P^1, P^5-Bis-(5′-adenosyl)pentaphosphate: Is this Adenylate Kinase Inhibitor Substrate for Mitochondrial Processes?

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1. P^1, P^5-Bis-(5′-adenosyl)pentaphosphate (Ap_5A) inhibits "soluble" adenylate kinase even when this enzyme is an integral part of the complete mitochondrion. The K_i is 10^{-5} M, *i. e.* about two orders of magnitude higher than the inhibitor constants determined for the purified adenylate kinase of rabbit muscle and an enzyme preparation separated from the mitochondrial intermembrane space. The weaker inhibitory effect is due to a lower accessibility of the enzyme.

2. As to be expected Ap_5A which is of the "multisubstrate analogue"-type does not affect mitochondrial nucleoside diphosphate kinase.

3. Though Ap_5A owns the structural elements of both ATP and ADP it is not a substrate of the adenine nucleotide carrier, *i.e.* neither it is exchanged across the inner mitochondrial membrane nor specifically bound.

4. Ap_5A is not metabolized by rat liver mitochondria.

Introduction

Shortly after Lienhard and Secemski [1] had shown that the multisubstrate analogue [2] P^1, P^5-bis-(5′-adenosyl)pentaphosphate (Fig. 1) is a potent inhibitor of pure rabbit muscle adenylate kinase (ATP: AMP phosphotransferase, EC 2.7.4.3), Ap_5A proved to be useful to investigate the metabolic role of this enzyme. Adenylate kinase catalyzes specifically the phosphate transfer reaction $ATP + AMP \rightleftharpoons 2\,ADP$ which is important for the regulation of adenine nucleotide concentrations in different cell compartments [3, 4].

The inhibitory action and the general experimental versatility of Ap_5A was pointed out by investigations of the Ap_5A properties in various cell extracts, organelles and erythrocytes [5]. In addition Lüstorff and Schlimme [6] could show that Ap_5A inhibits mitochondrial bound adenylate kinase without affecting oxidative phosphorylation.

This paper reports in detail on Michaelis-Menten kinetics of Ap_5A applied to adenylate kinase integrated into the mitochondrion. Furthermore, we examined binding and exchange properties of Ap_5A with respect to the adenine nucleotide carrier because Ap_5A encloses structural features of both substrates. Finally, we looked for metabolic pathways of Ap_5A because recently Ap_3A – and Ap_4A – splitting enzymes were detected in rat liver [7], each of which shows phosphate chain length specificity.

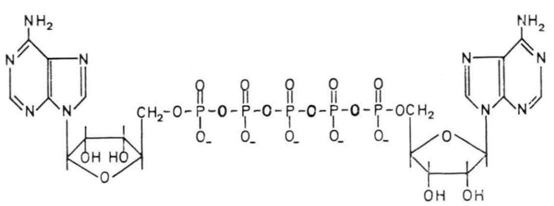

Fig. 1. Structure of P^1, P^5-Bis-(5′-adenosyl)pentaphosphate (Ap_5A), an adenylate kinase inhibitor of the "multisubstrate analogue"-type.

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Materials and Methods

[^{14}C]Ap_5A was synthezised by direct condensation of [^{14}C]ADP with activated ATP [8]. Characterization of the ^{14}C-labelled compound was performed by ^{31}P-NMR- and UV-spectroscopy as well as by chemical, chromatographic, electrophoretic and enzymatic methods [8] and revealed impurities of monoadenosine nucleotides up to about 3%, which are comparable to unlabelled Ap_5A (Boehringer, Mannheim). Other nucleotides, coenzymes and enzymes were purchased from Boehringer, Mannheim. All other substrates and chemicals were used in commonly available reagent grade.

Thin layer chromatography (TLC) was performed on PEI-cellulose plates from Schleicher and Schüll, Dassel, in the following solvent systems (I): 0.75 M KH_2PO_4 pH 4.1; (II): 1.5 M LiCl. These systems allow to distinguish between Ap_5A, Ap_5, Ap_4 and ATP [5]. Radioactivity was counted in a liquid-scintillation counter (Packard Tricarb, model 544) and with a TLC-Scanner from Berthold, Wildbad.

Mitochondria were prepared from rat liver (male Wistar rats, 150 – 200 g weight) following published procedures [9]. Digitonin particles were obtained according to a method reported by Hoppel and Cooper [10]. Rough extracts of mitochondrial adenylate kinase were obtained by collecting the supernatant containing the enzymes of the mitochondrial intermembrane space as well as outer membrane fragments after digitonin treatment of mitochondria. Protein was determined by the biuret method.

Respiratory control measurements with mitochondria and digitonin particles were performed polarographically with a commercially available Clark type oxygen electrode (Eschweiler, Kiel). Mitochondrial adenine nucleotide translocation studies were carried out at 5 °C according to [11].

Differentiation between carrier-linked, *i.e.* atractyloside sensitive, and non-carried-linked, *i.e.* atractyloside insensitive binding as well as between binding and exchange with the endogenous mitochondrial adenine nucleotide pool was carried out as described [12]. Ap_5A-metabolism was studied by incubation of [^{14}C]Ap_5A with mitochondria. After different incubation intervals mitochondria were separated from the medium by rapid centrifugation in a Beckman microfuge modell 152 through a silicon layer followed – when indicated – by denaturation in 15% perchloric acid. Aliquots of the supernatant and the homogenated neutralized sediment, repectively, were analyzed in TLC-systems as described above. Nucleotide containing spots were cut out for radioactivity measurements.

Incubation media: (1) for translocation experiments: (medium III) 70 mM sucrose, 210 mM mannitol, 1 mM triethanolamine, pH 7.2; (2) metabolism studies and respiratory control measurements: (medium IV) 250 mM sucrose, 10 mM triethanolamine, 0,2 mM EDTA, 10 mM KCl, 10 mM $MgCl_2$, 5 mM inorganic phosphate, pH 7.4.

Enzyme assays: In a total incubation volume of 0.5 ml at 25 °C. (a) pure adenylate kinase *in vitro*, back reaction (consuming ADP) [4, 13]: 60 mM triethanolamine pH 7.5, 5 mM $MgSO_4$, 20 mM glucose, 0.33 mM NADP, 10 mM KCl, hexokinase from yeast (ATP: D-glucose 6-phosphotransferase EC 2.7.1.1., 140 U/ml) = 0.7 U, glucose-6-P-dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase EC 1.1.1.49, 140 U/ml) = 1.4 U, ADP, start of the reaction with 0.02 U adenylate kinase (rabbit muscle). (b) Pure adenylate kinase *in vitro*, foreward reaction (generating ADP) [14, 15]: 60 mM triethanolamine pH 7.5, 55 mM KCl, 20 mM $MgSO_4$, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH; lactatdehydrogenase from rabbit muscle (L-lactate: NAD oxidoreductase EC 1.1.1.27, 5000 U/ml) = 30 U, pyruvatekinase from rabbit muscle (ATP: pyruvate-phosphotransferase EC 2.7.1.46, 1.000 U/ml) = 10 U, AMP and ATP, start of the reaction with 0.02 U adenylate kinase (rabbit muscle). (c) *In situ* adenylate kinase activities of rat liver mitochondria: instead of triethanolamine, $MgSO_4$ and KCL medium IV was taken in experiments with intact mitochondria (no effects on enzyme activities as shown in control experiments), 1 μg oligomycin (Serva Heidelberg) per mg mitochondrial protein was added to inhibit ATPase reactions and 4 μg atractyloside per mg mitochondrial protein to block adenine nucleotide translocation. Mitochondrial protein 0.5 mg/0.5 ml total volume. Both atractyloside and oligomycin do not affect the assayed enzymatic reactions [16–18]; all other ingredients unaltered with respect to (a) and (b). (d) Rough adenylate kinase extracts of rat liver mitochondria: Oligomycin concentration was elevated to 10 μg/mg mitochondrial protein due to increased ATPase-activity, all other ingredients unaltered with respect to (c). (e) Pure nucleoside diphosphate kinase (nucleoside diphosphate phosphotransferase EC 2.7.4.6) *in vitro*, foreward reaction [19–22]: components as described in (b), except that AMP is replaced by UDP, pyruvate kinase 0.2 U, start of the reaction with 0.4 U nucleoside diphosphate kinase (beef liver). (f) Pure nucleoside diphosphate kinase *in vitro*, back reaction: as described in (a), additional substrate is UTP which is added after ADP, start of the reaction with 0.4 U nucleoside diphosphate kinase. (g) *In situ* nucleoside diphosphate kinase activities of rat liver mitochondria: Medium IV was taken as in (c), additional to the reagents in (e) and (f) oligomycin and atractyloside were added according to (c), Ap_5A up to 1 mM was added to inhibit adenylate kinase reaction, mitochondrial protein concentration was 1 mg/0.5 ml total volume. Enzyme activities were linear with-(mitochondrial)-protein concentration in all assays. Orientating experiments showed that Ap_5A has no influence on hexokinase and pyruvate kinase reactions up to a fifty fold excess compared to other nucleotides (see also [23]). All enzymatic experiments were recorded with an Eppendorf spectrophotometer monitoring the NAD(P)H absorbance at 366 nm.

Results and Discussion

1. Interaction of Ap_5A with adenylate kinase

Table I shows the activities of adenylate kinase, which is localized in the mitochondrial intermembrane space [24, 25]. The inhibitory effect of Ap_5A in experiments with adenylate kinase separated from rat liver mitochondria by digitonin treatment is weaker ($K_i = 3 - 9 \times 10^{-7}$ M) with respect to purified rabbit muscle adenylate kinase ($K_i = 5 - 15 \times 10^{-8}$ M, in both directions), but up to about one respectively two orders of magnitude stronger ($K_i = 5 - 20 \times 10^{-6}$ M) compared to adenylate kinase which is structurally integrated into the mitochondrion (Tables II and III). The inhibition is competitive in all cases, no matter if ADP or ATP and AMP are externally offered substrates.

Table I. Distribution of activities of rat liver mitochondrial adenylate kinase. Digitonin solubilization was performed according to Hoppel and Cooper [10]. Enzymatic activities were determined as described in Materials and Methods (c–d).

Cell fraction	Activity of adenylate kinase		Protein content	Total protein
	[U/g protein]	Total activity in percent of the mitochondrial fraction	[g/1]	[g]
Intact rat liver mitochondria	257	100	39.5	0.178
Digitonin particles (mitoplasts)	15	2.3	56.3	0.068
Extract of adenylate kinase after digitonin solubilization	500	77	3.6	0.075

All inhibitor constants were determined by Dixon plots and were controlled arithmetically using the Lineweaver-Burk linearization with a range of confidence of 95% in the regression-analysis [26]. As proved for the rabbit muscle enzyme [27] a random-bi-bi-mechanism can be deduced, too, from our *in situ* measurements for the action of the mitochondrial integrated enzyme.

With respect to the amount of unspecifically bound $[^{14}C]Ap_5A$ by rat liver mitochondria (chapter 3, Table V) the concentration of Ap_5A in the intermembrane space was calculated to be as high as in the incubation medium when using an intermembrane space volume of 1 μl per mg mitochondrial protein [28]. Therefore, the kinetic experiments demonstrate very clearly that the inhibitory action of Ap_5A depends on the accessibility of adenylate

	Variable substrates AMP [M]	ADP [M]	ATP [M]
K_M	1.9×10^{-4} b	4.4 (2.6–8.9) a $\times 10^{-5}$	1.4×10^{-4} b
K_M[4]		1.6×10^{-3} $3-10 \times 10^{-5}$ c	
K_i Ap_5A	$9-15 \times 10^{-8}$ d	5.0 (4.9–5.3) a $\times 10^{-8}$	5.0×10^{-8} d
K_i Ap_5A [1, 5]		3×10^{-8}	

Table II. Kinetic properties of pure rabbit muscle adenylate kinase *in vitro*. K_M values were calculated using the Lineweaver-Burk linearization. (a) range of confidence (95%); (b) corrected with respect to cosubstrate concentration by secondary plots; (c) calculated for low Mg^{2+}-concentrations; (d) cosubstrate concentration = 1.2×10^{-3} M. Inhibitor constants were determined by Dixon plots.

Table III. Kinetic properties of mitochondrial adenylate kinase. For experimental details see Materials and Methods (c–d). (a) range of confidence (95%); (b) endogeneous ATP as cosubstrate; (c) cited authors used another test-system; (d) cosubstrate concentration (ATP = 1.8×10^{-3} M); (e) cosubstrate concentration (AMP = 2.0×10^{-4} M).

		Variable substrates AMP [M]	ADP [M]	ATP [M]
Adenylate kinase *(in situ)*	K_M app	8 (4–16) a $\times 10^{-5}$ b	5 (3–11) a $\times 10^{-4}$	—
	K_M app [41]		1.3×10^{-3} c	
	K_i Ap_5A	1×10^{-5} b	$5-20 \times 10^{-6}$	—
Adenylate kinase (digitonin solubilized)	K_M app	2 (1.2–3.3) a $\times 10^{-5}$ d	—	4 (3–6) a $\times 10^{-5}$ e
	K_M [42] (rat liver isoenzyme III purified)	1×10^{-4}	2×10^{-4}	4×10^{-4}
	K_i Ap_5A	$3-9 \times 10^{-7}$ d	—	$4-8 \times 10^{-7}$ e

Table IV. Kinetic properties of nucleoside diphosphate kinase. Ap_5A does not inhibit nucleoside diphosphate kinase reaction in concentrations up to 5 mM when the concentrations of variable substrates are in the range about 0.1 mM.

		Variable substrates ADP [M]	ATP [M]	UDP [M]	UTP [M]
Pure beef liver nucleoside disphosphate kinase	K_M	2×10^{-5}	3×10^{-5}	2×10^{-4}	3×10^{-4}
	K_M [19]	$4-10\times10^{-5}$	$4-6\times10^{-4}$		
Mitochondrial nucleoside diphosphate kinase *(in situ)*	K_M	1.1×10^{-5}	—	—	5×10^{-4}
	K_M [19]	1.5×10^{-5}	—	—	1.3×10^{-4}

kinase which differs in the solubilized compared to the integrated state. Comparable results are known for mitochondrial intermembrane localized creatine kinase, which reacts more slowly with externally offered ATP than with ATP generated by oxidative phosphorylation [29].

2. *Interaction of Ap_5A with nucleoside diphosphate kinase*

Whereas adenylate kinase reacts with adenine nucleotides in a random-bi-bi-mechanism [27], nucleoside diphosphate kinase does not catalyze direct transphosphorylation between the nucleoside tri- (NTP)- and -diphosphate (N'DP) but reacts in a ping-pong mechanism [30] according to: $NTP + E \rightleftharpoons NDP + E{\sim}P$ followed by $E{\sim}P + N'DP \rightleftharpoons E + N'TP$. Though possessing structural features of both nucleoside diphosphate substrates, Ap_5A as a multisubstrate analogue was not expected to inhibit this reaction, unless it is assumed that Ap_5A would "bridge" intra- or intermolecularly both substrate sites. Indeed Ap_5A is not an inhibitor, neither of the *in vitro-* nor of the *in situ* reaction (Table IV). These findings agree with experimental data reported by Grau [23] for human muscle and erythrocyte nucleoside diphosphate kinase.

3. *Interaction of Ap_5A with the adenine nucleotide carrier*

Ap_5A includes the structural elements of ATP and ADP, both substrates of the inner mitochondrial membrane integral adenine nucleotide carrier [31–33]. Thus the following question arises: Does this carrier also interact with Ap_5A which fulfils the molecular characteristics of both substrates? To clarify this question experiments were carried out with $[^{14}C]$-Ap_5A. In order to obtain true results it has to be taken into account that $[^{14}C]Ap_5A$ contains up to 3 percent of impurities of ^{14}C-labelled monoadenosinephosphates as ADP, ATP and Ap_4. Since these adenine nucleotides are exchangeable by the carrier system all experimental results have to be corrected, assuming that binding properties of these contaminations correspond to those of ATP.

The results obtained in this way with $[^{14}C]$-Ap_5A are summarized in Table V. Ap_5A neither is a substrate for carrier mediated transfer nor specifically bound though the following criterea for the translocation are fulfilled: (1) three or more negative charges [31], (2) an intact ribofuranoside ring system [34] and (3) no hinderance of an intact ribose ring puckering by the dinucleotide structure [34–37]. Even the intramolecular stacking of the two adenine groups in Ap_5A which is proved by circular dichroism [38] as well as by our findings (7% hypochromicity at $\lambda = 260$ nm [39]) could not be considered to prevent an ATP- or ADP analogous positioning at the carrier binding site because unter the chosen

Table V. Interaction of $[^{14}C]Ap_5A$ with the mitochondrial adenine nucleotide carrier. (a) Binding and exchange values are given for the native substrate ATP [43] at an externally offered nucleotide concentration of 170 μM which accords with saturation conditions; (b) for impurities see Results and Discussion, chapter 3.

Mode of interaction [nmol/mg mit.protein]	Externally offered $[^{14}C]Ap_5A$ concentration (corrected for impurities [b]) 170 μM	251 μM	403 and 476 μM
Carrier-linked specific binding of $[^{14}C]Ap_5A$	<0.10 (1.6) [a]	0.24	0.10
Carrier-mediated exchange of $[^{14}C]Ap_5A$ with the endogenous ANP-pool	<0.10 (1.6) [a]	<0.10	<0.10
Unspecific binding of $[^{14}C]Ap_5A$	0.71 (0.65) [a]	0.85	1.34

conditions about 30 percent of the Ap_5A-population is in an unfolded state.

Very probably, therefore, that part of the molecule which could act as a substrate is hindered to attain a proper positioning in the substrate binding site. This may be caused by the lack of a free phosphate chain end, *e. g.* ADP-ribose [12] and P^3-alkyl-ATP [40] are not or at best slightly bound to the carrier. On the other hand, unspecific interactions with other membrane components of either the nucleotide moiety or the five negative charges may impede specific binding. Unspecific binding properties of Ap_5A resemble those of ATP and modified adenine nucleotides [32, 34]. To test any competition between Ap_5A and ATP with respect to unspecific binding sites experiments with [^{14}C]ATP were carried out in the presence of Ap_5A, the concentration of which was varied in the range between 70 and 800 μM. No competitive effect of Ap_5A could be observed concerning the amount of unspecifically bound ATP, no matter if Ap_5A was added before or after ATP. Nevertheless results obtained for unspecific binding of Ap_5A (Table 5) suggest that the Ap_5A concentration in the intermembrane space is identical to that externally applied. Additional experiments [39] with energized, *i. e.* P_i-swollen and atractyloside free mitochondria did not show any alteration in binding properties of [^{14}C]Ap_5A.

4. *Is Ap_5A metabolized in rat liver mitochondria?*

Incubation of [^{14}C]Ap_5A (2 mM) with mitochondria (25 mg protein/ml) in medium IV was followed by centrifugation and TLC-analysis of the incubation medium, the homogenated mitochondrial pellet and the whole suspension. Samples were taken during an incubation range between 15 seconds and 2 hours. Analysis did not indicate any ^{14}C-metabolites of [^{14}C]Ap_5A compared to control experiments without mitochondria. No enrichment of mitochondrial bound total ^{14}C-nucleotides could be detected as a function of incubation time. This makes sure that Ap_5A itself and not Ap_5A-metabolites inhibit adenylate kinase *in situ*. Further evidence of Ap_5A not being metabolized were derived from the translocation experiments (chapter 3), because there was no time dependent increase of bound and translocated ^{14}C-labelled compounds. These experiments were carried out because specific enzymes splitting Ap_3A or Ap_4A, respectively, were found in rat liver homogenates recently [7]. Our experimental findings show that Ap_5-A splitting activities are not present in the outer mitochondrial membrane and the intermembrane space.

Conclusions

P^1, P^5-bis-(5′-adenosyl)pentaphosphate specifically inhibits adenylate kinase of various tissues *in vitro* and *in situ*, *e. g.* in intact mitochondria. The inhibitory action is dependent on enzyme accessibility and the "structural constraint" of adenylate kinase. All processes of oxidative phosphorylation are unaffected by Ap_5A since there is no interrelationship with the adenine nucleotide carrier. Furthermore, no "interfering" metabolites are formed. Experimental results with adenylate kinase point very clearly to a structural organization of reaction systems in the intermembrane space.

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