release of N-ADP by CATR and BA. Data in Figure 6 (traces A–F) illustrate the enhancing effect of micromolar concentrations of ADP on the slow release of N-ADP initiated by CATR and BA in heart mitochondria at 10 °C. In trace A, after part of the bound N-ADP has been rapidly removed from beef heart mitochondria by CATR, addition of 5 μM ADP just at the onset

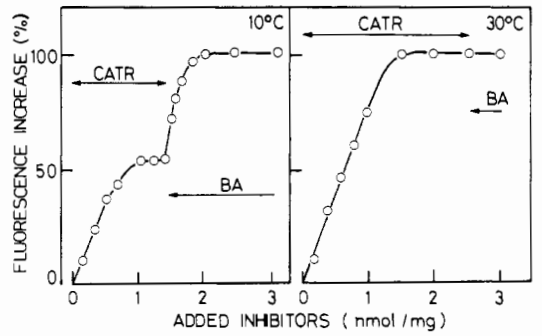

FIGURE 5: Titration of CATR and BA binding sites by means of the fluorescence response corresponding to the release of bound N-ADP. Beef heart mitochondria (2 mg of protein) were suspended in 2.5 mL of the standard saline medium, pH 6.5, at 10 °C with 5 μM N-ADP. After a 3-min period corresponding to the stabilization of the fluorescence base line, CATR or BA was added by increments, and the fluorescence response was recorded as described in the legend of Figure 1. It was checked that the sum of fluorescence increments resulting from fractional additions of CATR or BA was equal to the fluorescence increase obtained by the single addition of a saturating concentration of either inhibitor. In the assay carried out at 10 °C, after a saturation plateau was reached with CATR, titration of the remaining sites was performed with BA. At 30 °C, when the saturation plateau was attained with CATR, a further addition of BA did not result in further release of N-ADP.

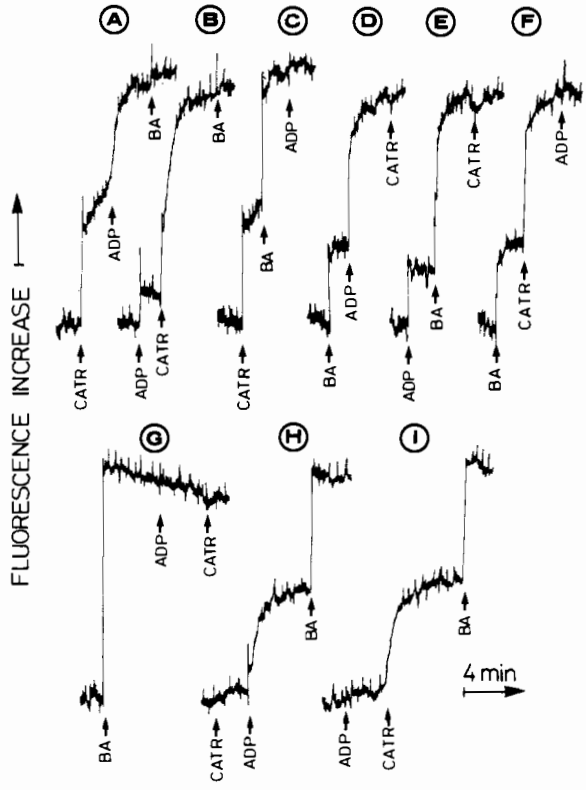

FIGURE 6: ADP-induced stimulation of release of bound N-ADP upon addition of CATR or BA. Frozen and thawed beef heart mitochondria (2 mg of protein), freshly prepared rat heart mitochondria (2 mg of protein), or sonic particles (1 mg of protein) were suspended in 2.5 mL of the standard saline medium, pH 6.5, at 10 °C, with 5 μM N-ADP. After 3 min, CATR (100 nmol), BA (10 nmol), and ADP (10 nmol) were added as indicated. Fluorescence was monitored as described in the legend of Figure 1. Traces A–C refer to beef heart mitochondria, traces D–F to rat heart mitochondria, and traces G–I to sonic particles.

of the slow phase resulted in immediate release of the residual carrier-bound N-ADP. In fact, a subsequent addition of BA was ineffective. When ADP was added first, only a small amount of N-ADP was released (trace B); addition of CATR following that of ADP resulted in rapid release of all the

remaining carrier-bound N-ADP as shown by the absence of effect of BA, as in the preceding experiment. The control trace (C) recalls that, in the absence of ADP, the effect of CATR is limited and that the subsequent addition of BA results in the release of the rest of bound N-ADP. After the release of N-ADP had attained completion upon addition of BA, there was no further effect of added ADP. Similar patterns of N-ADP release were observed when CATR was replaced by BA as first-added ligand (traces D–F). In these experiments, freshly prepared rat heart mitochondria were preferred to frozen beef heart mitochondria because the rapid response to BA added first was less extensive in the former; this allowed a better evaluation of the acceleration of ADP of the slow phase of N-ADP release. All these experiments were carried out with 5 μM N-ADP, a nonsaturating concentration, because of technical difficulties pertaining to the high fluorescence background when saturating concentrations of N-ADP are used. Control assays performed at 15 μM N-ADP, a subsaturating concentration, showed, however, that the responses were virtually the same. Not only ADP but also ATP and AMPPNP, which are transportable nucleotides, increased the rate of N-ADP release during the slow phase. In contrast, 3′-O esters of ADP and ATP (Lauquin et al., 1978; Boos & Schlimme, 1979; Block et al., 1982) and 8-bromo-ADP and ATP (Schlimme & Stahl, 1974), which are able to bind to the carrier but are not transported, were totally ineffective.

In sonic particles, as in mitochondria, the rapid and extensive release of bound N-ADP induced by BA at 10 °C (see Figure 3) was not enhanced by ADP or CATR (Figure 6, trace G). As already shown (Figure 3), the effect of CATR alone on sonic particles at 10 °C was negligible: however, when ADP was added subsequently or prior to CATR (Figure 6, traces H and I), 40–50% of the carrier-bound N-ADP was released, the rest being removed upon addition of BA. As shown in a preceding section (Figure 3), the same amount of the bound N-ADP in sonic particles was released by CATR at 30 °C in the absence of added ADP, most likely reflecting a population of sonic particles permeable to CATR. Thus, a temperature of 30 °C without added ADP has virtually the same effect as added ADP at 10 °C on the displacement of N-ADP by CATR in sonic particles. This will be discussed later.

The data in Figure 7 illustrate the effect of increasing concentrations of ADP on the slow release of N-ADP that follows the rapid one upon addition of CATR (cf. Figure 6, trace A). The reciprocal of the difference between the rate of N-ADP release in the absence of ADP (V_1) and that in the presence of ADP (V_2) (inset of Figure 7) was plotted against the reciprocal of the ADP concentration (Figure 7). The experimental points could be fitted by a straight line, indicating a saturable process. The concentration of ADP required for the half-maximal stimulation of N-ADP release was 3 μM, a value similar to the K_m found for ADP transport (Duée & Vignais, 1969).

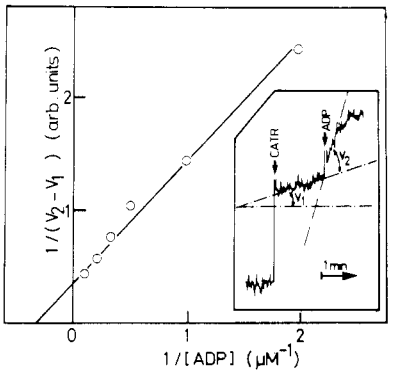

FIGURE 7: Effect of concentration of ADP on the rate of the second phase of N-ADP release. Beef heart mitochondria in 2.5 mL of the standard saline medium, pH 6.5, at 10 °C, were incubated for 3 min with 5 μM N-ADP. Then CATR (10 nmol) was added, resulting in fast fluorescence response (first phase of N-ADP release). At the onset of the second phase, ADP was added at increasing concentrations, and the rate of fluorescence increase, which is proportional to the rate of N-ADP release, was recorded. The rate of fluorescence increase prior to addition of ADP was refered to as V_1 and that measured after addition of ADP as V_2. The reciprocal of $V_2 - V_1$, i.e., the increase in rate due to ADP, was plotted vs. the reciprocal of the ADP concentration.

Discussion

In the present study, the fluorescent nucleotide analogue N-ADP was used to probe interactions of two antagonistic ligands, CATR and BA, and the natural substrate, ADP, with the membrane-bound ADP/ATP carrier protein. The starting observation was that, at 10 °C, the kinetics of release of bound N-ADP from heart mitochondria by saturating concentrations of CATR or BA could be resolved in two steps differing in their rates by at least 2 orders of magnitude (Figure 1). The first step was a rapid one, the plateau of N-ADP release being attained in less than 0.5 s; this step could not be resolved in our experimental conditions, and the corresponding period of time reflected more the homogeneous distribution of the ligand (CATR or BA) by mixing than the kinetics of N-ADP release. The second step was a slow one, the full removal of the remaining bound N-ADP requiring more than 20 min; this slow step was dramatically accelerated by BA when the first-added ligand was CATR and by CATR when the first-added ligand was BA (Figure 2). At first view, these results could be explained by assuming that each carrier unit contains two sets of N-ADP binding sites, one set being susceptible to BA binding and the other to CATR binding. This simple explanation is, however, unlikely for the following reasons. (1) CATR and BA compete with each other for binding (Vignais et al., 1973; Klingenberg & Buchholz, 1973; Lauquin & Vignais, 1976), and although a limited, simultaneous binding of [^{14}C]acetyl-CATR and [^{3}H]BA has been observed (Block et al., 1980), it is clear that CATR and BA are to a large extent mutually exclusive for binding. If one takes as an example the case of frozen beef heart mitochondria, where at 10 °C half of the carrier-bound N-ADP is susceptible to CATR and the other half to BA, when CATR and BA are used at saturating concentrations, the above hypothesis would mean that each carrier unit binds both CATR and BA in equal amounts: this possibility is excluded by the binding data. (2) The sequential titration at 10 °C of the CATR and BA binding sites followed by release of N-ADP is characterized by two end points, corresponding to subsaturating concentrations of the two inhibitors, namely, 0.8 nmol of CATR/mg of protein and 0.4–0.5 nmol of BA/mg of protein (Figure 5). The sum of these subsaturating concentrations is roughly equal to the concentration of either the high-affinity CATR sites or the high-affinity BA sites, which amounts to 1.2–1.5 nmol/mg of protein (Block et al., 1982). (3) The same K_d value for N-ADP is found, whatever the inhibitors used, CATR or CATR plus BA (Figure 4); this again strongly argues against heterogeneity of the N-ADP binding sites.

A more plausible explanation is that, in the absence of CATR or BA, the ADP/ATP carrier can exist in two conformations, both able to bind N-ADP; one conformation binds CATR (CATR conformation) and the other BA (BA conformation). In keeping with this explanation, it is supposed that in mitochondria, the carrier units are distributed between the CATR and BA conformations. The rapid release of

N-ADP upon addition of CATR reflects the immediate access and binding of CATR to those carrier units that possess the CATR conformation. The other carrier units, i.e., those in the BA conformation, are not directly reactive to CATR; they may, however, undergo a slow transition from the BA to the CATR conformation, and this is accelerated either by adding ADP or by increasing the temperature. The same reasoning holds when BA is the first-added ligand. In this case, BA rapidly binds to the fraction of carrier units in the BA conformation; the remainder undergoes a slow transition toward the BA conformation, which is likewise accelerated at higher temperature or by ADP. The distribution of the carrier units between the CATR and BA conformations depends upon the nature of the particle preparation; for example, 70–80% of the carrier units are in the CATR conformation in freshly made rat heart mitochondria, compared to 40–50% in frozen beef heart mitochondria. One may wonder what might be the cause of this difference. A possible explanation is that the CATR conformation depends on the presence of nucleotides outside the particles. The nucleotide content of freshly made heart mitochondria (beef or rat heart) is between 6 and 7 nmol/mg of protein whereas that of frozen and thawed mitochondria is 4–5 nmol/mg of protein; in the latter case, part of internal adenine nucleotides was released to the medium. Thus in thawed mitochondria, due to adenine nucleotides present both on the inside and outside of the mitochondrial membrane, transport is functioning, and the carrier units are statistically distributed, half of them in the CATR conformation and the other half in the BA conformation. On the other hand, it can be inferred from the extensive effect of BA on the release of N-ADP that most of the sonic particles are in the BA conformation.

In spite of the fact that ADP does not efficiently remove bound N-ADP even when added in large excess (Block et al., 1982), it markedly accelerates, at micromolar concentrations, the slow phase of N-ADP release induced by CATR and BA in mitochondria; it also accelerates the release of N-ADP induced by CATR in permeable sonic particles. It is probable that because of the subsaturating concentrations of N-ADP used, ADP added at micromolar concentrations can compete directly with N-ADP for binding and then trigger the CATR to BA conformational transition. This competition results in a fast but limited release of N-ADP. If CATR or BA is present in the medium, the carrier units are immediately trapped in either the CATR or BA conformation by the binding of CATR or BA, respectively; this is accompanied by the release of ADP, now available for a new cycle, and the process finishes with the complete release of the carrier-bound N-ADP.

In the absence of ADP, the CATR and BA conformations are probably not in rapid equilibrium, at least at low temperature. The effect of ADP is shared by transportable nucleotides. The half-maximal rate of transition is obtained at 3 μM ADP, a value close to the K_m for ADP transport; these results strongly suggest that the ADP-induced transition between the CATR and BA conformations is a molecular event linked to transport. The effect of the transportable nucleotides might be to lower the activation energy of the CATR to BA transition. This energy barrier could also be overcome by increasing the temperature; it is, however, equally possible that, upon raising the temperature, internal adenine nucleotides are released to the external medium, thereby inducing transport, as does externally added ADP or ATP.

As far as sonic particles are concerned, CATR addition together with ADP at 10 °C (Figure 6, traces H and I) or

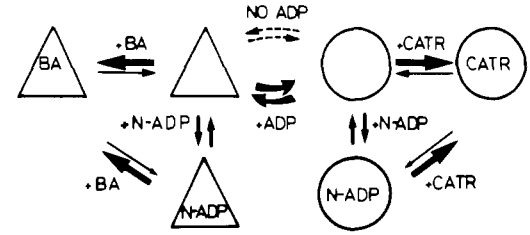

FIGURE 8: Schematic representation depicting transition between CATR and BA conformation. The CATR and BA conformations are supposed to exist in the absence of added CATR or BA. Upon binding of these ligands, the CATR– or BA–carrier complex is formed. ADP and other transportable nucleotides are supposed to facilitate or trigger the transition between the two preexisting conformations through probably a number of intermediary and transient steps that are intrinsic events of ADP/ATP transport. As shown in the scheme, both CATR and BA conformations are able to bind N-ADP.

CATR addition at 30 °C without added ADP (Figure 3) results in the release of 30–50% of the bound N-ADP, whereas no release is detected upon addition of CATR at 10 °C in the absence of added ADP (Figure 6, trace H). A plausible explanation is that, although most of the sonic particles in our preparations are inside out (Lauquin et al., 1977a), a substantial fraction (up to 50%) is leaky to CATR (Lauquin et al., 1977b); further, as stated above, in these sonic particles, nearly all the carrier units are in the BA conformation. The release of N-ADP therefore reflects the access of CATR that has penetrated the particles to those carrier units in the CATR conformation. This means that in sonic particles either a temperature of 30 °C or the addition of ADP at 10 °C triggers the transition from the BA conformation (that is accessible by BA from the outside) to the CATR conformation (that is accessible to CATR from the inside); the carrier units in particles permeable to CATR are the only ones that can react with internal CATR, resulting in the release of N-ADP.

It has also been demonstrated by immunological studies (Buchanan et al., 1976) and by investigation of the intrinsic fluorescence of the solubilized ADP/ATP carrier (Brandolin et al., 1981) that the CATR–carrier and BA–carrier complexes correspond to two different conformational states of the carrier protein. In the latter case, ADP or ATP was required for the BA conformation being fully expressed. The role of ADP or ATP in triggering conformational changes in the ADP/ATP carrier protein is exemplified by the ADP- or ATP-induced unmasking of SH groups in the membrane-bound carrier; however, in this case, the membrane has to be energized in order for full unmasking to occur quickly (Vignais & Vignais, 1972; Aquila et al., 1982; Michejda & Vignais, 1981). The present data, however, provide the first direct experimental evidence for the CATR and BA conformations to exist prior to addition of CATR or BA. This is illustrated by the scheme of Figure 8, which shows that any ADP/ATP carrier is either in the CATR or BA conformation and that the effect of CATR and BA is to stabilize the CATR or BA conformation by binding to their specific preexisting sites. An alternative possibility (not shown in the scheme) is that of a stable form of the carrier that contains two loosely reactive sites for CATR and BA; upon binding of one or the other of the inhibitors, the sites would adopt, by an induced-fit mechanism, a new geometry, resulting in firm binding of either CATR or BA. However, this latter alternative is not consistent with our present results.

The existence of two conformational states corresponding to the orientation of the substrate site to the outside (cytosolic or C state) or to the inside (matrix state or M state) was postulated by Klingenberg & Buchholz (1973), and it was

proposed that the same site was able to bind both the substrates ADP/ATP and the inhibitors CATR and BA. Since CATR attacks the carrier from the outside and BA from the inside, the carrier is expected to bind CATR in the C state and BA in the M state. The demonstration of CATR and BA conformations triggered by ADP apparently favors the view that the CATR conformations corresponds to the C state and the BA conformation to the M state. It should be recalled, however, that there are a number of observations based on chemical modifications (Block et al., 1981a,b) that argue against a single site for substrate and inhibitors. Moreover, the binding studies presented in the present paper (Figure 4) clearly indicate that the CATR and BA conformational states bind N-ADP with the same affinity. On the other hand, since externally added ADP stimulates the slow release of N-ADP induced by CATR alone (Figure 6A), it must be recognized by the carrier in the BA conformation. External ADP also stimulates the slow release of N-ADP induced by BA alone (Figure 6D); it must, then, also be recognized by the carrier in the CATR conformation. As a consequence, both conformational states can bind external ADP, a conclusion that is not supported by the single-site mechanism. Further, binding studies with N-ADP (Block et al., 1982; Dupont et al., 1982) and kinetic data on ADP transport (Duyckaerts et al., 1980; Barbour & Chan, 1981) strongly point to the existence of more than one binding site per carrier unit. In brief, whereas the transition between the CATR and BA conformations most probably occurs by steps that are involved in ADP/ATP transport, it does not reflect the reorientation of a single binding site from the outside to the inside of the mitochondrial membrane.

Registry No. N-ADP, 71160-02-6; CATR, 33286-30-5; BA, 11076-19-0; ADP/ATP carrier, 9068-80-8.

References

Aquila, H., Eiermann, W., & Klingenberg, M. (1982) *Eur. J. Biochem. 122*, 133–139.

Barbour, R. L., & Chan, S. H. P. (1981) *J. Biol. Chem. 256*, 1940–1948.

Beyer, R. E. (1967) *Methods Enzymol. 10*, 186–194.

Block, M. R., Pougeois, R., & Vignais, P. V. (1980) *FEBS Lett. 117*, 335–340.

Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1981a) *Biochemistry 20*, 2692–2699.

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

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

Boos, K. S., & Schlimme, E. (1979) *Biochemistry 18*, 5304–5309.

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

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

Chance, B., & Hagihara, B. (1963) *Proceedings of the International Congress of Biochemistry, 5th, Moscow*, Vol. 5, pp 3–37, Pergamon, London and New York.

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

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

Duyckaerts, C., Sluse-Goffard, C. M., Fux, J. P., Sluse, F. E., & Liebecq, C. (1980) *Eur. J. Biochem. 106*, 1–6.

Gottikh, B. P., Kraysesky, A. A., Tarussova, N. B., Purygin, P. P., & Tsilevich, T. L. (1970) *Tetrahedron 26*, 4419–4433.

Klingenberg, M., & Buchholz, M. (1973) *Eur. J. Biochem. 38*, 346–358.

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

Lauquin, G. J. M., Villiers, C., Michejda, J. W., Hryniewiecka, L. V., & Vignais, P. V. (1977a) *Biochim. Biophys. Acta 460*, 331–345.

Lauquin, G. J. M., Devaux, P. F., Bienvenüe, A., Villiers, C., & Vignais, P. V. (1977b) *Biochemistry 16*, 1202–1208.

Lauquin, G. J. M., Brandolin, G., Lunardi, J., & Vignais, P. V. (1978) *Biochim. Biophys. Acta 501*, 10–19.

Michejda, J. W., & Vignais, P. V. (1981) *FEBS Lett. 132*, 129–132.

Schlimme, E., & Stahl, K. W. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 1130–1142.

Shertzer, H. G., & Racker, E. (1974) *J. Biol. Chem. 249*, 1320–1321.

Smith, A. L. (1967) *Methods Enzymol. 10*, 81–86.

Vignais, P. V. (1976) *Biochim. Biophys. Acta 456*, 1–38.

Vignais, P. V., & Vignais, P. M. (1972) *FEBS Lett. 26*, 27–31.

Vignais, P. V., Vignais, P. M., & Defaye, G. (1973) *Biochemistry 12*, 1508–1519.