REVERSIBILITY OF THE CYANIDE INHIBITION OF ELECTRON TRANSPORT IN SPINACH CHLOROPLAST THYLAKOIDS

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SUMMARY

The prior treatment of thylakoids with cyanide (30 mM) was shown to inhibit plastocyanin-dependent electron transport reactions. We find that cyanide inhibition of electron flow from either water or diaminodurene to methyl viologen, but not from water to ferricyanide, is partially reversed when the thylakoids are collected by centrifugation and resuspended in a cyanide-free medium. However, methyl viologen reduction in thylakoids pretreated with cyanide is sensitive to cyanide (~1 mM) added to the reaction mixtures, whereas that in control thylakoids is unaffected. The cyanide must be added in the dark. Electron transport to methyl viologen in chloroplasts pretreated with cyanide is also sensitive to inhibition by EDTA and bathocuproine sulfonate. Thus, KCN inhibition of electron transport in thylakoids is partially reversible. Moreover, the accessibility of plastocyanin to various reagents is probably altered by the KCN treatment.

To determine the magnitude of the pH differential (ΔpH) across thylakoid membranes generated by electron flow through photosystem II alone, electron flow must be blocked between the two photosystems. Although dibromothymoquinone accomplishes this (1), it reacts with hexylamine[2] which is used in this laboratory in the estimation of ΔpH (2). Thus, we turned to the pretreatment of thylakoids with cyanide (3), which inactivates electron flow in the plastocyanin region of the electron transport chain (4,5,6), without inhibiting electron donation to photosystem II. Because incubations with cyanide are carried out with dilute thylakoid suspensions, the thylakoids were collected by centrifugation and resuspended in a smaller volume of cyanide-free solution. To our surprise, the inhibition of methyl viologen reduction partially disappeared, but could be restored by adding low concentrations of cyanide. The cyanide inhibition is, therefore, partially reversible.

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TABLE I

Partial Restoration of Methyl Viologen Reduction in KCN-Inhibited Thylakoids

Experiment	Presence of KCN in 90 min incubation	Sedimentation and Resuspended	Rate of Electron Flow[a]	% of -KCN Control
			$H_2O \rightarrow MV$[b]	
I	-	-	324	-
	+	-	18	5.6
	-	+	221	-
	+	+	88	39.8
			DAD[c] $\rightarrow$ MV	
II	-	-	1108	-
	+	-	71	6.4
	-	+	849	-
	+	+	357	42.1

[a] µmol O_2 reduced/hr/mg chlorophyll

[b] MV, methyl viologen

[c] DAD, diaminodurene

MATERIALS AND METHODS

Unwashed spinach thylakoids (7) were incubated in the dark at 0°C at a chlorophyll concentration equivalent to 0.2 mg/ml chlorophyll in a medium which contained 100 mM mannitol, 100 mM Tricine[3]-NaOH (pH 7.75), 2 mM $MgCl_2$, +/- 30 mM KCN, and 0.05 mM $K_3Fe(CN)_6$. When electron flow to methyl viologen, with water as donor, was inhibited 90% or more, the thylakoids were collected by centrifugation at 3,000 xg for 7 min and were resuspended in a small volume of a medium which contained: 0.3 M mannitol, 0.02 M Tricine-NaOH (pH 7.2), and 0.01 M NaCl.

Electron flow was assayed at 24.5° by following either oxygen consumption or evolution polarographically with a Clark-type electrode. All 1.75 ml reaction mixtures contained: 50 mM Tricine-NaOH (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM NH_4Cl, 1-2 µM gramicidin, and thylakoids equivalent to 10-40 µg of chlorophyll. For methyl viologen or anthraquinone sulfonate reduction with water as donor, 0.5 mM sodium azide and 0.1 mM methyl viologen or anthraquinone sulfonate were present. Diaminodurene (0.5 mM), ascorbate (2.5 mM), and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (10 µM) were added in addition to methyl viologen and azide for the assay of photosystem I-dependent electron flow. The assay mixture for ferricyanide reduction contained 2 mM $K_3Fe(CN)_6$, and, when added, 2,6-dimethylquinone (0.5 mM). The assay mixture for dichlorophenol indophenol reduction contained 0.25 mM indophenol dye. Saturating white light (about 10^6 ergs/cm^2/s) was used.

[3] Abbreviation: Tricine, [N-tris(hydroxymethyl)]-methyl glycine

TABLE II

Effects of Sedimentation and Resuspension on Electron Flow in the Presence of Various Electron Acceptors

Electron Acceptor	Rate of Electron Flow[a]			
	Before Sedimentation		After Sedimentation	
	-KCN[b]	+KCN	-KCN	+KCN
Methyl viologen	648	36	442	175
Ferricyanide	876	0	516	11
Dichlorophenol-indophenol	274	64	134	38
2,6-Dimethyl benzoquinone[c]	n.d.[d]	n.d.	107	106

[a] μmol O_2 consumed or evolved/mg chlorophyll/hour

[b] -KCN and +KCN refer to the presence or absence of 30 mM KCN in the 90 min incubation

[c] 1 μM dibromothymoquinone was also present

[d] n.d., not determined

RESULTS

The incubation of thylakoids for 90 min in the presence of 30 mM KCN severely inhibited electron flow from water to methyl viologen (Table I) relative to control thylakoids incubated under similar conditions in the absence of KCN. A partial reversal of this inhibition was observed when the thylakoids were sedimented and resuspended in a KCN-free medium. In six experiments in which electron flow was inhibited more than 90% by the KCN treatment, sedimentation and resuspension restored electron flow to 25-60% of that in control thylakoids. Restored electron flow was fully sensitive to 1 μM dibromothymoquinone or to 5 μM 3-(3,4-dichlorophenyl)-1,-dimethyl urea. The KCN inhibition of electron flow from diaminodurene to methyl viologen, a photosystem I-dependent reaction, was also partially relieved by sedimentation and resuspension of the KCN-treated thylakoids. Similar results were obtained when anthraquinone sulfonate was used as the electron acceptor in place of methyl viologen (data not show).

TABLE III

Effect of KCN Added to Reaction Mixtures on Electron Flow from Water to Methyl Viologen

Treatment	Rate of Electron Transport[a]	
	Control Thylakoids[b]	KCN-Treated Thylakoids[b]
No KCN added	335	138
Light, then 1 mM KCN	311	132
1 mM KCN added in the dark	311	24

[a] $\mu mol/O_2$ consumed/mg chlorophyll/hr

[b] Thylakoids were incubated with and without 30 mM KCN and were sedimented and resuspended.

Although ferricyanide and dichlorophenol indophenol reduction were inhibited to a similar extent as methyl viologen reduction by the KCN treatment, sedimentation and resuspension of KCN-treated thylakoids did not markedly relieve the inhibition (Table II). The reduction of 2,6-dimethyl quinone, a photosystem II-dependent reaction, was not affected by KCN treatment, followed by sedimentation and resuspension of the thylakoids.

The apparent reversibility of the KCN inhibition of electron flow through photosystem I appeared to preclude the use of KCN-inhibited thylakoids in the assay of ΔpH supported solely by photosystem II-dependent electron flow. However, electron flow from water to methyl viologen in thylakoids previously treated with KCN was sensitive to the addition of 1 mM KCN to the reaction mixture. Interestingly, the KCN had to be added to the thylakoids in the dark to be effective (Table III).

Half maximal inhibition of restored electron flow, in KCN-treated thylakoids was obtained with 0.4 mM KCN and the inhibition was greater than 90% at 2 mM KCN. The addition of KCN to reaction mixtures containing control thylakoids

TABLE IV

Effect of Various Inhibitors on Electron Flow From Water to Methyl Viologen in KCN-Treated Thylakoids[a]

Inhibitor Added to Reaction Mixture	Rate of Electron Flow[b]	% Inhibition
None	159	-
1.25 mM KCN	31	81
5 mM EDTA	39	76
30 μM Bathocuproine sulfonate	39	76

[a] Sedimented and resuspended. The rate of methyl viologen reduction in control thylakoids following sedimentation and resuspension was 413. The inhibitors had little to no effect on methyl viologen reduction in control chloroplasts.

[b] μmol O_2 consumed/mg chlorophyll/hr

had no effect on the rate of electron flow from water to methyl viologen. KCN also inhibited electron flow from diaminodurene to methyl viologen in thylakoids pretreated with KCN, but not in control thylakoids. Thus, the KCN inhibition of electron flow is partially reversible and full inhibition requires the continued presence of KCN. When aliquots of the thylakoid suspensions containing 30 mM KCN were assayed directly for electron flow, the concentration of KCN carried over (1 to 2 mM) was sufficient to supress the reversible component.

KCN treatment renders electron flow from water to methyl viologen sensitive not only to KCN, but also to the copper chelators, EDTA and bathocuproine sulfonate (Table III). The inhibitors were added to the reaction mixtures for electron flow and inhibition was observed without incubation of thylakoids with the inhibitors prior to illumination. In contrast, these reagents had little to no effect on electron flow in control thylakoids.

DISCUSSION

Our observation that the cyanide inhibition of photosynthetic electron flow is partially reversed when the thylakoids are resuspended in a cyanide-

free medium leads to a number of suggestions regarding the nature of the inhibition. From studies of the interaction between cyanide and plastocyanin in solution it has been inferred that the inhibition of plastocyanin-dependent activities in thylakoids by cyanide is due to the chelation of copper (3,6). Although irreversible inhibition due to copper chelation probably occurs (6), cyanide may also inhibit reversibly by acting as a ligand to the copper in plastocyanin. The continued presence of cyanide would thus be required to maintain the fully inhibited state. The failure of others to observe the reversible portion of the cyanide inhibition is probably due to the fact that the cyanide-treated thylakoids were always assayed in the presence of cyanide carried over from the incubations.

Our finding that the restored electron flow can be inhibited rapidly by KCN, EDTA or bathocuproine may suggest that the plastocyanin is rendered more accessible or sensitive to agents in the external solution by incubation with KCN (8). [See Horton and Cramer (9) for a discussion of the accessibility of cytochrome f to agents in the external medium.] This alteration in membrane properties after exposure of the thylakoids to 30 mM KCN cannot be due solely to incubation of the thylakoids in dilute solution (200 μg chlorophyll/ml) for extended periods of time (up to 2 hours), since these inhibitors are not effective with control thylakoids. The sensitivity of the restored electron flow to inhibition by relatively low concentrations of the inhibitors (10,11) may also be related to the number of functional electron transport chains in the KCN-treated and resuspended thylakoids.

The fact that cyanide inhibits restored electron flow only when it is added in the dark may be related to the redox state of the plastocyanin. Illumination of KCN-treated and resuspended thylakoids in the presence of an electron acceptor and uncoupling agents would lead to a reduction of the endogenous plastocyanin (12). The original cyanide incubation is performed in the dark in the presence of ferricyanide, a condition in which the plastocyanin would be primarily oxidized. The action of cyanide on thylakoid-bound plasto-

cyanin may require a more oxidized protein, though this does not appear to be the case with plastocyanin in solution (3). In the case of the mercuric chloride inhibition of plastocyanin-dependent electron transport reactions, Kimimura and Katoh (10) observed that inhibition was more pronounced when plastocyanin was in the reduced state.

The residual electron flow from water to ferricyanide in cyanide-treated thylakoids does not appear to involve plastocyanin since it is not sensitive to 1 μM dibromothymoquinone or to low concentrations of KCN. The absence of a photosystem I component of ferricyanide reduction in a system which is capable of reducing methyl viologen with water as the electron donor may suggest that plastocyanin in KCN-treated thylakoids is somehow inactivated by high potential oxidants. This action of ferricyanide may be related to its still unexplained role in potentiating the inhibitory effect of cyanide on electron flow (3). We suggest that 1-2 mM KCN be added to reaction mixtures which contain KCN-treated thylakoids to assure that the inhibition of electron flow between the photosystems is complete. Our preliminary experiments indicate that ΔpH may be estimated from hexylamine uptake driven by electron flow solely through photosystem II in KCN-inhibited chloroplasts.

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