Interaction of 3′-*O*-(1-Naphthoyl)adenosine 5′-Diphosphate, a Fluorescent Adenosine 5′-Diphosphate Analogue, with the Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier Protein in the Mitochondrial Membrane†

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ABSTRACT: 3′-*O*-(1-Naphthoyl)adenosine 5′-diphosphate (N-ADP), a fluorescent analogue of ADP, was established as a potent inhibitor of adenosine 5′-diphosphate/adenosine 5′-triphosphate (ADP/ATP) transport in mitochondria and inside-out sonic particles; the K_i value was about 5 μM. The inhibition was of a mixed type. On the other hand, N-ADP was not transported in a measurable way in either type of particles. Upon binding to the particles, the fluorescent intensity of N-ADP was decreased; the release of the bound N-ADP upon addition of carboxyatractyloside (CATR) to mitochondria and bongkrekic acid (BA) to sonic particles was reflected by increases of fluorescence. In parallel assays dealing with ^{14}C-labeled N-ADP, specifically bound [^{14}C]N-ADP was equated to [^{14}C]N-ADP released upon addition of either CATR (mitochondria) or BA (sonic particles). The specific binding of N-ADP corresponded to 1.4–1.6 nmol/mg of protein in mitochondria, with a K_d value of 3 μM, and to 1.5–1.6 nmol/mg of protein in sonic particles, with a K_d value of 6 μM. Essentially similar values were obtained for N-ATP binding. These values are at least twice as high as those found for specific ADP or ATP binding, suggesting that N-ADP or N-ATP binds to potential nucleotide binding sites that were not totally occupied by ADP or ATP. Whereas nearly all the specifically bound N-ADP in mitochondria was displaced by an excess of ADP (400 μM) at pH 7.4, only 30% could be removed from sonic particles under the same conditions. Furthermore at pH 6.5, no more than half of the specifically bound N-ADP could be removed by excess ADP in mitochondria and only 10–20% in sonic particles. These results indicate that each ADP/ATP carrier unit contains at least two types of nucleotide sites capable of interacting with N-ADP. Because of the hydrophobic nature of the naphthoyl moiety of N-ADP, the data suggest that difference in N-ADP binding in mitochondria and sonic particles are related to differences in the hydrophobic nature of their sites. Due to the special features of N-ADP (strong specific binding to the ADP/ATP carrier and no competence for transport), this ADP analogue was particularly suitable for investigating the sensitivity of the nucleotide binding sites of the carrier to chemical modifiers. Inactivation studies were therefore carried out with mitochondria and sonic particles to compare the sensitivity to UV light and butanedione of the binding of N-ADP, [^{3}H]BA, and [^{14}C]Ac-CATR, a radiolabeled substitute for CATR. Both in mitochondria and in sonic particles, UV light and butanedione inactivated more rapidly the binding of N-ADP than that of [^{3}H]BA. On the other hand, in mitochondria, UV light inactivated more rapidly the binding of [^{14}C]Ac-CATR than that of N-ADP; the reverse was true for the inactivation by butanedione. The inactivation data conclusively indicate that BA, CATR, and adenine nucleotides are recognized by different specific sets of amino acids.

A number of ribose-modified ADP and ATP analogues have been recently used to investigate the functioning of the two main ADP/ATP-dependent proteins of the inner mitochondrial membrane, namely, the F_1-ATPase and the ADP/ATP carrier. The 3′-O esters of ADP are strong inhibitors of oxidative phosphorylation exhibiting K_i values lower than 1 μM; one of the most potent of them is 3′-*O*-(1-naphthoyl)adenosine 5′-diphosphate (N-ADP)[1] (Schäfer & Onur, 1979). The 3′-O esters of ADP also interact with the ADP/ATP carrier; thus, photoactivable 3′-arylazido derivatives of ADP are recognized but not transported by the ADP/ATP carrier (Lauquin et al., 1978). The fluorescent 3′-*O*-[(dimethylamino)naphth-1-oyl]adenosine 5′-diphosphate analogue also interacts with the ADP/ATP carrier, as shown by fluorescence changes that are sensitive to transport inhibitors like bongkrekic acid (BA) (Schäfer & Onur, 1980). Using a number of ribose-modified ADP and ATP analogues, Boos & Schlimme (1979) were able to specify the basic steric, contact, and structural elements of the nucleotides that are necessary for specific binding to the ADP/ATP carrier and for transport.

In the course of a systematic investigation of the interaction of specific ligands with the mitochondrial ADP/ATP carrier, we found that N-ADP interacts in a specific way with the ADP/ATP carrier, by studying the response of bound N-ADP to the two well-known specific inhibitors of ADP/ATP transport, BA and carboxyatractyloside (CATR). As previously reported, CATR attacks the ADP/ATP carrier protein at the outer face of the inner mitochondrial membrane, whereas BA has to enter the mitochondrial membrane to interact with it; the reverse situation holds true for inside-out submitochondrial particles [for a review, cf. Vignais (1976)]. As shown in this paper, unlike 3′-*O*-[(dimethylamino)-

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[1] Abbreviations: sonic particles, inside-out vesicles obtained by sonication of beef heart mitochondria; N-ADP, 3′-*O*-(1-naphthoyl)-adenosine 5′-diphosphate; ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; Ac-CATR, acetyl carboxyatractyloside; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CDI, 1,1′-carbonylbis[imidazole]; N-GDP, 3′-*O*-(1-naphthoyl)guanosine 5′-diphosphate; N-AMP, 3′-*O*-(1-naphthoyl)adenosine 5′-monophosphate; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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naphth-1-oyl]adenosine 5′-diphosphate (Schäfer & Onur, 1980), N-ADP binds to the ADP/ATP carrier both in mitochondria and in sonic submitochondrial particles. Removal of bound N-ADP upon addition of CATR or BA is accompanied by an increase of the fluorescence intensity. On the other hand, N-ADP is not transported, and it inhibits ADP transport. These features are particularly useful in studies of the nucleotide binding site(s) in the ADP/ATP carrier protein.

The mechanism of ADP/ATP transport was previously discussed in terms of a common binding site for ADP or ATP, ATR or CATR, and BA (Klingenberg & Appel, 1980) or a common binding center with specific sites for substrates and inhibitors (Block et al., 1979, 1981a). On the other hand, kinetics of ADP transport recently reported by Duyckaerts et al. (1980) and Barbour & Chan (1980) could be interpreted by a two substrate site carrier model. The data reported here favor the view that the ADP/ATP carrier contains at least two binding sites for ADP or ATP and that the substrate binding sites differ in some amino acid residues from the binding sites for ATR or CATR and BA.

Experimental Procedures

Materials. [^{14}C]Adenosine 5′-diphosphate was purchased from New England Nuclear, adenosine 5′-diphosphate was from P-L Biochemicals, 1-naphthoic acid was from Fluka, butanedione and EEDQ were from Aldrich, and carboxyatractyloside was from Boehringer Mannheim.

[^{3}H]Bongkrekic acid and [^{14}C]acetyl carboxyatractyloside were prepared as described by Lauquin & Vignais (1976) and Block et al. (1980), respectively. Synthesis of 3′-*O*-(1-naphthoyl)adenosine 5′-diphosphate (N-ADP) included an activation step of naphthoic acid by a carboxyl reagent 1,1′-carbonylbis[imidazole] (CDI) (Gottikh et al., 1970). CDI (0.3 mmol) and 1-naphthoic acid (0.1 mmol) were dissolved in 0.1 mL of dimethylformamide dried over molecular sieves and calcium hydride. The mixture was stirred for 15 min prior to introducing 50 μmol of ADP dissolved in 0.5 mL of water. The reaction was allowed to proceed for 2 h at room temperature. The solvent was evaporated to dryness, and the residue was washed 3 times with acetone, followed by centrifugation to eliminate the excess of naphthoic acid and imidazole. The acetone-washed residue was dissolved in 1 mL of ethanol–water (1:1) and applied to a Whatman No. 3 paper. The chromatogram was developed with 1-butanol–water–acetic acid (5:3:2, v/v). Two major fluorescent spots of R_f 0.65 and 0.75 in addition to a nonfluorescent, UV-absorbing band (R_f 0.1) were obtained. The two fluorescent spots were identified as mononaphthoyl- and dinaphthoyl-ADP derivatives; their stoichiometric composition was established by using [^{14}C]ADP of known specific radioactivity and assessing the naphthoyl residue content by absorbancy at 300 nm. The molecular extinction coefficients of mononaphthoyl-ADP at pH 7 were 6200 M^{-1} cm^{-1} at 300 nm and 15 400 M^{-1} cm^{-1} at 260 nm.

Biological Preparations. Beef heart mitochondria were prepared according to Smith (1967) and rat heart mitochondria according to Chance & Hagihara (1963). Inside-out submitochondrial particles (sonic particles) were routinely obtained by sonication of frozen beef heart mitochondria as described by Beyer (1967) and modified by Lauquin et al. (1977).

Binding Assays of Radioactive Ligands. [^{14}C]Acetyl carboxyatractyloside ([^{14}C]Ac-CATR) and [^{3}H]bongkrekic acid ([^{3}H]BA) were added at the appropriate concentrations to the suspensions of mitochondria or sonic particles at 0 °C in the appropriate medium, and the incubation was carried out at 0 °C for 30 min. For assessment of specific [^{14}C]N-ADP binding, two parallel series of incubations were carried out. In both series, the medium contained 120 mM KCl, 10 mM Mes, pH 6.5, and 1 mM EDTA (standard saline medium), and the incubation was carried out at 30 °C for 5 min. For the first series of tubes, the incubation was continued without any further addition for a 5-min period, and the particles were collected by centrifugation; the bound radioactivity reflected the sum of the specifically and unspecifically bound [^{14}C]N-ADP. The medium in the second series of tubes was supplemented with 5 nmol/mg of protein of CATR or BA, and the incubation was continued for 5 min at 30 °C; the radioactivity bound to those pellets after centrifugation corresponded only to the unspecifically bound [^{14}C]N-ADP. The difference between the two series was ascribed to the specifically bound [^{14}C]N-ADP. The pellets were treated with 1 mL of 5% Triton X-100 and 0.5 M NaCl, and radioactivity was determined by liquid scintillation.

Transport Assays. N-ADP transport was assayed by direct and back-exchange procedures (Duée & Vignais, 1969). In the direct-transport assay, 100 μM [^{14}C]ADP was added to mitochondria or sonic particles in suspension in the standard saline medium (see above section) at a concentration of 1 mg/mL. For the back exchange, mitochondria or sonic particles suspended at a concentration of about 15 mg of protein/mL in a 0.25 M sucrose, 5 mM Tris-HCl, pH 7.2, and 1 mM EDTA medium were loaded with [^{14}C]ADP by incubation with 100 μM [^{14}C]ADP for 30 min at 0 °C and then washed twice with the suspension buffer to remove external [^{14}C]ADP. Finally, they were resuspended in the standard saline medium (see above section) to a concentration of 1 mg of protein/mL. The transport reaction was initiated by addition of ADP or N-ADP and stopped after incubation periods ranging from 15 s to 15 min by rapid filtration through a 0.45-μm Millex filter (Millipore) fitted to a 5-mL syringe. The radioactivity of an aliquot fraction of the filtrate was determined by liquid scintillation. Inhibition of [^{14}C]ADP transport by N-ADP was performed with the direct-transport assay in the standard saline medium (Duée & Vignais, 1969).

Fluorescence Assays. Fluorescence assays were achieved 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 30 °C. Routinely, 0.5–2 mg of membrane protein was suspended in a final volume of 2.5 mL of standard saline buffer, pH 6.5, unless indicated.

Protein Modifications. Treatment of mitochondria with butanedione was carried out as described by Block et al. (1981a) in a medium containing 70 mM borate, pH 7.8, and 50 mM KCl at 25 °C. After appropriate incubation periods, 1-mL fractions were withdrawn and added to 4 mL of a chilled medium made of 50 mM KCl and 70 mM Mes buffer, pH 6.5, supplemented with [^{14}C]Ac-CATR or [^{3}H]BA. At identical periods, 2-mL fractions were acidified to pH 6.5 with 50 μL of 1 M Mes; then N-ADP was added to a final concentration of 10 μM, and the fluorescence changes upon addition of BA were measured at 30 °C as described earlier. Irradiation by UV light was performed as previously described by Block et al. (1979, 1981b). The medium used was a standard saline medium containing 120 mM KCl, 10 mM Mes, pH 6.5, and 1 mM EDTA at 20 °C.

Results

Fluorescent Properties of N-ADP. The fluorescent properties of N-ADP were markedly different from those of the dimethylaminonaphthoyl derivatives described by Schäfer &

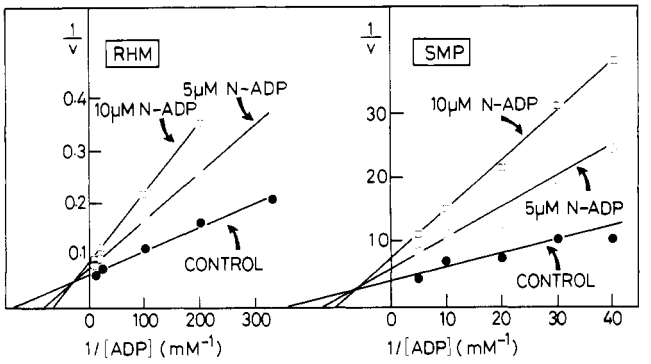

FIGURE 1: Inhibition of [^{14}C]ADP transport by N-ADP. Rat heart mitochondria (RHM) or sonic particles (SMP) were preincubated for 15 min at 0 °C with N-ADP in the standard saline buffer, pH 6.5, at a concentration of 1 mg/mL. Transport was initiated by the addition of [^{14}C]ADP and terminated with 10 μM CATR or BA (v is given in nanomoles per minute per milligram of protein).

Onur (1980). The fluorescence excitation spectrum of N-ADP in solution in water, or in buffered medium (from pH 6.5 to pH 7.4), or in dioxane was characterized by a single peak at 310 nm. The emission peak of N-ADP in water or buffer was centered at 395 nm; when N-ADP was in solution in dioxane, the fluorescence intensity was strongly quenched, and the emission peak was shifted to 368 nm. In kinetic fluorometric assays with N-ADP to be presented thereafter, the excitation wavelength was set at 310 nm and the emission wavelength at 395 nm.

Inhibitory Effect of N-ADP on ADP Transport. As shown by the direct-transport assay (cf. Experimental Procedures), there was no uptake of [^{14}C]N-ATP by mitochondria or sonic particles. With the more sensitive back-exchange technique, in which the particles are first loaded with [^{14}C]ADP, there was no release of radioactivity upon addition of N-ADP, even after an incubation of 20 min. This clearly demonstrated that N-ADP is not transported in mitochondria or sonic particles by the adenine nucleotide carrier.

Although N-ADP was not transported, it proved to be a potent inhibitor of ADP transport in mitochondria or sonic particles. The inhibition was of a mixed type (Figure 1) with K_i values of 5.5 μM and 4.5 μM for mitochondria and sonic particles, respectively. This similarity in K_i values has to be contrasted with the differences in K_M values for ADP transport in mitochondria and sonic particles (10 vs. 50 μM).

CATR- and BA-Induced Release of Bound [^{14}C]N-ADP. Upon incubation of [^{14}C]N-ADP with either mitochondria or sonic particles, a large fraction of radioactivity was recovered with the particles. The bound radioactivity increased with the amount of added [^{14}C]N-ADP, but the binding did not reach saturation (Figure 2). This was probably due to the large unspecific, low-affinity binding resulting from the hydrophobic nature of the naphthoyl moiety of [^{14}C]N-ADP and the subsequent partition of [^{14}C]N-ADP between the bulk aqueous medium and the lipid core of the membrane. Upon addition of CATR or BA to mitochondria and BA to sonic particles, a fraction of the bound [^{14}C]N-ADP was released. Because of the high specificity of CATR and BA for the ADP/ATP carrier, it is postulated that the fraction of bound [^{14}C]N-ADP released upon addition of CATR or BA corresponds to the [^{14}C]N-ADP specifically bound to the ADP/ATP carrier. The amount of released [^{14}C]N-ADP increased with the concentration of added [^{14}C]N-ADP up to a maximum of 1.6 and 1.5 nmol of [^{14}C]N-ADP/mg of protein for mitochondria and sonic particles, respectively. These numbers were found by extrapolation of Scatchard plots, corresponding to the data

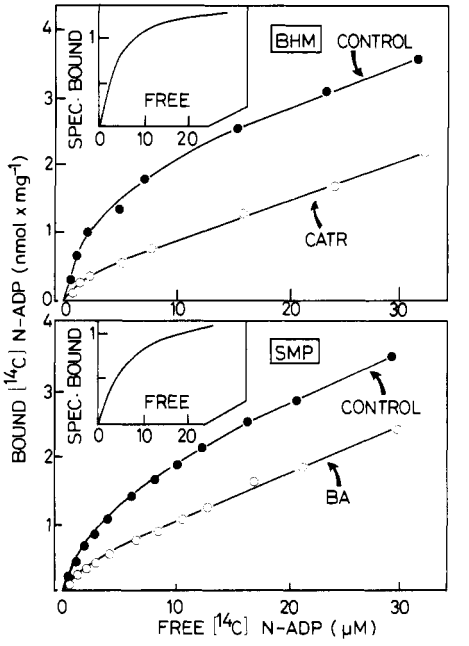

FIGURE 2: Specific binding of [^{14}C]N-ADP to the ADP/ATP carrier. Beef heart mitochondria (BHM) or sonic particles (SMP) (1.5 mg of protein) in 0.12 M KCl, 10 mM Mes, pH 6.5, and 1 mM EDTA were preincubated with increasing concentrations of [^{14}C]N-ADP at 30 °C for 5 min (final volume 3 mL). A control assay was carried out with aliquot fractions of 0.5 mg of protein without inhibitor. To other fractions of 0.5 mg of protein was added [^{14}C]N-ADP, followed by 5 nmol of CATR or BA. Incubation was performed for 5 min at 30 °C to attain full equilibrium between bound and free [^{14}C]N-ADP. The particles were then centrifuged. The pellets were digested with 1 mL of 5% Triton X-100 and 0.5 NaCl, and radioactivity was counted by liquid scintillation. The difference between the total bound [^{14}C]N-ADP and the bound [^{14}C]N-ADP after the chase by CATR or BA was taken as the specifically bound [^{14}C]N-ADP (inset).

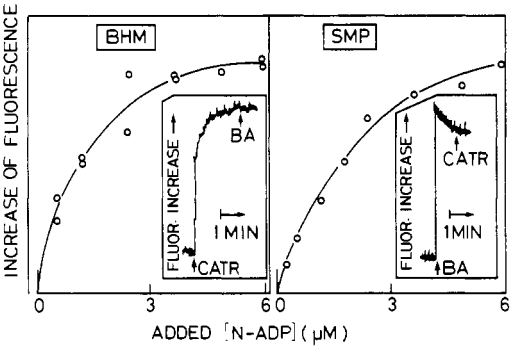

FIGURE 3: Fluorescence monitoring of N-ADP specific binding. Beef heart mitochondria (BHM) or sonic particles (SMP) (0.5 mg of protein) were suspended in 2.5 mL of the standard saline medium at 30 °C. Increasing amounts of N-ADP were added. The excitation light was set at 310 nm (2-nm band-pass), and the fluorescence emission was recorded at 395 nm (6-nm band-pass). After an incubation period of 5 min for stabilization of the fluorescence base line, CATR (5 nmol) and BA (5 nmol) were added (see inset). The increase in fluorescence emission shown in the inset correspond to about 5% of the initial fluorescence intensity.

of Figure 2, on the assumption of homogeneous binding sites with a K_d of about 3 μM for mitochondria and 6 μM for sonic particles.

Fluorescence Response of Specifically Bound N-ADP to CATR and BA. Following the addition of N-ADP to beef heart mitochondria or sonic particles, a plateau of fluorescence was attained in 2–3 min at 30 °C, indicating equilibrium between bound and free N-ADP. At that time, addition of CATR at a saturating concentration (5 nmol/mg of protein)

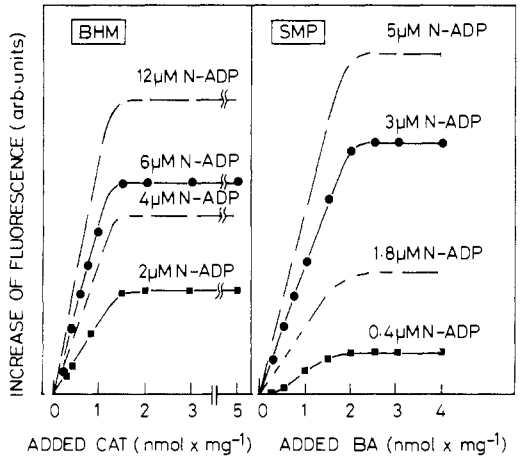

FIGURE 4: Titration of CATR and BA binding by fluorescence response of N-ADP. Beef heart mitochondria (BHM) or sonic particles (SMP) (2 mg of protein) were suspended in 2.5 mL of standard saline medium at 30 °C with increasing concentrations of N-ADP and left for 5 min to stabilize the fluorescence base line. Increasing amounts of CATR or BA were then added, and the fluorescence response was monitored as described in the legend of Figure 3. The sum of increments in fluorescence resulting from fractional additions of CATR or BA was equal to the maximal fluorescence increase obtained with a saturating concentration of CATR or BA.

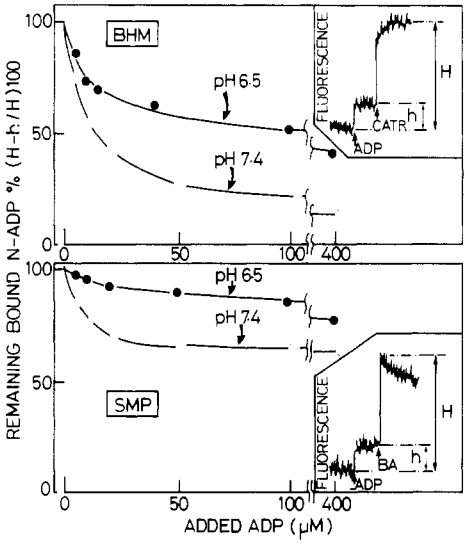

FIGURE 5: Removal of specifically bound N-ADP by ADP. Beef heart mitochondria (BHM) or sonic particles (SMP) were suspended in saline medium containing 0.12 M KCl, 1 mM EDTA, and 10 mM Mes (pH 6.5) or Mops (pH 7.4) at 30 °C at a final concentration of 0.8 mg of protein/mL. N-ADP was added at 10 μM. To aliquots of 2.5 mL were added increasing amounts of ADP, and the fluorescence response was monitored as shown in the inset, in the presence of either CATR (mitochondria) or BA (sonic particles). In the case of sonic particles, upon addition of BA, there was a quick increase of the fluorescence intensity that reached a peak and then slowly decreased to a stable intermediate value; H (see text) was chosen as the peak of the fluorescence response.

resulted in a rapid increase of the fluorescence intensity that stabilized in less than 1 min (Figure 3). The same observation holds true when CATR was substituted by BA (5 nmol/mg of protein). Once the plateau of fluorescence induced by either of the two inhibitors (CATR or BA) had been reached, no further increase was obtained upon addition of the other. It was confirmed that, at the concentrations used, CATR and BA did not interfere with the fluorescence emission of N-ADP. Consequently, by comparison of the radioactivity and fluorescence data, it may be concluded that the fluorescence increase resulting from addition of CATR or BA to the particles reflected the removal of specifically bound N-ADP. A plot of the fluorescence increase obtained at a fixed saturating concentration of CATR and increasing concentrations of added N-ADP is given in Figure 3. The fluorescence response was clearly saturable. Similar data were obtained for sonic particles (Figure 3), with the difference being that full fluorescence increase was produced by BA, but not by CATR, in accordance with the fact that the ADP/ATP carrier in sonic particles is accessible to added BA, but not to added CATR. The above results strongly suggest that the fluorescence response to CATR or BA is highly specific for the N-ADP bound to the ADP/ATP carrier. Further evidence that supported the specific nature of the fluorescence response came from titration of CATR and BA binding by the removal of the N-ADP bound to mitochondria and sonic particles. The mitochondria and sonic particles were first loaded with fixed concentrations of N-ADP, and then either CATR or BA was added at increasing concentrations to remove the bound N-ADP. Whatever the degree of saturation of the carrier by N-ADP, the end point of the titration was obtained with the same amount of CATR (1.3 nmol/mg of protein) in the case of mitochondria and of BA (2 nmol/mg of protein) in the case of sonic particles (Figure 4). Because of the very high affinity (K_d < 10 nM) and specificity of CATR for the carrier protein (Vignais et al., 1973), the concentration of CATR corresponding to the end point of the titration (1.3 nmol/mg of protein) is very close to the concentration of bound CATR at saturation, 1.1–1.2 nmol/mg of protein (Vignais, 1976; Block et al., 1980). This is not the case for BA; in fact, whereas BA binds with high affinity to the specific sites of the ADP/ATP carrier (Lauquin & Vignais, 1976), it also binds to unspecific sites that could not be differentiated in the above titration assay.

Interaction between ADP and N-ADP at the Level of the ADP/ATP Carrier. The partially competitive nature of the inhibition of ADP transport by N-ATP mentioned above strongly suggested interaction between N-ADP and the nucleotide site(s) of the ADP/ATP carrier. The ADP moiety appeared to be required for the inhibitory properties of N-ADP; for example, 3′-*O*-(1-naphthoyl)guanosine 5′-diphosphate (N-GDP) barely reacted with the ADP/ATP carrier as shown by the sluggish fluorescence response to CATR of mitochondria preincubated with N-GDP. The decisive role played by the ADP moiety in the recognition of N-ADP by the carrier is in accordance with the known restricted specificity of the carrier for ADP and ATP (Duée & Vignais, 1969). On the other hand, the K_d values of 20 μM and 15 μM found for the binding of [^{3}H]N-AMP to mitochondria and sonic particles were much lower than those found for [^{3}H]AMP under the same conditions (more than 100 μM), suggesting a possible strategic role of the naphthoic moiety in binding.

The identity of binding sites for N-ADP and ADP was checked by the chase experiment illustrated in Figure 5. The mitochondria or sonic particles were first incubated with a saturating concentration of N-ADP. Then aliquots were withdrawn and supplemented with increasing concentrations of ADP. The ADP-induced release of the bound N-ADP was assayed by fluorometry (Figure 5). The plateau corresponding to the stabilized release was noted (h). Then 5 μM CATR in the case of mitochondria and 5 μM BA in the case of sonic particles were added to elicit full release of the N-ADP bound to the carrier; this resulted in a new plateau of fluorescence (H). The $(H - h)/H$ ratio was taken as the fraction of N-ADP remaining specifically bound to the carrier. The ADP-induced

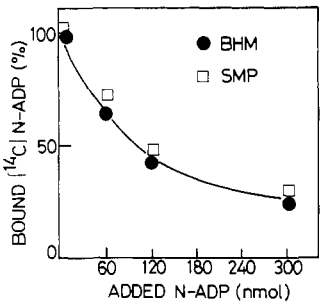

FIGURE 6: Removal of specifically bound [^{14}C]N-ADP by unlabeled N-ADP. Beef heart mitochondria (BHM) or sonic particles (SMP) (0.2 mg/mL) were incubated in the standard saline medium for 5 min at 30 °C with 60 nmol/mg [^{14}C]N-ADP. Aliquots of 5 mL (1 mg of protein) were dispensed into centrifuge tubes with increasing concentrations of unlabeled N-ADP and incubated for 5 min. Half of the volume, corresponding to 0.5 mg of protein was used as a control to determine the total amount of bound [^{14}C]N-ADP. To the other half was added 5 nmol of CATR or BA depending on whether mitochondria or sonic particles were used. After centrifugation the pellet was digested and the radioactivity counted. The difference between the control without inhibitor and the assay with CATR or BA was ascribed to the specifically bound [^{14}C]N-ADP.

release of bound N-ADP depended on pH and on the nature of the particles; it was more extensive at pH 7.4 than at pH 6.5 and with mitochondria than with sonic particles. The fact that N-ADP bound to mitochondria was released at pH 7.4 nearly to completion upon addition of an excess of ADP (400 μM) strongly suggested a genuine competition between N-ADP and ADP for binding. This was in contrast with the partial removal of bound N-ADP from mitochondria and sonic particles at pH 6.5 and from sonic particles at pH 7.4; the lack of displacement in the latter cases at high ADP concentrations could not be explained by an irreversible binding of N-ADP to the carrier, since upon addition of an excess of N-ADP, the bound [^{14}C]N-ADP was extensively released, virtually to the same extent in mitochondria and sonic particles (Figure 6).

Beef heart mitochondria bind between 0.6 and 0.7 nmol of ADP/mg of protein to specific sites on the ADP/ATP carrier (Weidemann et al., 1970; personal results). From the data of a preceding section, it is clear that beef heart mitochondria bind twice as much N-ADP (1.4–1.6 nmol/mg of protein) as ADP. In view of a possible half of the site reactivity of the carrier, an experiment was devised to check how the removal of half of the bound N-ADP by ADP in mitochondria saturated with N-ADP (Figure 5) can affect the efficiency of ADP transport. In the case of half of the site reactivity, no restoration of transport is expected. On the contrary, in the case of normal site reactivity, half-restoration of transport can be predicted. The experiment illustrated in Figure 7 was carried out in two consecutive steps. First, the mitochondria were incubated for 1 min at 30 °C and pH 6.5 with 20 μM N-ADP to saturate all the potential binding sites of the carrier protein. This was followed by an addition of 400 μM unlabeled ADP; the suspension was left to react with the added ADP for 1 min at 30 °C, a condition that results in the release of about half of the bound N-ADP (Figure 5). The mitochondrial suspension was then brought at 0 °C for assay of [^{14}C]ADP transport. The data of Figure 7 show that [^{14}C]ADP transport was not restored in spite of the fact that half of the bound N-ADP was chased; in fact, the initial rate of transport was still 90% inhibited. These results suggest but do not definitely prove that the ADP/ATP carrier functions according to a mechanism involving half of the site.

Effect of Chemical Modifications on the Binding of N-ADP. Previous reports have shown that a number of chemical

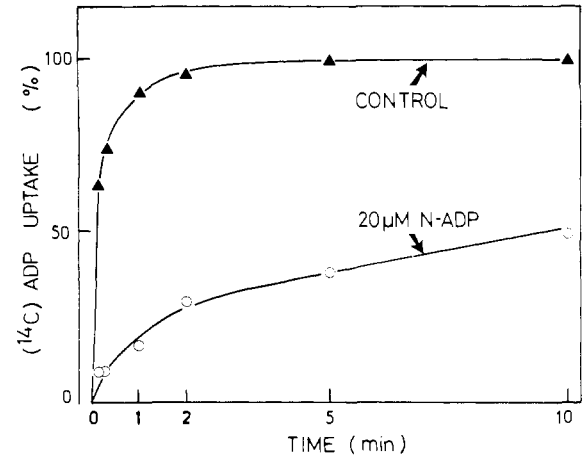

FIGURE 7: Residual inhibition of [^{14}C]ADP exchange, after removal of easily exchangeable bound N-ADP by ADP. Rat heart mitochondria at a concentration of 1 mg/mL in the standard saline medium were incubated 1 min at 30 °C with 20 μM N-ADP. Then 400 μM ADP was added, and after a further incubation of 1 min at 30 °C, particles were brought to 0 °C. At time zero, [^{14}C]ADP was added; aliquot fractions were withdrawn when indicated, and the nucleotide exchange was stopped with 10 μM CAT, followed by a centrifugation for 5 min at 22000g. A control experiment was carried out under similar conditions but without N-ADP. Although the kinetics of transport in control mitochondria was not well resolved, it is clear that the rate of [^{14}C]ADP transport in N-ADP-pretreated mitochondria was less than 10% of the rate in control mitochondria.

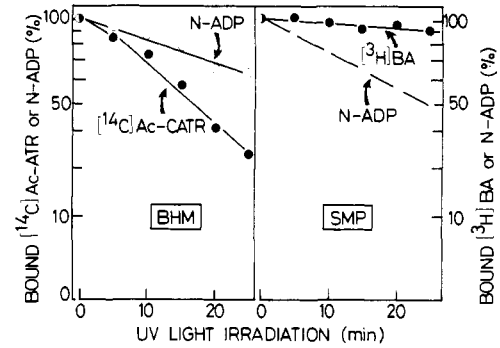

FIGURE 8: Time course of inactivation by UV light of the binding of [^{14}C]Ac-CATR, [^{3}H]BA, and N-ADP. Beef heart mitochondria (BHM) or sonic particles (SMP) were suspended in the standard saline medium at a final concentration of 1 mg of protein/mL and placed in a 10 cm diameter petri dish at 15 cm from a 15-W Philips T-UV lamp equipped with a reflector. After various periods of incubation, 1- and 2-mL aliquots were withdrawn. The 1-mL fractions were diluted with 4 mL of cold medium supplemented with 0.6 μM [^{3}H]BA or [^{14}C]Ac-CATR for binding assay (cf. Experimental Procedures). The 2-mL fractions were supplemented with 10 μM N-ADP, and the fluorescence response to BA addition was followed at 30 °C.

modifications of the membrane-bound ADP/ATP carrier induced differential inactivation of ATR and BA binding. In all cases, the ATR binding was more sensitive to the chemical modifiers (Block et al., 1979, 1981a,b). In the present work, we took advantage of the finding that the binding capacity for BA was only slightly changed by chemical modification to determine the amount of N-ADP specifically bound to the carrier protein; this was taken as the amount of N-ADP that could be removed by BA at 30 °C, as measured fluorometrically.

For inactivation by UV light, mitochondria and sonic particles were photoirradiated for different periods of time, and aliquots were withdrawn for direct-binding assays with [^{14}C]Ac-CATR (mitochondria) and [^{3}H]BA (sonic particles) and fluorometric assays of bound N-ADP (both particles) as

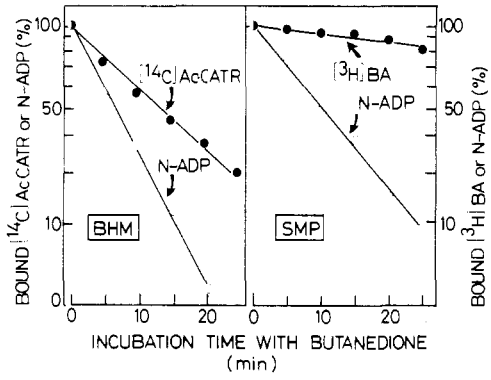

FIGURE 9: Time course of inactivation by butanedione of the binding of [^{14}C]Ac-CATR, [^{3}H]BA, and N-ADP. The incubation medium contained 70 mM borate buffer, pH 7.8, 50 mM KCl, beef heart mitochondria (BHM) or sonic particles (SMP) at a concentration of 1 mg of protein/mL, and 4 mM butanedione. After various periods of incubation at 25 °C, 1- and 2-mL aliquots were withdrawn as in the experiment of Figure 8 and used for [^{3}H]BA and [^{14}C]Ac-CATR binding and N-ADP binding, respectively.

described in the legend of Figure 8. In the case of mitochondria, the inactivation of [^{14}C]Ac-CATR binding was twice as fast as the inactivation of N-ADP binding. The kinetics of inactivation in both cases were of the pseudo-first-order type. The rates of inactivation of N-ADP binding in sonic particles and mitochondria were not very different (the half-times of inactivation were 35 and 25 min, respectively). As previously shown (Block et al., 1979), inactivation of BA binding by UV light was negligible.

As for UV light inactivation, the rates of inactivation by butanedione of N-ADP binding in mitochondria and sonic particles were not very different (the half-times of inactivation were 6 and 10 min, respectively) (Figure 9). Here again, the binding of BA was not much affected by butanedione. On the other hand, in contrast to what was observed for UV-light inactivation, butanedione treatment of mitochondria inactivated the binding of N-ADP nearly twice as fast as that of [^{14}C]Ac-CATR. In all cases, the kinetics of inactivation followed a pseudo-first-order pattern.

Discussion

In this paper we describe the binding properties of N-ADP, a fluorescent ADP analogue, with respect to the membrane-bound ADP/ATP carrier. N-ADP possesses a number of features that make it an interesting probe of the ADP/ATP carrier. (1) N-ADP is recognized by the ADP/ATP carrier but is not transported; (2) its binding to the carrier is associated with changes of the fluorescence yield; (3) the release of the specifically bound N-ADP may be achieved by inhibitors of ADP/ATP transport such as CATR, Ac-CATR, and BA and by the substrates ADP and ATP; (4) finally, N-ADP inhibits ADP/ATP transport, the inhibition being of a mixed type, competitive and noncompetitive. Because of these features, N-ADP is a convenient substitute for ADP to investigate in particular the unresolved problem of the unity or plurality of sites for substrates and inhibitors (Block et al., 1979, 1981a,b; Klingenberg & Appel, 1980).

Does the ADP/ATP Transport Unit Contain More Than One Substrate Site for ADP or ATP? In this work, the specific release of N-ADP from the ADP/ATP carrier was followed either by fluorometry, the release of bound N-ADP corresponding to a fluorescence increase, or by a radioactivity assay involving first the loading of the particles with [^{14}C]N-ADP, followed by specific release upon addition of CATR or BA. On the basis of the radioactivity assay, it was found that a maximal amount of 1.4–1.6 nmol of N-ADP/mg of protein binds to the carrier in either mitochondria or sonic particles. This value is roughly twice that found by the same differential method, with the labeled substrate [^{14}C]ADP (Weidemann et al., 1970; personal results). It is noteworthy that chase experiments reported here were carried out at 30 °C. At this temperature, the chases of bound N-ADP by CATR and BA were maximal. In other experiments to be reported later (personal unpublished results), it was shown that the chase by CATR was temperature dependent and typically biphasic at 10 °C.

The fact that the amount of specific binding sites determined for [^{14}C]N-ADP is nearly twice as high as that found for [^{14}C]ADP (Weidemann et al., 1970) could be due in part to the isotopic dilution of added [^{14}C]ADP by internal ADP. This, however, is unlikely. An alternative explanation, which is inherent to the mechanism of transport itself, is that the carrier protein exhibits a half-reactivity of the site with ADP and a normal one with N-ADP; in other words, on the assumption that the carrier is a dimeric protein (Brandolin et al., 1980; Hackenberg & Klingenberg, 1980) with two potential nucleotide sites,only one is reactive at a given time with the substrate ADP; but both of them can bind N-ADP. This would be consistent with some of the kinetic data presented in this paper (Figure 7). At this point, however, it must be mentioned that 1 mol of CATR appears to bind to 1 mol of carrier dimer (Klingenberg et al., 1978; personal unpublished results); although the number of N-ADP sites (1.4–1.6 nmol/mg of protein) is higher than the number of CATR sites (1.1–1.2 nmol/mg of protein) (Vignais, 1976; Block et al., 1980), it is not twice as much as it should be expected. In accordance with a half-reactivity of the substrate site, 2 mol of N-ADP would bind per carrier dimer. Clearly, further investigation is required to determine the possible half-reactivity of the nucleotide site in ADP/ATP transport. In the above hypothesis, the problem of the localization of the N-ATP sites was not examined; these sites can be located on the same side of the inner membrane or on opposite sides. Because of the high hydrophobicity of N-ADP, it is difficult to exclude the possibility that N-ADP is able to cross passively the mitochondrial membrane.

N-ADP binding sites are present in similar amounts in mitochondria and sonic particles; yet the binding sites in each type of particles differ by their affinity and readiness to displace bound N-ADP by added ADP. For example, at pH 7.4, more than 90% of the specifically bound N-ADP was displaced by ADP in mitochondria vs. 30% in sonic particles; at pH 6.5, the percentage of removable N-ADP were 50 and 10%, respectively. Thus there appears to exist at least two types of sites on the ADP/ATP carrier capable of binding N-ADP. These two types are present in different proportions in mitochondria and sonic particles. The sites of the first type, from which the bound N-ADP is readily released by ADP, are supposed to bind ADP and N-ADP equally well. The sites of the second type, which retain the bound N-ADP even at very high concentration of added ADP (400 μM), would interact more strongly with N-ADP than with ADP. The strong interaction of the latter sites with N-ADP may be related to their hydrophobic nature and the resulting hydrophobic interaction with the naphthoyl moiety of N-ADP. Acidic pH, which appears to increase the interaction of the carrier protein with N-ADP, could expose hydrophobic amino acid residues in or close to the nucleotide sites, favoring the hydrophobic interaction with N-ADP. It is reasonable to postulate that N-ADP binds to genuine ADP/ATP sites on the carrier. This

is in accordance with the nucleotide specificity of the sites (for example, the recognition of N-GDP is sluggish, and the affinity for N-AMP is less than that for N-ADP). Whether the nucleotide sites exhibiting strong interaction with N-ADP are normally present or are induced by N-ADP binding is not known at the present time.

Are ADP/ATP Sites Different from CATR and BA Sites? The ADP/ATP carrier binds firmly two specific inhibitors, CATR (or ATR) and BA. As shown by different approaches [for a review, cf. Vignais (1976)], CATR (or ATR) attacks the carrier in mitochondria from the outside and from the inside in sonic particles; on the other hand, BA attacks the carrier in mitochondria from the inside and in sonic particles from the outside. Two alternative interpretations have been put forward to explain this binding asymmetry. The first one by Klingenberg & Appel (1980) is that the carrier possesses a common binding site for ADP/ATP, CATR (or ATR), and BA; when this site is turned to the outside, it then has a conformation that recognizes and binds CATR (or ATR) [cytosolic (C) state]; when it is turned to the inside, it assumes a conformation that recognizes and binds BA [matrix (M) state]. The alternative explanation (Block et al., 1981a) is that CATR and BA binding sites are partially or totally independent and therefore reflect the asymmetric topography of the ADP/ATP carrier protein. The problem of the presence of one or several sites for substrate and inhibitors has been approached through the use of chemical modifiers and the analysis of their effect on binding of substrates and inhibitors. For a number of chemical modifiers, it was found that the binding of BA was less altered than that of CATR and that inactivation could be prevented by preincubation with the respective ligands; these and other data led us to conclude that CATR and BA bind to separate sites (Block et al., 1981b). This conclusion has been challenged by Klingenberg & Appel (1980). They propose on the basis of the single-site hypothesis that, upon chemical modification, the carrier site is moved to, and fixed in, the M state, which readily binds BA, but not CATR, which would explain the apparent inactivation of CATR binding. The carrier site in the M state can bind not only BA but also any recognizable nucleotide, e.g., N-ADP. Provided that the added N-ADP has access to the site in the M state (this is the case of sonic particles), it is possible to assay the effect of chemical modifiers on N-ADP binding. Under these conditions, it can be predicted that N-ADP binding will escape inactivation, as does BA binding. This prediction, which is inherent to the single-site theory as formulated by Klingenberg & Appel (1980), is not supported by our inactivation data that showed that in sonic particles, N-ADP binding was inactivated much more rapidly than BA binding. Moreover, the fact that in mitochondria, CATR binding is more sensitive than N-ADP binding to UV light and less sensitive to butanedione is also not consistent with a single binding site for CATR and N-ADP.

Although the chemical modifiers used may react with residues of amino acids at a distance from the specific binding sites for the different ligands envisaged, the inactivation effects observed are strongly in favor of distinct sites or of overlapping sites differing by at least a set of critical amino acid residues. It must be stressed that although the strategic amino acids involved in the binding of substrates and inhibitors are most likely different, they may be in a close neighborhood, which may result in overlapping sites. In short, the inactivation data presented in the present paper conclusively indicate that N-ADP (or any other recognizable nucleotide) and the inhibitors CATR (or ATR) and BA bind at sites that differ by at least a few amino acids.

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