ARTEFACTS IN THE ESTIMATION OF ADP ANALOGS AS PHOSPHATE ACCEPTORS IN MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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1. Introduction

It is generally accepted that the high specificity of intact mitochondria for ADP as phosphate acceptor in oxidative phosphorylation is mainly due to the transport system located in the inner membrane; the enzymatic machinery itself, responsible for 'oragification' of P_i, is able to use many other naturally occurring or chemically synthesized nucleoside diphosphates as acceptors [1–6]. However, we have shown that o^1ADP and ϵADP are not substrates for the oxidative phosphorylation catalyzed by rat liver mitochondrial membranes, in spite of the fact that they can replace ADP in various transfer reactions of the phosphoryl group [7–9]. As the specificity of the energy-generating or energy-consuming mitochondrial processes for adenine nucleotides is connected to the mechanism of oxidative phosphorylation [2,10] it is important to establish whether the existing discrepancies reflect some structural peculiarities of nucleotide analogs or are just artefacts.

2. Materials and methods

2.1. Chemicals

All purine nucleotides (except dADP), nicotinamide adenine nucleotides, substrates and coupling

Abbreviations: o^1ADP, o^1ATP, adenosine N^1-oxide di- and triphosphate; ϵADP, ϵATP, 1,N^6-ethenoadenosine di- and triphosphate; TuDP, TuTP, tubercidine di- and triphosphate; AMP-P(NH)P, adenylyl(β,γ-imido)-diphosphate; Ap_5A, P^1,P^5-di(adenosine 5')-pentaphosphate; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconic acid

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enzymes (a generous gift from Professor F. H. Schmidt) were commercial products of Boehringer (Mannheim). Tubercidin and dADP were products of Sigma Chem. Co. (St Louis, MO). $^{32}P_i$ was obtained from the Institute of Physics and Nuclear Engineering, Bucharest. Nucleotide analogs, o^1ADP, ϵADP and TuDP were synthesized as in [11–13]. Commercial samples of dATP, dADP, GDP, GTP and AMP-P(NH)P were purified by chromatography on DEAE–Sephadex A-25 [14].

2.2. Biological preparations and analytical methods

Rat liver [15], beef heart [16] mitochondria, sonicated submitochondrial particles [16,17] were prepared by the procedures cited. Measurement of respiration and oxidative phosphorylation, determination of individual enzyme activity and the chromatographic separation of nucleotides were described in [7,12,14,18]. Experimental details are given in the legends to figures and tables.

3. Results

3.1. Identification and assay of traces of adenine nucleotides in samples of their analogs

The presence of adenine nucleotides as 'contaminants' of the commercial guanine and inosine nucleotides has long been noticed as a potential source of error. In general, detection of contaminants amounting to <1% poses serious difficulties. Looking at the nucleotide specificity of a large number of phosphotransferase, it appears that the highest specificity for adenine nucleotides is shown by yeast hexokinase and muscle creatine kinase [19,20]. For nucleotide concentrations below the K_m, the efficiency of GTP (and
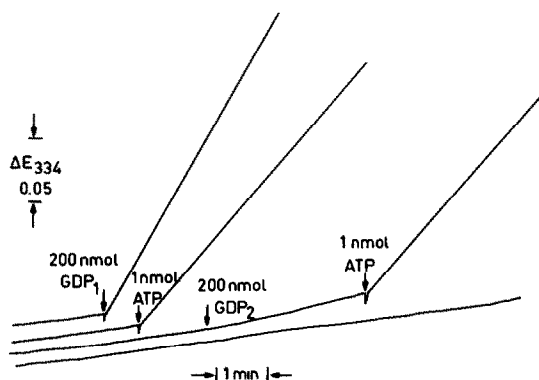
Fig.1. Identification and quantification of adenine nucleotides in commercial samples of GDP. The reaction medium contained in 1 ml final vol. at 25°C: 50 mM Tris–HCl (pH 7.6); 50 mM KCl; 5 mM $MgCl_2$; 5 mM creatine phosphate; 10 mM glucose; 0.4 mM $NADP^+$; 5 U creatine kinase; 1.5 U hexokinase; and 1.8 U glucose-6-phosphate dehydrogenase. Following the addition of ATP, GDP_1 (commercial, unpurified sample), or GDP_2 (commercial sample purified by DEAE–Sephadex chromatography), the increase in absorption at 334 nm is recorded for several minutes using an Eppendorf 1101 M spectralline photometer equipped with a W + W 4410 type recorder (full scale deflection 0.25 absorbance units). The relationship between ΔE_{334}/min and the ATP (or ADP) concentrations is linear over 0.1–5 nmol. For each determination a correction is made for the small increase in extinction due to the presence of adenine nucleotides in the commercial samples of $NADP^+$.

GDP, respectively) as substrate for hexokinase and creatine kinase is just 1% of that of ATP (and ADP, respectively). This particularity is also encountered with many other natural or synthetic nucleotides; one may thus estimate the presence of contaminating adenine nucleotides in GDP or GTP preparations, amounting to <1%, by coupling the reactions catalyzed by the two enzymes in a 'cyclic' system, using glucose-6-phosphate dehydrogenase and excess $NADP^+$ as indicator. As seen in fig.1, purified GDP samples have no effect upon the production of NADPH even at 200-times higher levels than that of ATP (or ADP). On the contrary, the unpurified commercial sample of GDP reacts with the above system, reflecting the presence of 0.7% ADP.

3.2. Incorporation of P_i in G-6-P or 6-PG in the presence of submitochondrial particles, nucleoside diphosphates and 'trapping' enzymes

Experiments on oxidative phosphorylation with submitochondrial particles do not allow one to follow

directly the formation of nucleoside triphosphates; one can, however, follow P_i incorporation into G-6-P using a trapping system with glucose + hexokinase [21]. There are, however, certain disadvantages;

(i) Mitochondrial membranes contain a measurable amount of adenine nucleotides (0.9 nmol ADP + ATP/mg protein in rat liver submitochondrial particles) responsible for significantly high 'controls' especially when determining low rates of phosphorylation;

(ii) The determination of G-6-[^{32}P]P by isobutanol extraction [21] is not specific, as any ^{32}P-labeled ester formed as a byproduct remains in the aqueous phase [13];

Table 1

Synthesis of G-6-P (rat liver) or 6-[^{32}P]PG (beef heart) by submitochondrial particles in the presence of ADP analogs and glucose-hexokinase as trapping system

Nucleotide	G-6-P or 6-[^{32}P]PG formed (nmol . min^{-1} . mg protein^{-1})	
	Rat liver	Beef heart
None	4.9	3.4
ADP	90.3 (100)	71.7 (100)
GDP (unpurified)	11.2 (7.4)	10.3 (10.1)
GDP (purified)	5.2 (<1)	3.6 (<1)
TuDP	16.8 (13.9)	6.0 (3.8)
dADP	20.5 (18.3)	11.4 (11.7)
o^{1}ADP	5.4 (<1)	3.7 (<1)
AMP-P(NH)P (0.05 mM)	10.7 (6.8)	12.6 (13.6)
ADP + AMP-P(NH)P (0.05 mM)	88.5 (97.9)	73.5 (102.6)

The incubation medium contained in 1 ml final vol.: 250 mM sucrose; 10 mM Tris–HCl (pH 7.4); 4 mM KP_i; 2.5 mM $MgCl_2$; 2 mg defatted bovine serum albumin; 0.5 mM EDTA; 5 mM glucose; 5 U hexokinase; 0.1 mM Ap_5A; 1 μg rotenone; and particles (0.3–0.5 mg protein). In experiments with beef heart particles $^{32}P_i$ was 2 mM (200 cpm/nmol), and the medium was supplemented with 0.5 mM $NADP^+$ and 1.8 U glucose-6-phosphate dehydrogenase. After 2 min preincubation at 25°C the reaction was started by the addition of 5 mM succinate and 0.4 mM nucleoside diphosphate. After 10 min incubation, the reaction was stopped with perchloric acid. The KOH-neutralized extract was subjected to the enzymatic assay of G-6-P or to column chromatography (0.55 × 50 cm) on DEAE–Sephadex A-25, using a linear gradient from 0–1.5 M Na-acetate in 0.05 M Tris-acetate buffer (pH 7) at a flow rate of 20 ml/h. Fractions (0.2 ml) corresponding to the radioactive peak of 6-[^{32}P]PG were used for counting with an Intertechnique (France) liquid scintillation counter. The relative values, shown in brackets, refer to the activity with ADP, taken as 100%.

(iii) At high concentrations of the analog, the presence of even <1% ADP causes appreciable increases of the $^{32}P_i$ 'organification' due to the small K_m-values (~5 μM) of the oxidative phosphorylation system for ADP [22]. As seen in table 1, the use of purified GDP does not lead to the formation of G-6-P or 6-[^{32}P]PG at significantly higher rates than in the controls even at 0.4 mM, whereas the unpurified commercial GDP shows a 'phosphorylating' activity which represents 7—10% of that of ADP, in agreement with [1,2]. In addition, o^1 ADP does not stimulate the formation of G-6-P, confirming [7].

Of the analogs which are phosphorylated both by intact mitochondria and by submitochondrial particles from rat liver or beef heart, dADP and TuDP present certain particularities. In rat liver the two nucleotides are phosphorylated at a rate which represents 18% and 14%, respectively, of that of ADP; these values are close to those obtained with intact mitochondria, where the formation of the corresponding nucleoside triphosphates could be followed directly (table 2). On the other hand, the phosphorylation of dADP by heart mitochondria or submitochondrial particles represents only 11.7%—12.4% of that of ADP, whereas the phosphorylation of TuDP is even smaller, only 2.3—3.8% of that of ADP. Our values for dADP are significantly smaller than those for beef heart mitochondria [2].

Table 2
Phosphorylation of TuDP and dADP by respiratory chain enzymes from intact rat liver and beef heart mitochondria

Nucleotide	[^{32}P]NTP (nmol . min^{-1} . mg protein^{-1})	
	Rat liver	Beef heart
ADP	134 (100)	109 (100)
TuDP	20.3 (15)	2.5 (2.3)
dADP	28.1 (21)	13.5 (12.4)

The incubation medium contained in 1 ml final vol.: 180 mM sucrose; 50 mM KCl; 25 mM Tris—HCl (pH 7.4); 0.5 mM EDTA; 2 mg defatted bovine serum albumin; 5 mM glutamate; 5 mM malate; 2 mM $^{32}P_i$ (200 cpm/nmol); and 0.6 mM nucleoside diphosphate. The reaction was initiated by addition of 0.4—1.2 mg mitochondrial protein. After incubating at 25°C for 5 min (ADP) or 10 min (TuDP, dADP), 1 ml 1 M perchloric acid was added and the KOH-neutralized extract was subjected to chromatographic separation of nucleotides as described in the legend of table 1. In parentheses are shown relative values, the activity of the natural nucleotide ADP being considered as 100%

3.3. Effect of AMP-P(NH)P upon the formation of G-6-P or 6-[^{32}P]PG

AMP-P(NH)P, a phosphate-modified ATP analog, is known to strongly inhibit ATP hydrolysis or ATP-utilizing reactions in whole mitochondria or submitochondrial particles [22—24]. This analog has no effect on ATP synthesis [18,22]. Preincubation of submitochondrial particles with AMP-P(NH)P results in a complete inhibition of ATP hydrolysis and an enhancement of G-6-P or 6-[^{32}P]PG formation when particles are incubated with respiratory substrates. On the other hand AMP-P(NH)P does not further increase the formation of G-6-P or 6-[^{32}P]PG via ADP (table 1). This effect of AMP-P(NH)P is considerably larger than that of TuDP or GDP. In view of the fact that the AMP-P(NH)P sample does not contain nucleotides that could act as phosphate acceptors in the oxidative phosphorylation (the analog being prepurified), the only valid explanation is a greater rate of 'recirculation' of membrane-bound adenine nucleotides through the oxidative phosphorylation and trapping hexokinase + glucose systems.

4. Discussion

The only unequivocal way of demonstrating the participation of ADP analogs in oxidative phosphorylation is the identification of the corresponding nucleoside triphosphates under conditions in which all other mitochondrial reactions involving transfer of phosphoryl groups are inhibited. At present this is only possible on intact mitochondria, in the absence of exogenous Mg^{2+}, which limits experiments to those analogs which are transported across the inner mitochondrial membrane. With the exception of tubercidine and deoxyadenosine nucleotides, the only such analogs are formycine and arabinosyladenine nucleotides [25,26]. As the hydrolysis of various nucleoside triphosphates by the membrane-bound or the soluble ATPase is a reaction with fairly broad substrate specificity [2,3,7,14] it is essential to establish to what extent the different nucleoside diphosphates are 'weak' substrates or do not take part at all in the oxidative phosphorylation:

(i) The quantitative differences regarding the participation of the analogs in nucleoside triphosphate hydrolysis or formation are compatible with the reversible character of the reaction catalyzed by the mitochondrial ATPase [10];

(ii) We would have to postulate the existence of two distinct centres within the ATPase, one specialized for ATP synthesis, another for ATP hydrolysis, possessing different specificities for the nucleotides [22,27,28].

Without being able to give a definite answer to this problem, our data outline the existence of at least three sources of error which could be encountered when assessing the capacity of certain ADP analogs to act as substrates in the oxidative phosphorylation:

(a) The purity of the analog;

(b) The formation of phosphorylated intermediates other than G-6-P [13];

(c) The inhibition of the mitochondrial hydrolytic reactions and the stimulation of the rate of 'recirculation' of membrane-bound adenine nucleotides through the oxidative phosphorylation and trapping systems.

Finally, by comparing the substrate capacity of TuDP with rat liver and beef heart mitochondria we observe great differences which cause us to be very cautious when extrapolating data obtained from various types of mitochondria. In fact, beef heart mitochondria are also distinguished by a greater specificity for the adenine nucleus in other mitochondrial reactions involving the participation of nucleotides (ATP-driven transhydrogenase or NAD^+ reduction by succinate). It becomes thus apparent why the simple replacement of the N_7 atom of the purine ring of ADP with a carbon atom in TuDP is accompanied by such a dramatic drop in the acceptor capacity in the oxidative phosphorylation.

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