Picrate Salt of a Cyclic Polyether, 18-Crown-6 (Ca-picrate-18-crown-6), Inhibits the Photosynthetic Electron Transport at the Acceptor Side of Photosystem II

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It has been demonstrated that cyclic polyether, K-picrate-18-crown-6 inhibited photosynthetic electron transport (Sabat *et al.*, 1991, Z. Naturforsch. **46c,** 87–92). We further analyzed the alterations induced in the fast chlorophyll *a* fluorescence and thermoluminescence pattern of pea thylakoids by calcium-18-crown-6 (crown-picrate). The results indicate that the site of action of calcium crown-picrate is at the acceptor side of photosystem II.

Introduction

The cyclic polyethers, referred to as crown ethers, possess a remarkable metal ion complexing property (Pedersen, 1967; Frensdorff, 1971). We found that one of the crown-ether, K-picrate-18-crown-6 (crown) selectively inhibits PS II oxygen evolving activity without affecting PS I (Sabat *et al.*, 1991). One of the likely interpretations of this effect of crown ether on PS II photochemistry was sought on the basis of its ability to chelate metal ions. We further characterized the action of Ca-crown-picrate on the thylakoids by independent techniques – by monitoring Chl *a* fluorescence and thermoluminescence. In this communication we report the possible mode of action of crown-Ca-picrate on the light reaction of photosynthesis.

Materials and Methods

Pea (*Pisum sativum* cv. Bonnevilla) seeds were obtained from National Seeds Corporation, IARI, New Delhi. Seedlings were germinated in acid-washed river sand and supplemented with half-strength Hoagland solution. Illumination was provided with fluorescent lamps positioned above the growing plants with an intensity of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$. Leaves from 10–12 day old plants were used. Thylakoids were isolated according to a method similar to Nakatani and Barber (1977). The leaves were homogenized in ice-chilled medium containing 0.4 M sorbitol, 15 mM tricine [N-tris(hydroxymethyl)-methylglycine] (pH 7.8) and 5 mM NaCl using a polytron homogenizer PT 3000 (Kinematica AG). The homogenate was filtered through four layers of mira-cloth, and centrifuged at $300 \times g$ for 1 min at 4 °C. The supernatant was spun again at $2100 \times g$ for 5 min. The pellet was washed in a buffer containing 10 mM tricine (pH 7.8), 10 mM NaCl and 5 mM $MgCl_2$ and finally suspended in the reaction buffer containing 0.1 M sorbitol, 10 mM tricine (pH 7.8), 10 mM NaCl and 5 mM $MgCl_2$ to a concentration of 2 mg Chl $\cdot$ ml^{-1}. The Chl concentration was determined according to Arnon (1949).

PS II oxygen evolution activity was measured polarographically using oxygen electrode assembly from Hansatech Limited, England (Model DW 2). Thylakoids equivalent to 10 µg of Chl were suspended in a buffer containing 0.1 M sorbitol, 10 mM tricine-KOH (pH 7.8), 5 mM $MgCl_2$, 0.1 mM DCBQ and 0.015 mM $K_3[Fe(CN)_6]$ at 25 °C. The intensity of actinic light used for exciting PS II was

Abbreviations: PS I, photosystem I; PS II, photosystem II; Chl, chlorophyll; Q_a (Q_a^-), Q_b (Q_b^-), primary and secondary stable electron acceptors of PS II in oxidized (reduced) state; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCBQ, dichlorobenzoquinone.

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5000 $\mu E \cdot m^{-2} \cdot s^{-1}$. We have noted that DCBQ supported PS II activities represent essentially uncoupled rates. The fast Chl *a* fluorescence was measured using a PAM Chl fluorometer (Schreiber *et al.*, 1986). Thylakoid membranes equivalent to Chl concentration of 100 $\mu g \cdot ml^{-1}$ were suspended in buffer containing 0.1 M sorbitol, 10 mM tricine-KOH (pH 7.8), 5 mM $MgCl_2$, at 25 °C and dark adapted for 5 min before measurement. Transient changes in Chl *a* fluorescence were recorded using a data acquisition programme DA-100 from Walz, Germany.

Leaves of *Scindapsus aureus* (pothos) was obtained from around 3 month old garden plants. The thermoluminescence pattern of leaves of these plants is very well characterized and is essentially similar to that of spinach (Desai *et al.*, 1975). We preferred the use of pothos leaf in this study as it provides very well resolved and intense 4 bands in the temperature range of −50 to 50 °C (Desai, 1992). One leaf provides enough discs of 10 mm diameter to have experimental repeats and this much area of leaf provided a significant thermoluminescence yield of each peak. For measurements, leaf discs of *Scindapsus aureus* were fitted into stainless steel planchet. Leaf samples were dark incubated for 5 min. Samples were exposed to two consecutive single turnover saturating flashes of white actinic light, and were then immediately cooled from ambient temperature to liquid nitrogen temperature. The glow curves were recorded from the samples according to the method as described by Desai *et al.* (1975). In this study we have used the nomenclature system as used by Sane *et al.* (1977) to denote different peaks in thermoluminescence pattern. For infiltration of crown-picrate or DCMU, the leaf discs were vacuum-infiltrated in a solution having the required concentrations of these inhibitors.

The crown-picrate ether (Ca-picrate-18-crown-6) used in this study was a picrate salt of 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6), and was a kind gift from Dr. V. Vijayavergiya, AIIMS, New Delhi.

Results and Discussion

Inhibition of oxygen evolution

The photosynthetic electron transport ($H_2O \rightarrow$ DCBQ) is inhibited by crown picrate. We measured inhibitory efficiency of crown picrate in the thylakoids isolated from pea. A probit analysis of inhibition of oxygen evolution at different concentrations of crown-picrate revealed that at a concentration of 5 μM crown-picrate (I_{50}), half of the PS II were inhibited.

Alteration of Chl a fluorescence transients

The Chl *a* fluorescence at room temperature emanates largely from PS II. The yield of Chl *a* fluorescence varies with the redox state of primary acceptor of PS II, Q_A. In the dark state, when all Q_A are in oxidized state, the yield of Chl *a* fluorescence is at its minimum, and is referred to as F_o. When the thylakoids are illuminated with actinic light, the yield of Chl *a* fluorescence changes to reach a maximum level (F_m) with a well known kinetics. Fig. 1 shows kinetics of fast Chl *a* fluorescence rise from F_o to F_m in the dark-adapted thylakoids and shows the Kautsky transients. When the thylakoids were incubated with the crown-picrate before the transients were measured, the fluorescence pattern altered. The rise of induction of fluorescence from F_o to F_m was quicker. Since the transient levels of fluorescence during the fast induction of Chl *a* fluorescence represent intermediary states of Q_A reduction, the altered pattern in crown-picrate treated thylakoids indicate that

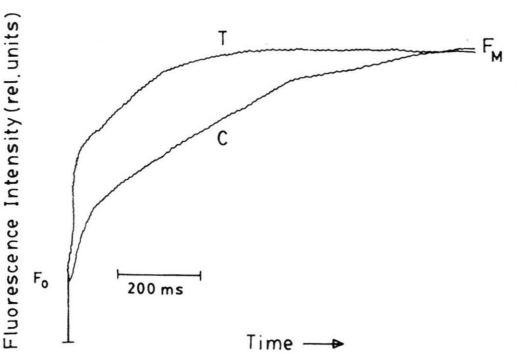

Fig. 1. The fast Chl *a* fluorescence induction of thylakoids without (C) or with (T) addition of 10 μM crown-picrate. Thylakoid membranes equivalent to Chl concentration of 100 $\mu g \cdot ml^{-1}$ were suspended in 0.1 M sorbitol, 10 mM tricine-KOH (pH 7.8), 5 mM $MgCl_2$, at 25 °C and dark adapted for 5 min before measurement. The fast Chl *a* fluorescence was measured using a PAM Chl fluorometer (Schreiber *et al.*, 1986). Transient changes in Chl *a* fluorescence were recorded using a data acquisition programme DA-100 from Walz, Germany.

crown-picrate mediate a block of electron transfer at PS II. Considering the nature of change, i.e. a faster rise to F_m level, the inhibitory action can be localized to the acceptor side of PS II. Since inhibition of electron flow at the oxidizing side induces lowering of Chl *a* fluorescence any crown-Ca-picrate dependent inhibition would have caused decrease in the Chl *a* fluorescence yield, which is not seen in the present case.

Alterations in thermoluminescence pattern

Pre-illuminated photosynthetic samples, when heated, emit light. The process known as thermoluminescence, occurs because the various intermediary charges created during the pre-illumination, recombine to produce an excited state of Chl *a* which finally deexcites to produce luminescence (Arnold and Sherwood, 1957). All the herbicides which interrupt electron transport at the acceptor side of PS II, cause a modification of the thermoluminescence pattern (Droppa *et al.*, 1981; Horvath, 1986). Since crown-picrate acts at the region of PS II, thermoluminescence (TL) was used for further investigation of its site of action. Fig. 2 shows a typical TL pattern obtained from leaf discs (control). The TL pattern exhibited four peaks, numbered as I, II + III, IV and V. When crown-picrate was infiltrated into the leaf discs prior to thermoluminescence measurements, the glow pattern showed remarkable change (Fig. 2). Crown-picrate caused a slight suppression in the intensity of peak II + III, and V, and abolished peak IV. Peak IV has been shown to originate in the recombination of charges present in the S_2/S_3 state of oxygen evolving complex (OEC) and Q_B (Rutherford *et al.*, 1982). Absence of peak IV in the crown-picrate ether treated leaf discs indicates an inhibition of formation of Q_B. Similar observations were also made when leaf discs were infiltrated with DCMU (Fig. 2), a well known inhibitor of electron transport between Q_A and Q_B. The changes induced to the glow curve by crown-picrate were almost similar to as induced by DCMU, except that DCMU leads to a slight increase in peak V. At present, we are unable to interpret this effect of DCMU on peak V.

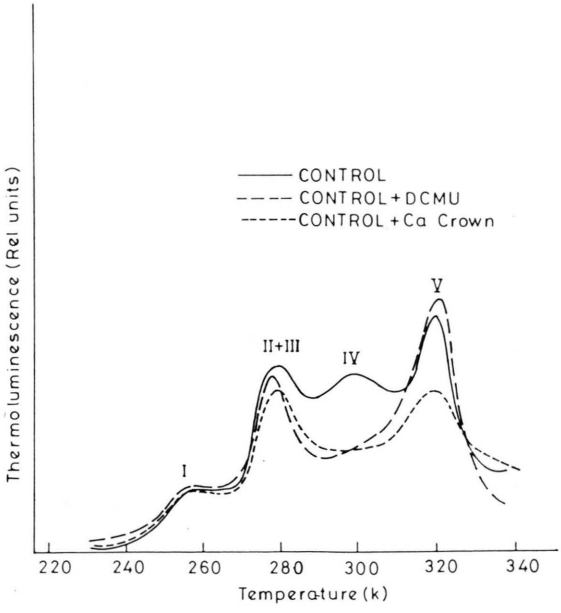

Fig. 2. The thermoluminescence pattern obtained from the leaves of control, DCMU or crown-picrate infiltrated leaf discs of *Scindapsus aureus* (pothos). The leaf discs were fitted into stainless steel planchet and dark incubated for 5 min. Samples were exposed to two consecutive single turnover saturating flashes of white actinic light, and were then immediately cooled from ambient temperature to liquid nitrogen temperature.

A proposed mode of action of crown-picrate ether

The process of oxidation of water to oxygen is catalyzed by water oxidizing complex containing tetra-nuclear manganese. In addition to Mn^{2+}, which is involved in charge accumulation process in photosystem II (PS II), other ions notably, Cl^- and Ca^{2+}, are also intimately involved in maintaining structural integrity of PS II and the water oxidizing system. These ions are also shown to be associated with the functional aspects of water oxidation process. Considering the metal chelating property of crown ethers, we were prompted to study its use in affecting an *in situ* metal ion depletion. The crown picrate effects on PS II activity, however, cannot be explained on the basis of its metal chelating properties. Any *in situ* metal ion depletion in thylakoids is expected to cause a general inhibition of PS II activity and cannot specifically inhibit the electron transport at the acceptor side of PS II.

Crown-picrate exhibited characteristics similar to DCMU, alters the fast Chl *a* fluorescence transient and the thermoluminescence pattern indicat-

ing it inhibits electron transport between Q_A and Q_B.

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