DIFFERENTIATION OF TWO STATES OF F_1-ATPase BY NUCLEOTIDE ANALOGS

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1. Introduction

H^+-ATPase in the membrane-bound state catalyzes the phosphorylation of ADP at the mitochondrial inner membrane when energy is provided by an electrochemical H^+ gradient. In the non-energized or anaerobic state the enzyme catalyzes the hydrolysis of ATP. Many models suggest (reviews [1–3]) that F_1, the catalytic part of H^+-ATPase may assume different conformational states, depending on energization of the membrane by a membrane potential. In a simplified manner the 2 states of the enzyme may be expressed as E and E^*, with E^* symbolizing the enzyme in the energized membrane state. These states were postulated to differ not only functionally, but also with regard to their affinities to various ligands as ADP, ATP or analogs [4]. So far, this could not be demonstrated directly for mitochondrial F_1.

This study provides direct evidence that F_1-ATPase is a 'two state' enzyme, and that either 1 of the 2 states can be specifically inhibited by certain 3′-substituted nucleotide analogs. This conclusion is based on the inverse behaviour of the analogs with ATP-synthesizing *vs* ATP-hydrolyzing inner membrane vesicles of beef heart mitochondria (SMPs). The analogs used here are 3′-*O*-(naphthoyl-1)-AD(T)P, 3′-*O*-(5-dimethylaminonaphthoyl-1)-AD(T)P and TNP · AD(T)P.

2. Methods

Oxidative phosphorylation of ADP by submitochondrial vesicles has been measured by ^{32}P-incorporation into ATP, or by direct monitoring of ATP-synthesis with luciferase/luciferin. The methods used are exactly as in [5–7] with two exceptions:

(1) Sonication of beef heart mitochondria for preparation of submitochondrial vesicles (SMPs) was done at pH 6.7 for 10 × 1 s. These preparations had high respiratory control (state 4 release by uncoupler) up to 5.3 and phosphorylation rates up to 350 nmol . min^{-1} . mg^{-1}.

(2) The luciferase assay medium [7] was supplemented with 0.3 mM 5′-AMP which stabilizes the light output also at higher [ATP]. ATPase activity of membrane-bound or isolated F_1 was measured by a coupled NADH-linked assay as in [6]. F_1-ATPase from beef heart was prepared essentially as in [8].

3′-Esters of adenine nucleotides were synthesized as in [5,6]; 2′(3′)-trinitrophenyl analogs were synthesized as in [9]; a first sample of TNP–ATP was a gift from M. Klingenberg (Munich). All chemicals and auxiliary enzymes were purchased from Merck, Boehringer and Sigma, respectively. ^{32}P (carrier-free) was obtained from Amersham-Buchler (Braunschweig).

3. Results

The strong inhibitory activity of 3′-esters like 3′-naphthoyl(1)-ADP or F · ADP has been described in [5]. The newly applied analogs, TNP · ADP or TNP · ATP, have been used to establish the presence of >1 catalytic site on isolated F_1-ATPase [10] to which they exhibit extremely high affinity; in contrast to the 3′-esters they contain a 3′(2′)-ether bond [9]. Nevertheless, because CPK models of these mole-

Abbreviations: F · ADP, 3′-*O*-(5-dimethylaminonaphthoyl-1)-ADP; TNP · AD(T)P, 3′(2′)-*O*-(2,4,6-trinitrophenyl)-AD(T)P; SMP, submitochondrial vesicles

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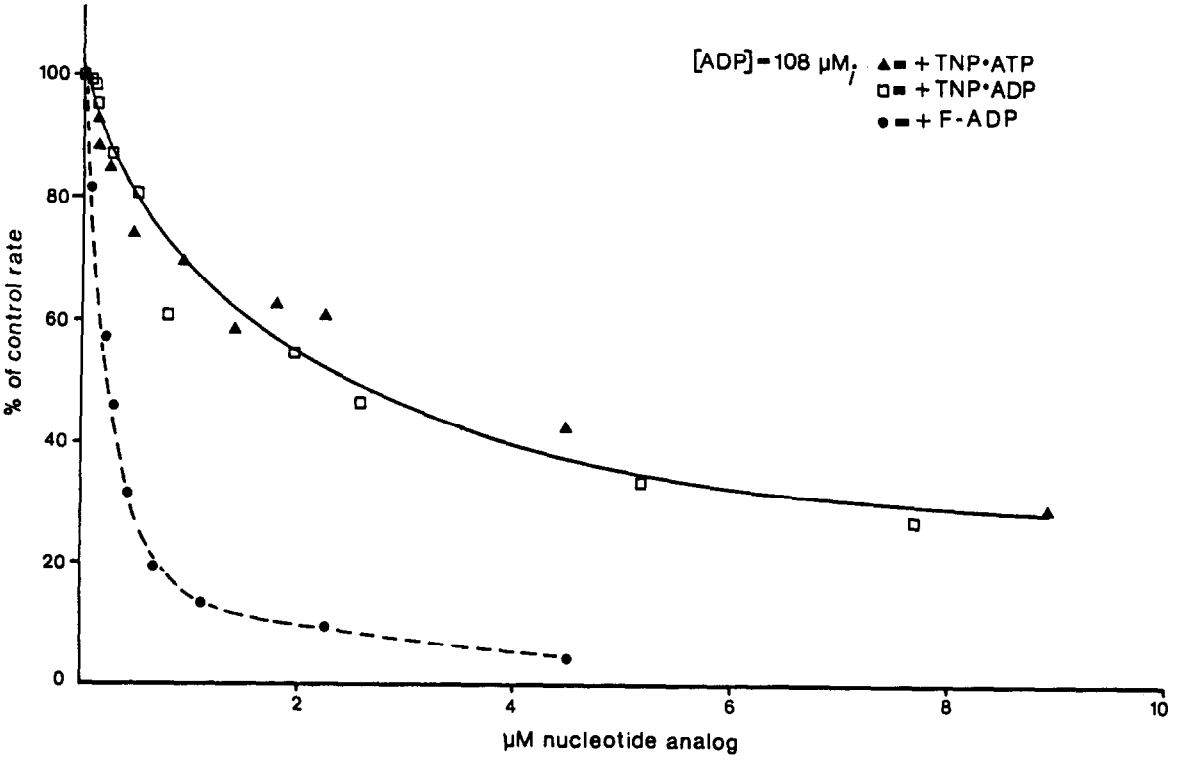

Fig.1. Inhibition of oxidative phosphorylation by TNP-analogs of ADP and ATP. ^{32}P-incorporation was measured in a medium containing: 220 mM sucrose, 10 mM Tris, 2.5 mM $MgCl_2$, 4.0 mM potassium phosphate, 1 mM EDTA; pH 7.4, 30°C. The medium was supplemented with 93 U/ml of hexokinase and 16 mM glucose as an ATP-trap. SMP was 0.21 mg/ml; 100% represents a rate of 316 nmol . mg^{-1} . min^{-1} at 108 μM ADP. [^{32}P]ATP was separated from $^{32}P_i$ as in [5,6]. To stress the difference to 3′-esters a titration with F · ADP has been included for comparison (●).

cules suggest some structural similarity to 3′(2′)-esters, it has been expected that they share the properties of the latter with regard to oxidative phosphorylation. Fig.1 shows that TNP-analogs inhibit in a concentration-dependent manner, achieving 50% inhibition at 2.4–3 μM of the analogs in presence of 108 μM ADP. However, there is no significant difference of inhibitory potency between TNP · ADP and TNP · ATP. This is in contrast to 3′-esters, of which only the ADP-analogs are very strong inhibitors ($K_i \leq 10^{-7}$ M) whereas the ATP-analogs are ≥100-times less effective [3,4].

Two other remarkable differences of TNP-analogs compared to 3′-esters could be observed:

(i) Their relative inhibitory effectiveness against ATP-synthesis and ATP-hydrolysis: TNP-analogs show a reciprocal activity as compared to 3′-esters. This can be measured simultaneously within the same sample of SMPs by direct monitoring of the process of ATP-synthesis and -hydrolysis by means of a luciferin/luciferase assay system (fig.2). ATP-formation by oxidative phosphorylation is initiated by injection of a small aliquot of ADP to respiring SMPs. Under these conditions the enzyme is in the energized state E*. When the ATP-level reaches a plateau, respiration is suddenly interrupted by injection of the respiratory inhibitors rotenone/antimycin A. This induces a rapid transition from the energized state E* to the non-energized state E of membrane-bound F_1-ATPase, accompanied by a first-order decay of ATP. The initial slopes of the traces (up or down) give the rates of ADP-synthesis or -hydrolysis, respectively.

The above analogs yield the results summarized in table 1. The 3′-ester F · ADP has only a negligible effect on the ATPase function of the particles whereas oxidative phosphorylation is drastically inhibited. In contrast, comparable concentrations of the 3′(2′)-

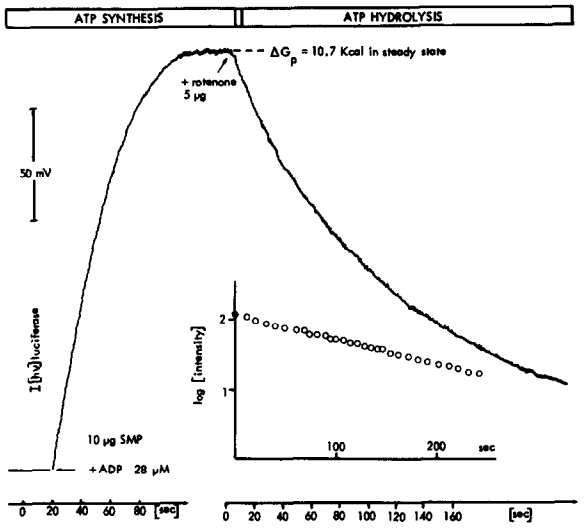

Fig.2. Continuous registration of ATP-synthesis and -hydrolysis by submitochondrial particles, using a luciferase assay. Dependent on the SMP preparation the phosphorylation potentials achieved in the steady state varied from 10.5–12.4 kcal. The incubation medium was as given in fig.1, supplemented with luciferin/luciferase as in [7]; pH 7.4, 22°C.

TNP-analogs exert only a moderate inhibition on oxidative phosphorylation in energized SMPs, while the ATPase function of non-energized particles is almost completely abolished. The type of inhibition is essentially competitive. A precise determination of K_i-values is given in table 2. In these experiments oxidative phosphorylation was measured by $^{32}P_i$ into ATP; ATPase activity of non-energized SMPs was determined by a coupled NADH-dependent assay as in [6]. These data agree well with the observations in table 1, as clearly expressed by the inverse ratios of the K_i-values for both types of nucleotide analogs.

Table 1
Differential inhibition of oxidative phosphorylation in SMPs by 3'-analogs of adenine nucleotides

Conditions	Ox. phos. rel. rate	% inhibn.	ATPase rel. rate	% inhibn.
Control	1	–	1	–
+ F · ADP 0.5 µM	0.265	73.5	0.96	4
+ TNP · ADP 0.4 µM	0.61	39	0.12	88
+ TNP · ATP 0.3 µM	0.63	36	0.02	98

The data were derived from direct registrations as in fig.2. Initial ADP was 7.5 µM; protein was 25 µg/ml

Table 2
Comparison of K_i-values for 3'-analogs of AD(T)P in oxidative phosphorylation and ATP-hydrolysis by beef heart SMPs

Compound	K_i-values (µM)		
	ATPase	Ox. phos.	Ratio
3'-NA · ADP[a]	11.7	0.02	587/1
3'-F · ADP	9.8	0.04	245/1
3'(2')-TNP · ADP	0.010–0.015	1.3	1/128–1/81
3'(2')-TNP · ATP	0.008–0.020	1.46	1/169–1/72

[a] NA · ADP denotes 3'-*O*-naphthoyl-ADP

Oxidative phosphorylation was measured by $^{32}P_i$-incorporation as in [5] and fig.1; ATPase was determined in a coupled NADH-linked assay as in [6]

(ii) Initial kinetics of ATP-hydrolysis of F_1-ATPase after transition from the energized to the non-energized state $E^* \rightarrow E$ of inner membrane vesicles. Fig.3 summarizes the results obtained with the monitoring system in fig.2. The various analogs were present from the beginning of each experiment. For simplicity, most traces are shown only for the time following de-energization of the membranes by sudden respiratory inhibition. A comparison of trace (a), a control experiment, with trace (b) shows that in presence of 0.5 µM F · ADP the rate of ATP-hydrolysis remains practically unaffected. When TNP · ADP (trace c) or TNP · ATP (trace d) were present, the initial rate of ATP-hydrolysis again was similar to the control. However, after a few seconds (Δt) the ATPase-activity was drastically diminished, resulting in a strong inhibition which was even more pronounced with the ATP-analog than with the ADP-analog. Thus, a transition time is necessary to establish full ATPase-inhibition by the TNP-analogs after de-energization of the membranes. It is important to note that this lag-time only occurs, when the inhibitory TNP-analogs were present already during the energized (respiring) state of the submitochondrial vesicles. When ATP was added to 'a priori' non-energized and respiratory inhibited membranes in presence of TNP · ATP, the inhibited state of ATP-hydrolysis was recorded from the very beginning without any lag-time (trace e). It is evident therefore

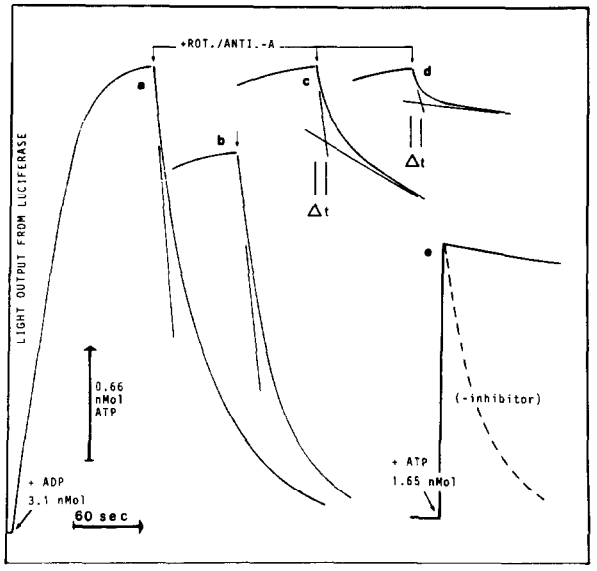

Fig.3. Effects of F · ADP, TNP · ADP and TNP · ATP on initial kinetics of F_1-ATPase of beef heart SMPs. The ATP-level was continuously monitored as given in [7] and fig.2. In experiments (a–d) 22 μg SMPs/ml were mixed with 7.3 μM ADP to start oxidative phosphorylation. When [ATP] reached a plateau, respiration and phosphorylation were stopped by injection of 2.5 μg rotenone and 2.5 μg antimycin A, initializing ATP-hydrolysis by F_1-ATPase. (a) Control; (b) with 0.5 μM F · ADP; (c) with 0.4 μM TNP · ADP; (d) with 0.3 μM TNP · ATP present. In (e) ATP-hydrolysis was induced by injection of ATP to non-energized SMPs in presence of 0.3 μM TNP · ATP to illustrate immediate onset of inhibition; (– – –) control without inhibitor.

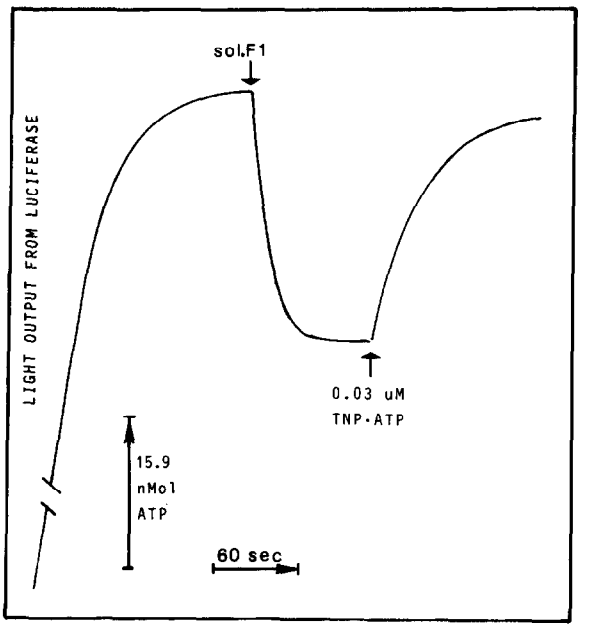

Fig.4. Effect of soluble F_1-ATPase and of TNP · ATP on the steady state ATP-level of phosphorylating SMPs. Oxidative phosphorylation was initialized and monitored as in fig.2. Where indicated 52.8 mU soluble beef heart F_1-ATPase, or TNP · ATP as an inhibitor, were added. Further description as given in the text.

that ATPase inhibition by TNP-analogs of AD(T)P requires a transition of the membrane from the energized to the non-energized state which occurs within the time interval Δt (fig.3). Direct proof of this postulate is given by the experiment of fig.4, using solubilized F_1-ATPase derived from beef heart submitochondrial particle membranes [8]. This enzyme is the same as that residing on the SMP-membrane; however, it can no longer undergo a transition from the non-energized to the energized state. When this soluble F_1-ATPase is added to respiring, ADP-phosphorylating SMPs, the ATP-concentration declines to a lower steady state, because the system is now composed of a fraction of ATP-synthesizing membrane-bound F_1-ATPase in the energized state E^*, and a fraction of non-energizable, ATP-hydrolyzing F_1-ATPase in state E. Addition of TNP · ATP leads to a rapid restoration of the original ATP-level; this can result only from a selective inhibition of the ATP-hydrolyzing, non-energized fraction of F_1-ATPase, while the ATP-synthesizing, energized fraction remains practically unaffected.

4. Discussion

It has been concluded from the preferential inhibition of ATP-synthesis by certain 3′-esters of ADP that these analogs represent 'conformation-specific ligands' [11,12], strongly inhibiting a form of F_1-ATPase characteristic for the 'energized' state of this enzyme; the effect on ATP-hydrolysis was considerably smaller (table 1,2).

In contrast, 3′(2′)-TNP-analogs of ADP and ATP are comparatively weak inhibitors of ATP-synthesis, but strongly inhibit ATP-hydrolysis by non-energized submitochondrial vesicles or solubilized F_1-ATPase [10]. Because presence or absence of a membrane potential is the main conditional difference for F_1-ATPase to function as an ATP-synthetase or as an ATPase, the conclusion is justified that TNP-analogs

preferentially inhibit the enzyme in its non-energized ATP-hydrolyzing conformation. Evidently, the two types of AD(T)P-analogs (3′-esters and 3′(2′)-TNP-ethers) provide a complementary set of 'conformation-specific' probes, allowing to demonstrate differential interactions with mitochondrial F_1-ATPase in its energized (E^*) and its non-energized (E) form. Thus, with respect to inhibitor function, F_1-ATPase is a 'two state' enzyme and reveals certain analogies to the adenine-nucleotide carrier of the mitochondrial membrane [13,14]; the latter also can exist in 2 distinct states. One of these (oriented to the cytosolic membrane side) is specifically inhibited by atractylate, the other (oriented to the mitochondrial matrix side) by bongkrekate or by F · ADP [14,15].

F_1-ATPase exhibits multiple nucleotide binding sites [1,2,4]. It remains to be established whether or not the E and E^* transition is reflected by accompanying binding changes of the 2 types of inhibitory analogs.

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References

[1] Senior, A. E. (1979) in: Membrane Proteins in Energy Transduction (Capaldi, R. ed) pp. 233–278, Marcel Dekker, New York.
[2] Cross, R. L. (1981) Annu. Rev. Biochem. 50, 681–714.
[3] Boyer, P. D. (1977) Annu. Rev. Biochem. 46, 957–966.
[4] Schäfer, G. (1978) in: Energy Conservation in Biological Membranes, (Schäfer, G. and Klingenberg, M. eds) pp. 243–246, Springer, Berlin, New York.
[5] Schäfer, G. and Onur, G. (1978) Eur. J. Biochem. 97, 415–424.
[6] Schäfer, G., Onur, G. and Schlegel, M. (1980) J. Bioenerg. Biomembr. 12, 213–232.
[7] Schäfer, G. (1981) FEBS Lett. 131, 45–50.
[8] Penin, F., Godinot, C. and Gautheron, D. C. (1979) Biochim. Biophys. Acta 548, 63–71.
[9] Hiratsuka, T. and Uchida, K. (1973) Biochim. Biophys. Acta 320, 635–647.
[10] Grubmeyer, Ch. and Penefsky, H. S. (1981) J. Biol. Chem. 256, 3718–3727.
[11] Schäfer, G. (1978) in: Frontiers of Biological Energetics (Dutton, P. L. et al. eds) pp. 484–493, Academic Press, New York.
[12] Schäfer, G., Onur, G. and Strotman, H. (1978) in: Energy Conservation in Biological Membranes (Schäfer, G. and Klingenberg, M. eds) pp. 220–227, Springer, Berlin, New York.
[13] Buchanan, B., Eiermann, W., Riccio, P., Aquila, H. and Klingenberg, M. (1976) Proc. Natl. Acad. Sci. USA 73, 2280–2284.
[14] Klingenberg, M., Aquila, H., Krämer, R., Babel, W. and Feckel, J. (1977) in: Biochemistry of Membrane Transport (Semenza, G. and Carafoli, E. eds) pp. 567–579, Springer, Berlin, New York.
[15] Schäfer, G. and Onur, G. (1980) FEBS Lett. 109, 197–201.