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Interaction of High-Affinity Nucleotide Binding Sites in Mitochondrial ATP Synthesis and Hydrolysis[1]

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Abstract

The present study contributes to the problem of the dynamic structure of mitochondrial F1-ATPase and the functional interrelation of so-called tight nucleotide binding sites. Nucleotide analogs are used as a tool to differentiate two distinct functional states of the membrane-bound enzyme, proposed to reflect corresponding conformational states; they reveal F1-ATPase as a "dual-state" enzyme: ATP-synthetase, and ATP-hydrolase. The analogs used are 3′-naphthoyl esters of AD(T)P, and 2′(3′)-*O*-trinitrophenyl ethers of AD(T)P. Both types of analogs act inversely to each other with respect to their relative effects on oxidative phosphorylation and on ATPase in submitochondrial vesicles. The respective ratios of K_i versus both processes are 250/1 compared to 1/170. It is also shown that in the presence of the inhibitory 3′-esters oxidative phosphorylation deviates from linear kinetics and that these inhibitors induce a lag time of oxidative phosphorylation depending on the initial pattern of nucleotides available to energized submitochondrial vesicles. The duration of the lag time coincides with the time course of displacement of the analog from a tight binding site. The conclusions of the study are: (a) the catalytic sites of F_1-ATP-synthetase are not operating independently from each other; they rather interact in a cooperative manner; (b) F_1-ATPase as a "dual-state" enzyme exhibits highly selective responses to tight binding of nucleotides or analogs in its "energized" (membrane-bound) state versus its "nonenergized" state, respectively.

Key Words: Oxidative phosphorylation; F_1-ATPase; nucleotide binding sites; cooperativity; nucleotide analogs; fluorescence; mechanism.

[1] Abbreviations used: N-AD(T)P, 3′-*O*-naphthoyl(1)-AD(T)P; DMAN-AD(T)P, 3′-*O*-(5-dimethylaminonaphthoyl(1))-AD(T)P, also termed F-AD(T)P in previous papers because of its fluorescence; TNP-AD(T)P, 2′(3′)-*O*-(2,4,6-trinitrophenyl)-AD(T)P; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone.

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Introduction

One of the most intriguing problems of biological energy transduction by mitochondrial H^+-ATPase is the function and mutual interrelation of the multiple nucleotide binding sites on this complex enzyme (for a review see Penefsky, 1979; Senior, 1978; Harris, 1978; Cross, 1981). In previous studies we have shown that certain 3′-*O*-modified adenine nucleotides have an apparently higher affinity to the catalytic center of this enzyme than the natural substrates but are nonreactive, i.e., they cannot participate in the catalytic cycle (Schäfer and Onur, 1979). Thus they act as potent inhibitors of ATP synthesis or hydrolysis and have been used as probes to elucidate some steric requirements at the catalytic site (Schäfer *et al.,* 1980). From the unexpected lack of fluorescence responses of some tightly binding 3′-analogs bearing fluorescent substituents, it was suggested that a tyrosyl residue at the catalytic site may be responsible for a fluorescence quench.

Another interesting property is the fact that the respective ADP and ATP analogs (e.g., 3′-*O*-naphthoyl-ADP) are almost equally effective as inhibitors of ATP hydrolysis, whereas in oxidative phosphorylation there exists a dramatic difference of efficacy, with the ADP analogs being at least 10 times more effective than the ATP analogs. Therefore, it has been postulated that only the ADP analogs may serve as "conformation-specific" probes, specifically interacting with F_1-ATPase in the energized state of the mitochondrial membrane (Schäfer, 1978). However, it has been found (Tiedge *et al.*, 1982) that the respective ADP analogs bind firmly also to "nonenergizable," isolated F_1-ATPase that has been depleted of nucleotides. More recently it was shown that 2′(3′)-TNP ethers of ATP and ADP firmly bind to and strongly inhibit isolated F_1-ATPase (Grubmeyer and Penefsky, 1981), acting comparatively weakly on oxidative phosphorylation.

The inverse behavior of the two types of analogs is compared in more detail in this paper. In addition, it will be shown that 3′-esters of ADP (but not of ATP) modulate the initial kinetics of ADP phosphorylation, inducing a lag time when added prior to ADP (Schäfer, 1981). Both a cooperative response of nucleotide binding sites in ATP synthesis and a selective inhibition of F_1-ATPase in its two functional (and presumably conformational) forms—as an ATP-synthetase or as an ATP-hydrolase—will be demonstrated. H^+-ATPase may therefore be considered a "dual-state" enzyme regarding its inhibitor sensitivity.

Methods and Materials

Beef heart mitochondria and submitochondrial vesicles (SMP) were prepared as repeatedly described (Schäfer and Onur, 1979; Schäfer *et al.,*

1980); the sonication medium contained 220 mM sucrose, 10 mM Tris-HCl (pH 6.8), 5 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM succinate, and 1 mM ATP. In contrast to previous procedures, sonication was carried out at 0°C for only 10 times 1 sec with 15-sec intervals at maximum power output of a Branson sonifier. The resulting preparations revealed excellent phosphorylation rates (up to 350 nmol/mg · min) and high respiratory control (state 4 release by uncoupler; up to 5.3). The final suspension was made up in 220 mM sucrose and 20 mM Tris (pH 6.8) at a concentration of about 70 mg protein/ml.

Oxidative phosphorylation was monitored by direct recording of ATP synthesis using a luciferase assay as described elsewhere in detail (Schäfer, 1981). Submitochondrial vesicles (10–30 μg) were incubated in 800 μl buffer containing 220 mM sucrose, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 4 mM P_i, 1 mM EDTA (pH 7.4), and 0.3 mM AMP; 40 mM ethanol, 133 μM NADH, and 120 units/ml of alcohol dehydrogenase (Boehringer 411523) were present as a NADH regererating system. The incubations were supplemented by 0.005 μg luciferase (Boehringer 102217; 10^7 units/mg) and 40 μM (−)-D-luciferin, ADP, and inhibitors as indicated in the legends to the figures. Final volume was 860 μl. An LKB luminometer was used. Batch incubations were carried out at 10 times the volume, and 500-μl samples were taken at the indicated times; the samples were quenched with 50 μl 3 M TCA and neutralized by KOH/Tris; thereafter 30-μl aliquots were analyzed for ATP by a luciferase assay under conditions where light output is strictly proportional to ATP concentration (Lundin *et al.*, 1976). If ^{32}P incorporation into ATP was used to measure ADP phosphorylation, the method described previously was applied (Schäfer *et al.*, 1980), using a hexokinase trap. Inorganic phosphate was separated from organic phosphates by precipitation (Sugino and Mioski, 1964), which had the advantage of serving as a deproteinizing step simultaneously.

ATPase activity was determined in a NADH-linked assay employing an ATP regenerating system as described (Schäfer and Onur, 1979). F_1-ATPase from beef heart was prepared essentially following the procedure described for pig heart by Penin *et al.* (1979) with minor modifications (Weber, 1980; Tiedge, 1980). Protein concentrations were determined by the usual Biuret or Lowry methods using bovine serum albumin as standard.

Fluorescence polarization studies were performed on an SLM 4800-S spectrofluorometer. The setup was essentially as described by Jameson *et al.* (1978). Fluorescence of 3′-*O*-naphthoyl-ADP was excited at 310 nm; emitted light was monitored at 400 nm. Binding of the analog to isolated F_1 was studied in 200 μl of a buffer containing 50 mM Tris-HCl, 50 mM KCl, and 2 mM $MgCl_2$ (pH 8). With submitochondrial vesicles the same buffer (2 ml) as in the experiments on oxidative phosphorylation was used; samples were constantly stirred magnetically. In these experiments total anisotropy is

composed of three fractions represented by the intrinsic anisotropy of submitochondrial vesicles and the anisotropy of bound and free 3′-*O*-naphthoyl-ADP, respectively. After measuring the anisotropy at any given concentration of the analog applied to the vesicles, an excess of AD(T)P (60 μM) was added, leading to dissociation of bound naphthoyl-ADP; the remaining signal could be arithmetically separated into the contribution from the particles and that from free naphthoyl-ADP. The degree of binding of naphthoyl-ADP was calculated from the difference between the anisotropy before and after addition of ADP.

Fitting of data was routinely performed on a PDP 11/23 computer by nonlinear regression methods, modifying a program as described by Duggleby (1981) for the specific purposes of this study.

3′-Esters of AD(T)P were synthesized as described by Schäfer *et al.* (1980) and Schäfer and Onur (1979); TNP-AD(T)P was synthesized according to Hiratsuka and Uchida (1973). All chemicals were of analytical grade from commercial sources.

Results

Initial Kinetics of Oxidative Phosphorylation

In the following experiments initial rate kinetics were determined via ^{32}P incorporation in the presence of a hexokinase trap.

With ADP as the variable substrate, the kinetics of oxidative phosphorylation are of the simple Michaelis–Menten type, yielding linear double reciprocal v vs. $[S]$ plots and apparent K_m values for ADP of about 15–20 μM (Hohnadel and Cooper, 1972; Kayalar *et al.*, 1976). In confirmation of the results of a preliminary study from our laboratory (Schäfer and Onur, 1980a), the kinetics may significantly deviate from linearity in the presence of certain

Table I. Apparent Hill Coefficients of Oxidative Phosphorylation in the Presence of Various ADP Analogs[a]

Compound	n_H
ADP	1
3′-Naphthoyl(1)-ADP	1.5
3′-(5-Dimethylaminonaphthoyl(1))-ADP	1.4
3′-Naphthoyl(1)-2′desoxy-ADP	1.4
3′-(5-Azidonaphthoyl(1))-ADP	1.4
αP-Naphthoyl(1)-ADP	1.7

[a]Phosphorylation rates were measured via $^{32}P_i$-incorporation in the presence of a hexokinase trap, varying the ADP concentration from 0.5 to 150 μM. Details as described in Methods and Materials.

inhibitory analogs, inducing sigmoidal v vs. [S] plots. This has been originally observed with an ADP analog acylated at the α-phosphate. Also with other 3′-*O*-naphthoyl analogs, nonlinear kinetics could be determined.

In Table I apparent Hill coefficients for a group of naphthoyl derivatives are summarized. The degree of apparent cooperativity varies with the type of analogs used.

Inhibitor-Induced Lag Phase of Oxidative Phosphorylation

As shown in Fig. 1, direct registration of ATP formation by means of a luciferase assay system also yields simple Michaelis–Menten kinetics for the phosphorylation of ADP; the K_m and V_{max} values are in good agreement with the above determinations where ^{32}P-incorporation was used as an indicator of ATP synthesis. In the presence of 3′-esters as inhibitors, however, kinetic measurements encountered difficulties, as it became obvious that the initial events of ATP formation did not proceed linearly with time. As has been reported elsewhere (Schäfer, 1981), a lag time τ of oxidative phosphorylation occurred, when submitochondiral vesicles were preincubated for a short time with 3′-naphthoyl-ADP, for example. This could be confirmed with other

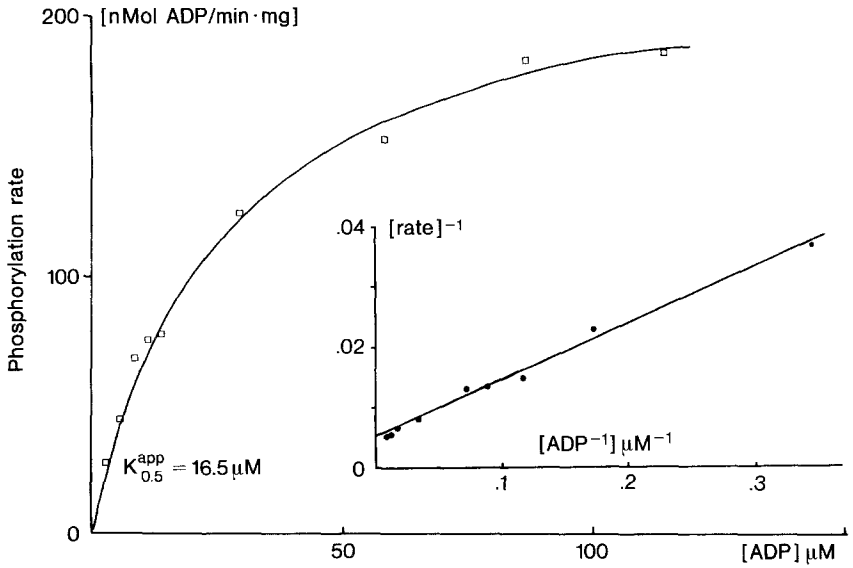

Fig. 1. Determination of the apparent K_m of oxidative phosphorylation by monitoring the initial rate of ATP formation with luciferase. The medium was as given in Methods and Materials; phosphorylation was started by injection of the indicated concentration of ADP to submitochondiral vesicles respiring with NADH; protein concentration was 17 µg/ml; the rate was determined from the initial slope of the registrations; the method was calibrated with ATP standard in the presence of SMPs; the experimental setup is described in Methods and Materials and in Schäfer (1981); T was 21°C.

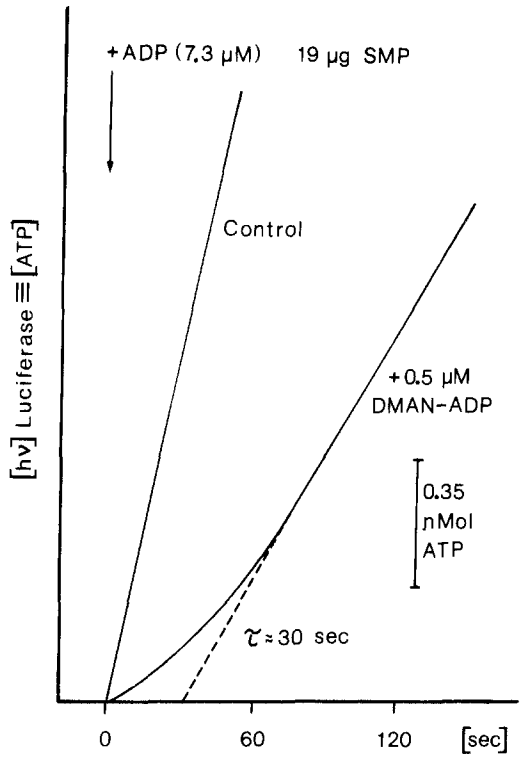

Fig. 2. Generation of a lag time τ of oxidative phosphorylation by preincubation of submitochondrial particles with the indicated concentration of DMAN-ADP for 60 sec prior to addition of ADP. Conditions as described in Fig. 1 and Methods and Materials. The control was run without addition of DMAN-ADP; protein concentration was 19 μg/ml; T was 21°C. The ordinate (light output) is an arbitrary scale; the calibration for ATP is given by the bar.

Table II. Influence of 1-min Preincubation of Respiring Submitochondrial Particles with Various ADP Analogs on the Time Course of ADP Phosphorylation and Generation of a Lag Phase[a]

Compound	c_i (μM)	τ (sec)
ADP 7.5 μM; (control)	—	0
+3′-Naphthoyl(1)-ADP	0.08	16
	0.17	49
+3′-Naphthoyl(1)-2′desoxy-ADP	3.8	15
	12	42
+3′-(5-Dimethylaminonaphthoyl(1))-ADP	0.2	7.5
	0.5	42

[a]Oxidative phosphorylation was initiated by injection of ADP (7.5 μM) to the incubation mixture as described in Methods and Materials; protein concentration was varied from 19–23 μg SMP/ml; T = 21°C. ATP formation was monitored by luciferase as described (Schäfer, 1981).

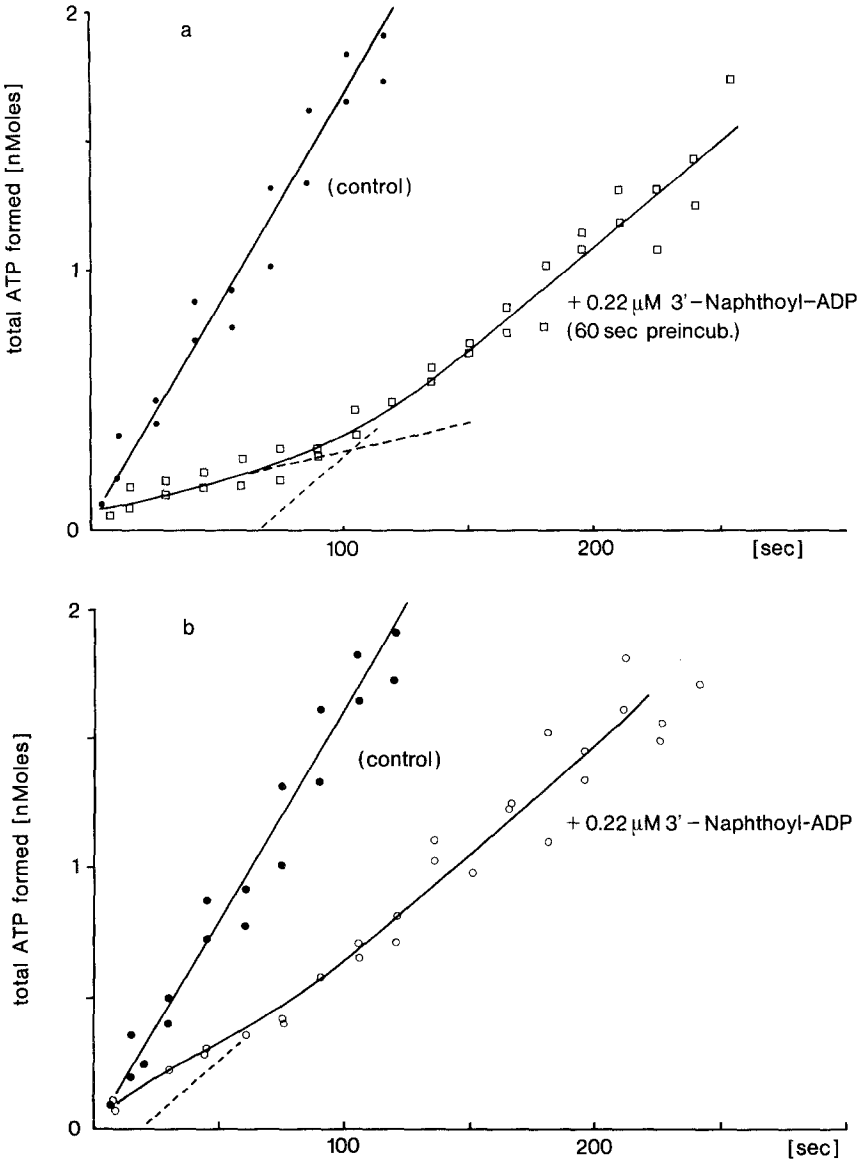

Fig. 3. Influence of sequence of inhibitor addition on development of the lag time τ. The figure gives an overlay of directly monitored ATP synthesis and of data obtained by sampling of batch incubations carried out under the respective conditions. ATP was analyzed in deproteinized aliquots as described in Methods and Materials. Where indicated, 0.22 μM 3'-naphthoyl-ADP was present. In experiment (a) submitochondiral vesicles were preincubated with the inhibitor for 60 sec before addition of ADP (14.6 μM); in experiment (b) the inhibitor and ADP were added simultaneously to the vesicles energized in both cases by respiration with NADH. Final protein concentration was 37 μg/ml.

3′-esters of ADP as given in Fig. 2 and Table II. Interestingly the time lag was absent, however, when the inhibitory analog was added together with or after ADP. This is documented in Fig. 3, giving superpositions of direct recordings with individual determinations of ATP from samples of batch experiments run in parallel. It may be added that it is necessary to verify directly recorded effects by independent methods due to the extreme sensitivity of the luciferase system against unexpected aberrations. In the above case the time lag was also verified by ^{32}P incorporation (not shown).

As to the origin of the time lag, a metabolic conversion of the respective 3′-esters may be excluded since the latter are not being phosphorylated in SMPs. From the fact that 3′-naphthoyl-(2′-desoxy-ADP) also generates the time lag, a 3′ → 2′ acyl migration can also be excluded; thus, a slow removal of the inhibitor by other factors has to be considered.

Properties and Modulation of the Lag Phase. Kinetic analysis of the inhibitor-induced lag time of phosphorylation made obvious that the latter is not due to a simple relaxation of an inhibited state (Schäfer, 1981). Similar to an autocatalytic process, the increase of the reaction rate during the lag phase follows a power function until a maximal rate is established.

Actually, it is assumed that the continuous accumulation of ATP (due to the absence of a hexokinase trap) may be responsible for a gradual release of the inhibition, as supported by the following experimental observations: An increase of protein concentration results in a decrease of the 3′-naphthoyl-ADP-induced lag time (Fig. 4). The time lag becomes shorter with increasing initial concentrations of ADP (Fig. 5). In the figure this is expressed by the ratio of final to initial phosphorylation rate, which assumes a value of 1 when the lag time disappears.

Despite the presence of a fixed concentration of the analog, all of the above conditions are equivalent to a higher capacity of the system for ATP synthesis and therefore to a more rapid availability of ATP as an alternate ligand. Finally the time lag is abolished or gradually diminished by the presence of very small concentrations of ATP added simultaneously with ADP

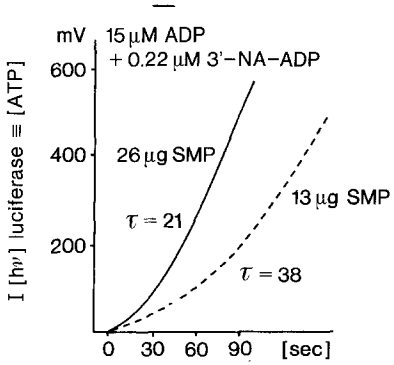

Fig. 4. Influence of protein concentration on the lag time of oxidative phosphorylation induced by preincubation with 3′-naphthoyl-ADP of energized submitochondrial vesicles. Conditions of the incubation and assay are as given in Figs. 1 and 2; specific data as indicated at the traces of the figure.

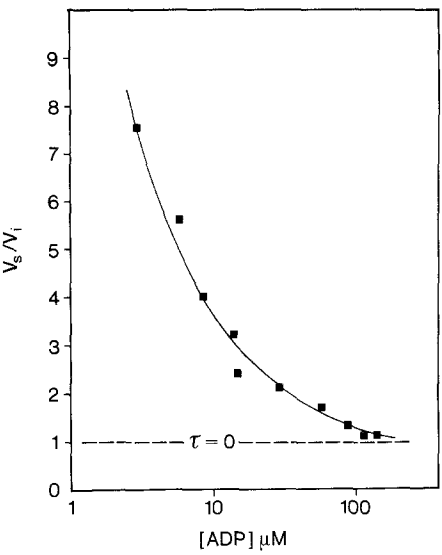

Fig. 5. Effect of initial ADP concentration on the duration of the 3′-naphthoyl-ADP-induced lag time of oxidative phosphorylation. Submitochondrial vesicles respiring with NADH were preincubated for 60 sec with 0.25 μM 3′-naphthoyl-ADP, and phosphorylation was started by addition of the indicated concentrations of ADP (abscissa); the ordinate gives the ratio of the maximal rate of ATP synthesis over the initial rate for each individual ADP concentration. Formation in ATP was directly monitored by luciferase as given in Methods and Materials; other conditions as described in Fig. 2.

to energized submitochondrial vesicles, preincubated for 60 sec with 3′-naphthoyl-ADP; this effect is illustrated in Fig. 6a. In terms of initial concentrations, ATP is obviously more effective than ADP in releasing the inhibition.

Although not shown explicitly, this finding is of importance from the context that to exert this release ATP can be replaced by analogs like 3′-naphthoyl-ATP or by TNP-ATP.

Figure 6b shows, in addition, that the time lag strongly depends on the concentration of the inhibitor. It is not dependent, however, on the period of preincubation, which has been investigated from 30 sec to 10 min.

Differentiation of Functional States

It is generally assumed that membrane-bound F_1-ATPase undergoes a conformational transition when a membrane potential or pH gradient "energizes" the membrane (Cross, 1981). This could not be directly monitored so far, but it may be reflected by the fact that 3′-esters of ADP specifically inhibit oxidative phosphorylation, exerting only weak inhibitory effects on isolated or on uncoupled membrane-bound F_1-ATPase.

2′(3′)-Trinitrophenylated analogs of AD(T)P have been described as strongly inhibiting high-affinity ligands of isolated ATPase (Grubmeyer and Penefsky, 1981). Their effects on oxidative phosphorylation, in contrast, are comparatively weak. This inverse behavior may be depicted from the K_i values of Table III. It may be interpreted to result from preferential interaction of the latter analogs with F_1-ATPase in the nonenergized state. This is strongly

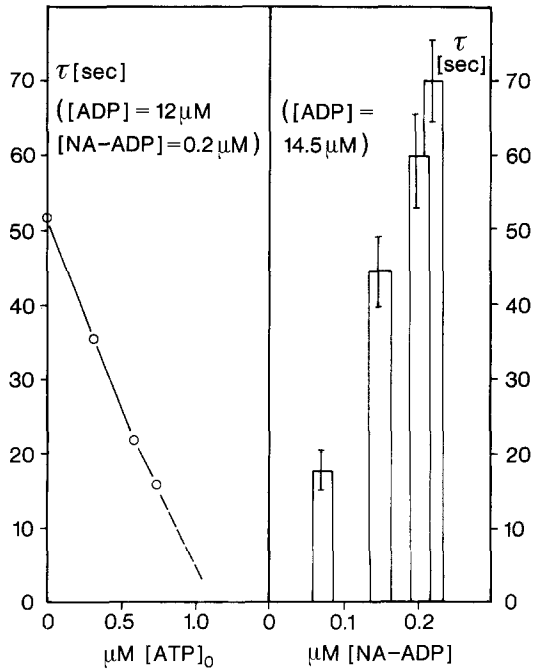

Fig. 6. Effect of ATP concentration and of inhibitor concentration on the duration of the lag time of oxidative phosphorylation. In (a) the indicated concentration of ATP was added together with 12 μM ADP when oxidative phosphorylation was started; the energized submitochondrial vesicles were preincubated for 1 min beforehand with the inhibitory analogs. In (b) the influence of increasing concentrations of 3'-naphthoyl-ADP on the lag time is given. Experimental details as given in Methods and Materials; SMP concentration was 21 μg/ml.

Table III. Comparison of K_i Values of Various AD(T)P Analogs Versus Oxidative Phosphorylation and ATPase, Respectively, Catalyzed by Beef Heart Submitochondrial Vesicles[a]

Compound	K_i (μM)	
	Oxidative phosphorylation	ATPase
3'-Naphthoyl(1)-ADP	0.02	11.7
3'-(5-Dimethylaminonaphthoyl(1))-ADP	0.04	9.8
3'-(5-Dimethylaminonaphthoyl(1))-ATP	0.3	12
3'(2')-(2,4,6-Trinitrophenyl)-ATP	1.4	≤0.02
3'(2')-(2,4,6-Trinitrophenyl)-ADP	1.3	≤0.015

[a]Oxidative phosphorylation was measured via $^{32}P_i$-incorporation as described in Methods and Materials and in Schäfer *et al.* 1980). ATPase activity of submitochondrial particles was determined according to Schäfer *et al.* (1980) in the presence of 0.5 μM uncoupler (FCCP). The K_i values were calculated from K_s and K_s' values on the basis of a competitive inhibition which was verified by the common ordinate intercept of the respective double reciprocal plots.

supported by the dynamics of the inhibition following transition of inner membrane vesicles from the energized to the nonenergized state, as illustrated by the experiments of Figs. 7 and 8.

The effects of the various analogs on ATP synthesis and hydrolysis could conveniently be measured within the same experiment, directly monitoring the ATP level by means of luciferin/luciferase. In all experiments the membrane vesicles were respiring with NADH, thus being in an energized state; phosphorylation was initiated as in the above experiments by injection of ADP. The ATP level reached a plateau when ADP phosphorylation and ATP hydrolysis were in a steady state; this was usually the case at a phosphorylation potential of 10.7–12.4 kcal, depending on the individual preparation. On the plateau, respiration was suddenly interrupted by rapid injection of

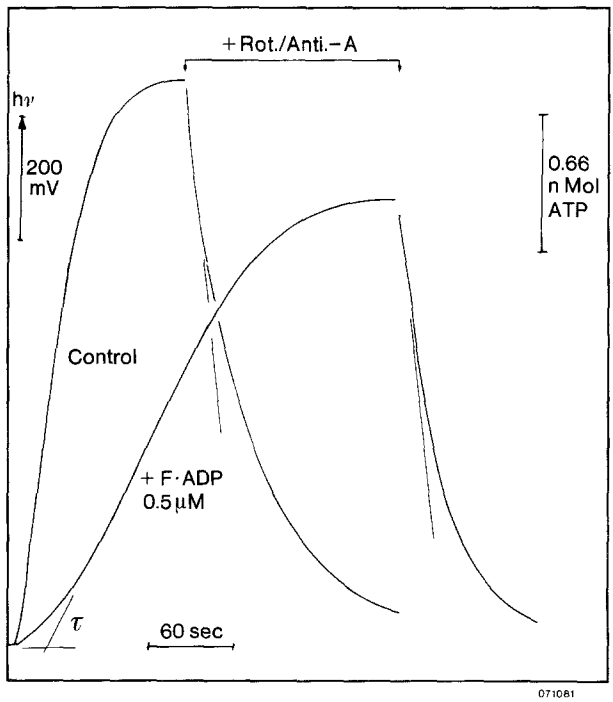

Fig. 7. Comparison of effects of 3′-(5-dimethylaminonaphthoyl(1))-ADP (in the figure termed F · ADP) on ATP synthesis and hydrolysis in submitochondrial vesicles. Conditions are as described in Methods and Materials and Fig. 2; ATP synthesis was initiated by injection of 7.3 μM ADP; where indicated, respiration was stopped by injection of 2.5 μg rotenone and 2.5 μg antimycin A, initiating ATP hydrolysis. Where the inhibitory 3′-esterified ADP analog was present, it was added 60 sec before ADP; protein concentration was 22 μg/ml. Details are described in the text.

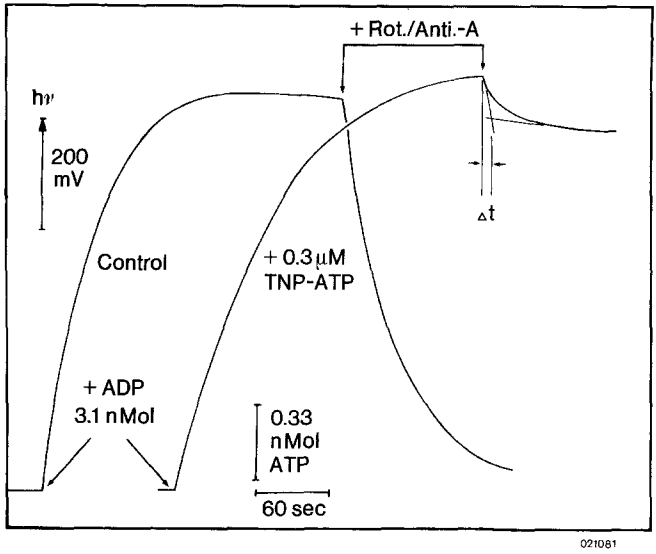

Fig. 8. Comparison of effects of TNP-ATP on ATP synthesis and hydrolysis. The experiments were carried out as given in Fig. 7, except that TNP-ATP was used as the inhibitory analog. Details are given with the traces and in the text.

rotenone/antimycin A, inducing a transition of the membranes to nonenergized conditions. A first-order decay of ATP followed.

An analysis of the events shown in Fig. 7 shows that the 3′-ester 3′-dimethylaminonaphthoyl-ADP (1) induces the described lag phase of oxidative phosphorylation, (2) exerts strong inhibition of ATP synthesis, and (3) on deenergization of the membranes, leaves ATPase activity uninhibited. The rate of ATP decay does not differ from that of the control significantly.

Conversely, an analysis of Fig. 8 shows that, using 3′(2′)-TNP-ATP, (1) no lag phase of oxidative phosphorylation can be observed, (2) the inhibition of ATP synthesis is negligible, and (3) following the "energized" → "nonenergized" transition, a dramatic inhibition of ATP hydrolysis is established. This inhibition, however, needs a transition time Δt to take effect; in the very initial phase a fast rate of ATP hydrolysis occurred, comparable to the control rate.

It is important to note that the transition time was absent when ATP was added in the presence of TNP-ATP to submitochondrial vesicles which were nonenergized and respiration inhibited *"a priori"*; an inhibited rate of ATP hydrolysis was recorded from the very beginning without any delay.

It is conceivable, therefore, that ATPase inhibition by TNP analogs of AD(T)P in fact requires the transition of the enzyme from an energized to a nonenergized form to become fully established.

The duration of the transition time Δt is well within the time range necessary to collapse the membrane potential, as can be proved by the time course of ANS fluorescence change, for example. Furthermore, the duration of the transition time was independent of the concentration of the analog: the latter only determined the degree of inhibition. With TNP-ADP, essentially the same results were obtained, the degree of ATPase inhibition being somewhat lower (cf. Table III).

The postulate that 3'(2')-TNP analogs selectively inhibit the membrane-bound enzyme in the nonenergized form could be directly demonstrated using mixed populations of ATP-synthesizing with ATP-hydrolyzing particles or with solubilized nonenergizable beef-heart F_1-ATPase (Fig. 9). Phosphorylating submitochondrial vesicles represent the enzyme in its "energized" form and maintain a certain ATP level. On addition of a small aliquot of soluble F_1-ATPase, the ATP level declines to a new steady state. Subsequent addition of TNP-ATP selectively inhibits the nonenergized, ATP-hydrolyzing fraction of ATPase, leading to a rapid resaturation of the original ATP level. The

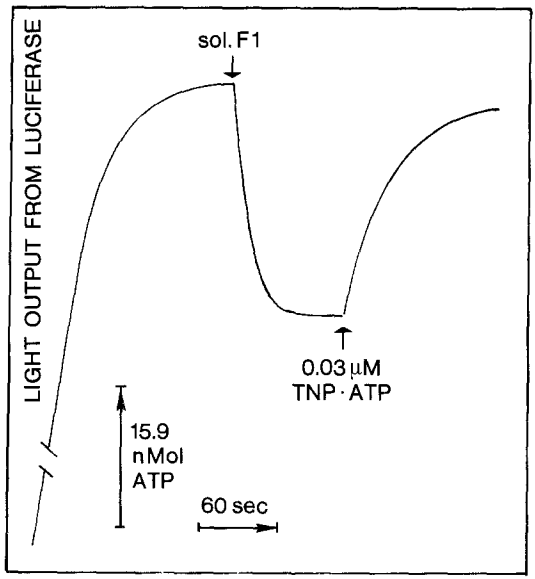

Fig. 9. Effect of soluble F_1-ATPase and of TNP-ATP on the steady-state ATP level of phosphorylating submitochondrial vesicles. The experiment was carried out as given in Fig. 2 with 28 μg SMPs present. Where indicated, 52.8 mU of beef heart F_1-ATPase or 31 nM TNP-ATP as an inhibitor were added. Oxidative phosphorylation was started by injection of 7.3 μM ADP. Further description is given in the text.

energized form of membrane-bound F_1 operating as an ATP-synthetase in the above system is almost insensitive to the inhibitor at the applied concentration. Thus, like 3′-esters, 3′(2′)-TNP analogs may also be considered to represent "conformation-specific" probes, which, however, inhibit the enzyme preferably in its nonenergized form.

Binding Changes of Nucleotides

Functional differentiation of enzymatic states by specific analogs lead to the question whether the selective inhibitory effects are reflected by corresponding binding characteristics. Whereas nucleotide binding can be conveniently studied with various methods on isolated F_1-ATPase, it is almost impossible to determine reliably K_d and, especially, n values with membrane-bound ATPase due to the presence of other nucleotide-binding proteins and to the usual inhomogeneity of vesicle preparations. For instance, the size of the particles would interfere significantly if nucleotides were distributed into the internal space of the vesicles.

With 3′-naphthoyl-AD(T)P, however, these difficulties could be elegantly overcome, using fluorescence depolarization as an indicator for binding of the analog. Its immobilization leads to a high degree of anisotropy if fluorescence is excited by linearly polarized light. In Fig. 10 polarized fluorescence titrations are shown with naphthoyl-ADP as the titrant. Computer-supported fitting of the titrations gave binding parameters which are in excellent agreement with the results from independent methods (K_d = 20–50 nM; n = 2) (Tiedge *et al.*, 1982). As seen from Fig. 10b and c, the method could be applied equally successfully to submitochondrial vesicles, clearly demonstrating that comparable K_d values (20–30 nM) were found. An exact determination of the stoichiometry is not possible in the latter case; based on the assumption that approximately 10–15% of the total membrane protein represents F_1-ATPase (Soper *et al.*, 1979), a value of n between 1.4 and 2 could be estimated. In these experiments interference with the adenine nucleotide carrier appears negligible, because the affinity of the latter to bind 3′-naphthoyl-ADP is more than 50 times lower than with F_1-ATPase.

Thus, it could be demonstrated that the inhibitory analog binds to the enzyme residing on the membrane with the same characteristics as to isolated F_1-ATPase. Attempts to detect an influence of energization on thermodynamic binding parameters has been unsuccessful so far (Fig. 10c). It could be shown, however, that addition of excess ADP or ATP results in a displacement of the bound inhibitory analog (Fig. 11). A very rapid liberation of a fraction of the analog (occurring within the mixing time) is followed by a very slow phase. The half-time of the slow phase is comparable to the lag time of oxidative phosphorylation shown above.

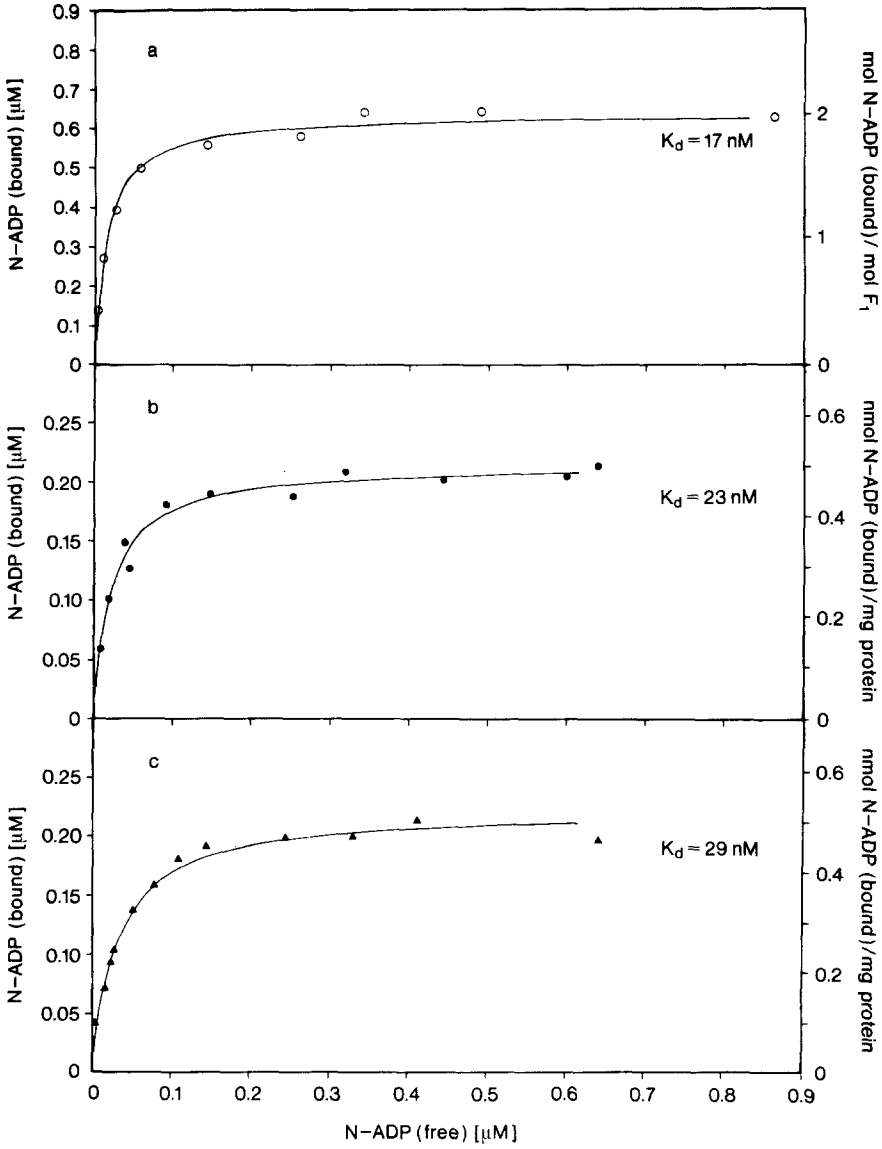

Fig. 10. Measurement of N-ADP binding to soluble and membrane-bound F1-ATPase by fluorescence polarization. (a) Titration of isolated beef heart F1-ATPase (0.32 μM); conditions as described in Methods and Materials. (b) Titration of submitochondrial vesicles (0.42 mg/ml) suspended in the medium used for oxidative phosphorylation measurements, except without an NADH regenerating system and NADH. (c) Titration of submitochondrial vesicles energized by 5 mM succinate; other conditions as in (b).

Discussion

By using modified adenine nucleotides, the present study contributes to the problem of the dynamic structure of mitochondrial ATP-synthetase. The experimental results may be discussed (1) under the aspect of catalytic site cooperativity, and (2) under the aspect of conformational and functional transformations depending on the energy state of the membrane.

Mitochondrial and bacterial F_1-ATPase is generally considered to contain 3α and 3β subunits (Baird and Hammes, 1979; Masasuke *et al.*, 1979; Bragg and Hou, 1975), the latter bearing the catalytic center (Esch and Allison, 1978; Ferguson *et al.*, 1975). Since only one gene is coding for the β-subunit (Senior *et al.*, 1979), one has to assume that these subunits represent functionally identical copies of the catalytic site within the assembled complex. In fact, the existence of at least two catalytic sites on isolated

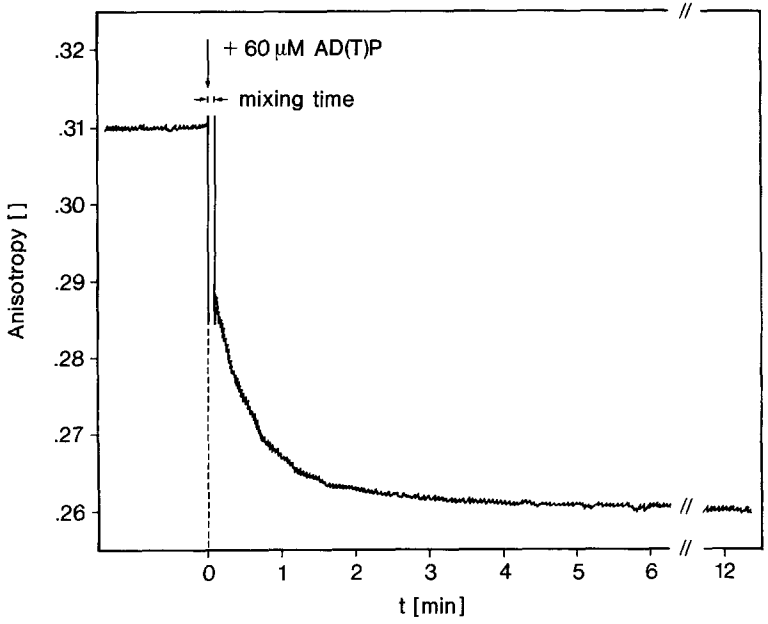

Fig. 11. Time course of displacement of 3'-naphthoyl-ADP from submitochondrial vesicles suspended in a buffer as used for measurements of ATP synthesis, except that NADH and the regenerating system were absent. The particles were preincubated for 3 min with 0.35 μM 3'-napththoyl-ADP. Fluorescence was excited with linearly polarized light at 310 nm, and anisotropy was directly monitored as the ratio of polarized fluorescence intensities perpendicular and parallel to the polarization of the excitation beam. SMP concentration was 0.42 mg/ml. Where indicated, an excess of 60 μM AD(T)P was added; the incubation mixture was rapidly stirred continuously at room temperature.

F_1-ATPase has recently been described (Grubmeyer and Penefsky, 1981). For the process of ATP synthesis a "dual-site" mechanism has been postulated (Kayalar *et al.*, 1977), and more recently this mechanism has been extended to a "triple-site" model (Boyer and Kohlbrenner, 1981), which fits the experimental findings more closely. Despite some evidence for a cyclic multisite mechanism of ATP synthesis, cooperativity of single reaction centers is usually not expressed in simple v vs. $[S]$ plots of oxidative phosphorylation. Instead, the system behaves as if all three reaction centers operate independently in parallel or in sequence. Confirming our previous findings with α-P-acylated ADP (Schäfer and Onur, 1980a), the above results demonstrate that under certain conditions sigmoidal v vs. $[S]$ plots can be observed, which is the case when 3′-*O*-naphthoyl analogs are used as inhibitors. The apparent Hill coefficients did not exceed 2, however, suggesting as a minimum hypothesis that more than one site has to cooperate in order to initiate the catalytic cycle of ATP synthesis. It does not disprove, on the other hand, that all three postulated centers may be involved in an alternating sequence.

Although under the applied conditions interaction of sites appears likely, a straightforward conclusion on the cooperative mechanism is not possible. A simplified interpretation would be that the inhibitory analog has to be displaced by ADP from one site before another site can synthesize or release newly synthesized ATP. Such a mechanism would assume the characteristics of a "slow transition" of the enzyme from an inactivated (inhibited by tightly bound analog) to an active state, especially if the "off rate" of the tightly bound inhibitor is very slow. This is strongly supported by the finding of a time lag of oxidative phosphorylation in the presence of inhibiting 3′-*O*-naphthoyl esters and by the dependence of this effect on the sequence of addition of the single nucleotides to the system. The time lag occurred only when the inhibitory ligand was present before addition of ADP as the substrate of oxidative phosphorylation. If all sites could be occupied by the natural ligands beforehand, it was essentially absent or drastically shortened. In fact, from the exchange studies it became evident that appropriate concentrations of ADP and ATP can displace 3′-*O*-naphthoyl-ADP completely. The time course of the slow phase of this exchange demonstrates that at least at one site the inhibiting analog exhibits a very slow "off rate" and correlates very well with the duration of the observed lag times (although it cannot be excluded that this is an accidental coincidence).

A slow replacement of naphthoyl-ADP from a tight binding site by ADP has also been observed with isolated F_1-ATPase; the half-times of this exchange are within the same range (Tiedge *et al.*, 1982). With regard to the ability of the enzyme to catalyze ADP phosphorylation, they may indicate the proposed slow transition from an inactivated to an activated state in the process of ATP synthesis. It should be noted in this context that the apparent

K_i values of 3′-*O*-naphthoyl-ADP in oxidative phosphorylation are in good agreement with the very low K_d values measured with isolated F_1 and with submitochondrial vesicles.

As has been observed previously, the lag time of ATP synthesis is drastically shortened by ATP (Schäfer, 1981). It may be postulated, therefore, that the inhibitory ligand is displaced from its site on energized submitochondrial vesicles by ATP rather than by ADP. This assumption would not only explain quasi-autocatalytic behavior of the system in the initial state of phosphorylation; it would also imply the necessity of ADP *and* of ATP to be present as ligands simultaneously, in order to maintain a continuous catalytic cycle. Thereby the presence of ATP at one site might facilitate the exchange of ligands at another site (Choate *et al.*, 1977; Hutton and Boyer, 1979). A similar role of ATP as a ligand at one of two sites on the enzyme has been put forward as part of the "dual-site" mechanism (Boyer and Kohlbrenner, 1981). An accelerating effect of ATP on catalytic conversion and on product release has been observed by others using a fluorescent, slowly reacting ATP analog as substrate (Matsuoka *et al.*, 1981). However, using the fluorescence depolarization technique, a preferential displacement of tightly bound 3′-naphthoyl-ADP by ATP could not be experimentally verified so far.

Summarizing the above, the results of the present study greatly support any model of ATP synthesis with interdependent catalytic activity of several sites which may occur in a sequential manner (Boyer and Kohlbrenner, 1981).

By means of the two types of analogs used in this study a differentiation of membrane-bound coupling factor F_1 in its two functional states was achieved. Taking 3′-*O*-dimethylaminonaphthoyl-ADP as an example, the ratio of K_i in ATP hydrolysis and in ATP synthesis, respectively, is 250/1, whereas the corresponding ratio with TNP-ATP was found to be 1/170 to 1/80. Both types of inhibitors act inversely to each other and behave in that sense like "one-way" inhibitors of a reaction. This is thermodynamically impossible as long as it is assumed that the reaction proceeds via the same intermediate states, however. The only reasonable explanation is an alteration of the catalyst's sensitivity to these inhibitors, depending on environmental conditions. Actually, the presence or absence of a membrane potential is the main factor determining whether F_1-ATPase functions as an ATP-synthetase or as an ATPase, respectively. In a simplified manner the two states may be expressed as [E] and [E*], with [E*] symbolizing the enzyme in the energized state of the membrane. Then the conclusion resulting from the above experiments is that TNP analogs preferentially inhibit the enzyme in state [E], which corresponds to the nonenergized conformation. Possibly the complex [E-I] has a higher stability than [E*-I], which has to be verified experimentally, however.

A simplified notation of the catalyzed overall reaction would be

$$ADP + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ATP + H_2O$$

By virtue of steady energy input in respiring submitochondrial vesicles, the catalyst is in state [E*], and $k_1 > k_{-1}$. Under these conditions submitochondrial vesicles are capable of maintaining a steady state of ATP with equal rates of synthesis and hydrolysis when a limiting amount of ADP is added to the system. Withdrawal of energization (blocking respiration) shifts the equilibrium to its normal position with $k_1 < k_{-1}$ ($k_1 = 0$, respectively), leading to a first-order decay of ATP (cf. Figs. 7 and 8); k_{-1} is the rate constant of this decay (at the concentration of SMPs in the above study the value of k_{-1} was 0.075–0.08 sec^{-1}). Simultaneously the enzyme undergoes the transition [E*] → [E]. The presence of TNP analogs does not affect the level of the energized steady state by any means in normally respiring particles. After deenergization, however, a time-dependent decrease of the rate constant k_{-1} to a much lower value occurs; ATPase is gradually switched off. Formation of the inactive complex [E-I] and establishment of the inhibition follows the time course of the transition from [E*] to [E].

In summary, these experiments give further evidence that membrane-bound F_1-ATPase is a "dual-state" enzyme, existing in two distinct states [E] and [E*]. Obviously 3′-naphthoyl esters and 2′(3′)-TNP ethers provide a complementary set of "conformation-specific" probes, allowing one to demonstrate opposite effects on the catalytic turnover in the respective states with a high degree of selectivity. Thus, with respect to inhibitor susceptibility, mitochondrial F_1-ATPase shows some similarities to the adenine nucleotide carrier which in its two possible orientations preferentially interacts with two different types of inhibitors (Klingenberg *et al.*, 1977; Schäfer and Onur, 1980b). Whether the transition of F_1-ATPase between [E] and [E*] is accompanied by binding changes of TNP analogs is presently under investigation.

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