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INHIBITION OF ATPase FROM CHLOROPLASTS BY A HYDROXAMIC ACID FROM THE GRAMINEAE

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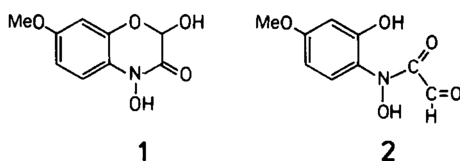
Key Word Index—Gramineae; resistance factor; 1,4-benzoxazin-3-ones; chloroplast coupling factor; thiols.

Abstract—DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), a hydroxamic acid from the Gramineae involved in the resistance of cereals to aphids, inhibits energy transfer reactions. In this paper the effect of DIMBOA on the ATPase activity of chloroplast coupling factor 1 (CF_1) was studied. A rapid inactivation of the enzyme was observed which increased with the concentration of DIMBOA. This inactivation was reversed by dilution and by filtration of the inhibitor. The inactivation was partially prevented by pre-treatment of the enzyme with iodoacetamide. A slower simultaneous inactivation was observed which followed pseudo-first order kinetics. It is suggested that part of the rapid inhibition of CF_1 ATPase caused by DIMBOA is due to its reaction with sulfhydryl groups on the enzyme. These reactions of DIMBOA may explain some of the inhibitory properties of DIMBOA on a wide range of organisms.

INTRODUCTION

Hydroxamic acids isolated from extracts of the Gramineae such as wheat, maize and rye [1] inhibit bacterial [2] and fungal [3] growth as well as insect development and reproduction [4–7]. These hydroxamic acids have been suggested as resistance factors of maize against the European corn borer *Ostrinia nubilalis* [4] and the aphid *Rhopalosiphum maidis* [5], and of wheat against other aphids [6, 7]. Recently DIMBOA (**1**), the main hydroxamic acid in maize extracts, was shown to inhibit energy transfer reactions [8]. The properties of DIMBOA as an energy transfer inhibitor may account for its widespread toxicity [8]. It was additionally shown that DIMBOA inhibits chloroplast coupling factor 1 (CF_1). DIMBOA reacts with sulfhydryl compounds forming addition and/or reduction products [9, 10]. In this paper the inhibition by DIMBOA of the ATPase activity of CF_1 from spinach chloroplasts is further characterized. It is suggested that this inhibition is partly due to the reaction of DIMBOA with sulfhydryl groups on the enzyme.



RESULTS

DIMBOA inhibited the ATPase activity of CF_1 from spinach chloroplasts. The inhibition was about 50% with 5 mM DIMBOA (Fig. 1 and Table 1). The reaction of DIMBOA with the enzyme was completed fast enough so that no further inhibition was appreciable during the assay period. A Hill plot of the inhibition of ATP hydrolysis by DIMBOA (Fig. 1, inset) gave an apparent *n* value of 1.03, suggesting that the reaction of 1 mol of hydroxamic acid per mol of active site is responsible for the enzyme inactivation. The inhibition was reversible since a 222-fold dilution of the inhibited enzyme or gel filtration restored the original activity (Table 1). This reversible inhibition was largely uncompetitive (Fig. 2), suggesting that the inhibitor binds mainly to the enzyme–substrate complex at a place other than the substrate binding site.

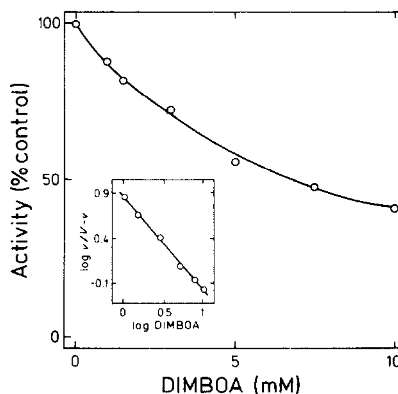


Fig. 1. Inhibition of CF_1 ATPase by DIMBOA. Tubes containing 1 ml of 40 mM Tricine–NaOH (pH 8), 5 mM ATP, 5 mM $CaCl_2$, different concentrations of DIMBOA and 3 μg of CF_1 were incubated at 37°. Inorganic phosphate was determined after 7 min as described in Experimental. The activity of the control was 13.8 μmol Pi/mg protein per min.

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Table 1. Reversibility of the inhibition of CF_1 ATPase by DIMBOA with short pre-incubation times

Treatment of samples	DIMBOA concentration (mM) Initial*	Final†	Activity (μmol Pi/mg protein per min)
No filtration	0	0	9.6
No filtration	0	0.02	9.4
No filtration	5	5	4.8
Filtration	0	0	9.6
Filtration	5	0	9.6
Inhibitor dilution	5	0.02	9.3

ATP, $CaCl_2$ and DIMBOA were added to tubes containing activated CF_1 (89 μg) in 100 μl of incubation medium (40 mM tricine–NaOH, pH 8, and 2 mM EDTA) in order to reach concentrations of 5, 7 and 5 mM, respectively. Similar control tubes without DIMBOA were set up. The tubes were incubated at 25° for 5 min and then filtered through Sephadex G-50 using the rapid centrifugation–filtration technique of Penefsky [18]. Protein determinations were carried out before and after filtration. ATPase activities before and after filtration were determined by transferring aliquots (4.5 μl) to 1 ml of a reaction medium containing 40 mM Tricine–NaOH (pH 8), 2 mM EDTA, 5 mM ATP and 7 mM $CaCl_2$, and incubating for 8 min at 37°. In addition, activities before filtration were measured in the tube with inhibitor in a reaction medium containing 5 mM DIMBOA, and in the tube without inhibitor in a reaction medium containing 0.02 mM DIMBOA (concentration after dilution in the reaction medium for the second incubation).

*DIMBOA concentration during the initial 5 min incubation.

†DIMBOA concentration during the second incubation in which activity was measured. In some treatments the concentration of DIMBOA was kept constant by adding the appropriate amount of the inhibitor after the dilution made for the second incubation.

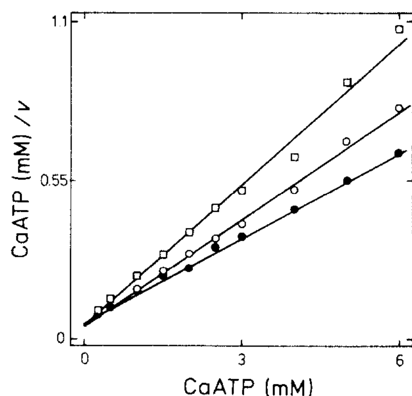

Fig. 2. Hanes plots [20] of substrate dependence of the ATPase inhibition by DIMBOA. Tubes containing 1 ml of 40 mM Tricine–NaOH (pH 8), different concentrations of CaATP and 3.4 μg of CF_1 were incubated at 37° in the absence (●) or presence of 2 (○) or 5 mM (□) DIMBOA. Inorganic phosphate liberated was determined after 8 min as described.

ADP inhibited the ATPase reaction, the saturation function with ATP becoming sigmoidal upon addition of the inhibitor [11]. The sigmoidicity was not altered by DIMBOA (Fig. 3), indicating that DIMBOA does not interfere appreciably with the allosteric site. Hill coefficients in the absence of ADP were 0.9 with DIMBOA and 1.1 without it, and in the presence of ADP were 1.6 with DIMBOA and 1.8 without it.

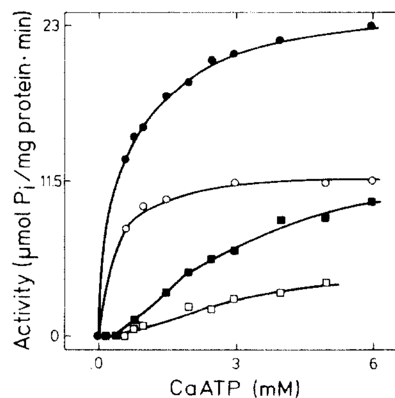

Fig. 3. Effect of DIMBOA on CF_1 ATPase in the presence and absence of ADP. ATPase activity of CF_1 was measured in the presence of different concentrations of CaATP in 1 ml of 40 mM Tricine–NaOH (pH 8) (control, ●) and 1 mM CaADP (■) or 5 mM DIMBOA (○) or 1 mM CaADP and 5 mM DIMBOA (□). The reaction was started with the addition of 1.5 μg of CF_1 and was carried out for 5 min at 37°. Inorganic phosphate was measured as described.

Since DIMBOA reacts with thiols [9, 10] and after activation CF_1 has four sulfhydryl groups [12, 13], the effect of iodoacetamide on the inhibition by DIMBOA was studied. Alkylation of the enzyme with iodoacetamide, although not inhibiting the enzyme significantly [12], appeared to protect the enzyme partially against the inhibition by DIMBOA (Fig. 4). The fact that protection of the enzyme by iodoacetamide was only partial suggests that cysteine residues are not the only groups reacting with DIMBOA to cause the observed inhibition.

In addition to the fast reversible reaction between CF_1 and DIMBOA described above, a slow inhibition was observed (Fig. 5). This inhibition was recognized as irreversible because it persisted in spite of the near 300-fold dilution of the inhibitor during the assay. This inhibition followed pseudo-first order kinetics (Fig. 5), suggesting that it is a single homogeneous process, i.e. only one group on the enzyme reacted with DIMBOA, or more than one group with comparable reactivities towards DIMBOA are reacting. This process was not further characterized.

DISCUSSION

DIMBOA equilibrates rapidly in aqueous solutions with the open-chain compound **2**. The aldehyde function of this species is a highly reactive electrophile since it has an adjacent carbonyl group draining electron density

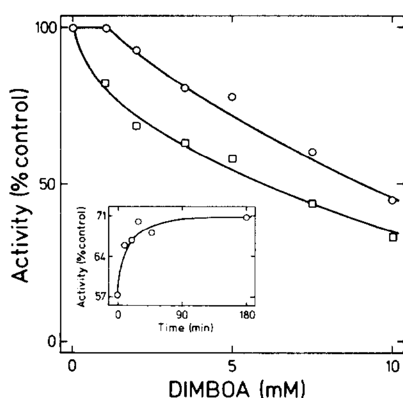

Fig. 4. Effect of treatment of CF_1 with iodoacetamide on the inhibition of CF_1 ATPase by DIMBOA. The time required for maximal protection by iodoacetamide from the inhibition by 5 mM DIMBOA is shown in the inset. CF_1 (220 μg) was added to 400 μl of incubation medium (40 mM Tricine–NaOH, pH 8, 2 mM EDTA and 10 mM iodoacetamide) at 25°. After different time intervals, 60 μl aliquots were withdrawn and filtered using the technique of Penefsky [18]. The enzyme reaction was carried out by adding aliquots of the filtrate into 1 ml of 40 mM Tricine–NaOH, pH 8, and 5 mM CaATP with and without added 5 mM DIMBOA, and after 5 min, inorganic phosphate was determined as described. Each tube contained 4.2 μg of CF_1 treated with iodoacetamide (○) or untreated CF_1 (□). A time equal to 90 min was chosen for studying the effect in the presence of other concentrations of DIMBOA. CF_1 (615 μg in 800 μl of incubation medium) was allowed to react for 90 min at 25° with and without 10 mM iodoacetamide. ATPase activities were then measured for 5 min in the presence of different concentrations of DIMBOA.

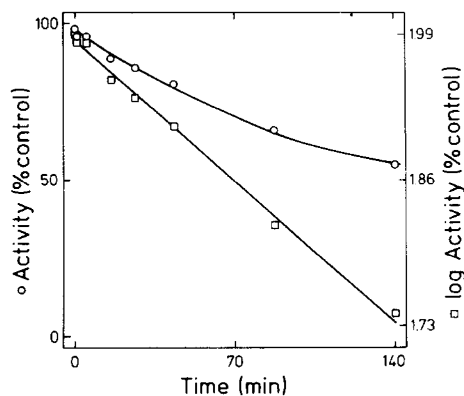

Fig. 5. Kinetics of CF_1 ATPase inhibition by DIMBOA. Two test-tubes containing 1.3 μg/ml of CF_1, 40 mM Tricine–NaOH (pH 8) and 2 mM EDTA were kept at 25°. A solution of DIMBOA in dimethyl sulfoxide was added to one of them making the solution 5 mM in DIMBOA. At different time intervals, an aliquot was withdrawn (3.5 μl) and added to tubes containing 1 ml of the ATPase assay medium where the activity was measured as described for 8 min. Activity at time zero was measured without inhibitor and with 17.5 μM DIMBOA (concentration after dilution in the assay medium), giving in both cases the same results. Activities of the controls were 7.8 μmol Pi/mg protein per min. ○ = activity; □ = log activity.

away from it. Furthermore, the nitrogen atom in **2** is prone to attack by reducing agents due to electron withdrawal from the adjacent carbonyl groups. In the presence of nucleophiles DIMBOA should thus form addition products quickly and reversibly. In the presence of reducing agents the hydroxamic nitrogen atom might be slowly reduced to an amide nitrogen. Thiols are both good nucleophiles and good reducing agents, and the reactions described above have been shown to occur between DIMBOA and thiols in aqueous solutions at pH 8 [9]. It is conceivable then that the enzyme inactivation caused by DIMBOA is partly due to its reaction with sulfhydryl groups in the enzyme. Sulfhydryl groups are normally more nucleophilic than amino and hydroxyl groups [14]. However, when the latter are present in an enzyme, they may also react with DIMBOA to cause additional inhibition.

The experiments described above show that DIMBOA caused a fast and reversible inhibition of the enzyme. The inhibition by DIMBOA was partly prevented by alkylation by iodoacetamide of sulfhydryl groups on the enzyme. In addition, the inhibition by DIMBOA was uncompetitive, consistent with the absence of cysteine residues in the substrate binding site. It is likely that reaction of DIMBOA with sulfhydryl groups on the enzyme induced a conformational change in the enzyme upon which it lost its normal activity. With longer reaction times between DIMBOA and the enzyme, an irreversible inhibition process appeared. The nature of this reaction remains to be clarified.

DIMBOA is deleterious to a wide range of organisms. Its activity as an energy transduction inhibitor [8] may account for its widespread toxicity. However, since DIMBOA reacts with thiols, other enzymes may also be expected to be inhibited by this molecule. The naturally-occurring concentration of DIMBOA (as glycosides) and related benzoxazinones (up to 5 mmol/kg fresh weight) in the Gramineae [7] as compared with those shown to be inhibitory in this and a previous study [8] suggest compartmentation at tissue and/or cellular levels in such a way that they would not affect metabolism.

EXPERIMENTAL

Isolation of DIMBOA. The inhibitor was isolated by a modification of the method in ref. [15]. Seeds of *Zea mays* L. cv T129s were sown in humus, watered with tap water, and grown under continuous light in a greenhouse at 28 ± 2°. Leaves of 7-day-old seedlings were extracted and the macerate was filtered through cheesecloth and adjusted to pH 3 by the addition of 0.1 M HCl. The acid extract was then centrifuged at 8500 g for 10 min and the supernatant partitioned against Et_2O (2 vols. × 3). The Et_2O phases were pooled and the solvent removed under vacuum. The crystalline residue was recrystallized from dioxane–petrol giving fine white needles which were characterized as DIMBOA by UV, IR and ^{1}H NMR spectroscopy.

Isolation and purification of CF_1. Class II chloroplasts were isolated from market spinach leaves (*Spinacia oleracea* L.) as described in ref. [16]. Thylakoid membranes were subjected to low ionic strength and CF_1 was extracted with EDTA. CF_1 was purified by precipitation with $(NH_4)_2SO_4$ and chromatography on DEAE-Sephadex, as described in ref. [17]. Electrophoresis on polyacrylamide gel showed it was at least 95 % pure. CF_1 was re-precipitated with $(NH_4)_2SO_4$ and stored at 3°.

Activation of ATPase. Activation was carried out as described in ref. [12]. Aliquots of purified CF_1 precipitated with

$(NH_4)_2SO_4$ were centrifuged for 3 min. The pellet was dissolved in a medium containing 40 mM Tricine–NaOH (pH 8), 50 ml dithioerythreitol and 2 mM EDTA. After incubating the mixture for 3 hr at 25°, it was filtered through Sephadex G-50 equilibrated with 40 mM Tricine–NaOH (pH 8) using the rapid centrifugation–filtration technique of ref. [18]. Protein determinations were carried out by the method of Lowry using bovine serum albumin as a standard.

ATPase activity. After 5–8 min of the enzyme-catalysed reaction at 37°, the reaction mixtures (1 ml) containing 1.5–5 μg of CF_1 were quenched with 1 ml of ice-cold trichloroacetic acid (TCA). Free inorganic phosphate was determined colorimetrically [19]. Blanks were made by delaying the addition of CF_1 until the aliquots of the reaction mixture had been mixed with TCA. The ATPase reaction was shown to be linear for at least 10 min at 37° with 5 mM CaATP and for at least 8 min with 0.4 mM CaATP.

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