Inhibitory Effect of Crown Compound on Photoelectron Transport Activity of Beet Spinach Thylakoid Membranes

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The effect of K-picrate-18-crown-6 (crown) on the photoelectron transport activity of beet spinach thylakoid membranes was investigated. Addition of micromolar concentration of crown to thylakoid preparation inhibited p-benzoquinone, chloride-indophenol, methyl viologen supported Hill activities maximally by 75 per cent in a concentration dependent manner. However, the photosystem I catalyzed reaction remained insensitive to crown suggesting that crown specifically inhibits photosystem II electron transport. Addition of exogenous electron donors like hydroxylamine or diphenylcarbazide failed to restore the crown induced inhibition of photosystem II electron transport and lowering of steady state chlorophyll *a* fluorescence yield. These observations suggest that crown also inhibits photosystem II catalyzed electron transport after the donation sites of these exogenous donors. Washing of the crown pre-treated thylakoids with isolation buffer, relieved the crown inhibited electron transport activity, indicating that this inhibition is reversible. Furthermore, in hydroxylamine washed thylakoids which are devoid of O_2 evolution capacity, the hydroxylamine induced increase in chlorophyll *a* fluorescence of variable yield was quenched by the addition of crown. These observations suggest that crown affects the oxygen evolution and inhibits at a site close to photosystem II reaction centres.

Introduction

Crown ethers, a group of macrocyclic polyethers are known to have mostly cation complexing property [1]. The complexing property of crown with cation is dependent on the cavity size of crown and ionic radius of the cation, but independent of the valency state of the cation. Because of this property, the crown ethers and their derivatives have been used for solvent extraction of alkali metals [2]. These compounds have also been employed for selective transport of cations across the synthetic model membranes [3].

Many metal ions like Mg^{2+}, Ca^{2+}, Mn^{2+}, and Cl^- are known to play a vital role in biological systems [4, 5] particularly in relation to the functional and structural regulations of thylakoid membranes [6]. The transiently oxidized tetranuclear Mn complex of photosystem (PS) II of thylakoid membrane participates in electron removal from water [7]. Chloride ions are required both for steady state electron transport [8, 9] and also for advancement of particular S-state of oxygen clock [10]. Calcium ions have been shown to be required for O_2 evolution at PS II [11, 12]. It has been proposed that 70 per cent of the PS II reaction centres possess high affinity for Ca^{2+} ions while the remaining 30 per cent centres have low affinity for this ion [13]. Thus besides Mn, both Ca^{2+} and Cl^- ions are essential for O_2 evolution. Magnesium helps not only in granal stacking [14] but also in regulating energy distribution between both the photosystems [15].

In view of the involvements of large number of ions in photoelectron transport, particularly water oxidation activity, we attempted a study to characterize the effect of crown ether on the photochemical activities of isolated thylakoid membranes. Potassium picrate (K-Pic) was complexed with 1,4,7,10,13,16-hexaoxacyclooctadecane(18-crown-6) to obtain the potassium picrate complex of crown (K-pic-18-crown-6, referred hereafter as crown) following the procedure as outlined previously [16] and used in this investigation. The results indicate that this complex, at low enough concentration, specifically inhibits PS II activity reversibly and the site of inhibition appears to be very close to the PS II reaction centers.

Abbreviations: Chl *a,* Chlorophyll *a;* DAD, 3,6-diaminodurene; DCIP, 2,6-dichlorophenolindophenol; DPC, Diphenylcarbazide; $K_3Fe(CN)_6$, Potassium ferricyanide; Hepes, (N-2 hydroxyethyl-piperazine- N′-2 ethanesulfonic acid); MV, Methyl viologen; NH_2OH, Hydroxylamine; pBQ, parabenzoquinone; PS, Photosystem.

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Materials and Methods

Thylakoid isolation

Broken chloroplasts (thylakoid membranes) were prepared from beet spinach (*Beta vulgaris* L.) leaves grinding them in ice-cold medium of 300 mM NaCl, 5 mM $MgCl_2$, 50 mM Hepes, buffered to pH 7.5 with KOH. The slurry was filtered through four layers of Mira-cloth and the filtrate was centrifuged at 300 × *g* for 3 min to remove the cell debris. The supernatant was centrifuged at 6000 × *g* for 5 min to sediment the thylakoids. The thylakoids were finally suspended in the same medium so as to obtain 1 mg equivalent Chl ml^{-1} of thylakoid suspension. Amount of Chl was estimated following Arnon [17]. All operations were done at 4 °C under very weak green light made by wrapping green cellophane papers around tube light.

Electron transport measurements

Electron transport activities of isolated thylakoids were measured both polarographically and spectrophotometrically [18, 19]. Unless otherwise mentioned all polarographic measurements were carried out under rate saturating intensity ($\simeq$480 W m^{-2}) at 25 ± 1 °C. The basal reaction mixture in 1 ml contained 100 mM sucrose, 5 mM $MgCl_2$, 10 mM NaCl, and 50 mM Hepes-KOH (pH 7.5). Thylakoids containing 20 µg Chl was used for all assays. Electron acceptors like p-benzoquinone (pBQ) and oxidized Cl-indophenol (DCIP) were used for assaying PS II catalyzed reaction. The whole-chain electron transport was monitored with MV as electron acceptor. Activity of PS I was measured with reduced-DAD, or reduced-DCIP as electron donors with MV as electron acceptor. The reduced duroquinone which feeds electron close to plastoquinone was used with MV to study the intersystem electron flow [20, 21]. Procedure for preparation of reduced-duroquinone was followed according to Izawa and Pan [20]. NH_4Cl (5 mM) was used as an uncoupler, when required. Light intensities were varied by inserting calibrated neutral density filters between the light source and reaction vessel. The light intensity was measured with a YSI radiometer. Details of the donor and acceptor concentrations used in reaction mixture are mentioned in appropriate figure and table legends.

Spectrophotometric and spectrofluorometric measurements

Room temperature absorption spectra were measured as described previously [22]. Low temperature (77 K) emission spectra were recorded in LS-5 spectrofluorimeter. The samples at Chl concentration of 3 µg ml^{-1} were excited at 440 nm with the excitation and emission slits at 10 nm and 5 nm respectively. Spectra were measured from 650 to 800 nm. Room temperature Chl *a* fluorescence of variable yield was measured with a pulse modulation fluorimeter (Walz Heinz see reference 23 for details). The intigrated intensity of weak modulated light (which measures the amplitude of dark fluorescence F_o) was 1 mW m^{-2} with modulation frequency of 1.6 KHz. The intensity of red actinic light (< 680 nm) used to evoke the maximum fluorescence (F_m) was 80 W m^{-2}. The extent of variable fluorescence F_v, was tabulated as $F_m - F_o$ by subtracting the fluorescence yield, F_o, obtained under weak modulated light from maximal fluorescence yield (F_m) excitated with red actinic light. The reaction medium in 0.4 ml contained 50 mM Hepes-KOH (pH 7.5), 10 mM NaCl, 100 mM sucrose, 5 mM $MgCl_2$ and 20 µg Chl equivalent thylakoids. The concentration of crown and NH_2OH used was 10 µM and 10 mM respectively.

Washing of thylakoid membranes

Thylakoid suspension containing 200 µg Chl was incubated with 10 µM crown in a total volume of 0.2 ml for 15 min in dark at 4 °C. The incubated thylakoids were finally washed twice with 5 volumes of isolation buffer and centrifuged at 10,000 × *g* for 10 min at 4 °C. The pellet was resuspended in fresh isolation buffer and assayed for photochemical activity. Washings of thylakoids with NH_2OH were performed in complete darkness according to Ort and Izawa [24]. All assays were repeated at least three to four times. The standard deviations have been shown in the tables.

Chemicals

For preparation of K-Pic-18-crown-6, 18-crown-6 from Sigma was used. The complex was dissolved in water and appropriately diluted. Dihydrochloride salt of DAD was recrystalized from charcoal treated alcohol solution by adding excess of concentrated HCl at 4 °C [25]. Fresh solutions

of DAD were made in 0.01 N HCl following reference [25]. pBQ was recrystalized through sublimation and fresh solution was used. The stock solution of DPC was prepared in methanol just before the use. Care was taken not to exceed the final methanol concentration in reaction mixture more than 0.5%. NH_2OH stock solution was prepared fresh and neutralized to pH 7. The addition of DPC or NH_2OH was carried out in complete darkness.

Results and Discussion

Addition of micromolar (μM) concentration of crown to beet spinach (*Beta vulgaris* L.) thylakoid membranes inhibited the pBQ and DCIP supported PS II catalyzed reactions (Fig. 1) in a concentration dependent manner. A maximal inhibition of electron transport was obtained at about 10 μM crown and this inhibition remained unaffected with further increase in crown concentration. The ferricyanide supported Hill reaction was also inhibited by crown (data not shown). At saturating concentration (10–20 μM), the maximal inhibition of electron transport was approximately 75 per cent of control. Similarly, the whole-chain electron transport ($H_2O \rightarrow MV$) reaction measured as MV dependent O_2 uptake was also inhibited by 75 per cent at 10 μM crown (Fig. 1). The PS I catalyzed electron transport activity however, remained insensitive to crown at concentration as high as 20 μM (Fig. 1). The electron transport rate measured with reduced-duroquinone to MV involving plastoquinone, cytochrome b_6f complex and PS I [20, 21] also remained insensitive. These data strongly suggest that the crown specifically inactivates PS II catalyzed electron transport reaction without affecting the PS I reaction or intersystem electron transport carriers. In NH_4Cl uncoupled thylakoids the extent of crown mediated inhibition was around same as in loosely coupled thylakoids (Table I). Neither 18-crown-6 nor potassium picrate, used for synthesis of K-Pic-18-Crown-6 showed any inhibitory effect on electron transport activities (data not shown). The inhibition by crown is not due to trivial colouration of reaction mixture and consequent inner filter effect during spectrophotometric assays.

Addition of crown (10 μM) did not alter the room temperature (25 °C) absorption characteristics of thylakoids (data not shown). Similarly low temperature (77 K) emission spectrum of thylakoids showed no major changes in the emission characteristics of PS II and PS I except for a very minor quenching of F_{695} band (data omitted).

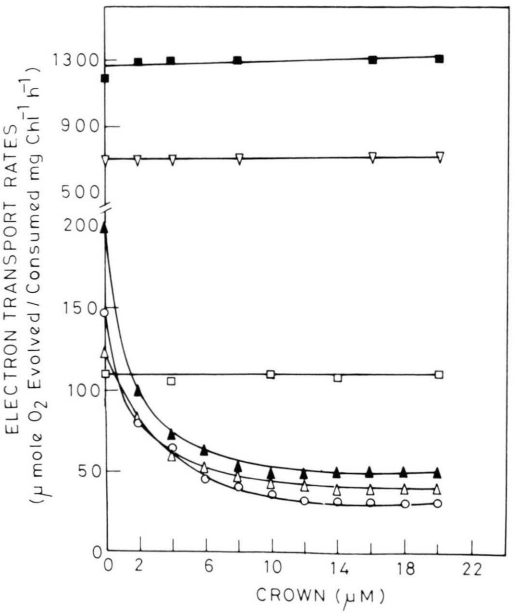


Fig. 1. Effect of addition of different μM concentrations of crown on the electron transport activity of beet spinach thylakoid membrane. Electron transport rates were measured in terms of oxygen evolution activity using various artificial electron acceptors like DCIP (○—○, 0.05 mM), pBQ (▲—▲, 0.5 mM) and MV (△—△, 0.05 mM). The reaction mixture for the above assays in 1 ml contained 50 mM Hepes-KOH buffer (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl, 100 mM sucrose and chloroplast suspension containing 20 μg chlorophylls. Fig. 1 also shows the PS I catalyzed electron transport activity in presence of different concentrations of crown, PS I catalyzed electron transport activity was measured in terms of oxygen concentration using $DCIPH_2$ (▽—▽, 0.1 mM) or $DADH_2$ (■—■, 0.05 mM) as donor and MV as electron acceptor (0.05 mM). The reaction mixture in total volume of 1 ml contained in addition to Hepes, NaCl, $MgCl_2$ and sucrose (as above). 2 mM ascorbate, 2 mM sodium azide and 5 μM DCMU. The amount of chlorophyll was 20 μg. The concentration of reduced duroquinone, when used as donor, was 0.1 mM (□—□). The electron acceptor used for this assay was 0.05 mM MV. The basal reaction ingredients for this reaction was same as for photosystem I but without ascorbate. All the electron transport assays were measured at 25 °C under 480 W m^{-2} white light illumination. The data represent the mean of five independent experiments. The mean variation was found to be not more than 7 per cent.

Table I. Effect of 10 μM crown on the pBQ (0.5 mM) and $K_3Fe(CN)_6$ (1 mM) supported Hill-activity in coupled ($-NH_4Cl$) and uncoupled ($+5$ mM NH_4Cl) thylakoid preparations. Figures in bracket indicate the percentage inhibition to their respective controls. Electron transport rate was measured as mentioned in Fig. 1. Mean value of three independent experiments and their variation is given as + SD.

Assay	Electron transport rate μmol O_2 evolved (mg Chl)$^{-1}$ h^{-1}			
	$-NH_4Cl$ $-$ Crown (Control)	$-NH_4Cl$ + Crown	$+NH_4Cl$ $-$ Crown (Control)	$+NH_4Cl$ + Crown
$H_2O \rightarrow pBQ$	200 ± 15	50 ± 08 (75)	244 ± 18	54 + 07 (78)
$H_2O \rightarrow K_3Fe(CN)_6$	107 ± 11	20 ± 05 (80)	223 ± 14	47 + 08 (79)

The lack of change in the thylakoid absorption and emission characteristics suggests that crown does not alter the pigment protein interaction of PS II (possibly of LHC II). The extent of inhibition with 10 μM crown of PS II catalyzed reaction with pBQ as electron acceptor was about same for both rate limiting and rate saturating light intensities (Fig. 2) suggesting that crown affects the electron transport by interacting with PS II reaction centres.

The inhibitory effect of crown on the PS II dependent DCIP photoreduction was also measured spectrophotometrically (Table II). Addition of crown (10 μM) inhibited DCIP photoreduction by 74 per cent. Exogenously added PS II electron donors like DPC or NH_2OH failed to relieve this inhibition. In NH_2OH washed thylakoids where O_2 evolving complex was inactivated [24, 26], both

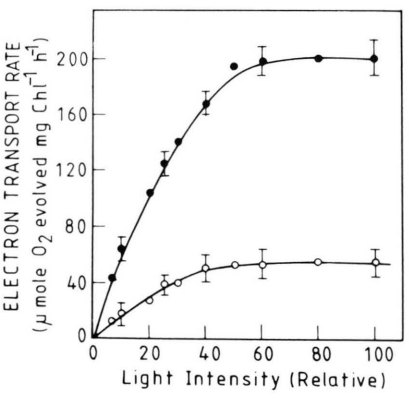


Fig. 2. Inhibition of pBQ (0.5 mM) supported Hill activity of 10 μM crown (○—○) treated and control (●—●) thylakoid membranes under different light intensities. 100 per cent light intensity, measured in a YSI radiometer, was 480 W m^{-2}. Electron transport rate was measured polarographically as in Fig. 1.

Table II. Effect of exogenously added electron donors on PS II electron transport activity of control and NH_2OH (5 mM) washed thylakoids in the presence and the absence of 10 μM crown. The data represent the mean of three independent observations.

Preparation	Electron donor used	Donor concentrations mM	Rate of DCPI reduction μmol DCPIP reduced (mg Chl)$^{-1}$ h^{-1}		Inhibition (%)
			$-$ Crown	+ Crown	
Control	None		84 ± 06	22 ± 03	74
	NH_2OH	10	93 ± 03	22 ± 05	76
	DPC	01	84 ± 10	22 ± 03	74
NH_2OH-washed	None		0	0	–
	NH_2OH	10	70 ± 08	21 ± 06	70
	DPC	01	55 ± 12	07 ± 11	87

DPC and NH_2OH supported PS II catalyzed electron transport rates (DPC/$NH_2OH \rightarrow$DCIP) were comparable to the unwashed thylakoid membranes. However, in NH_2OH washed thylakoids 70 per cent of NH_2OH and 87 per cent of DPC supported PS II activity was inhibited by the addition of 10 μM crown. The failure of these two exogenous donors to restore the DCIP photoreduction indicated that the site of inhibition of electron transport by crown is close to the donation site of these exogenous electron donors.

Changes in room temperature Chl *a* fluorescence of variable yield of thylakoid membranes in the presence of crown is shown in Table III. The changes in variable yield of Chl *a* fluorescence at room temperature, are associated with PS II photochemistry [27]. Usually, an inhibition of electron transport at acceptor side of PS II enhances Chl *a* variable fluorescence yield [27], whereas a block at the donor side lowers the yield of fluorescence [28]. Addition of 10 μM crown lowered the variable fluorescence yield of control thylakoids by 30 per cent (Table III). The possible reason for the small extent of lowering in Chl *a* fluorescence yield by crown which brings large inhibition in O_2 evolution is not clear to us at present and needs further investigation. However this may associated with cyclic electron flow around PS II.

Washing of thylakoid with 5 mM NH_2OH in dark removes functional Mn of PS II [26, 29]. Addition of 10 mM NH_2OH as exogenous PS II donor, enhanced the Chl *a* fluorescence yield and this yield was quenched to the extent of 70 per cent by 10 μM crown (Table III). Thus the lowering of variable fluorescence, can be argued due to a block of electron flow by crown at the donor side of PS II. The inhibition of electron transport by crown both with H_2O and of NH_2OH electron donation indicate crown possibly affects PS II reaction centre complex.

To ascertain if crown inhibition of electron transport activity is reversible, the crown pretreated thylakoids were washed with isolation buffer to remove crown and assayed for their O_2 evolution activity with 1 mM $K_3Fe(CN)_6$ as electron acceptor. The data presented in Table IV show that the washings marginally lowered ($\simeq$10 per cent) the Hill activity of control thylakoids. The washings of the 10 μM crown pre-treated thylakoids, the O_2 evolution activity was restored significantly suggesting that the crown inhibition of electron transport of PS II is reversible. The removal of crown by washings of crown treated thylakoids pre-exposed to light also yielded similar results.

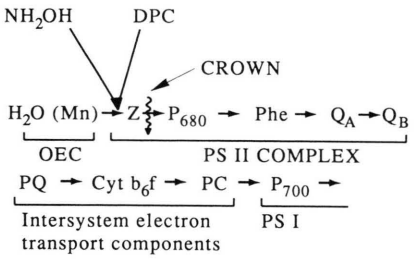
In summary, our results clearly indicate that crown (K-Pic-18-crown-6) in micromolar concentration very specifically inhibits the PS II electron transport without affecting the PS I. The site of action of crown appears to be very close to O_2 evolving complex, and the PS II reaction centre complex as NH_2OH fails to donate electrons to PS II as depicted in Scheme 1. Crown mediated inhibition is completely reversible. It is possible that initial crown induced loss of O_2 evolution capacity may be linked to cation, anion chelating property of this compound. However, the exact mechanism of crown inhibition of PS II electron transport ac-

Table III. Effect of 10 μM crown on variable fluorescence yield of control and 5 mM NH_2OH washed (treated) thylakoids. The table also depicts the effect of crown on NH_2OH washed thylakoid in presence of 10 mM NH_2OH. The variable fluorescence yield F_v, represents $F_m - F_o$ as monitored with a PAM fluorimeter. No change in F_o value was observed in the crown concentration used. F_v/F_o value for control thylakoids was 3.1 (which represents the 100 per cent).

Preparation	NH_2OH addition in mM concentration	Variable fluorescence (F_v) yield (Relative units)		Inhibition %
		− Crown	+ Crown	
Control (unwashed thylakoids)	none	87 ± 10	60 ± 06	31
Treated	none	16 ± 02	18 ± 04	
	10	82 ± 07	26 ± 05	68

Table IV. Effect of washings of thylakoids pre-incubated with 10 μM crown on electron transport activities. The washing was carried out as mentioned in Materials and Methods. The data represents the mean value of three independent experiments. The percentage of maximum deviation was found to be 4 per cent of the mean value.

Preparation	Rate of electron transport ($H_2O \rightarrow K_3Fe(CN)_6$) μmol O_2 evolved (mg Chl)$^{-1}$ h^{-1}	% activity
Control	96	100
Control washed	86	89
Crown (incubated)	21	22
Crown washed thylakoids	80	83



Scheme 1: Diagramatic representation of thylakoid electron transport components, site of electron donation by DPC or NH_2OH (↓) and inhibitory site of crown (⌿).

tivity and the possible interaction of crown with Cl^- or Ca^{2+} ions however, remains to be elucidated.

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