Energy Transfer Inhibition Induced by Nitrofen

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Z. Naturforsch. 37 c, 787 – 792 (1982); received April 30/June 8, 1982

Heredities, Nitrofen, Energy Transfer Inhibition, Photophosphorylation, Nucleotide Exchange

The herbicide nitrofen was shown to act as an energy transfer inhibitor. The results proved nitrofen to act by inhibiting nucleotide exchange on the chloroplast coupling factor. A strong correlation was found between the inhibition of phosphorylation, ATPase activity, and nucleotide exchange. These results are discussed in terms of a regulatory effect of tightly bound ADP on the enzymatic activity of the chloroplast coupling factor.

Introduction

Nitrofen is used to control annual weeds in cultures of parsley, onion, carrot, celery, and rice [1]. Until now its mode of action is a controversial question. The main herbicidal effect of nitrofen may probably be located in the photosynthetic apparatus [2]. Besides chlorophyll bleaching [3 – 5] and electron transport inhibition [2, 4] nitrofen causes energy transfer inhibition [4, 6]. From their own results Lambert et al. [6] concluded that nitrofen must be competitive to ADP but not to phosphate in the process of photophosphorylation. This result is rather surprising and therefore particularly interesting because, of the lack of structural similarity between the substrate ADP and the inhibitor nitrofen. However, a competitive type of inhibition could also be caused by other effects than substrate displacement from the catalytic site [7]. In this paper, the mechanism of energy transfer inhibition by nitrofen is studied in greater detail.

The terminal process of photophosphorylation may be subdivided into two partial reactions, I) H+ translocation through the CF0-part of the ATPase complex, and II) the ATP synthase reaction catalysed by CF1. Of the known energy transfer inhibi­tors, covalent binding of the carbodiimide DCCD to CF1 [8 – 10] and interaction with tributyltin chloride [11, 12] inhibit the former reaction, while others affect CF1, e.g. phlorizin [13 – 16], Dio-9 [17 – 19], tentoxin [20 – 27], and a number of 3’-esters of ADP [28].

Chloroplast ATPase is a latent enzyme which needs activation in order to display its catalytic activity. Physiological activation results from membrane energization [29, 30]. Recently it was shown that activation is paralleled by energy dependent release of tightly bound ADP from CF1 and deacti­vation to the reverse reaction [30 – 32]. Probably the rate of photophosphorylation is usually controlled by enzyme activation rather than by fatigue number of the enzyme [29, 33]. Inhibition of ATPase activation as well as ATPase reaction would of course affect the rate of photophosphorylation and produce the characteristics of energy transfer inhibition (i.e. inhibition of coupled electron transport).

The goal of the present study was to localize the effect of nitrofen with regard to the partial reactions mentioned earlier.

Methods

Chloroplast isolation from spinach leaves was carried out as described by Strotmann et al. [34]. For chlorophyll determination Arnon’s method [35] was employed.

Non-cyclic electron transport from water to fer­ricyanide was measured by following the decrease in 420 nm absorbance in a spectrophotometer (PMQ II, Zeiss) with cross illumination equipment for one minute (continuous registration). The cross illu-
mination equipment contains a 2 mm heat filter (Schott) within a cooled lens system. The red actinic light (filter RG 630, Schott) was 870 W/m², measured within the reaction cuvette, which was kept at a temperature of 20 °C. The reaction medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 5 mM Pi, pH 8.0, 0.5 mM ADP, 1 mM K₃[Fe(CN)₆], and 12 to 20 μg chlorophyll in a total volume of 2 ml.

For the measurement of non-cyclic phosphorylation the medium contained 5 mM ³²P-labeled phosphate (1.8 MBq/ml, Amersham Buchler). The reaction was stopped after one minute by addition of HClO₄ at a final concentration of 0.3 M. After centrifugation (2 minutes at 10,000 × g) an aliquot was analyzed for organic phosphate employing the method of Strotmann [36].

For measurement of uncoupled electron transport, CF₄ stripped chloroplasts were prepared as described by Tischer and Strotmann [37]. Cyclic phosphorylation was measured with 50 μM PMS instead of Fecy. Photosystem I dependent phosphorylation was followed in a system containing 20 μM DCMU, 5 mM ascorbate, 0.2 mM DCPIP, and 0.2 mM MV instead of Fecy. In the same system, electron transport was followed by measuring the consumption of O₂ with a Clark type electrode.

Trypsin activated ATPase activity of isolated CF₄ was measured as described by Strotmann et al. [38] and Hesse et al. [39]. Light triggered ATPase was measured as described by Schumann and Strotmann [31]: Chloroplasts were illuminated for one minute in a water bath at 20 °C with white light (1200 W/m²) of a commercial lantern-slide projector. The reaction medium contained 25 mM tricine buffer, pH 8.0, 5 mM MgCl₂, 50 μM PMS, 20 μM DCMU, and 0.13 to 0.15 mg chlorophyll per ml. After 5 seconds in the dark, a sample of the activated chloroplasts was injected into the ATPase test medium, containing no PMS but 0.5 mM ATP labelled by γ-[³²P]ATP (3—4 KBq/ml).

Kinetic measurements of light induced exchange of ADP on membrane bound CF₄ was measured using the method of Strotmann et al. [40]. Light dependent proton transport across the thylakoid membranes was measured with a glass electrode at 20 °C in a medium containing 50 mM NaCl, 5 mM MgCl₂, 50 μM PMS, and 60 to 80 μg chlorophyll in a final volume of 3 ml. Illumination was performed employing a commercial lantern-slide projector. The red actinic light (filter RG 630, Schott) was 935 W/m², measured inside the temperature controlled (20 °C) glass reaction chamber.

**Results**

1. Nitrofen as an inhibitor of photosynthetic electron transport

Energy transfer inhibition by nitrofen is superimposed by direct inhibition of electron transport [2, 41]. The latter effect can be isolated by following uncoupled electron transport from water to Fecy. Half maximal inhibition is achieved by 10 μM nitrofen. Congruent to earlier results [2] no inhibition of uncoupled photosystem I electron transport from

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**Fig. 1. Double-reciprocal plot of atrazine binding to CF₄ stripped chloroplasts competing with 4 μM (Δ), 2 μM (□), and 1 μM (○) nitrofen or without addition of nitrofen (○). Chlorophyll content was 27 μg/ml. Experimental conditions corresponded those of uncoupled electron transport measurement with the exception of the electron acceptor being omitted.**
Table I: Inhibition of electron transport through photosystem I. The reaction medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 5 mM ascorbate, pH 8.0, 0.2 mM MV, 0.2 mM DCPIP, 15 μg/ml chlorophyll, and 0.2 mM ADP and 5 mM phosphate, pH 8.0, when measuring phosphorylation. As the herbicide and DCPIP were dissolved in methanol, all samples contained 5% (v/v) methanol. Uncoupled chloroplasts were prepared as described by Tischer and Strotmann [37] from the same leaves in parallel. Electron transport was measured by following O₂ consumption in Clark type O₂ electrode.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Non-phosphorylating</th>
<th>Phosphorylating</th>
<th>Uncoupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 μM nitrofen</td>
<td>37.4</td>
<td>76.5</td>
<td>497.0</td>
</tr>
<tr>
<td>control</td>
<td>37.6</td>
<td>162.3</td>
<td>496.4</td>
</tr>
<tr>
<td>inhibition</td>
<td>–</td>
<td>52.9%</td>
<td>–</td>
</tr>
</tbody>
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DCPIP to MV was obtained (Table I), indicating that the site of nitrofen inhibition is around photosystem II. Fig. 1 shows that nitrofen is a competitive inhibitor in the binding of ¹⁴C-labelled atrazine. The calculated Kᵢ is 11 μM. It appears therefore that the site of electron transport inhibition by nitrofen is identical with the DCMU binding site.

2. Nitrofen acting as an energy transfer inhibitor

In contrast to the uncoupled system, photosystem-I dependent electron transfer in the presence of P₇ and ADP is diminished by the herbicide (Table I) with an I₅₀ of 1.5 μM. The same value is obtained all the same whether PMS mediated photophosphorylation or non-cyclic electron transport mediated phosphorylation in a water-ferricyanide system are measured. Accordingly, this effect of nitrofen may be ascribed to energy transfer inhibition. Lambert et al. [6] found that inhibition of phosphorylation by nitrofen is competitive to ADP. This result is confirmed by double-reciprocal plots of our own experiments. But a Dixon-plot of these experiments indicates partial competitive inhibition with respect to ADP interacting with CFᵢ (Fig. 2). As a working hypothesis derived from the Briggs-Haldane form of an enzyme reaction, the occurrence of a CFᵢ-ADP complex during ATP-synthesis is postulated. This is in good agreement with models based on experimental results of Boyer et al. [42] and Bickel-Sandkötter and Strotmann [43]. On the basis of this assumption, the shown results indicate that nitrofen binds as well to CFᵢ as to CFᵢ-ADP-complex without forming a ternary dead-end-complex but reducing turnover rate. On the other hand, the inhibition kinetics are complicated with regard to the substrate P₇. At P₇ concentrations less than 150 μM the effect of nitrofen is not manifest any more, indicating that phosphate binding and even phosphoryl transfer may be unaffected. The observed phosphorylation rates of 161 μmol ATP per mg chl and hour (150 μM P₇) down to 43 μmol ATP per mg Chl and hour (20 μM P₇) were not inhibited by addition of 12 μM nitrofen any more. Therefore our further interest was focused on nitrofen interactions with nucleotide dependent reactions.

3. Effect of nitrofen on exchange of tightly bound adenine nucleotides and on ATP hydrolysis

In addition to the catalytic ADP binding site, CFᵢ contains a regulatory site which binds ADP and
Correlation between the activation of ATPase activity (upper part) and liberation of tightly bound \( ^{14} \)C ADP (lower part) with (\( \Delta \)) and without (\( \circ \)) addition of 12 \( \mu \)M nitrofen, respectively, during the activation step. The reaction medium contained 25 mM tricine buffer, pH 8.0, 5 mM MgCl\(_2\), 50 \( \mu \)M PMS, 20 \( \mu \)M DCMU, and 28 \( \mu \)g/ml chlorophyll. The chloroplasts had been preloaded with \( ^{8-14} \)C ADP as described by Schumann and Strotmann [31]. Employing their method, each sample was split into two portions. One was applied in an ATPase experiment, the other one was analysed for liberated ADP.

Probably ATP too [34, 42–45]. This site changes its affinity to the ligand in energized and de-energized membranes. Tightly bound nucleotides present in the de-energized state are liberated or exchanged, respectively, when the thylakoid membranes are energized by light [34, 46], pH jump [34, 47] or external electrical fields [29]. It has been shown that energy induced release of bound ADP is related to the induction of ATPase activity ("light-triggered ATPase") and re-binding of ADP causes deactivation of the enzyme [31, 32, 48]. Probably the same mechanism is also involved in the control of phosphorylation [29, 49].

Fig. 3 shows the effect of nitrofen on the kinetics of the release of tightly bound \( ^{14} \)C ADP and concomitant activation of ATPase activity. Both reactions are inhibited in parallel. Under the employed conditions (PMS mediated electron transport) an inhibition of electron transport can be excluded (s. Table I) so that the observed effect must be attributed to a reaction related to the ATPase complex itself.

A nucleotide binding site of CF\(_{1}\) which has been depleted by membrane energization, can be re-filled with ADP yielding a tightly bound nucleotide again [40]. This reaction is independent of energy input and virtually irreversible in non-energized conditions [44–46]. Fig 4 shows that the rate of re-binding of \( ^{14} \)C ADP is inhibited by nitrofen, too.

In Fig. 5 the effect of nitrofen on the rate of ATP hydrolysis induced by pre-illumination is shown. The herbicide is added after the light pre-treatment in order to exclude superposition of the nitrofen

![Graph showing correlation between ATPase activity and ADP release](image)

![Graph showing binding of \( ^{8-14} \)C ADP to ADP depleted membranes](image)
effect on ATPase activation. Nevertheless inhibition of ATP hydrolysis is apparent under these conditions. However, maximum inhibition is not more than 50%.

Discussion

The presented results show nitrofen to inhibit the overall reaction of photophosphorylation at two sites:

I) nitrofen inhibits electron transport and shows typical reactions of a photosystem II herbicide;
II) nitrofen acts like an energy transfer inhibitor.

The experiments should yield some more insight into the mode of action resulting the latter effect. Nitrofen did not only cause an inhibition of photophosphorylation but also of light triggered ATPase activity and nucleotide exchange. Discussing the results a central reaction inhibited by nitrofen should be found.

The first experiments showed a partial competitive effect of nitrofen with respect to ADP taking part in photophosphorylation. The participation of phosphate in this reaction was not at all affected. To get an idea of the mechanism of inhibition, the following very simplified sequence of reactions leading to phosphorylation of ADP is introduced:

\[
\text{ADP} + \text{CF}_1 \rightleftharpoons \text{ADP-}\text{CF}_1 \rightleftharpoons \text{ADP-}\text{CF}_1 \text{ product} \rightleftharpoons \text{CF}_1 \text{ product} \rightleftharpoons \text{CF}_1 \text{ product} + \text{CF}_1
\]

Remembering the theoretical considerations concerning a partial competitive inhibition, one can demand nitrofen affecting the steps 1, 2 or 3. But if nitrofen should affect step 3, competition like Lineeweaver-Burk plots would not have been found. This means nitrofen inhibits step 1 or 2. Employing our methods, it is impossible to differentiate between these two steps. One only can see the overall reaction of these two steps in terms of nucleotide exchange on CF$_1$.

From our experience with phosphorylation experiments one would rather say nitrofen affects affinity of CF$_1$ to nucleotides (or the binding constants) than the maximal phosphorylation rate. But, due to the mechanism of partial competitive inhibition, another possibility has to be mentioned: Retarded activation of a portion of the CF$_1$ population of each thylakoid would pretend a reduced affinity. The experiments reported by Strotmann et al. [49] proved the necessity of an activation of CF$_1$ before ATP synthesis or hydrolysis, respectively, can be catalysed. As shown by the results presented, the postulated activation steps of both reactions are inhibited by addition of nitrofen. The activation of the enzyme is paralleled by a liberation of, up to that moment, tightly bound ADP. From these connections it seems reasonable that the \( I_{50} \) were determined to be in the range of 2 \( \mu \text{M} \) for inhibition of ATP synthesis, ATP hydrolysis, and nucleotide exchange respectively.

Another effect of nitrofen is the deletion of the regulatory effect of ADP on the size of the transmembrane proton gradient. As the experiments concerning measurement of proton gradient were carried out employing non-phosphorylating conditions, one can only speculate about the possible connections between enhancement of proton gradient and regulation of the enzymatic activity of CF$_1$. As a matter of fact, nitrofen concentrations in the range of...
2 μm were found to result 50% deletion of ADP-effect. This can be understood as a reference to a correlation between these effects. Further experiments concerning this problem will be carried out.

Acknowledgements

I return thanks to Professor Dr. H. Strotmann, University of Düsseldorf, for his interest in my experiments, for managing financial support, and critical reading of the manuscript. The author is grateful to Professor Dr. P. Böger, University of Konstanz, for a sample of nitrofen. I appreciate the excellent technical assistance of Mr. K. Edelmann. This study was supported by the Deutsche Forschungsgemeinschaft (grant Str. 103/14).