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Interaction of Fluorescent Adenine Nucleotide Derivatives with the ADP/ATP Carrier in Mitochondria. 1. Comparison of Various 3′-*O*-Ester Adenine Nucleotide Derivatives†

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ABSTRACT: Fluorescent 3′-*O*-acyl-substituted adenine nucleotide (dimethylamino)naphthoyl and trinitrophenyl groups were studied for binding to the ADP/ATP carrier in mitochondria and submitochondrial particles. The changes in fluorescence intensity and emission maximum are for the most part similar to those observed in nonaqueous solvents. The (dimethylamino)naphthoyl derivatives from a largely quenched aqueous state have a shortwave shift up to 85 nm and increase up to 90-fold (1,5 derivative), whereas the little quenched naphthoyl derivatives show a fluorescence decrease and the weakly fluorescent trinitrophenyl derivative shows only a small increase on binding. All derivatives are good inhibitors (K_I = 1–10 μM) of nucleotide transport. The fluorescence titrations have an apparent $K_{1/2}$ = 2–7 μM. The fluorescence of the 1,5-DAN nucleotide is fully suppressed by bongkrekate but only partially suppressed by carboxyatractylate. The fluorescence response is much stronger in submitochondrial particles than in mitochondria. Both facts suggest fluorescent binding to the "m" state of the carrier site at the inner face of the membrane. 1,5-DAN-AMP shows a slightly more efficient binding than DAN-ADP or DAN-ATP.

Binding studies to the ADP/ATP carrier of mitochondria have proved to be a treasure house full of information elucidating modes of carrier function (e.g., Klingenberg et al., 1972; Weidemann et al., 1970; Klingenberg, 1976). Due to the inherent changes in the binding center of a carrier, considerable information of the functional state of the carrier can be revealed by both static and dynamic binding studies. These have so far been based on isotopic-labeled ligands, and therefore kinetic studies have been difficult. Ligands with fluorescent reporter groups have been sought, which might reflect important conformational changes relative to transport. Neither the substrates ADP and ATP nor the tightly binding inhibitors ATR[1] and BKA have an optical or fluorescent signal that can be monitored in binding studies. Because of the high selectivity of the carrier binding center, well-known fluorescent derivatives of ADP or ATP such as the ethenoadenine or 2-aminopurine nucleotides are nonbinding (Graue & Klingenberg, 1979). Only the structurally closely related and fluorescent adenine isomers, formycin di- and triphosphate, interact with the ADP/ATP carrier However, the characteristics of this fluorescence are rather disadvantageous such as low quantum yield, very short fluorescence lifetime (0.8 ns), and a strong overlap of excitation and emission with the signals of tryptophan. In part these difficulties were overcome by fluorescence transfer from formycin to anthracene derivatives (Graue & Klingenberg, 1979).

It was therefore greatly welcomed when Schäfer & Onur (1980) observed that (dimethylamino)naphthoyl esters of ADP etc. bind to the nucleotide carrier in submitochondrial particles, exhibiting a strong fluorescence signal. Before employing and exploring the binding possibilities of this compound to the ADP/ATP carrier on a broader scale, we had to develop a chemical synthesis of the most interesting substituent, 5-(dimethylamino)-1-naphthoic acid. The studies reported here concentrate on the carrier still located in the mitochondrial membrane. A part of these results has been reported previously (Klingenberg, 1981).

Materials and Methods

Chemical Synthesis. N^6-(2,4-Dinitrophenyl)adenosine 5′-triphosphate (DNP-ATP) was synthesized at a 35% yield by the method of Hiratsuka et al. (1973) with the modification that the reaction was allowed to proceed for 48 h and two Sephadex LH-20 (3 × 80 cm) purification steps were employed. 2′(3′)-*O*-(2,3,6-Trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was synthesized at a 62% yield by the method of Hiratsuka & Uchida (1973) also with the modification that two Sephadex LH-20 (3 × 90 cm) steps were employed and that fractions containing homogeneous TNP-ATP were pooled and immediately adjusted to pH 7 with Tris base; the Meisenheimer complex undergoes a slow hydrolysis process, forming ATP and picric acid at −20 °C, necessitating

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[1] Abbreviations: ATR, atractylate; BKA, bongkrekate; BHM, beef heart mitochondria; BHSMP, beef heart submitochondrial particles; CAT, carboxyatractylate; DAN-AMP, [(dimethylamino)naphthoyl]-adenosine 5′-monophosphate; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; RLM, rat liver mitochondria; RLSMP, rat liver submitochondrial particles; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; ATPase, adenosinetriphosphatase.

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rechromatography prior to use.

4-(Dimethylamino)-1-naphthoic acid was synthesized from 4-(dimethylamino)naphthalene and phosgene at a 30% yield according to the procedure described by Friedlander (1878). 5-(Dimethylamino)-1-naphthoic acid and 5-(dimethylamino)-2-naphthoic acid were synthesized from the respective aminonaphthoates by methylation with dimethyl sulfate (Laurence, 1957). 4-Aminonaphthoic acid was prepared by reduction of the 5-nitro-1-naphthoic acid synthesized according to Ekstrand (1886a,b); reduction was achieved by addition of 35 g of powdered iron over a 1-min period to 2 g of the nitro compound dissolved in 20 mL of acetic acid, the temperature being kept below 60 °C in an ice bath; after the effect of the temperature release had subsided, the mixture was incubated at 55 °C for 5 min prior to extraction by benzene and precipitation of the amine by HCl gas (Hazlet & Dornfield, 1944). 5-Amino-2-naphthoic acid was prepared by an identical reduction procedure using 5-nitro-2-naphthoic acid synthesized according to Ekstrand (1980). The 5-(dimethylamino)-naphthoic acid derivatives were synthesized from the corresponding α- and β-naphthoates at an overall 8–12% yield and had the reported melting points and the anticipated NMR spectra and elemental analyses. All the (dimethylamino)-naphthoates were shown to be homogeneous by TLC on silica gel G in solvent systems 1–4 or on cellulose in solvent systems 5 and 6.

3′-*O*-(1-Naphthoyl)-AMP (NAPH-AMP), 3′-*O*-[5-(dimethylamino)-1-naphthoyl]-AMP (1,5-DAN-AMP), 3′-*O*-[4-(dimethylamino)-1-naphthoyl]-AMP (1,4-DAN-AMP), 3′-*O*-[5-(dimethylamino)-2-naphthoyl]-AMP (2,5-DAN-AMP), and their corresponding ADP or ATP analogues were synthesized from the respective naphthoic acids by the carbonyldiimidazole procedure described by Jeng & Guillory (1975) for the esterification of ATP with *N*-(*tert*-butoxycarbonyl)-α-alanine.

The synthesis was performed as follows. 1,1′-Carbonyldiimidazole (1120 μmol) and 1,5-DAN (480 μmol) dissolved in 600 μL of anhydrous *N*,*N*-dimethylformamide were stirred 15 min at room temperature. Then 120 μmol of nucleotide in 2 mL of H_2O was added, and the solution was stirred overnight. The solvent was removed by lyophilization, and the reaction products were washed with ether to remove unreacted 1,1′-carbonyldiimidazole and 1,5-DAN. The yield in the esterification reactions was usually 5–15%. The reaction products were purified on an LH-20 column (bed volume 100 mL). The fractions were eluted with distilled water at 25 °C. The fractions of the second peak were pooled, lyophilized, and purified on a diethylaminoethylcellulose column (bed volume 14 mL at 4 °C). The fractions were eluted by a gradient of 0–0.6 M triethylamine–bicarbonate (pH 7.2) saturated with CO_2 at room temperature.

All nucleotide derivatives were shown to be homogeneous and free of unreacted DAN by paper or TLC–cellulose chromatography in solvent systems 5–7 or on silica gel G in solvent 3.

The purity of the various adenine nucleotides was routinely assayed on thin-layer chromatography in system 5. The DAN derivatives of AMP, ADP, and ATP hydrolyze slowly at 0 °C (about 20% in 1 month); consequently preparations were rechromatographed on DEAE-Sephadex.

Thin-layer chromatography was performed on silica gel G in the following systems: (1) chloroform–ethanol–glacial acetic acid, 190:20:12; (2) benzene–pyridine–glacial acetic acid, 80:20:5; (3) isobutyric acid–ammonia–water, 66:1:33; (4) chloroform–methanol, 1:1. Paper or thin-layer chromatography was carried out in the following systems: (5) butanol–2-propanol–water, 1:1:1; (6) butanol–water–glacial acetic acid, 5:3:2; (7) isobutyric acid–1 M ammonium hydroxide–0.1 mM EDTA, 100:60:1.6.

Biochemical Experiments. Mitochondria from beef heart (BHM) and from rat liver (RLM) were separated as described previously (Smith, 1967). Submitochondrial particles from beef heart (BHSMP) were prepared by sonication of BHM for 10 × 5 s in 50 mM sucrose, 15 mM $MgCl_2$, and 10 mM Pipes, pH 7.4, at 0 °C and isolated from the 12 000 rpm supernatant by another centrifugation at 30 000 rpm for 30 min.

Fluorescence spectra were performed with a Schoeffel RRS 1000 fluorospectrometer. Usually 5 × 5 nm quartz fluorescent cuvettes were used with a sample volume of 0.3 mL. For the titrations, excitation wavelength was 350 nm and emission at 524 nm. Measurements were usually performed at 4 °C in the "standard incubation" medium consisting of 250 mM sucrose and 20 mM Tris buffer, pH 7.2.

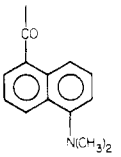
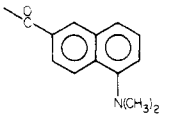
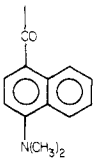
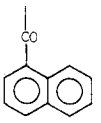
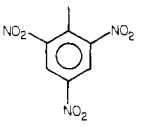
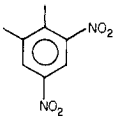
Results

Fluorescent Properties of Adenine Nucleotide Derivatives. Several ribose-ring-modified fluorescent adenine nucleotide *O*-acyl ester derivatives were synthesized as described under Materials and Methods. Table I lists some fluorescence properties of these derivatives in different solvents. Generally there is a blue shift of the emission when going from aqueous to nonaqueous solvents. In several cases the fluorescence is also enhanced by these solvents. 1,5-DAN-ATP has a large up to 90-fold enhancement in solvents of increasingly apolar composition, which relates to a strong quenching by H_2O. The emission and excitation spectra of 1,5-DAN-ATP in dioxane are identical with those previously reported (Schäfer & Onur, 1980). The fluorescence spectra of 1,5-DAN-ADP, 1,5-DAN-AMP, and 1-naphthoic acid are identical and have a solvent dependence similar to that of 1,5-DAN-ADP (not shown). In fact, the nucleotide-free fluorophor 1,5-DAN-methyl ester has similar fluorescent properties. In contrast, 2,5-DAN-ADP exhibits considerable fluorescence in aqueous solution with further fluorescence enhancement in 50% ethanol (red shifted) and in dioxane (blue shifted). Most remarkably this fluorescence is strongly quenched by dioxane. The nitrophenyl derivatives show comparatively weak fluorescence (see Table II). TNP-ATP has a considerable fluorescence enhancement in ethanol.

Substitution of fluorophoric groups at the γ-position of ATP by making γ-phosphoamidate was also pursued with the following groups: [[5-(dimethylamino)naphth-1-yl]-sulfonyl]ethylenediamine, 1-anthranoylamine, 1-naphthoylamine, fluoresceinylamine. All these compounds, however, did not give a carrier-specific response with the membrane. Their fluorescence was not changed on addition of BKA or CAT, nor did they exert a clearly competitive inhibition on the ADP/ATP exchange.

Fluorescent Properties on Binding to Mitochondrial Membranes. The fluorescence effect of these various nucleotide derivatives on binding to mitochondrial membranes was investigated. In order to differentiate the binding to the ADP/ATP carrier sites, the fluorescence decrease caused by the inhibitor ligands CAT and BKA was also recorded. As an example the fluorescence emission spectra registered on addition of 1,5-DAN-ADP are given in Figure 1. In the case of BHSMP a relatively strong emission spectrum with a blue-shifted maximum at 505 nm is recorded, with practically no noise level despite the considerable light scattering. It is clearly separated from the signal of the BHSMP alone which

Table I: Fluorescence Characteristics of Some ATP Derivatives

compound	R[a]	H_2O (%)	EtOH (%)	dioxane (%)	λ^{exc}_{max} (nm)	λ^{em}_{max} (nm)	λ shift from H_2O (nm)	intensity change from H_2O	τ (ns)
1,5-DAN-ATP		100			333	609		1	
		50	50		337	588	−21	7.7	
		0		100	345	524	−84	92	
		2		98	345	536	−70	48	
		50		50	341	580	−29	0.3	
2,5-DAN-ATP		100			345	550		1	
		50	50		352	536	−14	5.9	7.8
		0		100	352	501	−49	0.5	
		50		50	352	538	−12	0.7	
1,4-DAN-AMP		100			378	413		1	
		50	50		378	410	−3	4.6	4.2
1-NAP-ATP		100			334	381		1	
		0	100		335.5	369	−12	0.5	16.3
TNP-ATP		100			487	548		1	
		50	50		487.5	533	−15	7.7	
DNP-ATP		100			491	506		1	
		50	50		497	512	+6	1.1	

[a] R = 3′-*O*-acyl group on AMP, ATP, or ADP.

Table II: Fluorescence Response of Nucleotide Derivative Binding to Membranes[a]

O-acyl nucleotides	$\lambda_{ex}/\lambda_{em}$	F	F_{BKA}	ΔF_{BKA}	F_{CAT}	ΔF_{CAT}	$\Delta F_{BKA}/F$	$\Delta F_{CAT}/F$
			Beef Heart Mitochondria (BHM)					
1,5-DAN-3′-ADP	377/505	2.76	0.13	2.62	1.27	1.48	0.95	0.53
2,5-DAN-3′-ADP	352/500	7.1	4.61	2.49	3.44	3.66	0.35	0.51
1,4-DAN-3′-ATP	378/426	5.7	2.57	3.13	2.76	2.95	0.58	0.51
1-NAP-3′-ATP	336/374	6.6	7.66	−1.06	8.18	−1.58	−0.16	−0.24
TNP-ATP	487/545	0.57	0.41	0.16	0.41	0.16	0.28	0.28
			Submitochondrial Particles (BHSMP)					
1,5-DAN-3′-ADP		4.1	0.29	3.81	3.9	0.2	0.93	0.05
2,4-DAN-3′-ADP		9.2	4.8	4.4	8.5	0.6	0.46	0.07
1,4-DAN-3′-ATP		5.4	2.44	2.96			0.55	
1-NAP-3′-ATP		8.2	10.6	−2.4			−0.29	
TNP-ATP		0.56	0.41	0.15			0.04	

[a] F = fluorescence in arbitrary units standardized for equal concentration of derivative and of mitochondria. F_{BKA} = fluorescence after addition of 10 μM BKA. F_{CAT} = fluorescence after addition of 10 μM CAT. $\Delta F_{BKA} = F - F_{BKA}$. $\Delta F_{CAT} = F - F_{CAT}$. Wavelength difference chosen for maximum response. Additions of derivatives at 5–10 μM. BHM and BHMSP at about 1 mg of protein/mL in "standard medium".

exhibits a maximum at 435 nm. On addition of CAT no marked reduction of fluorescence is seen. However, on addition of BKA the fluorescence with its maximum at 505 nm is nearly abolished to the level of blank fluorescence, in agreement with the observation of Schäfer & Onur (1980). In BHM the fluorescence response on addition of 1,5-DAN-ADP is smaller. With a 3-fold amplification as compared to that of BHSMP the same maximum is observed, which is much less separated from the blank fluorescence. In this case addition of CAT can partly suppress the fluorescence, and BKA again suppresses it completely. The fluorescence responses of 1,5-DAN derivatives of AMP or ATP are very similar and will be further elaborated.

The addition of 2,5-DAN-ADP to BHM and BHSMP gives an about 2.5-fold stronger fluorescence signal than that of 1,5-DAN-ADP, partially due to the less quenched fluorescence of 2,5-DAN-ADP in H_2O. The maximum is blue shifted to 500 nm. Unlike the results with 1,5-DAN-ADP, the fluorescence of 2,5-DAN-ADP is suppressed more strongly by CAT than by BKA in BHM; in BHSMP, similarly as in the isomer, CAT is without effect, whereas BKA strongly suppresses the fluorescence. However, 30% of the signal resists

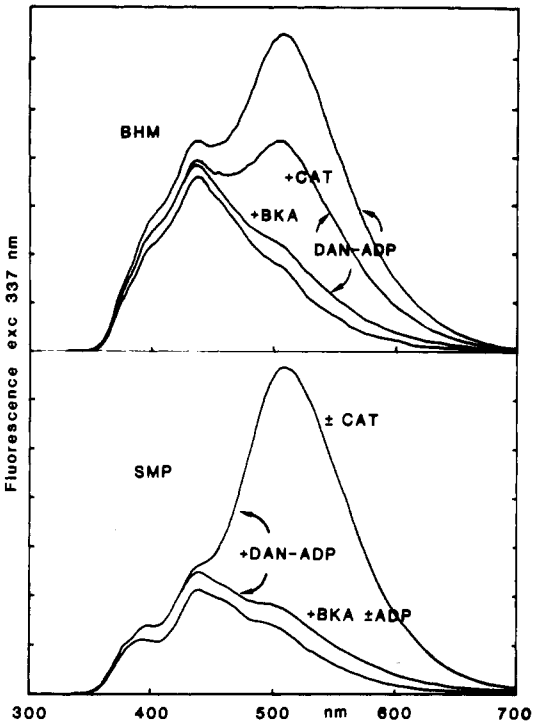


FIGURE 1: Fluorescence emission spectra of DAN-ADP added to beef heart mitochondria (BHM) and mitochondrial particles (SMP). Uncorrected spectra. Where indicated, the following additions have been made to mitochondria prior to addition of 7 μM 1,5-DAN-ADP: 25 μM CAT, 25 μM BKA, and 0.5 mM ADP; 5 mg of protein/mL of BHM and 3.3 mg of protein/mL of BHSMP were incubated in standard medium at 10 °C.

BKA addition with a red-shifted maximum. The fluorescence spectra of the various derivatives on addition to BHM and BHSMP were evaluated, and the results are listed in Table II. The fluorescence signal "*F*" is composed of contributions from the membrane and from free, from carrier-bound, and from otherwise bound derivatives. The wavelengths are those where maximum change on addition of the inhibitors is observed after which the fluorescent derivative is assumed to be released. The listed *F* values are standardized for equal carrier site and derivative concentration. Most important for our purpose is the fluorescence difference after addition of the inhibitors BKA and CAT which indicates fluorescence change on carrier binding. In general the fluorescence changes on binding are similar to those observed on transition from an aqueous solution to a hydrophobic solvent.

In BHM and BHSMP the changes have the same direction and are of similar size. In BHM, CAT gives about the same change as BKA, whereas in BHSMP, CAT is much less effective. The three different DAN derivatives exhibit a strong fluorescence increase on binding whereas the NAP derivatives show a small decrease. Most significant is the comparison of the relative fluorescence changes expressed as $\Delta F_{BKA}/F$ and $\Delta F_{CAT}/F$. 1,5-DAN-ADP fluorescence is nearly completely suppressed to 95% by BKA in both membranes but only to 53% by CAT in BHM and not at all in BHSMP. The fluorescence of the 2,5- and 1,4-DAN-ADP is decreased only by 35 or 46% and 58 or 55% by BKA. In contrast, in the case of NAP-ATP an increase, though relatively small, is observed on addition of the inhibitor. The trinitrophenyl derivative shows again a decrease of 28% with BKA and CAT; however, its fluorescence level is only small.

The time-dependent recording of the fluorescence, shown in Figure 2, was used for titration of the concentration dependence to follow the kinetics of the response and to examine the influence of various fluorescence-enhancing or -quenching

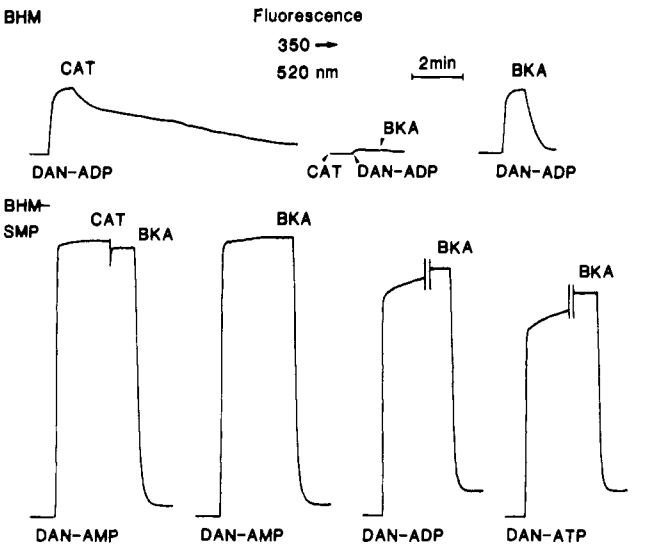


FIGURE 2: Fluorescence recording of DAN nucleotides when added to suspension of beef heart mitochondria (BHM) and particles (BHSMP). Subsequent inhibition of fluorescence by addition of inhibitor ligands to the ADP/ATP carrier. BHM (1 mg of protein/mL) in standard buffer: addition of 6 μM 1,5-DAN-ADP. BHSMP (1.3 mg protein/mL): addition each time of 6 μM 1,5-DAN-AMP, 6 μM 1,5-DAN-ADP, 6 μM 1,5-DAN-ATP, 20 μM BKA, and 20 μM CAT. Recording at 10 °C.

factors. The recordings with BHM and BHSMP show that the fluorescence response to DAN-ADP addition is rapid. The subsequent addition of CAT leads to a slow and complete reversion of the fluorescence in BHM but has only a minor effect in BHSMP. With BKA, however, rapid and nearly full suppression of the fluorescence is reached in both preparations.

Comparison of the three nucleotide derivatives of 1,5-DAN or AMP, ADP, and ATP shows a similar response on addition to the membrane and to BKA. The ratio between signal strengths of the AMP:ADP:ATP derivatives is 10:8.4:7.7. This is most surprising in view of the fact that only ADP and ATP but not AMP are substrates or ligands of the ADP/ATP carrier. The kinetics following the addition of the derivative have a fast and a slow phase. The slow phase increases, going from AMP to ADP to ATP derivative, i.e., from 3% to 16% to 22% of the BKA-sensitive signal (see Discussion).

Effects on Exchange and Half-Saturation Constants. The ability of the nucleotide derivatives to interact with the ADP/ATP carrier was assessed by examining their inhibitory effect on the ADP-promoted exchange rate in BHM as well as in ATP-loaded BHSMP. In these preparations the exchange measurements are less accurate because of the unavoidable heterogeneity of the preparation. Rat liver mitochondria are, therefore, also used, permitting more accurate measurements because of their higher degree of intactness. Experiments with BHSMP in which the concentrations of ADP varied over a wide range at different concentrations of DAN-ADP (Figure 3A) showed that this derivative is a competitive inhibitor of the nucleotide translocation. In this case the K_M value for ADP is 0.5 mM in the presence of 50 μM DAN-ADP. A titration with DAN-AMP of the exchange at 100 μM ADP gives the inhibition constant K_i = 5 μM for DAN-ADP (Figure 3B). The other derivatives also proved competitive inhibitors. Inhibition parameters extracted from the results of a number of experiments are summarized in Table III. All eight derivatives are potent inhibitors of the nucleotide exchange, possessing apparent K_i values from 0.6 to 12 μM. The strongest inhibitor is TNP-ATP followed by NAP- and 1,4-DAN-ATP. The DNA derivatives are slightly less effective with a $K_{i(1/2)}$ = 3–10 μM.

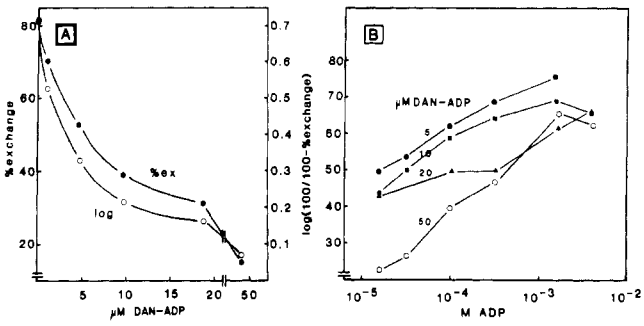


FIGURE 3: Inhibition by 1,5-DAN-AMP of nucleotide exchange in mitochondrial particles (BHSMP). ATP-loaded BHSMP were first charged with 10000 dpm/mg [^{14}C]ATP for back-exchange. Exchange was started by addition of ADP and stopped after 20 s by 20 μM BKA. The release of [^{14}C]ATP was determined in the supernatant. (A) Inhibition of the exchange by increasing concentrations as indicated. Start with 20 μM ADP. (B) Competitive inhibition between DAN-ADP and ATP. Incubation of SMP at various concentrations of ADP and DAN-ADP as indicated. For both experiments A and B incubation of 2 mg of protein/mL of BHSMP in standard buffer at 10 °C.

Table III: Apparent Inhibition Constants of the Nucleotide Derivatives

derivative	50% inhibition[a] (μM) RLM	BHM	BHSMP
1,5-DAN-ADP		12	8
1,5-DAN-AMP	2.8	8	7
2,5-DAN-ADP			4
2,5-DAN-ATP	10		4.2
1,4-DAN-ATP	2		
NAP-ATP	2		
TNP-ATP	0.6		
DNP-ATP	2		

[a] In the presence of 100 μM ADP. RLM, rat liver mitochondria; BHM, beef heart mitochondria; BHSMP, beef heart submitochondrial particles.

Table IV: Half-Maximum Concentration ($K_{1/2}$) for BKA-Displaceable Fluorescence Enhancement

derivative	$K_{1/2}$ (μM) BHM[a]	BHSMP[a]
1,5-DAN-ADP	3	1.8
2,5-DAN-ATP	3.5	2
1,4-DAN-ATP	1.7	3
NAP-ATP	5	

[a] BHM, beef heart mitochondria; BHSMP, beef heart submitochondrial particles.

In separate experiments the concentration dependence of the fluorescence was determined for the various fluorescent derivatives. The fluorescence increase on incremental additions was recorded both in the absence and in the presence of BKA. The half-maximum concentration for the BKA-displaceable fluorescence given in Table IV shows that these affinities are close to the inhibition constants of Table III. The latter are in fact somewhat elevated by the competition with 0.1 mM ADP. The value for NAP-ATP is rather inaccurate because of unfavorable fluorescent properties, i.e., a 23% decrease of fluorescence on binding (see Table II). The fluorescence change of TNP-ATP is too small to permit a meaningful evaluation.

Inhibition by Dyes and Quenching. Some planar aromatic dyes known as nucleotide analogues from studies with other nucleotide binding proteins (Neslund & Dahms, 1979), in particular fluoresceine derivatives, have been examined for their effect on the 1,5-DAN-AMP interaction with the carrier. All these dyes are able to diminish the fluorescence of 1,5-DAN-AMP added to BHSMP. From titration studies with these compounds $K_{(1/2)}$ values are evaluated as shown in Table V. Rose bengal, known as an inhibitor of the nucleotide transport, is the most effective competitor for 1,5-DAN-AMP binding, followed by tetraiodofluorescein and bromcresol green. Here 1,5-DAN-ATP fluorescence serves as a convenient tool for evaluating the interaction of these dyes with the ADP/ATP carrier. It can be assumed that these dyes remove the nucleotide derivative rather than quench the fluorescence by energy transfer.

Table V: Effects of Dye Nucleotide Analogues on 1,5-DAN-AMP Fluorescence

analogue	concn (μM) for 50% release of 1,5-DAN-AMP fluorescence
rose bengal	1.1
tetraiodofluorescein	2.2
bromocresol green	2.9
8-(4-anilino-5-sulfo-1-naphthylazo)-1-naphthalene-3,6-disulfonic acid	12
alizarin red S	72

In other results not shown here Stern–Volmer plots of I^- quenching of the fluorescence of 1,5-DAN-AMP and ADP indicate that in BHSMP the quenching by I^- is collisionally controlled. Bound DAN-AMP is considerably less accessible to solvent compared to ε-NAD bound to alcohol dehydrogenase (Gafni, 1979) but is more accessible to solvent than ε-ATP bound to G-actin (Harvey & Cheung, 1976). The 1,5-DAN-ADP binding sites in both uncoupled BHM and BHSMP have identical solvent exposure and I^- quenching. In RLSMP the 1,5-DAN-ADP site is more exposed to solvent than in BHSMP.

Discussion

The high specificity of the ADP/ATP carrier for its substrates leaves only little choice among nucleotide analogues that still have binding affinity. Substitution at the 2′- or 3′-*O*-position of the ribose moiety seems to offer the best chance of forming derivatives acceptable to the carrier sites, as borne out by the fluorophoric derivatives employed in the present study. Whereas substitution at the γ-phospho position with phosphoamidates failed to provide derivatives that interact with the ADP/ATP carrier, most of the *O*-acyl derivatives exhibit a surprisingly strong affinity, as witnessed by the strong inhibitory power of the nucleotide transport. Originally naphthoyl derivatives were synthesized by Schäfer & Onur (1979, 1980) and found to be good ligands to the F_1-ATPase and, in the case of the 1,5-DAN derivatives, also to the nucleotide carrier.

Introduction of the classical dansyl [5-(dimethylamino)-naphthalene-1-sulfonyl] group into ADP and ATP has not been achieved so far, since dansylation obviously requires reaction in nonaqueous media such as pyridine in which ADP and ATP are nonsoluble. Only the preparation of dansyladenine has been reported, which was applied as fluorescent ligand to adenine deaminase (Skorka et al., 1981). The dansyl group has, however, been coupled to 2′-amino-ADP and -ATP, and these derivatives have been applied to fluorescence studies of the myosin ATPase (Watanabe et al., 1981; Matsuoka et al., 1981). On the other hand, substitution by esterification at the 2′- or 3′-ribose group by the carbonyldiimidazole method permits handling of the unmodified nucleotides in aqueous

solution (Gottikh et al., 1967). this is important for derivatizing ADP and ATP, which are little soluble in nonaqueous media.

The difference in fluorescence of the naphthoyl derivatives in aqueous and nonaqueous media is remarkably high and of particular interest because of similar differences observed on binding of these derivatives to the nucleotide carrier. What are the causes for the change in the fluorescence yield? Apart from the usual dynamic quenching in aqueous solutions observed with many aromatic fluorophors, a conformational change may be responsible. In aqueous solutions a sandwichlike structure may bring the naphthoyl and adenine moiety into close proximity, causing fluorescence quenching [see Watanabe et al. (1981)]. In nonaqueous low dielectric solvents the molecule has a more stretched and mobile shape, allowing for little interaction between the two ring structures. The 1,5-DAN should have particularly favorable steric conditions for this quenching whereas the 2,5- and 1,4-DAN derivatives may have less favorable steric prerequisites as they show considerable fluorescence also in aqueous solution.

The fluorescence change observed on binding to the nucleotide carrier sites corresponds well to the transition to nonaqueous media. Again it is noted that whereas the fluorescence of all DAN derivatives is enhanced, the fluorescence of the unsubstituted NAP derivative decreases on binding to the nucleotide sites. It can be visualized that the sterical change occuring on binding to the carrier is similar to that incurred by transition to nonaqueous media. For example, in the bound state the DAN 3′-*O*′-acyl nucleotide derivatives may have a more stretched conformation than in aqueous solution. Judging from the fluorescence data presented, and also in view of their high affinity to the nucleotide carrier, the 1,5-DAN-adenine nucleotides seem to be the most useful fluorescent derivatives for studying the ADP/ATP carrier, particularly for the following reasons: the fluorescent enhancement on binding is much stronger than that of other probes, fluorescence produced upon binding to BHM was completely abolished by BKA, the 1,5-DAN-adenine nucleotides are bound to the carrier with high affinity, the fluorescence decay time has a magnitude appropriate for fluorescence polarization studies, and fluorescence excitation emission is far removed from that of intrinsic tryptophan.

The identification of the 1,5-DAN nucleotide fluorescence signal exclusively through binding to the ADP/ATP carrier relies heavily on the specific interactions with BKA. In fact, to put it more precisely, it shows that this interaction takes place only with the "m"-state conformation of the carrier. The question whether the derivatives also interact with the "c"-state side will be dealt with in the following paper (Klingenberg et al., 1984).

When the three derivatives of 1,5-DAN are compared, it is obvious that the fluorescence yield decreases somewhat going from DAN-AMP to DAN-ATP, since we can assume, as shown in the following paper (Klingenberg et al., 1984), that the three nucleotides bind to the same extent. Possibly the hydrophobic interaction is stronger with DAN-AMP and slightly disturbed by the higher ionic charges of DAN-ATP.

The biphasic fluorescence increase which is most pronounced with DAN-ATP provides in our opinion an interesting glimpse on the interaction of the derivatives also with the c state. The interaction with the c state of the carrier is believed to increase going from the AMP to the ATP derivative. However, as discussed in the following paper (Klingenberg et al., 1984), this binding is nonfluorescent. The slow-phase fluorescent increase is visualized to reflect the transition of the nonfluorescent c-state DAN-ATP/carrier complex into the fluorescent m-state complex.

The surprising finding that the DAN-AMP derivative binds with at least the same efficiency as the DAN-ADP and DAN-ATP derivatives seems at first to argue against binding of the 1,5-DAN-nucleotides to the nucleotide site since the latter had been shown not to accept free AMP. This finding may suggest that the binding sites for DAN-AMP and BKA are identical but different from the nucleotide binding site. It would then also argue against our original concept (Klingenberg, 1974; Klingenberg & Buchholz, 1973) that the carrier contains a single binding center which exhibits a well-defined affinity to the various ligands. The question to what degree the DAN-AMP sites are identical with or different from the nucleotide binding sites will be more closely examined in the following paper (Klingenberg et al., 1984).

It is remarkable that 1,5-DAN-ADP and -ATP, when binding to the ATPase in BHSMP, form a tight nonfluorescent complex (Schäfer & Onur, 1979, 1980). Therefore, the fluorescence quenching of the endogenous tryptophan on binding of DAN-ADP has been used in this case for following the interaction with ATPase. NAP derivatives of ADP and ATP have been applied to study the ADP/ATP carrier despite their disadvantages (Block et al., 1979, 1982). Here the signal starts at a high level of fluorescence caused by the free NAD-ADP, and on binding one observes a relatively small decrease. This not only does give a high noise level and therefore less accuracy but also makes it very difficult to differentiate between the free and bound nucleotides on the basis of fluorescence alone. Probably the problems of the synthesis protocols of 1,5-DAN were the reason that led Block et al. (1982) to use the unsubstituted NAP derivatives although the advantages of the 1,5-DAN derivatives had been already briefly documented by Schäfer & Onur (1980) and by us (Klingenberg, 1981).

Registry No. DNP-ATP, 50909-88-1; 2′-TNP-ATP, 50826-97-6; 3′-TNP-ATP, 50826-99-8; 1,4-DAN, 78062-03-0; 1,5-DAN, 86042-10-6; 2,5-DAN, 89232-18-8; NAPH-AMP, 71160-04-8; 1,5-DAN-AMP, 72947-53-6; 1,4-DAN-AMP, 89232-19-9; 2,5-DAN-AMP, 89232-20-2; 5′-AMP, 61-19-8; 5′-ADP, 58-64-0; 5′-ATP, 56-65-5; 1,5-DAN-ATP, 72947-54-7; 2,5-DAN-ATP, 89232-21-3; 1-NAP-ATP, 76152-01-7; 1,5-DAN-ADP, 72947-52-5; 2,5-DAN-ADP, 89232-22-4; 1,4-DAN-ATP, 89232-23-5; BKA, 11076-19-0; CAT, 35988-42-2; rose bengal, 11121-48-5; tetraiodofluorescein, 15905-32-5; bromocresol green, 76-60-8; 8-(4-anilino-5-sulfo-1-naphthylazo)-1-naphthalene-3,6-disulfonic acid, 89232-24-6; alizarin red S, 130-22-3.

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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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