Site of Inhibition by 2-Amino-1,1,3-tricyanopropene of Photosynthetic Oxygen Evolution by Spinach Leaf Chloroplasts

By E. MARGARET BURDEN and ALAN A. HORTON

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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2-Amino-1,1,3-tricyanopropene inhibits photosynthetic O_2 evolution, but, unlike 3-(3,4-dichlorophenyl)-1,1-dimethylurea, has little effect on the steady-state fluorescence of chlorophyll. In chloroplasts prepared from spinach leaves and inhibited by 2-amino-1,1,3-tricyanopropene, a 3-(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive photoreduction of ferricyanide may be restored by addition of semicarbazide. It is concluded that 2-amino-1,1,3-tricyanopropene acts at a point close to the photo-oxidation of water.

The mechanism of O_2 evolution is one of the most fundamental problems remaining in the study of photosynthesis. The discovery of inhibitors of this process provided useful aids for the investigation of its mechanism, and a variety of compounds, including hydroxylamine (Losada & Arnon, 1963), the substituted phenylureas, e.g. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Wessels & Van der Veen, 1956), and carbonyl cyanide *m*-chlorophenylhydrazone (Cheniae, 1970), have been shown to act in this way.

Photosynthetic O_2 evolution is also inhibited by 2-amino-1,3-tricyanopropene (Horton & Hall, 1967), and the aim of the present study has been to locate its site of action. The present paper reports that 2-amino-1,1,3-tricyanopropene, unlike 3-(3,4-dichlorophenyl)-1,1-dimethylurea, does not inhibit the reoxidation of fluorescence quencher Q reduced by electrons from photosystem II, but appears to act at a point before quencher Q in photosystem II close to the photo-oxidation of water.

Chloroplasts were isolated from fresh spinach (*Spinacea oleracea*) leaves obtained commercially from locally grown produce by a previously described method (Horton & Hall, 1968). Chloroplasts were uncoupled by washing with EDTA by the method of Jagendorf & Smith (1962). Chlorophyll was determined by a spectrophotometric method (Arnon, 1949). Chlorophyll fluorescence excited by 475 nm light in a Farrand spectrofluorimeter (Farrand Optical Co., New York, N.Y., U.S.A.) was measured at 675 nm with a broad-band red filter placed before the photomultiplier to minimize the effect of scattered light.

O_2 evolution was measured by a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) inserted into a cuvette and connected to the electrode input of a GME Oxygraph (model KM; Gilson Medical Electronics, Middleton, Wis., U.S.A.). Ferricyanide photoreduction was measured at 400 nm in 1 cm Thunberg-

type cuvettes made anaerobic by evacuation and filling with argon three times. The cuvettes used for measurements of O_2 evolution and ferricyanide photoreduction were surrounded by a transparent jacket through which water at 15°C circulated. The reaction mixture (specific conditions are given in the legends to Fig. 1 and Table 1) was mixed by use of a Teflon-covered bar operated by a magnetic stirrer located below the cuvette. Side illumination was provided by a tungsten lamp yielding 10800 lx at the position of the cuvette (O_2 measurement), and red actinic light (4300 lx) for ferricyanide photoreduction was obtained with a 648 nm interference filter.

2-Amino-1,1,3-tricyanopropene was kindly provided by the Upjohn Co., Kalamazoo, Mich., U.S.A., and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (K & K Laboratories, Plainview, N.Y., U.S.A.) was supplied by Kodak Ltd., Kirkby, Lancs., U.K. All other reagents used were of A.R. grade.

Measurements of the steady-state fluorescence yield of chloroplasts in the presence and in the absence of 2-amino-1,1,3-tricyanopropene at concentrations sufficient to inhibit photosynthetic O_2 evolution were compared with the results of similar experiments with 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The results show that, whereas 3-(3,4-dichlorophenyl)-1,1-dimethylurea caused an increase in the steady-state fluorescence, the presence of 2-amino-1,1,3-tricyanopropene resulted in a small decrease, and the addition of semicarbazide, a photosystem II electron donor, was without effect, which indicated different sites of action for the two inhibitors. This effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea led to the conclusion that it acts at a site after quencher Q in photosystem II (Duysens & Sweers, 1963), and that it is the blocking of electron transport between the two 'light' reactions that results in the complete reduction of quencher Q, which in turn leads to the loss of capacity to evolve O_2 . In view of the lack of any marked effect of 2-amino-1,1,3-tricyanopropene on steady-state fluorescence and the

Fig. 1. Effects of 2-amino-1,1,3-tricyanopropene and 3-(3,4-dichlorophenyl)-1,1-dimethylurea on the steady-state fluorescence yield of and O₂ evolution by spinach leaf chloroplasts

For fluorescence measurements (□) the complete reaction mixture included, in a final volume of 3.0 ml, chloroplasts containing 6 μg of chlorophyll, and the following: Tricine [*N*-tris(hydroxymethyl)methylglycine] buffer adjusted with KOH to pH 7.8, 90 μmol; sucrose, 0.3 mmol; NaCl, 30 μmol; K₃Fe(CN)₆, 2 μmol; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, when used, 6 nmol; 2-amino-1,1,3-tricyanopropene, when used, 3 μmol. Results are expressed as the means of 30 assays. Results for column 1 fluorescence (no addition) were arbitrarily fixed at 100. Mean values ± s.d. of fluorescence measurements in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and 2-amino-1,1,3-tricyanopropene on this numerical basis were 198.8 ± 16.5 and 86.65 ± 6.42 respectively. For measurements of O₂ evolution (■) the complete reaction mixture included, in a final volume of 3.0 ml, chloroplasts containing 120 μg of chlorophyll, and the following: Tricine buffer adjusted with KOH to pH 7.8, 90 μmol; sucrose, 0.3 mmol; NaCl, 30 μmol; K₃Fe(CN)₆, 4 μmol; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, when used, 6 nmol; 2-amino-1,1,3-tricyanopropene, when used, 3 μmol. The mean value ± s.d. of ten assays of O₂ evolution (no addition) was 150 ± 19.25 . Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TCAP, 2-amino-1,1,3-tricyanopropene.

reactions of photosystem I (Horton & Hall, 1967) at concentrations sufficient to inhibit O₂ evolution, it seemed likely that 2-amino-1,1,3-tricyanopropene acted at a point before quencher Q in photosystem II.

Additional information on the site of inhibition by 2-amino-1,1,3-tricyanopropene of O₂ evolution has been obtained by use of alternative electron donors to photosystem II. In chloroplasts in which O₂ evolution has been inhibited by washing with 0.8 M-Tris buffer, pH 8.0, a 3-(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive photoreduction of ferricyanide may be restored under anaerobic conditions by the addition of semicarbazide (Yamashita & Butler, 1969). A similar restoration of ferricyanide photo-

reduction by semicarbazide under anaerobic conditions has now been demonstrated in chloroplasts inhibited by 2-amino-1,1,3-tricyanopropene (Table 1), although Mn²⁺, an electron donor preceding photosystem II (Ben-Hayyim & Avron, 1970), was without effect. This implies that the site of action of 2-amino-1,1,3-tricyanopropene is before the point at which semicarbazide donates electrons to photosystem II. In view of this, and the fact that 2-amino-1,1,3-tricyanopropene does not greatly affect the steady-state fluorescence of chlorophyll, the site of inhibition of electron transport is probably on the oxidizing side of photosystem II at a point close to the photo-oxidation of water.

Although a number of inhibitors of photosynthetic

Table 1. *Restoration by semicarbazide of the photoreduction of ferricyanide by spinach leaf chloroplasts after inhibition by 2-amino-1,1,3-tricyanopropene*

The complete reaction mixture included, in a final volume of 3.0ml, uncoupled chloroplasts containing 30 μ g of chlorophyll, and the following: potassium phosphate buffer, pH 7.8, 150 μ mol; NaCl, 60 μ mol; MgCl₂, 12 μ mol; K₃Fe(CN)₆, 0.1 μ mol; semicarbazide, when used, 9 μ mol; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, when used, 6nmol; 2-amino-1,1,3-tricyanopropene, when used, 3 μ mol. The duration of the assay was 10min. Results are expressed as the means $\pm$ s.d. of five assays. Ferricyanide photoreduction is recorded as the change in percentage transmission at 400nm [ΔT (%)].

Additions to reaction mixture	ΔT at 400nm (%)	
	Light	Dark
None	6.6 $\pm$ 0.2	0.4 $\pm$ 0.1
2-Amino-1,1,3-tricyanopropene	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1
2-Amino-1,1,3-tricyanopropene + semicarbazide	9.1 $\pm$ 0.2	0.7 $\pm$ 0.1
2-Amino-1,1,3-tricyanopropene + semicarbazide + 3-(3,4-dichlorophenyl)-1,1-dimethylurea	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1

O₂ evolution have been reported, not all of them appear to act at the same site. 2-Amino-1,1,3-tricyanopropene appears to belong to a small group of inhibitors that act at a point close to the photo-oxidation of water. From a consideration of molecular structures the unifying characteristics of this group of inhibitors are the possession of an amino group and the fact that all are non-cyclic compounds.

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