Biochimica et Biophysica Acta, 724 (1983) 224–229
Elsevier

BBA 41342

EFFECTS OF NITROFEN ON CHLOROPLAST COUPLING FACTOR-DEPENDENT REACTIONS

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(Received March 2nd, 1983)

Key words: Photophosphorylation; Herbicide; Coupling factor; Nitrofen; (Spinach thylakoid)

Nucleotides induce a conformational change in the proteins of the CF_0-CF_1 complex. They give rise to reduced proton permeability of the thylakoid membranes. This reaction is paralleled by an enhanced yield of the steady-state proton uptake and a reduced nonphosphorylating electron-transport rate. Nitrofen acts as an energy-transfer inhibitor. It inhibits the rate of nucleotide exchange on CF_1 both at 'loose' and 'tight' binding sites. During illumination the percentage of nucleotide-free CF_0-CF_1 complex seems to be enhanced in the presence of nitrofen. This results in a prevention of the described ADP effects on proton uptake and electron transport. These similar effects of nitrofen on loose and tight nucleotide-binding sites correspond with the idea that both types are different states of identical sites.

Introduction

Photophosphorylation can be inhibited in three fundamentally different ways: by uncoupling electron transport from ATP synthesis, preventing electron transport, and inhibiting ATP synthesis itself. Inhibition of the last type is called energy-transfer inhibition. This means that the transfer of energy, accumulated by reactions driven by electron transport, to ATP is blocked [1].

The herbicide nitrofen (2,4-dichloro-4′-nitrophenyl ether) was found to act as an energy-transfer inhibitor [2,3]. In a preceding paper [4] we observed similar dependences on nitrofen concentration for inhibition of: (a) photosynthetic electron transport and ATP synthesis, (b) the rate of nucleotide exchange on chloroplast coupling factor, and (c) activation as well as activity of light-triggered ATPase activity. From our results we concluded the inhibition of nucleotide exchange to be the primary effect of nitrofen. The other CF_1-dependent reactions seemed to be regulated to some extent by nucleotide exchange.

In the present study we examined the effects of nitrofen, observing two different CF_1-dependent reactions in parallel tests. Thus, we wanted to investigate the cause of the reactions. Our results verified our conclusions in the preceding paper [4]. From our data we conclude the most important effect of nitrofen (with respect to energy-transfer inhibition) to be inhibition of nucleotide exchange on CF_1. The significance of our result for the understanding of energy conservation will be discussed.

Materials and Methods

The herbicide nitrofen was a gift from Professor Böger, University of Konstanz. Nucleotides, hexokinase, DCMU, DCIP, methyl viologen and phenazine methosulfate were purchased from Sigma Chemie GmbH. [8-^{14}C]ADP and $^{32}P_i$ were bought from Amersham Buchler Corp. Forced dialysis measurements were performed employing

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; Tricine, *N*-tris(hydroxymethylglycine; Chl, chlorophyll.

plastic syringes connected with 13-mm filter holders (Schleicher und Schüll, type FP). Membrane filters with a pore size of 0.01 μm (Schleicher and Schüll, type AC62) were used. Filtration was driven by nitrogen pressure.

Isolation of thylakoids from spinach leaves was carried out as described by Strotmann et al. [5]. For chlorophyll determination the method of Arnon [6] was employed. All reactions were measured at 20°C. Light intensity was 870 W/m^2. The standard reaction medium contained: 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 50 μM phenazine methosulfate, 20 μM DCMU, 2% (v/v) methanol $\pm$5 μM nitrofen, and thylakoids corresponding to 20–25 μg Chl per ml.

When measuring photophosphorylation, 5 mM phosphate, pH 8.0, labelled by $^{32}P_i$ (about 2 MBq/ml), and 0.2 mM ADP were added to the incubation mixture. The reaction was stopped by addition of $HClO_4$ at a final concentration of 0.3 M. Organic phosphate was determined as described by Sugina and Miyoshi [7].

Electron transport was measured by observing O_2 consumption in a Clark-type O_2 electrode. The reaction medium contained 5 mM ascorbate, pH 8.0, 0.2 mM DCIP, and 0.2 mM methyl viologen instead of phenazine methosulfate.

Proton uptake by illuminated thylakoids was followed by observing the proton concentration in the incubation medium. For better resolution the buffer concentration was reduced to 0.5 mM Tricine buffer, pH 8.0, in these experiments.

Nucleotide binding to chloroplast coupling factor was measured employing two different methods: (a) The centrifugation technique described by Bickel-Sandkötter and Strotmann [8] and (b) a filtration technique similar to the method of Tischer and Strotmann [9]. We used plastic syringes containing the reaction medium mixed rapidly by a strong magnetic stirrer (Colora Messtechnik). For determination of free nucleotides a sample was forced through a membrane filter by nitrogen pressure. To avoid unspecific binding preincubation with 1 mM AMP was performed. The sample was fractionated and only the second fraction was used for determination. The first fraction contained preincubation solution. The filtration technique enabled us to perform binding studies within periods of less than 5 s. The filtration apparatus made it possible to perform parallel measurement of nucleotide binding and proton uptake. Light intensity was measured inside the reaction chambers. The two methods gave identical results.

Results and Discussion

From their results, Lambert et al. [2] concluded nitrofen to act as a competitive inhibitor to ADP in the reactions of ATP synthesis. Therefore, we did not expect a direct effect on P_i interacting with the ATP synthase. This was confirmed by the results shown in Table I. The left-hand part of the table shows the effect of nitrofen on ATP synthesis in the presence of 1 mM phosphate. The right-hand part shows that phosphorylation in the presence of the highest nitrofen concentration (20 μM) still depends on phosphate concentration. In parallel experiments (data not shown) we found that at each constant nitrofen concentration tested the phosphorylation rate was still dependent on phosphate concentration. This shows that nitrofen does not affect phosphate binding or even phosphoryl transfer during ATP synthesis, but probably inhibits ADP exchange at the catalytic binding site.

As described in a preceding paper [4], nitrofen was found to act as a partially competitive inhibitor of photophosphorylation to ADP. To gain some more information about the mechanism of inhibition, measurement of interactions between CF_1 and ADP was carried out.

It is accepted that electron transport leads to proton translocation across thylakoid membranes [10]. The result is an activated state controlling the rate of electron transport [11]. Without addition of phosphate the leakage of proton out of the thylakoids is controlled by the conformational state of the CF_0-CF_1 complex. The conductivity of the complex is reduced by addition of free nucleotides. The result is an increased proton gradient across the thylakoid membranes [12–14]. It is accepted that this depends on a nucleotide-induced conformational change in the protein of the CF_0-CF_1 complex.

Fig. 1 shows an experiment where electron transport and the extent of steady-state proton uptake were measured simultaneously in the absence of added phosphate. It can be seen that

TABLE I

EFFECTS OF NITROFEN AND LIMITING PHOSPHATE CONCENTRATIONS ON PHOTOPHOSPHORYLATION

Two different experimental conditions are shown: (Left). The P_i concentration was kept constant (1 mM). ATP synthesis is inhibited by increasing concentrations of nitrofen. (Right) The inhibitory nitrofen concentration (20 μM) was kept constant. ATP synthesis is limited by lowering the phosphate concentration.

Phosphate concentration: 1 mM		Nitrofen concentration: 20 μM	
[Nitrofen] (μM)	μmol ATP/mg Chl per h	[Phosphate] (μM)	μmol ATP/mg Chl per h
0	435.8	1000	162.2
0.25	432.3	500	159.7
0.50	418.9	400	156.3
1.00	373.7	300	148.3
1.50	335.6	250	141.3
2.00	314.7	200	138.3
2.50	293.3	150	139.8
5.00	236.1	130	133.4
7.50	206.8	100	129.6
10.00	188.5	75	107.4
20.00	162.2	50	93.5

proton uptake can be enhanced by addition of ADP. This enhancement can be referred to reduced permeability of the CF_0-CF_1 complex for protons as described above. In the parallel measurement an inhibition of these electron-transport rate was observed. The ADP concentration resulting in 50% of the maximal effect was determined. Identical ADP concentrations for both reaction described were found and termed $K_{50\%}$. When employing comparable experimental conditions, $K_{50\%}$ was found to resemble K_s of the tight nucleotide-binding sites [15,16]. As shown in Fig. 1, nitrofen abolishes the effect of ADP on proton uptake and electron transport. This means that nitrofen prevents the induction of a conformational state of the CF_0-CF_1 complex less permeable to protons.

If there is a correlation between the described effect and energy-transfer inhibition the same dependence on nitrofen concentration should exist. Therefore, the effects of different nitrofen concentrations on electron transport through Photo-

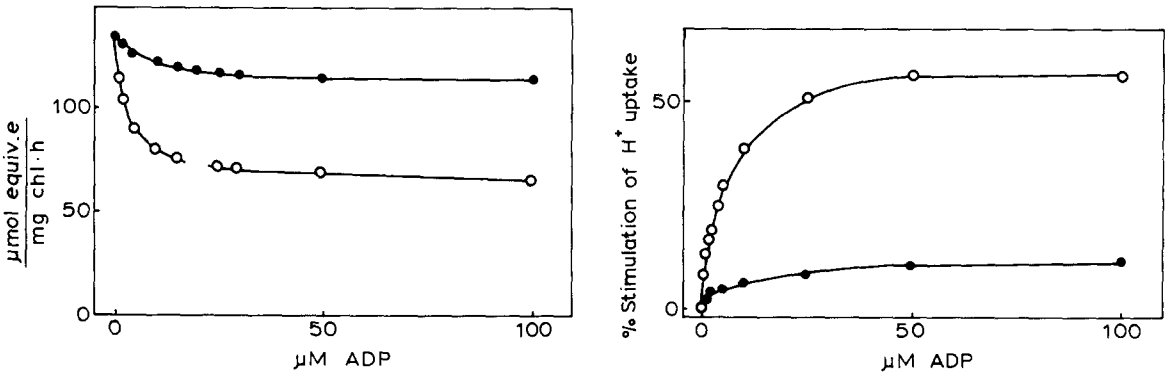

Fig. 1. Parallel measurement of electron transport and proton uptake by thylakoid membranes. After isolation thylakoids were illuminated for 2 min in the standard reaction medium without addition of DCMU, methanol, and nitrofen to reduce the concentration of bound nucleotides. Chlorophyll concentration was 10 μg/ml. Illumination was immediately followed by a centrifugation. In the absence of added phosphate the effects of increasing ADP concentrations on electron transport (left) and steady-state proton uptake (right) were studied. Filled symbols indicate addition of 5 μM nitrofen.

system I in the absence of phosphate have been measured. No effect was observed unless free nucleotides were present. In the absence of added phosphate and ADP an electron-transport rate of 141 μmol equiv./mg Chl per h was found. Addition of 1 mM ADP resulted in 46.4% inhibition. Similarly to phlorizin [1] nitrofen was found to abolish this ADP effect. The nitrofen concentration resulting in 50% of maximal stimulation of the electron-transport rate was determined. This concentration was similar to the I_{50} (2 μM) for inhibition of phosphorylation [4].

In another experiment, parallel measurement of proton uptake by illuminated thylakoid membranes and the binding of ADP to CF_1 were performed. As shown in Fig. 2 the process of nucleotide binding seems to reach completion much earlier than the steady state of proton uptake is established. The enhancement of the size of the steady-state proton gradient is apparently controlled by the degree of nucleotide binding. The second result of these experiments is that nitrofen inhibits the rate of nucleotide binding. The total number of nucleotide-binding sites does not seem to be altered. During steady-state binding in the light the amount of bound nucleotides is reduced by addition of nitrofen. Nevertheless, the total amount of rebound ADP in the dark is not affected by addition of nitrofen. A third result is that both tight and loose binding of ADP are affected by addition of nitrofen in the same way. In context with the results published in other papers [8,17,18] we understand these results as follows: The tight and loose nucleotide-binding sites represent two different states of the same site. The CF_0-CF_1-ADP complex shows lower proton conductance than the nucleotide-free complex [14]. Nitrofen inhibits the exchange rate of nucleotides on CF_1. This results in an enrichment of the nucleotide-free species and an increased leakage of protons.

Fig. 3 shows the effect of external pH on (a) binding of ADP following an acid-base transition employing a constant pH jump, (b) stimulation of the extent of proton uptake by illuminated thylakoids induced by addition of ADP, and (c) inhibition of electron transport by addition of ADP in the absence of phosphate. The parallel effect of medium pH on these three reactions leads

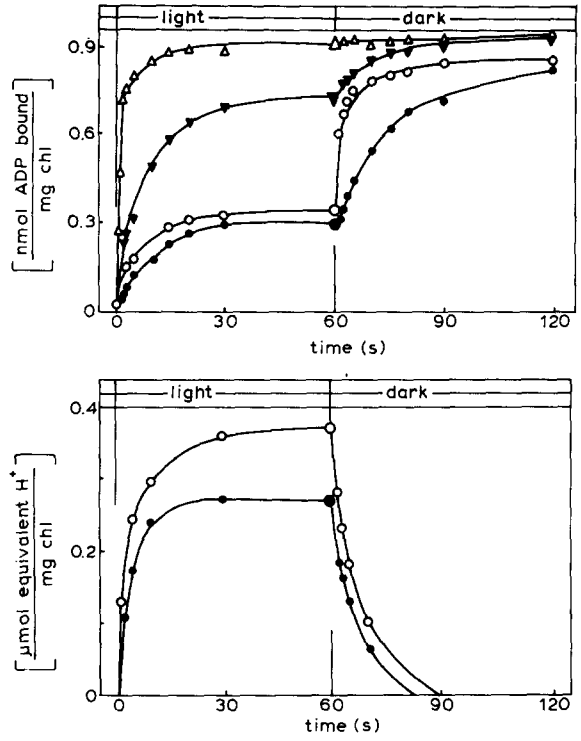

Fig. 2. Parallel measurement of nucleotide binding to CF_1 and proton uptake by thylakoid membranes. After isolation thylakoids were pretreated as described in Fig. 1. The upper panel shows a nucleotide-binding experiment, the lower the kinetics of proton uptake by thylakoid membranes. Both experiments were performed in syringes. Tightly bound ADP (○/●) means nucleotides which could not be exchanged for 10 mM unlabeled ADP added. Subtracting tightly bound ADP and total amount of bound ADP (△/▼) results in the amount of nucleotides bound under steady-state conditions. The control experiments are shown employing open symbols (○/△) while the results of measurements in the presence of 5 μM nitrofen are shown by filled symbols (●/▼).

to the conclusion that, as proposed by Gräber et al. [14], nucleotide binding regulates proton conductivity of the CF_0-CF_1 complex. This also causes a control of the transmembrane proton gradient and electron-transport rate.

In the next experiment the effect of nucleotides on the conformational state of the CF_0-CF_1 complex in the presence of phosphate was tested. Steady-state rates of electron transport and ATP synthesis have been measured in parallel. Fig. 4 shows that at low ADP concentrations ATP synthesis takes place very slowly. Therefore, an

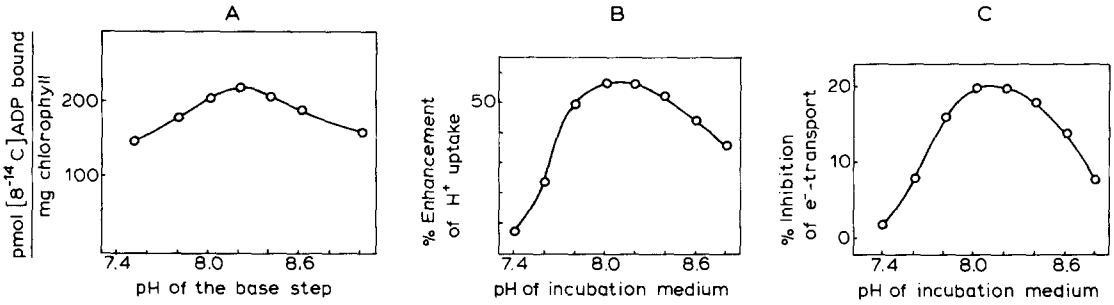

Fig. 3. Comparison of the pH effects on electron transport, nucleotide binding, and proton uptake by isolated thylakoid membranes. Panel A shows the binding of ADP to CF_1 induced by an acid-base transition. Starting with different pH values of the acid step, pH jumps of constant 3.5 pH units were employed using the method of Huchzermeyer and Strotmann [22]. In panel B the stimulation of the extent of the steady-state proton uptake by thylakoids induced by addition of 1 mM ADP was measured. The inhibition of nonphosphorylating electron transport by addition of 1 mM ADP is shown in panel C. As shown, the extent of the inhibition depends on the pH of the incubation medium. As the electron-transport rate is pH dependent itself, the % inhibition is shown. At pH 7.4, the electron-transport rate of the control was 98 μmol equiv./mg Chl per h.

increase in ADP concentration at first results in an inhibition of electron transport due to an increased concentration of the CF_0-CF_1-ADP complex. When ATP synthesis is accelerated with increasing ADP concentrations, a stimulation of electron transport parallel to an increased proton leakage through the phosphorylating pathway can be observed. An addition of nitrofen abolishes the inhibitory effect of low ADP concentrations. 50% recovery of the electron-transport rate was achieved by addition of 3 μM nitrofen. This points to an inhibition of nucleotide exchange being the reason for the observed effect.

Our results confirm the thesis that nucleotide binding to CF_1 affects the conformational state of the CF_0-CF_1 complex. This reaction is indicated by a reduced proton conductance of the proton channel, resulting in an increased transmembrane proton gradient in the steady state [14,19]. The nucleotide effect takes place whether or not free phosphate is present. Employing phosphorylating conditions, the nucleotide effect on the size of the proton gradient normally is obscured by proton consumption due to ATP synthesis. One inhibitory effect of nitrofen seems to be based on abolishing the nucleotide effect. As nucleotide-binding sites have been found on CF_1 only [19], nitrofen is thought to interact with this protein. We observed a parallel effect of nitrofen on the rates of nucleotide exchange, proton uptake, and electron trans-

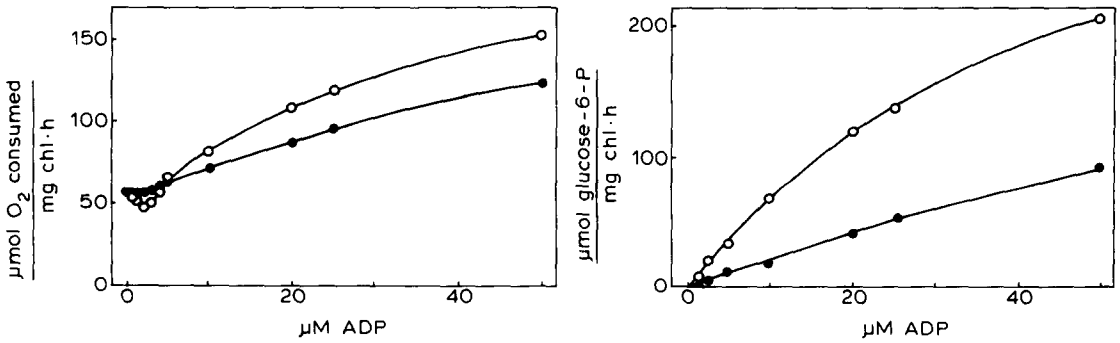

Fig. 4. Parallel measurement of electron transport and ATP synthesis. Chloroplasts were pretreated as described in Fig. 1. The incubation medium contained additionally 10 mM glucose and 30 U/ml hexokinase. The effects of increasing ADP concentrations on steady-state rates of electron transport (left) and ATP synthesis (right) are shown. Open symbols indicate the control experiments, filled ones show the results measured in the presence of 5 μM nitrofen. glucose-6-P, glucose 6-phosphate.

port. Therefore, we conclude a regulation of proton translocation and electron transport by nucleotide binding to CF_1 in agreement with other publications [10,12–14,19–21]. But, when considering the extent of nucleotide exchange being regulated by the energetic state of the membranes [8], it becomes difficult to understand the significance of the regulatory effect of nucleotide binding.

The fact that we observed similar effects of nitrofen on tight and loose nucleotide-binding sites would correspond with the idea that both types are different states of identical sites. It is still a controversial question as to whether one of these states represents the catalytically active site of ATP synthesis. This would imply an explanation for a more direct inhibition of ATP synthesis by nitrofen: The herbicide inhibits interaction between CF_1 and one of its substrates (nucleotides). This explanation corresponds with the finding [4] that energy-transfer inhibition of phosphorylation is paralleled by an inhibition of coupled electron transport.

Acknowledgements

The authors are grateful to Professor Dr. P. Böger, University of Konstanz, for a sample of nitrofen. We wish to thank K. Edelmann for excellent technical assistance and construction of the filtration apparatus. This study was supported by the Deutsche Forschungsgemeinschaft (Grant Hu 332/1-2).

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