Conformationally Restricted Adenine Nucleotide Analogs in Mitochondrial Adenine Nucleotide Transport

Karl Siegfried Boos and Eckhard Schlimme

Laboratorium für Biologische Chemie im Fachbereich Naturwissenschaften der Universität, Gesamthochschule, Paderborn

and

Morio Ikehara

Faculty of Pharmaceutical Science, University of Osaka, Osaka, Japan

The conformationally restricted adenine nucleotide analogs 8,3'anhydro-8-oxy-9-(β-D-xylofuranosyl) adenine-5'-O-tri(di)-phosphate (**I**), and 8,2'-anhydro-8-oxy-9-(β-D-arabinofuranosyl) adenine-5'-O-tri(di)-phosphate (**II**), were prepared chemically as their α-^{32}P-labelled compounds and compared with syn-structured 8-bromo AT(D)P in mitochondrial adenine nucleotide translocation.

The experimental findings demonstrate that the heterocycle-ribose orientation affects the carrier mediated adenine nucleotide transport very strongly, *i. e.* a non fixed adenine heterocycle in the anti region is prerequisite for the bound nucleotide to induce the transfer action of the adenine nucleotide carrier.

1. Introduction

The adenine nucleotide carrier is an integral lipo-protein of the inner mitochondrial membrane and catalyses the transport of adenine nucleotides across it [1 – 3].

This transfer system is highly specific for ATP and ADP as substrates [4 – 6].

To characterize structural features involved in carrier specific adenine nucleotide binding and transfer with respect to the nucleobase-ribose orientation, *i. e. syn-anti* conformation, the conformationally restricted adenine nucleotide analogs **I** (Fig. 1 a) and **II** (Fig. 1 b) were used and compared with syn-structured 8-bromo AT(D)P.

The experimental findings demonstrate that specific changes in the heterocycle-ribose orientation affect carrier mediated transport very strongly.

2. Methods and Materials

2.1. Chemical syntheses

8,3'-Anhydro-8-oxy-9-(β-D-xylofuranosyl) adenine was prepared starting from 3'-triisopropylbenzene-sulfonyl-8-bromo adenosine which was converted to the 8-oxyderivate by treatment with sodium acetate in acetic acid. 8-Oxy-3'-triisopropylbenzenesulfonyl-
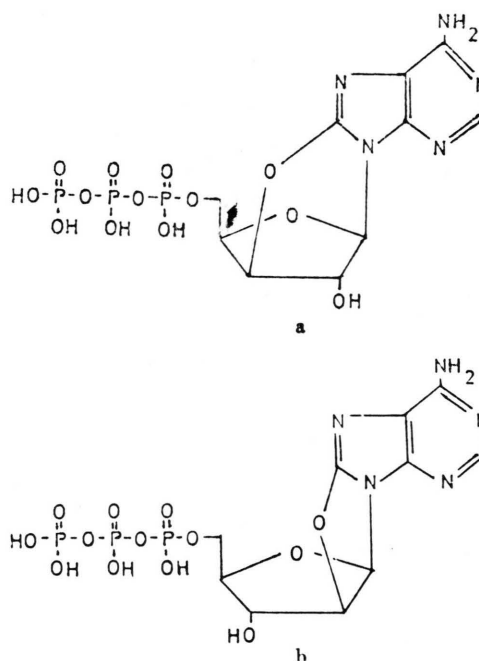
Fig. 1. Chemical structure of the investigated adenine nucleotide analogs.
a 8,3'-anhydro-8-oxy-9-(β-D-xylofuranosyl) adenine-5'-O-triphosphate (**I**),
b 8,2'-anhydro-8-oxy-9-(β-D-arabinofuranosyl) adenine-5'-O-triphosphate (**II**).

adenosine was then cyclized by heating with sodium acetate in dimethylformamide to yield 8,3'-anhydro-8-oxy-9-(β-D-xylofuranosyl) adenine [7]. The [^{32}P]

5'-monophosphate of this nucleoside (37 μmol) was prepared according to Sowa and Ouchi [8] using 0.16 mmol [^{32}P]phosphorus oxychloride (Code No. PB 5, Amersham-Buchler, Braunschweig, Germany). After neutralisation the reaction mixture was applied to a DEAE-cellulose column (Whatman, DE 52 cellulose, 3×80 cm). The [^{32}P]labelled 5'-monophosphate was separated using a linear gradient of 0 to 0.3 M triethylammonium bicarbonate [(Et)$_3$NH$_4^+$HCO$_3^-$] pH 7.3, 2.4 l total volume. It was eluted at 0.24 M (Et)$_3$NH$_4^+$HCO$_3^-$. The corresponding α-[^{32}P]labelled triphosphate, I, was synthesized by a modified procedure of Hoard and Ott [9], starting with 20 μmol of the [^{32}P]monophosphate. α-[^{32}P]labelled I (eluted at 0.3 M (Et)$_3$NH$_4^+$HCO$_3^-$) was fully separated from side products by using a linear gradient of 0.2 to 0.35 M (Et)$_3$NH$_4^+$HCO$_3^-$ pH 7.3, 2 l total volume. Total phosphate analysis according to ref. [10] resulted in an adenine to phosphate ratio being equal to 1 : 3. Specific activity: α-[^{32}P]labelled adenine nucleotide analog I $= 5.2 \times 10^5$ cpm/μmol.

8,2'-Anhydro-8-oxy-9-(β-D-arabinofuranosyl) adenine was obtained according to the reaction sequence described for 8,3'-anhydro-8-oxy-9-(β-D-xylofuranosyl)adenine, however, with 2'-triisopropylbenzenesulfonyl-8-bromo adenosine [7] as starting material. For further characterization of the fused ring nucleosides see Ikehara and Kaneko [7]. The synthesis of α-[^{32}P]labelled II was identical to that of α-[^{32}P]labelled I. Specific activity: α-[^{32}P]labelled adenine nucleotide analog II $= 6.6 \times 10^5$ cpm/μmol.

[^{14}C]8-bromo ATP was prepared from [^{14}C]ATP ([^{14}C]ATP: 196 mCi/mmol, Buchler-Amersham, Braunschweig, Germany) by bromination with bromine in 0.2 M sodium acetate buffer pH 3.8 at room temperature for 24 h [11]. Specific activity: [^{14}C]8-bromo-ATP $= 2.3 \times 10^5$ cpm/μmol.

Preparation of the corresponding nucleoside diphosphates was achieved by treatment with yeast hexokinase (E.C. 2.7.1.1). For incubation assay see ref. [12]. Purity was controlled by thin layer and high pressure liquid chromatography according to ref. [13]. The data are summarized in Table I.

2.2. Translocation measurements

Mitochondria were prepared from rat liver (male Wistar rats, 150 – 200 g weight, Versuchstierzuchtbetrieb Winkelmann, Borchen) following published

Table I. Analytical properties of the investigated nucleotides.

	R_F-values		R_t-values
	System I	System II	[min]
Analog I			
nucleoside	n.d.	0.18 (1/8)	n.d.
5'-diphosphate	0.16	0	31.6 [b]
5'-triphosphate	0.04	0	79.2 [b]
Analog II			
nucleoside	n.d.	0.22 (1/8)	n.d.
5'-diphosphate	0.29	0	15.4 [c]
5'-triphosphate	0.11	0	39.2 [c]
8-bromo adenosine	n.d.	0.73 (3/7)	n.d.
8-bromo ADP	0.28; 0.48 [a]	0	26.4 [c]
8-bromo ATP	0.12; 0.19 [a]	0	140.0 [c]
adenosine	n.d	0.60 (3/7)	n.d.
ADP	0.35; 0.59 [a]	0	5.7 [c]; 28.0 [b]
ATP	0.16; 0.25 [a]	0	34.0 [c]; 96.4 [b]

n.d. = not determined; system I: 1 M ammonium acetate/ethanol = 1/8 and 3/7 (V,V) respectively, on cellulose plates (Merck, Darmstadt, Germany); system II: 0.75 M KH$_2$PO$_4$ pH 4.1 on polyethyleneimine-impregnated cellulose plates (PEI cellulose F 1440 UV, Schleicher and Schüll, Dassel); a = PEI cellulose plates (Polygram CEL 300 PEI/UV, Macherey and Nagel, Düren); b = anion exchange liquid chromatography on Nucleosil 5 SB (Macherey and Nagel, Düren), mobile phase: 0.3 M LiNO$_3$, 0.02 M KH$_2$PO$_4$ pH 2.7, column: 300×5 mm, flux rate: 11 ml/h, 85 bar, 24 °C; c = anion exchange liquid chromatography on Ionex 5 B (Macherey and Nagel, Düren), mobile phase: 0.1 M KNO$_3$, 0.02 M KH$_2$PO$_4$ pH 2.6, column: 300×5 mm, flux rate: 21 ml/h, 67 bar, 24 °C.

procedures [14]. Protein was determined by the biuret method. Mitochondrial adenine nucleotide translocation studies were carried out at 5 °C according to Pfaff and Klingenberg [15]. Differentiation between carrier-linked, i.e. atractyloside sensitive (specific), and non-carrier-linked, i.e. atractyloside insensitive (unspecific), binding as well as between binding and exchange with the endogenous mitochondrial adenine nucleotide pool was performed as described [16]. Mitochondria (2.5 mg protein) were incubated in a medium containing 70 mM sucrose, 210 mM mannitol, 1 mM triethanolamine, pH 7.2 in a total volume of 250 μl at 5 °C; for further details in assaying see [17]. In inhibition experiments sampling for rate measurements was performed within the linear phase (15 sec) of [^{14}C]adenine nucleotide uptake according to ref. [17] at 5 °C. Radioactivity was counted in a liquid scintillation counter (Packard Tricarb model 544). Readily available reagent grade chemicals were used.

3. Results

3.1. Translocation properties

The adenine nucleotide analog **II** exhibits carrier specific binding to rat liver mitochondria whereas the analog **I** does not. Neither of these analogs show any detectable carrier mediated exchange with the endogenous adenine nucleotide pool across the inner mitochondrial membrane. The external concentration of the nucleotide analogs was varied in a range up to 25 μM. Values are given in Table II.

Table II. Mitochondrial binding and exchange. Values were taken at 250 μM external nucleotide concentration, *i. e.* at saturation conditions for the natural substrate (5 °C).

Nucleotides	Specific binding [μmol$\times$g (mitochondrial protein)$^{-1}$]	Exchange	Unspecific binding
ATP	1.65 ± 0.21 *	1.76 ± 0.21	0.61 ± 0.07
I	0.02 ± 0.002	0.02 ± 0.002	1.74 ± 0.22
II	0.36 ± 0.04	0.01 ± 0.001	3.42 ± 0.42
8-bromo ATP	0.51 [18]	0.02 [18]	1.5 [18]

* Range of confidence of 95 percent. Values reported are taken from 4 independent experiments.

3.2. Inhibition studies

Inhibition experiments were carried out with the analog **II** which was found to bind specifically to the adenine nucleotide carrier to evaluate the effect on [14C]ATP uptake by rat liver mitochondria. The inhibitor constant K_I was derived from a Dixon plot (Fig. 2) and controlled arithmetically using the Lineweaver-Burk linearisation with a range of confidence of 95 percent in the regression analysis [19]. The analog **II** turns out to be a competitive inhibitor with a $K_I = 227$ $(185 - 291) \times 10^{-6}$ M for external ATP in an ATP exchange at 5 °C. This exchange is characterized by $K_M(\text{ATP}) = 41$ $(29 - 83) \times 10^{-6}$ M and $V_{max}(\text{ATP}) = 7.0 (5.7 - 9.1)$ μmol$\times$ min$^{-1}\times$g (mitochondrial protein)$^{-1}$ at 5 °C [17].

3.3. Conformational aspects

To facilitate the discussion about structural requirements of the adenine nucleotide carrier binding site with respect to its substrate some essential conformational data of the analogs are given in this section. In the nucleotide **I** the heterocycle is centered by the anhydro linkage in an anti position around the N-glycosidic bond with $\Phi_{CN} = -27°$ [20], *cf.* Fig. 3 a. Introduction of a covalent linkage via a bridging oxygen atom between the 2'carbon of the sugar moiety and the C8 atom of the purine fixes the adenine base in the nucleotide **II** in the syn/anti boundary region ("high-anti") with $\Phi_{CN} = -122°$ [23], *cf.* Fig. 3 b.

8-Bromination of ATP leads to a sterically preferred *syn* conformation of the nucleobase around the N-glycosidic bond with a torsion angle $\Phi_{CN} = +120°$ [24], *cf.* Fig. 3 c.
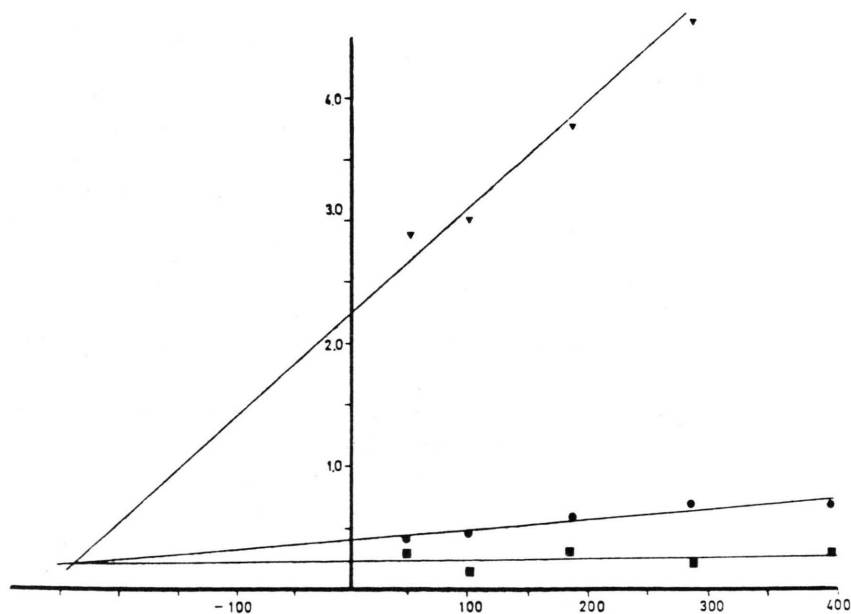

Fig. 2.
Effect of compound **II** on the adenine nucleotide uptake by rat liver mitochondria.
Ordinate: v^{-1}, v is expressed as μmol ATP$\times$min$^{-1}\times$g mitochondrial protein^{-1}; abscissa: inhibitor concentration with [I] as μM; ($\blacktriangledown - \blacktriangledown$) [14C]ATP uptake (6.04 μM), $r = 0.983$, $y = (0.009 \pm 0.001) \times + (2.214 \pm 1.015)$; ($\bullet - \bullet$) [14C]ATP uptake (62.1 μM), $r = 0.987$, $y = (0.001 \pm 0.0001) \times + (0.396 \pm 0.157)$; ($\blacksquare - \blacksquare$) [14C]ATP uptake (211.4 μM), $r = 0.989$, $y = (0.0001 \pm 0.00001) \times + (0.203 \pm 0.071)$, $K_I = 227 (185 - 291) \times 10^{-6}$ M; V=5.0 (3.2−7.5) μmol$\times$min$^{-1}\times$g mitochondrial protein^{-1} at 5 °C; range of confidence 95 percent. Values reported are taken from 4 independent experiments.
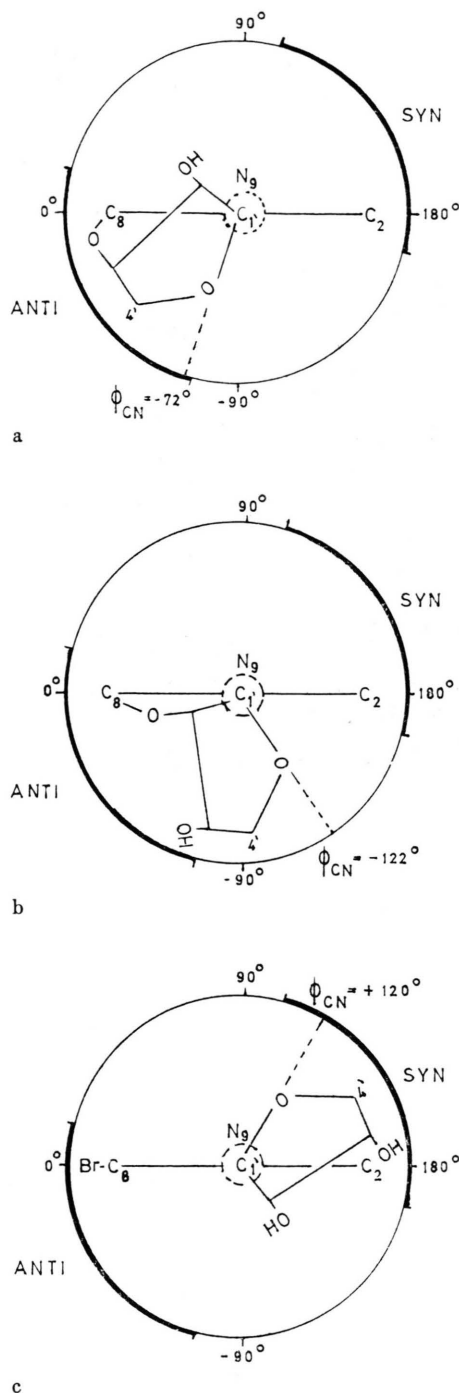
Fig. 3. Conformational aspects of the investigated adenine nucleotide analogs. a **I**; b **II**; c 8-bromo ATP.
The position of the adenine base relative to the pentose is described by the torsion angle Φ_{CN} (O1'-C1'-N9-C8). The base can be *anti-* or *syn* positioned; for an *anti* conformation the angle Φ_{CN} is defined to $-30 \pm 45°$ and for a *syn* conformation to $150 \pm 45°$ [21, 22].

4. Discussion

Nucleotides are built up by three chemical features: (1) the ionized phosphate chain, (2) the hydrophilic sugar and (3) the hydrophobic heterocycle thereby generating distinct nucleotide conformations leading to their biofunctional versatility. Conformational alterations concerning primarily the heterocycle-ribose orientation — as described in this paper — were introduced into the adenine nucleotide molecule either by bridging these two nucleotide segments or by substitution of the C8-hydrogen by a bulky group, *e. g.* bromine. Such analogs, therefore, are useful tools to characterize structural requirements of the mitochondrial adenine nucleotide carrier for substrate binding and transmembrane exchange.

It is known that an adenine base positioned in the anti region as in ATP ($\Phi_{CN} = -20°$ [25]) and a base restricted to the syn range as in 8-bromo ATP (Fig. 3 c) both allow carrier specific binding of the appropriate nucleotides. A covalent fixation of the nucleobase, on the other hand, even in an anti position as it exists in the fused ring compound **I** (Fig. 1 a, 3 a) impedes specific binding. This may be due to the loss of rotational freedom of the heterocycle leading to an overall structure of the nucleotide that cannot be accomodated in the carrier binding site.

Exclusion of a covalently fixed anti positioning of the adenine ring, on the other hand, as present in **II** (Fig. 1 b, 3 b) still allows carrier-specific binding which is also possible when the heterocycle is sterically stabilized in the syn region as found in 8-bromo ATP. Both analogs do not attain such a positioning in the carrier binding site that the transfer step is induced, but they are both competitive inhibitors. K_I of the adenine nucleotide analog **II** (Fig. 2) and 8-bromo ATP [18], both in the range of 10^{-4} M, are about one to two orders of magnitude higher than K_D for ATP [4]. This difference in binding is due to the covalently fixed adenine heterocycle in the syn/anti boundary in compound **II** and the stabilized syn conformation in 8-bromo ATP, respectively.

In conclusion, the experimental findings with the conformationally restricted adenine nucleotide analogs point out that the induction of the transfer step depends primarily on a non fixed nucleobase in the anti region. Moreover, an unblocked 2'-ribo hydroxyl group is necessary for the bound nucleotide

to induce the catalytic action of the adenine nucleotide-carrier [26] probably by means of hydrogen bonding interaction to the complementary position in the adenine nucleotide carrier protein.

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