Inhibition study of ADP,ATP transport in mitochondria with trinitrophenyl-modified substrates

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The ADP,ATP carrier of rat liver mitochondria is specifically inhibited by Meisenheimer-type trinitrophenyl (TNP) derivatives of ADP and ATP. Due to a systematic inhibition study we could show that the TNP-moiety itself, even in the 2′, 3′-*O*-cyclic Meisenheimer complex, revealed no inhibition of mitochondrial ADP,ATP transport. Nucleosidic TNP-compounds are weak inhibitors. Introduction of a phosphate group at the 5′-position, however, enhances the inhibitory power markedly. Our findings point to a synergistic effect of the 5′-phosphate chain and the TNP-moiety. Irreversible inhibition by TNP-nucleotides can be ruled out due to the fact that the inhibited ADP,ATP-transport system is fully reactivated by addition of albumin.

ADP,ATP transport *Inhibition* *2,4,6-Trinitrophenyl(TNP) compound* *Rat liver mitochondria*

1. INTRODUCTION

2,4,6-Trinitrophenyl(TNP)-nucleotides have become important tools in the field of bioenergetics [1–5], especially in the investigation of the mitochondrial ATPase [1,3]. 2′,3′-*O*-(2″,4″,6″-trinitrophenyl)-derivatives of ADP (TNP-ADP) and ATP (TNP-ATP) are competitive inhibitors in the ATPase reaction catalyzed by the isolated enzyme [1] as well as by submitochondrial inside-out vesicles [3] due to their high affinity and extremely low turnover rate at the catalytic site [1]. TNP-ATP preferentially inhibits the F_1-ATPase in its non-energized ATP-hydrolyzing conformation [3]. However, a structure–inhibitor activity relationship of these useful analogs has neither been developed nor discussed.

We have found that the ADP,ATP carrier system of rat liver mitochondria is also specifically inhibited by TNP-derivatives of ADP and ATP. We thus performed a systematic inhibition study by employing a variety of TNP-derivatized substrate and non-substrate analogs. The structure–activity relationship derived is discussed in the context of structural data of the TNP-moiety obtained by ^{1}H-NMR spectroscopy and our findings on bonding interactions of the nucleotide at the binding domain.

Some results have been presented in part as a lecture [6].

2. MATERIALS AND METHODS

Syntheses of TNP-derivatives of nucleotides, nucleosides and ribose-5-phosphate were performed with 2,4,6-trinitrobenzenesulfonic acid (TNBS) as reactant following [7,8]. After completion of the reaction, the solution was evaporated to dryness and a mixture of acetone/methanol (3:1) was added. By this treatment, the TNP-nucleotides were precipitated, whereas the non-phosphorylated TNP-derivatives were dissolved. Further purification and characterisation of the TNP-compounds was performed as in [8]. The TNP-compounds were purified by preparative thin layer chromatography on activated silica gel plates

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(SIL G-200, Macherey and Nagel, FRG) using tetrahydrofuran: H_2O (9:1, v/v) as mobile phase. Proton NMR spectra were recorded with a Bruker WP 250 F8.

Binding and translocation measurements were performed with rat (male Wistar rats Bor: WISW, SPF TNO; 150–200 g) liver mitochondria prepared as in [9]. Mitochondria (2.5 mg Biuret-protein) were incubated at 5°C in a medium containing 70 mM sucrose, 210 mM mannitol, 1 mM triethanolamine, 40 μM P^1,P^5-(bis-5′,5″-adenosyl)-pentaphosphate (Ap_5A) and oligomycin (4 μg/mg protein) (pH 7.2) in 290 μl total vol. Separation of mitochondria from the incubation mixture was done by rapid centrifugation through a silicone oil layer (AR 200, Wacker Chemie). Differentiation between carrier-linked (specific) and non-carrier-linked (unspecific) binding as well as exchange with the endogenous adenine nucleotide pool was done as in [10,11].

3. RESULTS AND DISCUSSION

At neutral and alkaline conditions typical AB-spectra for the H_a and H_b protons (fig.1) of the TNP-moiety were obtained with resonances at ν = 8.21 and 8.26 ppm (pH 7, D_2O), indicating that the TNP-derivatives of ribose-containing nucleotides, nucleosides and ribose-5-phosphate exist as cyclic 2′,3′-complexes of the Meisenheimer type [7,8,12]. By lowering the pH, the Meisenheimer complex undergoes a rearrangement to the ether form (2′-or-3′) (fig.1), where the H_a- and H_b-protons are equivalent, as indicated by the respective resonance band at δ = 9.19 ppm (pH 3.5, D_2SO_4). This arrangement has pK ~5, confirming [8].

The data of table 1 demonstrate that TNP-ADP

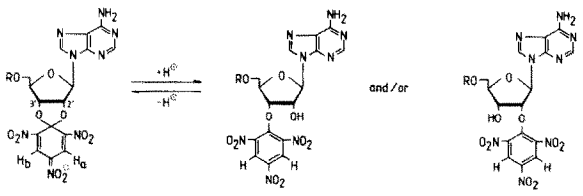

Fig.1. 2,4,6-Trinitrophenyl-derivatives of adenosine and adenine ribonucleotides. pH-Dependency of the Meisenheimer complex formation. R = H (adenosine); PO_3H_2 (AMP); $P_2O_6H_3$ (ADP); $P_3O_9H_4$ (ATP).

prevents carrier-mediated [^{14}C]ADP uptake into the mitochondria due to inhibition of carrier-specific binding of [^{14}C]ADP (expt.2). The remaining amount of bound [^{14}C]ADP in the presence of TNP-ADP corresponds to that obtained by preincubation of mitochondria with carboxyatractyloside (expt.3). Furthermore, the TNP-ADP displaces the same amount of carrier-bound [^{14}C]ADP as does carboxyatractyloside (expt.4,5). These findings show that TNP-ADP interacts specifically with the mitochondrial adenine nucleotide carrier protein. The TNP-derivatives of AMP, ATP, adenyl-5′-yl-imidodiphosphate (AMP-PNP) and CDP exhibit the same properties (not shown). The other TNP-compounds investigated caused neither a significant inhibition of mitochondrial ADP uptake nor a displacement of carrier bound ADP.

Table 2 summarizes I_{50}-values for the inhibition of carrier-mediated uptake of ADP by different TNP-compounds. The data show that the starting reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS), picric acid and TNP-ribose-5-phosphate do not affect ADP transport at all. Interestingly, the TNP-moiety itself, even in the 2′,3′-*O*-cyclic Meisenheimer complex as realized in TNP-ribose-5-phosphate, revealed no inhibition of mitochondrial [^{14}C]ADP transport.

The 2′,3′-*O*-cyclic TNP-complexes of adenosine (fig.1) and cytidine, however, turned out to be weak inhibitors of [^{14}C]ADP uptake. This enhancement of the inhibitory potency obviously is due to the introduction of a heterocycle. These results fit earlier findings obtained with a variety of base-modified nucleotide analogs. By means of these analogs we know that an amidine-structured heterocycle [14] must be present in order to contact the nucleotide binding domain of the protein.

Introduction of one phosphate group at 5′-position as in TNP-AMP increases even more the inhibitory power. This is in line with our finding that the cytosolic substrate requires at least one negative charge at 5′-position in order to be bound by the nucleotide carrier [13,14]. The most potent inhibitors are TNP-compounds derived from ADP, ATP, AMP-PNP and CDP. The inhibitory activity of TNP-CDP points to a synergistic effect of the 5′-phosphate chain and the TNP-moiety due to the fact that CDP does not bind to the

Table 1

Effect of TNP-ADP on mitochondrial carrier-mediated [^{14}C]ADP transport

Expt.	Sequence of addition				[^{14}C]ADP nmol/mg protein
1			ADP	– 30 s – C	3.52 ± 0.13
2	TNP-ADP	– 30 s –	ADP	– 30 s – C	0.25 ± 0.02
3	CAT	– 30 s –	ADP	– 30 s – C	0.23 ± 0.02
4	ADP	– 30 s –	TNP-ADP	– 30 s – C	1.73 ± 0.10
5	ADP	– 30 s –	CAT	– 30 s – C	1.60 ± 0.08

Incubation of mitochondria was done as in section 2 and terminated (C) by centrifugation: [^{14}C]ADP, 60 μM; carboxyatractyloside (CAT), 250 μM; TNP-ADP, 250 μM. The differences yield from: expt.1 and 5, 1 and 4, inhibitor-sensitive (carrier-specific) binding of [^{14}C]ADP; expt.5 and 3, 4 and 2, carrier-mediated uptake; expt.2 and 3, inhibitor-insensitive (carrier-unspecific) binding. Data represent mean ± SE for 4 separate determinations; range of confidence is 95%

Table 2

Mitochondrial carrier-mediated [^{14}C]ADP uptake (I_{50}-values of TNP-derivatized compounds)

Compound	I_{50} [μM]
TNBS	>1.0
Picric acid	>1.0
TNP-ribose-5-phosphate	~1.0
TNP-adenosine	92
TNP-cytidine	124
TNP-AMP	35
TNP-ADP	18
TNP-ATP	30
TNP-AMP-PNP	43
TNP-CDP	50

Incubation sequence: Inhibitor – 1 min – [^{14}C]ADP (60 μM) – 10 s – carboxyatractyloside (500 μM) – 30 s – centrifugation. For further details see section 2

ADP,ATP-carrier at all [11,15,16].

In principle, the mode of inhibition by TNP-derivatives could be due to a nucleophilic attack of an amino acid residue of the carrier protein at the bridging C_1''-atom of the 2′,3′-cyclic TNP-moiety. This reaction would generate an irreversible Meisenheimer type complex between the analog and the binding center of the carrier protein. Covalent bond formation also could be possible by an electrophilic attack of an amino acid side chain at the O_2'- or O_3'-atom of the Meisenheimer complex; i.e., an electrophilic group mimics a proton-like effect (fig.1) whereby the 2′,3′-cyclic TNP-complex undergoes a rearrangement to the ether form. These modes of irreversible inhibition, however, can be ruled out by the results shown in table 3.

The inhibited ADP-transport system is fully reactivated by addition of serum albumin. Albumin obviously successfully competes for TNP-derivatized nucleotides with the carrier protein under the conditions employed. Moreover, preincubation with albumin prevents the inhibition exerted by TNP-compounds. A similar effect was demonstrated for TNP-ADP and TNP-ATP induced inhibition of MF_1-ATPase [3] and azo-dye mediated inhibition of the ADP,ATP carrier [17].

Taking into account that 2′,3′-cyclic TNP-adenine nucleotides are strong, active site-directed inhibitors with respect to the ADP,ATP carrier, new aspects are provided to the mode of inhibition of 2′- and/or 3′-acyl-, aryl-, alkyl- and alkylidene derivatives of ADP. 2′,3′-Cyclic ADP analogs as 2′,3′-*O*-methoxy-methylidene- and 2′,3′-*O*-isopropylidene-ADP do not show any displacement of carrier-bound ADP [18]. 3′-*O*-alkyl-ADP analogs exhibit weak carrier-specific binding [18] whereas 3′-*O*-esters of ADP [19–22] (e.g.,

Table 3

Prevention and abolition of inhibition

Expt.	Sequence of addition	[^{14}C]ADP nmol/mg protein
1	CAT – 1 min – ADP – 30 s ——— C	0.23 ± 0.02
2	ADP – 10 s – CAT – 30 s – C	1.01 ± 0.08
3	TNP-ADP – 1 min – ADP – 30 s ——— C	0.26 ± 0.02
4	Albumin – 1 min – TNP-ADP – 1 min – ADP – 10 s – CAT – 30 s – C	0.91 ± 0.07
5	TNP-ADP – 1 min – Albumin – 1 min – ADP – 10 s – CAT – 30 s – C	0.96 ± 0.08

Incubation of mitochondria was done as in section 2 and terminated (C) by centrifugation: [^{14}C]ADP, 60 μM; carboxyatractyloside (CAT), 250 μM; TNP-ADP, 250 μM; bovine serum albumin, 2.5 mg. Data represent mean ± SE for 4 separate determinations; range of confidence is 95%

3′-arylazido-derivatives [20], 3′-*O*-[5-(dimethylamino)-naphthoyl-1]-ADP [21] and 3′-*O*-(naphthoyl-1)-ADP [21]) interact strongly with the ADP,ATP-transport system.

The different binding properties of 3′-*O*-alkyl- compared to 3′-*O*-acyl-ADP analogs provide evidence that a 2′⇌3′-*O*-acyl transfer reaction [23,24] is involved in the inhibition. The inhibitory action is assumed to depend on a 2′,3′-*O*-alkylhydroxy-methylidene transition-state intermediate which is stabilized by protein interaction. Such a 2′,3′-*O*-cyclic system is present in TNP-compounds.

This point of view is furthermore substantiated by the fact that 3′-tosyl-2′-dADP which cannot undergo the aforementioned cyclisation due to the lack of the 2′OH-group, is a 10-fold poorer inhibitor [6].

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