# 8-AZIDO-ADP, A COVALENT-BINDING INHIBITOR OF MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSLOCATION

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## 1. Introduction

The translocation of adenine nucleotides across the mitochondrial inner membrane is catalyzed by an extremely specific carrier, which only binds to atractylate and bonkrekic acid as high affinity ligands other than ADP and ATP [1,2]. Modified nucleotides as topochemical analogues provided some insight into stereochemical requirements of substrates for interaction with the carrier, but no specific information could be obtained on the nature of the binding sites [3,4]. A break-through was achieved by Klingenberg et al. [5], when tagging the carrier with labeled atractylate and isolation of the carrier protein. However, the other trail of investigation, using modified nucleotides was directed to a search for covalently binding probes which not only should allow the isolation of the carrier, but also the identification of the specific binding site.

In this regard substrate analogues carrying an azido-group are of interest, which on irradiation form highly reactive nitrenes capable of rapid covalent binding at their originally non-covalent binding sites.

This communication identifies 8-azido-ADP (8-$N_3$ADP) as an analogue interacting with the adenine nucleotide-carrier of rat liver mitochondria in a light-dependent reaction, causing irreversible inhibition of adenine nucleotide exchange.

* For minor studies 8-$N_3$ADP can be synthesized from 8-$N_3$-AMP [6] using Michelsons method [7,8]. A more detailed method for large scale preparation will be reported from us in Liebigs Ann. Chem.

## 2. Methods and materials

Mitochondria were prepared from male Wistar rat livers according to standard procedures by differential centrifugation in isotonic sucrose/manitol medium. Respiration was measured polarographically using succinate as substrate in presence of rotenone. The medium contained 0.25 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 5 mM $K_2HPO_4$, 0.2 mM EDTA, 10 mM triethanolamine. Cytochrome *b* reduction was measured in a dual-wavelength spectrophotometer (Phoenix) at 430–410 nm. Pyridine nucleotide reduction was monitored in an Eppendorf-Fluorimeter. 8-$N_3$ADP was prepared from 8-$N_3$-AMP by conversion to the diphosphate*. U.v. irridiation with light from a 400 W Xenon lamp (Zeiss) equipped with a broad band filter (250–400 nm) was carried out under stirring and cooling between 0–10°C. Mitochondrial swelling was followed by O.D. registration at 546 nm as described by Guerin and Klingenberg [9]. ATPase activity was either measured by registration of $H^+$-liberation in an unbuffered sucrose medium with a recording pH-meter (calibration by aliquots of HCl added after each experiment), or enzymatically according to Pullman et al. [10].

ADP-exchange was measured using the atractylate-differentiation method as described in [1]. Separation of mitochondria from the incubation mixture was achieved by centrifugation through a silicone layer (CR-20, Wacker-Chemie) in microvials using a Beckman Microfuge, cooled with $CO_2$.

All chemicals and enzymes were obtained from commercial sources, [$^{14}$C]ADP from Buchler (Braunschweig).

## 3. Results and discussion

### 3.1. *Influence of 8-$N_3$ADP on respiratory functions*

As a prerequisite for the aforementioned purpose a covalent binding analogue should bind to specific catalytic sites and replace for the natural substrate. Therefore ADP-dependent mitochondrial functions have been investigated in presence of 8-$N_3$ADP. In contrast to other purine ring-modified ADP analogues, 8-$N_3$ADP inhibits the normal state 4 → 3 transitions of mitochondrial respiration induced by ADP (fig.1 and 2). The rate of ADP-stimulated respiration is diminished in a concentration dependent way; the inhibition can be released by uncouplers, suggesting a direct interference with the energy conserving pathway. Both, respiration and the redox state of cytochrome *b* or pyridine nucleotides (not shown), indicate that 8-$N_3$ADP itself does not induce phosphorylation cycles, however. At this point it can not be decided wether it is not translocated or it does not serve as a phosphate acceptor as compared to normal ADP. Actually, when added after completion of a normal phosphorylating cycle 8-$N_3$ADP induced a partial oxidation of respiratory pigments and an intermediate state of activated respiration. The same result is observed with small aliquots of ATP added prior to 8-$N_3$ADP. This strongly suggests that 8-$N_3$-ADP is phosphorylated extramitochondrially generating normal ADP via a bypath involving myokinase or nucleoside-diphosphate kinase, which are both present on the outside of the mitochondrial membrane. In fact, 8-$N_3$ADP proved to be an excellent substrate of purified nucleoside-diphosphate kinase, but not of myokinase.

This allows the conclusion that 8-$N_3$ADP does not necessarily penetrate the inner membrane, but at least it interacts with the carrier decreasing the rate of translocation of normal ADP.

U.v. irradiation of mitochondria in presence of the

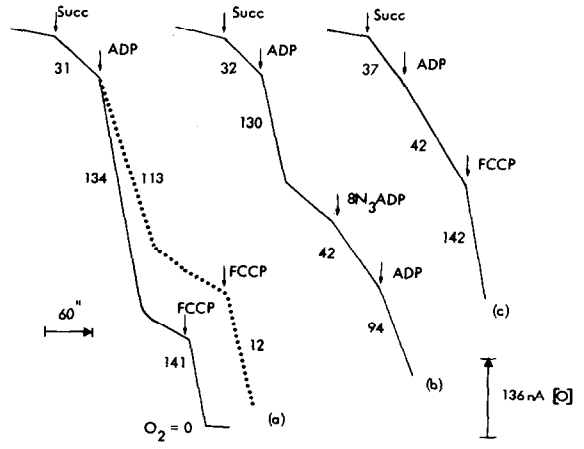


Fig.1. Respiratory control of rat liver mitochondria with succinate. (a) Untreated mitochondria; dashed line: 2 min preincubation with 8-$N_3$ADP ($1.8 \times 10^{-4}$ M). (b) 8-$N_3$ADP ($4.7 \times 10^{-4}$ M). added after one phosphorylating cycle. (c) Mitochondria preincubated with $6.5 \times 10^{-4}$ M 8-$N_3$ADP and 90 sec u.v. irradiation. Figures at the traces represent the respiratory rate as natoms [O]/min·mg protein. Additions: Succinate $3.8 \times 10^{-3}$ M; ADP $1.9 \times 10^{-5}$ M (a) or $1.1 \times 10^{-5}$ M (b,c); FCCP $1.14 \times 10^{-8}$ M; total volume 2.62 ml.

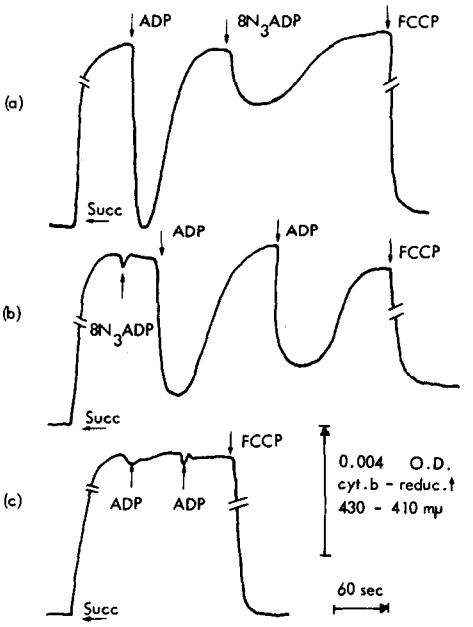


Fig.2. Cytochrome *b* redox cycles of rat liver mitochondria. (a) Untreated mitochondria. (b) 8-$N_3$ADP ($3.0 \times 10^{-4}$ M) added before ADP. (c) Mitochondria 120 sec u.v. irradiation with 8-$N_3$ADP ($7.6 \times 10^{-4}$ M) before succinate addition. Additions: Rotenone 4 μg; succinate $1.9 \times 10^{-3}$ M; ADP $1.15 \times 10^{-4}$ M; FCCP $1.9 \times 10^{-7}$ M. Total volume 2.6 ml.

Table 1
Influence of 8-$N_3$ADP on active respiration of rat liver mitochondria; effect of u.v. irradiation

| Addition of Inhibitor (M) | rel. state-3 respiration −h·υ | R.C. ratio | rel. state-3 respiration + h·υ (90 min) | R.C. ratio |
|---|---|---|---|---|
| – | 1.0[a] | 5.9 | 1.0[a] | 4.7 |
| $1.8 \times 10^{-4}$ | 0.82 | 3.4 | 0.46 | 2.1 |
| $3.2 \times 10^{-4}$ | 0.75 | 3.0 | 0.40 | 1.5 |
| $4.7 \times 10^{-4}$ | 0.68 | 2.7 | 0.29 | 1.3 |
| $6.5 \times 10^{-4}$ | – | – | 0.12 | 1.1 |

[a] 1.0 Represents an activity of 138 natoms [0]/min·mg protein with succinate as substrate.

Table 2
Quantity of ADP-induced transitory oxidation of cytochrome *b*. Effect of 8-$N_3$ADP and of u.v.-irradiation.

| 8-$N_3$ADP [M/l] | Redox change $-\Delta$O.D. $\times 10^{-3}$ (430–410 nm) | Treatment of mitochondria prior to addition of ADP |
|---|---|---|
| ∅ | 5.9 | Control; no inhibitor added |
| $1.9 \times 10^{-4}$ | 5.9 | Inhibitor illuminated prior to addition |
| $1.9 \times 10^{-4}$ | 5.7 | Inhibitor added to mitochondria in the darkness |
| $1.9 \times 10^{-4}$ | 4.1 | Inhibitor illuminated 120 sec with mitochondria |
| $3.8 \times 10^{-4}$ | 3.4 | Inhibitor illuminated 120 sec with mitochondria |
| $7.5 \times 10^{-4}$ | 4.1 | Inhibitor added to mitochondria in the darkness |
| $7.5 \times 10^{-4}$ | ∅ | Inhibitor illuminated 120 sec with mitochondria |

inhibitor largely increased its inhibiting effect as summarized in table 1, giving the relative state-3 respiratory rates with succinate. The corresponding effect on the extend of cytochrome b redox-cycles is shown in table 2.

### 3.2. *Influence of 8-$N_3$ADP on ATP-linked mitochondrial swelling*

In presence of mersalyl as an inhibitor of phosphate efflux ATP-hydrolysis causes drastic swelling of uncoupled mitochondria [10]. This process involves exchange transport of ATP and ADP across the mitochondrial inner membrane. Table 3 illustrates the inhibiting effect of 8-$N_3$ADP on ATP-induced swelling and the enhancement of this inhibition by preillumination. The result is interpreted by a decreased accessibility of ATP-ase for ATP via an inhibition of adeninenucleotide transport. Inhibition of ATP-ase as alternative is rather unlikely as shown in the next paragraph.

Table 3
Rate of ATP-induced phosphate swelling of rat liver mitochondria in presence of mersalyl. Effect of 8-$N_3$ADP and u.v. light. (Expt. 011275)

| 8-$N_3$ADP $\frac{\text{nmoles}}{\text{mg protein}}$ | Swelling rate (− ΔO.D./min) 546 nm | u.v. irradiation (min) |
|---|---|---|
| ∅ | 0.33 | 4.0 |
| 1.48 | 0.28 | no light |
| 0.743 | 0.115 | 4.0 |
| 1.48 | 0.085 | 4.0 |

Table 4
Activity of ATPase of beef heart submitochondrial particles in presence of 8-$N_3$ADP. (Expt. 271175)

| 8-$N_3$ADP [M/l] | ATPase spec. activity | u.v. irradiation |
|---|---|---|
| ∅ | 1.0[a] | – |
| $7.5 \times 10^{-4}$ | 0.92 | – |
| $1.9 \times 10^{-3}$ | 0.91 | – |
| $3.8 \times 10^{-3}$ | 1.10 | – |
| $7.5 \times 10^{-4}$ | 1.06 | 30 sec |
| $7.6 \times 10^{-4}$ | 1.08 | 60 sec |

[a] 1.0 Represents an activity of 0.75 U/mg protein.

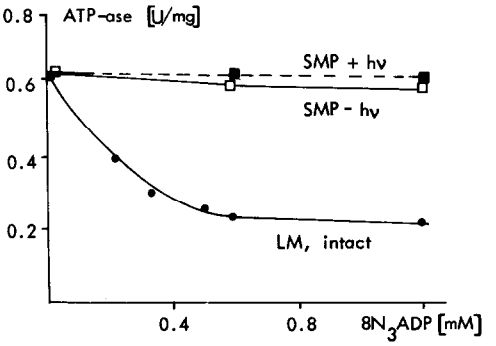


Fig.3. Influence of 8-$N_3$ADP on uncoupler stimulated ATPase of rat liver mitochondria and of sonicated mitochondria (SMP); for better comparison the latter was adjusted to the same initial activity (inhibitor absent) as that of intact mitochondria.

### 3.3. *Effect of 8-$N_3$ADP on mitochondrial ATPase*

Neither in the dark, nor after u.v. irradiation 8-$N_3$ADP exerts any inhibiting effect on ATPase activity of beefheart submitochondrial particles as shown in table 4. In order to discriminate between an interaction with either ATPase or the adenine-nucleotide carrier a comparative titration of ATPase activity was performed with intact and with sonicated rat liver mitochondria respectively. With intact mitochondria the analogue causes a concentration dependent inhibition, which was absent after sonication of the same mitochondrial suspension (fig.3), resembling the result with beef heart particles.

It becomes evident from these experiments that an intact mitochondrial membrane is absolutely necessary for 8-$N_3$ADP to develop its inhibitory effect. This strongly supports the view point that this analogue recognizes the carrier, although its affinity may be very low. An enhancement of the inhibition observed in all cases after irradiation makes it most likely that a portion of the weakly bound ADP-analogue reacts covalently with the carrier decreasing the total capacity of the membrane for adenine-nucleotide exchange.

### 3.4. *Effect of 8-$N_3$ADP on mitochondrial [$^{14}$C]ADP exchange*

Direct proof of an interaction of 8-$N_3$ADP with the carrier is derived from measurements of the exchange of [$^{14}$C]ADP with the endogenous ADP of

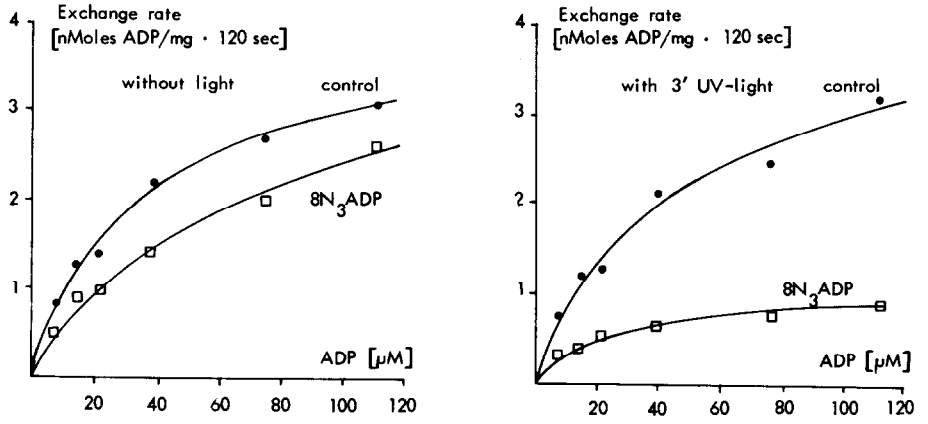


Fig.4. Exchange of exogenous ADP with endogenous ADP of rat liver mitochondria; incubation at 4°C for 120 sec; irradiation when applied: 3 min. at 0°C. 8-$N_3$ADP $3.4 \times 10^{-4}$ M, when present.

rat liver mitochondria. The translocation was studied as a function of exogenous ADP concentration; it was terminated by addition of excess atractylate as described [1]. Under both conditions, without and with illumination in presence of the inhibitor, 8-$N_3$ADP diminishes the exchange of ADP (fig.4). Irradiation generates a pronounced increase of the inhibiting effect. In absence of the inhibitor the effect of irradiation is negligible.

According to reciprocal plots of these data 8-$N_3$-ADP lowers the affinity of the carrier system for ADP by a factor of 0.6 at the applied concentration, whereas the maximum exchange rate is only slightly affected (from 1.63 to 1.51 nmol/min/mg protein). This indicates a competitive type of interaction between ADP and the analogue with the carrier. However, when light is applied together with the inhibitor, the maximum rate of exchange is significantly lowered (from 1.63 to 0.51 nmol/min/mg protein), indicating a decrease of the availability of carrier binding sites, presumably due to covalent binding of the inhibitor and irreversible loss of exchange capacity. In the latter case the affinity for ADP remains almost unaffected; this is a logical result, taking into account that only those inhibitor molecules which are loosely bound to the carrier are capable to react covalently, whereas free inhibitor molecules simultaneously are destroyed by irradiation, equivalent to the removal of excessive active inhibitor from the reaction mixture.

In summary, 8-$N_3$ADP revealed as a promising tool for covalent labeling of the active site of the adenine nucleotide carrier, although the affinity of the inhibitor is low ($K_i \approx 400\ \mu M$ for the overall exchange reaction). Due to the high content of endogenous adenine nucleotides and the low number of specific binding sites in liver mitochondria [1], carrier-specific binding data have to be determined with ADP-depleted mitochondria. These data and labeling experiments with [$^{14}$C]8-$N_3$ADP will be reported in a subsequent communication.

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