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ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION IN CHLOROPLASTS AS A FUNCTION OF THE ELECTRON ACCEPTOR

II. ACCEPTOR-SPECIFIC INHIBITION BY KCN

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SUMMARY

1. Dark pretreatment of chloroplasts with high concentrations of KCN in the presence of a trace of ferricyanide completely blocks subsequent transfer of electrons from water to hydrophilic acceptors such as ferricyanide and methylviologen. In the KCN-treated chloroplasts there is still a largely uninhibited electron flow from water to lipophilic oxidants such as *p*-benzoquinonediimide (oxidized *p*-phenylenediamine), duroquinonediimide (oxidized diaminodurene) and 2,5-dimethyl-*p*-benzoquinone. The cyanide-insensitive electron transport supports phosphorylation with a P/e_2 ratio of 0.3 to 0.4. It is concluded (a) that these lipophilic oxidants can accept electrons from an unknown intermediate carrier, X, which precedes the KCN-inhibition site and is inaccessible to hydrophilic oxidants, and (b) that a phosphorylation site is associated with the electron pathway $H_2O \rightarrow X$.

2. Electron transport from diaminodurene to methylviologen and the associated phosphorylation are both inhibited by KCN. The same is true of cyclic photophosphorylation reactions catalyzed by diaminodurene, reduced dichlorophenolindophenol, pyocyanine, and low concentrations of *N*-methylphenazonium methosulfate. However, cyclic photophosphorylation can be largely restored by high concentrations of PMS.

3. Isolated plastocyanin reacts readily with high concentrations of KCN (>10 mM) but only at the relatively high pH values (>7.5) required for effective KCN treatment of chloroplasts. It is suggested that this copper protein is the site of KCN inhibition in chloroplasts.

4. Photoreduction of *p*-benzoquinonediimide is extremely sensitive to Photosystem II inhibitors, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea, and the inhibition is completely independent of light intensity. In contrast, although the reduction of ferricyanide is almost equally sensitive at very low light intensities, the sensitivity decreases as the light intensity increases. It is suggested that the lipophilic reduction site, X, may be located close to Photosystem II.

Abbreviations: CMU, 3-(4-chlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-*p*-benzoquinone; P/e_2, ATP molecules formed for each pair of electrons transported; PMS, *N*-methylphenazonium methosulfate.

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INTRODUCTION

Saha *et al.*[1] discovered a group of Hill oxidants which they called "Class III" acceptors. *p*-Benzoquinonediimide (oxidized *p*-phenylenediamine), duroquinonediimide (oxidized diaminodurene) and 2,5-dimethylquinone (DMQ) are Class III acceptors. These lipophilic oxidants are photoreduced at unusually high rates regardless of the presence or absence of ADP and phosphate. The electron transport nevertheless supports phosphorylation with a P/e_2 ratio of 0.5 to 0.6. Conventional electron acceptors such as ferricyanide, viologens, $NADP^+$–ferredoxin, *etc.* have been grouped under "Class I". These are all ionic, polar substances with negligible lipid solubility, or are low potential oxidants. As is well known, the much slower reduction of these acceptors is stimulated by ADP and phosphate, and ATP is produced with a P/e_2 ratio of 1.0 to 1.3.

To explain the remarkable difference in behavior between Class I and Class III acceptors, Saha *et al.*[1] proposed a model of chloroplast electron transport which postulated two phosphorylation sites in a linear electron transport chain. It was assumed that Class I acceptors have access only to the end of the chain whereas lipophilic Class III oxidants penetrate the thylakoid membrane and accept electrons from an intermediate electron carrier which is located between the two phosphorylation sites.

The present paper deals mainly with inhibition studies designed to demonstrate the key premise of the above scheme — that Class III oxidants intercept electrons at the level of an intermediate carrier, X. We reasoned that an inhibition which blocked electron transport at a point after X should have little effect on the reduction of Class III acceptors. Cyanide proved to be such an inhibitor. However, the cyanide experiments disclosed some new facts which necessitated a minor modification of the original model.

MATERIALS AND METHODS

Biological materials

Chloroplasts (unfragmented, naked lamellae) were prepared from commercial spinach (*Spinacia oleracea* L.) as described before[1]. Cytochrome *f* was prepared from fresh leaves of parsley (*Petroselinum crispum* L.) by the method of Davenport and Hill[2], as modified by Forti *et al.*[3]. Fraction III was used as experimental material after it was dialyzed against 10 mM Tris–HCl (pH 8.0). Plastocyanin was extracted from the spinach chloroplasts used in this study, by the method of Anderson and McCarty[4]. An eluate from the second DEAE-cellulose column was dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.0) at 0 °C. Further purification of these substances was considered unnecessary for the present purpose (test of reactivity with KCN).

Chemicals and reaction mixtures

Freshly recrystallized, colorless dihydrochloride salts of *p*-phenylenediamine and diaminodurene were dissolved in 10 mM HCl to make up an amine concentration of 10 mM (stock solution). The oxidized forms of these amines were prepared in the reaction mixture (2 ml), immediately before the reaction, by adding 0.8 μmole

of the amine and 2.4 μmoles of $K_3Fe(CN)_6$. (Before adding the chloroplasts, the reaction mixture containing oxidized *p*-phenylenediamine or oxidized diaminodurene should appear colorless except for the faint yellow due to the excess ferricyanide.) Since the lifetime of oxidized *p*-phenylenediamine was limited under the conditions employed, quick manipulations were necessary. After 4–5 min a brownish green color developed, indicating the formation of secondary products. Oxidized diaminodurene is much more stable. The standard reaction mixture (2 ml) contained: 0.1 M sucrose, 40 mM Tricine (pH adjusted to 8.2 with NaOH), 2 mM $MgCl_2$, 10 mM $Na_2H^{32}PO_4$, 1 mM ADP, 0.4 mM $K_3Fe(CN)_6$, 0.4 mM oxidized *p*-phenylenediamine or oxidized diaminodurene or DMQ (if used) and chloroplasts containing 20 to 40 μg chlorophyll.

Measurements

Red light (600–700 nm, 780 kergs$\cdot s^{-1}\cdot cm^{-2}$) was used as the actinic light. Ferricyanide reduction was followed by continuously monitoring the absorbance decrease of the reaction mixture at 420 nm. The reduction of quinonediimides and quinones was followed similarly, using the excess ferricyanide as an indicator. At the reaction pH (8.2) the oxidation of *p*-phenylenediamines by ferricyanide is rapid and stoichiometric. Reactions were terminated by shutting off the light before not more than 50% of the ferricyanide was reduced (15–60 s depending on the reaction rate). Photoreduction of methylviologen, either with water or with an exogenous reductant as the electron donor, was assayed as the O_2 uptake resulting from the aerobic oxidation of the reduced viologen[5]. For these studies a Clark-type oxygen electrode was used. No H_2O_2 trap was necessary, since the chloroplast preparations used were virtually free of catalase activity. ATP formation was assayed by the method of Avron[6].

RESULTS

(1) Differential inhibition by KCN

The aim of this experiment was to show that a block can be inserted between the site of oxidized *p*-phenylenediamine reduction and the site of ferricyanide reduction. In one typical experiment chloroplasts were pretreated in sealed test tubes at 0 °C as described in Table I and their activities tested subsequently. (Fresh addition of KCN to the reaction mixture to compensate for dilution was not necessary, since the inhibition is practically irreversible.) These procedures were basically similar to those employed by Bishop and Spikes[7] when they first showed that the Hill reaction can be inhibited by high concentrations of KCN.

As Table I shows, treatment with KCN *plus* ferricyanide nearly obliterated the chloroplasts' ability to reduce ferricyanide without lowering their ability to reduce oxidized *p*-phenylenediamine by more than 30%. Clearly the presence of ferricyanide in the preincubation medium enhances the effect of KCN. Yet the ferricyanide is not essential since the same inhibition can be obtained with KCN alone by simply extending the time of preincubation (*cf.* Fig. 1). In most of the experiments given in this paper, however, ferricyanide was routinely included so as to minimize the pretreatment time.

The effectiveness of the KCN treatment depends critically upon the pH of

TABLE I

EFFECT OF PRETREATMENTS OF CHLOROPLASTS WITH KCN AND FERRICYANIDE ON THE FERRICYANIDE- AND THE OXIDIZED *p*-PHENYLENEDIAMINE-REDUCING SYSTEMS

Preincubation mixtures in tightly sealed test tubes (1 ml each) contained chloroplasts equivalent to 300 μg chlorophyll, 0.1 M sucrose, 1 mM $MgCl_2$ and the following ingredients: (1) 90 mM Tricine–30 mM KOH buffer (pH 7.8), (2) Tricine–KOH buffer *plus* 50 μM potassium ferricyanide, (3) 90 mM Tricine *plus* 30 mM KCN (pH 7.8), (4) Tricine–KCN *plus* ferricyanide. After 60 min incubation at 0 °C in the dark, aliquots (0.1 ml each) were added to the reaction medium (total 2 ml). For reation mixtures and assay conditions, see Materials and Methods. Rates are μequiv (or μmoles ATP)/h per mg chlorophyll.

Preincubation condition	*Rates of electron transport (E.T.) and phosphorylation (ATP)*					
	Ferricyanide as acceptor			*Oxidized p-phenylenediamine as acceptor*		
	E.T.	*ATP*	(P/e_2)	*E.T.*	*ATP*	(P/e_2)
(1) Control	413	212	(1.03)	1380	364	(0.53)
(2) Ferricyanide treatment	362	180	(1.00)	1324	328	(0.50)
(3) KCN treatment	125	61	(0.97)	1107	215	(0.39)
(4) KCN + ferricyanide treatment	20	3	(—)	1010	172	(0.34)

the treatment medium; between pH values 7 and 8 the higher pH values strongly favor a rapid development of inhibition. Thus in one set of experiments, where

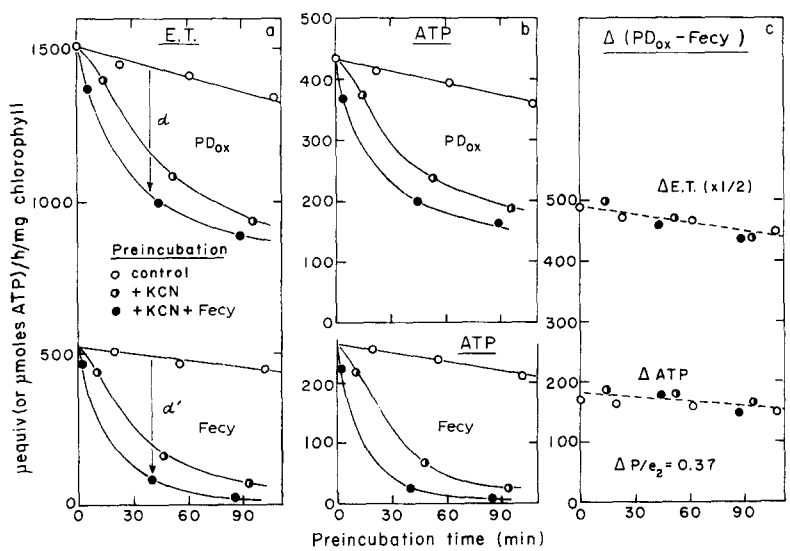

Fig. 1. Effect of preincubation of chloroplasts with KCN on electron transport (E.T.) and phosphorylation (ATP) with oxidized *p*-phenylenediamine (PD_{ox}) and ferricyanide (Fecy) as electron acceptors. Preincubation conditions were essentially the same as in Table I. Assay conditions and the compositions of reaction mixtures are given in Materials and Methods. For explanations, see text.

chloroplasts were incubated with 30 mM KCN and 50 μM ferricyanide, the time required for 90% inhibition of ferricyanide reduction were 90, 50 and 30 min at pH values 7.3, 7.6 and 8.0, respectively.

The preferential inhibition of ferricyanide reduction by KCN constitutes virtually irrefutable evidence that oxidized *p*-phenylenediamine is mainly reduced at a site that precedes the site of ferricyanide reduction. However, the effects of KCN on oxidized *p*-phenylenediamine reduction and on the associated phosphorylation are by no means insignificant. The nature of these partial inhibitions became clear when the time course of the development of the KCN inhibition was followed. It can be clearly seen in Figs 1a and 1b that the absolute KCN effects on oxidized *p*-phenylenediamine reduction and the associated phosphorylation are very nearly parallel to the effects on the ferricyanide system (compare arrows d and d'), as if the oxidized *p*-phenylenediamine reduction system were really a mixture of two systems, one of which (KCN-sensitive component) is identical to the ferricyanide system in every respect. It thus appears that oxidized *p*-phenylenediamine withdraws electrons from the transport chain at two points — a point (X) which precedes both the KCN-sensitive site and one of two sites of phosphorylation, and at another site which is indistinguishable from the ferricyanide reduction site (probably the end of the chain). These two reduction pathways are assumed to be kinetically independent (*i.e.* additive) despite the fact that they share common intermediates. As will be explained in the discussion, this situation is quite plausible.

If these interpretations are correct, it follows that the characteristics of the electron transport genuinely attributable to the partial pathway ending in X should be those observed after eliminating the concurrent normal Hill reaction. This elimination can be achieved by actually treating chloroplasts with KCN (as already shown) or, as an approximation, by subtracting the rates of electron transport and phosphorylation of the ferricyanide system from those of the oxidized *p*-phenylenediamine system. Plots in Fig. 1c were obtained by a combination of these two methods. It is clearly seen that a phosphorylation reaction with a P/e_2 ratio of 0.37 is associated with the rapid transfer of electrons from water to oxidized *p*-phenylenediamine, *via* a partial pathway $H_2O \rightarrow$Photosystem II$\rightarrow$X.

(2) Effects of KCN with various electron acceptors

In Table II a number of acceptors are listed in order of decreasing Class I activity and increasing Class III activity. The order is based on two diagnostic parameters: (a) an overall P/e_2 ratio decreasing toward 0.5 and (b) increasing rates of electron transport and phosphorylation. If we assume that increasing Class III character, thus defined, means an increasing proportion of the substance reacting at the X site rather than at the normal Class I reaction site, we should expect to find a corresponding decrease in KCN sensitivity. Thus KCN should inhibit methylviologen reduction as severely as it inhibits ferricyanide reduction but the inhibition should diminish sharply with the three lipophilic oxidants in the order given in the table.

The data (Table II, columns 1–6) verify these predictions. Approximately 50% of the total electron flux observed with 2,5-dimethyl-*p*-benzoquinone is attributable to reduction at X. The value increases to 60–70% in the oxidized diaminodurene-reducing system and to 70–80% in the oxidized *p*-phenylenediamine-reducing

TABLE II

EFFECT OF KCN TREATMENT OF CHLOROPLASTS ON THE ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION WITH VARIOUS ELECTRON ACCEPTORS

Chloroplasts were treated for 60 min at 0 °C with buffer alone (control) or with 30 mM KCN *plus* 50 μM ferricyanide as described in Table I. Photoreduction of DCIP (50 μM) was followed optically at 560 nm. For reaction mixtures and other assay conditions see Materials and Methods. The rates are μequiv (or μmoles ATP)/h per mg chlorophyll.

Expt No.	*Class**	*Electron acceptor***	*Rates of electron transport (E.T.) and phosphorylation (ATP)*								
			Control			*KCN-treated*			*Difference*		
			(*1*) *E.T.*	(*2*) *ATP*	(*3*) (P/e_2)	(*4*) *E.T.*	(*5*) *ATP*	(*6*) (P/e_2)	(*7*) Δ*E.T.*§	(*8*) Δ*ATP*§	(*9*) ($\Delta P/e_2$)§§
1	I	Fecy	426	230	(1.08)	20	7	—	406	223	(1.10)
	I	MV	440	239	(1.09)	25	4	—	415	235	(1.13)
	III	DMQ	1020	322	(0.63)	582	95	(0.33)	438	227	(1.01)
	III	DAD_{ox}	1285	331	(0.52)	355	113	(0.26)	430	218	(1.01)
	III	PD_{ox}	1980	502	(0.50)	1560	265	(0.34)	420	237	(1.13)
2	I	Fecy	453	250	(1.10)	30	8	—	423	242	(1.14)
	III	DMQ	892	297	(0.66)	434	69	(0.32)	458	228	(1.00)
	III	DAD_{ox}	1044	300	(0.57)	586	78	(0.27)	456	222	(0.97)
	III	PD_{ox}	1420	393	(0.55)	1005	182	(0.36)	415	211	(1.02)
	II	DCIP	718	—		128	—		590†		

* See Introduction and ref. 1.

** Fecy, ferricyanide; MV, methylviologen; DMQ, 2,5-dimethyl-*p*-benzoquinone; DAD_{ox}, oxidized diaminodurene (duroquinonediimide); PD_{ox}, oxidized *p*-phenylenediamine (*p*-benzoquinonediimide); DCIP, 2,6-dichlorophenolindophenol.

§ Values for "control" *minus* values for "KCN-treated".

§§ $2\,\Delta ATP/\Delta E.T.$

† This high value is probably due to the uncoupling effect of DCIP.

system (Columns 1 and 4). However, the amounts of electron transport and phosphorylation sensitive to KCN are very similar with all of the acceptors including ferricyanide and methylviologen. Only that part of the reaction which utilized the KCN-resistant pathway varies markedly with the acceptor (Columns 4 and 5). Nevertheless the phosphorylation efficiency of all of the KCN-resistant reactions is similar. If we take into consideration a demonstrated slight uncoupling by oxidized diaminodurene, we are led to the conclusion that the apparent P/e_2 ratio of the pathway $H_2O \rightarrow$ Photosystem II$\rightarrow$X is 0.3 to 0.4 regardless of the acceptor.

As can be seen in Table II, 2,6-dichlorophenolindophenol (DCIP) is reduced for the most part through the KCN-sensitive part of the electron transport, although a small portion of the reaction by-passes the KCN block.

(3) Effects of KCN on Photosystem I-dependent reactions

Cyclic photophosphorylation reactions catalyzed by diaminodurene, reduced DCIP or pyocyanine and the phosphorylation reactions associated with the non-cyclic transport of electrons from diaminodurene to methylviologen are all very strongly inhibited by the KCN treatment. So is the electron transport from diaminodurene to methylviologen itself. The electron transport from reduced DCIP to methylviologen seems somewhat resistant to KCN, but the associated phosphorylation is obliterated (Table III).

Interestingly, the effect of KCN treatment on PMS-mediated cyclic photophosphorylation was found to be a function of the concentration of *N*-methylphenazoniam methosulfate (PMS). With low concentrations ($< 10\ \mu$M), the reaction is almost completely inhibited but the inhibition diminishes as the concentration of PMS is raised. With 0.2 mM PMS the inhibition is only 30% (Table IV).

TABLE III

EFFECT OF KCN TREATMENT OF CHLOROPLASTS ON VARIOUS PHOTOSYSTEM I-DEPENDENT REACTIONS

Chloroplasts were treated for 90 min at 0°C with buffer alone (control) or with 30 mM KCN *plus* 50 μM ferricyanide as described in Table I. The concentrations of electron carriers used were: diaminodurene, 0.6 mM; methylviologen, 0.1 mM; reduced DCIP, 0.1 mM; pyocyanine, 10 μM; ascorbate, 4 mM (used as electron reservoir for Expts 2, 3 and 4). DCMU (1 μM) was present in all experiments except for the pyocyanine system. The reaction pH for reduced DCIP systems was 7.6, for other systems 8.2. For other conditions, see Materials and Methods. Rates are μequiv (or μmoles ATP)/h per mg chlorophyll.

System	*Phosphorylation rate*		*Electron transport rate*	
	Control	*KCN-treated*	*Control*	*KCN-treated*
Diaminodurene (cyclic)	532	5		
Diaminodurene → methylviologen	606	5	2880	14
Reduced DCIP (cyclic)	44	0		
Reduced DCIP → methylviologen	44	0	130	60
Pyocyanine (cyclic)	830	66		

TABLE IV

EFFECT OF KCN TREATMENT OF CHLOROPLASTS ON PMS-MEDIATED CYCLIC PHOTOPHOSPHORYLATION

Chloroplasts were treated for 90 min at 0 °C with buffer alone (control) or with 30 mM KCN *plus* ferricyanide as described in Table I. In this particular case strong white light was used as actinic light. Other conditions are as described under Materials and Methods except that 1 μM DCMU was present. Rates are μmoles ATP/h per mg chlorophyll.

PMS (μM)	*Phosphorylation rate*		
	Control	*KCN-treated*	*% of control*
10	563	32	6
100	640	125	20
200	650	463	71

(4) The reactivities of isolated cytochrome f and plastocyanin with KCN

Since it seemed probable that KCN was reacting with a metal-containing electron carrier and, moreover, the inhibition of donor reactions (preceding section) strongly suggested a blocking of electron transport near Photosystem I, we tested the effects of high concentrations of cyanide on isolated cytochrome *f* and plastocyanin.

Cytochrome *f* appears to be quite immune to KCN. Incubation of the cytochrome (oxidized or reduced) at room temperature with 30 mM KCN at pH 7.8 did not cause any detectable change in the absorption spectra in the α–β regions. Nor was there any apparent change in the ferri–ferrocytochrome ratio when a 1:1 mixture (poised with ferri–ferrocyanide) was treated with KCN.

In contrast, plastocyanin does react with KCN at a very appreciable rate, provided that the reaction conditions are very similar to those employed in the treatment of chloroplasts (KCN > 10 mM, pH > 7.5). Under conditions identical to those used routinely for treatment of chloroplasts (30 mM KCN *plus* 50 μM ferricyanide, pH 7.8 at 0 °C) the characteristic blue color of plastocyanin disappeared completely in less than 1 min. The color was not restored by the addition of excess ferricyanide, which shows that the KCN-induced bleaching is not due to a reversible reduction reaction such as those caused by ascorbate or salicylaldoxime[8].

Experiments in which isolated plastocyanin was bleached by KCN at 20–23 °C are shown in Fig. 2 and Table V. The bottom curve of Fig. 2 shows that KCN reacts with the ascorbate-reduced form of plastocyanin as rapidly as it reacts with the oxidized form. Table V shows that the rate of bleaching of plastocyanin is a critical function of the pH of the medium. Within the range of pH values tested (6.8–8.0) the rate of bleaching is almost proportional to $[OH^-]$. As described before, in this range the higher pH's also strongly favor the development of KCN inhibition in chloroplasts (Section 1 of Results).

It therefore seems very probable that plastocyanin is the primary target for KCN inhibition in chloroplasts. The slow development of the inhibition could be

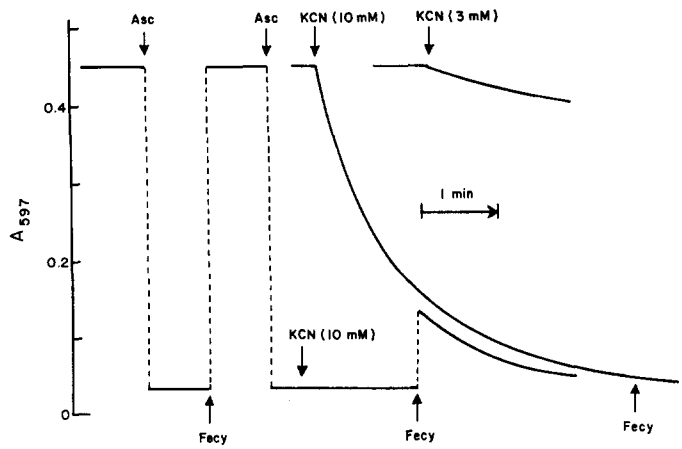

Fig. 2. Reaction between isolated plastocyanin and KCN as followed by the absorbance change at 597 nm (absorption peak of the oxidized form of plastocyanin). Plastocyanin was extracted from the spinach chloroplasts employed in this study and partially purified as described in Materials and Methods. The reaction mixture (1 ml) contained 50 mM Tricine–NaOH buffer (pH 7.8) and an amount of plastocyanin which gave the absorbance indicated (approx. 0.1 mM). The final concentrations of ascorbic acid (Asc) and ferricyanide (Fecy) were 0.4 mM and 5 mM, respectively. The temperature was 23 °C.

TABLE V

THE pH DEPENDENCE OF KCN-INDUCED BLEACHING OF ISOLATED PLASTOCYANIN

The bleaching was followed as the absorbance disappearance at 597 nm as described in Fig. 2. The reaction was first order with respect to plastocyanin. The reaction mixture (1 ml) contained plastocyanin ($A_{597\ nm}=0.4$), 0.2 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–NaOH buffer and 10 mM KCN. Temperature, 20 °C.

pH	$[H^+]\times 10^6$ (*M*)	*Half-time (min) for bleaching*
8.00	1.00	2.0
7.45	3.55	7.8
6.85	14.1	29.0*

* Extrapolated value.

due to many factors, for instance some unique conformational state of the protein *in situ* and/or a compartmentation which provides a localized acidic environment. It does not seem likely that the penetration of the cyanide is limiting since at the pH values used the cyanide is mostly in its membrane-permeating form, HCN. The increase in the rate of development of inhibition due to ferricyanide, an effect not encountered in the reaction of free plastocyanin with KCN, may reflect such structural factors. It is known that ferricyanide exerts diverse effects on the thylakoid membrane[9].

(5) Effect of DCMU on oxidized p-phenylenediamine reduction

Under routine experimental conditions (high light intensity) the reductions of oxidized *p*-phenylenediamine and oxidized diaminodurene were found to be extremely sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Minimum concentrations of DCMU which have little effect on ferricyanide reduction cause a very sharp drop in the rate of oxidized diaminodurene reduction (Fig. 3).

In order to explain this difference we reasoned as follows: Perhaps in the slower ferricyanide-reducing system a mild inhibition introduced near Photosystem II has little effect on the overall reaction rate because a severely limiting step occurs between the plastoquinone pool (Pool A) and cytochrome f[10,11]. The same mild inhibition might then express itself much more conspicuously in any fast reaction resulting from the relaxation or by-passing of the rate limitation. If this explanation is basically correct, the differences should disappear if both reaction systems are limited by a common step. With such reasoning in mind, we examined the DCMU inhibition as a function of light intensity.

The results, shown in Fig. 4, verified the prediction but in an unexpected manner. A weak inhibition of ferricyanide reduction caused by a low concentration of DCMU becomes greater as the rate of electron transport is lowered by lowering the light intensity. Eventually, at very low intensities, the inhibition of ferricyanide reduction approaches the level of the inhibition of oxidized *p*-phenylenediamine reduction. In striking contrast, the level of inhibition of the oxidized *p*-phenylenediamine-reducing system is completely independent of light intensity. Similar results were obtained when 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) and atrazine were

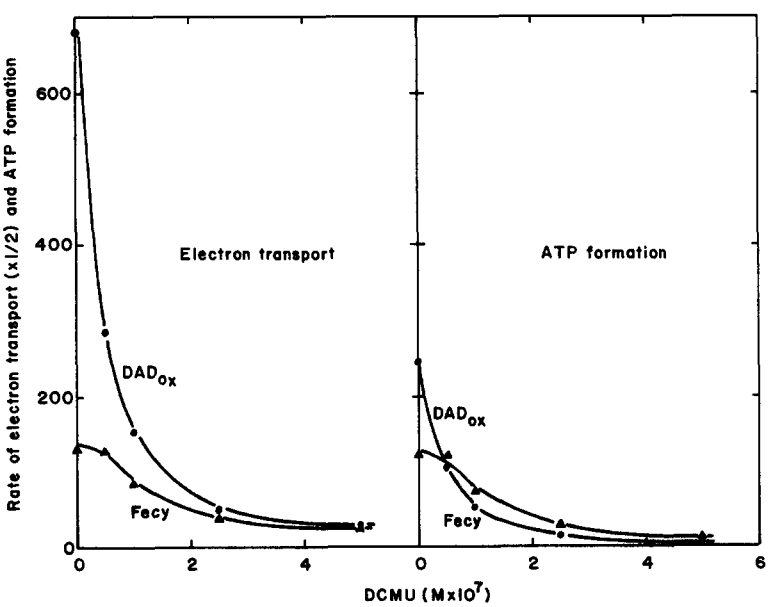

Fig. 3. Effect of DCMU on electron transport and phosphorylation with oxidized diaminodurene (DAD_{ox}) and ferricyanide (Fecy) as acceptors at high light intensity. Assay conditions are given in Materials and Methods. Rates are μequiv (or μmoles ATP)/h per mg chlorophyll.

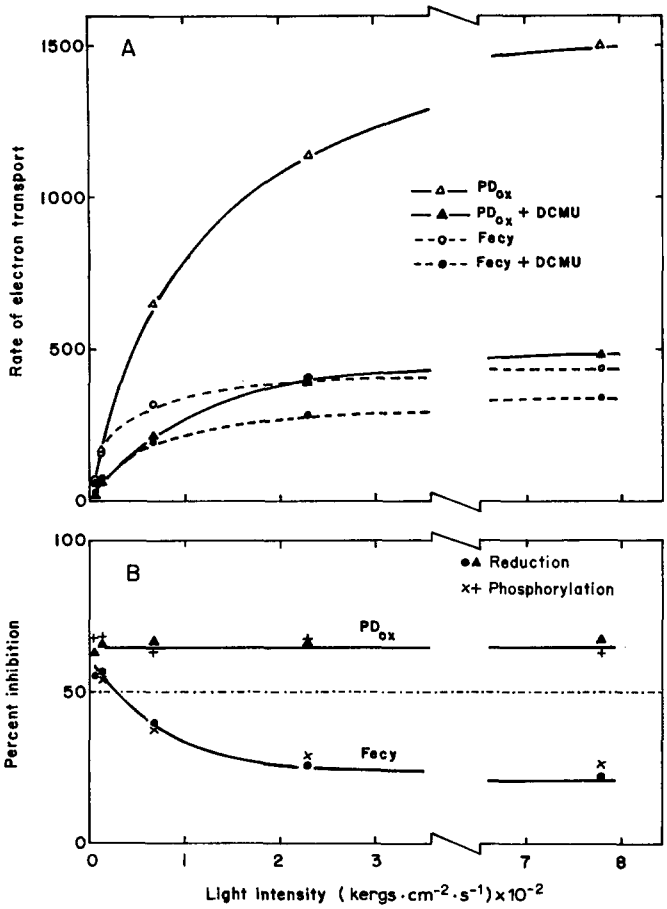

Fig. 4. Inhibition of oxidized *p*-phenylenediamine (PD_{ox}) reduction and ferricyanide (Fecy) reduction by a low concentration of DCMU (30 nM) as a function of the light intensity. Assay conditions are given in Materials and Methods. Rates are μequiv/h per mg chlorophyll.

used instead of DCMU. It should be noted here that a simple relaxation (by uncoupling) of the major rate-limiting step in ferricyanide reduction does not give rise to this phenomenon of intensity-independent inhibition[12]. We may thus reasonably assume that the intensity-independent DCMU inhibition of oxidized *p*-phenylenediamine reduction is in some way related to the fact that oxidized *p*-phenylenediamine is reduced mainly at the level of an intermediate electron carrier (see Discussion).

DISCUSSION

Pathway of reduction of lipophilic oxidants (Class III acceptors) and the sites of phosphorylation

The main conclusions we have drawn from the present study are summarized in the scheme presented in Fig. 5. In this scheme X, the lipophilic reduction site, is

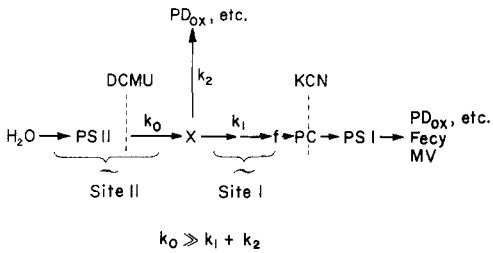

Fig. 5. A scheme for non-cyclic electron transport and phosphorylation in chloroplasts. PS I and PS II represent Photosystem I and Photosystem II, respectively. PD_{ox}, PC, Fecy, MV stand for oxidized *p*-phenylenediamine, plastocyanin, ferricyanide, methylviologen, respectively. k_0, k_1 and k_2 represent electron transport steps as well as the apparent rate constants of the corresponding steps. Step k_2 is the terminal reaction of the KCN-resistant partial electron transport pathway. The symbol ~ represents an energy conservation step in the phosphorylation mechanism. For details, see text.

placed before the primary rate-limiting step (k_1) of the normal noncyclic pathway. This is done because the rate of flow of electrons from X can be as high as 2000 μequiv/h per mg chlorophyll (*i.e.* when oxidized *p*-phenylenediamine is reduced *via* k_1 and k_2). The efflux from X toward the rest of the chain through k_1 is limited to about 500 (the rate of ferricyanide or methylviologen reduction). The latter rate can be enhanced by uncoupling but only up to about 1000. The reduction of Class III acceptors *via* the by-pass k_2 is very probably limited by k_2 itself, the terminal reduction step. This is suggested by the fact that the rate of electron flow through the KCN-resistant pathway depends very much on the nature of the acceptor used. Therefore, it seems reasonable to assume that, when Class III acceptors are reduced at two sites simultaneously, the overall reduction rates are still limited by k_1+k_2. Thus, when KCN blocks the electron flow through k_1, its effect is equivalent to the elimination of the ferricyanide reduction rate. The same applies to the phosphorylation rates (Fig. 1 and Table II).

The reduction of Class III acceptors *via* the KCN-resistant pathway $H_2O \rightarrow X$ supports phosphorylation. However, the coupling between this phosphorylation reaction and electron transport seems to be "loose" since electron flow is independent of phosphorylation. The phosphorylation efficiency is accordingly low, but is approximately constant with all of the three Class III acceptors tested ($P/e_2 = 0.3$–0.4). Hence our belief in the reality of Site II (Fig. 5). A necessity for postulating this (second) phosphorylation site near Photosystem II is also stressed by the conclusion that X precedes the primary rate-limiting step k_1 which is the only step that so far has been associated with phosphorylation[13,14]. This rate-limiting phosphorylation step is tentatively designated Site I. The electron flow through Site I is responsive to ADP/phosphate and uncouplers. The P/e_2 ratio inherent in this site is unknown, but logically we assume it to be less than 1.0. The relation of site I to DCMU-insensitive, Photosystem I-dependent phosphorylation reactions has not been explored in this study.

The location of the lipophilic reduction site X: DCMU experiments

The conclusion that X precedes the primary rate-limiting step inevitably places X in the Q→ plastoquinone region (Q: primary electron acceptor for Photosystem II).

In fact, the experiments of Kok *et al.*[15] with DCIP suggest that, besides the reducing end of Photosystem I, the above region may be the segment of the transport chain which is most easily accessible to lipid soluble oxidants. In line with this we found that an appreciable portion of DCIP reduction can by-pass the KCN block (Table II).

In this connection we should recall the DCMU experiments described in Section 5 of Results. As pointed out already, the light intensity-dependent inhibition of ferricyanide reduction[12,16] could be explained simply in terms of the relative positions of the inhibition site and the rate-limiting step. However, in fact, an intensity-independent inhibition such as observed for oxidized *p*-phenylenediamine reduction seems more consistent with the widely accepted concept of the unit structure of the electron transport chain ("photosynthetic unit"), since a block placed at any point in an independent chain should render the whole chain inoperative regardless of the light intensity. In order to accommodate the intensity-dependent inhibition of ferricyanide reduction, one would therefore have to postulate some kind of interchain connection or common electron pool before the rate-limiting step. Thus with ferricyanide as acceptor, a full effect of blocking of a small number of chains before the pool would be observed only when the input to the pool is already limited by low light intensity. However, the same maximum effect would also be observed, regardless of the light intensity, when electrons are intercepted before the pool. In both cases the inhibition would be strictly proportional to the number of chains blocked. Now there is good reason to believe that the plastoquinone pool constitutes just such a common pool[17,18] and, moreover, there is evidence that the Photosystem II units (O_2 producing units) are indenpedent of each other[19]. Thus the light intensity-dependent inhibition of ferricyanide reduction is easily explained in terms of a common electron pool and the known rate-limiting step, while the intensity-independent inhibition of oxidized *p*-phenylenediamine reduction is as readily explained in terms of its reaction in the Q→plastoquinone region.

Plastocyanin as the site of KCN inhibition

Katoh and San Pietro[8] have reported that none of the metal-complexing agents they tested, including salicylaldoxime and KCN, had sufficient reactivity with plastocyanin to be useful as an electron transport inhibitor. This observation has been taken as decisive evidence against the previous suggestion of Trebst[20] that the inhibitory effects of salicylaldoxime and KCN on chloroplast reactions might be due to inactivation of plastocyanin. However, we have demonstrated here that KCN does react quite rapidly with plastocyanin under the very conditions which cause an inhibition of electron transport. We have also shown that KCN blocks Photosystem I-dependent electron transport from diaminodurene to methylviologen without inhibiting the electron transport from water to oxidized *p*-phenylenediamine. Furthermore, one of us (S.I.) has recently obtained spectroscopic evidence that KCN interferes with electron transfer between cytochrome *f* and P700. A description of these spectroscopic studies which strongly implicate plastocyanin as the target for KCN inhibition will be published elsewhere*.

Hauska *et al.*[21] have shown that an antibody to plastocyanin can be useful, if applied to finely fragmented chloroplasts, for detecting plastocyanin involvement in

* Research conducted jointly with R. Kraayenhof, E. K. Ruuge and D. DeVault at the Johnson Research Foundation, University of Pennsylvania.

electron transport. They found that in their subchloroplast particles ferricyanide was reduced largely *via* a pathway which did not involve plastocyanin. This does not seem to be the case with our unfragmented chloroplasts. The only oxidants we find able to by-pass KCN-blocked plastocyanin to a measurable extent appear to be those with lipid solubility and fairly strong oxidizing potentials.

Brand *et al.*[22] have recently described another specific inhibition of Photosystem I reactions. In the absence of salts, histones and other polycations seem to bind with an electron carrier (problably plastocyanin) and thereby inhibit Photosystem I reactions.

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