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ACETAZOLAMIDE INHIBITION OF PHOTOSYSTEM II IN ISOLATED SPINACH CHLOROPLASTS

J A SWADER and BRUCE S JACOBSON

Department of Plant Sciences and Biology respectively, University of California, Riverside, Calif 92502, U S A

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Abstract—Acetazolamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide), a compound commonly used to inhibit carbonic anhydrase has been found to inhibit the photoreduction of 3-phosphoglyceric acid, NADP, and methyl viologen by isolated spinach chloroplasts Acetazolamide had no effect on the photoreduction of methyl viologen by chloroplasts using ascorbate plus 2,6-dichloroindophenol, as an electron donor, indicating the site of the acetazolamide inhibition was not in photosystem I There was no acetazolamide effect on NADP reduction by isolated chloroplasts using hydroxylamine instead of water as the electron donor The results indicate that the site of acetazolamide inhibition is near the water-splitting side of photosystem II

INTRODUCTION

RECENTLY the role of carbonic anhydrase in green plants has been examined in greater detail [1] A method commonly used to study the biological significance of an enzyme is to use an inhibitor of that enzyme and in the case of carbonic anhydrase, acetazolamide is thought to specifically inhibit the activity of this enzyme in animals.[2] Thus, it became obvious that acetazolamide could provide a valuable tool in elucidating the role of plant carbonic anhydrase.[1]

The inhibitory effects of various anions and sulfonamides including acetazolamide have been investigated with plant carbonic anhydrase.[1] The most potent inhibitor of carbonic anhydrase activity was acetazolamide In addition, acetazolamide partially inhibited CO_2 fixation in chloroplasts and it was concluded that carbonic anhydrase enhanced CO_2 fixation by facilitating bicarbonate movement into the chloroplasts [1] However, since acetazolamide shares some chemical properties with other photosynthetic electron transport inhibitors such as 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), in that all three compounds have a methylated carbonyl amide attached to an unsaturated ring structure, inhibition of CO_2 fixation could have arisen from blockage of the photoact rather than an inhibition of carbonic anhydrase. In this paper we report on the probable site of acetazolamide inhibition of photosystem II

RESULTS AND DISCUSSION

In addition to inhibiting spinach carbonic anhydrase[1] acetazolamide inhibits photosynthetic electron transport (Fig 1) The concentration of acetazolamide required for 50% inhibition of the electron transport system is 25 times greater than that reported for the inhibition of the enzyme carbonic anhydrase, but the amount of inhibition of CO_2 fixation in isolated chloroplasts by 1·0 mM acetazolamide with HCO_3^- concentrations of 0·5 and 1·0 mM observed by Everson[1] was almost identical to the amount of inhibition of the same concentration of acetazolamide on the photosynthetic electron transport (Fig. 1). The fact

[1] R G EVERSON, *Phytochem* **9**, 25 (1970)
[2] T H MAREN, *Physiol Rev* **47**, 595 (1967)

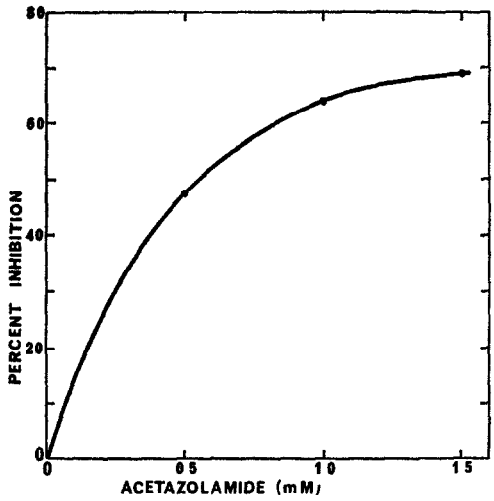

FIG 1 EFFECT OF ACETAZOLAMIDE ON THE PHOTOOXIDATION OF METHYL VIOLOGEN BY ISOLATED SPINACH CHLOROPLASTS REACTION MIXTURE (2 0 ml) CONTAINED 0 1 mM METHYL VIOLOGEN, 1 5 mM ADP, 1 5 mM P_i, 10 0 mM KCl, 5 0 mM $MgCl_2$, 1 0 mM KCN, 0 05 M Hepes pH 7 6, AND CHLOROPLASTS (51 0 μg CHLOROPHYLL)

that 1·0 mM acetazolamide inhibits photosynthetic electron transport and CO_2 fixation in isolated chloroplasts by approximately the same amount, obfuscates any conclusions regarding the role of carbonic anhydrase in photosynthetic CO_2 fixation (see Ref 1) with the use of this inhibitor However, it is interesting to note and difficult to reconcile that acetazolamide at concentrations which should have markedly inhibited electron transport and which inhibited carbon fixation at 0 5 and 1 0 mM HCO_3^- was without effect when fixation was conducted under conditions of higher, 5 0 mM, HCO_3^- concentrations [1] It is possible, since acetazolamide does not completely inhibit electron transport, that enough electron flow occurred and that saturating quantities of HCO_3^- overcame the inhibition of fixation

In order to provide some indication that acetazolamide permeates the outer membrane of the chloroplast we measured the effect of the inhibitor on the rate of 3-PGA mediated O_2 evolution The chloroplasts were a mixture of broken and intact plastids, but only the intact ones have the components necessary for the photoreduction of 3-PGA [3] Since acetazolamide inhibited the reduction of 3-PGA (Table 1) it is apparent that the inhibitor penetrates the cloroplasts' outer envelope, but since there was approximately a minute time lag before acetazolamide inhibition could be observed in the light, it is likely that the membrane is not completely permeable to acetazolamide

An alternate explanation for the acetazolamide inhibition of 3-PGA mediated O_2 evolution is that the outer membranes of the chloroplasts were disrupted by the inhibitor, however, acetazolamide could be washed from the plastids and activity restored This was done by incubating the chloroplasts in the assay medium (same as in Table 1) plus 1·0 mM acetazolamide for 5 min, centrifuging at 2000 *g* for 50 sec, pouring off the supernatant, and resuspending the chloroplast pellet in the assay medium. The rates of O_2 evolution by these plastids in the presence of 3-PGA were identical to the controls handled in the same manner

[3] J M ROBINSON and C R STOCKING, *Plant Physiol* 43, 1597 (1968)

TABLE 1 EFFECT OF ACETAZOLAMIDE ON OXYGEN EVOLUTION BY ISOLATED SPINACH CHLOROPLASTS USING EITHER 3-PGA OR NADP AS AN ELECTRON ACCEPTOR

Treatment	Oxygen evolved μEquiv /mg chlorophyll/hr 3-PGA*	NADP†
Control	105 6	235 2
Acetazolamide	63 2	156 4

* Reaction mixture (2 0 ml) contained 1 0 mM 3-PGA, 1 5 mM ADP, 1 5 mM P_i, 0 33 M sorbitol, 2 0 mM $NaNO_3$, 2 0 mM Na isoascorbate, 1 0 mM $MnCl_2$, 1 0 mM $MgCl_2$, 5 0 mM Na_4 pyrophosphate, 0 05 M Hepes pH 7 6, chloroplasts containing 94 8 μg chlorophyll, and where added 1 0 mM acetazolamide

† Reaction mixture (2 0 ml) contained 1 5 mM NADP, 1 5 mM ADP, 1 5 mM P_i, 10 0 mM KCl, 5 0 mM $MgCl_2$, saturating amounts of ferredoxin, chloroplasts (71 0 μg chlorophyll), 0 05 M Hepes pH 7 6, and where added 1 0 mM acetazolamide

Acetazolamide also inhibited the photoreduction of NADP (Table 1) in broken chloroplasts. Even in these chloroplasts that no longer have an intact outer envelope, however, the acetazolamide inhibition was enchanced about 10 per cent by sonification The observations that there was a lag time in the acetazolamide inhibition and that the acetazolamide inhibition in broken chloroplasts was enhanced by sonification suggests that acetazolamide had some difficulty passing through the chloroplast membranes

As a means of narrowing down where the acetazolamide inhibition is, we first determined whether acetazolamide inhibited photosystem II to I by the simple technique previously used by Izawa *et al*[4] Methyl viologen transfers electrons from photosystem I to O_2 with the formation of H_2O_2 If photosystem II is blocked, for example with DCMU, O_2 is not taken up (Table 2) However, since ascorbate plus 2,6-dichloroindophenol (DCIP) donate electrons to photosystem I, the DCMU inhibition is by-passed[4] as shown in Table 2 Similarly, it was found that acetazolamide inhibited photosystem II but not I since ascorbate with DCIP overcame the inhibition of O_2 uptake (Table 2)

Once it was determined that acetazolamide inhibited photosystem II another procedure was used to provide evidence that acetazolamide interrupted photosynthetic electron transport near the water splitting act and that this inhibition was not like that evoked by DCMU This conclusion is based on the fact that while hydroxylamine inhibits electron transport on the oxidizing side of photosystem II, at high concentrations it also donates electrons to the photosystem [5,6] Acetazolamide was found to have no effect on the photooxidation of hydroxylamine with NADP as the electron acceptor, while the photooxidative reaction was strongly inhibited by DCMU (Table 3) Therefore, acetazolamide inhibition is

[4] S IZAWA, T N CONNOLLY, G D WINGETAND and N E GOOD, in *Energy Conservation by the Photosynthetic Apparatus*, p 169, Brookhaven Symposia in Biology No 19 (1966)
[5] S IZAWA, R. L HEATH and G. HIND, *Biochim Biophys Acta* **180**, 388 (1969)
[6] S VAKLINOVA, *Compt Rend Acad Bulg Sci* **17**, 283 (1964).

TABLE 2 EFFECT OF ACETAZOLAMIDE ON OXYGEN UPTAKE BY ISOLATED SPINACH CHLOROPLASTS USING METHYL VIOLOGEN AS AN ELECTRON ACCEPTOR

Electron donor	Oxygen uptake μEquiv /mg chlorophyll/hr		
	Control	DCMU	Acetazolamide
H_2O	322 0	4 0	96 0
Abscorbate, DCIP	344 0	348 0	344 0

Reaction mixture (2 0 ml) contained 0 1 mM methyl viologen, 1 5 mM ADP, 1 5 mM P_i, 10 0 mM KCl, 5 0 mM $MgCl_2$, 1 0 mM KCN, 0 05 M Hepes pH 7 6, chloroplasts (44 6 μg chlorophyll), and where added 0 5 mM DCIP, 2 0 mM ascorbate, 1 0 mM acetazolamide and 0 02 mM DCMU

not like that caused by DCMU Furthermore, since NH_2OH donates electrons to photosystem II and by-passes the inhibitory effects of acetazoalmide, it is probable that acetazolamide interrupts photosynthetic electron flow on the oxidative side of photosystem II

The rate of NADP reduction was much lower when the chloroplasts were using hydroxylamine as an electron donor, only about 45 per cent of the rate observed when water was the electron donor (Table 3) The report of Izawa *et al* [5] showed the rate of indophenol dye reduction by spinach chloroplasts using hydroxylamine as an electron donor was about 67 per cent of the rate when water was used as an electron donor. Even though hydroxylamine concentrations were increased from 50 to 100 mM in the NADP reduction reaction

TABLE 3 EFFECT OF ACETAZOLAMIDE ON NADP REDUCTION BY ISOLATED SPINACH CHLOROPLASTS USING HYDROXYLAMINE AS AN ELECTRON DONOR

Treatment	μEquiv NADP reduced/mg chlorophyll/hr Electron donor	
	H_2O	NH_2OH†
Control	124 2	55 4
DCMU*	13 8	9 6
Acetazolamide	53 4	62 2

Reaction mixture (3 0 ml) contained 1 5 mM NADP, 1 5 mM ADP, 1 5 mM P_i, 10 0 mM KCl, 5 0 mM $MgCl_2$, saturating amounts of ferredoxin, chloroplasts (50 3 μg chlorophyll), 0 05 M Hepes pH 7 6, and where added 2 0 mM acetazolamide, 1 67 μM DCMU, and 50 0 mM hydroxylamine

* Values are corrected for the presence of acetone in which the DCMU was dissolved

† Values are corrected for the sulfate inhibition in the hydroxylamine sulfate

the reaction rate was still only about 45 per cent of the rate obtained with water as the electron donor, indicating the 50 mM concentration of hydroxylamine was not the limiting factor. Katoh and San Pietro[7] found that NADP photoreduction with *Euglena* chloroplasts using ascorbate as the electron donor was inhibited by hydroxylamine This would indicate that ascorbate donates electrons to the chain between hydroxylamine inhibition and the water splitting reaction or that hydroxylamine inhibits at another site, perhaps NADP reductase, as suggested by Izawa *et al.*[5] If hydroxylamine inhibits at another site such as NADP reductase, this would account for the greater difference in rate of NADP reduction with hydroxylamine and water as electron donors compared to the difference in rates of indophenol dye reduction with these two electron donors previously employed [5]

In conclusion, it is important to be congnizant of the multiple inhibitory effects of acetazolamide on photosystem II and carbonic anhydrase prior to using this inhibitor as a tool in investigating the role of carbonic anhydrase in photosynthetic CO_2 fixation

EXPERIMENTAL

Chloroplasts Isolation

The method of Jensen and Bassham[8] as modified by Robinson and Stocking[3] was used to isolate chloroplasts from spinach (*Spinacia oleracea* L) bought in the market 10 g of deveined spinach leaves were homogenized in a Waring blendor in 30 ml of 0 33 M sorbitol, 2 0 mM $NaNO_3$, 2 0 mM EDTA, 2 0 mM Na isoascorbate, 1 0 mM $MnCl_2$, 1 0 mM $MgCl_2$, 0 02 M NaCl and 0 05 M 2-(*N*-morpholino) ethanesulfonic acid (Mes) buffer pH 6 1 The homogenate was filtered through nylon mesh and the filtrate was centrifuged at 2000 *g* for 50 sec The chloroplast pellet was resuspended in 5 0 ml of 0 33 M sorbitol, 2 0 mM $NaNO_3$, 2 0 mM Na isoascorbate, 1 0 mM $MnCl_2$ 1 0 mM $MgCl_2$, 0 02 M NaCl, and 0 05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer pH 6 7 The chloroplast resuspension used in all experiments, except where 3-PGA was added as an electron acceptor, was sonicated for 3 × 10 sec, with a 5-sec interval, at 0° with a Bronwill Scientific Biosonik III at 1/3 the maximum intensity Chlorophyll was determined by the method of Arnon [9]

Reagents

NADP, ADP, and the Mes and Hepes buffers were obtained from Calbiochem. Acetazolamide was purchased from K & K Laboratories NH_2OH H_2SO_4 was dried *in vacuo* at 24° The NH_2OH solutions were prepared just before use The DCMU was recrystallized from MeOH Inorganic phosphate (P_i) was added to the reaction mixture as Na_2HPO_4.

Acetazolamide was prepared in a stock solution identical to the assay media except the pH was adjusted to 9 2, since acetazolamide is insoluble at a lower pH 10 μl aliquots were added to the acetozolamide treatments and 10 μl aliquots of the assay media pH 9 2 were added to the controls The stock solution of DCMU was prepared in 100% acetone and added to the reaction media in 10 μl aliquots Ten μl aliquots of 100% acetone were added to the controls

Isolation of ferredoxin Ferredoxin was isolated from spinach leaves as described by San Pietro [10] The precipitate from the acetone treatment was resuspended in 5 0 mM Tris-HCl, pH 8 0, and centrifuged at 10,000 *g* for 20 min The 10,000 *g* supernatant was dialysed overnight against 5 0 mM Tris-HCl, pH 8 0, to remove the acetone The ferredoxin was collected on a DEAE-cellulose column by the technique used by Shin *et al* [11] Ferredoxin was eluted from the column with 0 35 M NaCl, 0 1 M Tris-HCl, pH 7 6,[12] dialysed overnight against 0 05 M Hepes, pH 7 6 and stored at −20°

Assays Changes in oxygen concentration of the reaction media were determined with a Gilson Medical Electronics Oxygraph, Model KM, with a vibrating Pt electrode The glass reaction cell was held at 25° Where O_2 evolution was studied, N_2 gas was bubbled through the reaction media at the beginning of each experiment to remove O_2 from solution CO_2 free solutions were used to determine O_2 evolution by isolated chloroplasts using 3-PGA as an electron acceptor No O_2 was evolved by the chloroplasts in these experiments in the absence of exogenously added 3-PGA

[7] S KATOH and A SAN PIETRO, *Arch Biochem Biophys* **122,** 144 (1967)
[8] R G JENSEN and J A BASSHAM, *Proc Natl Acad Sci* **56,** 1095 (1966)
[9] D I ARNON, *Plant Physiol* **24,** 1 (1949)
[10] A SAN PIETRO, in *Methods in Enzymology* VI (edited by S P COLOWICK and N O. KAPLAN), p 439, Academic Press, New York (1963).
[11] M. SHIN, K TAGAWA and D I ARNON, *Biochem Z* **338,** 84 (1963)
[12] N NELSON and J NEUMANN, *J Biol Chem* **244,** 1926 (1969)

The reduction of NADP was determined spectrophotometrically The samples were incubated in the light or in the dark at 25° for 10 min, centrifuged at 10,000 *g* for 5 min, and the absorbance of the supernatant determined at 340 nm

The data reported in the tables are the difference in the rates in light minus the rates in the dark Two 300 W, 120 V, General Electric Reflector Flood lamps, mounted 15 0 cm from the reaction media, each with an intensity of 27,000 lx, were used as the light source in all experiments

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Key Word Index—Spinacea oleracea, Chenopodiaceae, spinach; chloroplasts, photosystem II, acetazolamide, inhibition