Exploration of the Nucleotide Binding Sites of the Isolated ADP/ATP Carrier Protein from Beef Heart Mitochondria. 2. Probing of the Nucleotide Sites by Formycin Triphosphate, a Fluorescent Transportable Analogue of ATP†

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ABSTRACT: Formycin triphoshate (FTP), a fluorescent transportable analogue of ATP, was used to probe the nucleotide binding sites of the isolated ADP/ATP carrier protein from heart mitochondria. The binding data were compared to those obtained with 3′-*O*-naphthoyladenosine 5′-triphosphate (N-ATP), a nontransportable nucleotide [see Dupont, Y., Brandolin, G., & Vignais, P. V. (1982) *Biochemistry* (preceding paper in this issue)]. When FTP was bound to the carrier protein, the nucleotide fluorescence was increased, reaching 140% of its initial value. Conversely, the release of bound FTP resulted in fluorescence quenching. The specifically bound FTP was assessed by release upon addition of the specific inhibitor carboxyatractyloside (CATR). Although the same number of sites per milligram of carrier protein was found for FTP and N-ATP, four classes of sites were demonstrated for FTP in contrast to two classes for N-ATP. One of the four FTP sites exhibited a very high affinity for FTP (K_d < 10 nM), contrasting with the low affinity of the others (0.5–2 μM). The four FTP sites could also be differentiated by their unequal sensitivity to CATR and bongkrekic acid (BA). The chase of bound FTP by increasing concentrations of CATR indicated the existence of two types of CATR binding sites differing in their affinity for CATR, one type (CATR α sites) being characterized by a very high affinity (K_d < 10 nM) and the other (CATR β sites) exhibiting a much lower affinity ($K_d \simeq 5$ μM). The total number of CATR sites was half the total number of FTP sites; half the CATR sites were of the α type and the other half of the β type. The binding of one molecule of CATR to the α site resulted in the release of two molecules of FTP from high- and low-affinity FTP sites, respectively. On the other hand, the binding of one molecule of CATR to the CATR β site was accompanied by the release of two molecules of FTP, both from low-affinity FTP sites. FTP binding exhibited sensitivity to BA. One site of high affinity for BA per four FTP sites was identified (K_d < 10 nM). The binding of BA to this site was responsible for the release of only one-fourth of the total amount of FTP bound at saturation level; the released FTP belonged most likely to one of the low-affinity FTP sites of the carrier. Increasing the concentration of BA to 10 μM resulted in further release of bound FTP up to 50–60%; however, total release of all specifically bound FTP could not be achieved by addition of BA, even at exceedingly high concentrations of this inhibitor (30 μM). ATP was found to compete for binding to both high-affinity and low-affinity FTP sites, consistent with the view that FTP binds to the nucleotide sites of the carrier. The peculiar binding properties of the isolated ADP/ATP carrier protein with respect to N-ATP and FTP, and the related effects of CATR and BA, could be explained by assuming that FTP, but not N-ATP, induces a specific conformational change of the carrier protein. This change of conformation might be responsible for the ability of FTP to be transported by the membrane-bound ADP/ATP carrier.

Recent studies on the kinetics of ADP/ATP exchange catalyzed by the mitochondrial ADP/ATP carrier have indicated that the mechanism of nucleotide transport involves the cooperation of two or more nucleotide binding sites (Duyckaerts et al., 1980; Barbour & Chan, 1981). These results involve the necessary formation of a ternary $(Adn)_2$–carrier complex and are at variance with the view that the ADP/ATP carrier contains only one ADP/ATP binding site which is alternatively oriented to the inside and the outside (Klingenberg & Appel, 1980). The first direct evidence for the existence of at least two classes of nucleotide binding sites on the ADP/ATP carrier has been described in the preceding paper (Dupont et al., 1982) in which the nucleotide binding sites of the isolated carrier in detergent were investigated with 3′-*O*-naphthoyladenosine 5′-triphosphate (N-ATP),[1] a fluorescent, nontransportable, ATP analogue.

The purpose of the present paper is to report on a similar study with formycin triphosphate (FTP), another fluorescent analogue of ATP (Ward et al., 1969a,b), which differs from N-ATP in that it is transported by the ADP/ATP carrier (Schlimme et al., 1980). The binding properties of FTP and the antagonistic effects of specific inhibitors were found to be significantly different from those described for N-ATP. Comparison of the binding properties of N-ATP and FTP provides further information on the mechanism of action of the ADP/ATP carrier.

Experimental Procedures

FTP was purchased from Calbiochem. Other reagents and the ADP/ATP carrier preparation in laurylamido-*N*,*N*-dimethylpropylamine oxide (LAPAO)[1] were obtained as described in the preceding paper (Dupont et al., 1982). Fluorescence measurements were performed by using the same

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[1] Abbreviations: FTP, formycin triphosphate; N-ATP, 3′-*O*-naphthoyladenosine 5′-triphosphate; CATR, carboxyatractyloside; BA, bongkrekic acid; LAPAO, laurylamido-*N*,*N*-dimethylpropylamine oxide; AdN, adenine nucleotide ADP or ATP.

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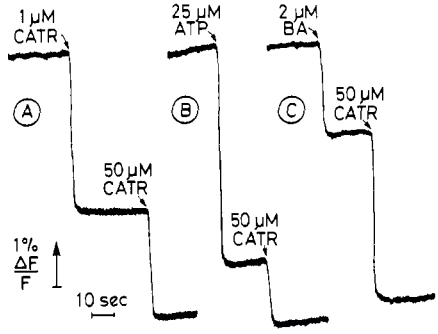

FIGURE 1: Fluorescence changes of bound FTP upon addition of ATP, CATR, and BA to the carrier protein. FTP fluorescence was measured at an emission wavelength of 370 nm, the excitation light being centered at 312 nm. Temperature was 10 °C. The experiments were performed in a medium containing 80 mM glycerol, 40 mM Mops, 4×10^{-5} M EDTA, and 0.2% w/v LAPAO at a final pH of 7.0. The concentration of purified ADP/ATP carrier protein was 0.04 mg/mL. FTP was used at the final concentration of 5 μM. Other concentrations are indicated in the figure.

methodology as for N-ATP. Formycin triphosphate fluorescence was excited at 312 nm with a 75-W Xe (Hg) arc lamp. The emitted light was measured at right angle through a 0.52 filter (corning) coupled to an ultraviolet light filter. The resulting band-pass was centered at 370 nm with a peak transmission of 63% and a width of 40 nm at half-maximum of the peak. As previously shown, changes in the intrinsic fluorescence of the ADP/ATP carrier protein occur upon addition of ATP and other ligands in the range 320–350 nm (Brandolin et al., 1981). In the present work, control assays carried out without FTP showed that the changes in the emitted light at 370 nm upon addition of ATP, CATR, or BA were negligible as compared to those due to FTP fluorescence.

Evaluation of the FTP binding sites was performed with the fitting procedure described by Dupont et al. (1982). Transport assays were performed following a back-exchange technique (Duée & Vignais, 1969) with rat heart mitochondria preloaded with [^{14}C]ADP.

Results

Principle of Measurement of Specific FTP Binding Sites on the Isolated ADP/ATP Carrier Protein. FTP is a fluorescent nucleotide which like N-ATP binds to the carrier protein; in addition it is transported across the inner mitochondrial membrane (Schlimme et al., 1980). The transport rate obtained by using FTP as external substrate and rat heart mitochondria loaded with [^{14}C]ADP was about half that measured with external ATP; the K_M value was in the range 20–50 μM.

Like for N-ATP, the specific FTP binding to the carrier protein was measured by an indirect technique based on the release of bound FTP upon addition of specific ligands (cf. Dupont et al., 1982). After FTP was mixed with the protein and stabilization of the fluorescence (1–2 min) was allowed for, a rapid quenching of fluorescence was induced by addition of either CATR, BA or ATP (Figure 1). These specific ligand-induced responses of fluorescence recall those obtained with N-ATP and were likewise ascribed to the release of the bound FTP into the aqueous medium. However, a significant difference in the responses that pertained to the nature of the probes used was that the release of N-ATP was reflected by fluorescence enhancement whereas that of FTP was accompanied by fluorescence quenching. High concentrations of CATR and ATP induced equivalent falls in fluorescence, and the effects of CATR, BA, and ATP were complementary when used at nonsaturating concentrations (Figure 1).

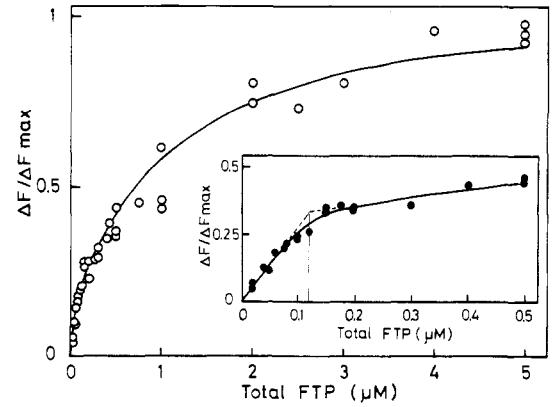

FIGURE 2: Titration of the specific FTP sites on the ADP/ATP carrier protein. Same experimental conditions as in Figure 1. Specifically bound FTP was measured by total displacement by a large excess of CATR (50 μM), the displacement corresponding to a decrease in fluorescence intensity. The curve was a fit obtained by the procedure described in the preceding paper (Dupont et al., 1982). Parameters are listed in Table I.

Results obtained with BA on FTP binding were at variance with those reported for N-ATP. In fact, BA induced the dissociation of a significant amount of bound FTP, whereas the response of bound N-ATP to BA was small; nevertheless saturating effects of BA were difficult to achieve. This point will be considered later.

Titration of Specific FTP Binding Sites. Although 2 μM CATR was sufficient to displace all the bound N-ATP (Dupont et al., 1982), a concentration of CATR as high as 50 μM was required for complete removal of bound FTP (Figure 1). It is noteworthy that the effect of CATR was typically saturating, indicating specific binding. In the present work, release of bound FTP was routinely assayed by addition of 50 μM CATR. As for N-ATP, the binding curve for FTP was biphasic, and at a low concentration of the probe, a linear titration of high-affinity binding sites for FTP was evident (Figure 2).

The fitting procedure detailed in the preceding paper (Dupont et al., 1982) was applied to describe the FTP binding parameters; two species of FTP sites only (high and low affinity) were assumed, since the accuracy of the data did not allow a more refined analysis. The experimental data were satisfactorily fitted for low concentrations of FTP; a concentration of 0.11 μM was calculated for the high-affinity FTP sites; the K_d value relative to these sites was close to zero (<10 nM) (Table I). Saturation of the low-affinity sites was attained at much higher concentrations of FTP, >4 μM. At these high concentrations of FTP, the accuracy of fluorescence measurements was limited, and an approximate value of 0.30–0.50 μM was obtained for the concentration of low-affinity FTP sites; this value was roughly 3–4 times higher than that of the high-affinity sites. The K_d value of the low-affinity FTP sites was between 0.8 and 2 μM. The total calculated concentration of FTP binding sites, 0.4–0.6 μM, was similar to that obtained, 0.44 μM, with N-ATP as substrate and the same carrier protein sample (see Table I). Since the titration of N-ATP sites was more accurate than that of FTP sites, the total concentration of FTP sites in the sample used (Table I) could be equated to that of N-ATP sites, i.e., 0.44 μM. The ratio of high- to low-affinity FTP sites being virtually equal to 1/3, this implied that there were four classes of nucleotide binding sites per carrier unit. The concentration of carrier protein used in the present experiment was 0.04 mg/mL, which is equivalent to 1.3 μM, based on a molecular weight of 30000. It is therefore clear that only a fraction of the carrier molecules

Table I: Comparison of Titration Data of Nucleotide and Inhibitory Sites in the Isolated ADP/ATP Carrier Protein, Using FTP and N-ATP as ATP Analogues and CATR and BA as Inhibitory Ligands[a]

substrate[b]	sites titrated[c]	concentration μM	concentration normalized	K_d
FTP	FTP, $n = 2$	0.11 ± 0.02[d]	1.0	<10 nM[e]
		0.30–0.50	2.8–4.5	0.08–2 μM
	CATR, $n = 2$	0.11 ± 0.01	1.0	<10 nM[e]
		0.11[f]	1.0	5 μM
	BA, $n > 1$[g]	0.10 ± 0.03	1.0	<10 nM
N-ATP	N-ATP, $n = 2$	0.22	2.0	<10 nM[e]
		0.22[h]	2.0	0.45 μM
	CATR, $n = 1$	0.22	2.0	<10 nM[e]
	BA[i]	nd		nd

[a] The concentrations of N-ATP and FTP binding sites and the corresponding K_d values as well as inhibitor data were determined with the same preparation of ADP/ATP carrier protein. [b] Substrate for the nucleotide site. [c] n = number of classes of sites. [d] Errors are standard errors calculated from the least squares fit method. [e] $K_d < 10$ nM refers to sites that were filled by all the added ligand. Their affinity was too high to be measured accurately, and only a maximal value of K_d can be given. [f] This value corresponds to the difference between the total concentration of CATR sites obtained by titration with N-ATP (0.22 μM) and the concentration of high-affinity CATR sites obtained by titration with FTP (0.11 μM). [g] Because of the sluggish and uncomplete chase of FTP by exceedingly large concentrations of BA, the binding parameters for low-affinity BA sites could not be determined; only the parameters for the high-affinity BA site are given. [h] The total concentration of N-ATP sites in this carrier protein preparation was 0.44 μM. The total concentration of N-ATP sites reported for another sample in the preceding paper (Dupont et al., 1982) was 0.26 μM. It is understood that the site concentration may vary from preparation to preparation. [i] No significant effect of BA on N-ATP binding was found.

is competent for FTP binding as previously shown for N-ATP binding [see Dupont et al. (1982)]. On the other hand, from the total concentration of FTP binding sites, it was estimated that binding of FTP to the carrier protein was accompanied by a fluorescence increase of 140% with respect to its initial value.

Titration of CATR Binding Sites. Titration of CATR binding sites was performed by releasing bound FTP by consecutive increments of CATR, a procedure similar in principle to the chase of bound N-ATP described in the preceding paper (Dupont et al., 1982). In the experiment illustrated by Figure 3, the total concentration of FTP binding sites on the carrier protein was identical with that found in the experiment of Figure 2, i.e., 0.44 μM. The profile of the chase of bound FTP as a function of the concentration of CATR was more complex than that of bound N-ATP. In fact, when the profile of the removal of FTP by CATR was examined at a saturating concentration of FTP (5 μM), two regions could be identified with respect to the CATR concentration, indicating the existence of two types of CATR sites differing by affinity for CATR. The high-affinity sites were referred to as α sites and the low-affinity sites as β sites. Each of these regions corresponded to the release of about half of the total concentration of bound FTP, i.e., 0.22 μM. The first half of bound FTP was released by low concentrations of CATR (less than 1 μM); this corresponded to a linear titration of high-affinity CATR sites designated as CATR α sites ($K_d < 10$ nM). The concentration of CATR α sites (0.11 μM) was equal to that of the high-affinity FTP binding sites, i.e., approximately one-fourth of the total concentration of FTP sites (Table I). Whatever the concentrations of FTP used, the concentration

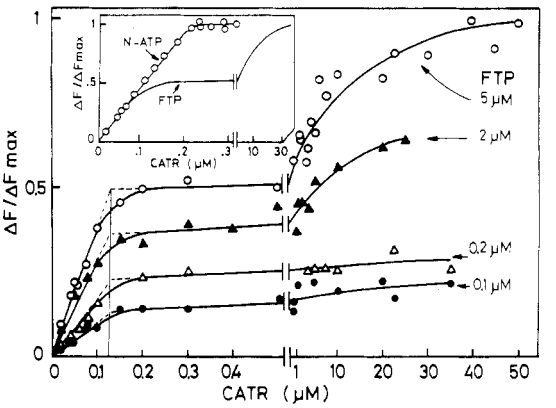

FIGURE 3: Displacement of specifically bound FTP by added CATR. Same experimental conditions as in Figure 1. The fluorescence decrease produced by release of FTP was measured after each consecutive addition of CATR, for the indicated FTP concentrations. The total concentration of FTP sites in this preparation was the same as in the experiment of Figure 2 (0.44 μM). (Insert) Displacement of bound N-ATP (5 μM added N-ATP) by CATR. The titration was made on the same sample of ADP/ATP carrier protein as that used for displacement of bound FTP by CATR. For comparison, the CATR titration curve corresponding to 5 μM FTP is shown in the insert.

of CATR α sites titrated was the same, and the titration curves remained linear (Figure 3). This provided evidence for the noncompetitive interaction between FTP and the CATR α sites. Because of the very high affinity of the α sites for CATR, it will be assumed that all the added CATR below 0.11 μM is equal to bound CATR.

When a saturating concentration of FTP, for example, 5 μM, and a concentration of CATR high enough to saturate the CATR α sites, for example, 0.4 μM were used, the ratio of FTP released (0.22 μM) to CATR bound (0.11 μM) was 2 (Figure 3) (it is anticipated that added at concentrations higher than 1 μM, CATR binds not only to the high-affinity CATR α sites but also to the low-affinity CATR β sites). At very low concentrations of FTP, for example, 0.1 μM, which saturated only high-affinity sites, addition of CATR at the same concentration, i.e., 0.1 μM, resulted in the complete release of the bound FTP. In other words, when the concentration of FTP added to the carrier protein was decreased from saturating to a very low level, the ratio of FTP released to CATR bound to the α sites decreased from 2 to 1 ($[FTP]_{1/2}$ = 1 μM). From these data, it can be concluded that the CATR α sites interact with half of the total FTP binding sites and that to each CATR α site corresponds two FTP sites susceptible to CATR. One of them is the high-affinity FTP binding site demonstrated in Figure 2, and the second is a low-affinity binding site filled at micromolar FTP concentrations.

The release of the second half of bound FTP required much higher concentrations of CATR than the first half ($[CATR]_{1/2}$ = 2 μM). Due to this relatively low affinity, direct titration of this second pool of CATR sites referred to as CATR β sites was not feasible. However, as seen in the inset of Figure 3, the concentration of CATR binding sites titrated by using N-ATP as ligand was 0.22 μM, a value twice as high as that of the CATR α sites (0.11 μM) titrated by using FTP as ligand; thus only half of the total CATR sites are recognized as high-affinity CATR α sites. A reasonable assumption therefore is that the high-affinity CATR α sites and the low-affinity CATR β sites are present in equal amounts, i.e., 0.11 μM. Each CATR β site would be able to interact with two low-affinity FTP sites. This can be compared to the interaction of each CATR α site with two FTP sites, one of high affinity

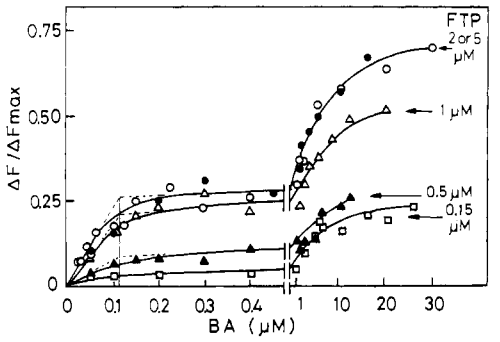

FIGURE 4: Amplitude of the FTP chase induced by BA. Same conditions as in Figure 3, except that BA was used instead of CATR.

and the other of low affinity (see above).

Effect of BA. The antagonistic effect of BA on the binding of FTP to the ADP/ATP carrier protein was demonstrated in Figure 1. A complete titration of the response to BA for various fixed concentrations of FTP is illustrated in Figure 4. As already observed for CATR, the BA-induced release of FTP was clearly biphasic. At saturating concentrations of FTP (>2 μM), high- and low-affinity BA binding sites were revealed. From the titration obtained for low concentrations of BA (<0.4 μM) using saturating concentrations of FTP, a concentration of high-affinity BA sites close to 0.12 μM was derived. This concentration was similar to that of the high-affinity CATR α binding sites and of the high-affinity FTP binding sites. Binding of BA to this site resulted in the release of FTP from a quarter of the total FTP binding sites. It is noteworthy that the high-affinity BA site could not be revealed at low concentrations of FTP, for which only the high-affinity FTP sites were saturated. In summary, one high-affinity BA site per four FTP sites was titrated; this BA site presumably interacted with a low-affinity FTP site.

Concentrations of BA higher than 1 μM were able to release more bound FTP; for example, when a saturating concentration of FTP was used, 50% of bound FTPs were released by 5 μM BA. Even at nonsaturating concentrations of FTP, for which only the high-affinity FTP sites were filled, a very significant release of bound FTP could be achieved at 5 μM BA. However, full release of all bound FTP could not be achieved even at exceedingly high concentrations of BA, for example 30 μM. A small effect of BA was also found with the N-ATP liganded carrier under any experimental conditions [see Dupont et al. (1982)].

Comparison of the CATR- and BA-Sensitive FTP Binding Sites. In Figure 5A are compiled the amplitudes of the release of bound FTP induced by binding of BA to high-affinity sites ([BA] < 0.4 μM) vs. the FTP concentrations used. On the other hand, the extent of the release of bound FTP induced by binding of CATR to α and β sites are plotted vs. FTP concentrations in Figure 5B. The sensitivities of FTP binding to CATR and BA were clearly different. In fact, the half-saturating concentration of FTP for the BA-sensitive FTP binding sites was roughly 0.5 μM, a value significantly different from those found for the CATR α-sensitive FTP binding sites, <10 nM and 2 μM. Less striking differences were observed between the binding of FTP to the BA-sensitive FTP sites and to the CATR β-sensitive FTP sites. Table II attempts to summarize the sensitivities of the four FTP binding sites to CATR and BA and the affinities of these sites for FTP according to the data of Figures 3–5. Three classes of FTP binding sites differing in K_d are postulated: high-affinity sites (FTP_1), intermediate-affinity sites (FTP_3), and low-affinity sites (FTP_2 and FTP_4). It may be recalled that, because of

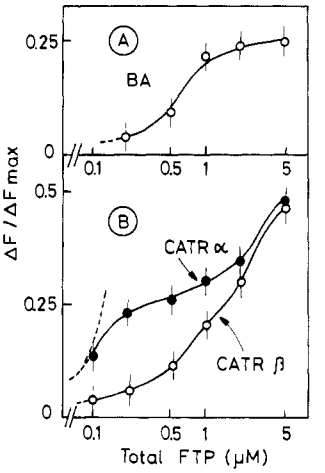

FIGURE 5: Comparison of the release of FTP by CATR and BA. The efficiency of CATR and BA to remove bound FTP from the carrier protein was derived from experiments similar to those described in Figure 3 or 4. The data are plotted as a function of the concentrations of added FTP. CATR α refers to FTP binding sites highly sensitive to CATR ([CATR] < 0.5 μM) and CATR β to FTP binding sites of low CATR sensitivity ([CATR] > 0.5 μM) (see Results for details). The BA-dependent release of bound FTP was for BA < 0.5 μM. The bars are the standard errors of the mean for five experiments. Note the logarithmic scale for the abscissa. The high-affinity region of the CATR α sites is extrapolated by a dashed line.

Table II: Sensitivity to CATR and BA of the Four FTP Binding Sites

sites	K_d (FTP)	CATR α	CATR β	BA (high affinity)
FTP 1	<10 nM	+[a]	−	−
FTP 2	≃2 μM	+	−	−
FTP 3	0.5 μM	−	+	+
FTP 4	≃2 μM	−	+	−

[a] (+) or (−) indicates that the FTP bound to the specific sites on the carrier is sensitive or not to the inhibitory ligands CATR or BA.

the limited accuracy of the experimental data, the fit of Figure 2 was calculated assuming only two classes of binding sites and that no attempt was made to perform a more refined fit.

Competition between FTP and ATP for Binding Sites on the Carrier Protein. The data reported in the above sections can be interpreted on the basis of a carrier unit which would contain four distinct potential nucleotide binding sites, each of them being characterized by a typical binding constant with respect to FTP. There are several lines of evidence indicating that FTP and ATP share the same binding sites: (1) the structures of the two nucleotides are closely related; (2) FTP is transported through the inner mitochondrial membrane by the ADP/ATP carrier ; (3) FTP binding to the isolated carrier is sensitive to the specific inhibitors, CATR and BA. Thus, it is not surprising that FTP and ATP compete for binding to the carrier. Figure 6 shows that the high-affinity FTP binding (FTP < 0.1 μM) is antagonized by low concentrations of ATP, indicating that ATP competes against FTP for binding to the high-affinity FTP sites. On the other hand, the fact that ATP binds competitively to the low-affinity FTP sites is clearly indicated by the progressive shift of the FTP binding curve reflecting the decrease of the apparent affinity for FTP, when ATP is added at increasing concentrations.

Discussion

The mechanism of reactions catalyzed by a number of enzymes using adenine nucleotides as substrates has been in-

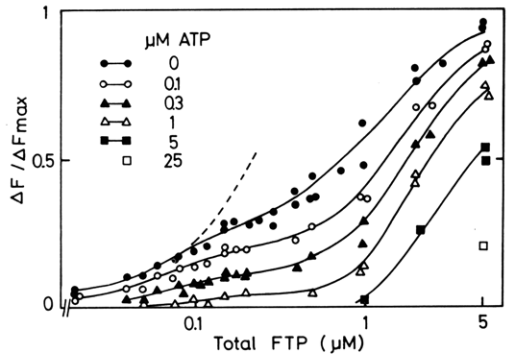

FIGURE 6: Competition between ATP and FTP binding. Same conditions as in Figure 1. $\Delta F/\Delta F_{max}$ is proportional to the amount of bound FTP in the presence of increasing concentrations of ATP. It was measured after complete release of FTP induced by 50 μM CATR.

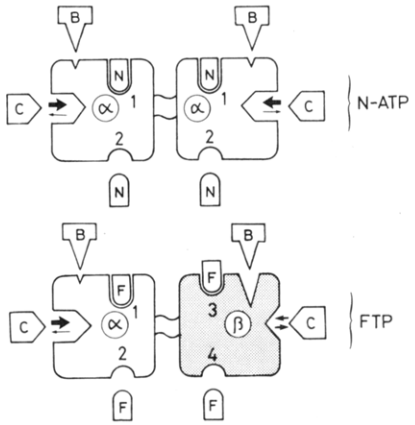

FIGURE 7: Hypothetical model of binding sites for ATP analogues and inhibitors in the ADP/ATP carrier. N = N-ATP; F = FTP; B = BA; C = CATR; N1 = high-affinity N-ATP binding sites; N2 = low-affinity N-ATP binding sites; F1 = high-affinity FTP binding sites; F2, F3, and F4 = low-affinity FTP binding sites; α and β refer to different states of the carrier protein (see text), the transition $\alpha \rightarrow \beta$ catalyzed by FTP binding. This model summarizes the data reported in this paper and the preceding one (Dupont et al., 1982). A dimeric structure is assumed. The model is a schematic illustration of the reactivities of the binding sites in the isolated carrier; it does not intend to describe the topography of the sites in the membrane-bound carrier.

vestigated, by means of the fluorescent formycin nucleotides (cf. Karlish et al., 1978; Ward et al., 1969a,b). FDP and FTP are transported by the ADP/ATP carrier across the mitochondrial membrane with K_M and V_{max} values similar to those obtained for ADP and ATP. They have been used in the present work as fluorescent probes of the nucleotide sites in the isolated ADP/ATP carrier protein. The present data complement those obtained in the preceding paper (Dupont et al., 1982) with N-ATP, a nontransportable nucleotide. The fact that ATP competitively inhibits N-ATP and FTP binding demonstrates the specific recognition of N-ATP and FTP by the nucleotide sites of the carrier protein and thus supports the relevance of the present data. It must be stressed that the difference between N-ATP and FTP resides essentially in the fact that N-ATP is recognized by the carrier protein but is not transported, in contrast to FTP which is not only recognized but also transported. A plausible interpretation of the data obtained with FTP is that the two sets of nucleotide binding sites revealed by N-ATP binding studies do in fact contain four binding sites. A similar splitting is also observed for the CATR binding sites titrated in the presence of FTP; the data are consistent with two sets of CATR sites that are present in equal concentrations and differ in their affinities for CATR. A further interesting difference between the binding of N-ATP and that of FTP concerns the effect of BA. Whereas BA is virtually ineffective in displacing bound N-ATP, it effectively displaces a significant amount of bound FTP. In spite of these differences, the total concentrations of N-ATP and FTP sites are the same (Table I). To explain the apparent differential behavior of the carrier protein with respect to N-ATP, FTP, and the inhibitors, we propose that FTP binding to the carrier protein induces a substantial conformational change which affects only half of the nucleotide and CATR binding sites, leaving the other half unchanged. N-ATP would be unable to promote this conformational change of the protein, possibly because of the presence of the bulky naphthoyl moiety in the molecule. Since FTP and N-ATP differ in that the former is transported whereas the latter only binds to the carrier without being transported, it is tempting to relate the postulated conformational change of the protein in the presence of FTP to molecular events of the transport process.

In this paper, we referred to the high- and low-affinity CATR sites as CATR α and CATR β sites. Since each CATR site interacts with two nucleotide sites, we shall use the superscripts α and β to designate the two putative substructures of the carrier protein associated with the respective CATR sites. It is, however, premature to ascribe any significance to these substructures in terms of the likely dimeric organization of the carrier protein (Hackenberg & Klingenberg, 1980; Brandolin et al., 1980). An alternative hypothesis which is less attractive but cannot be dismissed is that the two substructures are part of a monomeric carrier protein. The results presented in this paper with FTP as ligand and in the preceding paper (Dupont et al., 1982) with N-ATP are assembled in a model illustrated in Figure 7. In the case of N-ATP, the two substructures are indistinguishable and are both in the α state. The α–α carrier protein apparently behaves as a single α protein that contains high-affinity CATR α sites, high-affinity N-ATP sites (sites 1), and low-affinity N-ATP sites (sites 2) in a stoichiometric ratio of 1/1/1. This α–α structure shows low affinity for BA. When FTP is used instead of N-ATP, half of the carrier sites undergoes a conformational change depicted as an α to β shift, and the carrier takes the α–β conformation. The β state is postulated to differ from the α state by a large increase of the CATR and FTP dissociation constants (2 or 3 orders of magnitude). Another consequence of the α to β transition is that one of the FTP sites, associated to the β structure, acquires a very high sensitivity to BA. That the BA-sensitivity FTP site is located in the newly formed β substructure is inferred from the plot shown in Figure 5, which indicates that the concentrations of FTP, at which the chases of FTP by CATR (CATR α) and BA are revealed, are markedly different. The sensitivities of the four FTP sites to the inhibitors CATR and BA are summarized in Table II.

The α–α conformation of the carrier protein would be stabilized by the binding of the nontransportable N-ATP and the α–β conformation by that of the transportable FTP, and probably of any transportable nucleotide. In a previous work (Brandolin et al., 1981), it was shown that addition of ATP (or ADP) to the isolated carrier protein resulted in an increase of the intrinsic fluorescence emission; the fluorescence increase was virtually doubled upon addition of a saturating concentration of BA and inhibited by CATR. In terms of the scheme of Figure 7, these data can be explained by an ATP-induced transition from the α to β conformation; the α conformation would bind CATR with high affinity and the β conformation

BA, which strongly points to the existence of a transitory ternary complex, inhibitor–substrate–carrier, in contrast with the proposal of Klingenberg & Appel (1980), who advocated a single site capable of binding substrates and inhibitors.

In the model of Figure 7, the α and β substructures of the carrier protein are associated. An alternative hypothesis would be that the α and β substructures represent two species of a heterogeneous mixture of carrier protein molecules, a fraction of which only is able to undergo the α to β transition upon FTP binding. This explanation, however, is unlikely since the CATR α site to CATR β site ratio has been reproducibly found to be 1 in more than 20 carrier protein preparations.

The model only summarizes the interactions between the substrate and inhibitor sites, as they are revealed by binding studies with the carrier protein in detergent; it, of course, does not intend to describe the situation of the carrier protein in the mitochondrial membrane with respect to its topology and functioning. However, from the present conclusion, it would be tempting to speculate that the membrane-bound ADP/ATP carrier is a dimer, with four potential interconvertible nucleotide binding sites, so that each subunit possesses a pair of sites. At a given time, the high-affinity site located on one side of one of the two subunits would take in charge ADP or ATP for transport; one may imagine that another site located on the second subunit, opposite to the first one, also interacts with ADP or ATP, but in that case with low affinity. The transport would then be sequential, in accordance with the kinetic data of Duyckaerts et al. (1980) and Barbour & Chan (1981), i.e., the nucleotide firmly bound by the first subunit would be transported and delivered to the other side of the membrane; concomitantly, the loose binding of the other nucleotide to the second subunit would become tighter, and transport of the second nucleotide would proceed in the opposite direction.

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Solvent Deuterium Isotope Effects in the Catalysis of Oxygen-18 Exchange by Human Carbonic Anhydrase II†

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ABSTRACT: By measuring the rate of exchange at chemical equilibrium of ^{18}O between HCO_3^- and H_2O catalyzed by human carbonic anhydrase II in the absence of buffers, we have determined the rate of release from the enzyme of water bearing substrate oxygen. The ratio of this rate measured in H_2O to the rate measured in D_2O, the solvent deuterium isotope effect, is between 4 and 9 in the range of pH(D) from 5.8 to 8.0, with a value of 8.0 ± 0.7 at pH(D) 6.6 (uncorrected pH meter reading). The magnitude of this isotope effect at pH(D) 6.6 has an exponential dependence on the atom fraction of deuterium in solvent water. We conclude that an intramolecular proton transfer between a proton shuttle group on the enzyme and the active site is rate limiting for the release from the enzyme of water bearing substrate oxygen and involves a change in bonding of more than one proton. In contrast, the solvent deuterium isotope effect on the intermolecular proton transfer between the external buffer imidazole and the active site (or proton shuttle group) of the enzyme is small, 2.3 at pH(D) 7.0, as determined from initial velocity experiments. With a rate constant near 9×10^8 M^{-1} s^{-1}, this intermolecular transfer is limited to a significant extent by diffusion processes.

Carbonic anhydrase is a zinc-containing metalloenzyme that catalyzes the hydration of CO_2, as given in eq 1, in a process

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \quad (1)$$

that involves at least one proton transfer, that between the enzyme and its solution environment. Measurement of the catalysis in H_2O and D_2O by Steiner et al. (1975) using human carbonic anhydrase II (the high-activity isozyme) and by Pocker & Bjorkquist (1977) using the kinetically equivalent bovine carbonic anhydrase from red cells gave a solvent deuterium isotope effect between 3 and 4 for V_{max} and an isotope effect very close to unity for V_{max}/K_m in both the hydration and dehydration directions. A solvent deuterium isotope effect on V_{max}, for example, is the ratio of V_{max} measured in H_2O

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