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THE SITE OF KCN INHIBITION IN THE PHOTOSYNTHETIC ELECTRON TRANSPORT PATHWAY

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SUMMARY

Treatment of chloroplasts with high concentrations of KCN inhibits reactions which involve Photosystem I (*e.g.* electron transport from water or diaminodurene to methylviologen), but not those assumed to by-pass Photosystem I (*e.g.* electron transport from water to quinonediimides). The spectrophotometric experiments described in this paper showed that KCN inhibits the oxidation of cytochrome *f* by far-red light without blocking its reduction by red light. Both optical and EPR experiments indicated that KCN does not inhibit the photooxidation of P700 but markedly slows down the subsequent dark decay (reduction). Reduction of P700 by Photosystem II is prevented by KCN. It is concluded that KCN blocks electron transfer between cytochrome *f* and P700, *i.e.* the reaction step which is believed to be mediated by plastocyanin. In KCN-poisoned chloroplasts the slow dark reduction of P700 following photooxidation is greatly accelerated by reduced 2,6-dichlorophenolindophenol or by reduced *N*-methylphenazonium methosulfate (PMS), but not by diaminodurene. It appears that the reduced indophenol dye and reduced PMS are capable of donating electrons directly to P700, at least partially by-passing the KCN block.

INTRODUCTION

It has long been recognized that high concentrations of KCN inhibit the Hill reaction in isolated chloroplasts[1]. Trebst[2] found that salicylaldoxime and KCN inhibit not only the Hill reaction but also Photosystem I-dependent photophosphorylation, and suggested that a blocking of electron transport may have occurred rather close to Photosystem I. However, the data were also explainable by assuming that

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMS, *N*-methylphenazonium methosulfate.

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those compounds induced a Photosystem II inhibition and uncoupling simultaneously. This seems to have been indeed the case with salicylaldoxime, since Katoh and San Pietro[3] later demonstrated that isolated plastocyanin does not react with salicylaldoxime at any significant rate. They also presented evidence that salicylaldoxime inhibition probably takes place close to Photosystem II. The site of salicylaldoxime inhibition in the electron transport chain has in fact been located before cytochrome *f* (refs 4–6).

It has been shown very recently, however, that under proper conditions KCN does block Photosystem I specifically[7]. Reactions which involve Photosystem I such as the transfer of electrons from water to methylviologen or from diaminodurene to methylviologen, and diaminodurene-mediated cyclic photophosphorylation are almost completely inhibited, while reactions which are assumed to largely by-pass Photosystem I such as the transfer of electrons from water to lipid-soluble oxidants (*e.g.* oxidized phenylenediamines[8]) are scarcely affected. Furthermore, isolated plastocyanin was shown to react with KCN quite rapidly under the conditions required for effective KCN treatment of chloroplasts, thus strongly implicating plastocyanin as the site of inhibition[7].

This paper deals primarily with location of the site of KCN inhibition in the electron transport chain. We conclude that KCN interferes with electron transfer between cytochrome *f* and P700, that is the reaction step which is believed by most workers to involve plastocyanin.

MATERIALS AND METHODS

Preparation and KCN treatment of chloroplasts

Chloroplasts (unfragmented, naked lamellae) were prepared from commercial spinach (*Spinacia oleracea* L.) as described before[8] and finally suspended in a medium containing 0.1 M sucrose, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH buffer (pH 7.4) and 2 mM $MgCl_2$ (chlorophyll, approx. 2 mg/ml). KCN treatment of chloroplasts was carried out as follows: 3 ml of the above chloroplast suspension were transferred to an ice-chilled test tube containing a mixture of 1 ml each of 0.15 M KCN and 0.3 M Tricine (acid) and 25 μl of 10 mM potassium ferricyanide. The final pH of the mixture was 7.8–7.9. The trace of ferricyanide included was not an essential ingredient but it accelerated the development of KCN inhibition. The test tube was sealed and allowed to stand at 0 °C in the dark. After 60–90 min incubation the mixture was exposed to room light, and the treatment was terminated by diluting and lowering the pH of the mixture with 10 ml of a medium containing 0.1 M sucrose, 30 mM HEPES–NaOH buffer (pH 7.2) and 2 mM $MgCl_2$. This procedure was effective in preventing the development of a secondary effect of KCN (Photosystem II inhibition) which would otherwise become appreciable after 90 min incubation. At no stage was it necessary to add fresh KCN to compensate for dilution since the KCN inhibition is essentially irreversible.

Measurements

Oxidation–reduction of cytochrome *f* (cytochrome c_{554}) and P700 was observed with a Hitachi–Perkin Elmer dual-wavelength spectrophotometer (Model 356). Reactions were run in a standard 1-cm cuvette thermostated at 20 °C. The actinic light

was provided by a pair of 100-W quartz–iodine lamps. Monochromatic red light (660 nm; half-band width 10 nm) and far-red light (714 nm; half-band width, 10 nm) were obtained by means of interference filters. The oxidation–reduction of cytochrome *f* was followed by observing the absorbance change of the α-band at 554 nm (reference wavelength, 540 or 565 nm). The oxidation–reduction of P700 was observed at 701 nm (reference 730 nm). The P700 changes were also observed by following the EPR signal (signal I; $g=2.0025$)[9,10] with a Varian E4 EPR spectrometer routinely operated at a microwave power of 5 mW. The peak-to-peak modulation amplitude used was 4 G for field scans and 8 G for kinetic experiments. In the latter experiments, changes in the height of the low-field derivative peak were followed with a time constant of 0.5 to 0.03 s. The deflections recorded were 1/5 to 1/4 of the full scale.

RESULTS

The effect of KCN on oxidation–reduction of cytochrome f

The responses of cytochrome *f* to red (660 nm) and far-red (714 nm) light in KCN-treated chloroplasts were compared with those in 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned chloroplasts (Fig. 1). The electron acceptor used was methylviologen. In these chloroplasts the Hill reaction activity with methylviologen was inhibited by more than 90%. The top trace of Fig. 1A represents the familiar pattern of cytochrome *f* oxidation–reduction in untreated (control) chloroplasts which has been documented in detail by Avron and Chance[11]. The middle trace of Fig. 1A clearly shows that KCN severely inhibited the oxidation of cytochrome *f* by 714-nm light. However, once the cytochrome was photooxidized at a diminished rate, it could be readily reduced by 660-nm light, suggesting that the pathway of electrons from water to cytochrome *f* was not blocked. In sharp contrast, DCMU stimulated the oxidation of cytochrome *f* by 714-nm light, as one would expect from blocking of the flow of electrons from Photosystem II (which is weakly activated by 714-nm light). Similarly, 660-nm light, now largely acting as Photosystem I light, oxidized cytochrome *f* instead of reducing it (Fig. 1A, bottom trace). An impeded oxidation of cytochrome *f* in KCN-treated chloroplasts was also clearly observed in a series of experiments given in Fig. 1B in which the effect of 714-nm light was examined in the presence of a weak 660-nm background light. Fig. 1C shows light-*minus*-dark difference spectra obtained under the conditions of Fig. 1A. There was no evidence that any chloroplast component other than cytochrome *f* was contributing to the spectra, except for the tail end of the 518-nm shift or/and the "570-nm shift"[12,13].

Experiments were also conducted using chloroplasts in which the Hill reaction activity with methylviologen was completely inhibited by a prolonged KCN treatment (2 h). In these experiments the reference wavelength was chosen at 565 nm to minimize the influence of the 518-nm shift. The mild reductant ascorbate (5 mM) was included in the reaction mixture so as to ensure the reduced state of cytochrome *f* before illumination, and DCMU to block Photosystem II and provide the optimal condition for observing the photooxidation of cytochrome *f*. The effectiveness of ascorbate was evident from the experiments with untreated chloroplasts shown by the top row traces of Fig. 2. The traces depict that the very slow reduction process (due to exhaustion of electron pools) of cytochrome *f* after oxidation by 714-nm light was markedly accelerated by the presence of ascorbate. Clearly, however, the donation of

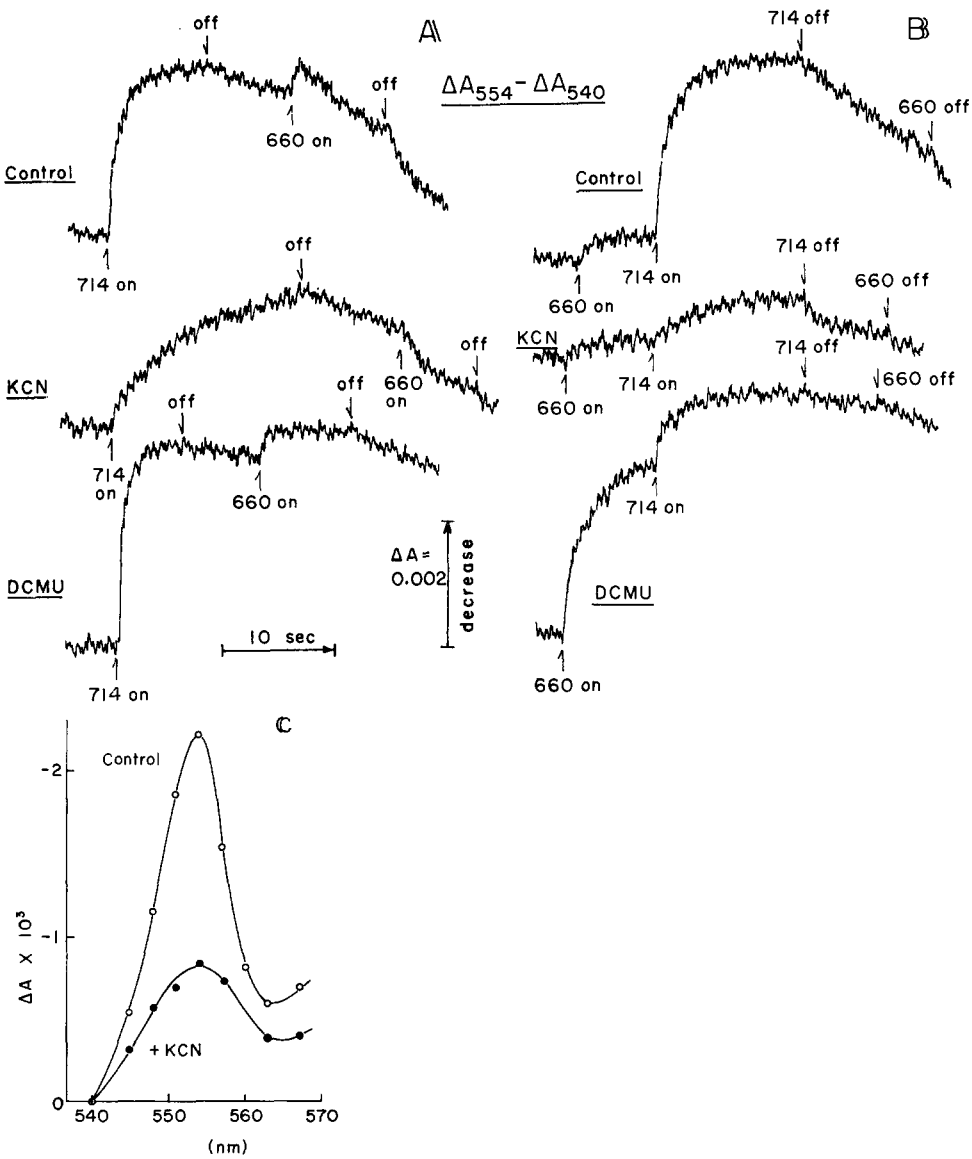


Fig. 1. Inhibition of cytochrome *f* oxidation by KCN treatment as contrasted with the inhibition of cytochrome *f* reduction by DCMU. The reaction mixtures (2 ml) contained 0.1 M sucrose, 40 mM Tricine–NaOH buffer (pH 7.8), 2 mM $MgCl_2$, 10 μM methylviologen (MV) and, if used, 1 μM DCMU. The concentration of chlorophyll was 62 μg/ml in the experiments of Fig. 1A or 75 μg/ml in Fig. 1B. The intensities of actinic light in kergs·s^{-1}·cm^{-2} were: (A) 12 (660 nm) and 8 (714 nm); (B) 2 (660 nm) and 8 (714 nm). The light-*minus*-dark spectra of C were obtained under the conditions of A. For KCN treatment of chloroplasts, see Methods.

electrons from ascorbate was still far too slow to counterbalance the withdrawal of electrons from cytochrome *f* by the 714-nm light. In KCN-treated chloroplasts, no trace of cytochrome *f* photooxidation was observed regardless of the presence or

absence of ascorbate and DCMU (Fig. 2, middle row traces). Yet in the same chloroplasts the photoreduction of cytochrome *f* was clearly observed when the cytochrome had been partially oxidized beforehand chemically with the lipid-soluble oxidant duroquinonediimide (oxidized diaminodurene), indicating that the pathway of electrons from water to cytochrome *f* was still open (Fig. 2, bottom traces). A slight photoreduction of cytochrome *f* was also detected in the presence of ferricyanide (0.4 mM) but only 3–4 min after the addition of ferricyanide. Apparently this hydrophilic oxidant does not react with cytochrome *f in situ* as readily as does oxidized diaminodurene.

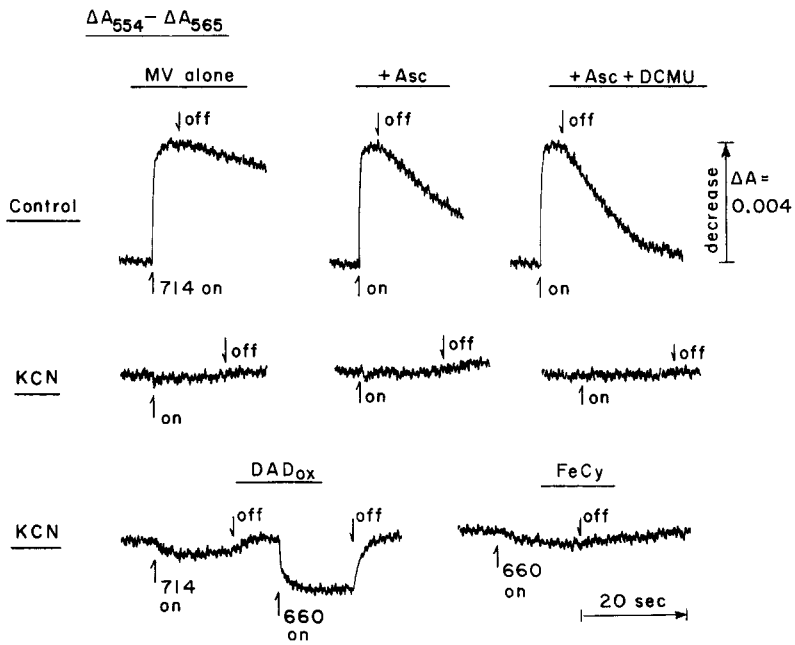


Fig. 2. Complete inhibition of cytochrome *f* oxidation by prolonged (2 h) KCN treatment of chloroplasts. The basic composition of reaction mixtures was as in Fig. 1. Oxidised diaminodurene (duroquinonediimide; final 0.4 mM) was prepared immediately before the reaction by mixing 0.8 μmole of diaminodurene and excess potassium ferricyanide (2.4 μmoles) in the buffered reaction mixture (2 ml). When ferricyanide was used alone, the concentration was 0.4 mM. The chlorophyll concentration was 80 μg/ml. The intensities of actinic light (red and far-red) were as in Fig. 1A. The light-*minus*-dark difference spectra of these changes in the 540–570-nm region confirmed that they were primarily due to cytochrome *f* changes. MV, methylviologen; Ass, ascorbate; DAD_{ox}, oxidised diaminodurene.

The effect of KCN on oxidation–reduction of P700

The effect of KCN treatment of chloroplasts on P700 was first examined spectrophotometrically by observing the absorbance changes at 701 nm (reference 730 nm) after flashes of 660-nm light (Fig. 3A). In control chloroplasts, no P700 shift remained when the fluorescence caused by the flashes had disappeared (top trace), indicating that photooxidized P700 was re-reduced as soon as the light was shut off or, more likely, it stayed reduced during the 660-nm flashes which strongly activate Photosystem II as well as Photosystem I (see also Fig. 4). In KCN-treated chloroplasts,

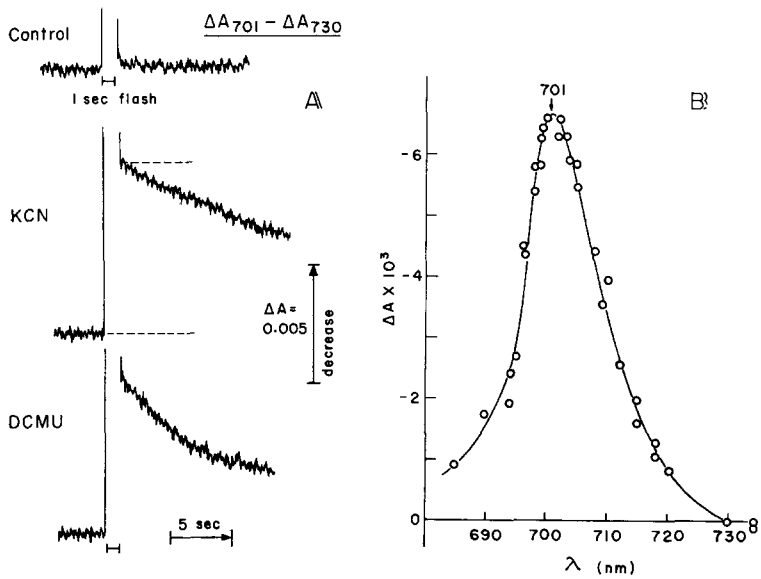
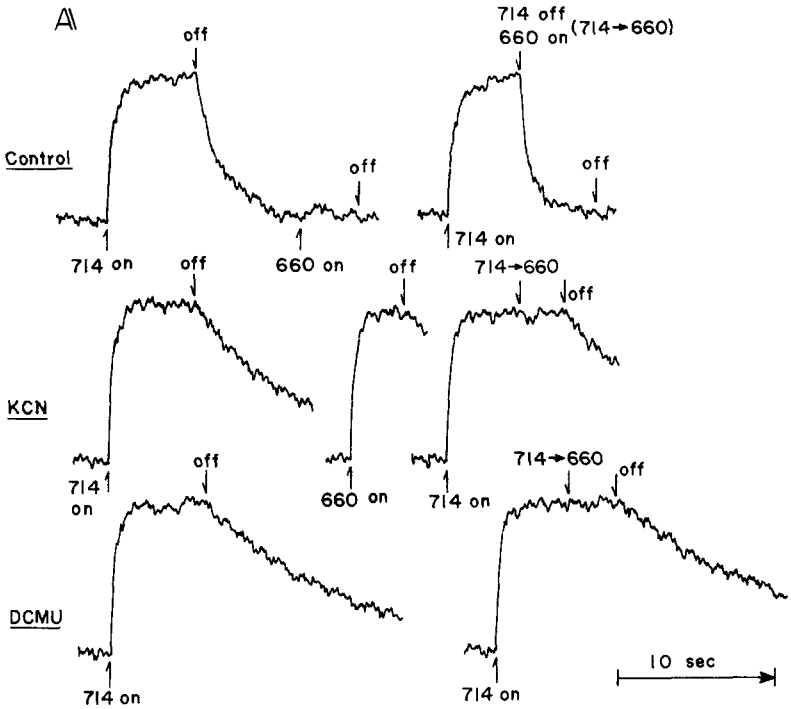


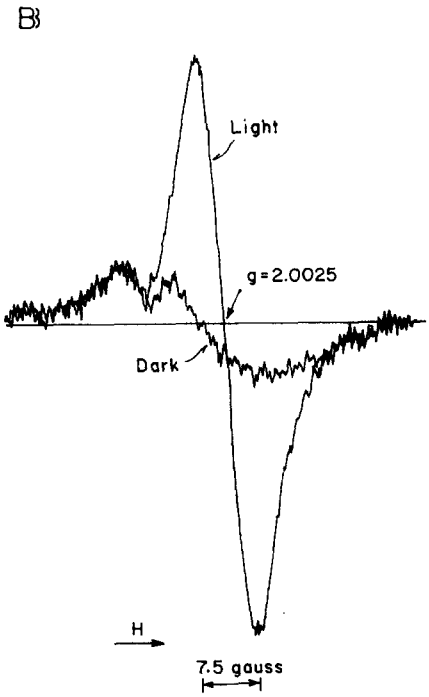
Fig. 3. Inhibition of P700 reduction by KCN treatment and by DCMU as observed spectrophotometrically by the dark decay of P700 after flashes. The basic composition of reaction mixtures was as in Fig. 1A. The chlorophyll concentration was 52 μg/ml. The intensity of actinic light (660 nm) was 24 kergs·sec^{-1}·cm^{-2}. The photomultiplier was guarded with a Corning CS2-64 filter and Kodak Wratten filters No. 36 and No. 70. The large signals observed during flashes were due to chlorophyll fluorescence.

however, a slow process of dark reduction of P700 was clearly observed after the flashes (Fig. 3A, middle trace). The decay kinetics were indeed so slow that there was practically no need for extrapolating the trace to estimate the oxidation level of P700 achieved in the light. (A typical light-*minus*-dark difference spectrum thus obtained is given in Fig. 3B.) Very similar results were obtained with DCMU-poisoned chloroplasts (Fig. 3A, bottom trace). Thus, viewing from P700, there is no clear distinction between the effects of KCN and DCMU: both inhibitors block the pathway of electrons from Photosystem II to P700.

The inhibition of P700 reduction by KCN was also confirmed by following the light-induced EPR signal (Signal I)[9,10] which originates from the oxidized form of P700 (Fig. 4). The "push–pull" effect of red and far-red light on P700 was very clearly observed in control chloroplasts (Fig. 4A, top row traces). This two-light effect, which is very similar to that on cytochrome *f*, was first demonstrated by Kok and Beinert[14] and was confirmed recently by Bochman *et al.*[15]. Both KCN and DCMU abolished this effect. The red light was no longer able to reduce P700; instead, it acted simply as Photosystem I light, always causing an oxidation of P700. Again a slowed-down process of reduction of P700 was evident (Fig. 4A, middle and bottom traces).

Thus, comparing the effects of KCN and DCMU on cytochrome *f* with their effects on P700, an unambiguous picture emerges: DCMU blocks electron transport at a point that precedes both cytochrome *f* and P700 (as is well-known), whereas KCN interferes with the transfer of electrons from cytochrome *f* to P700.


A
Control
KCN
DCMU
off
714 off
660 on (714→660)
714 on
660 on
714→660
10 sec


B
Light
g=2.0025
Dark
H
7.5 gauss

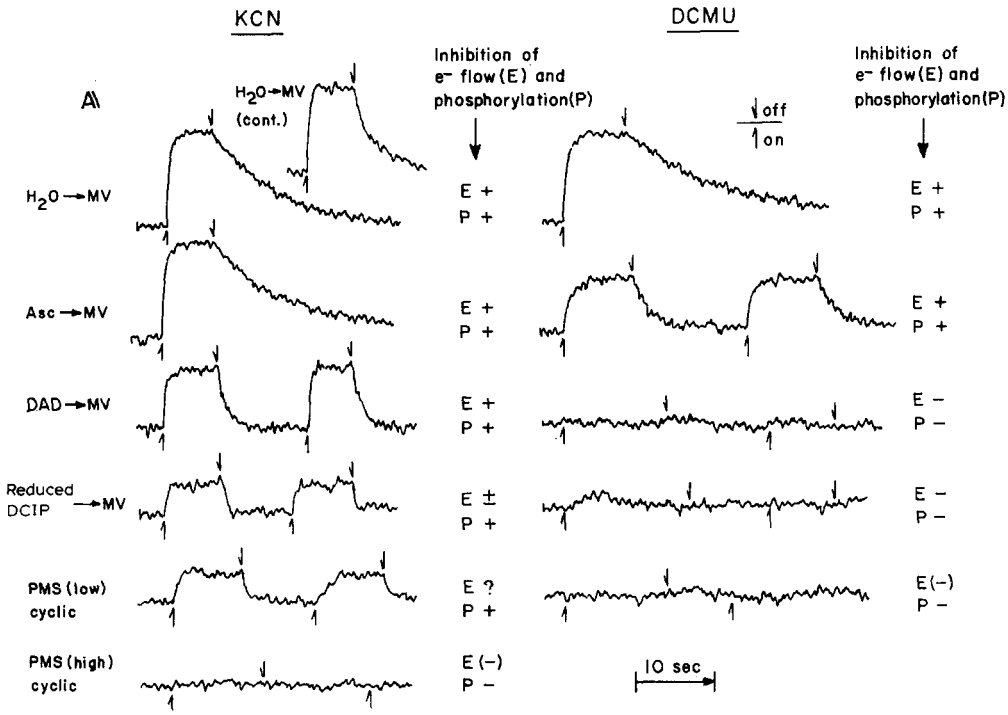
Accessibility of P700 to artificial electron donors in KCN-treated chloroplasts

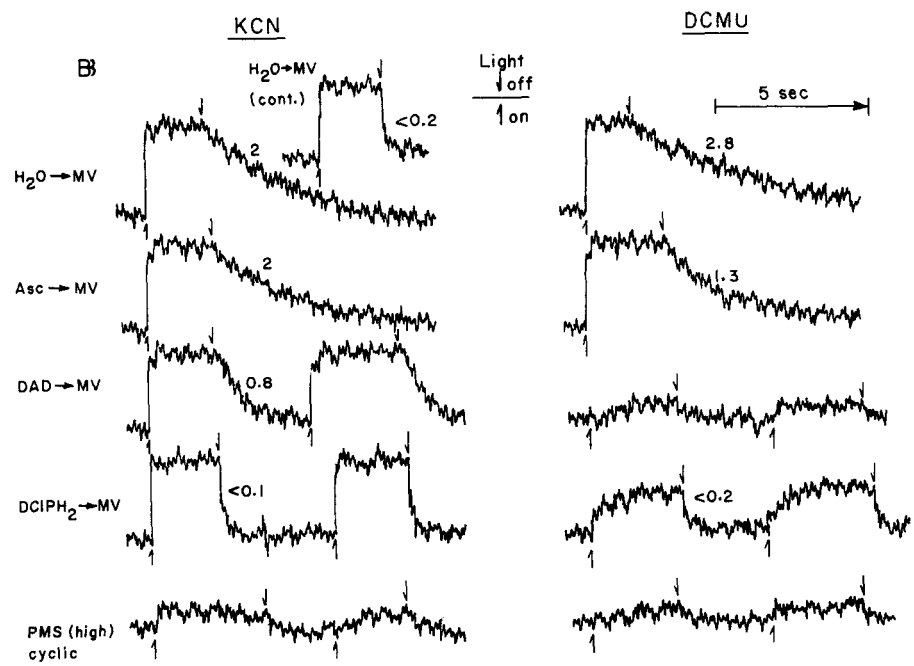
In order to further differentiate the site of KCN inhibition from the known site of DCMU inhibition, the behavior of P700 (as EPR signal) in KCN-treated chloroplasts was compared with that in DCMU-blocked chloroplasts in the presence of various artificial electron donors. It should be noted here that in these "inhibited" chloroplasts Photosystem II is functionally disconnected from Photosystem I, and therefore the effect of light on P700 is always in the direction of oxidation.

Fig. 5A represents experiments with relatively weak far-red light. When DCMU blocked the electron transport chain near Photosystem II, P700 was still easily accessible, through the rest of the chain, to diaminodurene, reduced 2,6-dichlorophenolindophenol (DCIP) and reduced *N*-methylphenazonium methosulfate (PMS) (high or low concentrations). This is suggested by the fact that in the presence of any of these reductants the actinic light failed to oxidize P700. In sharp contrast, when KCN blocked the chain near Photosystem I, only high concentrations of PMS (0.2 mM; partly reduced with 0.1 mM ascorbate) were able to keep P700 completely reduced in the light. Qualitatively, these observations are quite consistent with the fact that DCMU inhibits none of the reactions supported by these reductants, while KCN inhibits all of the reactions except the cyclic photophosphorylation supported by high concentrations of PMS[7] (see also inhibition data cited in Fig. 5A). Ascorbate, when given alone (5 mM) had virtually no access to P700 when the electron transport chain was blocked by either of these inhibitors.

Fig. 5B depicts an independent series of experiments with a different batch of chloroplasts, in which a much higher light intensity and a faster instrumental time constant (resolution, approx. 0.1s) were employed. It can be seen in DCMU experiments that the donation of electrons from reduced DCIP was no longer able to keep pace with an increased rate of P700 photooxidation. This is clearly a reflection of the fact that reduced DCIP is a much poorer donor compared with diaminodurene and (reduced) PMS. In KCN-treated chloroplasts a full photooxidation of P700 was now achieved even in the presence of diaminodurene or reduced DCIP. Only high concentrations of partially reduced PMS continued to prevent the photooxidation of P700. However, the kinetics of the dark reduction of P700 by reduced DCIP (dark decay) in KCN-treated chloroplasts were relatively fast ($t_{1/2} \leqslant 0.1$ s), in line with the fact that the transfer of electrons from reduced DCIP to methyviologen contains an appreciable portion which is insensitive to KCN[2,7]. (In the chloroplast preparations utilized in these studies the control and KCN-inhibited rates of the reduced DCIP $\rightarrow$methylviologen reaction were typically 400 and 150 μequiv $\cdot h^{-1} \cdot mg^{-1}$ chlorophyll, respectively.)

Fig. 4. (A) Effects of KCN treatment and DCMU on the light-induced oxidation–reduction of P700 as observed by the EPR signal (Signal I). The height of the low-field peak of the first derivative EPR spectrum of oxidized P700 (Fig. 4B) was followed with a time constant of 0.5 s. The upward deflections (appearance of signal) indicate the oxidation of P700 and the downward deflections (disappearance of signal) indicate reduction. The peak-to-peak modulation amplitude used was 8 G for kinetics or 4 G for spectrum. The microwave power was 5 mW. The reaction mixture (0.5 ml) in a flat quartz sample cell (thickness 0.25 mm) contained 0.1 M sucrose, 50 mM HEPES–NaOH buffer (pH 7.6), 2 mM $MgCl_2$, 10 μM methylviologen and, if used, 2 μM DCMU. The chlorophyll concentration was 500 μg/ml. The intensities of red (660-nm) and far-red (714-nm) light were 26 and 12 kergs $\cdot s^{-1} \cdot cm^{-2}$, respectively. MV, methylviologen.


KCN
DCMU
A
Inhibition of e⁻ flow (E) and phosphorylation (P)
Inhibition of e⁻ flow (E) and phosphorylation (P)
H2O→MV (cont.)
off
on
H2O→MV
Asc→MV
DAD→MV
Reduced DCIP →MV
PMS (low) cyclic
PMS (high) cyclic
E + P +
E + P +
E + P +
E ± P +
E ? P +
E (−) P −
E + P +
E + P +
E − P −
E − P −
E(−) P −
10 sec


KCN
DCMU
B
H2O→MV (cont.)
Light
off
on
5 sec
H2O→MV
Asc→MV
DAD→MV
DCIPH2→MV
PMS (high) cyclic
2
<0.2
2
0.8
<0.1
2.8
1.3
<0.2

DISCUSSION

Plastocyanin as the site of KCN inhibition

In this paper we have presented spectroscopic evidence that KCN blocks the transfer of electrons from cytochrome *f* to P700. This location of the KCN inhibition site is in harmony with the inhibition data previously obtained for various partial reactions[7]. The fact that KCN inhibition occurs closer to P700 than does DCMU inhibition has also been confirmed by the experiments with artificial electron donors described in the preceding section. As for the identity of the target for the KCN inhibition, isolated plastocyanin has already been shown to react with KCN quite rapidly under the conditions required for electron transport inhibition[7]. Katoh's dialysis experiment suggests that KCN may remove copper from plastocyanin[16]. No reaction between isolated cytochrome *f* and KCN has been detected[7]. These observations, taken together with the various lines of evidence which indicate the involvement of plastocyanin in electron transfer between cytochrome *f* and P700 (refs 17–21), lead us to the following conclusion. The KCN inhibition of chloroplast electron transport arises primarily from a blocking of electron transfer between cytochrome *f* and P700, and this blocking is due to an inactivation of plastocyanin involved therein. Conversely, it could be said that our observations speak strongly in favor of the view which places plastocyanin between cytochrome *f* and P700, rather than the view advanced by Knaff and Arnon[22] which places plastocyanin before cytochrome *f*.

The inhibitors of chloroplast reactions which have so far been established to block the entry of electrons to Photosystem I are very few: an antibody to plastocyanin[23], polycations[24,25] and the cyanide dealt with here. All of these agents appear to react specifically or primarily with plastocyanin *in situ* but all under limited conditions: in extensively sonicated chloroplasts (antibody), in the absence of salts (polycations), or after 60 min preincubation (KCN). For studying reactions that involve phosphorylation or require membrane integrity, KCN seems to have advantage over the other two in that it is applicable to well coupled chloroplasts without frag-

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Fig. 5. Effects of KCN treatment and DCMU on the oxidation–reduction of P700 in the presence of various artificial electron donors (EPR experiments). The upward deflections (appearance of signal) indicate the oxidation of P700 and the downward deflections (disappearance) indicate reduction. The basic composition of reaction mixtures was the same as in Fig. 4 except for the presence of various reductants. The concentration of reductants were: ascorbate (in the ascorbate →methylviologen system) 5 mM; diaminodurene, 0.2 mM (with 5 mM ascorbate); reduced DCIP, 0.24 mM (with 5 mM ascorbate), low PMS, 10 μM (with 10 μM ascorbate) and high PMS, 0.2 mM (with 0.1 mM ascorbate). In A the inhibition data for electron transport (E) and phosphorylation (P) are from ref. 7 except for the ascorbate→methylviologen system which are from unpublished data of one of the authors (S.I.). The + sign indicates strong inhibition; −, no or little effect; ±, partial inhibition. The signs in parentheses for electron transport are deductions from phosphorylation data. In Fig. 5A the chlorophyll concentration was 520 μg/ml. The actinic light used was 714-nm light (12 kergs·s^{-1}·cm^{-2}). Other assay conditions were as in Fig. 4. B represents a series of experiments conducted separately from those of A using a different batch of chloroplast preparation. The compositions of reaction mixtures were as in A except for the chlorophyll concentration which was 500 μg/ml. The actinic light used was relatively strong broad-band red light (640–700 nm; 180 kergs·s^{-1}·cm^{-2}). In these experiments the changes in signal height were followed with a time constant of 30 ms. The figures, 0.2, 2, 2.8, *etc.*, represent approximate half-times (s) of dark decay (P700 reduction). In both A and B the top trace labelled "cont." is for control (uninhibited) chloroplasts. MV, methylviologen; Asc, ascorbate; DAD, diaminodurene.

menting or exposing them to unfavorable ionic environment. There has been no indication of KCN affecting the mechanism of phosphorylation in any way. In fact, KCN inhibition has already been successfully applied in attempts to resolve phosphorylation sites[7], and also in a membrane-probe study designed to assess the relative energy output from various segments of the electron transport chain[26].

Interpretation of KCN-resistant Photosystem I reactions

The phosphorylation reaction associated with the transfer of electrons from reduced DCIP to methylviologen is abolished by KCN, but the electron transport itself is only partially inhibited[7]. The EPR experiments of Figs 5A and 5B revealed that KCN in fact does not completely block the pathway of electrons from reduced DCIP to P700, suggesting that a part of electron donation from reduced DCIP takes place directly at P700. The implication seems clear: the electrons entering the transport chain at P700 do not support phosphorylation. Only that portion of electrons which enters the chain at a point that precedes the KCN-sensitive site (plastocyanin) supports phosphorylation, presumably because this point also precedes a site of phosphorylation associated with the main pathway of electron transport (*cf.* refs 12 and 27). This interpretation also offers an explanation to the well-known but poorly understood fact that the phosphorylation associated with reduced DCIP reactions often appears to be totally unrelated to the observed electron flux[28].

Cyclic photophosphorylation catalyzed by PMS represents a unique case. It is almost as sensitive to KCN as is the diaminodurene-mediated system when catalyzed by 10 μM PMS, but becomes highly insensitive when the PMS concentration is raised to 0.2 mM (ref. 7). The EPR experiments of Figs 5A and 5B suggest that P700 may be easily accessible to the electrons from high concentrations of reduced PMS even when the electron transfer between cytochrome *f* and P700 is blocked by KCN. This finding is reminiscent of the observations of several workers[29–31] that at least in some chloroplast preparations (long-aged chloroplasts or detergent-prepared particles) P700 can accept electrons from reduced PMS (>30 μM) directly and very rapidly. These facts may seem to suggest that in the PMS cyclic system the phosphorylation reaction can be supported by the electrons entering the transport chain at P700. However, Fork and Murata[32] clearly showed that in chloroplast fragments the dark reduction of P700 by reduced PMS was much more efficient when the native state of the linkage between P700 and cytochrome *f* was partially preserved than when it was disrupted by detergents. Thus, in unfragmented chloroplasts, quite possibly a large portion of electrons from (photo-)reduced PMS may enter the main transport chain well before the plastocyanin–P700 region, and it may be this cyclic electron flow that supports phosphorylation. There might not be any important difference between this PMS cycle and the cycle mediated by diaminodurene. The fact that high concentrations of PMS relieve KCN inhibition of cyclic photophosphorylation could be explained if one considers the possibility that PMS, besides mediating cyclic electron transport, tends to create a by-pass in the plastocyanin region. The reported ability of PMS to complex with P700 (ref. 31) seems to suggest such a possibility. Some variable portions of the electrons from reduced PMS may well enter the chain at P700 simultaneously, and form a second cycle which, however, is probably nonphosphorylating as in the case of reduced DCIP. This may explain why the quantum efficiency of PMS-mediated

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