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Exploration of the Nucleotide Binding Sites of the Isolated ADP/ATP Carrier Protein from Beef Heart Mitochondria. 1. Probing of the Nucleotide Sites by Naphthoyl-ATP, a Fluorescent Nontransportable Analogue of ATP†

Yves Dupont,* Gérard Brandolin, and Pierre V. Vignais

ABSTRACT: The ADP/ATP carrier protein was extracted and purified from beef heart mitochondria, and its binding parameters with respect to 3′-*O*-naphthoyladenosine 5′-triphosphate (N-ATP), a fluorescent nontransportable analogue of ATP, were studied. The binding of N-ATP to the isolated carrier protein was accompanied by a decrease in fluorescence. Conversely, the release of bound N-ATP upon addition of carboxyatractyloside (CATR) or ATP resulted in a fluorescence increase. The bound N-ATP that was released upon addition of an excess of CATR or ATP was referred to as specifically bound N-ATP, i.e., N-ATP bound to the nucleotide sites of the carrier protein. Two classes of binding sites for N-ATP could be identified; the number of high-affinity sites (K_d < 10 nM) was equal to the number of low-affinity sites (K_d = 0.45 μM). CATR behaved apparently as a noncompetitive inhibitor of the binding of N-ATP. The amount of N-ATP released increased linearly with the amount of CATR added, indicating an extremely high affinity of the carrier protein for CATR. The number of CATR binding sites was equal to half the total number of N-ATP binding sites (high- and low-affinity sites); at saturating concentrations of N-ATP, the binding of 1 mol of CATR resulted in the release of 2 mol of bound N-ATP, one from the high-affinity site and the other from the low-affinity site, showing unambiguously that each CATR site is interacting with a pair of probably interdependent N-ATP sites. A clear competition between N-ATP and ATP for binding to the carrier protein was demonstrated. The K_d values of the high- and low-affinity sites for ATP were <50 nM and 5 μM, respectively. In the presence of high concentrations of ATP, the two classes of N-ATP binding sites became indistinguishable, suggesting interconversion. It is proposed that the asymmetry in affinity for N-ATP binding is induced by the binding step itself, the carrier protein exhibiting a negative cooperativity for N-ATP binding.

Upon addition of substrates or inhibitors, the mitochondrial ADP/ATP carrier exhibits conformational changes which are reflected by unmasking of SH groups (Leblanc & Clauser, 1972; Vignais & Vignais, 1972) or antigenic determinants (Buchanan et al., 1976) and by modification of the intrinsic fluorescence of tryptophanyl residues (Brandolin et al., 1981). These conformational changes can be observed not only with the ADP/ATP carrier in the mitochondrial membrane but also with the isolated ADP/ATP carrier protein (Brandolin et al., 1981; Aquila & Klingenberg, 1982); they are probably related to the transport mechanism of ADP and ATP. To obtain more information about the molecular mechanism of ADP/ATP transport, we have studied nucleotide binding to the isolated carrier protein, by using fluorescent analogues of ATP. The binding of these fluorescent analogues to the nucleotide sites of the carrier can be readily assessed in fluorometric assays by chase with specific inhibitors, e.g., carboxyatractyloside (CATR)[1] and bongkrekic acid (BA).

In this paper we describe the binding properties of 3′-*O*-naphthoyladenosine 5′-triphosphate (N-ATP) to the isolated ADP/ATP carrier protein. In preliminary experiments (unpublished results), it was found that N-ATP is not transported in mitochondria or in inside-out submitochondrial particles but is able to bind to the carrier protein with high affinity. A similar study was performed with formycin triphosphate, another fluorescent analogue which differs from N-ATP in that it is transported (Brandolin et al., 1982). The data are interpreted in terms of interaction between several nucleotide binding sites located on the carrier.

Experimental Procedures

Materials. Nucleotides and carboxyatractyloside (CATR)[1] were purchased from Boehringer, 1-naphthoic acid was from

† From the Laboratoire de Biophysique Moléculaire et Cellulaire (CNRS/ER 199) (Y.D.) and Laboratoire de Biochimie (CNRS/ERA 903 et INSERM U.191) (G.B. and P.V.V.), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, Grenoble, France. *Received July 6, 1982.* This investigation was supported by a research grant from the Fondation pour la Recherche Médicale and from the Centre National de la Recherche Scientifique (ATP: Conversion de l'Énergie dans les Membranes Biologiques).

[1] Abbreviations: N-ATP, 3′-*O*-naphthoyladenosine 5′-triphosphate; CATR, carboxyatractyloside; BA, bongkrekic acid; LAPAO, lauryl-amido-*N*,*N*-dimethylpropylamine oxide; Mops, 3-(*N*-morpholino)-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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Fluka, and hydroxylapatite was from Bio-Rad. Bongkrekic acid was prepared as described in a previous report (Lauquin & Vignais, 1976). 3′-*O*-Naphthoyl-ATP (N-ATP) was synthesized by the method of Schäfer & Onur (1979), based on the use of the imidazolide derivative of 1-naphthoic acid (Gottikh et al., 1970). Laurylamido-*N*,*N*-dimethylpropylamine oxide (LAPAO) was synthesized as described by Brandolin et al. (1980). The ADP/ATP carrier protein from beef heart mitochondria was prepared by chromatography on hydroxylapatite (Krämer & Klingenberg, 1977). Purified LAPAO was used as the detergent instead of aminoxyde WS35, and further purification of the carrier protein was performed by chromatography on a column of AcA 202 (LKB) equilibrated in a medium containing 50 mM Mops, 0.1 mM EDTA, and 0.5% (w/v) LAPAO, at a final pH of 7.0. The protein fraction was eluted in the void volume. On the basis of the response of the intrinsic fluorescence of tryptophan in the isolated carrier in LAPAO to added ADP or ATP, it was found in a preceding paper (Brandolin et al., 1981) that only a fraction of the carrier molecules (20–40%) survived purification in detergent and responded homogeneously to the added nucleotides.

Fluorescence Techniques and Site Titration. N-ATP fluorescence was measured in a 1 × 1 cm fluorescence cuvette with continuous stirring in 2 mL final volume. Reagents were injected with Hamilton syringes in small volumes (2–20 μL). 3′-*O*-Naphthoyl-ATP fluorescence was excited at 312 nm with a 75-W Xe (Hg) lamp through a Zeiss M4QII monochromator. The emitted light was measured at right angle through a K1 (410 nm) Balzers filter.

Dissociation of bound N-ATP from the ADP/ATP carrier protein was induced by ATP or by antagonists like CATR. This dissociation was accompanied by a fluorescence increase, ΔF, which was measured over a large range of N-ATP concentrations. Since the N-ATP fluorescence quenching induced by binding to the protein was difficult to quantify, the concentration of free and bound N-ATP could not be directly calculated, and therefore the number of binding sites and the values of the binding constants could not be simply derived from the fluorescence data. In the case of a single type of sites and provided that the site concentration is within the same range as the dissociation constant, an appropriate graphical expression of the data is expected to result in a linear plot from which the above parameters can be calculated (Gutfreund, 1972). This method could not be applied, however, to the binding data presented below, since these data yielded nonlinear plots, indicating the existence of at least two types of binding sites. Consequently we chose to fit directly the fluorescence data with the analytical expression of $R = S_{bound}/(E_1 + E_2)$ obtained under the assumption that the substrate, S, is bound to two types of sites of total concentrations E_1 and E_2, characterized by the dissociation constants K_1 and K_2, respectively:

$$K_1 = e_1 s/\mathrm{Se}_1 \quad (1)$$

$$K_2 = e_2 s/\mathrm{Se}_2 \quad (2)$$

e_1, e_2, and s being respectively the concentrations of free sites and free substrate and Se_1 and Se_2 being the concentrations of liganded sites.

$$E_1 = e_1 + \mathrm{Se}_1$$

$$E_2 = e_2 + \mathrm{Se}_2 \quad (3)$$

$$S_0 = s + \mathrm{Se}_1 + \mathrm{Se}_2 = s + S_{bound}$$

Combining (1), (2), and (3) leads to

$$\mathrm{Se}_1 + \mathrm{Se}_2 = S_0 - s = E_1 s/(K_1 + s) + E_2 s/(K_2 + s) \quad (4)$$

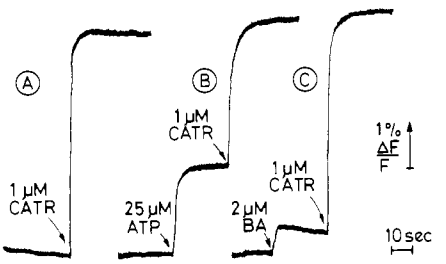

FIGURE 1: Fluorescence changes induced by addition of ATP, CATR, and BA, to the carrier protein–N-ATP complex. N-ATP fluorescence was measured at 410 nm with an excitation wavelength of 312 nm at 10 °C. The medium contained 80 mM glycerol, 40 mM Mops, pH 7.0, 4×10^{-5} M EDTA, and LAPAO 0.2% (w/v). The isolated ADP/ATP carrier protein was purified as described under Experimental Procedures. Its final concentration in the cuvette was 0.04 mg/mL. N-ATP was added at a final concentration of 2 μM. The concentrations of CATR, ATR, and BA are given in the figure. The fluorescence increases, $\Delta F/F$, reflected the release of N-ATP to the medium and were expressed relative to the initial fluorescence emission, F, of the added probe.

The free substrate concentration, s, is then the solution of the cubic equation

$$s^3 + as^2 + bs + c = 0 \quad (5)$$

with

$$a = E_1 + E_2 + K_1 + K_2 - S_0$$

$$b = E_1K_2 + E_2K_1 + K_1K_2 - S_0(K_1 + K_2)$$

$$c = -S_0K_1K_2$$

The analytical solution of eq 5 was calculated and introduced into a least-squares fit program referred to as "Maximum Likelihood Program" (MLP) (Ross, 1980). The MLP program provided the best adjustment of the fractional saturation $R = (S_0 - s)/(E_1 + E_2) = S_{bound}/(E_1 + E_2)$ to the relative fluorescence changes that were experimentally determined, $\Delta F/\Delta F_{max}$, the parameters adjusted being E_1, E_2, K_1, and K_2.

Results

Measurement of Specific Binding of N-ATP to the Isolated ADP/ATP Carrier Protein. The specific N-ATP sites in the carrier protein were explored by recording the fluorescence changes occurring when bound N-ATP was released from the carrier–N-ATP complex upon addition of specific ligands like CATR or ATP. For technical reasons, this indirect procedure was preferred to the direct binding assay. In brief, the carrier protein was mixed with N-ATP, and after a few seconds, when the fluorescence decrease was stabilized, CATR or ATP was added. A rapid fluorescence increase was observed that was ascribed to the release of bound N-ATP to the medium. Indeed, in previous binding experiments carried out with mitochondria and [^{14}C]N-ADP, the release of bound [^{14}C]N-ADP assayed by radioactivity counting was found to be correlated with fluorescent enhancement (Block et al., 1982). As shown in Figure 1 (trace A), addition of 1 μM CATR to a medium containing the carrier–N-ATP complex resulted in a rapid rise of fluorescence, which reached a stable plateau in less than 10 s. Further additions of CATR had no further effect, indicating a saturation effect. ATP used at saturating concentrations was able to induce the same fluorescence increase as CATR. When used at nonsaturating concentrations, CATR and ATP exhibited complementary effects (Figure 1, trace B).

The large amplitude of the fluorescence response to CATR or ATP addition contrasted with the small fluorescence enhancement resulting from addition of BA (Figure 1, trace C). Appropriate control assays were performed to ascertain that

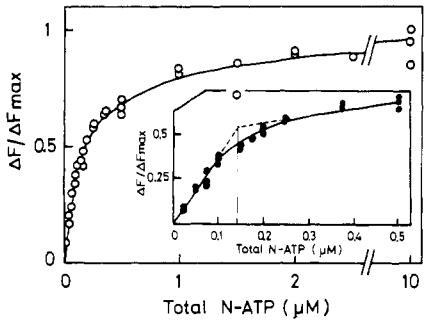

FIGURE 2: Specific binding of N-ATP to the ADP/ATP carrier protein. Experimental conditions as in Figure 1. For each N-ATP concentration, the specifically bound N-ATP was determined after release induced by addition of 2 μM CATR. The best set of parameters derived from the fitting procedure described under Experimental Procedures was $E_1 = 0.13 \pm 0.01$ μM, $E_2 = 0.14 \pm 0.10$ μM, $K_1 < 10$ nM, and $K_2 = 0.45 \pm 0.10$ μM. These values allowed calculation of the fractional saturation data that were used to build the curve shown in the figure.

the fluorescence changes of N-ATP were not produced by direct interactions with either ATP, CATR, or BA.

Characteristics of the Specific N-ATP Binding Sites in the Isolated ADP/ATP Carrier Protein. The extent of the increase in fluorescence induced by addition of saturating concentrations of CATR was measured over a large range of N-ATP concentrations, and the data were plotted in terms of relative fluorescence increase (Figure 2). At the low concentration of carrier protein used in the experiment of Figure 2, the shape of the binding curve was compatible with that of a binding process involving the presence of two types of binding sites. The linearity of the curve for added N-ATP concentrations below 0.1 μM suggested that all of the added N-ATP was bound to the carrier protein because of very high affinity for specific sites. At higher concentrations of N-ATP, a second class of binding sites of lower affinity became apparent. The fitting technique described under Experimental Procedures and applied to the binding data indicated that these two classes of N-ATP binding sites were present in equal concentrations, namely, 0.13 μM for the carrier protein preparation used in the experiment illustrated in Figure 2. One of them showed an extremely small dissociation constant (K_1 < 10 nM), contrasting with the much higher K_d value of the other (K_2 = 0.45 μM). From the total concentration of N-ATP sites in the carrier protein preparation, n = 0.26 μM, it was inferred that upon binding of N-ATP to the carrier protein the fluorescence of the probe was reduced to 60% of its initial value. When a minimal molecular weight of 30 000 is taken as reference, the carrier protein concentration of 0.04 mg/mL corresponds to 1.3 μM. When the concentrations of sites and carrier protein were compared, it was clear that only a fraction of the isolated carrier molecules was competent for binding N-ATP, most likely because it survived purification in detergent. It is inferred that the loss of the binding properties is an all-or-none process and that the surviving molecules are fully competent for nucleotide and CATR binding. Increasing the concentration of the carrier protein up to 4 times that used in routine assays did not change the binding characteristics of the specific N-ATP sites.

Titration of CATR Binding Sites by Release of Bound N-ATP. Titration of CATR binding sites was performed by measuring the amplitude of the relative increase in fluorescence $\Delta F/\Delta F_{max}$ caused by the release of bound N-ATP upon addition of increasing concentrations of CATR. ΔF_{max} was the maximal fluorescence increase obtained when the carrier protein incubated with a saturating concentration of N-ATP

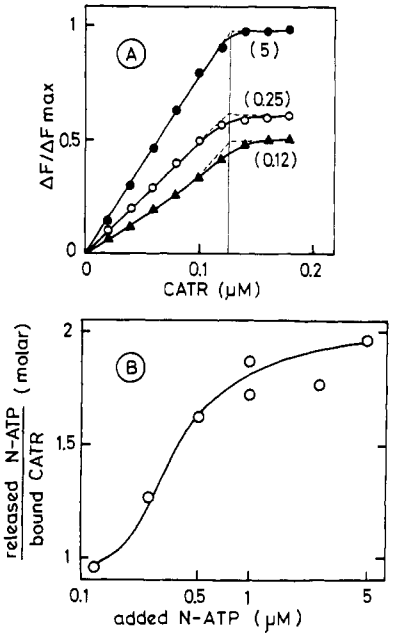

FIGURE 3: CATR-induced release of N-ATP from the ADP/ATP carrier protein. Experimental conditions as in Figure 1. (A) Three representative titration curves are given, showing the displacement of bound N-ATP by increments of 20 nM CATR. The total N-ATP concentration (μM) is indicated in parentheses. Note the strict linearity of the plot for concentrations of N-ATP higher than 0.12 μM and the appearance of a slight curvilinearity for 0.12 μM N-ATP. In the experiment shown, the CATR site concentration was 0.13 μM. (B) Molar ratio of released N-ATP to bound CATR as a function of added N-ATP. The concentration of CATR added, 0.13 μM, was sufficient to saturate the CATR sites; because of the very high affinity binding, the amount of the bound CATR could be equated to that of added CATR. The measurement of the concentration of N-ATP released was based on the fluorescence change of N-ATP upon binding (40% decrease) and on a total N-ATP site concentration of 0.26 μM. Note the logarithmic scale of the abscissa.

is treated by a saturating concentration of CATR. As shown in Figure 3A, for two largely different concentrations of N-ATP, a saturating concentration of 5 μM and a nonsaturating one of 0.25 μM, the titration was strictly linear up to 0.13 μM CATR which is the end point of the titration; from the peculiar shape of the titration curve, it could be inferred that the carrier protein displays a very high affinity for CATR binding with a dissociation constant of the carrier–CATR complex much lower than 10 nM. The concentration of the CATR sites was equated to the end point of the titration, i.e., 0.13 μM, since all the added CATR up to 0.13 μM was bound to the protein. This concentration was equal to half the total concentration of N-ATP sites, 0.26 μM, determined with the same protein preparation (see preceding section). Varying the N-ATP concentration between 0.2 and 10 μM had no effect on the linearity of the CATR effect, suggesting that CATR inhibits N-ATP binding noncompetitively. At or below 0.12 μM N-ATP, the CATR titration became nonlinear; it is likely that, at these low concentrations of N-ATP, a number of high-affinity N-ATP binding sites are not filled and that CATR preferentially binds to these free sites.

Whereas increasing the N-ATP concentration from 0.12 to 5 μM had no effect on the concentration of titrated CATR sites that remained equal to 0.13 μM (Figure 3A), the molar ratio N-ATP released/CATR bound exhibited a clear transition from 1 to 2, indicating the filling of the low-affinity N-ATP binding sites ($[N\text{-}ATP]_{1/2}$ = 0.45 μM) (Figure 3B). It could also be concluded that in the isolated carrier protein, a pair of N-ATP sites (high and low affinity) interacts with one single CATR binding site.

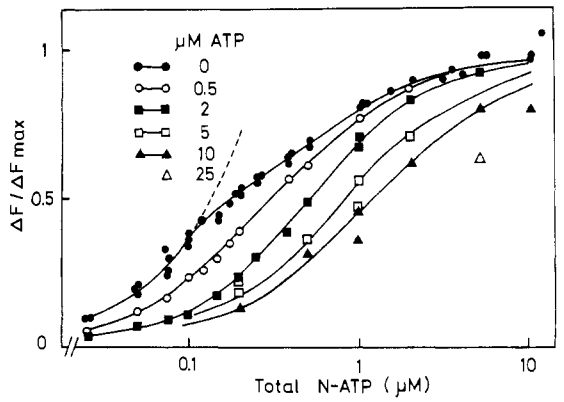

FIGURE 4: Competition between ATP and N-ATP for binding to the carrier protein. Experimental conditions as in Figure 1. Note the logarithmic scale of the abscissa. The remaining bound N-ATP in the presence of various fixed concentrations of ATP was measured after release by 2 μM CATR. Curves are the best fits obtained by the fitting procedure described under Experimental Procedures, as previously explained in Figure 2. The parameters used to compute these curves were also used in Figure 5. In the control N-ATP curve in the absence of added ATP, a high-affinity region was clearly distinguishable (extrapolated by a dotted line).

Effect of BA. As shown in Figure 1, the efficiency of BA to displace bound N-ATP from the ADP/ATP carrier protein was much lower than that of CATR. Displacement of bound N-ATP by BA depended on N-ATP concentration. At low concentrations of N-ATP (less than 1 μM), a significant fraction of bound N-ATP could indeed be released from the protein. For example, by use of 0.25 μM added N-ATP, about 60% of bound nucleotide was released upon addition of 10 μM BA. At higher concentrations of N-ATP, there was no further release of bound N-ATP. In contrast to the pH dependence of BA binding to mitochondria (Lauquin & Vignais, 1976), no pH effect was observed in the case of the isolated protein in a range of pH from 6.3 to 7.8. In all cases, CATR added after BA displaced completely the bound N-ATP.

Competition between ATP and N-ATP for Specific Binding Sites. ATP added at a saturating concentration to the carrier protein previously saturated with N-ATP induced full release of the bound N-ATP. A detailed investigation was conducted to test the concentration effects of the two ligands, ATP and N-ATP. For displacement of N-ATP, ATP was added, resulting in a fluorescence increase. The remaining bound N-ATP was determined by addition of an excess of CATR; this resulted in a second fluorescence increase, ΔF. The fractional saturation of N-ATP sites was assumed to be equal to $\Delta F/\Delta F_{max}$. In a preceding section, ΔF_{max} was defined as the maximal fluorescence increase obtained with a saturating concentration of CATR added to the carrier protein previously saturated with N-ATP.

The binding of N-ATP was measured at concentrations of ATP ranging between 0.5 and 10 μM (Figure 4). The observed effects of ATP were as follows: (1) ATP bound competitively to the N-ATP sites. This was demonstrated by the fact that the concentrations of N-ATP sites measured at saturating concentrations of N-ATP remained constant whatever the concentration of added ATP. Thus, the ATP-induced chase of bound N-ATP is clearly different from the CATR-induced chase that was apparently noncompetitive. (2) In the presence of very low concentrations of ATP, the N-ATP binding curve below 0.1 μM N-ATP deviated significantly from the characteristic titration slope. In accordance with the postulated competition between N-ATP and ATP, this indicated that the apparent K_d of the high-affinity binding sites for N-ATP increased, reaching measurable values.

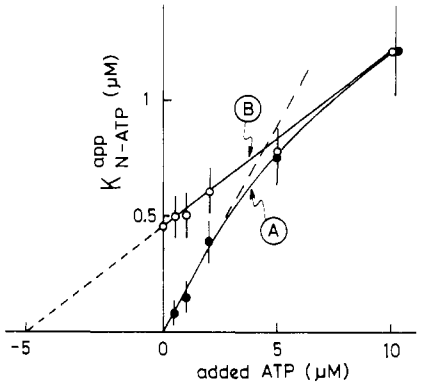

FIGURE 5: Variation of the apparent dissociation constant for N-ATP binding, ($K_{N\text{-}ATP}^{app}$), in the presence of increasing concentrations of ATP. Curve A refers to the high-affinity N-ATP sites E_1 and curve B to the low-affinity N-ATP sites E_2. The apparent dissociation constants were obtained from the binding data of Figure 4 by the fitting technique described under Experimental Procedures. The bars correspond to standard errors calculated by the least-squares fit method. Due to the low N-ATP site concentration used in these experiments, concentrations of added ATP were equated to concentrations of free ATP (see text).

Under the assumption that ATP and N-ATP compete for binding to the same site, the apparent binding constant for N-ATP can be expressed by the simple relationship $K_{(N\text{-}ATP)}^{app} = K_{(N\text{-}ATP)}(1 + [ATP]/K_{(ATP)})$, where $K_{(N\text{-}ATP)}$ and $K_{(ATP)}$ are the true dissociation constants for N-ATP and ATP, $K_{(N\text{-}ATP)}^{app}$ is the apparent dissociation constant for N-ATP, and [ATP] is the concentration of free ATP. Due to the low concentration of the N-ATP binding sites in the carrier protein preparation used, the concentration of added ATP which ranged between 0.5 and 10 μM (Figure 4) could reasonably be equated to the concentration of free ATP. The values of $K_{(N\text{-}ATP)}^{app}$ for different concentrations of added ATP were determined, by the fitting technique described under Experimental Procedures, from the experimental data of Figure 4, and were plotted against ATP concentrations (Figure 5). When the low-affinity N-ATP sites were considered, there was a linear relationship between the apparent dissociation constant, $K_{2(N\text{-}ATP)}^{app}$, and the concentration of added ATP, in accordance with a strict competition. The intercept with the abscissa gave an estimate of the true dissociation constant for the binding of ATP to low-affinity sites: $K_{2(ATP)} = 5$ μM. In the case of the high-affinity N-ATP sites, the intercept with the abscissa was close to zero, indicating that the dissociation constant $K_{1(ATP)}$ was too low to be determined. The only available value was the ratio $K_{1(ATP)}/K_{1(N\text{-}ATP)}$ which was found equal to 5.5 by measuring the slope of the plot near the origin in Figure 5. From this, it could be deduced that the upper estimate for $K_{1(ATP)}$ is 50 nM. At higher ATP concentrations (>5 μM), the plot of $K_{1(N\text{-}ATP)}^{app}$ vs. ATP concentration deviated clearly from linearity, so that $K_{1(N\text{-}ATP)}^{app}$ and $K_{2(N\text{-}ATP)}^{app}$ became indistinguishable. Indeed under these conditions, the N-ATP binding curves were correctly fitted with one single set of binding sites (see Figure 4). The curvilinearity of the plot corresponding to the high-affinity sites in Figure 5 indicated that competition of ATP and N-ATP for binding to the high-affinity sites of the carrier protein involved a complex mechanism. A plausible interpretation is that the change in the characteristics of the high-affinity N-ATP binding sites when ATP concentration increases is a consequence of the progressive filling of the low-affinity binding sites by ATP.

Discussion

The binding data reported in this paper show conclusively that N-ATP binds with high affinity to the isolated ADP/ATP

carrier protein from beef heart mitochondria. That the isolated carrier in detergent solution is able to recognize adenine nucleotides was previously demonstrated by specific and fast intrinsic fluorescence changes of the carrier protein induced by low concentrations of ATP or ADP (Brandolin et al., 1981). Because of its fluorescence properties, N-ATP is a useful probe to investigate the properties of the nucleotide binding sites in the isolated carrier. Several lines of evidence indicate that the binding sites occupied by N-ATP on the carrier protein can be identified as those associated with ATP: (1) An apparently noncompetitive antagonistic effect of CATR on N-ATP binding was observed, similar to that of CATR on ADP/ATP transport or ADP/ATP binding to the carrier protein in mitochondria (Vignais et al., 1973) or in a reconstituted system (Brandolin et al., 1981). (2) ATP and N-ATP were found to compete for two classes of sites differing in affinity. (3) The chases of N-ATP by CATR and by ATP had the same maximal amplitude when CATR and ATP were added at saturating concentrations; they were additive when CATR and ATP were used at nonsaturating concentrations. Of particular interest is the finding that BA is a weak competitor for N-ATP binding. In earlier studies, synergistic effects of BA and ATP (ADP) were reported (Lauquin & Vignais, 1976) and interpreted on the basis that the binding and the inhibitory effect of BA are facilitated by an ATP- or ADP-induced conformational change of the carrier. This hypothesis was supported by recent data dealing with the effect of BA on the tryptophanyl fluorescence of the isolated carrier protein (Brandolin et al., 1981). The fact that N-ATP is not transported by the carrier accounts for its inability to induce a key conformational change of the protein essential for the transport process and may explain the sluggish effect of BA on N-ATP binding.

The major finding of this study is that the solubilized ADP/ATP carrier protein possesses at least two pools of N-ATP binding sites (high and low affinity) of equal size which most likely correspond to two classes of ADP/ATP sites. In addition, the data concerning the chase of bound N-ATP by CATR clearly indicate that each CATR binding site is interacting with a pair of N-ATP sites made of one high-affinity site and one low-affinity site; these sites are most probably interdependent. The noncompetitive behavior of the chase by CATR provides further evidence for the existence of an inhibitor site that does not coincide exactly with the nucleotide binding site(s) in the carrier protein (cf. Block et al., 1981). That high- and low-affinity N-ATP sites reflect a heterogeneous population of carrier molecules is hardly probable since in a large number of carrier protein preparations, it was found that the stoichiometric ratio of high- to low-affinity N-ATP sites is 1 and that 1 mol of CATR is able to chase 2 mol of N-ATP, one bound with high affinity and the other with low affinity.

It was proposed on the basis of hydrodynamic studies (Hackenberg & Klingenberg, 1980) and electron microscopic data (Brandolin et al., 1980) that the ADP/ATP carrier protein is organized as a dimer. Under the assumption that the active site is shared by the two subunits, it was postulated that steric hindrance would prevent the binding of more than one substrate per dimer (Klingenberg, 1981); on kinetic grounds, this proposal would involve a ping-pong mechanism. However, as reported by Duyckaerts et al. (1980) and Barbour & Chan (1981), the kinetic behavior of the ADP/ATP exchange does not obey a ping-pong mechanism but rather involves the simultaneous binding of two nucleotides. This is consistent with our finding of two associated types of nucleotide binding sites per CATR site in the carrier protein. A way to reconcile our results with the model proposed by Klingenberg would be to assume an intrinsic asymmetry of the N-ATP binding sites; thus only one class of sites should be involved in the transport of ATP or ADP, the second one having a putative regulatory role. However, the results obtained in competition experiments dealing with N-ATP vs. ATP do not favor this hypothesis. Indeed, in the presence of high concentrations of ATP, the two classes of sites become indistinguishable, suggesting that they are interconvertible. A plausible consequence of this assumption is that the asymmetry in affinity for N-ATP binding is induced by the binding step itself, the ADP/ATP carrier protein exhibiting a negative cooperativity at least for N-ATP binding. Further experiments are necessary to substantiate this conclusion and to extend it to the natural substrates ADP and ATP. However, the fact that binding of ATP to the low-affinity sites is able to modify the affinity of the other sites for N-ATP binding is a clear indication that the characteristics of ATP binding depends on site–site interactions.

Acknowledgments

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