UNMASKING COOPERATIVITY OF OXIDATIVE PHOSPHORYLATION BY A NEW α-PHOSPHATE ACYLATED ADP-ANALOG

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1. Introduction

Aromatic 3′-esters of ADP are surprisingly strong inhibitors of oxidative and photophosphorylation, whereas their inhibitory activity on uncoupled membrane-bound or isolated F_1-ATPase is ⩾200-times lower [1,2]. Therefore they have been tentatively assigned conformation-specific probes of the catalytic site, assuming that only in 'energized' membrane-bound coupling factors the catalytic site is 'open' for these analogs [1,3]. Accidently it was observed, that synthesis of 3′-esters can be directed to yield larger amounts of side-product acylated at the α-phosphate of ADP instead of acylation of ribose. This is shown here for the fluorescent derivatives 3′-*O*-(5-dimethylamino-naphthoyl-1)-ADP and the respective α-P-acyl-derivative. Unexpectedly, also the α-P-acylated ADP-analog strongly inhibits oxidative phosphorylation, and moreover unmasks cooperativity of this fundamental process of biological energy conversion. Results of kinetic studies suggest, that the mechanism involves two interdependent nucleotide binding sites.

2. Methods and materials

Beef heart submitochondrial particles were prepared as in [1] and oxidative phosphorylation was followed via ^{32}P-incorporation into ATP using a hexokinase trap [1,2]. The buffer contained 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 hexokinase in 0.6 ml total vol.; protein was 0.2–0.3 mg/ml submitochondrial particles. ATPase was measured in a coupled assay employing an ATP-regenerating system [4]. Fluorescence measurements were done in a Schoeffel RS-1000 spectrofluorometer. ^1H NMR spectra were recorded in a Brucker-WH 270 spectrometer, ^{31}P NMR in a Varian XL 100-spectrometer. Synthesis of 3′-*O*-(5-dimethylaminonaphthoyl-1)-ADP (I) was as in [1,5]; ~20% of the α-P-acylated analog (II) were obtained when the reaction was carried out in dioxane/water (1:1) for 12 h with an initial ratio of ADP to the imidazolide of 5-dimethylamino-1-naphtoic acid of 2:1. It was purified by thin-layer chromatography on cellulose (Merck 5716) in *n*-butanol/*i*-propanol/water (1:2:1). The structure was determined by IR-, UV-, ^1H NMR- and ^{31}P NMR-spectroscopy and by phosphate analysis.

Abbreviations: I, 3′-*O*-[5-dimethylaminonaphthoyl-1]-ADP; II, α-P-[5-dimethylaminonaphthoyl-1]-ADP

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3. Results

Fig.1 shows the structure of (II) together with ^{31}P NMR spectra of regular ADP (a), and of (II), demonstrating the dramatic downfield shift of the α-phosphate in the new analog (b). ^1H NMR spectra of (II) show chemical shifts, not deviating from those of normal ADP (H_2, H_8, H_1', H_2', H_3').

The new compound (II) shows similar fluorescence behaviour to the isomeric 3′-ester ($\lambda^{ex.}_{max}$ = 358 nm, $\lambda^{em.}_{max}$ = 512 nm). However, the quantum yield in dioxane is lower (~0.1, versus 0.28, respectively). Fluorescence of both analogs is highly sensitive to polarity with negligible emission in aqueous solution.

Also in biological systems both compounds exhibit similar properties. Like the 3′-ester (I), the α-phosphate

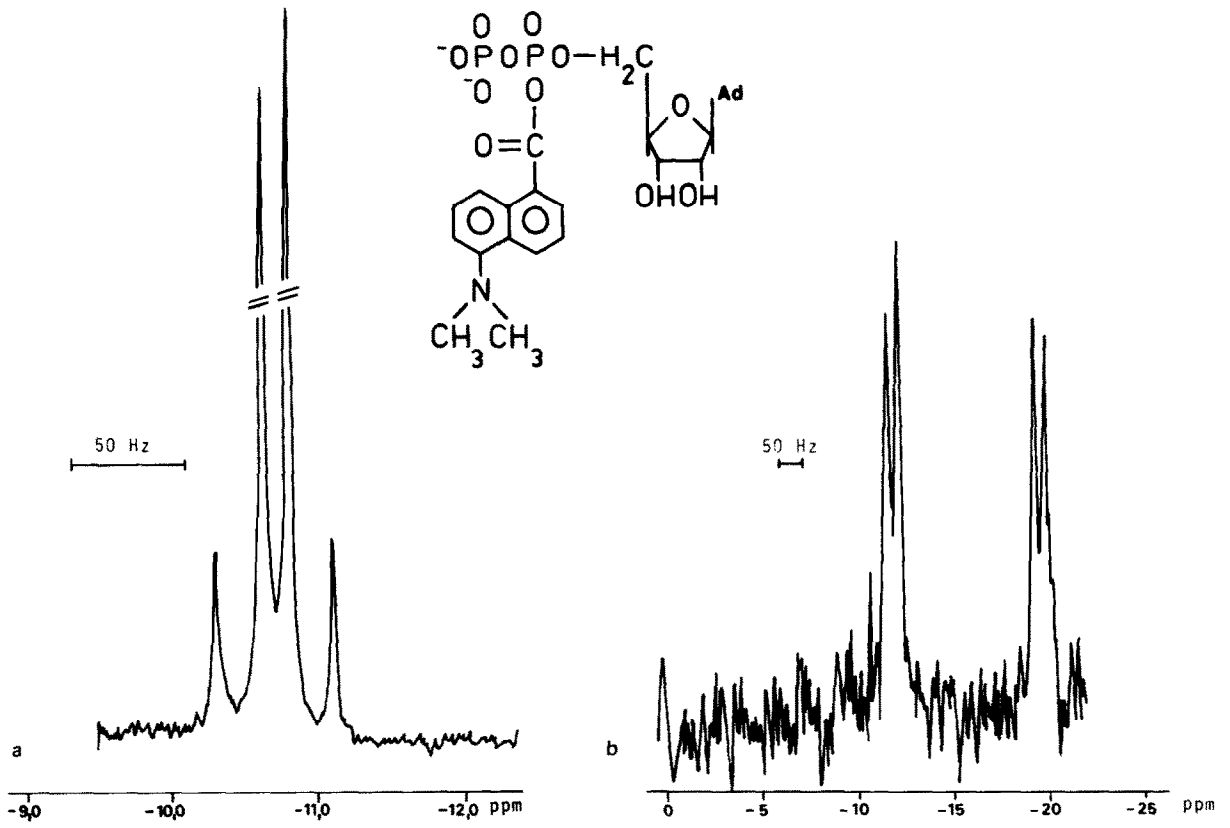

Fig.1. ^{31}P NMR spectra of ADP and of (II). (a) 60 mM ADP in D_2O (pH 6.5); 100 scans, 0.1 Hz digital resolution; chemical shifts of α- and β-phosphates are −11.0 and −10.4 ppm, respectively (J_{pp} = 20.1 Hz). (b) 5 mM (II) in D_2O (pH 6.5); 3200 scans, 0.5 Hz digital resolution; chemical shifts of α- and β-phosphates are −19.6 and −11.8 ppm (J_{pp} = 22.5 Hz). Proton-decoupled ^{31}P NMR spectra were recorded at 37°C; chemical shifts were determined from 85% H_3PO_4 as external standard.

acylated analog (II) binds to the membranes of submitochondrial particles with increased fluorescence. Titrations show saturation kinetics with an app. K_d = 21.1 μM. Fluorescence increase of (I) and (II) is immediately quenched by addition of bongkrekic acid, indicating that they bind to the same site on the carrier; this site is exposed to the inner surface of the mitochondrial inner membrane [5]. (II) has no effect on ADP-stimulated respiration of intact mitochondria, making it clear that the analog does not inhibit when applied from the outside of the mitochondrial inner membrane; this implies also that the analog is not translocated itself into the mitochondria.

When oxidative phosphorylation in submitochondrial particles is titrated by (II), half maximal inhibition at saturating ADP-levels is obtained at 4.3 μM (fig.2). Table 1 compares effects of (I) and (II) with regard to oxidative phosphorylation, ATPase inhibition and fluorescence titrations of submitochondrial particles. The phosphoacyl-compound generally acts like the isomeric 3′-ester of ADP and like (I), it is not phosphorylated itself. Thus, although the affinity to the ATP-synthesizing enzyme is lower, it resembles the properties of 3′-esters.

The most interesting result, however, is derived from kinetics of ADP-phosphorylation. Reciprocal plots of phosphorylation rates versus ADP as variable substrate exhibit a common ordinate intercept with and without this inhibitory analog, suggesting a competitive type of inhibition; from those plots the K_i values of table 1 were derived. The plots, however, deviate from linearity in a way typical for positive cooperativity in presence of (II).

Fig.3 gives plots of v vs [S] for oxidative phosphorylation in presence of different concentrations of the analog. Obviously, kinetics of mitochondrial ATP-

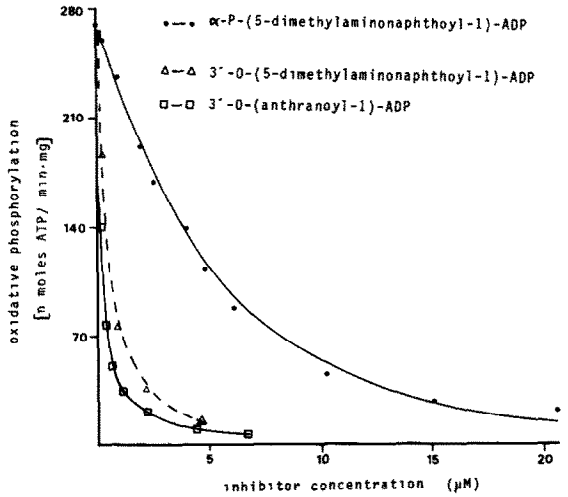

Fig.2. Inhibitor titration of oxidative phosphorylation in beef heart submitochondrial particles. ADP-phosphorylation was measured at 30°C under standard conditions as in section 2. Each point is an average of 2–4 single measurements.

synthesis become sigmoidal under these conditions. Whereas with increasing inhibitor concentration the $K_{0.5}$ is successively increased, the Hill-coefficient always assumes values of 1.4–1.7, suggesting that at least 2 interdependent ADP-binding sites are involved in the catalytic mechanism.

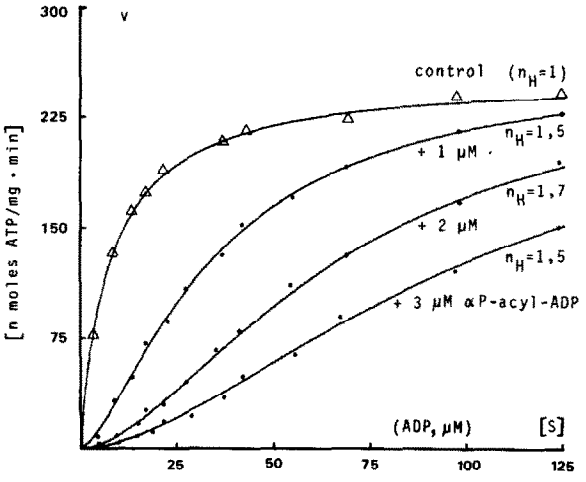

Fig.3. v vs [S] plots of oxidative phosphorylation; influence of (II). The curves were drawn by computer, using the parameters n_H and $K_{0.5}$ determined from Hill-plots of the same data. In the control experiment (II) was omitted; all data points are the average of 2–4 single determinations. Oxidative phosphorylation was measured at 30°C under standard conditions as in section 2.

Table 1
Comparison of inhibitory and binding constants of (I) and the α-P-acylated analog (II) with beef heart submitochondrial particles

Compound	Oxidative phosphorylation K_i (μM)	ATPase K_i (μM)	Fluorescence K_d (μM)
(I)	0.04	9.8	7.3
(II)	0.26	≥450	21.1

K_i-values of oxidative phosphorylation were determined in experiments as in fig.3. Membrane-bound ATPase of submitochondrial particles was measured as in section 2; these data are taken from [2]. K_d refers to fluorescence titrations [5] of submitochondrial particles (excitation 340 nm; emission at the respective maximum wavelengths of the analogs)

Careful reinvestigation of the kinetics with the isomeric 3′-ester (I) or with 3′-(naphthoyl-1)-ADP made it clear that with these analogs sigmoidal curves could be verified also; this effect was clearly established for the first time, however, with (II). Table 2 shows comparative data, regarding inhibitory efficiency and the degree of cooperativity.

4. Discussion

It is surprising that an ADP-analog bearing such a bulky substituent at the α-phosphate group is accepted by the enzyme, acting as an inhibitor with remarkably high affinity. It seems unlikely, therefore that the α-phosphate plays a predominant role in specific binding of the nucleotide as might be concluded from results obtained with α-S-analogs of ADP in chloroplasts [6]. Space-filling CPK-models show that the new α-P-acylated analog (II) in fact can assume a conformation which is farily similar to that of (I),

Table 2
Comparative kinetic data of several modified adenine nucleotides in oxidative phosphorylation

Compound	K_i (μM)	$\bar{n}_H$	R_S
(I)	0.04–0.08	1.35	25.9
(II)	2.26	1.65	14.3
3′-*O*-naphthoyl-ADP	0.02	1.45	20.7
ADP	–	1	80

The data have been determined from experiments as in fig.3. Oxidative phosphorylation was measured at 30°C under standard conditions (see section 2)

regarding the position of the naphthoyl-ring relative to the phosphate chain and the ribose moiety. This similarity supports our view [2,3], that screening of the phosphate chain against attack of activated P_i is an essential event in the inhibitory mechanism.

The phosphate-acylated analog is practically inactive against uncoupled F_1-ATPase, making it another analog behaving like a 'one-way' inhibitor of energy conservation. This strongly suggests that only in energized particles can the catalytic center interact with the modified nucleotide.

Whereas several conditions have been described for F_1-ATPase to induce positive or negative cooperativity [7–10], this is the first report to show this on oxidative phosphorylation. Hill-coefficients of 1–2 indicate that in the functional unit 2 types of interactive nucleotide binding sites are operating. This is in line with findings on isolated F_1-ATPase from yeast [10], as well as with the function of 'flip–flop' mechanisms or of a 'dual-site' model, based on a binding-change mechanism [11].

Assuming 2 nucleotide binding sites to participate in the catalytic mechanism, a simplified model would suggest that the inhibitory analog has to be displaced by ADP from one site before the other site can synthesize and release ATP. In absence of the inhibiting analog and with ADP being the only nucleotide offered, all sites would be occupied by ADP as the correct ligand ab initio, and non-linear kinetics therefore cannot be detected.

We cannot distinguish yet whether the new nucleotide analog interacts with the so-called 'tight' binding sites assumed to exert control functions (reviewed [12]).

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