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Effects of Hydroxamic Acids Isolated from Gramineae on Adenosine 5′-triphosphate Synthesis in Chloroplasts[1]

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ABSTRACT

Two hydroxamic acids isolated from maize extracts, 2,4-dihydroxy-7-methoxy-1,4-(2H)-benzoxazin-3(4H)-one (DIMBOA) and the 2-*O*-β-D-glucopyranoside of DIMBOA, inhibit photophosphorylation by spinach chloroplasts. Both cyclic and noncyclic photophosphorylations were inhibited to the same extent. The concentrations producing 50% inhibition for DIMBOA and its glucoside were about 1 and 4 millimolar, respectively. These compounds inhibit coupled electron transport but do not affect basal or uncoupled electron transport. Both acids inhibit the ATPase activities of membrane-bound coupling factor 1 (CF_1) and of purified CF_1. On the basis of these results, it is concluded that DIMBOA and its glucoside act as energy transfer inhibitors of photophosphorylation.

ATP synthesis in chloroplasts is catalyzed by an enzyme complex associated with the thylakoid membrane. It is composed of two parts: CF_1[2], which protrudes from the membrane towards the stroma, and F_o, which is embedded in the membrane. One of the main experimental approaches to study the mechanism of photophosphorylation is the use of specific inhibitors. Conversely, from the type of effects produced by an inhibitor, its mode of action may be deduced. Inhibitors affect light-dependent ATP synthesis in chloroplasts by blocking electron transport coupled to ATP synthesis, by uncoupling ATP synthesis from electron transport, or by directly inhibiting phosphorylation reactions. This latter inhibition (energy transfer inhibition) may be localized at F_o or at CF_1. For example, while phloridzin (8), efrapeptine (11), DI0-9 (12), *N*-ethylmaleimide (13), and several alkaloids (1, 18) appear to inhibit isolated CF_1, dicyclohexylcarbodiimide (14) and triphenyltin chloride (6) seem to interact with F_o.

Cyclic hydroxamic acids isolated from extracts of certain Gramineae exhibit inhibitory effects in a wide range of organisms (2, 25). The most abundant of these acids is DIMBOA (Fig. 1), which is found as a glucoside in intact tissue (7). The mode of action of DIMBOA and its glucoside is presently unknown. In this paper, we report on the effect of DIMBOA and DIMBOA-Glc on ATP synthesis in class II chloroplasts.

MATERIALS AND METHODS

Isolation of Compounds. Compounds were isolated from 7-day-old seedlings of *Zea mays* L. cv. LH Rinconada grown under continuous light at 28 ± 3 C. DIMBOA was isolated from ether extracts of plants macerated at room temperature and was characterized by a procedure described previously (2). DIMBOA-Glc was isolated from aqueous extracts of boiled leaves, by passing the extract through an SP-Sephadex-Fe column (3) and, further, through a Sephadex G-10 column (7). The UV, IR, and nuclear magnetic resonance spectra of DIMBOA and DIMBOA-Glc were the same as reported previously (5, 22).

Chloroplast Isolation. Chloroplasts were isolated from market spinach leaves (*Spinacia oleracea* L.), as described (18), and resuspended in 250 mM sucrose, 20 mM Tris-HCl (pH 7.8), and 5 mM $MgCl_2$. Chl content in the chloroplasts was determined according to Whatley and Arnon (24).

ATP Synthesis and Electron Transport. ATP synthesis was determined in the same medium used for chloroplast resuspension with the addition of 2 mM ADP, 3 mM K-phosphate containing 10^6 cpm of ^{32}P, and either 50 μM phenazine methosulfate or 100 μM methyl viologen and 500 μM NaN_3 (1 ml total volume). Chloroplasts equivalent to 10 μg Chl were used per test tube. Duplicate experiments were performed. Incubations were carried out at 25 C, in the dark or in saturating light provided by one 500-w halogen lamp. The reaction was stopped after 2 min by selectively precipitating Pi as phosphomolybdate with triethylamine (20). The tubes were centrifuged, and 0.5 ml of the supernatant was diluted with 10 ml H_2O and counted in a Beckman LS-233 liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Electron transport from H_2O to methyl viologen was measured as O_2 uptake with a Clark electrode and a Gilson oxygraph. The reaction medium (1.50 ml) contained 100 mM sucrose, 10 mM NaCl, 25 mM Tricine/NaOH (pH 8), 5 mM $MgCl_2$, 100 μM methyl viologen, 1 mM NaN_3, and an amount of chloroplasts equivalent to 60 μg of Chl. Basal electron transport was measured in the presence of 150 μM added ATP, coupled electron transport in the presence of 2 mM ADP and 2 mM Pi, and uncoupled electron transport in the presence of 150 μM ATP and 10 mM methylamine.

Ca^{2+}-ATPase Activity. Trypsin-activated Ca^{2+}-ATPase activity of chloroplasts was determined according to Lien and Racker (10). The activation with trypsin was carried out in a reaction medium (1 ml) containing 40 mM Tris-HCl (pH 8), 2 mM EDTA, 1 mM

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[2] Abbreviations: CF_1, chloroplast coupling factor 1; F_o hydrophobic membrane portion of the ATPase complex; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3(4H)-one; DIMBOA-Glc, 2-*O*-β-D-glucopyranoside of DIMBOA.

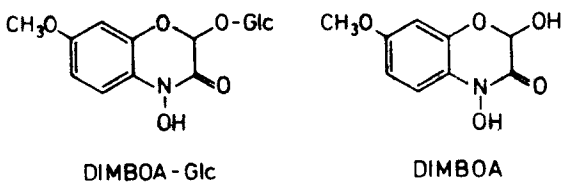

FIG. 1. Cyclic hydroxamic acids isolated from maize extracts.

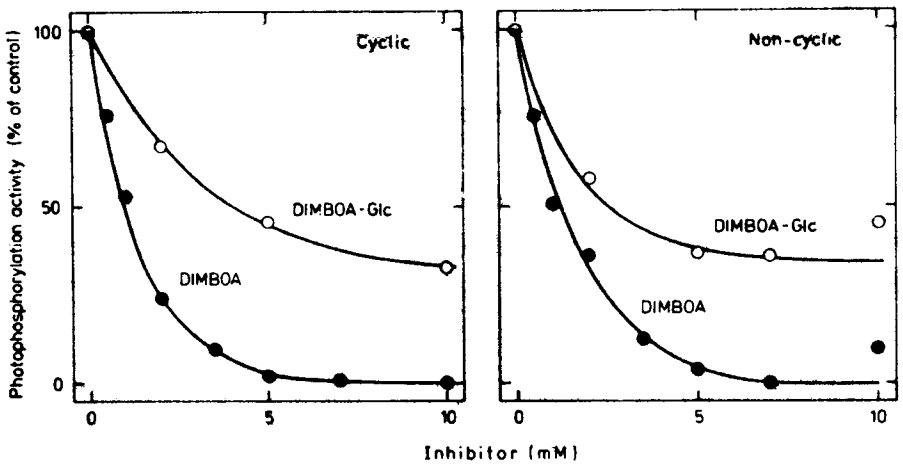

FIG. 2. Inhibition of cyclic (catalyzed by phenazine methosulfate) and noncyclic (coupled to electron transport between H_2O and methyl viologen) photophosphorylations in spinach class II chloroplasts by DIMBOA (●) and its glucoside (○). Activities for controls were 230 and 40 μmol ATP/mg Chl·h, respectively.

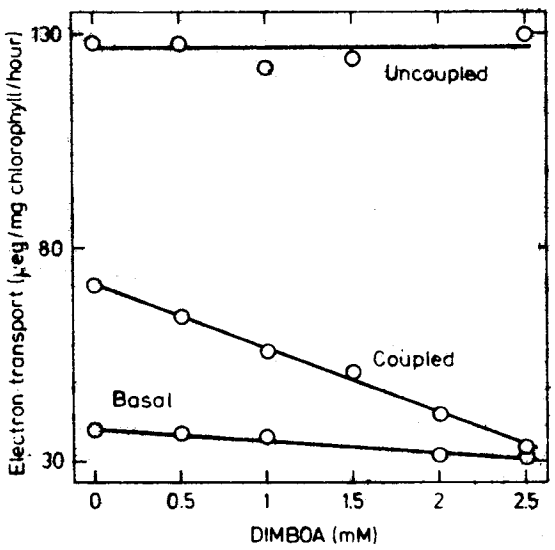

FIG. 3. Effect of DIMBOA on photosynthetic electron transport from H_2O to methyl viologen. Conditions are described in "Materials and Methods."

ATP, chloroplasts equivalent to 200 μg Chl and 800 μg trypsin. After 10 min at 25 C, 1.6 mg trypsin inhibitor were added. Thereafter, 0.1-ml aliquots were transferred to 0.9 ml of a reaction medium containing 40 mM Tris-HCl (pH 8), 5 mM $CaCl_2$, and 5 mM ATP. After 10 min at 37 C, the reaction was stopped by addition of 1 ml 0.5 N TCA. The reaction medium was centrifuged, and free Pi in the supernatant was determined colorimetrically (21).

CF_1 was prepared from chloroplasts as described (10). Electrophoresis on polyacrylamide gels showed that it was at least 95% pure. Its activation by trypsin or by heat was carried out as described (10) in a medium (1 ml) containing 40 mM Tris-HCl (pH 8), 5 mM $CaCl_2$, 5 mM ATP, and 5 μg of the activated enzyme.

In all assays, the inhibitors were added immediately before the addition of chloroplasts or enzyme preparations.

RESULTS

Inhibition of Cyclic and Noncyclic Photophosphorylations by DIMBOA and DIMBOA-Glc. Cyclic photophosphorylation, catalyzed by phenazine methosulfate, and noncyclic photophosphorylation, coupled to electron transport from H_2O to methyl viologen, were inhibited to the same extent by DIMBOA (Fig. 2). DIMBOA-Glc also inhibited both processes. The concentration of glucoside necessary to produce 50% inhibition was approximately 4 times greater than that of DIMBOA.

Effect of DIMBOA and DIMBOA-Glc on Photosynthetic Electron Transport. The inhibition of photophosphorylation described may be due to an effect of the inhibitors on either electron transport or energy transfer reactions. Figure 3 shows that DIMBOA affects neither basal electron transport nor electron transport uncoupled by methylamine. In addition, Figure 3 shows that electron transport from H_2O to methyl viologen coupled to ATP synthesis is inhibited to the basal level in a concentration range similar to that affecting photophosphorylation. A similar behavior was observed with the glucoside (Fig. 4). Figure 4 also shows that electron transport inhibition by DIMBOA-Glc is reversed by addition of the uncoupler. Therefore, according to Izawa and Good (8), these compounds may be classified as energy transfer inhibitors.

Inhibition of ATPase activity of CF_1. CF_1, bound to the thylakoid membrane, has a latent ATPase activity that may be triggered by illumination in the presence of thiol reagents or by treatment with trypsin (15). When CF_1 is detached from the membranes, its

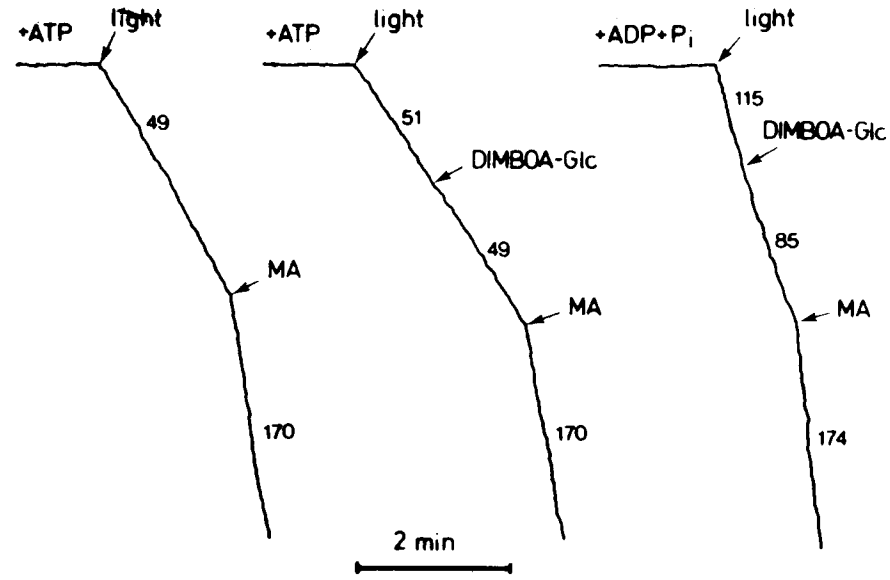

FIG. 4. Effect of DIMBOA-Glc on photosynthetic electron transport from H_2O to methyl viologen. Numbers over the slopes indicate the rate of electron transport in μmol O_2/mg Chl·h.

Table I. *Effect of DIMBOA and its Glucoside on ATPase Activities of Spinach Class II Chloroplasts and Isolated CF_1*

Preparation	Inhibitor	Concentration	ATPase[a]
		mM	*activity*
Chloroplasts activated by trypsin	Control	0	78.0
	DIMBOA	2	17.2
	DIMBOA	5	6.2
	DIMBOA-Glc	5	46.8
CF_1 activated by trypsin	Control	0	12.5
	DIMBOA	2	7.7
	DIMBOA	5	5.5
	DIMBOA-Glc	5	9.8
CF_1 activated by heat	Control	0	13.7
	DIMBOA	2	8.5
	DIMBOA	5	4.8
	DIMBOA-Glc	5	11.5

[a] Activities in chloroplasts are expressed in μmol Pi/mg Chl·h and in CF_1 in μmol Pi/mg protein·min.

ATPase activity can be unmasked by heating (23), by treatment with trypsin, or by prolonged incubation with thiols (4, 15). Table I shows that DIMBOA and its glucoside inhibit ATPase activity of chloroplasts and of soluble CF_1 activated by different treatments.

DISCUSSION

DIMBOA and its glucoside inhibited ATP synthesis and coupled electron transport but affected neither basal nor uncoupled electron transports in spinach class II chloroplasts. These results show that these compounds act as energy transfer inhibitors. Furthermore, it was shown that part of the inhibition was due to an interaction with the CF_1 component of the enzyme. DIMBOA inhibited ATP synthesis in chloroplasts of spinach, a plant that lacks this hydroxamic acid. The effects of this compound on chloroplasts of Gramineae are not known. The naturally occurring concentrations of these hydroxamates (up to 7 mmol/kg fresh weight) (2) suggest compartmentation at tissue and/or cellular level in such a way that they would not affect chloroplast ATP synthesis *in vivo* in Gramineae.

The activity of DIMBOA as an energy transfer inhibitor could explain some of its inhibitory activity on a wide range of organisms. Preliminary experiments with mitochondrial preparations show that these organelles have about one-half of the sensitivity of chloroplasts to DIMBOA. The ATPase complexes that catalyze ATP synthesis in mitochondria and chloroplasts are similar with respect to mol wt, subunit composition, and coupling activity (16, 17). However, they differ immunologically and in sensitivity towards other inhibitors. For example, the two well known inhibitors of oxidative phosphorylation, oligomycin and aurovertin, do not affect photophosphorylation (19), although aurovertin inhibits the soluble ATPase (9). It would be desirable to test the activity of DIMBOA on the ATPase complex of bacterial membranes and of mitochondria from different sources, in particular from plant pathogens.

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