

Diphenyl Ether Herbicides, a Tool to Elucidate the Mechanism of Photophosphorylation

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At least two different classes of ADP binding sites on chloroplast coupling factor are described in the literature. High-affinity sites are assumed to entail regulatory functions while low-affinity sites are involved in catalysis.

Diphenyl ether herbicides, acting as energy transfer inhibitors, interfere with nucleotide exchange on both categories of ADP binding sites. Their inhibitory potency varies depending on their substitution. We found that each diphenyl ether assayed revealed identical I_{50} values for inhibition of nucleotide interaction with both classes of binding sites.

We show here that diphenyl ether inhibition of energytransfer is primary based upon the interference with ADP binding to high-affinity binding sites. Thereby the control of proton permeability through the coupling factor complex is affected. Moreover, we found that the three β -subunits are not absolutely fixed in one conformational state: After covalently blocking the high-affinity site by an azido-label, a new high affinity state appeared on another β -subunit.

Introduction

Diphenyl ether herbicides cause three independent inhibitory effects: I) inhibition of photosynthetic electron transport between the two photosystems [1–4]; II) light induced bleaching [5–7]; III) energy-transfer inhibition by blocking the nucleotide exchange on the ATP-synthesizing enzyme, the chloroplast coupling factor (CF₁) [4, 8, 9]. In our research project we wanted to use these energy-transfer inhibitors as tools to elucidate the mechanism of photophosphorylation.

In two preceding papers [4, 9] we described the mechanism of energy-transfer inhibition produced by diphenyl ethers. It is completely different from the one described for other inhibitors like DCCD, tentoxin, phloridzin, Dio-9, and arylestere of nucleotides [10–12]. We will show that our most recent results fit in well with the proposed reaction sequence leading to phosphorylation, though at first some of the results seem to disagree with the current understanding of the mechanism of ATP synthesis. Moreover they prove the existence of at

least two different types of nucleotide binding sites.

Experimental procedures

Materials

Nitrofen was purchased from Dr. S. Ehrensdofer, Augsburg. 8-azido-nucleotides, buffers, and column materials were obtained from Sigma Chemie GmbH, Deisenhofen. 8-azido-[α]- 32 P ATP was purchased from ICN Biomedicals GmbH, Eschwege. 14 C-labeled nucleotides and 32 P_i were obtained from Amersham Corp., Braunschweig.

Methods

Chloroplast isolation from spinach leaves was carried out as described by Strotmann *et al.* [13]. For chlorophyll determination Arnon's method [14] was used. Non-cyclic electron transfer from water to ferricyanide was measured by following the decline of 420 nm absorbance as described in a previous paper [4]. Phosphorylation was followed by the incorporation of 32 P_i into ATP using the method of Sugino and Miyoshi [15] with slight modifications [16]. P/e₂ ratios were determined by parallel measurements of the rate of electron transport from reduced DCPIP to MV and ATP synthesis [16]. Nucleotide binding to chloroplast coupling factor was examined as described by Huch-

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zermeyer [17]. Thiol modification of CF₁ was performed as published by Ketcham *et al.* [18]. Using such modified thylakoid preparations, ATPase activity was determined from the hydrolysis of [γ]-³²P ATP from aliquots taken at intervals of 3 sec [17].

Results and Discussion

After prior activation of chloroplast coupling factor (CF₁) binding of nucleotides induces conformational changes in the protein. This causes a reduced proton permeability of thylakoid membranes and therefore is paralleled by an enhanced yield of the steady state proton uptake and a reduced nonphosphorylating electron transport rate [10, 19]. Diphenyl ether herbicides interfere with nucleotide exchange on CF₁. As shown in Table I each of the diphenyl ethers assayed shows similar *I*₅₀ values for the nucleotide dependent reactions. In agreement with the literature [20] apparently two types of nucleotide binding sites can be distin-

guished: "tight" and "exchangeable" sites. Both of them have catalytic capacity. Tight binding sites have a high affinity to ADP. Nucleotide binding to these sites has regulatory effects: reduction of proton leakage through the CF₀CF₁ complex (s.a.) and inhibition of ATPase activity. Exchangeable sites show a ten-fold lower affinity to ADP, but nucleotide exchange on these sites under phosphorylating as well as under non-phosphorylating conditions takes place at a high rate comparable to the rate of phosphorylation. For inhibition of nucleotide exchange on these sites we determined the same *I*₅₀ values as for photophosphorylation with all tested diphenyl ethers. From the sequence of diphenyl ethers shown in Table I it is suggested that for energy-transfer inhibition electronegativity is dominant over the lipophilic parameter. But some irregularities indicate that additionally other (steric) parameters play an important role.

In agreement with Lambert *et al.* [8] we published that a complete inhibition of the catalytic functions of CF₁ (ATP synthesis and hydrolysis as

Table I. Effect of different diphenyl ethers on nucleotide-dependent reactions.

Position of substituent					p <i>I</i> ₅₀ Values		Sum of lipophilicity constants (π)	Sum of sigma-Hammett values (σ)
2	4	6	2'	4'	Regulatory nucleotide binding	ATP synthesis		
	Br			Br	3.87	3.91	3.93	1.71
				NO ₂	4.65	4.71	4.63	-0.55
	Cl			NO ₂	4.83	4.82	4.85	0.23
	Cl		NO ₂		5.11	5.06	5.04	0.23
Cl	Cl			NO ₂	5.70	5.65	5.63	1.14
	Cl		NO ₂	Cl	5.32	5.34	5.31	1.14
Cl	Cl	Cl		NO ₂	5.28	5.32	5.29	1.85
			NO ₂	NO ₂	4.52	4.53	4.54	-0.56
Cl	Cl		NO ₂	CF ₃	4.83	4.85	4.84	2.02
NO ₂	CF ₃			NO ₂	no exp.	3.78	3.81	0.31

Regulatory nucleotide binding: Two parallel experiments have been performed giving identical results. In the first experiment reduction of basal electron transport rate after addition of 1 mM ADP has been measured by a Clark electrode. In the second one the stimulation of proton uptake by illuminated thylakoids after addition of 1 mM ADP has been measured. The standard incubation medium contained 30 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 0.2 mM methyl viologen, 2.5 mM ascorbate, pH 8.0, 0.2 mM diaminodurene and thylakoids corresponding to 30 μ g chlorophyll per ml. When measuring proton uptake, tricine buffer was omitted.

ATP synthesis: Thylakoids corresponding to 23 μ g chlorophyll per ml have been illuminated in a temperature-controlled cuvette at 20 °C for 20 sec using white light from a slide projector (820 W/m² measured inside the cuvette). The incubation mixture was the same as described under Fig. 1.

ATP hydrolysis: Thiol modified thylakoids were reactivated by 30 sec of illumination. When the light was switched-off 0.2 μ M gramicidine and different concentrations of the diphenyl ethers were added. After another 5 sec in the dark [γ -³²P]ATP at a final concentration of 500 μ M was added. ATPase activity was determined by taking aliquots at intervals of 5 sec. In these experiments the chlorophyll concentration was adjusted to 30 μ g/ml.

well as the regulation of coupled electron transport rate) can not be found with nitrofen [4, 9]. Most of the other diphenyl ethers showed even poorer inhibitory effects. Nevertheless the reduction of proton permeability through the CF_0CF_1 complex induced by ADP binding was completely diminished by nitrofen [9]. This result indicates that different types of CF_1 -ADP interactions are affected by diphenyl ethers. — In succeeding experiments we used nitrofen as an example. — As the mechanism of photophosphorylation still is an open question, the current state of knowledge is summarized as follows.

It is generally accepted that the catalytically active sites are located on the three β -subunits of the CF_1 portion of the enzyme complex. These sites are identical in their amino acid sequence [20]. But with respect to their asymmetric arrangement within the total protein with a subunit composition of $\alpha_3\beta_3\gamma\delta\epsilon$ different states of the β -subunits within the same protein are predicted [21]. After several hypotheses have been discussed in the past, currently an indirect way of participation of protons in ATP synthesis is supposed. Boyer [22] and Slater [23] have proposed mechanisms in which conformational changes in the CF_0CF_1 complex are essential. They are attained by controlled protonation/deprotonation reactions producing changes in substrate and product binding affinities. In the latest version of Boyer's "binding change mechanism for ATP synthesis" three catalytic subunits of the enzyme complex are assumed to cooperate [24, 25]. It is strictly ruled out that 1) one ADP binding site may have regulatory functions and that 2) any structural changes are induced by proton motive force other than those for release of tightly bound nucleotides from catalytic sites. These statements are questioned by other authors (see Strotmann and Bickel-Sandkötter for a review [20]). They believe that one β -subunit, differing from the two others in its orientation with respect to the central mass of the three smaller subunits, might possess regulatory functions. Nevertheless this hypothesis lacks further experimental prove, too. The existence of a functionally heterogenic population of β -subunits has already been observed in Boyer's lab [26].

We tried to reduce the discussions concerning the mechanism of photophosphorylation to a simple question solvable experimentally: If the critics

of Boyer's hypothesis are right, high affinity binding of ADP to one site on one of the three β -subunits of CF_1 is essential for the regulation of the enzyme. Due to the observation that proton permeability is reduced in the presence of ADP (s. a.), we concluded that nucleotide binding might regulate the efficiency of phosphorylation. Therefore we measured P/e_2 ratios with increasing concentrations of added nitrofen. As shown in Fig. 1 we found a reduction of the P/e_2 ratios in the presence of the herbicide. 50% of the maximal effect was observed with $2.7 \mu\text{M}$ nitrofen. This value is close to the I_{50} for phosphorylation of $2 \mu\text{M}$ (Table I). We think this result indicates an inhibition of the ADP induced conformational change in the coupling factor. This interpretation implies that there is a "slip in the pump" without "regulatory binding" of ADP. To prove this assumption we tried to block the high affinity ADP binding site by covalent binding of 8-azido-ADP. Indeed we

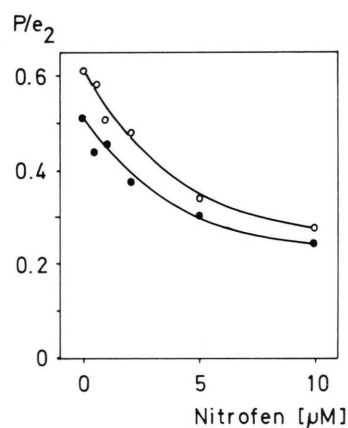


Fig. 1. Reduction of P/e_2 ratios of PS I driven photophosphorylation by nitrofen. Thylakoids corresponding to $25 \mu\text{g}$ chlorophyll per ml have been illuminated in a Clark electrode at 20°C for 1 min. Then a sample of the incubation medium was removed and analyzed for synthesized $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using the method of Sugino and Miyoschi [15]. The incubation medium contained 30 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM MgCl_2 , 0.2 mM methyl viologen, 2.5 mM ascorbate, 0.2 mM diamino-durene, 0.5 mM ADP, 5 mM phosphate labeled by $^{32}\text{P}_i$ ($0.2 \text{ MBq}/\mu\text{mol}$), 1% (v/v) methanol, $25 \mu\text{g}/\text{ml}$ chlorophyll, and nitrofen at indicated concentrations. Maximal rate of O_2 consumption was $558 \mu\text{mol O}_2 \cdot (\text{mg chlorophyll} \cdot \text{h})^{-1}$. Maximal P/e_2 ratio measured in this experiment was 0.61 (○). In a parallel experiment (●) high-affinity binding sites for ADP were blocked by labeling with 8-azido-ADP prior to the experiment as described under Fig. 2a.

found a reduction of proton permeability comparable to the effect of ADP with 2 mM 8-azido-ADP. But during the measurement of basal electron transport rate or the proton gradient, increasing amounts of the label were covalently bound to the enzyme and, as shown in control experiments, the catalytic capacity of CF_1 was severely inhibited. After two minutes of illumination with actinic light for electron transport about 1.5 to 1.7 mol of 8-azido-ADP were covalently bound per mol of CF_1 and the rate of phosphorylation was inhibited to about 50%. Therefore we decided to introduce the label into the high-affinity site more specifically. As described in the legend of Fig. 2 we first generated a nucleotide-depleted form of the coupling

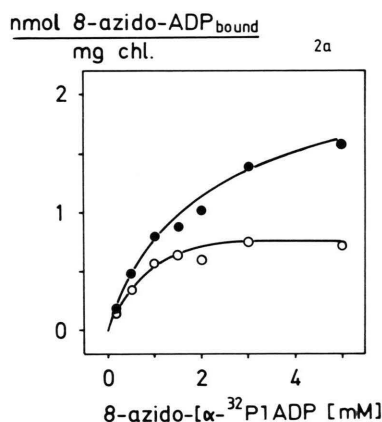
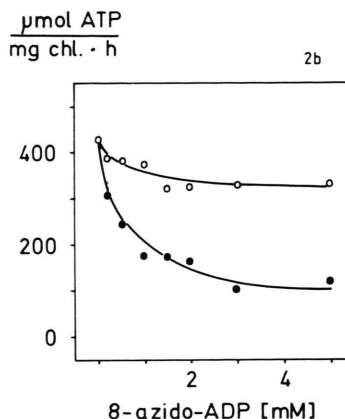
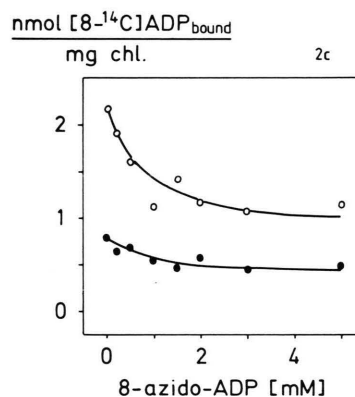


Fig. 2. Effects of covalent labeling of high affinity nucleotide binding sites. a. Binding of 8-azido-[α - ^{32}P]ADP to thylakoid membranes. Thylakoids corresponding to 100 μ g chlorophyll per ml were illuminated for 2 min at 20 °C in a medium containing 30 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 50 μ M PMS (phenazine methosulfate). Then the mixture was centrifuged in an Eppendorf centrifuge. The pellets were resuspended using a medium containing 1 mM AMP and indicated concentrations of 8-azido-ADP instead of PMS. After 1 min of incubation in an ice bath ADP at a final concentration of 10 mM was added. The samples were centrifuged and washed two times using the same medium without added nucleotides. The samples were diluted to give a chlorophyll concentration of about 20 μ g/ml. Then they were illuminated to activate the photolabel (see ref. [29]). After a centrifugation and another washing step the suspension was analyzed for radioactivity in presence of a scintillation cocktail (Beckman EP). The chlorophyll content of the samples was determined from the channel ratio (○). In a parallel experiment (●) the addition of ADP after preincubation with the azido analog was omitted.



b. Inhibition of ATP synthesis by covalent binding of 8-azido-ADP. Thylakoids have been treated as described above. In the first experiment (○) binding of 8-azido-ADP to low affinity sites was prohibited by addition of 10 mM ADP before activation of the photolabel. In the parallel experiment (●) this step was omitted. The reaction mixture in the phosphorylation experiment contained 30 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM ADP, 5 mM phosphate labeled with $^{32}P_i$ (0.18 MBq/ μ mol), 50 μ M PMS, and thylakoids corresponding to 12 μ g chlorophyll/ml.



c. [^{14}C]ADP binding after prior labeling of the high affinity site with 8-azido-ADP. Thylakoids have been labeled with 8-azido-ADP prior to measurement of [^{14}C]ADP binding. Low-affinity binding sites were protected by addition of unlabeled ADP as described above. Total binding of [^{14}C]ADP (○) was measured using the method of Huchzermeyer [17]. For determination of high-affinity binding (●) the method of Strotmann *et al.* [13] was used. The incubation mixture in the binding experiment contained 30 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 50 μ M PMS, 100 μ M [^{14}C]ADP (0.18 MBq/ μ mol), and thylakoids corresponding to 37 μ g chlorophyll per ml.

factor, known to bind nucleotides to its high-affinity site in a non-exchangeable manner [20]. The photolabel was activated after washing away the surplus label. We believed to have blocked the high-affinity ADP binding sites of the coupling factor by this method. If the inhibition of the regulatory effects by nitrofen was due to its inhibitory effect on nucleotide exchange, nitrofen should not affect P/e_2 ratios with such preparations. But we found that the P/e_2 ratios measured after covalently blocking the high-affinity sites for ADP still were sensitive to nitrofen (Fig. 1).

We first suspected that in our experiments we did not succeed in blocking the high-affinity sites. Therefore we checked our method of covalent labeling of nucleotide binding sites. As shown in Fig. 2a we found that we obviously were able to label a high-affinity site covalently. Severe inhibition of phosphorylation was found as soon as more than one site per coupling factor was blocked by binding of 8-azido-ADP (Fig. 2b). But the result of a parallel nucleotide binding experiment was a surprise to us: Our data indicate that there still was a high-affinity binding site for ADP and another one showing a lower affinity to ADP (Fig. 2c).

These results indicate that the asymmetry of the nucleotide binding sites on the β -subunits of the coupling factor may alter. Similar results have been published already by Shapiro and McCarty [27]. Catalysis proceeds pretty well as long as two sites may cooperate. If more than one nucleotide

binding site is covalently blocked, photophosphorylation is severely inhibited. This result is comparable to earlier findings of Wagenvoort *et al.* [28]. From computer simulations it appears to us that for complete inhibition of ATP synthesis covalent blocking of three nucleotide binding sites is necessary. But as the calculated residual rate of phosphorylation after binding of two mols 8-azido-ADP per mol of CF_1 is only 2 to 5% of the control rate, such results need experimental prove using an analog with a higher affinity to the nucleotide binding sites.

The effect of diphenyl ether herbicides may be summarized as follows: Herbicides like nitrofen primary act on the high-affinity type of the ADP binding sites. The control of proton permeability through the CF_0CF_1 complex exerted by nucleotide binding is diminished by nitrofen. At a lower extent, the nucleotide exchange on low-affinity binding sites is affected as well. But while the effects of nucleotide binding to the high-affinity sites on the conformation of the CF_0CF_1 complex can be diminished by nitrofen, catalysis cannot be inhibited by diphenyl ether herbicides completely.

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