ENERGY TRANSFER INHIBITION IN PHOTOSYNTHESIS BY 3′-aryl-N_3-ADP, AN ADP ANALOG

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1. Introduction

The effects of nucleotide analogs on mitochondrial and chloroplast ATP-synthesizing systems have been used to approach a functional attribution of nucleotide binding sites in energy coupling [1–5]. More recently covalent labeling of the polypeptide subunits of the mitochondrial adenine nucleotide carrier [6–8] and the coupling ATPase has been achieved by 8-azido-adenine nucleotides and by arylazido analogs [9,10]. The former revealed to be unsuitable for photoaffinity labeling of membrane-bound or isolated CF_1, due to the unfavorable conformation of the adenine ring system in relation to the ribose moiety (almost fixed *syn* conformation). Arylazido esters of ribose-3′-hydroxyl appear to be preferential candidates, since chemical alteration at this position of the nucleotide does not severely affect its properties with respect to photophosphorylation and binding to the tight nucleotide-binding site of CF_1 [5]. Actually, we found 3′-aryl-N_3-ADP having a high affinity to the tight site of CF_1. Moreover this compound interferes with the catalytic site of ATP synthetase although it is not phosphorylated. In fact, it acts as a powerful reversible inhibitor of photophosphorylation, exhibiting the characteristics of a pure energy transfer inhibitor. Most probably energy transfer inhibition is due to competition of 3′-aryl-N_3-ADP with both ADP and P_i at the catalytic site. Thus this derivative seems to be an interesting probe for mechanistic studies of substrate interactions at the catalytic site of ATP synthetase in chloroplasts, independent of its photolabeling capability.

Abbreviations: 3′-aryl-N_3-ADP, 3′-*O*-(4-(N,4-azido-2-nitrophenyl)-aminobutyryl)-ADP; aryl-N_3-butyric acid, N(4-azido-2-nitrophenyl-4-aminobutyric acid

2. Materials and methods

Chloroplasts were isolated from spinach leaves as in [11]. Light-induced incorporation into membrane-bound CF_1 of [^{14}C]ADP was described in [11].

Electron transport was followed by photoreduction of ferricyanide in a Zeiss PMQ II spectrophotometer with cross illumination equipment. Photophosphorylation was measured by incorporation of $^{32}PP_i$ into the organic phosphate fraction, either simultaneously with electron transport or separately. In the latter case, incubation was performed in small test tubes which were inserted into a water bath (20°C). The deproteinized samples were extracted by isobutanol/benzene using a method in [12] as modified [13].

3′-Aryl-N_3-ADP was synthesized from ADP and aryl-N_3-butyric acid [14] by carbonyldiimidazol catalysis as in [15].

3. Results

3′-Aryl-N_3-ADP was synthesized for covalent labeling of adenine nucleotide binding sites on thylakoid membranes. In order to perform specific photolabeling, the interaction of the photoreactive compound with the corresponding binding sites has to be ascertained. This can be achieved under conditions which avoid photoactivation, in our case by using red light as the actinic energy source for photosynthesis.

It is well known that membrane-bound CF_1 contains tightly-bound ADP which rapidly exchanges with free ADP only on energization of the chloroplasts [16–19]. Energy-induced exchange can be conveniently followed by the incorporation of [^{14}C]ADP [11,18]. As shown in fig.1, 3′-aryl-N_3-ADP effectively competes with ADP for this site (K_i 6.0 μM); its affinity is comparable with that of the parent compound (K_s 2.5 μM) but is much higher than that of 8-N_3-ADP (K_i 190 μM). Therefore 3′-aryl-N_3-ADP seems to be much more suitable as a photolabel for CF_1.

Most probably the tight nucleotide-binding site of CF_1 is not identical with the catalytic ADP-binding site in the process of photophosphorylation [5,20]. In order to study the affinity of 3′-aryl-N_3-ADP to the catalytic site, another set of experiments was performed. Table 1 shows that this derivative is virtually not phosphorylated. The result however, does not exclude an interference with the catalytic ADP site. Therefore the effect of 3′-aryl-N_3-ADP on ADP phosphorylation was investigated.

Figure 2 shows titration curves of photophosphorylation and photosynthetic electron transport with 3′-aryl-N_3-ADP. It is obvious that phosphorylation is severely inhibited by 3′-aryl-N_3-ADP in a μM range. Concomitantly, coupled electron transport is decreased approaching the level of basal electron transport at about 50 μM. Basal electron flow itself (in presence of ADP) is not at all affected by 3′-aryl-N_3-ADP.

Since inhibition of coupled electron transport is released by an uncoupler (fig.3) and uncoupled electron transport is not injured by the arylazido analog (table 2), this compound acts as a pure energy-transfer inhibitor in photosynthesis. It should be emphasized that this type of energy transfer inhibition is

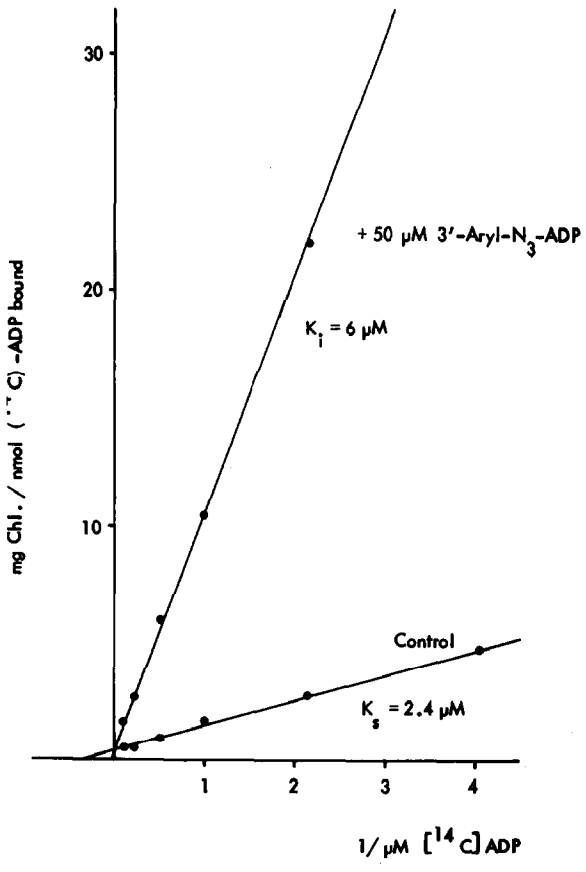

Fig.1. Effect of 3′-aryl-N_3-ADP on light-induced binding of [^{14}C]ADP by washed broken chloroplasts. The medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM methylviologen and [^{14}C]ADP at varying concentrations (2.5–10 μM). In one series additional 50 μM 3′-aryl-N_3-ADP was present. Light intensity (red light, filter RG 630, Schott) was 4.0 × 10^5 ergs/cm^{2}s. The samples were illuminated for 1 min at 20°C. Total vol. 0.5 ml; chlorophyll content 0.237 mg/ml.

reversible. In 3′-aryl-N_3-ADP-treated chloroplasts photophosphorylation is fully restored by subsequent washes.

The mode of action of 3′-aryl-N_3-ADP in energy transfer inhibition may be elucidated by kinetic studies. Table 3 shows that the degree of inhibition at a fixed concentration of 3′-aryl-N_3-ADP depends on ADP as well as on phosphate concentration. A more careful analysis revealed that the type of inhibition is complex. The inhibitor appears to act competitively to both ADP and phosphate.

Table 1
Photophosphorylation of 3′-aryl-N_3-ADP

Phosphate acceptor	μmol ^{32}P incorporated/mg chl × h	
	– Hexokinase trap	+ Hexokinase trap
None	1.6	2.5
3′-Aryl-N_3-ADP 5 μM	2.5	8.8
3′-Aryl-N_3-ADP 25 μM	9.5	10.0
3′-Aryl-N_3-ADP 200 μM	9.9	11.1
ADP 100 μM	–	224.3

The incubation medium contained 25 mM tricine buffer; pH 8.0. 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $^{32}PP_i$, 0.5 mM methylviologen, and chloroplasts equivalent to 35.7 μg chlorophyll/ml. In the case where a hexokinase trap was employed, the medium contained additional 10 mM glucose and hexokinase (54 units/ml). The samples were illuminated with red light (4.5 × 10^5 ergs, filter RG 630, Schott) at 20°C

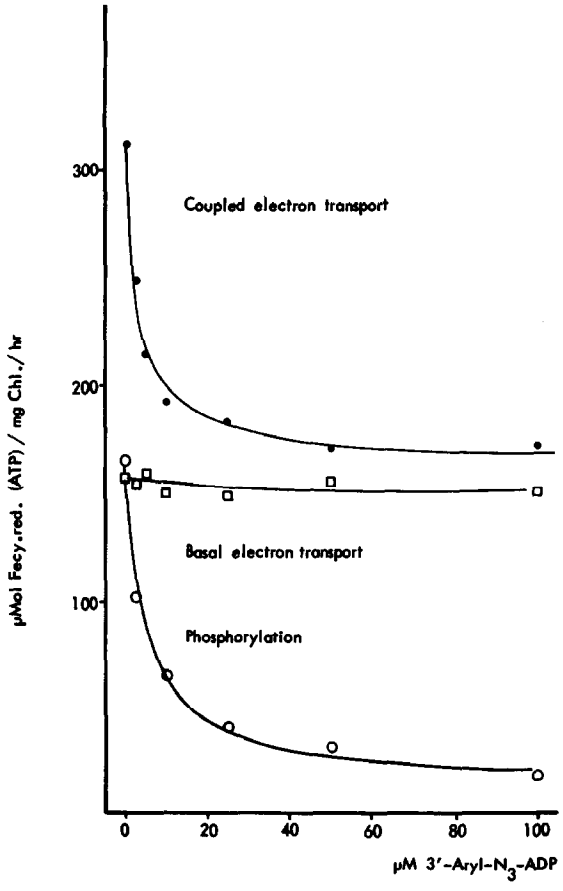

Fig.2. The effect of 3′-aryl-N_3-ADP on photophosphorylation, coupled and basal electron transport. The incubation medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM ADP and 1 mM $K_3[Fe(CN)_6]$. For simultaneous measurement of coupled electron transport and phosphorylation additional 5 mM $^{32}PP_i$, 10 mM glucose and 17.9 units/ml hexokinase (ammonium sulfate-free, Sigma) were present. The reactions were performed in a tempered glass cuvette at 20°C using a Zeiss PMQ II spectrophotometer. The intensity of the red actinic light (RG 630 filter, Schott) was 8.7 × 10^5 ergs/cm²s. Reaction vol. 2.0 ml; chlorophyll content 5.2 μg/ml. For phosphorylation measurements, light was extinguished after 2 min and an aliquot (1 ml) was deproteinized by $HClO_4$ (final conc. 0.3 M).

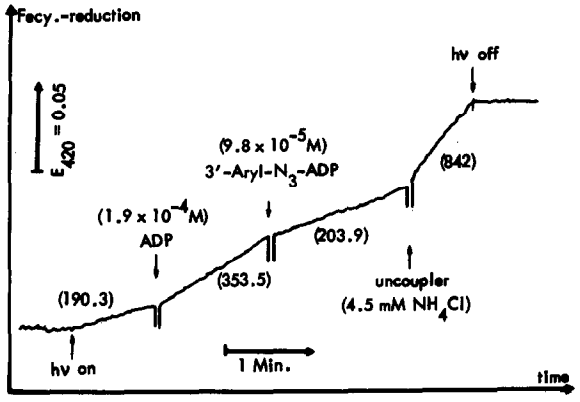

Fig.3. Inhibition of coupled electron transport by 3′-aryl-N_3-ADP and release of inhibition by uncoupling. For experimental details see fig.2 legend. The medium did not contain a hexokinase trap.

Table 2
Effect of 3′-aryl-N_3-ADP on uncoupled electron transport

Addition	μmol fe.cy. red/mg chl. h^{-1}
None	1060
3′-Aryl-N_3-ADP 20 μM	1049
3′-Aryl-N_3-ADP 200 μM	946

Uncoupled chloroplasts were prepared by removal of CF_1 according to the method described in l.c. [21]. The incubation medium (total vol. 2.0 ml) contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM ferricyanide and the indicated concentrations of 3′-aryl-N_3-ADP. Chlorophyll conc. 13.4 μg/ml; temp. 20°C. Ferricyanide reduction was followed directly as described in fig.2 legend

Table 4
Efficiencies of some energy transfer inhibitors in photosynthesis

Compound	I_{50}	Ref.
Dio-9	1 μg/ml	[23]
Phlorozin	0.3 mM	[24]
Synthalin	0.2 mM	[25]
N-Ethyl-3-thiocyanatoindole	50 μM	[26]
Discarine B	50 μM	[27]
Tentoxin	1.5 μM (spinach)[a]	[28]
3′-Aryl-N_3-ADP	5 μM	this paper

[a] I_{50} depends on plant species

4. Discussion

In studies on the utilization of ADP analogs, it was established that the base moiety on one hand and the phosphate residue on the other hand are the essential structural parts of the ADP molecule with respect to photophosphorylation and binding to the tight nucleotide binding site of CF_1 [5]. On the contrary the ribose moiety is much less important for the interaction with chloroplast ATP synthetase. In particular, modifications at 3′-position do not change the properties of the ADP molecule very much. Accordingly 3′-deoxy-ADP [21] and 3′-*O*-methyl-ADP (H. S. and E. Schlimme, unpublished) were found to be good substrates in photophosphorylation and good substitutes for ADP at the tight site. Thus from its structural features 3′-aryl-N_3-ADP should also be able to interact with these ADP-binding sites of CF_1. This was directly demonstrated by the above experiments.

However, 3′-aryl-N_3-ADP is not phosphorylated but acts as a powerful energy transfer inhibitor. Thus we may conclude, that the base and phosphate moieties of the molecule are essential for its recognition at the ADP-binding sites, and that the arylazido

Table 3
Inhibition of ADP phosphorylation by 5 μM 3′-aryl-N_3-ADP

Condition	μmol ATP/mg chl. h^{-1}	% Inhibition
0.5 mM P_i		
ADP 10 μM control	109.5	0.0
+ 5 μM 3′-aryl-N_3-ADP	25.9	76.3
ADP 100 μM control	191.6	0.0
+ 5 μM 3′-aryl-N_3-ADP	116.0	39.5
10 mM P_i		
ADP 10 μM control	110.0	0.0
+ 5 μM 3′-aryl-N_3-ADP	51.6	53.1
ADP 100 μM control	199.1	0.0
+ 5 μM 3′-aryl-N_3-ADP	167.9	15.7

The incubation medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM methylviologen, 10 mM glucose, 27 units/ml hexokinase (Sigma, ammonium sulfate-free) ADP and $^{32}PP_i$ as indicated. The chlorophyll content was 29.2 μg/ml. Other experimental conditions see table 1

residue is responsible for its property to act as an energy transfer inhibitor. In this context it is noteworthy that aryl-N_3-butyric acid itself does not inhibit phosphorylation at all.

The effect of 3′-aryl-N_3-ADP on phosphorylation can be partially released by increasing the phosphate concentration. In view of this fact and considering the possible conformations of 3′-aryl-N_3-ADP, as derived from molecular models, it is suggested that this compound, besides occupying the ADP site, simultaneously covers the P_i binding site at the catalytic center. Logically, on the enzyme these two sites should be in close proximity.

Compared with other known energy transfer inhibitors, the effectivness of 3′-aryl-N_3-ADP is considerably high (table 4). Moreover, its ADP-analogous structure allows a precise localization of its site of action. Studies are in progress to derive by further modification of the 3′-substituent an optimum structure of a nucleotide analog to block energy transduction at the level of ATP synthetase.

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