# PHOTOLABELING OF THE ADENINE NUCLEOTIDE CARRIER BY 8-AZIDO-ADP

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**Received August 9,1977**

SUMMARY

[$^{14}$C] $8N_3ADP$ was synthesized from [$^{14}$C] 8BrADP. It shows atractylate sensitive specific binding to the adeninnucleotide carrier of mitochondria, but is only a weak inhibitor of the translocator. Via nitrene formation uv-irradiation allows covalent labeling of the carrier protein and induces irreversible inhibition of transport. The labeled carrier protein can be isolated; the stoichiometry of labeling is 0.5 moles $N_3ADP$/mole carrier subunit which has a molecularweight of 31000, suggesting a dimer structure of the carrier in situ. Labeling is specific for $8N_3ADP$; $8N_3AMP$ is inactive as an inhibitor or as photolabel.

The intracellular compartimentation of adenine nucleotides in eucariotic cells is achieved by a highly specific carrier-protein which has been functionally characterized (1,2) and more recently purified to a state allowing the induction of antibodies specific to different functional or conformational states of this protein (3,4,5). For studies of the molecular architecture of the substrate-binding peptide sequence of specific anion carriers covalent binding substrate-analogues or inhibitors are of great interest. Especially azido-derivatives capable of photoaffinity-labeling of the binding site provide a useful tool. In a previous investigation (6) we have observed that $8N_3ADP$ is a weak inhibitor of adenine nucleotide transport in rat liver mitochondria in the dark. After uv-irradiation, however, a pronounced and irreversible inhibition occured.

This report describes the covalent incorporation of [$^{14}$C]$N_3ADP$ and the isolation of the labeled carrier protein





*ISSN 0006-291X*

from beef heart mitochondria providing some new information on the carrier structure in situ.

## METHODS

Beef heart mitochondria were isolated as described elsewhere (7). Binding experiments were carried out essentially as described by Weidemann et al. (8) using atractylate or carboxyarractylate for differentiation of specific binding. Mitochondria were separated from the incubation mixture by silicone layer centrifugation. For photolabeling the mitochondria suspensions were illuminated either in a slowly rotating cylindric quarz-cuvette horizontally immersed into an ice-bath to one half of its diameter, or in small portions of incubation mixture in micro-diffusion disks where the layer of the solution did not exceed 1,5mm; the disks were placed on a cooled aluminum-block (0°) a uv-lamp (fluorotest) with an appropiate cut-off filter was placed above at an average distance of 10cm. The buffer contained 0.25M sucrose, 2mM EDTA, 20µg/ml rotenone. The same buffer was used for washing experiments, supplemented with 0.2mM ADP. TCA-precipitates were washed with 7.3% TCA. Mitochondrial pellets and TCA-precipitates were dissolved in 4% Triton-X-100 solution or in hyamine, respectively, and aliquots were used for liquid scintillation counting. Chromatography of the carrier protein on hydroxyapatite and on sepharose 6B was performed as described by Riccio et al. (3). Synthesis of cold and of $^{14}C$ labeled 8-azido-adeninenucleotides was carrier out according to Schäfer et al. (9) and Penades et al. (10). SDS-gelelectrophoresis was carried out on 8% polyacrylamide according to Weber and Osborn (11) using a set of calibration proteins. All radioactivity measurements were done in a TRICARB liquid szintillation counter.

## RESULTS AND DISCUSSION

Together with uv-irradiation $8N_3ADP$ exerts inhibitory effects on adenine nucleotide transport and respiratory activity of beef heart mitochondria. Using $[^{14}C]8N_3ADP$ its binding- and translocation properties were determined, therefore. By means of the atractylate-differentiation method it was found that $8N_3ADP$ exhibits carrier specific binding. The number of specific binding sites amounts to 0.85 per mole cytochrome $a/a_3$ which is in the same range as for ADP, suggesting that the analogue binds to the same sites. The affinity to the carrier is two orders of magnitude lower with a $K_s$ = 68µM. The analogue is not translocated through the mitochondrial membrane, however; this is in line with structural considerations, suggesting

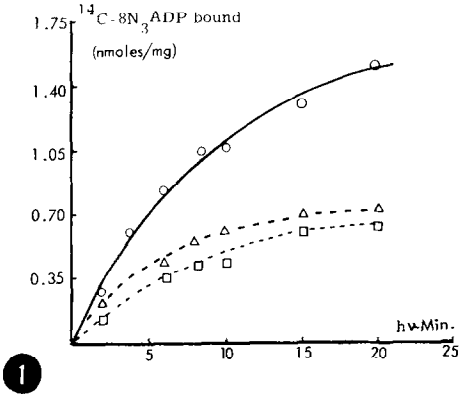
Table 1: Replacement of [$^{14}$C]8$N_3$ADP from beef heart mitochondrial membranes by atractylate ($5.8\times10^{-5}$M) or ADP ($9.8\times10^{-5}$M), and protective effect against light-dependent incorporation. Mitochondria were incubated 20 min. with $7.1\times10^{-5}$M [$^{14}$C]8$N_3$ADP, spec. activity 0.303 mCi/mMol, and were separated by filtration centrifugation.

| | nMoles 8$N_3$ADP bound/mg protein | | |
|---|---|---|---|
| uv-irradiation for 20min., 0°C | - | + | + |
| no additions | 1.86 | 1.94 | 2.03 |
| Atractylate added after incub. | 0.91 | 1.80 | - |
| ADP added after incubation | 0.93 | 1.56 | - |
| Atractylate added before inc. | - | - | 0.98 |
| ADP added before incubation | - | - | 0.75 |

that the anti-conformation of the nucleotide is a prerequisite for a mobile carrier-substrate complex (12). This latter conformation is very unlikely for 8$N_3$ADP.

Binding of 8$N_3$ADP is inhibited by ADP, ATP, atractylate or carboxyatractylate, respectively. Carrier-bound [$^{14}$C] 8$_3$ADP can be replaced by these agents. Replacement is largely abolished however, after uv-irradiation of mitochondria with the azidonucleotide, as shown in table 1. ADP or atractylate added prior to illumination protect against incorporation and only unspecific sites become labeled, representing about 50% of the total binding sites.

The time course of photo-labeling of mitochondrial membranes is shown in figure 1, demonstrating that a plateau is reached after about 20 minutes; again the protective effect of atractylate and ADP becomes obvious. The mitochondrial pellets have been washed by trichloracetic acid (TCA) several times before solu-



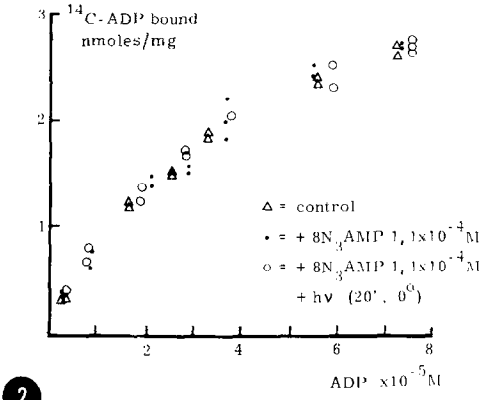




Figure 1: Time course of photoaffinity labeling of beef heart mitochondria with [$^{14}$C]8N$_3$ADP. Conditions as given in methods. Mitochondria were irradiated either with 80μM [$^{14}$C] 8N$_3$ADP either in absence o.....o, or in presence of 100μM ADP Δ.....Δ or 10μM carboxyatractylate □.....□ , respectively.

Figure 2: Influence of 8N$_3$AMP on binding of [$^{14}$C]ADP to beef heart mitochondria. Δ.....Δ control with ADP only; •.....• 1.1x10$^{-4}$M 8N$_3$AMP added 20min. before incubation; o...o 1.1x10$^{-4}$M 8N$_3$AMP added and 20min. uv-irradiation at 0°C before incubation.

bilization and counting for [$^{14}$C] radioactivity. In the dark-controls the total label could be washed off either with isotonic solutions or with TCA, or could be removed by prolonged dialysis.

Regarding the specificity of binding and of labeling the properties of 8N$_3$ADP and of 8N$_3$AMP were compared. Using concentrations from 2.5 to 95mM [$^{14}$C]8N$_3$AMP neither carrier-specific binding, nor atractylate sensitive photolabeling with 8N$_3$AMP could be detected. In addition it could be shown that regardless of uv-illumination 8N$_3$AMP does not interfere with carrier binding of [$^{14}$C]ADP, as given in figure 2. From this result confidence is obtained that the atractylate sensitive photolabel 8N$_3$ADP in fact is a reliably specific marker of the carrier protein.

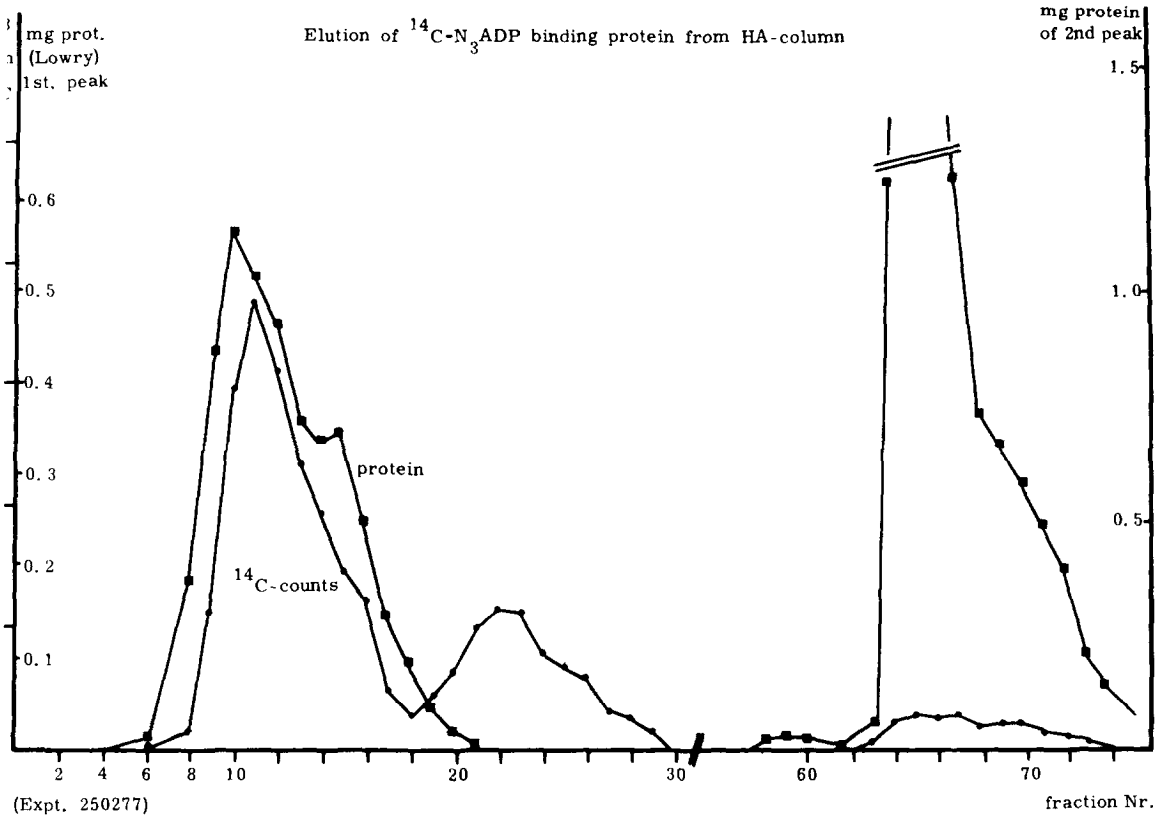



Figure 3: Hydroxyapatite-chromatography of triton-solubilized beef heart mitochondrial membrane proteins (3), after photolabeling with [$^{14}$C]8$N_3$ADP; labeling by 20min. irradiation at 0°C as given in methods. Elution by 0.1M NaCl, 0.1M MOPS pH 7.2, 0.5% Triton; after bufferchange: 0.09M NaCl, 1mM EDTA, 110mM $Na_2HPO_4$ pH 7.2, 0.5% Triton.

After labeling mitochondrial membranes with [$^{14}$C]8$N_3$ADP the carrier protein can be isolated by hydroxylapatite chromatography of Triton-X-100 extracts. The elution pattern of figure 3 shows that the label appears with the protein fraction containing the translocator protein which has been shown to be eluted shortly after the solvent front (3). In dark-controls no radioactivity appears with the respective protein fraction. When atractylate was present to protect the carrier against labeling, the radio-

Table 2: Purification of [$^{14}$C]8N$_3$ADP-labeled protein from beef heart mitochondria; photolabeling under conditions of figs. 1 or 3, respectively.

| fraction | protein (mg) | nmoles [$^{14}$C-] $N_3$ADP | %of total | nmoles/mg protein | relative enrichment |
|---|---|---|---|---|---|
| inc.mix. + h. | 100 | 1736 | 100 | 1.97 | 1.00 |
| 1.wash | - | 1250 | 72 | - | - |
| 2.wash | - | 270 | 16 | - | - |
| Tritonex-tract of sediment | 54.1 | 172 | 10 | 3.1 | 1.57 |
| UZ-super-natant | 43.0 | 167 | 9.6 | 3.8 | 1.98 |
| Peak 1 from HA-column | 4.9 | 67.5 | 3.9 | 13.7 | 6.95 |

activity of the eluted protein was largely suppressed. This supports the conclusion that the labeled protein is identical with the atractylate-binding protein of the inner membrane. By rechromatography on sepharose 6B the label stays with a homogeneous protein fraction.

The efficiency of the purification of the carrier labeled with [$^{14}$C] 8N$_3$ADP is illustrated in table 2. SDS-gelelectrophoresis of the purified labeled protein was performed, following concentration by pressure dialysis. The radioactive protein migrates as a homogeneous band; from it's relative mobility versus calibration-proteins a molecular weight of 31000 $\pm$ 1000 can be calculated, (figure 4) which is in excellent agreement with the data reported for the carrier protein by others (3,5).

Based on this molweight the stoichiometry of photolabeling never exceeded more than 0.5 moles 8N$_3$ADP/mole of carrier. From this we conclude that the minimum supramolecular

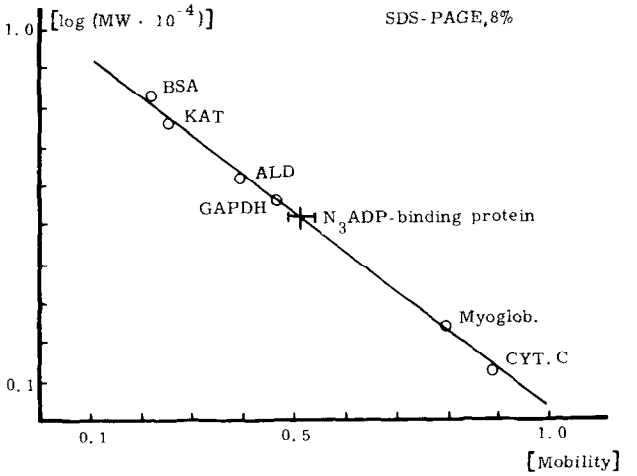



Figure 4: SDS-polyacrylamide gelelectrophoresis of purified $8N_3ADP$-labeled membrane protein, mobility relative to calibration proteins indicates a molecularweight of 31000 ± 1000 (average of 5 independent determinations).

structure of the native carrier in the membrane is a dimer. Since the membrane is impermeable for $8N_3ADP$, one may assume that only half of the binding sites of the intact carrier system are exposed to the outer surface of the inner membrane, thus being accessible to the photolabel. The $8N_3ADP$-loaded carrier is immobilized in the membrane preventing reorientation and exposure of the inner binding sites thereafter. An alternate assumption would be a model requiring an assembly of atleast two subunits, which together form the specific binding area at the entrance of a pore-type carrier system. Covalent tagging of the carrier system has also been achieved with the arylazido-labeled inhibitor atractylate (13). No data are availabel, however, wether this latter label binds to the same peptide sequence of the molecule as $8N_3ADP$ does. Further investigations using nucleotide derivatives carrying an azido-group in the ribose mojety are in progress. From the comparative use

of these different labels a mapping of the substrate binding site and an understanding of the transport mechanism can be expected. The above approach also shows that by use of appropiate photolabels the molecular identity of anion carrier systems may be investigated in general.

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