Huber, S. C., & Edwards, G. E. (1977a) *Biochim. Biophys. Acta 462*, 583.
Huber, S. C., & Edwards, G. E. (1977b) *Biochim. Biophys. Acta 462*, 603.
Kotyk, A., & Janacek, K. (1975) *Cell Membrane Transport. Principles and Techniques*, pp 80–89, Plenum Press, New York.
Lehner, K., & Heldt, H. W. (1978) *Biochim. Biophys. Acta 501*, 531.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 2*, 431.
Ogren, W. L. (1984) *Annu. Rev. Plant Physiol. 35*, 415.
Robinson, S. P. (1982) *Plant Physiol. 70*, 1032.
Robinson, S. P. (1984) *Plant Physiol. 75*, 425.
Sicher, R. C. (1984) *Plant Physiol. 74*, 962.

Interaction of Fluorescent 3′-[1,5-(Dimethylamino)naphthoyl]adenine Nucleotides with the Solubilized ADP/ATP Carrier†

Martin Klingenberg,* Ingeborg Mayer, and Maria Appel
Institut für Physikalische Biochemie der Universität München, 8000 München 2, Federal Republic of Germany
Received July 26, 1984

ABSTRACT: The binding of the 3′-[1,5-(dimethylamino)naphthoyl] (DAN) derivatives of AMP, ADP, and ATP to the solubilized ADP/ATP carrier is studied, evaluating primarily the fluorescence enhancement and ^{3}H-labeled compound binding. DAN nucleotides also fluoresce when adsorbed to Triton X-100 micelles that are used for solubilization of the carrier. The partition of DAN-AMP between water and Triton X-100 micelles is measured, and it is shown to be shifted toward a higher content in Triton micelles with increasing salt concentration. In order to maintain a low level of fluorescence, the Triton content is decreased. The fraction of DAN nucleotide fluorescence due to carrier binding is determined by the suppression with bongkrekate (BKA). In contrast to the membrane-bound carrier, the solubilized preparation shows an increase of total BKA-sensitive fluorescence by 30–60% upon addition of ATP or ADP. In the solubilized atractylate–protein complex, the ADP-stimulated fluorescence amounts even to 80%. The suppression of fluorescence by BKA is independent of the presence of ADP or ATP, while that by carboxyatractylate (CAT) depends on ADP or ATP. The quantitation with [^{3}H]BKA and [^{3}H]CAT of these ligand interactions with DAN-AMP fluorescence shows that DAN-AMP fluorescence reflects the "m"-state carrier population and its redistribution under the influence of ADP or ATP. Thus, besides the "c"/"m" distribution, the kinetics of the c to m transition in the solubilized carrier also can be determined. The m share is increased to 80% when SO_4, P_i, or pyrophosphate is present during solubilization. The rate of the ADP- or ATP-stimulated transition to the m state is markedly dependent on pH and on the presence of various anions, whereas the extent is little varied. The affinity decreases 4-fold going from DAN-AMP to DAN-ADP and to DAN-ATP (K_D = 0.9, 1.6, and 3.2 μM). Comparison with physical binding of [^{3}H]DAN nucleotides shows that the fluorescence yield of bound DAN-AMP is about 1.4 times higher than that of bound DAN-ATP. DAN substitution causes more than a 100-fold affinity increase for AMP and a 50-fold increase for ADP or ATP, probably because of interaction of the DAN group with a hydrophobic niche. A less specific, low-affinity displacement of DAN nucleotides by GDP, ADP, GTP and ATP (K_i = 1–2 mM) probably reflects primarily the ionic interactions at the binding center.

The 3′-[1,5-(dimethylamino)naphthoyl] derivatives of AMP, ADP, and ATP are valuable fluorescent probes for the ADP/ATP carrier in mitochondrial membranes (Schäfer & Onur, 1980; Schäfer et al., 1980; Klingenberg, 1981a; Mayer et al., 1984). The DAN[1] nucleotides make possible visualization of changes in the binding center of the ADP/ATP carrier associated with the translocation process, which have so far been examined primarily in binding studies of labeled ligands. The DAN nucleotides emit a strong fluorescence on binding to the ADP/ATP carrier only when the binding center is in the "m" state, directed to the m (matrix) side, whereas it does not fluoresce on binding to the center in the "c" state when it is directed to the c (cytosol) side; this was shown both by the sensitivity to the carrier state specific inhibitors CAT and BKA and by studying the access of the DAN nucleotides to the carrier sites in mitochondria vs. submitochondrial particles (Klingenberg et al., 1984).

Whereas the results on the ADP/ATP carrier in the membrane in its original environment are of considerable interest, they are complicated by the presence of other nucleotide binding proteins, by the membrane barrier and uncertain membrane orientation, particularly in submitochondrial particles. Thus, the effects of ADP and ATP on DAN nucleotides binding indicated functional heterogeneity of the binding sites for exchange and for binding. It is likely that this heterogeneity was introduced by the sonication of the membranes.

By using the solubilized and purified ADP/ATP carrier, these complications can be avoided, and less equivocal results on the interaction of the DAN-ATP with the carrier can be

† This work was supported by grants from the Deutsche Forschungsgemeinschaft (Kl 134/22 and 23).

[1] Abbreviations: ATR, atractylate; BKA, bongkrekate; CAT, carboxyatractylate; DAN-AMP, 3′-[1,5-(dimethylamino)naphthoyl]-adenosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; LAPAO, lauroylpropylamine oxide; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-3650$01.50/0

obtained. As a result, new information about the dependence of the c- and m-state distribution on a variety of environmental factors and a more precise insight into the transition between these states have been obtained. Furthermore, the ligand interaction forces at the binding center have been elucidated and rationalized in terms of a single binding center with an optional hydrophobic niche in the m state used by DAN nucleotides and BKA. A brief report on some of these results was given in Klingenberg (1981a).

Materials and Methods

The 3′-[1,5-(dimethylamino)naphthoyl]adenine nucleotides and the 1,5-DAN derivatives of [^{3}H]AMP, [^{3}H]ADP, and [^{3}H]ATP were synthesized as described previously (Mayer et al., 1984). The preparations were stabilized against hydrolysis by dissolving the fractions obtained from the last purification step after the DEAE column in absolute ethanol and storage at −180 °C. Thus, hydrolysis could be restricted to less than 5% in 2 months. Hydrolysis is faster with 1,5-DAN-AMP than with DAN-ADP and DAN-ATP. The critical synthesis of 1,5-(dimethylamino)naphthoic acid was worked out by Stephen Dahms in 1980 in this laboratory [see details in Mayer et al. (1984)]. In the meantime, a synthesis of this material by a somewhat different procedure was described briefly by Schäfer's group (Onur et al., 1983).

Amberlite NAD-4 was obtained from Fluka, Buchs; [^{3}H]CAT was synthesized according to Klingenberg et al. (1975); [^{3}H]BKA was prepared according to Babel et al. (1976).

Solubilized enriched ADP/ATP carrier was prepared as follows [see also Krämer et al. (1977)]: Isolated beef heart mitochondria were stored in liquid nitrogen. In a typical case, 1 mL of thawed mitochondria containing 60 mg of protein was added to 2.5 mL of solution containing 8% Triton X-100, 10 mM Tris, pH 8.0, 0.2 mM EDTA, and 0.15 M Na_2SO_4. After being shaken for 10 min at 10 °C, the solubilized mitochondria are mixed by homogenization with 4 mL of a pasty hydroxylapatite suspension prepared according to Bernardi (1971), followed by centrifugation at 800g for 2 min. The supernatant is then treated by addition of 4.3 g of wet amberlite beads to 4.5 mL of supernatant for partial removal of excess Triton X-100. After being shaken for 15 min, the supernatant is removed and again treated with 2.8 g of wet amberlite. This preparation typically contained 1.2 mg of protein/mL and 0.75% Triton.

The carrier content was determined by binding of [^{3}H]CAT. This binding was measured by passage through a Sephadex G-75 column, as described previously (Krämer et al., 1977). Incubation of the carrier preparation was typically in a "standard" medium containing 0.2 mM EDTA and 15 mM Tris buffer, pH 7.2, at 4 or 12 °C. About 200 μL of the carrier preparation was added to 400 μL of incubation medium resulting in a final concentration of about 0.4 mg of protein/mL, 30 mM Na_2SO_4, 0.25% or 4 mM Triton X-100, and 10 mM Tris buffer. Fluorescence measurements were performed in a Hitachi MPF 44a spectrofluorometer in 5 × 5 mm cuvettes with 0.4-mL samples.

Binding Measurement of [^{3}H]DAN Nucleotides by Small Gel Column Centrifugation. The binding of [^{3}H]DAN nucleotides was determined by gel chromatography with Sephadex G-50. In order to avoid dissociation of the DAN nucleotides from the carrier during gel passage, and in view of the scarcity of the [^{3}H]DAN nucleotides, a method was developed using forced passage through small columns by centrifugation. Centrifuge cups (Eppendorf) were opened and supplied with a polyethylene filter support, onto which 0.6 mL of Sephadex G-50 was layered 1 cm high. These "columns" were stuck into 1.5-mL cups for collecting the effluent from the centrifugal-forced passage. The column was preequilibrated with 20 mM Tris, pH 7.4, and freed from excess fluid by centrifugation at 650 rpm for 1 min in a swing-out centrifuge. A total of 100 μL of the sample was layered on the gel column and centrifuged for 30 s at 650 rpm. The protein with bound [^{3}H]DAN nucleotides was measured in 100 μL of the effluent by scintillation counting. In the presence of BKA used as the blank, there was a binding of about 5–15% compared to the samples, probably caused by trapping in Triton. The contamination of the effluent by free [^{3}H]DAN nucleotides was less than 4% of the total applied.

The evaluation of the concentration dependence of fluorescence is complicated by the binding of fluorescent DAN nucleotides to both the carrier and the Triton. This required separate determination of the relation between fluorescence and binding to Triton, as demonstrated in Figure 1. The calibration of the carrier binding fluorescence is achieved by determining the CAT binding capacity (C_0) in the presence of 20 μM ADP, which ensures maximum binding.

The dissociation constant K_D was evaluated from mass action plots with the use of eq 1. Here, D = DAN nucleotide

$$\frac{\Delta F}{\phi} = C_0 - \frac{\Delta F}{\phi D_0 - \Delta F} K_D(1 + \beta[TX]) \qquad (1)$$

(D_0 = total concentration), C = carrier sites (C_0 = total concentration), β = binding coefficient of DAN nucleotide to Triton micelles (mM^{-1}) (see Figure 1), F = fluorescence, $\Delta F = F - F(+BKA)$ (corresponding to the carrier-specific fluorescence), $\phi = \Delta F_{max}/C_0$, and [TX] = Triton concentration (mM). The slope S of a plot of $\Delta F/\phi$ vs. $\Delta F/(\phi D_0 - \Delta F)$ gives K_D:

$$K_D = \frac{S}{1 + \beta TX} \qquad (2)$$

This measurement requires determination of TX concentration and of the "molar" fluorescence change $\phi = \Delta F_{max}/C_0$. ΔF_{max} is extrapolated from the plot of $1/\Delta F$ vs. $1/D_0$. For a more accurate determination of ϕ, $1/\Delta F$ vs. $1/D_0$ is plotted at several protein (carrier) concentrations to give ΔF_{max} (cf. Table I). A subsequent plot of ΔF_{max} vs. increasing carrier concentration (C_0) gives a good linear relation. From this slope, an average ϕ is extracted.

For the "Hill" plots, log $[\Delta F/(\Delta F_{max} - \Delta F)]$ vs. log D_{free}, the concentration of free DAN nucleotides, had to be calculated according to the equation:

$$D_{free} = -a/2 + [(a/2)^2 + b]^{1/2} \qquad (3)$$

with $a = (C_0 - D_0)/(1 + k[TX]) + K_d$ and $b = K_D D_0/(1 + k[TX])$. K_D is taken from the mass action plots.

Results

Influence of Detergents. First, conditions had to be established for preparing solubilized nucleotide carrier protein to which DAN nucleotides could be successfully applied. Carrier protein was extracted from beef heart mitochondria (not preloaded with inhibitors) with Triton X-100 plus salt and was then enriched by treating the solubilizate with a batch of hydroxylapatite. Essentially the same procedure was first introduced for preparing nucleotide carrier for reconstitution with phospholipid vesicles (Krämer et al., 1977). The choice of detergent was limited to Triton X-100. DAN nucleotides also bind to the Triton micelles in a fluorescent mode, although the binding is considerably weaker than that to another de-

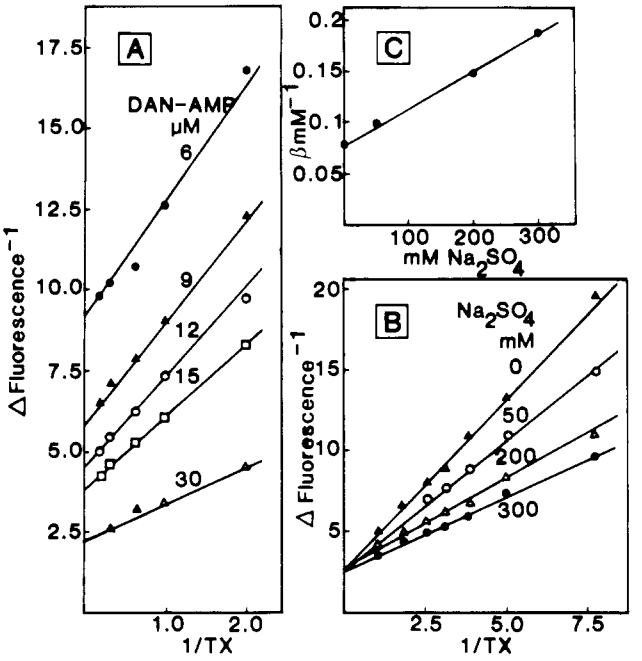

FIGURE 1: Influence of Triton X-100 and salt on the DAN-AMP fluorescence. (A) Dependence of the fluorescence on the reciprocal of the Triton concentrations (1/%) at various DAN-AMP concentrations. Incubation medium including 100 mM Na_2SO_4, 0.2 mM EDTA, and 1.5 mM Tris, pH 7.2, 4 °C. (B) Influence of salt concentration on the DAN-AMP fluorescence bound to the Triton micelles. Medium as in (A) except for Na_2SO_4 as indicated; 10 μM DAN-AMP was added throughout. (C) Dependence of the binding coefficient β of DAN-AMP to Triton micelles on the salt concentration.

tergent used for the batch procedure, lauroylpropylamine oxide (LAPAO) (Krämer et al., 1977). The fluorescence from DAN nucleotides in the micelles can interfere with measurements of the binding to the carrier protein. Therefore, the Triton content has to be decreased in the hydroxylapatite extract by treatment with amberlite.

For a quantitative assessment of the fluorescence due to DAN nucleotide partition in Triton micelles, the dependence on Triton concentration was investigated. At a given DAN-AMP concentration the fluorescence increases according to an absorption isotherm, and a plot $1/F$ against $1/[TX]$ gives straight lines (Figure 1):

$$\frac{1}{F} = \frac{1}{\beta[TX]F_{max}} + \frac{1}{F_{max}} \quad (4)$$

F = fluorescence, [TX] = Triton concentration (mM), and β = binding constant for DAN-AMP to Triton. In the plot $1/F$ vs. $1/[TX]$, the intercept $1/F_{max}$ and the slope $1/(\beta F_{max})$ give the binding coefficient β.

The plots for different concentrations of DAN-AMP (Figure 1A) are obtained from assays done in the absence of salt and therefore requiring higher amounts of Triton. From the five lines an average value of $\beta = 0.12$ mM^{-1} is calculated. The uptake of DAN-AMP into Triton is increased by salts. This factor is important in view of the presence of Na_2SO_4, originating from the medium used for the extraction and the hydroxylapatite passage. Plots of the fluorescence/Triton relation give slopes decreasing in accordance with the salt concentration, but with a common intercept (Figure 1B). The binding constant β increases from 0.06 to 0.16 mM^{-1} at 300 mM Na_2SO_4 (Figure 1C).

The binding of DAN-AMP was also determined for the detergent lauroylpropylamine oxide (LAPAO). Here at 20 mM Na_2SO_4, $\beta = 0.25$ mM^{-1} was found; i.e., the affinity of DAN-AMP for LAPAO micelles is 2 times stronger. Although this detergent has optical advantages, as the region below 210 nm is not obscured, it is therefore less easily applied in DAN-AMP binding studies with the solubilized ADP/ATP carrier.

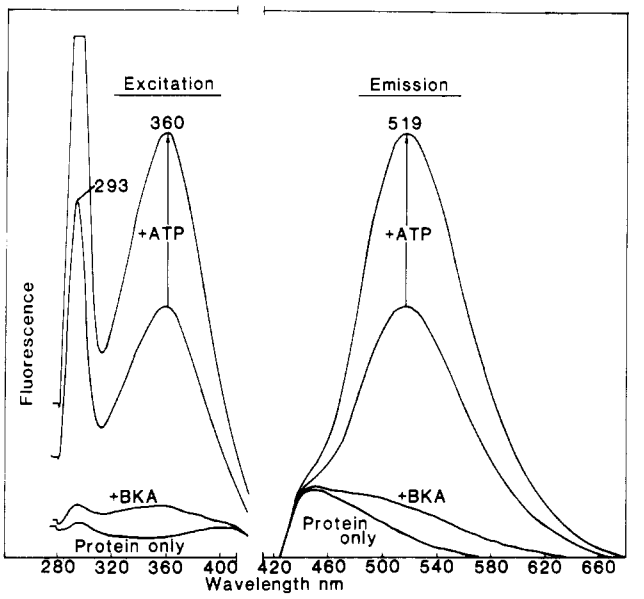

FIGURE 2: Fluorescence excitation and emission spectra of 1,5-DAN-AMP bound to the Triton-solubilized ADP/ATP carrier. DAN-AMP (20 μM) is added to the unliganded ADP/ATP carrier preparation (see Materials and Methods), which contains 0.4 mg of protein/mL and 0.6% Triton X-100 in the standard incubation medium at 12 °C. Additions of 30 μM ADP and 10 μM BKA are as indicated.

General Fluorescence Properties of Binding. Typical fluorescence excitation and emission spectra obtained on addition of DAN-AMP to a solubilized and enriched ADP/ATP carrier preparation are shown in Figure 2. The clear extracts make it possible—in contrast to the situation with submitochondrial particles—to obtain the excitation spectrum of the DAN-AMP response to binding on the ADP/ATP carrier. The excitation maximum at 360 nm coincides with the absorption maximum of DAN-AMP. The peak at 293 nm originates partially from a resonance energy transfer from Triton X-100 to DAN-AMP and is also observed in the absence of protein. It also reflects energy transfer from the aromatic amino acids, although this is largely obscured by the strong absorbance of Triton X-100.

The emission spectrum at 4 °C has a maximum at 519 nm and is close to that of DAN-AMP in dioxane solution (λ_{max} = 524 nm) (Mayer et al., 1984). The signal with protein alone is due mostly to light scattering. It distorts the spectrum much less than observed previously for DAN-AMP fluorescence in submitochondrial particles (Schäfer & Onur, 1979) (Figure 2). The DAN-AMP fluorescence is strongly enhanced on addition of ATP, in contrast to the absence of an ATP influence reported for submitochondrial particles. This effect will play a major role in our further investigations. With BKA, the fluorescence is largely suppressed to the same level in the presence and absence of ATP. The existence of a distinct BKA-insensitive fluorescence is in contrast to the complete BKA sensitivity in mitochondrial particles. This BKA-insensitive fluorescence depends on the content of Triton X-100 and can surpass the BKA-sensitive portion when Triton is not depleted from the hydroxylapatite extract with amberlite. The fluorescence yield and the emission maximum of the carrier-bound DAN-AMP is temperature dependent. Going from 4 to 30 °C the emission maximum is shifted from 519 to 524 nm and the fluorescence yield decreases 1.4-fold (not shown).

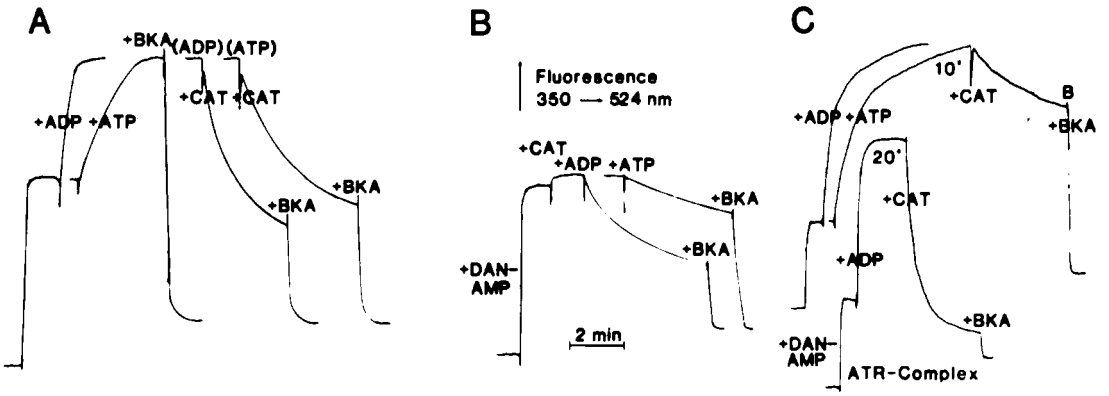

FIGURE 3: Fluorescence recordings of binding of DAN-AMP to solubilized ADP/ATP carrier and influence of various ligands, indicating that DAN-AMP binds primarily to the m state of the carrier. (A) Unliganded ADP/ATP carrier preparation, 0.5 mg/mL in standard medium at 12 °C. Addition of 15 μM DAN-AMP, 30 μM ADP, 30 μM ATP, 10 μM CAT, and 10 μM BKA. (B) Same preparation and conditions as in (A). (C) ATR–carrier complex preparation, 0.3 mg/mL at 10 and 25 °C. Additions of 20 μM DAN-AMP, 50 μM ADP, 10 μM CAT, and 10 μM BKA.

For studying the influence of various parameters such as DAN nucleotide concentration, inhibitors, pH, etc. and for following the kinetics, time-dependent measurements of the DAN-AMP fluorescence were made. The initial fluorescence response on addition of DAN-AMP is rapid, but the subsequent increase on addition of ADP is slower (Figure 3A). There is a marked difference between the rates of ADP and ATP, that with ATP being distinctly slower. BKA addition rapidly abolishes most of the fluorescence, and we assign this difference to the carrier-bound DAN-AMP. Addition of CAT after ADP or ATP decreases the fluorescence much more slowly. This rate is faster in the presence of ADP than with ATP. BKA addition after CAT causes a more extensive decrease. CAT alone is unable to decrease the fluorescence markedly (Figure 3B); upon further addition of ATP (or ADP), the fluorescence decreases to the same level as when ATP is added prior to CAT, whereas with BKA the same level is reached, independent of ADP.

The fact that ADP or ATP can both enhance and reduce the DAN nucleotide fluorescence rules out the suggestion of a fluorescence enhancement by ADP of the bound DAN nucleotides. It rather agrees with the proposal that the fluorescence changes reflect quantitatively binding changes at the m site of the carrier, as already derived from studies with the DAN nucleotides at the mitochondrial membrane (Klingenberg et al., 1984). Therefore, we conclude that up to 50% of the carrier molecules in the Triton-solubilized ADP/ATP carrier preparations appear to require ADP or ATP for fluorescence binding of DAN nucleotides. Also, ADP or ATP is required for the displacement by CAT of the fluorescent DAN-AMP binding whereas the effect of BKA is independent of the presence of ADP or ATP. Since ADP or ATP has been shown to catalyze the transition between the c and m state of the carrier in both directions (Klingenberg & Buchholz, 1973; Aquila et al., 1978; Klingenberg et al., 1977, 1983), these findings can be well rationalized in terms of the fluorescent binding of DAN-AMP exclusively to the m state of the carrier.

When the carrier is prepared from mitochondria loaded with ATR, most of the carrier population can be expected to be in the c state as the ATR complex. Accordingly, DAN-AMP addition produces only little fluorescence above that of the BKA-insensitive level (Figure 3C). The subsequent addition of ADP and ATP correspondingly further increases fluorescence. The increase stimulated by ADP or ATP is somewhat slower than that in the unloaded carrier preparation, presumably because it is limited by the dissociation of ATR from the carrier. Studies using [^{3}H]ATR indicate that about 80% of [^{3}H]ATR is removed by DAN-AMP under these conditions (not shown).

Concentration Dependence. The increase of fluorescence on binding of DAN nucleotides was carefully titrated in numerous experiments, an example of which is given in Figure 4A. Here, only the BKA-sensitive portion of the fluorescence is presented for all three DAN nucleotides. The maximum ΔF (at 1.6 μM DAN nucleotides) is considerably higher for DAN-AMP by the factors 1.43 over DAN-AMP and 1.2 over DAN-ADP. The concentrations for half-maximum ΔF ("$K_{1/2}$") are 1.8 μM for DAN-AMP, 2.1 μM for DAN-ADP, and 2.8 μM for DAN-ATP. For evaluating the dissociation constants, the free DAN nucleotide concentration has to be calculated, taking into account the binding not only to the protein but also to the Triton micelles. From the amount of total nucleotides and the Triton content, the equivalent free DAN nucleotides in terms of the fluorescent units can be calculated and used for the mass action plot (Figure 4B). Here approximately linear relations are obtained only for the lower concentration range. At higher concentration, the fluorescence increase seems to be saturated too early. Probably this is explained by binding of DAN-AMP also to the c side in a nonfluorescent mode, as discussed already for the binding studies with DAN nucleotides to the carrier in the mitochondrial membranes (Klingenberg et al., 1984). From the lower linear range, a larger gradation of K_D for the three DAN derivatives is evaluated than was indicated by the $K_{1/2}$ values. The K_D values calculated from these fluorescent titrations are 0.9 μM for DAN-AMP, 1.6 μM for DAN-ADP, and 3.2 μM for DAN-ATP.

Experiments were performed also in which the protein concentration for evaluating the K_D was varied but the Triton concentration was kept constant in order to eliminate possible complications of DNA nucleotide binding to the Triton micelles. A typical experiment as evaluated from titrations of the binding sites with increasing amounts of DAN-AMP is given in Table I. The maximum BKA-sensitive fluorescent increase was proportional to the amount of protein present. The DAN-AMP concentrations for half-maximum fluorescence also varied according to the amount of protein. However, the K_D values calculated from mass action plots such as in Figure 4B are independent of the amount of protein present if adequate corrections for binding of DAN-AMP to Triton are made. In this case, an average K_D = 0.5 μM is calculated. In several other similar titration experiments, the K_D varies between 0.85 and 0.5 μM for DAN-AMP.

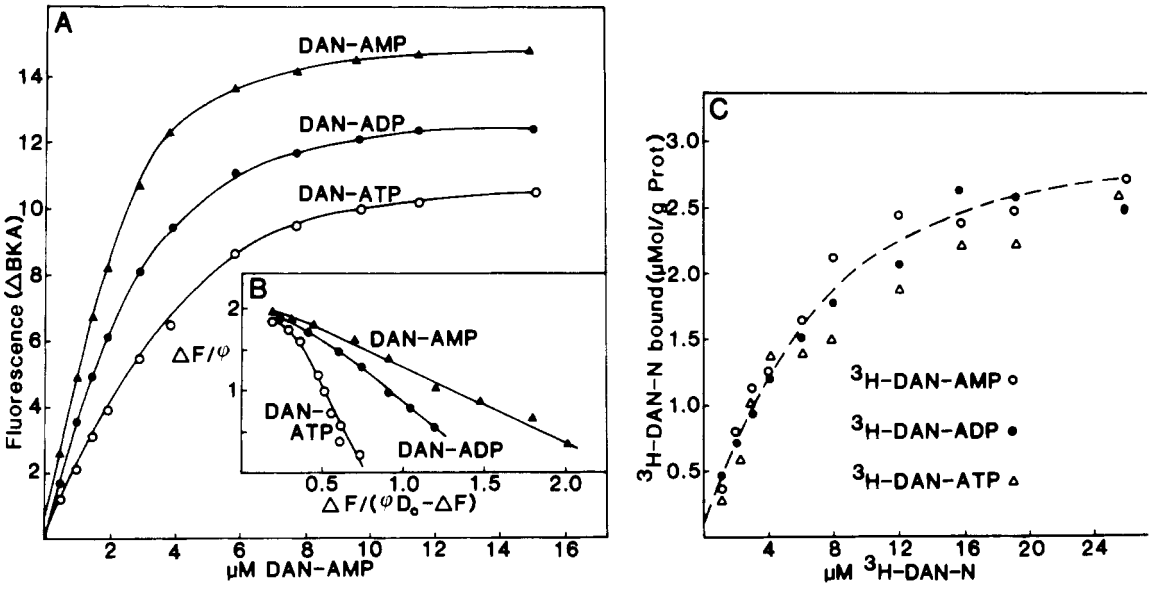

FIGURE 4: Concentration dependence of the DAN nucleotide binding. Titration of the fluorescence and determination of physical binding of [^{3}H]DAN nucleotides. (A) Titration of unliganded ADP/ATP carrier preparation with increasing concentrations of DAN-AMP, DAN-ADP, and DAN-ATP. Titrations both in the absence and presence of 10 μM BKA. Only the difference ±BKA in the fluorescence response is plotted. (B) Evaluation of fluorescence binding in a mass action plot, taking into account the distribution of the unliganded DAN nucleotides between Triton micelles and solution. ΔF is taken from part A; ϕ is the conversion factor between fluorescence and micromoles per gram of protein nucleotides bound. D_0 is added DAN nucleotide concentration. In the abscissa, $\Delta F/(\phi D_0 - \Delta F)$ is the equivalent for bound/free. Incubation of carrier preparation containing 0.53 mg of protein/mL and 0.14% Triton with a binding capacity of 3.7 μM [^{3}H]CAT/mg of protein. (C) Physical binding of DAN nucleotides to unliganded ADP/ATP carrier preparation with DAN-[^{3}H]AMP and DAN-[^{3}H]ADP. Incubation of 0.85 mg of protein carrier preparation in 0.2-mL samples; addition of increasing amounts of DAN nucleotides in standard medium at 15 °C. Determination of binding by small gel column centrifugation (see Materials and Methods). Only the difference between binding ± prior addition of 20 μM BKA is plotted.

Table I: Influence of Protein (Carrier) Concentration on Titration with DAN-AMP[a]

protein (mg/mL)	ΔF_{max} (±BKA)	S (μM)[b]
0.14	4.2	−0.67
0.22	6.3	−0.60
0.29	8.5	−0.65
0.36	11.2	−0.72
0.43	13.9	−0.75

[a] Evaluation of mass action plots $\Delta F/\phi$ vs. $\Delta F(\phi D - \Delta F)$ (see Materials and Methods). $\phi = \Delta F_{max}/C_0 = 8.6$ μmol^{-1} averaged from the slope of plot ΔF_{max} vs. increasing C_0, based on C_0 = CAT binding capacity = 3.5 μmol/g of protein. Average slope $\bar{S} = -0.70$ μM; $K_D = \bar{S}/(1 + 0.77 \times 5.61) = 0.50$ μM. Incubation of carrier preparation containing 0.72 mg of protein/mL and 0.55% Triton and binding capacity of 3.5 μM [^{3}H]CAT/mg of protein in standard medium at 4 °C to the protein concentration given. The final concentration of Triton was kept at 0.35% or 5.6 μM. The final salt concentration varied from 6 to 35 mM Na_2SO_4. [b] See eq 1.

These titrations were also evaluated in Hill plots of log $[\Delta F/(\Delta F_{max} - \Delta F)]$ vs. log $[\text{DAN-AMP}]_{free}$ (Figure 5). For this purpose the $[\text{DAN-AMP}]_{free}$ was calculated by taking into account binding to carrier and Triton according to eq 1–3 given under Materials and Methods. Straight lines could only be approximated in the lower range of DNA nucleotide concentration, from which a slope of $n = 1.3$ was evaluated for all protein concentrations. This Hill coefficient agrees with that obtained in submitochondrial particles (Klingenberg et al., 1984). Possible reasons for the nonlinearity will be given under Discussion.

In order to verify the fluorescent-type binding in terms of "physical" binding of the DAN nucleotides, the DAN derivatives of [^{3}H]AMP, -ADP, and -ATP were synthesized. In these binding measurements some technical problems were encountered. Equilibrium dialysis is not possible because during the 4–5 h of equilibration time the ADP/ATP carrier will deteriorate. Therefore, a rapid gel chromatography by centrifugation through a small column was developed (see Materials and Methods). This method has more errors than the fluorescence titration, and these are amplified in the differences between the binding values in the absence and presence of BKA. Therefore, as shown in Figure 4C, the points are considerably scattered. This makes evaluation in the mass plot difficult since calculations of the free concentration increases the scattering still more.

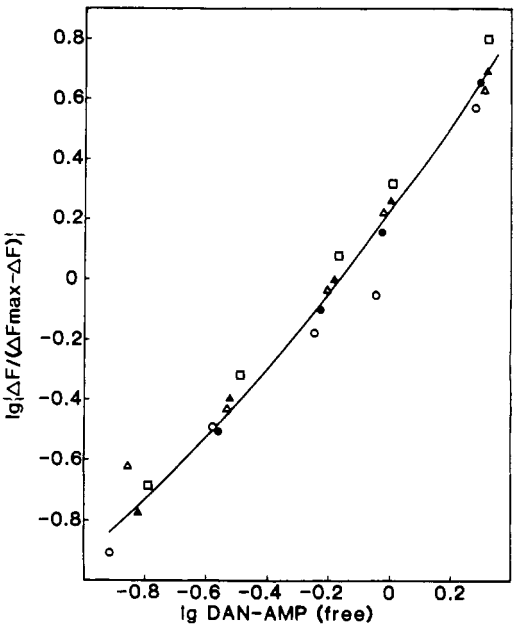

FIGURE 5: Hill plot of fluorescence titration with DAN-AMP at various protein concentrations. Same experiment as in Table I. The data are evaluated by calculating the free DAN-AMP concentration according to Materials and Methods. The symbols correspond to curves at the following protein concentration (mg/mL): (□) 0.43; (▲) 0.36; (△) 0.29; (●) 0.22; (○) 0.14.

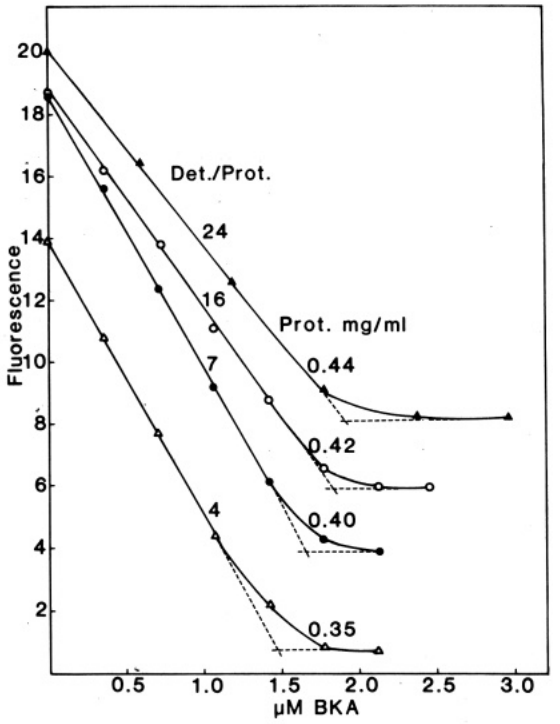

FIGURE 6: Decrease of DAN-AMP bound to the ADP/ATP carrier on addition of increasing amounts of BKA. Variation of detergent/protein ratio. The carrier preparation was treated in four steps with amberlite for the removal of Triton. At each step the content of Triton X-100 and protein was determined, and a sample was diluted 1:1 with 20 mM Tris, pH 7.2, to give the following final contents of Triton and protein per milliliter (mg/mg): 21/0.44; 13/0.42; 5.6/0.41; 2.5/0.36. The fluorescence was recorded after addition of 21 μM DAN-AMP and 20 μM ATP and sequential additions of BKA at concentrations indicated.

The importance of these physical binding studies lies in the quantitative evaluation of the fluorescence in terms of the binding. As is to be expected, the three DAN nucleotide derivatives have about the same maximum binding of 2.6 μmol/g of protein. Extrapolation in a mass action plot gives a binding capacity of 3.3 μmol of DAN nucleotide bound per gram of protein in this carrier preparation. Furthermore, the fact is remarkable that all three nucleotides reach about the same binding level whereas the fluorescence maxima differ widely. The possible reasons for this difference between fluorescence and [^{3}H]DAN nucleotide binding measurements will be explained under Discussion.

Quantitative Relation between Inhibitor Binding and Fluorescence Change. By titrating the fluorescence with increasing concentrations of BKA, a quantitative assay of the carrier sites present in these preparations can be obtained. This convenient assay benefits from the high affinity of BKA for the carrier. Evaluation of such an experiment is shown in Figure 6, where the dependence of the fluorescence decrease on the BKA concentration is plotted for preparations with different contents of detergent but approximately constant content of protein. Before addition of BKA, the carrier sites are largely converted to the m state by binding with DAN-AMP in the presence of ATP. The decrease of fluorescence is linear in BKA, up to approximately 1 equiv of BKA per DAN nucleotide binding site, as a result of the high affinity of BKA to the predisposed m-state carrier sites. The titration gives fairly sharp breaks at fluorescence levels that increase in height according to the detergent concentration. The slopes decrease slightly at higher detergent concentration, presumably because some BKA is diverted into the Triton micelles instead of to the carrier sites. The extrapolated end values correspond

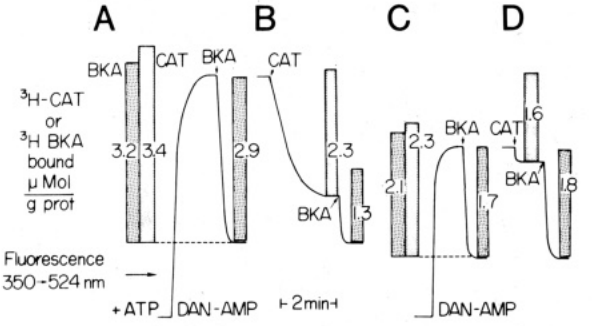

FIGURE 7: Quantitative comparison of fluorescence response of DAN-AMP binding and of physical binding of [^{3}H]BKA and [^{3}H]CAT. In (A) and (B) 50 μM ATP is present for facilitating transition between c and m state. Samples were divided for fluorescent recording and for separate determination of [^{3}H]BKA and [^{3}H]CAT binding. The fluorescenc sample was finally analyzed after addition of [^{3}H]BKA. In (B) and (D) separate samples received either [^{3}H]CAP or unlabeled CAT and then [^{3}H]BKA. Incubation in standard medium at 12 °C. Determination of the binding of [^{3}H]BKA and [^{3}H]CAT by the direct procedure (see Materials and Methods).

to the following ratios (μmol of BKA/g of protein) in the sequence of detergent depletion: 4.3, 4.3, 4.1, and 4.3. The average value of 4.3 μmol of BKA/g of protein should correspond to the number of BKA-titratable carrier sites. Thus, the fluorescence of DAN-AMP can be used for recording the binding of BKA, which is not possible directly due to a lack of optical signals originating from BKA itself.

As shown in previous studies with mitochondrial membranes (Mayer et al., 1984; Klingenberg et al., 1984), a valuable application of DAN-AMP fluorescence consists in the visualization of the carrier distribution in the c and m states. In the soluble protein, the c/m distribution is of special interest since here the conformation changes are independent of the asymmetric constraints present in the membrane, of the membrane potential, and of other membrane-related factors. Inhibitor binding has been the main parameter used for determining the c/m distribution (Klingenberg et al., 1972, 1975, 1983; Klingenberg & Buchholz, 1973). Therefore, a quantitative comparison between the degree of fluorescence and the binding capacity for BKA and CAT is most desirable, making use of the fact that binding to the carrier of CAT is specific for the c state and of BKA to the m state. For this purpose, the measurements of binding and fluorescence are combined by withdrawing samples from the cuvettes after addition of [^{3}H]BKA and [^{3}H]CAT (Figure 7). In the presence of ATP, redistribution between c and m states should be possible, and the carrier can follow the pulling action of the tightly binding inhibitors. In the absence of ATP, the c and m states remain segregated, and the c/m distribution is measured by the ligand binding.

In the presence of ATP, addition of either BKA or CAT results in approximately equal binding of about 3.4 μmol/g of protein, corresponding to the total carrier site content (Figure 7A). When DAN-AMP is added first, the subsequent binding of BKA (2.9 μmol/g of protein) is nearly the same as that without DAN-AMP. When CAT is added instead, the extent of binding is smaller, in accordance with the smaller fluorescence decrease. Subsequently, some BKA is bound, accompanied by a further fluorescence depression (Figure 7B). Presumably, some CAT has been removed by BKA due to the equilibration between c and m states in the presence of ATP. In the absence of ATP with segregated c and m states, the binding of [^{3}H]BKA or [^{3}H]CAT is lower (Figure 7C). In principle, here the sum of [^{3}H]CAT and [^{3}H]BKA binding should give the same result as the binding of each inhibitor

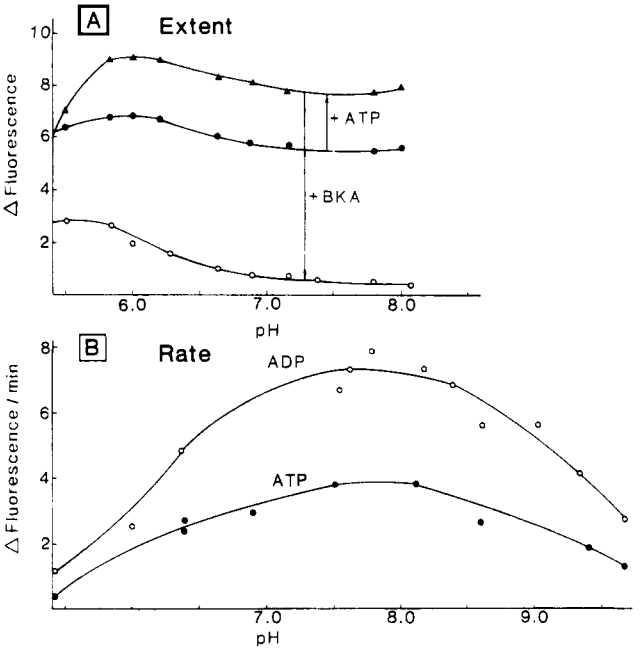

FIGURE 8: Influence of pH on the fluorescent binding of DAN-AMP to unliganded ADP/ATP carrier preparation. (A) Extent of fluorescence response with or without ATP addition in dependence on pH. (B) Rate of fluorescence increases on addition of ADP or ATP in dependence on pH. Unliganded ADP/ATP carrier preparation in standard medium at 4 °C. Addition of 10 μM DAN-AMP, 20 μM ATP, 20 μM ADP, and 10 μM BKA.

alone in the presence of ATP. The larger sum observed may indicate that the segregation between the c and m states is not complete despite the absence of added ATP. The binding of BKA after addition of DAN-AMP is proportional to the smaller fluorescence responses without ATP. Addition of CAT after DAN-AMP still gives large binding to the "nonfluorescent" c-state carrier molecules and can be followed by the binding of BKA to a portion of the m-state population and displacement of the DAN-AMP (Figure 7D).

This quantitative analysis of the inhibitor binding verifies that the fluorescence of DAN-AMP binding enables us to follow the transition between the c and m states in the presence of ATP and to determine the carrier distribution between the c and m states in the absence of ATP.

Influence of the Ionic Environment and pH. The pH dependence of ADP/ATP carrier functions is important in view of a suggested H^+ involvement in the ADP/ATP exchange (Mitchell & Moyle, 1969; Klingenberg, 1972). The DAN-AMP fluorescence provides a flexible tool for studying the pH influence. The fluorescence generated on binding to the solubilized carrier has only a small pH dependence (Figure 8). It becomes smaller only at pH below 6. At the same time, the BKA-insensitive fluorescence increases, indicating that the amount of free DAN-AMP available to the carrier decreases. The ATP-stimulated fluorescence increase is more pH sensitive. The rate of this increase has a broad maximum at about pH 7.8 with either ADP or ATP. Although the rate strongly decreases at the extreme pH values, it is more significant that the variation between pH 6.4 and 8.6 is only 30%. Since this rate is assumed to reflect the central translocation step, the results agree well with the earlier observation that the transport in mitochondria as well as in the reconstituted system has only a small pH dependence.

The influence of salt or anions on the ATP-stimulated DAN-AMP fluorescence was noted first when varying the nature and concentration of the salt used in the extraction and purification steps, from which salt is carried over to the final

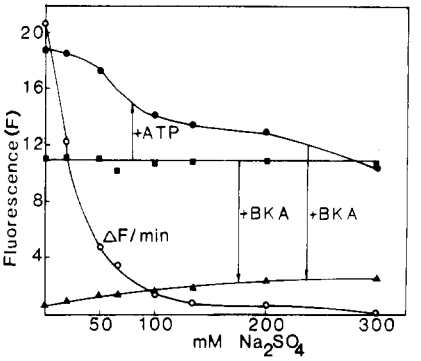

FIGURE 9: Sensitivity of the DAN-AMP to salts, as exemplified by Na_2SO_4. The extent of fluorescence response with or without ATP addition is plotted. The carrier-binding portion is differentiated by the addition of BKA. The unliganded ADP/ATP carrier preparation is depleted of salts originating from the solubilization by passage through a Dowex column. A total of 0.3 mg of protein is incubated in 8 mM Tris-HCl buffer, pH 7.2, with the indicated amount of Na_2SO_4 and 10 μM DAN-AMP, 20 μM ATP, and 10 μM BKA, at 7 °C.

Table II: Influence of Extracting Ions on the c/m Distribution

additions	ADP increase[a]
600 mM NaCl	60, 64
150 mM Na_2SO_4	55, 57
20 mM Na_xPO_4	28, 32
20 mM $Na_xP_2O_7$	27
150 mM Na_2SO_4 + 8 mM ATP	22

[a] Percent of total BKA-sensitive fluorescence. The concentrations of these salts were present in the extraction medium (see Materials and Methods) in addition to 8% Triton X-100, 10 mM Tris buffer, pH 8.0, and 0.2 mM EDTA.

incubation. A systematic study of the Na_2SO_4 influence is shown in Figure 9. The extent of the BKA-sensitive fluorescence decreases only slightly at higher salt concentrations. However, the ATP-stimulated fluorescence diminishes and is fully suppressed at 300 mM Na_2SO_4. Even more sensitive to Na_2SO_4 is the rate of the ATP-stimulated fluorescence increase, which decreases to half at about 30 mM Na_2SO_4. Similar effects were observed with NaCl and sodium phosphate.

The effect of salts or anions on the c- to m-state transition is important not only for practical purposes when dealing with the isolation of free ADP/ATP carrier but also in view of the recent finding by Krämer (1984) that anions stimulate transport activity in the reconstituted system. The influence of three different anions used during the extraction and purification procedure was studied. Since the yield and purity of the carrier preparation varies with the salt, only the relative changes in the ADP-stimulated fluorescence can be compared, and only these data are given in Table II. There is a clear decrease in capacity for the ADP enhancement going from chloride to the more highly charged anions phosphate or pyrophosphate. The smallest increase is observed when mitochondria are extracted in the presence of ATP. These data suggest that the anions shift the distribution toward the m state of the carrier. Although much weaker than ADP or ATP, there is sufficient time for them to act when the carrier is exposed to the anions during the isolation procedure.

Competition between Nucleotides and DAN-AMP. A competitive inhibition by ADP and ATP binding is to be expected if the DAN-AMP binding site is identical with the nucleotide binding center at the carrier. However, as shown previously in the studies on DAN nucleotide binding in submitochondrial particles, this competition is rather ineffective

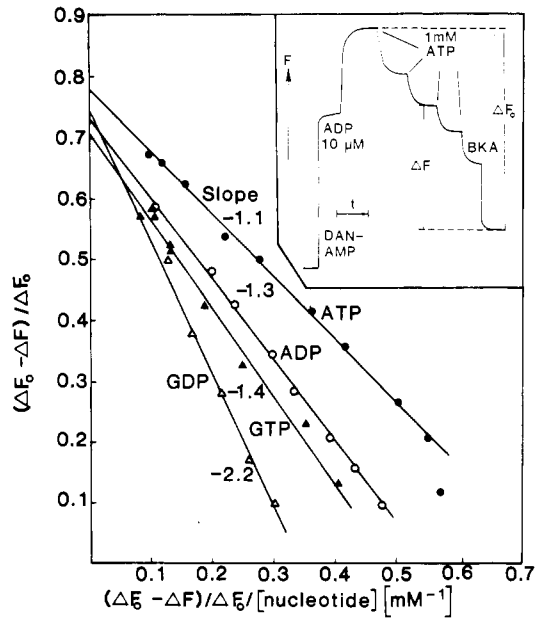

FIGURE 10: Decrease of the BKA-sensitive DAN-AMP fluorescence by competition with nucleotides. First, 10 μM DAN-AMP and 20 μM ADP are added for full fluorescence response. Subsequently, increasing amounts of nucleotides are added, and the fluorescence decrease $\Delta F_0 - \Delta F$ is evaluated. ΔF_0 is the maximum fluorescence difference without and with BKA. Preparation incubated in standard medium at 0.4 mg of protein/mL, pH 7.2, 7 °C. The plot corresponds to the equation $(\Delta F_0 - \Delta F)/\Delta F_0 = 1 - [K_i D/(K_D N)](\Delta F_0 - \Delta F)/\Delta F_0$ with D = [DAN-AMP] and N = [ATP] etc. The slope = $-K_i D/K_D = -11K_i$, with D = 10 μM and K_D = 0.9 μM (see above). K_i = inhibitor constant = dissociation constant for competing nucleotides; K_D = dissociation constant for DAN-AMP.

even at high nucleotide concentration. Clearer results on this important point can be obtained with the solubilized carrier, as shown in Figure 10. The decrease of the DAN-AMP fluorescence that has been first augmented by a comparatively small amount of ADP is titrated with increasing concentrations of nucleotides. This is possible as the concentration range required for the displacement is much higher and is well separated from the activation range. This decrease reached about 70–80% of the fluorescence decrease by BKA at up to 7 mM nucleotides. The data are converted into a mass action plot for the evaluation of K_i (Figure 10). Linear relations are obtained with slopes that have the same value whether or not one corrects the fluorescence ΔF_0 for the residual nucleotide-resistant portion. Two aspects are prominent, the relatively high concentrations of nucleotides required for effective removal of DAN-AMP and the nucleotide base unspecificity. The K_i values vary only by a factor of 2 between ATP (0.1 mM) and GDP (0.2 mM). Whereas ADP is more effective in the activation, ATP is stronger in the displacement. Although GTP is stronger than GDP, the displacement by the guanine nucleotides contrasts with their ineffectiveness in the activation. This suggests that a displacement through ionic forces is more important than that by the nucleotide base. Pyrophosphate is also able to remove the DAN-AMP, although with a much higher K_i ($\simeq$3 mM). In this context the inhibition of DAN-AMP fluorescence by sulfate with a $K_i >$ 30 mM should also be noted (Figure 8). All these results point to a predominantly anionic type of competition with DAN nucleotide binding.

Discussion

DAN Nucleotides and Triton Interaction. We shall first discuss the technical problems that arise when employing the DAN nucleotides as a probe for the isolated ADP/ATP carrier. One has to take into account that here the protein exists in a detergent–protein micelle surrounded by additional excess detergent micelles (Hackenberg & Klingenberg, 1980). DAN nucleotides obviously bind to these micelles, presumably because the hydrophobic (dimethylamino)naphthoyl moiety is attracted to the hydrophobic core of the detergent micelles. As a result, the DAN nucleotides exhibit the same fluorescent enhancement which they have when they are dissolved in nonaqueous media (Mayer et al., 1984). A partition equilibrium appears to exist between the aqueous and the micellar phase, as shown by the titration studies. Fortunately, the partition with Triton X-100 is not so unfavorable as to interfere seriously with the binding of DAN nucleotides to the carrier, provided the Triton content is sufficiently decreased. The increased partition into Triton with salt probably has two reasons—a decrease of the solubility of the amphiphilic DAN nucleotide in the aqueous solution and an increase of the micelle size (Tanford, 1980) together with a concomitant stabilization of the hydrophobic core and increase of hydrophobicity. Therefore, a low salt concentration is also advantageous for decreasing the solubility in the Triton micelles. Most of the "BKA-insensitive" fluorescence, which can be as low as 10% of the total, is due to binding in the Triton micelle and is accounted for in calculating the content of free DAN nucleotides in he binding equilibrium.

Carrier m-State Binding. The sensitivity to BKA is not only a most convenient and convincing assay for the carrier binding, it also identifies the m-state binding of the DAN nucleotides. This conclusion, which is substantiated by the influence on the DAN nucleotide binding of the other ligands CAT and ADP or ATP, is more convincingly reflected in the results with the isolated carrier than in those obtained with the membranes [see reaction scheme in Figure 8 in Klingenberg et al. (1984)]. Here the membrane barrier against the ligands, particularly CAT and the nucleotides, and the uncertainty of membrane sidedness in submitochondrial particles are avoided. In the particles, CAT removes only about 40% of the DAN nucleotide, while it displaces up to 90% of the BKA-sensitive fluorescence in the solubilized preparation, provided that ADP or ATP are present; this is in agreement with an exclusive m-state fluorescent binding of DAN nucleotides. In retrospect, these results also support the notion that all the DAN nucleotide binding in submitochondrial particles happens at carrier binding centers, although the criteria of a competition with ADP or ATP failed for a large portion of the DAN nucleotide binding (Klingenberg et al., 1984). Obviously in these particles and in the solubilized carrier, the affinity for ADP and ATP is strongly decreased.

The identity of the DAN nucleotide binding site with the postulated common but adaptive binding center for nucleotides, and for the inhibitor ligands, BKA and CAT, is further borne out by the stoichiometry of binding. The binding capacity of 3.2 μmol for [^{3}H]DAN nucleotides agrees quite well with the binding of 3.4 μmol of [^{3}H]BKA or [^{3}H]CAT found in these preparations. This argues not only for one DAN nucleotide binding site per dimeric carrier molecule but also for the shared carrier binding center.

The binding of the DAN nucleotides as analyzed by the concentration dependence deviates to some degree from a linear mass action relation (Figure 4A,B). At this stage, it is difficult to explain this phenomenon, which we feel is not surprising in view of the complexity of the solubilized carrier preparation. Moreover, in the membrane the mass action plots were linear (Klingenberg et al., 1984). One possible reason could be the nonfluorescent weak binding to the c state of the

Table III: Classification of Nucleotide Interaction with the Carrier Binding Center

state		nucleotide ligand	affinity K_D	segment specificity			dominant binding forces
				base	ribose (label)	phosphate	
c/m	translocation active	ADP, ATP	30 μM	high		high	H-bond, ionic
m*	inhibited	DAN-AMP, -ADP, and -ATP	2 μM	high	high	low	hydrophobic
	inhibited	AXP, GXP, PP	1–2 mM	low		low	ionic

carrier, which was shown to occur in membrane-bound carrier from the c side to which DAN-AMP does not find a barrier in the solubilized preparation (Klingenberg et al., 1984). For example, a premature nonhyperbolic saturation of the fluorescent binding to the m state could result from an influence of the DAN nucleotides on the distribution ratio between the c and m states by autocatalyzing a slow transition, instead of relying only on ADP or ATP. This may be also one reason for nonlinearity in the Hill plot. An in-depth investigation of this question is necessary to draw more definite conclusions. At any rate, these rather weak deviations do not seem to justify speculations about cooperative effects between presumably two binding sites per carrier molecule (Dupont et al., 1982).

Specificity and Hydrophobic Interactions. Already in submitochondrial particles DAN-AMP was found to be at least as good a ligand as DAN-ADP or DAN-ATP, although AMP is no substrate for the ADP/ATP carrier (Klingenberg & Pfaff, 1966). When we worked with the soluble protein, the more quantitative evaluation shows that DAN-AMP exhibits a 4-fold higher affinity than DAN-ATP. This may be related to the finding that DAN-AMP has a higher fluorescent yield than the ADP and ATP derivatives, as shown by the comparison of fluorescence with physical binding. Since the DAN group fluorescence reflects a hydrophobic environment, it indicates tighter hydrophobic binding, probably facilitated by the decreased charge of DAN-AMP^{2-} as compared to DAN-AMP^{3-} and DAN-ATP^{4-}. In fact, these hydrophobic forces seem even to overcome the deficit of the ionic forces in AMP^{2-}, because they are less restricted here by strong charges. Also, the affinity of ADP and ATP is strongly increased by the additional DAN moiety ($K_D \simeq 10^{-6}$ M) considering that the affinity of ADP and ATP in soluble carrier preparations is so low ($K_D > 2 \times 10^{-5}$ M) that it cannot be measured with sufficient accuracy. As discussed previously in context with the binding of DAN nucleotides to the membrane-bound carrier (Mayer et al., 1984; Klingenberg et al., 1984), the fact that the weak binding in the c state is without fluorescence argues for a major contribution of the fluorescent and hydrophobic interaction of the DAN moiety with the carrier to the strong binding in the m state.

The ready displacement of the DAN nucleotides by BKA suggests that the same hydrophobic binding niche at the carrier may be shared by the two ligands. Both DAN nucleotide and BKA possess a hydrophobic moiety that is missing in ADP and ATP, and both are "inhibitors" because their affinity is much higher than that of ADP or ATP. On binding, BKA has been visualized (Klingenberg et al., 1973, 1976) to have folded the three carboxylic groups together, thus forming a cluster of three anionic charges, just as they exist in ADP, and an opposite apolar segment in the molecule. Also, this hydrophobic interaction with the carrier seems to be rather unspecific since 3′-*O*-trinitrophenyl and 3′-*O*-(dinitrophenyl) substituents generate an even higher affinity than the DAN substitution (Mayer et al., 1984).

Nucleotide Competition. A more unspecific interaction of nucleotides with the binding site is revealed by the competition with DAN nucleotides. ADP and ATP, as specific ligands to the carrier site, might be expected to compete with DAN-AMP much more effectively than GDP and GTP. When applied to membranes, however, even at more than 10 mM ADP, only 30% of DAN nucleotides were displaced [Figure 4 in Klingenberg et al. (1984)]. This limited competition was interpreted to be due to restricted accessibility but not to different types of binding sites. This agrees with an 80% displacement by 5 mM nucleotides in the solubilized carrier (Figure 10). However, the high concentration required, as well as the base unspecificity, argues for a different type of interaction than that of ADP or ATP in the translocation. Also, the superiority of ATP to ADP is in contrast to the translocation affinities. The fact that even pyrophosphate and to some extent sulfate can oppose DAN-AMP binding indicates competition primarily by ionic forces of low specificity. These conclusions are summarized in Table III.

All these results seem to argue against an identity of the DAN-AMP and the translocator site. However, this does not necessarily mean that ADP or ATP and the DAN nucleotides bind to different sites of the carrier. The difference consists rather in an enlargement of the carrier binding center by a hydrophobic niche, which may involve a ligand-induced fit. Unless this binding is abolished by competing hydrophobic ligands such as BKA, the hydrophilic nucleotides can only intercept at concentrations so high that the specific base–protein interaction is inferior to more unspecific ionic interactions.

Comparison to Naphthoyl (N) Nucleotide Derivatives. A few comments are necessary on the reported studies with the fluorescent naphthoyl derivatives of ADP or ATP (N-ADP) (Dupont et al., 1982; Block et al., 1982, 1983). We have abstained from using these naphthoyl derivatives extensively because on binding the fluorescence decreases by only about 10–20% and because these derivatives do not markedly differentiate between c- and m-state binding [see Table I in Mayer et al. (1984)]. These disadvantages may explain why the reported results give a quite different picture from that obtained with the DAN nucleotides, both in the solubilized preparation (Dupont et al., 1982) and in the mitochondrial membrane (Block et al., 1982, 1983). On the other hand, there is no good reason to assume that they interact with the carrier in a basically different manner with only the 5-(dimethylamino) group missing. The reported demonstration of two binding sites for N-ADP per one CAT-binding site differs from our results with the DAN nucleotides. Also the very high affinity calculated for N-ADP ($K_D = 10^{-8}$ M) is surprising and at variance with its facile displacement by only 10 μM ATP. It rather seems that N-ADP has a lower affinity than DAN-ATP because the latter requires millimolar amounts of ATP for displacement.

The high- and low-affinity sites for N-ADP are interpreted as two anticooperative sites at the same carrier molecule (Dupont et al., 1982). This is a crucial difference, compared to our concept of one binding center for all ligands. However, it seems to us that the N-ADP binding may be overestimated due to the inherently difficult calibration of the N-ADP fluorescence changes. Moreover, as with the DAN nucleotides, we suggest that the fluorescence yield of N-ADP is different for the high- and low-affinity sites. In fact, in later studies

on the membrane-bound carrier (Block et al., 1983), the binding is analyzed more in terms of two states of one binding site, so-called BKA and CAT states, similar to our results with the DAN nucleotides.

Critically applied, N-ADP or N-ATP can be valuable tools despite their technical shortcomings, supplementing the results obtained with the DAN nucleotides, particularly in connection with what we interpret as the c-state binding. With this in mind and a critical reassessment of the experiments reported with N-ATP, we do not regard them as disproving but rather as supporting the reorienting single-site mechanism, which has turned out to be a powerful model for explaining the interaction of DAN nucleotides with the ADP/ATP carrier.

Registry No. DAN-AMP, 72947-53-6; DAN-ADP, 72947-52-5; DAN-ATP, 72947-54-7; ADP, 58-64-0; ATP, 56-65-5; GDP, 146-91-8; GTP, 86-01-1; BKA, 11076-19-0; CAT, 35988-42-2; Triton X-100, 9002-93-1.

REFERENCES

Aquila, H., Eiermann, W., Babel, W., & Klingenberg, M. (1978) *Eur. J. Biochem. 85*, 549–560.

Babel, W., Aquila, H., Beyer, K., & Klingenberg, M. (1976) *FEBS Lett. 61*, 124–127.

Bernardi, G. (1971) *Methods Enzymol. 22*, 325–339.

Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1982) *Biochemistry 21*, 5451–5457.

Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1983) *Biochemistry 22*, 2202–2208.

Dupont, Y., Brandolin, G., & Vignais, P. V. (1982) *Biochemistry 21*, 6343–6347.

Erdelt, H., Weidemann, M. J., Buchholz, M., & Klingenberg, M. (1972) *Eur. J. Biochem. 30*, 107–122.

Hackenberg, H., & Klingenberg, M. (1980) *Biochemistry 19*, 548–555.

Klingenberg, M. (1972) in *Mitochondria: Biomembranes*, pp 147–162, Elsevier, Amsterdam.

Klingenberg, M. (1974) in *Dynamics of Energy-Transducing Membranes* (Ernster, L., Estabrook, R. W., & Slater, E. C., Eds.) pp 511–528, Elsevier, Amsterdam.

Klingenberg, M. (1984a) in *Structural and Functional Aspects of Enzyme Catalysis* (Eggerer, H., & Huber, R., Eds.) pp 202–212, Springer-Verlag, Berlin and New York.

Klingenberg, M. (1981b) *Nature (London) 290*, 449–454.

Klingenberg, M., & Pfaff, E. (1966) in *Regulation of Metatolic Processes in Mitochondria* (Tager, J. M., et al., Eds.) pp 180–201, Elsevier, Amsterdam.

Klingenberg, M., & Buchholz, M. (1973) *Eur. J. Biochem. 38*, 346–358.

Klingenberg, M., Buchholz, H., Erdelt, H., Falkner, G., Grebe, K., Kadner, H., Scherer, B., Stengel-Rutkowski, L., & Weidemann, M. J. (1972) in *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., et al., Eds.) pp 465–486, Academic Press, New York and London.

Klingenberg, M., Scherer, B., Stengel-Rutkowski, L., Buchholz, M., & Grebe, K. (1973) in *Mechanisms in Bioenergetics* (Azzone, G. F., et al., Eds.) pp 257–284, Academic Press, New York and London.

Klingenberg, M., Grebe, K., & Scherer, B. (1975) *Eur. J. Biochem. 52*, 351–363.

Klingenberg, M., Riccio, P., Aquila, H., Buchanan, B. B., & Grebe, K. (1976) in *The Structural Basis of Membrane Function* (Hatefi, Y., & Djavadi-Ohaniance, Eds.) pp 293–311, Academic Press, New York and London.

Klingenberg, M., Aquila, H., Krämer, R., Babel, W., & Feckl, J. (1977) in *Biochemistry of Membrane Transport* (Semenza, G., & Carafoli, E., Eds.) pp 567–579, Springer-Verlag, Berlin, Heidelberg, and New York.

Klingenberg, M., Appel, M., Babel, W., & Aquila, H. (1983) *Eur. J. Biochem. 131*, 647–654.

Klingenberg, M., Mayer, I., & Dahms, A. S. (1984) *Biochemistry 23*, 2442–2449.

Krämer, R., & Kürzinger, G. (1984) *Biochim. Biophys. Acta 765*, 353–362.

Krämer, R., Aquila, H., & Klingenberg, M. (1977) *Biochemistry 16*, 4949–4953.

Mayer, I., Dahms, A. S., Riezler, W., & Klingenberg, M. (1984) *Biochemistry 23*, 2436–2441.

Mitchell, P., & Moyle, J. (1969) *Eur. J. Biochem. 7*, 471–484.

Onur, G., Schäfer, G., & Strotmann, H. (1983) *Z. Naturforsch., C: Biosci. 38C*, 49–59.

Schäfer, G., & Onur, G. (1980) *FEBS Lett. 109*, 197–201.

Schäfer, G., Onur, G., & Schlegel, M. (1980) *J. Bioenerg. Biomembr. 12*, 213–232.

Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd ed., Wiley-Interscience, New York.