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Interaction of Fluorescent Adenine Nucleotide Derivatives with the ADP/ATP Carrier in Mitochondria. 2. [5-(Dimethylamino)-1-naphthoyl]adenine Nucleotides as Probes for the Transition between c and m States of the ADP/ATP Carrier†

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ABSTRACT: The binding to the ADP/ATP carrier in mitochondrial membranes of the 3′-*O*-(dimethylamino)naphthoyl (DAN) derivatives of AMP, ADP, and ATP was quantitatively analyzed. The sidedness of the fluorescent type binding to the "m" side only was shown comparing the mitochondrial membranes in various stages of integrity and surface orientation. In particles displacement by bongkrekate (BKA) is direct, whereas in the case of carboxyatractylate (CAT) the requirement for ADP and ATP demonstrates the transition from the "m" to the "c" state. Quantitatively the "physical" binding of [^{3}H]DAN-AMP and fluorescence are well correlated, allowing for a little nonfluorescent binding to the c side. For DAN-AMP K_D is 1.6 μM, for DAN-ADP K_D is 0.8 μM, and in the Hill plot a straight line with $n = 1.25$ is obtained. The maximum number of binding sites for [^{3}H]DAN-AMP (1.5 μmol/g of protein) is about equal to the sites found for [^{3}H]BKA if the unspecific binding of both ligands is differentiated by blocking carrier sites with CAT. [^{3}H]CAT binding is somewhat lower in accordance with the limited access of CAT to inverted vesicles. ADP is able to decrease fluorescence only by about 35% at high concentrations (10 mM) whereas GDP has virtually no effect. With ADP, DAN-AMP binding decreases by 30% of the total binding sensitive to BKA. Binding to ATPase is low because of the absence of Mg^{2+}. The a priori identity of the 10-30% ADP-sensitive and therefore also exchange-active carrier sites with the 70-90% ADP-insensitive sites was established in comparative titrations of the exchange, of binding, and of fluorescence with DAN-AMP, ADP, and BKA. DAN-AMP binding to whole mitochondria includes uptake which can be back-exchanged against external ADP. This implies binding of DAN nucleotides also to the c state of the carrier. In rat liver mitochondria (RLM) an energy-dependent regulation of DAN-ATP uptake, similar to that known for ATP, is observed. These results indicate fluorescent, strong DAN nucleotide binding to the carrier in the m state and nonfluorescent, weak binding to the c state.

In the preceding paper (Mayer et al., 1984) it was shown that by 3′-O-acylation of adenine nucleotides fluorescent derivatives that interact with the ADP/ATP carrier of mitochondria can be obtained. The most suitable fluorescent probes are the 5-(dimethylamino)-1-naphthoyl (DAN) derivatives of AMP, ADP, and ATP. When free in aqueous solution, they are nearly fully quenched, but on binding to carrier sites in mitochondria or submitochondrial particles, they produce a strong fluorescence upon addition of inhibitors of the ADP/ATP carrier, particularly when BKA[1] is added.

In the present paper the interaction of the 1,5-DAN-adenine

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[1] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; BHM, beef heart mitochondria; BHSMP, beef heart submitochondrial particles; BKA, bongkrekate; CAT, carboxyatractylate; DAN-AMP, [(dimethylamino)naphthoyl]adenosine 5′-monophosphate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; RLM, rat liver mitochondria; SMP, submitochondrial particles; ATPase, adenosinetriphosphatase; SDS, sodium dodecyl sulfate.

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Table I: Relative BKA-Reversible Fluorescence of 1,5-DAN-AMP with Intact, Uncoupled, and Detergent-Treated Mitochondria

condition	ΔF/mg
BHM[a]	
fresh	17 ± 2
frozen–thawed	35 ± 5
subtilisin treated	8 ± 2
Brij 36 treated	34 ± 5
RLM[a]	
fresh	0 ± 0.5
frozen–thawed	7.5 ± 1
fresh, aged 7 °C, 12 h	7.5 ± 2
fresh, sonicated 1 min	6 ± 2
Brij 36 treated, fresh	8 ± 1

[a] BHM, beef heart mitochondria; RLM, rat liver mitochondria.

nucleotides (henceforth called DAN nucleotides) with the ADP/ATP carrier will be studied with special emphasis on the following aspects: identification of the binding site for DAN nucleotides; determination of the binding parameters; binding to the "c" and "m" state of the carrier; the use of DAN nucleotides as probes for the transition between the two carrier states catalyzed by ADP and ATP; kinetics of binding and of the c-state to m-state transition studied by means of the fluorescence signal.

Materials and Methods

For details of material and preparation of mitochondria, submitochondrial particles, and the (dimethylamino)naphthoyl derivatives, as well as for fluorescence measurements, see Mayer et al. (1984). [^{3}H]DAN-AMP, [^{3}H]DAN-ADP, and [^{3}H]DAN-ATP were prepared from [^{3}H]A(M,D,T)P at a specific activity of 100 000 dpm/nmol applying the preparative procedure described in the preceding paper (Mayer et al., 1984) but on a small scale (4 mg of ATP).

Binding and Exchange Measurements. For determination of binding, beef heart mitochondria (BHM), rat liver mitochondria (RLM), or beef heart submitochondrial particles (BHSMP) were incubated in standard medium at about 1 mg of protein/mL. [^{3}H]DAN-AMP (-ADP, -ATP) was added at the concentration indicated in the legends by using specific activities of about 1×10^4 dpm/nmol. After incubation for the time intervals indicated in the legends of the figures, the BHSMP were centrifuged at 60000*g* for 30 min and the BHM or RLM at 800*g* for 15 min. The pellets were dissolved in 5% SDS. The [^{3}H]DAN nucleotide radioactivity was determined in both the supernatants and the pellets.

Results

Membrane Sidedness of Fluorescent Interaction with DAN Derivative. There are essentially two ways, supplementing each other, for differentiating the sidedness of binding to the ADP/ATP carrier: one by approaching the carrier with the substrate from either the outside or the inside and the other by probing the carrier site with inhibitors specific for either the c or the m side. The BKA-dependent elimination or reversal of fluorescence on binding of the DAN derivatives suggests not only that the fluorescence is specifically associated with binding to the ADP/ATP carrier but also that this binding is occurring only to the m state of the carrier.

Additional support for this interpretation was provided by comparing the fluorescence yield on addition of DAN-AMP to mitochondrial preparations which exhibit varying degrees of structural integrity, i.e., with varying accessibility to the m side. Freshly prepared BHM consistently have a more than 50% lower fluorescence with DAN-AMP than beef heart mitochondria which have undergone a freeze-thaw cycle and are largely uncoupled (Table I). BHM prepared with subtilisin treatment of the muscle homogenate in order to obtain more intact mitochondria give a 2-fold lower fluorescence than non-subtilisin-treated BHM. Detergent (Triton X-100 or Brij 36) treatment of fresh BHM (1.4 mg/mg of protein) also resulted in a 2-fold amplification of DAN-AMP-dependent fluorescence; there was no apparent change in affinity for DAN-AMP due to any of these treatments (not shown). Detergent treatment of BHSMP gives no increase of the BKA-reversible fluorescence.

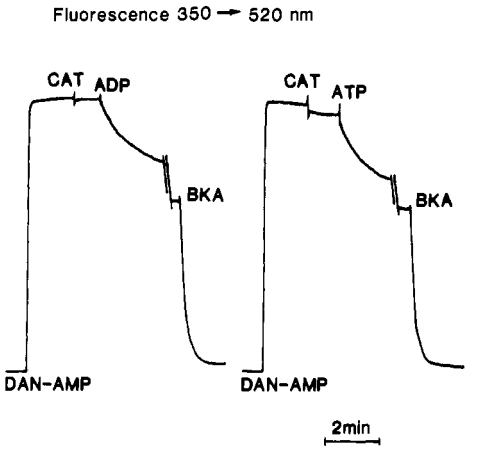

FIGURE 1: Influence of ADP and ATP on the CAT-induced suppression of DAN-AMP binding. Fluorescence recording of DAN nucleotides added to submitochondrial particles (BHSMP). BHSMP and 1.2 mg of protein/mL in standard buffer at 4 °C: addition of 5.5 μM DAN-AMP. Further additions 20 μM CAT and 50 μM ATP or ADP.

Freshly isolated RLM exhibited no fluorescence upon addition of DAN derivatives. Freeze–thawing or brief sonication produced a fluorescent response upon addition of DAN-AMP of about 20% of that observed under identical conditions with BHM. Treatment of RLM with detergents also gave an enhancement of DAN-AMP fluorescence. After solubilization of RLM with Triton X-100 (>2 mg of TX-100/mg of protein) fluorescent response was still present. However, it was relatively labile, $t_{1/2}$ = 20 min at 4 °C.

The sidedness question was further approached by studying the influence of ADP and ATP on fluorescence. Addition of ADP and ATP did not change and the fluorescence response with the DNA derivatives in BHM and BHSMP. The fluorescence was diminished by CAT to about 40–50% only if also ATP or ADP were added; neither of the two ligands alone was able to decrease the fluorescence (Figure 1). In contrast, BKA suppressed the fluorescence nearly completely, independent of ADP addition. In freshly prepared BHM the relatively small fluorescence of DAN-AMP could be fully repressed by CAT and BKA without significant dependence on ADP or ATP [Figure 1 in Mayer et al. (1984)].

All these results can be consistently explained by assuming that the fluorescent binding of DAN nucleotides occurs to the m state of the carrier. In particular the interaction of CAT requires the c state, and therefore ADP or ATP are necessary in order to facilitate the transition from the m to the c state (see also Disussion).

Inhibition by Nucleotides and Nucleotide Analogues of DAN Nucleotide Fluorescence. It was an unexpected finding that prior subsequent addition of ADP or ATP in concentrations up to 300 times higher than that of DAN-AMP or DAN-ADP reduced the fluorescence in BHSMP only to a small extent (Table II). Since the K_M for ADP uptake into intact BHM or BHSMP is 10 μM (Klingenberg, 1976) and 7.5 μM (Klingenberg, 1977), respectively, it was anticipated

Table II: Effect of Various Nucleotides and Analogues on DAN-AMP Fluorescence in BHSMP

additions	concn	relative fluorescence[a]
ADP	2.5 mM	62
ATP	2.5 mM	63
AMP	2.5 mM	98
NAPH	0.04 mM	100
AMPPNP	1.25 mM	64
GDP	5 mM	97
IDP	5 mM	97
palmitoyl-CoA	6 μM	47

[a] 100 with no addition.

that 150 μM ADP and ATP would effectively compete with 2–6 μM DAN nucleotides. We shall return to the effect of ADP and ATP further below.

AMPPNP also reduced DAN-ADP fluorescence with an apparent K_I of 500 μM. GDP and IDP exhibited little or no effect at 5 mM. Significantly, palmitoyl-CoA effectively abolished DAN-AMP fluorescence. Quantitative studies with palmitoyl-CoA in BHSMP and intact BHM gave a $K_I \simeq 1$ μM and 0.5 μM, respectively, considerably less that its critical micelle concentration of 4 μM (Zahler et al., 1969). This suggests that no detergent effect of palmitoyl-CoA is involved.

Concentration Dependence of the Binding and Fluorescence of DAN Derivatives. The fluorescence enhancement on addition of the DAN derivatives seems to be specific only for the m-side interaction. The fact that the DAN nucleotides inhibit exchange from the c side in mitochondria, as shown by Mayer et al. (1984), suggests that the DAN derivatives bind also to the c side, albeit in a nonfluorescent mode. It is to be expected that DAN derivatives also interact with other nucleotide binding sites in mitochondria or SMP in a nonfluorescent mode, e.g., nonfluorescent binding to F_1 (Schäfer & Onur, 1980); this binding would, of course, be BKA and CAT insensitive.

In order to analyze quantitatively the total extent of binding, measurements with radioactivity labeled DAN derivatives were carried out. To this end, binding and fluorescence were measured simultaneously in dependence on the concentration of nucleotides. After addition of isotopically labeled DAN nucleotides, the fluorescence increase was recorded, and then the content of the cuvette was spun down to determine the binding of radioactivity in the sediment. From these data, the fluorescence and binding curves were constructed as a function of the concentration of DAN-AMP and DAN-ADP (Figure 2A,B). The fluorescence is nearly totally suppressed by BKA; therefore, no correction for BKA-independent fluorescence is necessary. On the other hand, the binding has a BKA-insensitive portion which increases linearly with the DAN-AMP concentration. DAN-AMP clearly shows stronger fluorescence and binding throughout the concentration range than DAN-ADP. In contrast, the BKA-insensitive binding portion is higher in fluorescence with DAN-AMP and DAN-ATP. The plots of fluorescence against BKA-sensitive binding give a straight line up to 1.2 μmol/g of protein, indicating that fluorescence and binding at the BKA-sensitive carrier site are well correlated. The binding continues slightly beyond the end point of fluorescence. This extra binding probably takes place to carrier sites in the c state from which DNA-AMP or DAN-ADP can be removed by BKA after switching these sites to the m state, as will be further elucidated below.

In the mass action plots (Figure 2) only approximately linear relations are obtained both for binding and for fluorescence (not shown). The Hill plots of the binding and fluorescence

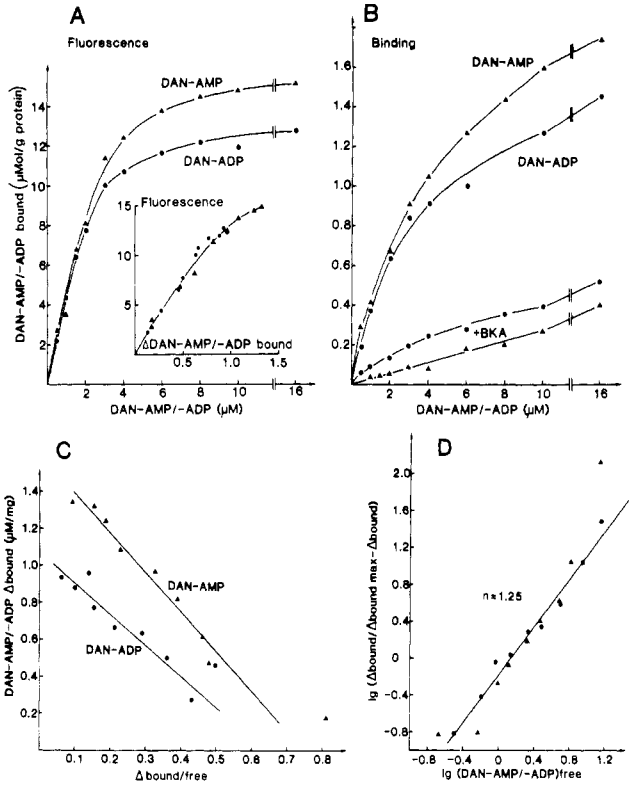

FIGURE 2: Comparison of fluorescence and binding of [^{3}H]DAN-AMP and [^{3}H]DAN-ADP added to BHSMP. Concentration dependence and differentiation by BKA addition. (A and B) Subsequent measurements of fluorescence and binding on the same sample. Incubation of 1.2 mg of protein/mL of BHSMP in standard medium. After fluorescence recording from each 0.8-mL sample, an aliquot of 0.3 mL was taken before and after addition of 12.5 μM BKA for binding measurements. Fluorescence after BKA addition is virtually zero. A plot of fluorescence against BKA-sensitive DAN-AMP/ADP binding is given in the inert of (A). (C) Mass action plot of [^{3}H]-DAN-AMP/ADP binding. The free DAN-AMP was calculated as $DAN\text{-}AMP_{free} = [^3H]DAN\text{-}AMP_{total} - [3H]DAN\text{-}AMP_{bound}$. (D) Hill plot of the binding experiment. Both fluorescence increase and Δ[^{3}H]DAN-AMP binding are plotted.

(Figure 2D) can be fitted by the same straight line for DAN-AMP and DAN-ADP. The slope corresponds to a Hill coefficient of $n = 1.25$ which may indicate a slight cooperativity.

Relationship between DAN Nucleotide Binding Sites and Nucleotide Binding Sites. Considerable effort was invested to gain an understanding of the relationship between binding of DAN nucleotides and the unsubstituted nucleotide at the carrier site and other nucleotide binding sites in BHSMP. The fluorescence of bound DAN-AMP is only partially insensitive to addition of ADP and ATP, as shown in Table II, but not to addition of AMP, GDP, and IDP. This selectivity agrees with the carrier specificity toward nucleotides. Additional, more quantitative evaluation of binding to noncarrier sites is obtained by comparing the decrease by ADP and GDP both of DAN-AMP binding and of DAN-AMP fluorescence over a wide concentration range. Competition with GDP, which binds to ATPase with a K_D of 30 μM (Mitchell & Moyle, 1971), might permit differentiation between binding to the ATPase and binding to the carrier. At least two complications may arise here: ADP is rapidly degraded by adenylate kinase whereas GDP is not, and ADP also stimulates binding of DAN-AMP to ATPase, as will be shown in the following. In order to avoid degradation of ADP by the combined action of adenylate kinase and ATPase, AP_5A and EDTA were added.

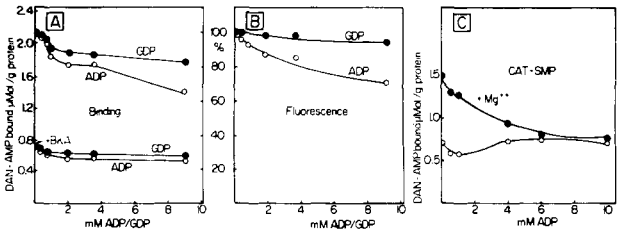

FIGURE 3: Influence of ADP and GDP on DAN-AMP binding and fluorescence in BHSMP. Direct comparison of binding and fluorescence in the same samples. (A) Residual [^{3}H]DAN-AMP binding is found in the presence of 10 μM BKA, whereas in (B) fluorescence is virtually zero. In (C) the carrier sites are blocked by CAT in order to analyze the noncarrier portion of DAN-AMP binding. BHSMP are used (CAT-SMP), which have been prepared from BHM loaded with CAT. Binding both in the presence and in the absence of 2 mM Mg^{2+}. Incubation of 1.1 mg of protein/mL of BHSMP and 1.2 mg of protein/mL of CAT-SMP in standard buffer. Addition of 50 μM AP_5A at 0 °C and at 10 °C and of 5 mM $MgCl_2$ as indicated.

Results from experiments in which binding and fluorescence were measured in the same samples are presented in Figure 3. Fluorescence is decreased by ADP only by 30% when the concentration of ADP reaches 9 mM. The effect of GDP is nearly negligible whereas DAN-AMP binding is decreased also by GDP. Up to 15% of DAN-AMP binding is sensitive to 0.5 mM of both nucleotides and more is removed only at a higher concentration of ADP. Compared to the BKA-sensitive binding, the removal amounts to 35% with ADP and 20% with GDP. This binding decrease is larger than the corresponding fluorescence decrease. On the other hand, as shown above, the fluorescence is completely suppressed by BKA whereas the binding is retained to one-third. The BKA-insensitive portion is largely resistant to ADP or GDP.

For further identification of the noncarrier binding sites, the effect of ADP on bound DAN-AMP was analyzed in BHSMP where all carrier sites are a priori blocked by adding CAT to the mitochondria prior to sonication (Klingenberg, 1977). Under the usual conditions up to 10 mM ADP did not appreciably affect DAN-AMP binding (Figure 3C). However, it is noteworthy that some DAN-AMP is removed at low ADP concentration (below 4 mM). This effect has been clearly reproduced in several experiments and obviously causes the complex behavior of the ADP-dependent decrease of DAN-AMP binding in normal BHSMP, shown in Figure 1A, i.e., the "bump" in the binding at 2–2.4 mM ADP. It may reflect a hitherto not yet reported cooperative effect of ADP on DAN-AMP binding to ATPase.

In the presence of Mg^{2+}, DAN-AMP binding is more than twice as large in the CAT-loaded particles and is now strongly diminished by ADP, reaching the same level as in the presence of EDTA. This binding portion clearly represents DAN-AMP at ATPase F_1 sites. The nature of the Mg^{2+}-independent binding which corresponds largely to the BKA-insensitive portion remains unknown. Possibly it represents unspecific adsorption to the membrane since it is not saturated even at 16 μM DAN-AMP (Figure 2).

The puzzling relative insensitivity towards ADP and ATP of the BKA-sensitive DAN-AMP binding raises the question of to what extent DAN-AMP binds to the carrier sites involved in ADP and ATP translocation. In order to consider this point, the competition between ADP and DAN-AMP for the exchange activity in BHSMP and the parallel effects of BKA on DAN-AMP binding were studied. For this purpose the exchange is activated in the presence of DAN-AMP with increasing concentrations of ADP to about half of the uninhibited activity (Figure 4A). In the same samples 15% of

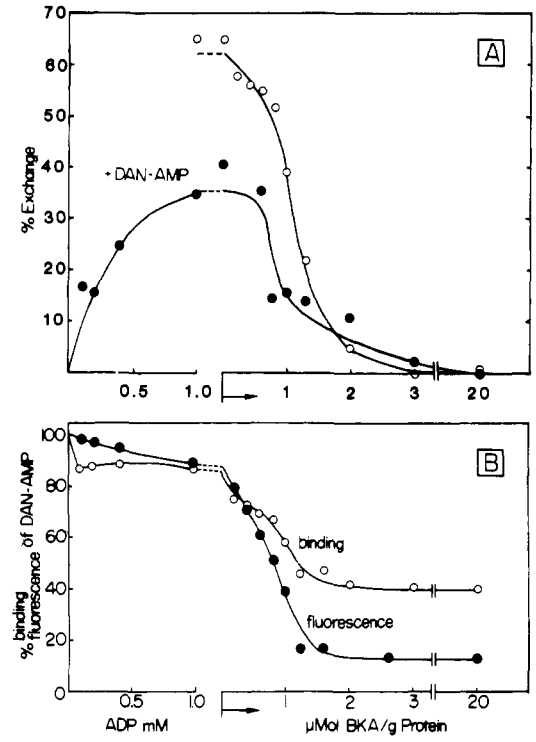

FIGURE 4: Competition between ADP and DAN-AMP for exchange and binding in BHSMP. Comparison of BKA influence on transport, binding, and fluorescence. Experiments with [^{14}C]ATP-loaded BHSMP incubated at 1 mg of protein/mL in standard medium at 10 °C. In the left-hand portion first 17 μM DAN-AMP was added, and then by addition of increasing concentrations of ADP the exchange was started. After 12 s the exchange was stopped by 12 μM BKA. For subtraction of unspecific leakage from SMP, BKA was added to parallel samples 4 min prior to ADP addition. In the right-hand portion BHSMP with and without 17 μM DAN-AMP were incubated with increasing amounts of BKA 4 min prior to addition of 1 mM ADP for starting the exchange. The binding of [^{3}H]DAN-AMP and its fluorescence was measured in all these samples.

DAN-AMP is removed by addition of up to 1 mM ADP, as measured both by fluorescence and by binding of [^{3}H]DAN-AMP (Figure 4B). The disparity between the effect of ADP on binding and percent exchange appears exaggerated. In fact, the rate of exchange $v = k \times \log 100/(100 - \text{percent exchange})$ is only activated by ADP to the extent of 40%. The residual disparity may indicate that a considerable fraction of DAN-AMP binds at carrier sites with a strongly decreased affinity for ADP, caused by sonication.

In the second part of the experiment we examined whether the removal of DAN-AMP by increasing amounts of BKA is correlated to the inhibition of the exchange by BKA. When BHSMP are titrated with small amounts of BKA, the exchange becomes increasingly inhibited. Both removal of DAN-AMP and inhibition of exchange run parallel, indicating that the sites to which BKA binds by removing DAN-AMP include the exchange active sites. Thus, a large amount of the BKA-sensitive DAN-AMP binding has an affinity equal to that of the exchange sites. On the other hand, only ADP-sensitive DAN-ATP binding should occur at active exchange sites for which a K_M = 10 μM for ADP was found in BHSMP (Klingenberg, 1977). The small effect of ADP on DAN-AMP binding and the still smaller effect on the fluorescence indicate that only a small portion of DAN-AMP binding sites is transport competent. The major portion (80%) seems not to be transport active although it has a high affinity for BKA.

Relationship between the Number of Binding Sites for DAN-AMP, BKA, and CAT. For a quantitative comparison of [^{3}H]DAN-AMP binding with that of [^{3}H]BKA and

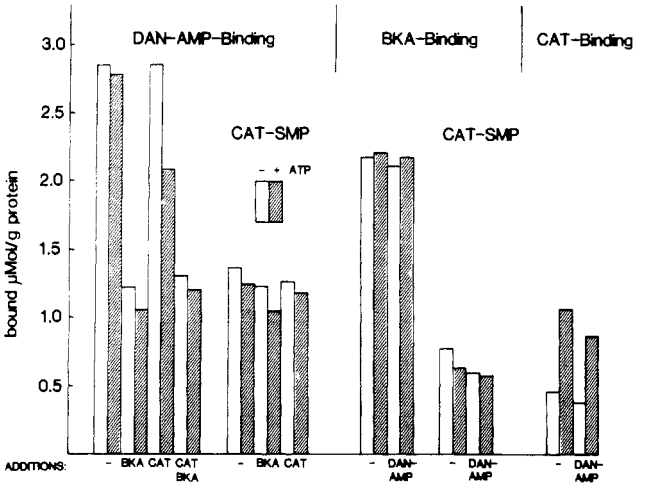

FIGURE 5: Comparison of binding of [^{3}H]DAN-AMP, [^{3}H]BKA, and [^{3}H]CAT to BHSMP and the interaction between these ligands under the influence of ATP. For control BHSMP with CAT-occupied carrier sites were used (CAT-SMP) prepared from BHM loaded with CAT; 1 mg of protein/mL of BHSMP is incubated in standard medium at 0 °C. Addition of 20 μM DAN-AMP or [^{3}H]DAN-AMP, 5 μM BKA or [^{3}H]BKA, 5 μM CAT or [^{3}H]CAT, and 50 μM ATP as indicated.

[^{3}H]CAT the establishment of a blind base with no free carrier sites is important. For this purpose BHSMP prepared from CAT-loaded mitochondria are used. Residual DAN-AMP binding to CAT-loaded BHSMP can be assumed to be at sites other than the carrier. As seen in Figure 5, total DAN-AMP binding measured near saturation with 30 μM DAN-AMP is about 2.8 μmol/g of protein in BHSMP and about 1.22 in CAT-loaded BHSMP, showing a difference of 1.6 μmol/g of protein binding at the carrier sites. After addition of BKA, DAN-AMP binding is as low as in the CAT-loaded SMP. The excellent agreement between the amount of DAN-AMP binding sensitive to CAT loading or that to subsequent BKA addition strongly supports the contention that DAN-AMP binding takes place at the same sites as binding of CAT and BKA, i.e., at the ADP/ATP carrier binding center.

Subsequent addition of CAT removes DAN-AMP only in the presence of ATP and only about half as effectively as prior CAT addition. This is well understandable on the basis of the ATP-linked switching between c and m state and the accessibility of CAT to only a part of the c sites in SMP (Klingenberg, 1974).

The [^{3}H]BKA binding to BHSMP contains a small unspecific portion given by the [^{3}H]BKA binding in the CAT-loaded SMP. The CAT-sensitive difference indicates a carrier-dependent binding of 1.6 μmol of BKA bound/g of protein which is in very good agreement with the BKA-sensitive DAN-AMP binding. About the same amount of [^{3}H]BKA binding is observed with and without DAN-AMP, reflecting the considerably lower affinity of DAN-AMP.

All binding of [^{3}H]CAT can be assumed to take place at the carrier sites. Binding in the presence of ATP is shown to be higher than in its absence (1.05 vs. 0.38 μmol/g of protein) due to the initial prevalence of the m state in BHSMP. In the presence of DAN-AMP the amount of CAT binding is slightly lower at 0.8 μmol/g of protein, again in good agreement with the amount of DAN-AMP removed (0.7 μmol/g of protein). The fact that CAT binding is considerably lower than that of BKA is attributed to the impermeability of a large portion of BHSMP to CAT.

Binding of DAN-AMP to Mitochondria. In order to resolve the question whether DAN-AMP can also bind to the carrier

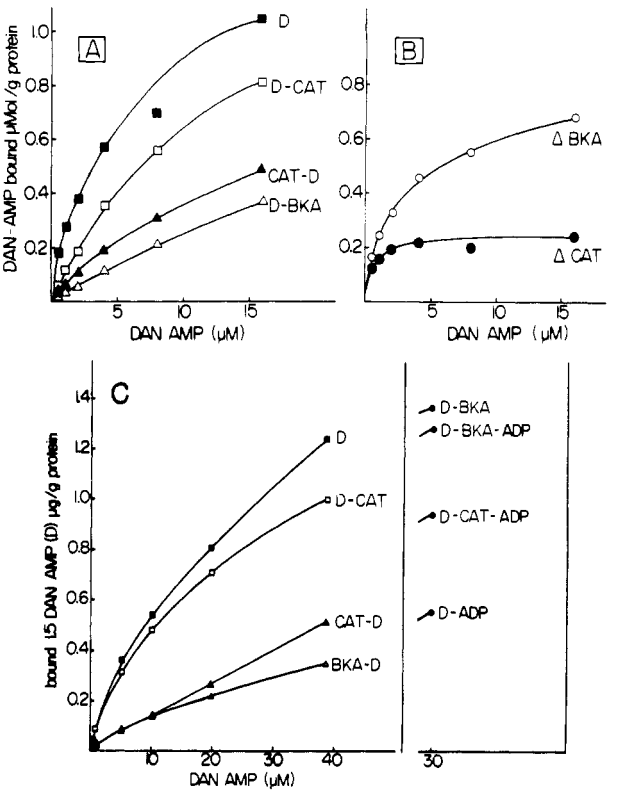

FIGURE 6: Binding of [^{3}H]DAN-AMP to mitochondria. (A) BHM incubated at 1 mg/mL protein in standard medium at 4 °C with addition of [^{3}H]DAN-AMP at increasing concentrations as indicated in the abscissa. Time intervals between the addition of either first DAN-AMP and CAT (D-CAT) or vice versa (CAT-D) are 2 min. In part B the DAN-AMP binding portions suppressed by BKA addition or CAT addition are plotted (D minus D-CAT or D minus D-BKA). (C) Binding of [^{3}H]DAN-AMP to rat liver mitochondria (RLM). RLM were incubated at 2 mg of protein/mL with addition of 1 mM ADP and if indicated with 15 μM CAT and 4 μM BKA in the sequence given. Time intervals between the sequential additions are 2 min.

in the c state, the binding of DAN-AMP was measured in BHM and RLM. This also bears on the question whether the DAN nucleotides bind to the nucleotide binding site at all. As nucleotide analogues, they can be expected to bind also to the c state. In order to minimize binding to the m state, fresh BHM were employed. The locus of binding was differentiated by addition of CAT and BKA. When CAT is added after DAN-AMP, binding of DAN-AMP to the c state should be inhibited. When CAT is added prior to DAN-AMP, a potential DAN-AMP uptake into the internal space by exchange and binding to the m state should be prevented (Weidemann et al., 1970). On subsequent BKA addition, binding at both outer and inner sites may be affected, since there is sufficient endogenous ADP and ATP available for catalyzing the transition between the c and m state.

The results in Figure 6A show that subsequent addition of CAT removes about 22% of the total binding whereas BKA removes more than 65%. Prior addition of CAT prevents more than half of the DAN-AMP binding. The total binding and in particular the CAT- and BKA-insensitive residual binding are not saturated at 15 μM DAN-AMP. However, the CAT-sensitive portion reaches a maximum already at 5 μM, and the BKA-sensitive portion tends to level off at more than 15 μM.

We can conclude from these results that there is a definite, though small, amount of DAN-AMP binding to the c side. This binding is indeed nonfluorescent, since on CAT addition no change in the fluorescence of DAN-AMP is seen. The large

binding deficiency, when CAT is added first, can be attributed to DAN-AMP taken up into the matrix in exchange for internal ADP. This portion is partially nonfluorescent as will be seen more clearly when RLM is used (Figure 6B). BKA removes both the c- and m-binding portion from the mitochondria as well as the internal nonbound DAN-AMP. The major portion of the internalized DAN-AMP in BHM is fluorescent and fully suppressed by BKA, indicating binding to carrier sites in the m state.

Fresh RLM are the best example for intact mitochondria where DAN-AMP should not have direct access to the m side but only to the c side. Here DAN-AMP binding is relatively high. While only about 10–15% is removed by CAT when added after DAN-AMP (Figure 6B), prior addition of CAT suppresses binding to a large extent, indicating that a large partion of DAN-AMP is taken up into the liver mitochondria. It is deposited there in a nonfluorescent manner, since binding of DAN-AMP to RLM is all nonfluorescent. Thus, binding to RLM is even higher than to BHM although the number of carrier sites in RLM is known to be about 5 times lower than in BHM. Accordingly, the removal of DAN-AMP by subsequent CAT addition is considerably smaller in RLM than in BHM. Even more striking is the different effect of BKA on DAN-AMP binding in BHM and RLM. BKA removes only a small portion of DAN-AMP from RLM. This agrees with the absence of fluorescent-type binding, i.e., binding to m sites. Obviously the high endogenous pool of ADP and ATP effectively competes with the internalized DAN-AMP for binding to the m sites.

The explanation that high amounts of DAN-AMP are transported into RLM receives further support from the data shown in the right-hand section of Figure 6B. Here ADP has been added after DAN-AMP. The greater amount of DAN-AMP is then driven out of the mitochondria by exchange, obviously by reversing the entrance pathway which therefore can be argued to follow the pattern of the carrier exchange pathway. This ADP effect is of course prevented by CAT and BKA.

Further support for a transport of DAN nucleotides into mitochondria is obtained by following the uptake kinetics for the three DNA nucleotides in RLM (Figure 7). Two respiratory states of the mitochondria are compared, keeping in mind that energized mitochondria favor the uptake of ADP in preference to that of ATP, whereas uncoupled mitochondria do not discriminate between the two nucleotides (Pfaff & Klingenberg, 1968). In the coupled state DAN-AMP is accumulated most rapidly, followed by a much slower uptake of DAN-AMP that eventually reaches about the same level. DAN-ATP, however, is taken up only very slowly and even after 10 min has not yet reached the final value. In the uncoupled state DAN-ATP accumulates nearly as fast as DAN-ADP whereas DAN-AMP uptake reaches only a low level. The behavior of DAN-ATP is strikingly similar to the exchange pattern of ATP whereas there is no analogy in the case of DAN-AMP since AMP is inactive. At any rate, these results support the view that the DAN derivatives bind to the c state. Furthermore, as true nucleotide analogues, they are transported by the carrier, although only slowly and to a limited extent.

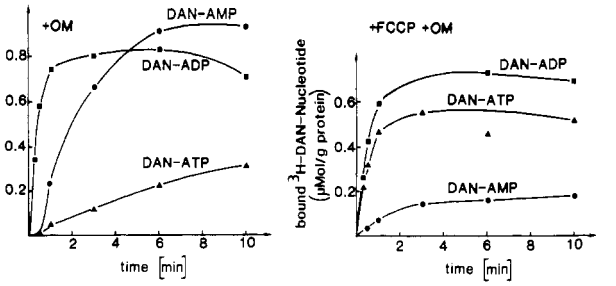

FIGURE 7: Influence of energization on the time-resolved uptake of DAN nucleotide derivatives into RLM. Comparison of the AMP, ADP, and ATP derivatives. Mitochondria incubated at 1.2 mg of protein/mL in standard medium at 4 °C with O_2 saturation and with addition of 2 mM succinate, 2 mM glutamate, 1 mM phosphate, 2 μg of oligomycin (OM), and 0.5 μM FCCP if indicated. Addition of 20 μM each of DAN-AMP, DAN-AMP, or DAN-ATP at time zero. Total volume of one experiment 5 mL. At the time indicated samples of 0.5 mL were withdrawn and injected into cups preloaded with 5 nmol of CAT for stopping the binding reaction. After centrifugation the binding of [^{3}H]DAN nucleotides was measured in the sediments.

Discussion

The application of the 1,5-DAN derivatives of AMP, ADP, and ATP to mitochondrial membranes provides a wealth of possibilities for studying the nucleotide carrier by means of a continuous signal-recording method. However, there are several unsettling questions concerning the nature of the nucleotide binding and, therefore, the identity of the binding site. First, the high binding affinity of DAN-AMP seems to suggest that the DAN derivatives are not substrate analogues for the carrier, since the carrier site does not interact with AMP; they rather mimic inhibitors such as BKA. Taking into account the assumption favored by others (Lauquin & Vignais, 1976; Block et al., 1979) that BKA binds to sites different from the binding sites of ADP and ATP, it would follow that DAN nucleotides do not bind to the substrate sites either. Our original postulate (Klingenberg et al., 1972) that substrates for the binding center must have at least three negative charges, which holds for all inhibitors and substrates known so far, also seems to be invalidated by DAN-AMP. However, the additional binding energy resulting from the close interaction of the DAN moiety with the protein, as evidenced by the fluorescence, possibly overcompensates the deficient ionic force.

Another argument for different sites may be seen in the relative insensitivity of DAN nucleotide binding to ADP and ATP. A more detailed analysis of the binding studies, however, argues in favor of direct competition between ADP and ATP and the DAN nucleotides. There is agreement with findings in previous experiments showing that in BHSMP only a small part of the binding sites seems to be active in transport and that a large portion of the carrier binding sites has lost the affinity for nucleotides but retained that for BKA and thus also for the DAN nucleotides (Klingenberg, 1977; Klingenberg et al., 1982). Possibly relatively small environmental changes in the membrane strongly decrease the affinity for ADP and ATP binding. Also in hypotonically or phosphate-treated membranes (Klingenberg et al., 1975) the ratio between nucleotide displacement ([^{3}H]-ADP) and CAT binding ([^{35}S]-CAT) is strongly decreased. After all, the isolated carrier has a strongly decreased affinity for nucleotides (Klingenberg et al., 1978).

This would agree with the finding that by addition of up to 1 mM ADP the exchange can be activated to nearly half of the uninhibited activity although only 10% of the DAN-AMP is removed from binding (Figure 4). On the other hand, the a priori homogeneity of the sites is indicated by an equal affinity for BKA, for both of the exchange active sites, and of all DNA-AMP binding sites.

The binding site conformation requirements for DAN-AMP obviously resemble more those for BKA than those for ADP or ATP. This agrees with our concept of the flexibility of the

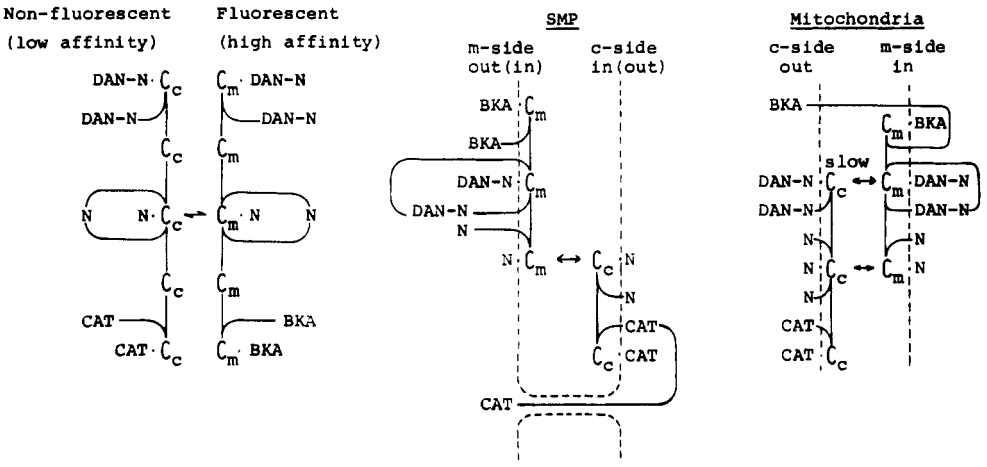

FIGURE 8: Modes and pathways of DAN nucleotide binding to the membrane-bound ADP/ATP carrier. Fluorescent and tight binding only to the m site; nonfluorescent and loose binding to the c site. In BHSMP the interaction of the inhibitors CAT and BKA with DAN nucleotide binding may depend (for CAT) on the access and the availability for ATP or ADP for facilitating the m- to c-state transition. Part of BHSMP are "broken", i.e., permeable to CAT. Mitochondria take up DAN nucleotides by exchange.

binding center and the overlapping of inhibitor sites with substrate sites (Klingenberg, 1976). A further argument in favor of the identity of sites for all ligands is the good agreement between the number of binding sites for DAN-AMP, BKA, and even CAT, obtained by employing isotope-labeled ligands (Figure 5). It is important to correct for unspecific binding by preparing BHSMP in which all carrier sites have been blocked by the highest affinity ligand (CAT). Although there is good agreement between the differentiated portions of DAN-AMP binding and BKA binding, CAT binding in BHSMP approaches 70% of DAN-AMP binding. The decrease is well understandable in view of the limited permeability of these particles to CAT. In fact the extent of BHSMP to which CAT is accessible is easily determined by measuring the degree of fluorescence suppression on addition of CAT plus ATP, as shown in Figure 1. It amounts to about 50% of the total sites which are located probably in "open" vesicles. Thus, a convenient method for assessing the intactness of BHSMP is obtained. The large portion of broken (open) vesicles is in agreement with the estimation based on transport studies in BHSMP (Klingenberg, 1977).

DAN-AMP Binding to the c and m Site. There is abundant evidence that the fluorescent binding of DAN-AMP is the m site of the carrier. Maximum fluorescence is observed in BHSMP, and in mitochondria the fluorescence response depends on breakage of the inner membrane by freezing or detergents. Moreover, the sensitivity to BKA is not only helpful in identifying binding to the carrier sites but also is most characteristic for the m-site interaction. In particular, the fact that CAT can suppress DAN-AMP fluorescence only when ATP is added accords with the m-site interaction. ATP is required for "switching" the sites from the m to the c state. The scheme based on the reorienting carrier-site mechanism (Erdelt et al., 1972; Klingenberg & Buchholz, 1973; Scherer & Klingenberg, 1974) (Figure 8) illustrates the logistics of the interaction between DAN-AMP binding and the inhibitors BKA and CAT. So far most of the information obtained with DAN nucleotides can be well rationalized by this mechanism.

The best indication that the DAN nucleotides interact with the c site comes from their transport via a CAT-sensitive path into the mitochondria (Figure 6). Other more direct evidence comes from the CAT-displaceable binding, which is only small but distinct. The fact that in RLM no fluorescence signal is recorded shows that binding of DAN nucleotides to the c site is nonfluorescent. This nonfluorescent interaction of DAN-AMP with the c site is apparently weak, since it is easily prevented by ADP, in contrast to the fluorescent, most ADP-resistant interaction in BHSMP. Obviously the absence of fluorescence means that the DAN group in the c state has a different environment, which is probably more hydrophobic than in the m state. As a result, the interaction with the protein is weaker, and therefore the total affinity of DAN-AMP is much lower.

It seems surprising that the relatively large amounts of internalized DAN nucleotides in RLM do not fluoresce, although they should have access to the m site. Probably in mitochondria the relatively high levels of endogenous ADP and ATP can effectively compete for the m site, all the more so since the carrier has a high affinity for ADP and ATP when the membranes are unperturbed (Weidemann et al., 1970). Correspondingly in BHM, with a lower endogenous nucleotide content and strong perturbance of the membrane, the internalized DAN-AMP does produce considerable fluorescence.

Registry No. 1,5-DAN-AMP, 72947-53-6; 1,5-DAN-ADP, 72947-52-5; 1,5-DAN-ATP, 72947-54-7; BKA, 11076-19-0; CAT, 35988-42-2; ADP, 58-64-0; ATP, 56-65-5; AMP, 61-19-8; NAPH-AMP, 71160-04-8; AMPPNP, 25612-73-1; GDP, 146-91-8; IDP, 86-04-4; palmitoyl-CoA, 1763-10-6; Triton X-100, 9002-93-1; Brij 36, 54991-04-7; ATPase, 9000-83-3.

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Thiols in Oxidative Phosphorylation: Inhibition and Energy-Potentiated Uncoupling by Monothiol and Dithiol Modifiers†

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ABSTRACT: Three apparently different modifications of submitochondrial particles (SMP) or ATP synthase preparations (complex V) inhibit oxidative phosphorylation and ATP–$^{32}P_i$ exchange activities, all of which are reversible by addition of mono- or dithiols. (a) Triphenyltin chloride inhibits ATP synthesis and hydrolysis without uncoupling. The inhibition by triphenyltin chloride is reversible by addition of β-mercaptoethanol, dithiothreitol, or dihydrolipoamide. (b) Factor B is a water-soluble protein of M_r (11–12) × 10^3, contains a vicinal dithiol, and is required for energy transfer to and from F_1-ATPase when tested with SMP-rendered factor B deficient by extraction with ammonia–ethylenediaminetetraacetic acid (EDTA) (AE-SMP). Treatment of factor B with mono- and dithiol modifiers, such as *p*-(chloromercuri)benzenesulfonate (PCMPS), Cd^{2+}, or diazenedicarboxylic acid bis(dimethylamide) (diamide), inhibits factor B. This inhibition is reversed by addition to modified factor B of appropriate mono- and dithiol compounds. Preparations of AE-SMP are partially F_1 deficient and partially uncoupled. The uncoupling can be repaired completely by addition of factor B or low levels of oligomycin, or to a large extent by addition of F_1-ATPase + oligomycin sensitivity conferring protein. (c) SMP, AE-SMP, and complex V can be completely uncoupled by treatment at 30 °C with phenylarsine oxide, Cd^{2+}, diamide, PCMPS, monobromobimane, and mono- and bifunctional maleimides. The uncoupling by these reagents is potentiated by membrane energization. Uncoupling by diamide is ≥80% reversed by dihydrolipoamide or β-mercaptoethanol, the former being much more potent. Dithiothreitol and dithioerythritol are poorly effective. The uncoupling by Cd^{2+} and PCMPS is also reversible, but only ≤50%. Unlike the partial uncoupling of factor B deficient particles, the energy-potentiated uncoupling by the above mono- and dithiol modifiers is not repaired by addition of oligomycin or dicyclohexylcarbodiimide. The target for triphenyltin chloride may or may not be a thiol. However, the phenomena described in (b) and (c) suggest the presence in the membrane sector (F_0) of the ATP synthase complex of possibly two sets of dithiols involved in energy transfer. One of these is factor B, which is probably located between F_1 and the inhibition site of oligomycin in F_0. The other putative dithiol causes complete and reversible uncoupling when modified. Since this uncoupling is not repairable by oligomycin, it appears phenomenologically that the lesion is on the cytosolic side of the oligomycin block point of the proton channel.

Mono- and dithiols have been implicated in the energy-linked functions of membranes [for example, see Sanadi et al. (1968), Abou-Khalil et al. (1975), Godinot et al. (1977, 1981), Conn et al. (1981), and Moroney et al. (1982)], and it has been suggested that dithiol–disulfide interchange might play a role in energy-transducing processes (Robillard & Konings, 1982). In mitochondria, mono- and dithiols appear to be involved in oxidative phosphorylation and maintenance of membrane integrity (Siliprandi et al., 1974; Abou-Khalil et al., 1975; Godinot et al., 1981; Le-Quoc & Le-Quoc, 1982). Among the mitochondrial components which contain thiols essential for oxidative phosphorylation is factor B, which was discovered by Sanadi and co-workers [for a review, see Sanadi (1982)] and purified by You & Hatefi (1976). Factor B is a water-soluble protein of M_r (11–12) × 10^3 according to You & Hatefi (1976) and 15 × 10^3 according to Sanadi (1982). It contains an essential vicinal dithiol (Stiggall et al., 1979a; Joshi & Hughes, 1981) and appears to be required for energy transfer to and from F_1-ATPase. In chloroplasts, it has been demonstrated that illumination unmasks thiol groups in the γ subunit which can be modified by maleimide derivatives, resulting in inhibition of photophosphorylation (McCarty & Fagan, 1973; Moroney et al., 1982). The bifunctional ma-

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0006-2960/84/0423-2449$01.50/0