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Use of Modified Adenine Nucleotides in Mechanistic Studies on Oxidative Phosphorylation: Structure and Space at the Catalytic Site[1]

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Abstract

This report summarizes structure/activity investigations on 3'-O-substituted adenine nucleotides derived from 3'-O-naphthoyl-ADP. Among these are fluorescent nucleotides, which allow one to differentiate between two types of binding sites on the inner surface of the mitchondrial inner membrane. One type of site is highly fluorescent but is not located on F_1. It is attributed to the nucleotide carrier, because it stays on the membrane when F_1 is removed. The other type of sites, giving no or only very low fluorescence, is located on F_1 and shows high affinity to these analogs, which is modulated by the energy state of the membrane. On the basis of kinetic data, stability of magnesium complexes, and fluorescence properties, conclusions are drawn on the probable conformation of these nucleotides in the bound state. They allow one to explain why these nucleotide analogs are extremely strong inhibitors of oxidative phosphorylation and photophosphorylation, why the ADP derivatives cannot be phosphorylated, and why the ATP analogs are no substrates of ATPase. Furthermore, the results allow some insight into the mechanism of phosphorylation and the structural properties at the catalytic site.

Introduction

Coupling factors of oxidative phosphorylation or photophosphorylation (F_1,CF_1) belong to the most complex enzymes, being composed of at least five different subunits. An understanding of their catalytic mechanism in ATP synthesis is rendered difficult by the presence of different nucleotide-binding

[1]Abbreviations: AP_5A = 5',5'-diadenosinepentaphosphate; SMP = submitochondrial particles; F-AD(T,M)P = fluorescent AD(T,M)P = 3'-O-(5-dimethylaminonaphthoyl-1)-AD(T,M)P; FCCP = carbonylcyanide 4-trifluoromethoxyphenylhydraxone.

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sites phenomenologically assigned "tight" binding sites and "catalytic" or rapidly reversible binding sites. Their true interrelation, however, is difficult to understand, as has been discussed in previous communications [1, 2]. In principle two models may be considered: First, that multiple binding sites exist which are functionally interconvertible in a cyclic manner, similar as proposed in the dual site model [3, 4]; second, that on these enzymes permanently distinct catalytic and control sites exist [5–7].

This problem has been repeatedly approached by use of modified nucleotides [8–10]. The conclusions, however, are confined to certain limits, because they are based on experiments conducted under widely different conditions (solubilized, membrane-bound, energized, or nonenergized coupling factors; phosphorylating or nonphosphorylating conditions) which imply that the enzyme itself was present in a variety of functional and conformational states, altering its binding properties. Thus, no clear-cut identification of the interrelation between the two types of sites was achieved so far.

More recently a group of analogs have been detected [11, 12] in our laboratory, which exhibit an apparently higher affinity to the enzyme than the natural substrates ADP and ATP themselves. These are 3′-O-esters or ethers of adenine nucleotides. Among other analogs the 3′-ester derivatives are the most potent inhibitors ($K_i < 1\ \mu M$) of energy-linked phosphorylation and, moreover, by virtue of their high affinity and specificity, may serve as vehicles into the nucleotide binding sites for a variety of probe molecules, carried as the 3′-O-substituent as, for example, covalent binding groups, spin labels, or fluorescence labels. Thus, they deserve considerable interest as promising tools in mechanistic studies on transphosphorylation during ATP synthesis and on the function of nucleotide binding sites.

The present report focuses on analogs derived from 3′-O-naphthoyl-(1)-AD(T)P which proved to be especially effective. From this analog, fluorescent probes have been derived. By use of these derivatives it became obvious that they bind to different sites on the inner mitochrondrial membrane, yielding high or low fluorescence efficiency, respectively. In contrast to previous expectations, the quantity interacting with F_1 sites is subject to a large fluorescence quench, which will be discussed mainly from conformational aspects as derived from space-filling molecular models.

Methods

Submitochrondrial particles (SMP) were prepared as described in detail [12] by sonication of beef heart mitochondria in a buffer containing 220 mM sucrose, 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM

succinate, and 1 mM ATP. Immediately after preparation they were kept at room temperature for 30–45 min at high protein concentration (70–100 mg/ml) and then stored on ice until use. Each experiment was carried out with particles freshly prepared from a stock of a large-scale beef heart mitochondria preparation frozen at −70°C.

Liposomes from synthetic dimyristoyl lecithin, palmitoyl lecithin, or egg-yolk lecithin were prepared by sonic dispersion of the lipids in 250 mM sucrose, 10 mM Tris (pH 7.5) following standard procedures [13]. The suspensions were prepared and stored at temperatures above the phase-transition temperature of each lipid and used within 12–24 h for fluorimetric binding studies.

Oxidative phosphorylation in SMPs was measured by incorporation of ^{32}P-labeled inorganic phosphate into ATP, using a hexokinase trap, at 30°C in a total volume of 0.6 ml containing 220 mM sucrose, 10 mM Tris (pH 7.2), 2.5 mM $MgCl_2$, 1.5 mM EDTA, 4 mM potassium phosphate, 10 mM succinate, 10 mM glucose, and 70 units/ml of hexokinase. ADP and inhibitors were present as given in tables and figures. Inhibitor titrations for determination of half-maximal inhibition were conducted with 150 μM ADP present; for K_i determinations the range of ADP concentrations was varied from 2 to 150 μM without (control) or with inhibitor present. Phosphorylation rates were calculated from the ratio of ^{32}P in extracted versus nonextracted aliquots of the samples after denaturation, as described [12].

Continuous registration of ATP synthesis by oxidative phosphorylation was achieved by monitoring the light emission of luciferin/luciferase as an indicator of ATP concentration [14, 15]. The measuring device was a high-sensitivity quantum counter with analog output (Lumacounter) and a micro reaction cell. The total volume was 640 μl containing a buffer as described above, and in addition 10 μg of luciferase-protein and 0.1 mM of D-luciferin. For inhibition of contaminating adenylate kinase 50 μM of AP_5A [16] were present; particles used for this assay were prepared in the presence of 15 μM atractylate which stays trapped within the particles and inhibits the adenine nucleotide carrier. Highly purified luciferace was obtained from Abimed (Düsseldorf).

ATPase activity of SMPs was measured in preliminary experiments by determination of P_i liberation from ATP [17]. For determination of kinetic data, continuous registration of NADH consumption in an ATP-regenerating system was used [18], composed of 40 mM Tris/HCl (pH 7.5), 5 mM KCN, 5 mM $MgCl_2$, 0.35 mM NADH, 0.5 mM phosphoenolpyruvate, 10 units of pyruvate kinase, and 27.5 units of lactate dehydrogenase (enzymes were purchased from Boehringer, Mannheim); temperature was 37°C; in this assay ATPase shows linear Lineweaver–Burk plots. The reaction was started by

addition of the enzyme (SMP dilution); the final volume was 1.0 ml. Under the applied conditions the particles are maintained in a nonenergized state. Addition of uncoupler did not increase the rate of ATP synthesis.

Stability constants of Mg^{++} with adenine nucleotides and several 3'-substituted derivatives were assessed by a competition titration of the Mg^{++}-complexing agent eriochrome blue. Spectral changes during titration with $MgSO_4$ were recorded in a Phoenix dual-wavelength spectrophotometer (604–566 mμ). Titration was carried out in a high ionic strength medium of high buffer capacity containing 0.1 M tricine (pH 8.0), 0.1 M NaCl. The data were analyzed by computer fitting to a kinetic model based on mass-action equilibria, assuming a 1:1 stoichiometry of the dye complex and the nucleotide complex with Mg^{++}. The K_d values given in Table II are means of three to five titrations.

Fluorescence spectra were measured in a Schoeffel (RRS-1000) ratio-fluorimeter with double monochromators. Fluorescence titrations were carried out with the same instrument or, if lower sensitivity was sufficient, with an Eppendorf fluorimeter, using appropriate filter combinations. Fluorescence quench titrations of F-ADP were performed in dioxane or dioxane/water mixtures. When quenches by phenylalanine, tyrosine, or tyrosine methyl ester were determined, the data were corrected for quench effects by the solvent used for the quenchers by analog experiments without the quencher.

The synthesis of 3-esters of nucleotides has been described in detail in a preceding report [12]. In most cases the carboxylic acids for esterification were available from commercial sources. In other cases they were freshly synthesized (by G. Onur), as described in an independent report.[3] Their structure has been verified by IR and NMR spectroscopy, partly after digestion of the nucleotides by alkaline-phosphatase to yield the corresponding nucleosides.

Results

General Properties of the Analogs

The principal structure of the analogs is given in Fig. 1. The residue R can be varied over a wide range of alkyl or aralkyl chains. The most potent inhibitory compounds are derived from the naphthoyl analogs as shown in the lower part of the figure.

Although the effects of 3'-esters of ADP on coupled electron flow in chloroplasts [11] suggested that these analogs are true energy-transfer

[3]Onur and Schäfer (1980), to be published.

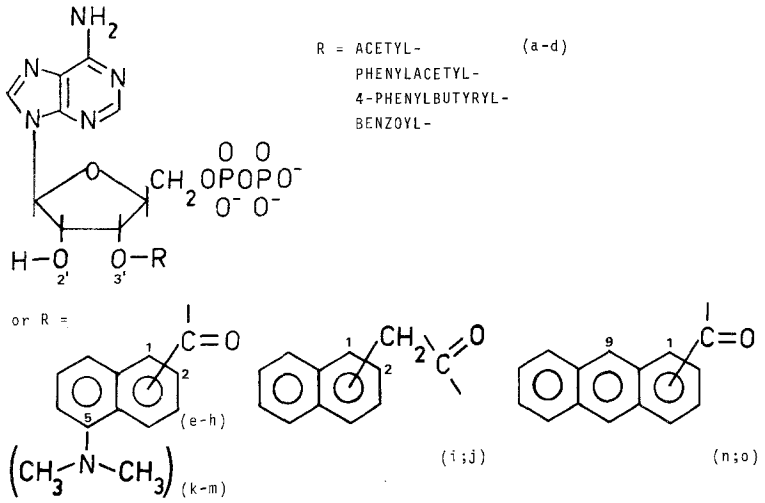

Fig. 1. Chemical structures of the ester analogs of adenine nucleotides used in this study. The letters in parentheses refer to the list of compounds in Table I; F-AD(T,M)P contains a dimethylamino group in position 5 of the naphthoyl (1) residue (left of lower line).

inhibitors, corresponding experiments were not available so far with submitochondrial particles, because the latter do not exhibit acceptor control of respiratory electron flow. By means of a luciferase assay, however, the rate of ATP formation could be directly recorded, confirming also for submitochondrial particles from beefheart that 3′-esters of ADP are inhibitors of ATP synthesis and not uncouplers of phosphorylation.

As shown in Fig. 2 initiation of phosphorylation by addition of ADP causes a steady increase of light emission via the luciferin/luciferase system. Addition of 3′-O-naphthoyl-ADP stops ATP formation, and light emission maintains a steady state, corresponding to the concentration of ATP developed prior to addition of the inhibitor. ATP synthesized during this period is hydrolyzed rapidly after addition of an uncoupler (FCCP). The dashed line shows the effect of uncoupler added prior to the energy-transfer inhibitor. It was ascertained in independent experiments that naphthoyl-ADP does not interfere with the luciferase reaction itself at the applied concentrations. The inhibitory action of 3′-naphthoyl-ADP on oxidative phosphorylation exemplifies a general property of all 3′-esters of ADP. ATP esters also inhibit, but are at least 10 times less effective, whereas the respective AMP analogs are without any effect [12, 19]. 3′-Esters of ADP are not substrates of phosphorylation [11, 12]. Accordingly 3′-esters of ATP are not substrates of F_1- or CF_1-ATPase; contrary reports in the literature [20–22] will be treated below.

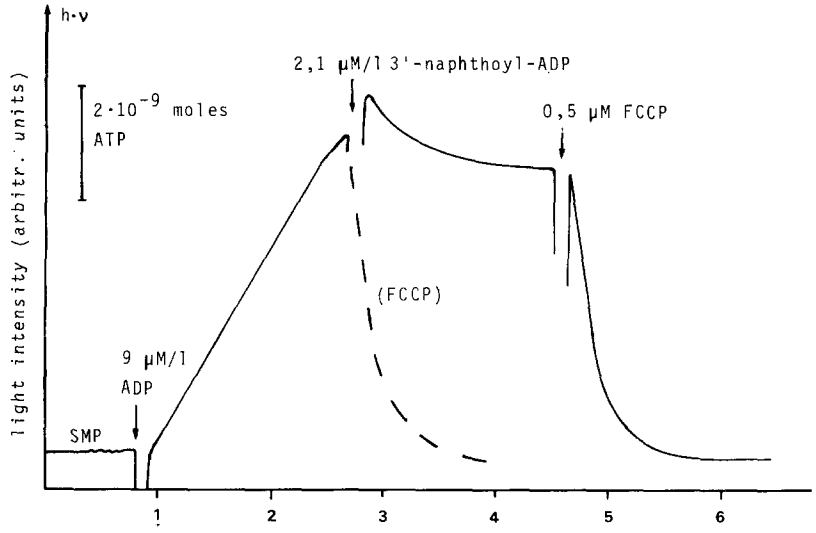

Fig. 2. Continuous monitoring of light emission by luciferin/luciferase during ATP synthesis by beef heart submitochondrial particles. Effect of 3'-naphthoyl-ADP and of uncoupler (FCCP). 80 μg of SMPs were suspended in 640 μl of buffer as given in Methods; the buffer was oxygen saturated and supplemented with 10 mM succinate; 50 μM AP_5A was added for inhibition of adenylate kinase; temperature was 20°C. Additions are given with the trace of light emission. The abscissa gives the time in minutes.

The effects of 3'-esters on oxidative phosphorylation can only be measured with submitochondrial particles. With intact mitochondria no inhibition can be observed because these analogs are not translocated into the mitochondria via the adenine-nucleotide carrier. They inhibit the carrier only at concentrations much greater than 100 μM, which is about 1000 times above their average K_i in phosphorylating submitochondrial particles. This observation is of special interest with regard to the response of the fluorescent analogs, as discussed below.

Structure/Activity Studies

Titrations of oxidative phosphorylation in beef heart submitochondrial particles have been carried out with a large series of 3'-esters of adenine nucleotides. In these titrations ADP was kept constant at 150 μM. The results of these experiments are summarized in Table I, depicting only a part of the analogs synthesized in the course of this study. Their relative activity is expressed in terms of the half-maximal inhibitory concentrations under the conditions applied. As to the type of inhibition, a competitive behavior versus ADP was found in all cases tested, demonstrating that the analogs directly compete with the substrate of phosphorylation [11, 12] at the catalytic center.

Table I. Concentrations of 3′-O-substituted Adenine Nucleotides Causing 50% Inhibition in Oxidative Phosphorylation[a]

Number	3′-O-Substituent	Nucleotide	c_i (μM)
a	acetyl	ADP	85
b	phenylacetyl	ADP	3.6
c	phenylbutyryl	ADP	4.6
d	benzoyl	ADP	6.0
e	naphthoyl-(1)	ATP	2.0
f	naphthyol-(1)	ADP	0.3
g	naphthoyl-(1)	AMP	inactive
h	naphthoyl-(2)	ADP	5.0
i	naphthyl-(1)-acetyl	ADP	0.8
j	naphthyl-(2)-acetyl	ADP	0.8
k	5-dimethylaminonaphthoyl-(1)	ATP	2.1
l		ADP	0.25
m		AMP	inactive
n	anthranoyl-(1)	ADP	0.56
o	anthranoyl-(9)	ADP	5.9

[a]Phosphorylation was measured as given in Methods. ADP was 150 μM in all titrations, and phosphate was 4 mM. Compounds k–m are termed F-AT (D,M)P in the text.

The K_i values were found as small as $\leq 10^{-7}$ M for the most active compound.

As demonstrated by the example of the 3′-acetyl analog, short-chain alkyl derivatives are rather weak inhibitors. Introduction of a phenyl group (compound b in Table I) brings about a 20-fold increase of inhibitory activity. Thus the mobility of the aromatic ring seems to be of some importance, because phenylacetyl- or phenylbutyryl analogs are slightly better inhibitors than the benzoyl analogs. A drastic further increase of activity is achieved by introduction of condensed aromatic systems, as seen from the naphthoyl and anthranoyl analogs of ADP (compounds f, n). These latter compounds are of particular interest for several reasons:

1. It is confirmed that ADP analogs are always stronger inhibitors than ATP analogs, which are approximately 10 times less effective; AMP analogs are inactive.
2. Addition of a 5-dimethylamino group (compounds k, l, m) to the naphthalene ring does not diminish or alter the inhibitory properties. However, these latter compounds [termed F-AD(T,M)P] are highly fluorescent.
3. The position of the aromatic substituent relative to the plane of the ribose ring and its mobility are of significant importance. This is documented by the large difference of inhibitory activity of naphthoyl-(1) and anthranoyl-(1) analogs versus their isomers naphthoyl-(2) and anthranoyl-(9) analog, respectively (compounds f, n, h, o). The efficiency of inhibition differs by at least a factor of 10

between corresponding isomers. As can be seen from space-filling models, in the case of the less inhibitory compounds the substituent is rather fixed in a position where its ring plane is kept in an orientation roughly perpendicular to the ribose ring (compare Fig. 7). Rotational mobility around the carboxyl linkage is strongly limited. That this conformational fixation is the cause for decreased inhibitory activity becomes clear from the fact that the difference between naphthoyl-(1) and naphthoyl-(2) residues disappears when a spacer is introduced between the carboxyl group and the aromatic ring, as in case of the naphthylacetyl analogs (compounds i, j). In these derivatives the rotational freedom of the aromatic substituent is regained and is comparable to that of the naphthoyl-(1) or anthranoyl-(1) analogs, yielding closely similar inhibitory efficiencies.

To further elucidate structural requirements for a best fit of these probes into the active site of the enzyme, it would be of interest to compare analogs with the same residue either in the 3′- or 2′-position of ribose, respectively. This is difficult with esters, since pure 2′-esters are not available because of an equilibrium between 3′ ⇌ 2′-esters by acyl-migration, the 3′ derivative being the predominant form [23, 24]. Corresponding ethers should circumvent this difficulty because substituent migration is excluded. Two compounds of this type have been synthesized with naphthyl-(1)-methyl as an O-substituent (details of synthesis, structure, and function will be reported separately).[4] With these compounds two preliminary observations have been made:

a) 3′-O ethers are significantly weaker inhibitors of oxidative phosphorylation than corresponding esters.
b) There exists a marked difference between the two isomers (2′ or 3′); one of them, preliminary assigned as the 2′-O-(naphthoyl-(1)-methyl) ether of ADP, shows much less inhibition than the other. This difference is even more pronounced in photophosphorylation of spinach chloroplasts.[5]

Mg^{++} Complexes

For all transphosphorylating enzymes Mg^{++} ions are an obligatory cofactor, and it has been suggested that the Mg^{++} complexes of nucleotides are the actual substrates. Therefore stability constants of the Mg^{++} complexes of several of the analogs have been determined, assuming that differences might occur, being related to the inhibitory properties.

[4]G. Onur and G. Schäfer (1980), to be published.
[5]H. Strotmann, personal communication.

Table II. Stability Constants of Mg^{++} Complexes of Adenine Nucleotides and Some 3′-O-Esters[a]

3′-O Substituent	Nucleotide	$-\log K_d$
—	ATP	4.66
—	ADP	3.22
—	AMP	2.54
Naphthoyl-(1)	ATP	4.24
Naphthoyl-(1)	ADP	3.01
Naphthoyl-(1)	AMP	2.61
5-dimethylaminonaphthoyl-(1)[b]	ATP	4.5
	ADP	4.08
Naphthyl(1)acetyl	ADP	3.43

[a]The constants were determined by competition titrations of eriochrome blue, as given in Methods.
[b]Compounds are termed F-AT(D)P in the text.

As seen from Table II, the stability constants do not differ significantly from those of unmodified nucleotides. Like ATP and ADP, they are separated by about one order of magnitude between the ATP and ADP analogs. There is one important exception, however, regarding F-ATP and F-ADP, which exhibit fairly identical stability constants.

Obviously there is no correlation at all between inhibitory activity and the stability of Mg^{++} complexes; this is especially supported by the large difference of K_d [Mg] between 3′-naphthoyl-ADP and F-ADP, although their respective K_i values in oxidative phosphorylation are almost identical (compounds f, l). Moreover, the close similarity of the K_d [Mg] for F-ADP and F-ATP (in contrast to the naphthoyl-(1) analogs) strongly suggests that the dimethylamino nitrogen of the substituent participates in the formation of the ionic complex. This is in support of the structural concept discussed below.

Although Mg^{2+} was shown to be involved in nucleotide binding [25], this does not necessarily imply that the nucleotide–Mg complex is the bound species; Mg^{2+} might well be equally necessary to maintain an enzyme conformation competent for nucleotide binding.

Effects on Membrane-Bound F_1-ATPase

It has been shown elsewhere [12] that 3′-esters of AD(T)P besides oxidative phosphorylation also inhibit energy-linked functions as well as uncoupled ATPase of submitochondrial particles, but to a much smaller extent. More detailed investigations have now shown that inhibition of membrane-bound ATPase by naphthoyl-ADP, F-ADP is definitely competitive.

In contrast to oxidative phosphorylation, however, the large discrepancy of inhibitory activity between corresponding ADP and ATP analogs disap-

Table III. Comparison of Inhibitory Activity of 3′-O-Esters of Adenine Nucleotides (Compounds k–m of Table I) in Oxidative Phosphorylation and Uncoupled ATPase of Submitochondrial Particles[a]

Nucleotide analog	Oxidative phosphorylation K_i (μM)	ATPase K_i (μM)
F-ADP	0.04	9.8
F-ATP	0.30	12–27
F-AMP	inactive	inactive

[a]Experimental details for determination of enzymatic rates are given in Methods. The K_i values were calculated from the apparent K_m values resulting from Lineweaver–Burk diagrams; inhibitions were competitive.

pears. This change predominantly concerns the inhibitory efficiency of the 3′-ADP derivatives, which is decreased to practically the same value as that of the 3′-ATP derivatives. This is clearly visible from the K_i values of Table III. It should be stressed that none of the ATP analogs behaved as a substrate of mitochondrial ATPase.

Fluorescent Analogs

Summarizing previous experiences with 3′-O analogs, it was especially surprising that 3′-substituents were tolerated, as large as the size of the purine

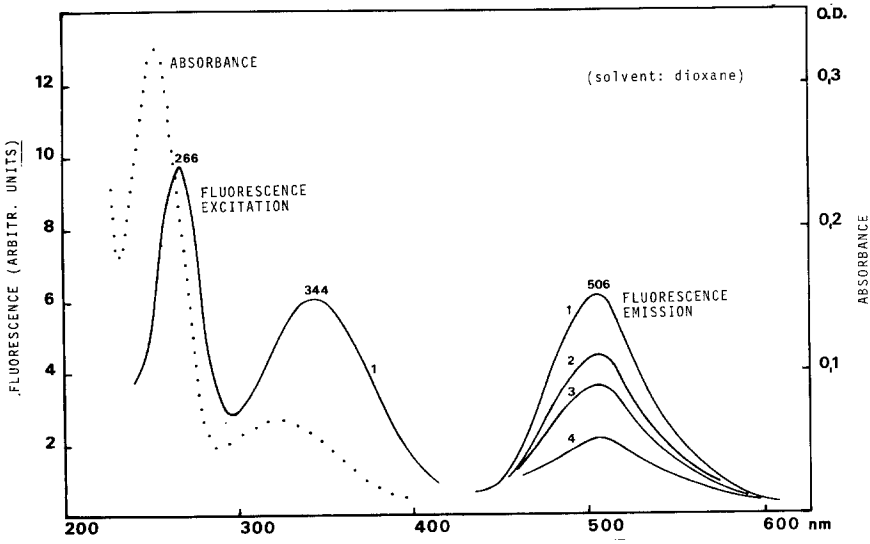

Fig. 3. Absorbance and fluorescence spectra of F-ADP (compound 1 in Table I). (· · · · ·) absorbance of 2.15 × 10^{-5} M F-ADP in 0.1 M K-phosphate buffer, pH 7.2; (———) fluorescence of 2.15 × 10^{-5} M F-ADP in dioxane; left: excitation spectrum; right: emission spectrum; (1) dioxane; (2) 2.2 vol.% H_2O; (3) 3.6 vol.% H_2O; (4) 5.4 vol.% H_2O in dioxane.

ring itself. Since all of these are predominantly hydrophobic, it was concluded that a large hydrophobic cavity may be present at the active site of energized mitochondrial F_1 or chloroplast CF_1, capable of accepting such a large hydrophobic residue [2, 12]. Accordingly, it was expected that in the case of a fluorescent substituent in the 3′-position a large increase of fluorescence yield occurs after binding to the active site.

F-ADP (compound I of Table I) is an analog which fluoresces negligibly in aqueous solutions but has significant fluorescence in apolar media like dioxane. Its spectral properties are shown in Fig. 3, demonstrating that increasing polarity by successive addition of water diminishes fluorescence dramatically. Fluorescence properties of the ATP, ADP, and AMP analogs are identical under the applied conditions; some of these properties have been communicated recently [26]. Figure 4a shows a fluorescence titration, illustrating that binding to submitchondrial particles in fact is accompanied by a large fluorescence increase of F-ADP. The respective adenosine derivative or 5-dimethylaminonaphthoic acid methyl ester (the nucleotide-free fluorophore) do not exhibit significant binding. Thus, binding with increased fluorescence requires a complete nucleotide structure. Fluorescence of F-ADP or of F-ATP revealed another interesting feature, namely that the analogs with intact mitochondria, albumin solutions, or with liposome suspensions show only negligible fluorescence and no saturating characteristics of binding. From this it becomes obvious that the analogs only bind specifically to the inner side of the mitochondrial membrane with enhanced fluorescence,

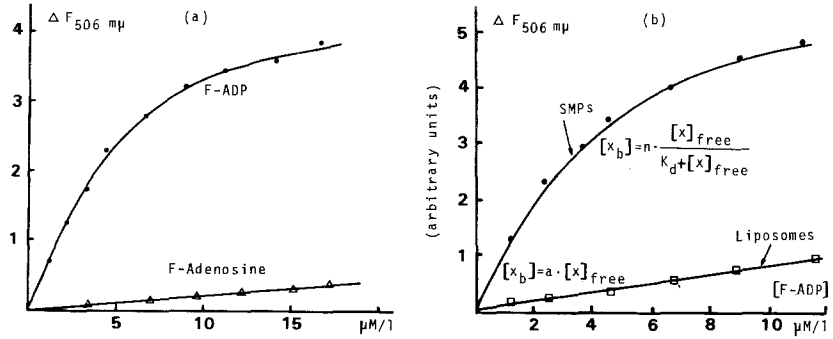

Fig. 4. (a) Fluorescence changes on binding of F-ADP or F-adenosine to submitochondrial particles. Ordinate gives the change in fluorescence in arbitrary units; abscissa gives the concentration of F-ADP (compound 1 for Table I) or of the respective 3′-substituted adenosine; titrations with the methyl ester of 5-dimethylaminonaphthalene-1-carboxylic acid yield the same result as with adenosine. SMP concentration was 0.94 mg/ml in a buffer containing 250 mM sucrose and 10 mM Tris (pH 7.4) at room temperature. (b) Fluorescence changes during titration of SMPs or of liposomes from lecithin (dimyristoyl-) by F-ADP (compound 1 from Table I) in the same buffer as given under (a). Particle concentration was 0.75 mg/ml (SMPs) or 1% (w/w) lipid dispersion, respectively. $[x_b]$ and [x] free signify bound and free ligand, respectively.

and thus can differentiate between the two surfaces of this membrane. This is illustrated in Fig. 4b, where the titration of submitochondrial particles is compared to that of liposomes. The latter totally resemble the results obtained with intact mitochondria, demonstrating that only a linear phase distribution occurs with other membranes or with lipid dispersions, except submitochondrial particles.

The result of fluorescence titrations, however, revealed some discrepancies with kinetic data from oxidative phosphorylation that need serious consideration. Anticipating the final result, it has to be assumed that the highly fluorescent binding sites are not identical with catalytic or "tight" sites on F_1. This will be documented by the experiments below.

Figure 5 shows a titration of the analog's fluorescence by submitochondrial particle protein. It is obvious not only that all three analogs (F-ADP,

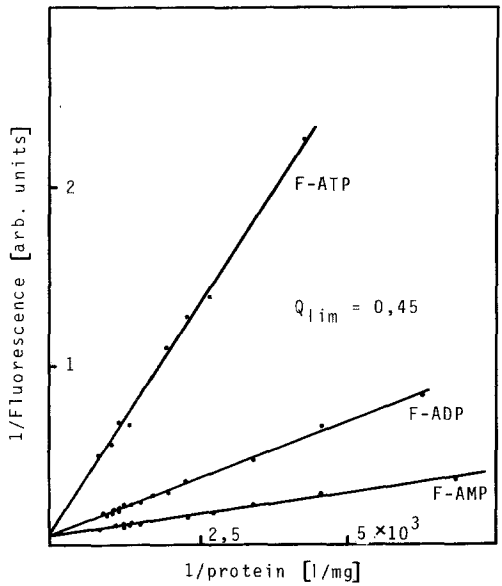

Fig. 5. Reciprocal plot of fluorescence titrations of F-AM(D,T)P by submitochondrial particle protein. Data are corrected for endogenous fluorescence of the particles in the absence of nucleotide analogs. The concentrations of nucleotides were 4.9 μM (F-ATP), 3.5 μM (F-ADP), and 3.1 μM (F-AMP). Titration was performed in 250 mM sucrose, 10 mM Tris (pH 7.4) at 20°C. The limiting fluorescence (quantum yield) was calculated from fluorescence of identical concentrations of nucleotides and their known quantum yield in dioxane or ethanol.

ATP, and AMP) bind to the particles with enhanced fluorescence, but also that the limiting fluorescence, i.e., the quantum yield of all three analogs, is identical, as seen from the common ordinate intercept in the double reciprocal plot.

The K_d values derived from fluorescence titrations are about 10 times above the apparent K_i values measured in oxidative phosphorylation. In addition, F-AMP with respect to fluorescence shows at least the same affinity as F-ADP, although it does not have any effect on oxidative phosphorylation.

As we have shown recently [26], the fluorescence of membrane-bound F-AD(T)P is immediately quenched by about 80% on addition of bongkrekic acid. The effect of bongkrekic acid on fluorescence is noncompetitive. These studies show clearly that in addition to F_1, the fluorescent analogs also bind to the adenine nucleotide carrier as it is exposed to the surface of submitochondrial particles. The presence of bongkrekate, however, has no influence on inhibitor sensitivity of oxidative phosphorylation, as shown in Table IV; identical half-maximal inhibitory concentrations were found as in the absence of bongkrekate or of atractylate.

From these results it is clear that binding to the catalytic center of oxidative phosphorylation (F_1) is totally different and independent from the quantity of F-ADP bound to the high-fluorescent, bongkrekate-sensitive sites on the membrane.

This is further supported by comparative fluorescence titrations of urea-treated submitochondrial particles, which have been deprived of at least ¾ of their F_1 as revealed by the decreased activity of ATPase (1.4 → 0.3 U/mg). Neither the affinity for F-ADP nor the total number of highly fluorescent binding sites has been significantly changed by removal of F_1 from the membrane.

Table IV. Influence of Bongkrekic Acid and of Atractylate on the Rate of Oxidative Phosphorylation and on the Inhibition of F-ADP in Beef Heart Submitochondrial Particles

Conditions of SMPs	Relative rate of oxidative phosphorylation (%)
Control (no inhibitors)	100[a]
+ Bongkrekic acid (2.4 nmoles/mg)	100
+ Atractylate (20 nmoles/mg)	100
+ F-ADP (0.5 μM)	56
+ F-ADP (0.5 μM) and bongkrekic acid (2.4 nmoles/mg)	55
+ F-ADP (0.5 μM) and atractylate (20 nmoles/mg)	55

[a] 100% phosphorylation rate (control) was 180 nmoles/min/mg. 150 μM ADP and 4 mM P_i were present in all experiments. Other experimental details as given in Methods. All figures are means of two to six determinations.

Model Experiments on Fluorescence

The preceding experiments show that the highly fluorescent sites have an apparent affinity of 4–9 μM/liter, which is equal to the K_d for F-ADP, whereas the K_i for inhibition of oxidative phosphorylation for these analogs is 10 to 100 times lower (K_i 0.04–0.3 μM). A corresponding high-affinity site on submitochondrial particles could not be detected by fluorescence. Thus, the question arose why binding to the coupling factor F_1 is not accompanied by a concomitant increase of fluorescence.

Two possibilities may be considered. One is that the binding site on F_1, hitherto considered to provide a rather hydrophobic environment, is much more polar than expected; the other is that fluorescence of the probe, bound with high affinity to F_1, is quenched by specific amino acid residues present in their vicinity.

Both possibilities have been verified in model experiments. Figure 3 shows the effect of increasing polarity, causing a decrease of quantum yield

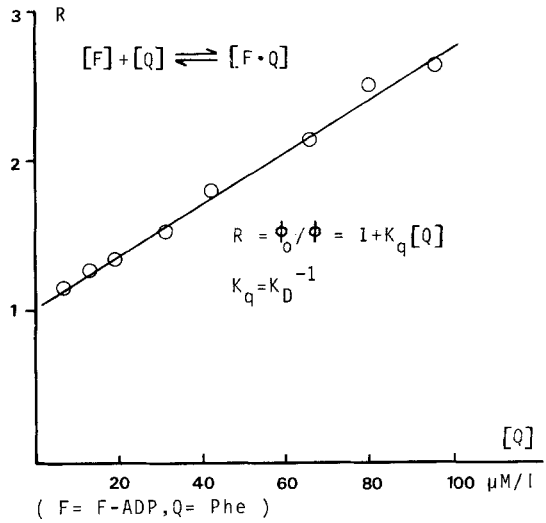

Fig. 6. Effect of a quencher on relative fluorescence of a fluorophore. The experiment shows titration of F-ADP fluorescence by phenylalanine; F-ADP 2.8 $\times$ 10^{-5} M was dissolved in dioxane; phenylalanine was used as aqueous solution (6.03 mM); spectral changes were corrected for the change induced by water alone. Total volume was 2.03 ml at 20°C. ϕ_0 refers to the initial fluorescence, ϕ is the resulting fluorescence after addition of quencher. [Q] refers to concentration of phenylalanine. The data were obtained from integrations of the total emission spectra for each concentration of Phe.

Table V. Efficiency of Quenchers of F-ADP Fluorescence[a]

Quencher	K_q	K_D (M)
H_2O	$7.01 \cdot 10^{-1}$	1.42
Phe	$8.01 \cdot 10^{3}$	$1.24 \cdot 10^{-4}$
Tyr	$2.73 \cdot 10^{4}$	$3.66 \cdot 10^{-5}$
Tyr-CH_3	$6.43 \cdot 10^{3}$	$1.56 \cdot 10^{-4}$

[a]The data were obtained from fluorescence quench titrations as described in Fig. 6. The same quench constants were found with F-AMP, F-ATP, or the methyl ester of 5'-dimethylamino-naphthoic acid.

by dipole–dipole interactions without a significant shift of the emission maximum.

From the relative fluorescence intensity remaining after addition of a quencher, an apparent dissociation constant between fluorophore and quencher can be calculated according to Fig. 6. This plot shows the results of a fluorescence quench titration with phenylalanine as the quencher.

By comparison of the apparent K_d values obtained from this type of experiment, the relative effectiveness of different quenchers can be visualized. Table V gives the respective constants for water and for aromatic amino acids. It is interesting to see that tyrosine and phenylalanine are by far more potent quenchers of F-ADP fluorescence than water. This result lends support to the idea that strong interactions of the aromatic substituent with aromatic amino acids like tyrosine might be responsible for the absence of detectable fluorescence at the catalytic site.

Discussion

Nucleotide analogs of the 3'-ester type have been used as photoaffinity labels for F_1-ATPase [20, 21, 27, 28] before we have detected that these compounds represent a group of potent effectors of ATP synthesis and ATP hydrolysis without the necessity of covalent linkage to coupling factors [11, 12]. Although results of photolabeling experiments helped to localize nucleotide binding sites on the β- and the α-subunits of these enzymes [21, 22, 27], the functional relationship of these nucleotide binding sites is still obscure. In the present study attention was directed not on this particular problem but rather on the catalytic and fluorescence properties of 3'-O-analogs of adenine nucleotides, sensing the molecular architecture and microenvironment of substrate-binding sites in oxidative phosphorylation.

Summarizing present knowledge on these analogs, it is clear that they are not phosphate acceptors of oxidative or photophosphorylation, and that

their strong inhibitory effect is competitive to ADP and noncompetitive to inorganic phosphate [12]. From this it has beeen concluded that 3′-esters of ADP can specifically replace unmodified ADP at the catalytic site and that the 3′-substituent, most likely by steric hindrance, affects the transfer of inorganic phosphate to the β-phosphate of the nucleotide. This view would imply that activated phosphate presumably approaches the β-phosphate from a direction to which the 3′-O-substituent extends.

The systematic configurational variation of aromatic 3′-O-substituents presented here lends support to these conclusions on the basis of conformational aspects. Comparison of 3′-esters and 2′-esters, for example with the naphthoyl residue as a substituent, clearly shows that only in the case of the 3′-esters does the substituent come into close proximity to the phosphate chain of the ADP analog. In addition, models make obvious that an exo/endo transition of the ribose ring yields totally different conformational results with regard to the oxygen substituents. Whereas in the case of the 2′-O-analogs there is only a minor change in orientational freedom of the naphthoyl moiety and of its distance to phosphate, in the case of the 3′-O-analogs the proximity between the substituent and the phosphate chain is maintained. Furthermore, the 3′-substituent as a result of this transition can describe a half-circle around the α-phosphate, always screening the phosphate chain from an attack by other molecules. The extreme positions of the 3′-naphthoyl residue during an endo/exo transition of the ribose ring are illustrated in Fig. 7.

The assumption that the 3′-O-substituent easily extends to a position similar to that shown on the left of Fig. 7 (3′-exo) is strongly supported by the fact that the stability constants of the Mg complexes of F-ADP and F-ATP are almost identical, due to participation of the 5-amino nitrogen in complex formation.

The postulated screening effect of the aromatic 3′-O-substituent on the phosphate moiety is considered as the main reason why these analogs are not phosphorylated. Simply because the substituent abolishes a proper contact of the phosphate chain with the catalytic surface structure of the enzyme, it is conceivable that the corresponding 3′-ATP-analogs should not be substrates of ATPase. This is exactly what has been found in this and preceding investigations [12]. Contrary findings by others [21, 22] using 3′-O-N-(4-azido-2-nitrophenyl-β-alanyl)-ATP are erroneous due to contamination by ATP, because this particular compound is extremely labile at the 3′-ester bond against hydrolysis and cannot be used when contaminated (3–5%). This instability was inevitably introduced by the presence of an amino group close to the ester bond. Compounds derived from aromatic carboxylic acids, as used in this study, are much more stable against hydrolysis and therefore—since

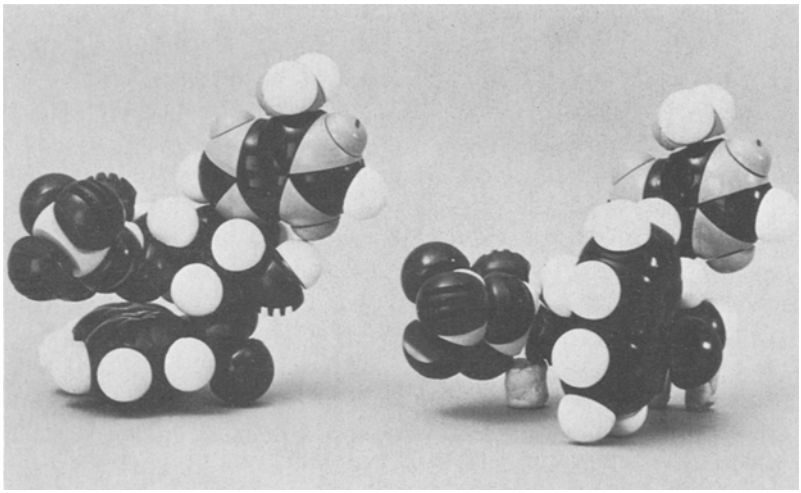

Fig. 7. Space-filling CPK models of 3′-O-naphthoyl-ADP under two steric conditions. Left: 3′-exo conformation with closest possible approximation of the substituent to the phosphate chain; right: 3′-endo conformation with largest possible separation of the substituent from the phosphate chain.

ATP contamination is absent—are suitable for showing that 3′-ATP analogs are not substrates of F_1 or CF_1 ATPases.

The properties of the above analogs are of particular interest also with regard to the structure of the enzyme in different functional states. From former experiments we conclude that 3′-arylazido-ADP and other 3′-O-esters of ADP are "conformation-specific" probes because they inhibit oxidative phosphorylation or photophosphorylation at least 10 times stronger than ATPase under nonenergized conditions. One reasoning is that in energized membranes the enzyme assumes a conformation at the catalytic site, exhibiting much higher affinity or accessibility to the 3′-O-ADP analogs than under nonenergized conditions. The present study strongly supports this idea, demonstrating that the large difference of inhibitory potency of ADP versus ATP analogs in oxidative phosphorylation is absent with uncoupled ATPase. This occurs mainly by a decrease of affinity of the ADP analog to the enzyme. The affinity of the ATP analog is much less affected.

While it is generally observed that modifications of nucleotides in the base moiety drastically reduce their affinity to nucleotide-dependent enzymes, it was surprising that introduction of substituents into the 3′-position of ribose, as space filling as the purine system itself, was not only tolerated by F_1 or CF_1, but also generated analogs with an apparently higher affinity to the catalytic site than the parent nucleotides. It is clear from the

above-mentioned aspects that besides steric and conformational requirements the presence of an aromatic substituent greatly enhanced the activity of these analogs as inhibitors. It therefore seems that not only are hydrophobic properties essential but also the ability of the substituent for π/π interactions. This might be the reason for ground-state quenching of the fluorescence of F-ADP(T)P when bound to the functional high-affinity sites of energized F_1.

In the light of present knowledge it cannot be excluded that the low fluorescence reflects a much more polar nature of the catalytic site than previously suggested [12]. However, since evidence has been accumulated that there is an essential tyrosine residue in the catalytic site [29–31], the possibility has to be considered that this very tyrosine is the quencher of F-ADP fluorescence. The results of the above model experiments are in favor of this possibility.

Actually, fluorescence energy transfer experiments of NBD-modified CF_1 and bound ϵ-ATP suggest that there is a tyrosyl residue in the vicinity of the nucleotide-binding enzyme surface [22]. The estimated distance, however, is 35 Å and it was stated that the bound nucleotide and the modified tyrosine need not be on the same subunit. Moreover, recently published CD spectra indicate that there occurs a tyrosyl-specific spectral change on binding of nucleotides to the β-subunit of TF_1 [32]. Thus it seems likely that the 3′-substituent is capable of interacting with this very tyrosine.

However, loss of fluorescence would also be expected as a result of intramolecular interaction of the 3′-substituent with the phosphate chain, as suggested by the proposed structure of the Mg^{++} complexes. Therefore the absence of fluorescence in the F_1-bound state provides additional evidence that the substituent at the active site screens the phosphate chain from interacting with the catalytic surface structure of the enzyme.

Finally it has been shown by the use of F-AD(T)P that there are other nucleotide binding sites on the inner side of the mitochondrial inner membrane, which bind these analogs with lower affinity but with high fluorescence. These sites are different from F_1 and have to be attributed to the adenine nucleotide carrier. The fluorescence at these sites may be useful for dynamic studies on conformational transitions of the carrier. One aspect regarding the quantum yields is of interest: the quantum yield in water is 0.018, in dioxane it is 0.245, whereas the limiting fluorescence with submitochondrial particles shows a quantum yield of about 0.45 [26]. This latter result suggests that fluorescence on these membrane sites does not merely reflect distribution into an environment of low polarity. The increase of quantum yield above that in dioxane most likely indicates that, by interaction of the dimethylamino group, conformational constraint may be exerted on the

3′-substituent of the analog, causing a more favorable overlap of the free N-orbitals or carbonyl orbitals with those of the aromatic π-system.

In summary, the nucleotide analogs described in this study proved to be useful tools, sensing structure and space at the catalytic site of oxidative phosphorylation and at other nucleotide-binding sites of membranes.

Addendum

Two important aspects should be added: one regarding the mechanism of phosphorylation, the other regarding the interaction of F-AD(T,M)P with the mitochondrial adenine nucleotide carrier. (1) It has been discovered that 3′-O-naphthoyl-ADP and related compounds induce sigmoidal kinetics of ADP-phosphorylation as we have shown recently also for an α-P-acylated ADP analog [33]. From the apparent Hill-coefficients it is suggested that in fact a dual-site mechanism of oxidative phosphorylation may be operating [4]. (2) The fluorescence of F-AD(T,M)P due to binding to the adenine nucleotide carrier of SMP can be quenched by bongkrekate or by palmitoyl-coenzyme-A; there is no influence or competition of ATP or ADP, however. Together with the fact that the fluorescent AMP-analog also binds (even with the highest affinity) these results suggest that the bongkrekate-sensitive binding sites are presumably not identical with the binding sites competent for translocation of nucleotides.

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