# PROPERTIES OF ADP- AND ATP-1-*N*-OXIDE IN THE ADENINE NUCLEOTIDE TRANSLOCATION IN RAT LIVER MITOCHONDRIA

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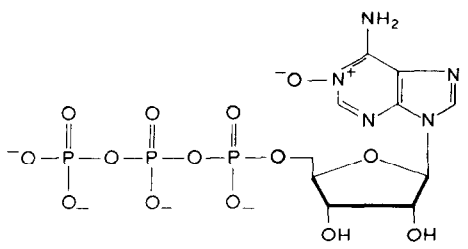
## 1. Introduction

It has been shown that the adenine nucleotide exchange across the inner mitochondrial membrane is catalyzed by a highly specific carrier [1–3]. In addition to that the role of specific translocation inhibitors like atractyloside [3–5] and bongkrekic acid [6, 7] has been investigated. Up to now, only some experimental results have become known about the properties of adenine nucleotide analogues. Adenosine 5′-tetraphosphate, ATPOP; adenosine 5′–*O*–(1, 2-methylenediphosphonate), AMPCH$_2$P and other phosphonic acid analogues, adenosine 5′–*O*–(diphosphoimido phosphate) ADPNHP, and dADP have been found to be active in adenine nucleotide exchange to some extent [3, 7, 8]. A change of the base, though, leads to a total inactivation in the translocation process as reported for guanine-hypoxanthine-, cytosine- and uracil-nucleotides [3]. From this point of view it is interesting to investigate the contribution of the adenine base to the carrier binding, with respect to what extent chemical modifications will be tolerated. As far as we are aware no modification of the adenine base with regard to the translocation activity has been reported.

This paper describes the role of adenine nucleotide-1-*N*-oxides (ox$^1$ADP, ox$^1$ATP, see formula) in the mitochondrial adenine nucleotide translocation.




## 2. Methods and materials

Mitochondria were prepared from rat liver according to common methods [9]. Mitochondrial protein was determined by the Biuret-method. ADP- and ATP-1-*N*-oxides (ox$^1$ADP; ox$^1$ATP) were generated from ADP and ATP by oxidation with monoperphthalic acid in aqueous buffer at pH 7.0, separated by DEAE-cellulose and Sephadex G-10 column chromatography and characterized by Boskamp low voltage electrophoresis and thin layer chromatography after *E. coli* alkaline phosphatase (EC 3.1.3.1) digest as described previously [10–13]. $^{14}$C-ox$^1$ADP (1 μmole = 6.2 × 10$^4$ cpm); $^{14}$C-ox$^1$ATP (1 μmole = 3.48 × 10$^6$ cpm) were prepared from $^{14}$C-ADP (50 μCi/ml; 494 mCi/mmole) and $^{14}$C-ATP (50 μCi/ml; 196 mCi/mmole), products of Amersham (England). *E. coli* alkaline phosphatase (1 mg/ml) was obtained from Boehringer Mannheim GmbH (Germany). Atractyloside was a generous gift of Boehringer Biochemica Tutzing (Germany). The incubation medium contained 0.07 M sucrose, 0.21 M mannit, 1 mM Tra (triethanolamine)-HCl pH 7.2, 10 μg oligomycin, 0.2 mg 3′AMP (both to block ATPase- and adenylate kinase-activity [3]), and 2.5 mg mitochondrial protein per 250 μl assay volume. All experiments were done at 5°. Differentiation of the specific (atractyloside-sensitive) and unspecific (atractyloside-insensitive) binding sites as well as of the exchanged portion with endogenous adenine nucleotides were achieved according to Weidemann et al. [3].

Table 1
Specificity of ADP- and ATP-1-*N*-oxide binding rat liver mitochondria after addition of a 50-fold excess of various compounds.

| Assay concentration | | Nucleotide bound (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ADP | ATP | ox$^1$ADP | ox$^1$ATP | GTP | ITP | Atractyloside |
| $^{14}$C-ADP (4 μM) | 100* (0.16)* | 36 | 42 | 82 | 81 | 99 | 91 | 56 |
| $^{14}$C-ADP (100 μM) | 100 (1.3) | 12 | 39 | – | – | 95 | 94 | 48 |
| $^{14}$C-ox$^1$ADP (108 μM) | 100 (1.8) | 28 | 28 | – | – | 56 | 58 | 69 |
| $^{14}$C-ATP (10 μM) | 100 (0.59) | 29 | 43 | – | 68 | 96 | 100 | 73 |
| $^{14}$C-ATP (40 μM) | 100 (1.4) | 16 | 28 | – | – | 104 | 105 | 66 |
| $^{14}$C-ox$^1$ATP (4 μM) | 100 (0.10) | – | – | – | 70 | – | 86 | 72 |
| $^{14}$C-ox$^1$ATP (8 μM) | 100 (0.23) | 26 | 29 | – | 55 | 64 | 59 | 56 |
| $^{14}$C-ox$^1$ATP (40 μM) | 100 (0.55) | 25 | 18 | – | – | 68 | 59 | 67 |

* Total amount of bound $^{14}$C-nucleotide (nmole/mg protein), implying specific and unspecific binding as well as exchanged $^{14}$C-nucleotide after equilibration with the endogenous nucleotide pool, was taken as 100%.
Rat liver mitochondria (1 mg protein/100 μl) were incubated in a total vol of 250 μl with $^{14}$C-labelled nucleotides for 2 min at 5°. Then a 50-fold excess of unlabelled nucleotides was added for 2 min, followed by rapid centrifugation. Atractyloside (100 μM) removable binding of $^{14}$C-nucleotide was tested after an incubation time of 1 min.

## 3. Results and discussion

### 3.1. *Specificity of ADP- and ATP-1-N-oxide binding by rat liver mitochondria*

From replacement studies we have found (table 1) that ox$^1$ADP and ox$^1$ATP are able to substitute for ADP and ATP and show atractyloside-sensitive and insensitive binding to rat liver mitochondria as well. This binding behaviour implies [3] carrier-specific and unspecific binding. Bound $^{14}$C-ox$^1$ADP and also $^{14}$C-ox$^1$ATP can be replaced by unlabelled ADP and ATP as well as by atractyloside. However, $^{14}$C-labelled adenine nucleotides are replaceable only to a lower extent after addition of a 50-fold excess of unlabelled ox$^1$ADP or ox$^1$ATP, respectively. These properties indicate lower binding affinities of the 1-*N*-oxides to the mitochondrial membrane.

Contrary to the natural adenine nucleotides, $^{14}$C-ox$^1$ADP and $^{14}$C-ox$^1$ATP can be removed from the membrane binding sites by a 50-fold excess of GTP and ITP up to about 40%. Using the atractyloside-differentiation-method [3], we observed that the amount of GTP- and ITP-replaceable ox$^1$ADP and ox$^1$ATP is due not only to the unspecifically bound portion of N-oxidized adenine nucleotides. Probably the association constant of ox$^1$ADP and ox$^1$ATP to the nucleotide carrier is low enough to permit even the replacement of a certain amount of 1-*N*-oxidized adenine nucleotides by GTP and ITP at the specific binding sites as well.

### 3.2. *Effect of ATP-1-N-oxide on binding and exchange properties of ATP by rat liver mitochondria*

The effect of ox$^1$ATP on atractyloside-sensitive and insensitive binding of $^{14}$C-ATP by rat liver mitochondria is shown in the following figures. As can be seen in fig. 1, the addition of an equimolar amount of ox$^1$ATP causes a slightly decreased "binding" in each of the three experiments. From investigations of $^{14}$C-ATP concentration dependency in absence and presence of a constant amount of ox$^1$ATP, we obtained no significant decrease of specific binding,

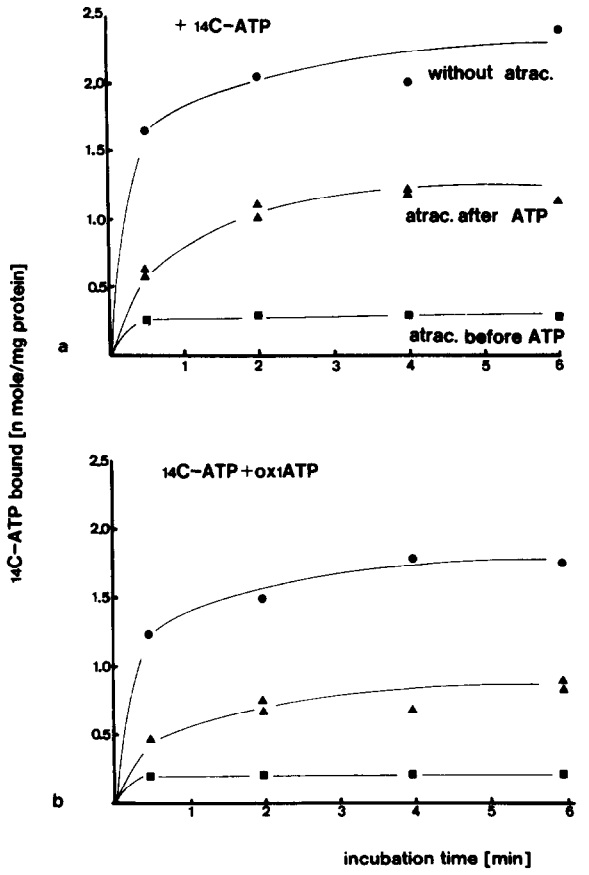



Fig. 1. Time dependence of $^{14}$C-ATP binding by rat liver mitochondria and the effect of ox$^{1}$ATP. Incubation was started by addition of 40 $\mu$M $^{14}$C-ATP in absence (a), and in presence of 44 $\mu$M ox$^{1}$ATP (b). (●–●–●) Experiments without actractyloside; (■–■–■) with 50 $\mu$M atractyloside added 1 min before starting experiments; (▲–▲–▲) $^{14}$C-nucleotide binding stopped with atractyloside various time intervals after starting with nucleotide addition.

whereas unspecific $^{14}$C-ATP binding is reduced to about 50%, caused by a competition for the same binding sites as supposed by the experiments of the next paragraph.

### 3.3. *Comparison of ATP- and ATP-1-N-oxide-binding to specific and unspecific mitochondrial binding sites*

The experimental results described in this paragraph were obtained from the concentration dependency of nucleotide binding. As shown in fig. 2,

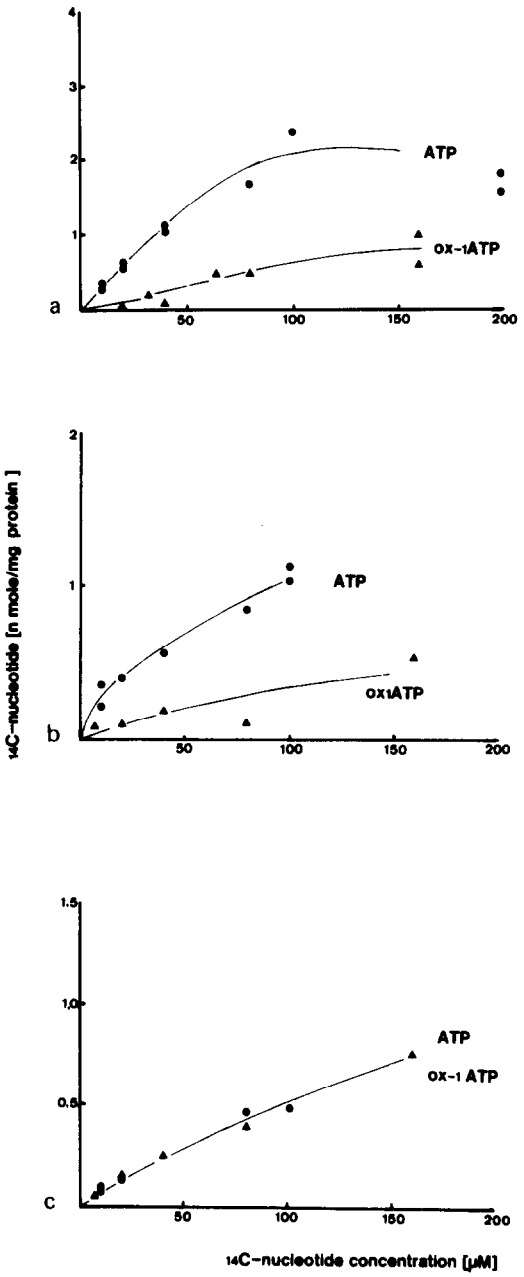



Fig. 2. Properties of $^{14}$C-ATP- and $^{14}$C-ox$^{1}$ATP-binding by rat liver mitochondria. Binding experiments were done with $^{14}$C-ATP (●–●–●) and $^{14}$C-ox$^{1}$ATP (▲–▲–▲), respectively. a) $^{14}$C-nucleotide specifically bound; b) exchanged portion of $^{14}$C-nucleotide; c) $^{14}$C-nucleotide unspecifically bound

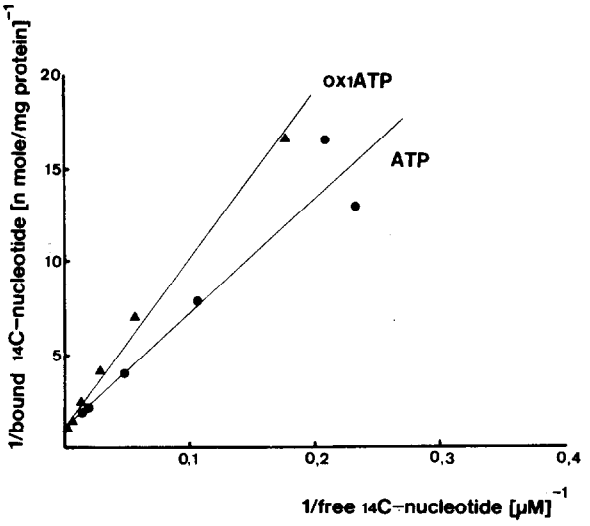



Fig. 3. Reciprocal plot of unspecifically bound $^{14}$C-ATP and $^{14}$C-ox$^1$ATP by rat liver mitochondria. Values for $^{14}$C-ATP (●–●–●) and $^{14}$C-ox$^1$ATP (▲–▲–▲) were mainly taken from fig. 2c. $K_{ass}$ (●–●–●) = 0.02 $\mu M^{-1}$; total number of binding sites $N_{max}$ (●–●–●) = 0.84 (nmole/mg protein); $K_{ass}$ (▲–▲–▲) = 0.01 $\mu M^{-1}$; $N_{max}$ (▲–▲–▲) = 0.89 (nmole/mg protein). Binding data were evaluated from the intercepts of the regression curves. r (●–●–●) = 0.96; r (▲–▲–▲) = 0.99.

$^{14}$C-ox$^1$ATP binds to the adenine nucleotide carrier, but to a lower extent than $^{14}$C-ATP, as already expected from findings described in sect. 3.2.

Parallel to this lowered binding affinity to the carrier a decrease in the translocation activity of ox$^1$ATP compared to ATP was measured (fig. 2b).

Electrophoretic analysis of the labelled ox$^1$ATP after incubation under assay conditions shows all radioactivity to be located in the ox$^1$ATP spot, whereas no radioactivity was detected in the ox$^1$AMP location of the electropherogram. This fact indicates that no interconversion of ox$^1$ATP into ox$^1$AMP took place. Thus, translocation of ox$^1$ATP across the inner mitochondrial membrane was not simulated by ox$^1$AMP penetration.

The experimental results found in the unspecific binding studies are demonstrated in fig. 2c. There was no significant difference in the amounts of unspecifically bound ATP and ox$^1$ATP.

Quantitative evaluation of binding constants and capacities was derived from Scatchard and reciprocal plots [14], indicating more than a single type of carrier-specific binding [15], as already reported for AT(D)P with beef and rat heart mitochondria by Weidemann et al. [3].

Carrier-specific binding parameters are difficult to estimate because of the high endogenous nucleotide pool and the low content of binding sites of rat liver mitochondria. The specific binding to high affinity sites of ox$^1$ATP is about 10-fold lower than that of ATP ($K_{diss}$(ATP) $\leq$ 1 $\mu$M was found about twice as high compared to [3] ), whereas binding to low affinity sites is about 20-fold lower. The capacity of these latter sites appears nearly identical. The ratio of high/low association constants was found to be 36 with ATP (compare [3]), and 90 with ox$^1$ATP, respectively.

Binding characteristics at the atractyloside-insensitive sites are given in fig. 3. The number of binding sites for ATP and ox$^1$ATP does not differ significantly, whereas the association constant of the natural adenine nucleotide is about twice as high as that of the 1-*N*-modified product.

In conclusion, our data suggest that carrier-specific binding of natural and chemically modified nucleotides follows the order: $K_{ass}$(AT(D)P) $\geqslant$ $K_{ass}$ (ox$^1$AT(D)P) $\geqslant$ $K_{ass}$(GT(D)P); (IT(D)P) [15]. However, introduction of an 1-*N*-oxide group into the adenine base leads to a reduction of the translocation rate and specific carrier binding, but does not introduce the non-exchangeability across the inner mitochondrial membrane as observed with guanine-(GDP; GTP) and hypoxanthine nucleotides (IDP, ITP) [3].

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