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Plastocyanin as the Possible Site of Photosynthetic Electron Transport Inhibition by Glutaraldehyde[1]

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ABSTRACT

Treatment of spinach chloroplasts with glutaraldehyde causes an inhibition in the electron transport chain between the two photosystems. Measurements of O_2 flash yields, pH exchange, and fluorescence induction show that the O_2 evolving apparatus, photosystem II and its electron acceptor pool are not affected. The behavior of P700 indicates that its reduction but not its oxidation, is severely inhibited. Cytochrome *f* is still reducible by photosystem II but also slowly oxidizable by photosystem I. The sensitivity of isolated plastocyanin to glutaraldehyde further supports the conclusion that glutaraldehyde inhibits at the plastocyanin level and thereby induces a break between P700 and cytochrome *f*.

In a preceding paper we described attempts to stabilize the photochemical activity of chloroplasts *in vitro* (7). We reported that GA[2]-treated chloroplasts, in which electron transport from water to viologen or ferricyanide (18, 21, 25) is inhibited, evolve O_2 with high rate in the presence of lipophilic electron acceptors (23).

A similar discrimination between the two types of electron acceptors has been observed in chloroplasts which were inhibited by KCN (20), DBMIB (8), or polycations (3, 19). These various agents appear to affect electron transport between the two photosystems so that the reduction of hydrophylic acceptors, which are presumably reduced by PSI, is inhibited. The reduction of lipophylic acceptors is unimpaired, presumably because they are reduced by PSII directly, possibly via plastoquinone. We have assumed that GA causes a similar inhibition and this paper presents additional attempts to locate the site of inhibition.

MATERIALS AND METHODS

Spinach chloroplasts fixed by GA and reaction mixtures for assaying electron transport activities were prepared as described in our preceding paper (7).

Techniques used for the various measurements, such as flash yields of O_2 or pH, fluorescence induction, and absorption changes have been described earlier (4, 5, 13, 17). Routinely, the chloroplasts used in the present study were fixed by a 5-min exposure to 5% GA at 4 C. $HgCl_2$-inhibited chloroplasts were prepared as described earlier (11). Isolated horse heart Cyt *c* as well as the GA were purchased from Sigma. All experiments were done at roughly 22 C.

RESULTS

Rates of Electron Transport. The data presented in Table I, column 1 show the effect of GA fixation on the rates of various electron transport activities measured with rate-saturating light. The results show a severe inhibition of O_2 evolution if it is coupled to a hydrophilic (PSI) acceptor, but only a mild inhibition when lipophilic (PSII) acceptors are used (7). Table I also includes data reported in the literature concerning four other inhibitors which have similar behavior (3, 8, 11, 12, 19, 20). The action of GA appears to be similar to that of KCN and polylysine. $HgCl_2$ seems to act more drastically, allowing only a low rate of O_2 evolution coupled to lipophilic acceptors and no electron donation to PSI at all. In contrast, DBMIB does not inhibit electron donation to PSI. Except for DBMIB which presumably acts at the reducing side of the plastoquinone pool (24), the other various inhibitors are thought to inactivate or remove plastocyanin.

O_2 Flash Yields. As shown in our preceding paper, the inhibition of electron transport in fixed chloroplasts decreases at very low light intensities, which indicates that a dark reaction step is involved, and that the photochemical traps may be fully active. This conclusion was confirmed by using illumination with saturating flashes given at a low rate (two flashes/sec). Figure 1, A and B shows the O_2 yields induced by such a series of flashes in (30 min) dark-adapted chloroplasts in the absence of an electron acceptor. The O_2 flash yield shows the typical oscillations (13), with a cycle of four lasting about three cycles, with the fixed as well as with the untreated chloroplasts. The average yield of the first 10 flashes in the two sequences was similar, but in the treated material the oscillation is more damped, indicating a somewhat higher number of "misses" (~20% *versus* 13%). We conclude that GA treatment has little effect on PSII. The steady-state yield, attained after depletion of the pools, dropped to ~16% of the initial average in the fixed chloroplasts as compared to ~30% in the control. Apparently even in intensities as weak as two hits/sec·trap, a GA-induced retardation of electron transport from PSII is noticeable.

Proton Exchange Processes. Table II shows some observations concerning light-induced proton exchange in fixed and nonfixed chloroplasts. The pH changes were induced either by a strong continuous light to study the build-up of a proton pool inside the thylakoids, or by short saturating flashes to study the pH changes coupled to single turnovers of the photoacts. As seen in this table, the extent of the proton uptake induced by continuous light in the presence of FeCn or MV is inhibited to about 25% of the control. The true degree of inhibition of electron transport must have been more severe than indicated by this number because: (*a*) in the absence of an uncoupler the

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[2] Abbreviations: Asc: ascorbate; DAD_{ox}; oxidized diaminodurene; DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DPIP: 2,6-dichlorophenolinodophenol; DMQ: 2,5-dimethyl-*p*-benzoquinone; FeCy: potassium ferricyanide; GA: glutaraldehyde; MV: methylviologen; PD_{ox}: oxidized *p*-phenylendiamine; PMS: N-methylphenazonium methosulfate; TMPD: N-tetramethyl-*p*-phenylendiamine.

Table I. Comparison of the effects of various inhibitors on the rate of photosynthetic electron transport in chloroplasts.

(A) Electron transport from water to hydrophilic acceptors, MV and FeCn. Average original activity was 960 and 1220 μeq/mg Chl·hr, respectively.

(B) Electron transport from water to lipophilic acceptors PD, DAD and DMQ (oxidized by excess of FeCn). Average original activity was 1800, 1480, 1400 μeq/mg Chl·hr, respectively.

(C) Electron transport from DPIP, DAD or TMPD (reduced by excess of ascorbate) to MV (PS I only). Average original activity was 760 and 2050 μeq/mg Chl·hr, respectively.

Assays (GA column) were carried at pH 7.4, using 30 mM methylamine as uncoupler. The other data in the table are taken from indicated references

Reaction System	GA	KCN[a]	Poly-cations[b]	$HgCl_2$[c]	DBMIB[d]
	% of Original Activity				
(A)					
$H_2O \rightarrow$ MV	3	5	1	0	7
$H_2O \rightarrow$ FeCn	11	7	4	5	13
(B)					
$H_2O \rightarrow$ PD	70	70	70	20	63
$H_2O \rightarrow$ DAD	55	45	60	--	52
$H_2O \rightarrow$ DMQ	35	48	55	20	33
(C)					
DPIPH $\rightarrow$ MV	25	45	10	0	95
DADH $\rightarrow$ MV (or TMPD)	12	10	10	--	100

a Taken from Refs. 8, 19, 20.
b Taken from Refs. 3, 19.
c Taken from Refs. 11, 12.
d Taken from Ref. 8.

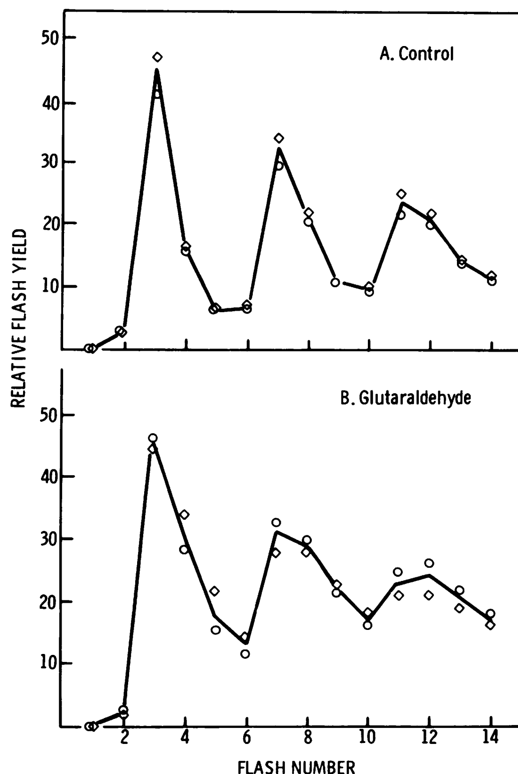

FIG. 1. O_2 flash yields in control and in GA-fixed chloroplasts. Same Chl concentration in both samples. The samples were 30-min dark-adapted prior to the flashing, in absence of electron acceptor. Flashing rate 2/sec. The different symbols refer to two experiments.

maximum rate also is low in the control chloroplasts; and (*b*) in the fixed chloroplast, the rate of proton efflux in the dark is slowed down by a factor of 1.5 to 2.

Again, the inhibition is much less in a weak illumination such as a sequence of flashes spaced at 1-sec intervals. The proton uptake induced by single flashes in the absence of an uncoupler and the proton release seen in the presence of methylamine are little affected by GA treatment (Table II, lines 3, 4). The data of Table II (line 5) confirm an earlier observation (25) that in the presence of PMS, which presumably mediates a cyclic electron flow in PSI, the inhibition is minimal. These data imply that GA fixation has little influence on the (photochemical and) proton exchange processes, although it inhibits the over-all electron transport.

Pool Sizes from the Fluorescence Induction Measurements. The data in Table III pertain to the effect of fixation on the size and reactivity of the PSII acceptor pools—as observed via fluorescence induction curves (15). In these experiments the fluorescence-exciting beam was applied to raise the fluorescence yield to a maximum level, interrupted during a measured dark time, and then applied again to record another rise curve. The area bounded by the rise curve served as an index of the acceptor pools. The rise curves observed with control chloroplasts after a 2-min far red illumination or with GA-treated material after a 10-min dark period, in both cases followed by the addition of 10 μM DCMU, were assumed to reflect a single equivalent/PSII trap "Q". GA fixation did not significantly change the parameters F_0, F_{max}, and the unit area, again supporting the view that the photochemistry of PSII is barely affected. In both the treated and the control chloroplasts, a 1-min dark period sufficed for the reoxidation of Q plus four to five acceptor equivalents which comprise the "A_2" pool (22); dark oxidation of the total pool required more than 10 min. However, whereas in normal chloroplasts pool oxidation could be accelerated photochemically by a 2-min exposure to far red light, this was not possible in treated material. Apparently, the oxidizing power generated by PSI could not, or could only ineffectively, reach the PSII acceptor pools. $HgCl_2$ treatment affected the chloroplasts in a similar way. Also, in this case, the reaction chain connecting the two photoacts appeared to be interrupted (11).

P700 Absorption Changes. Figure 2A shows the light-induced P700 changes in control chloroplasts in the presence of MV and in the absence of uncoupler. In strong red light (λ >650 nm), P700 is rapidly oxidized. When the light is turned off $P700^+$ is rapidly reduced by the pool of PSII electron acceptors which was reduced during the previous light period (see Table III). The 0.3-sec response time of the instrument did not allow for following the details of the kinetics in these instances. If a weak far red (720 nm) beam is then introduced, one observes a slow photooxidation of P700 with a distinct initial lag. This lag reflects the reduced pool, which maintains P700 in its reduced state until all intermediates in the chain are photooxidized via PSI. If the 720 light then is turned off, $P700^+$ is reduced very slowly ($t_{0.5}$ ~17 sec), presumably by unidentified reducing agents in the suspen-

Table II. Extent of proton uptake induced by flashes and by strong continuous light.

Electron acceptors	Illumination	Control chloroplasts	Glutaraldehyde-fixed chloroplasts
		Arbitrary units	
Ferricyanide	continuous light	35	9.5 (27%)[b]
Methylviologen	continuous light	24.5	5.5 (22%)
Ferricyanide	1 flash/sec	7.7	6.8 (88%)
Ferricyanide + methylamine	1 flash/sec	-8.7[a]	-6.5 (75%)
PMS	continuous light	23.5	17.0 (72%)

[a] Minus refers to flash induced proton release.
[b] % of control.

Table III. Fluorescence induction in GA fixed and in control chloroplasts.

The table shows the number of equivalents in the pools A_2 and A_{total}, calibrated against the pool seen in the presence of 10 μM DCMU, which is taken as 1. Numbers are averages of 2 to 5 experiments. Chloroplasts containing about 20 μg Chl/ml were suspended in STN pH 7.4 medium

Pretreatment	Dark		Light
	1 min	10 min	2 min far red light
Control	7	15	15
GA fixed	6	5	18
$HgCl_2$ inhibited	7	5	20

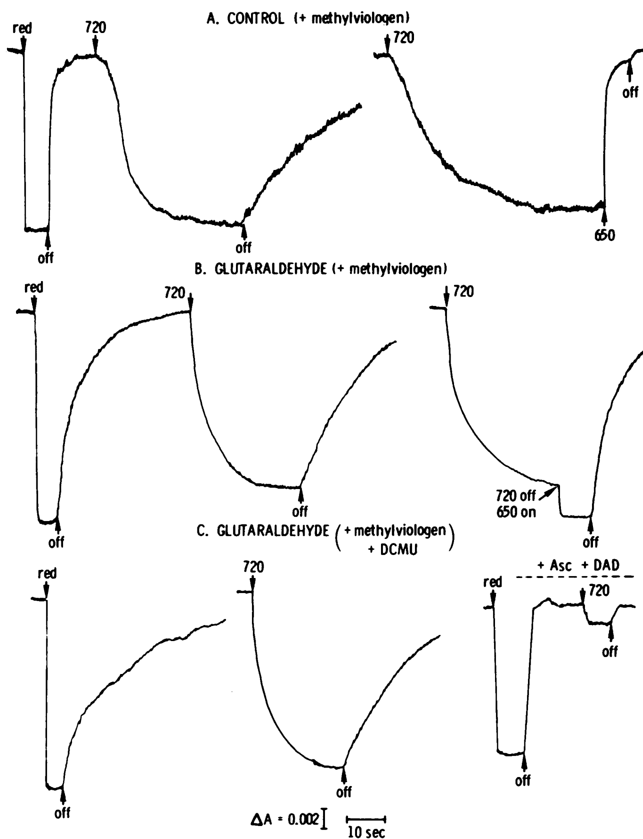

FIG. 2. P700 absorption changes in control and in GA-fixed chloroplasts in presence of MV and absence of uncoupler. Optical path length 3 mm. Chl concentration in A and B 100 μg/ml; in C 120 μg/ml. Concentration of MV 20 μM; DCMU 10 μM; ascorbate 10 mM; DAD 0.5 mM. Weak 650 light was obtained by using an interference filter with a band width of 10 nm and strong red light by using a cut-off (>650 nm) filter. Measuring and reference beams were 703 and 720 nm, respectively.

sion. If, after the far red oxidation, a weak 650 beam is given, PSII is activated preferentially and most of P700 is photoreduced ($t_{0.5}$ ~1 sec).

In fixed chloroplasts (Fig. 2B) the rapid photooxidation by strong red light resembles that in the control, showing that P700 photooxidation is unaffected by GA. However, when the red light is turned off, the reduction is slow ($t_{0.5}$ ~4 sec). The 720 beam introduced subsequently causes immediate photooxidation and no pool can be detected in the trace; in subsequent darkness the reduction by endogenous agents is very slow ($t_{0.5}$ ~12 sec). Upon switching from 720 to 650 light, the redox state of P700 changes little. In some experiments the pigment became more oxidized, while in others, slightly more reduced. The 650 beam provides more PSI photons than the 720 beam and evidently the reducing power generated by its PSII quanta has very limited access to P700, Upon darkening, the reduction is slow ($t_{0.5}$ ~4 sec, as was seen after the red illumination), but faster than seen after 720 light, which did not fill the pool. The behavior of P700 was not different when 100 μM ferricyanide instead of MV was used either in control or in fixed chloroplasts.

As can be seen, DCMU has little or no effect on the P700 behavior in fixed chloroplasts (compare Fig. 2, B and C). Also in this case the initial reduction of P700 is faster after red light than after 720 light. Considering that in the presence of DCMU, the pool is not being reduced and that upon darkening, Q^- is reoxidized in a less than 1-sec backreaction (2), it follows that some reducing power must have been stored in an intermediate "outside" the chain. This intermediate shares some features with "C" (14). It now appears that its reduction by PSII, like that of Q, is not influenced by DCMU; its route to P700 might bypass the quinone pool.

The rate of dark reduction by exogenous materials (which is insensitive to DCMU) can be greatly accelerated by the addition of ascorbate and DAD, for example. After such an addition, the 720 actinic light is too weak to change the oxidation state of P700. This implies that reduced DAD has (limited) access to $P700^+$ in fixed chloroplasts. Figure 3 shows similar experiments, in which we compared the effects of three mediators of cyclic electron transport—DPIP, PMS, and DAD—added in concentrations described elsewhere (20). In the presence of 0.1 mM DPIP (Fig. 3A), strong red light oxidizes P700 completely in control chloroplasts. When the red light is turned off, the DPIPH quickly reduces $P700^+$. The 720 beam is too weak to cause any net oxidation of P700 because the rate of reduction is much faster. In the fixed chloroplasts, the 720 light is relatively more efficient in oxidizing P700. Evidently the rate of reduction by DPIPH is slowed down, because of inhibition on the donor side of $P700^+$. In the presence of 0.2 mM PMS (Fig. 3B), the 720 light does not induce any net oxidation of P700, and even in the strong red light, oxidation is only partial. This behavior is not changed by GA, which is in agreement with the results on proton uptake (Table II). The site of entry of reduced PMS, therefore, must be closer to $P700^+$ than that of DPIPH. In the presence of 0.6 mM DAD (Fig. 3C), the 720 and the red lights are both quite ineffective in the control chloroplasts, which reflects very rapid electron donation. In the fixed material, the events resemble those seen with DPIPH: the rate of reduction has slowed down, presumably because one of the site(s) of entry of DADH has been removed.

As shown in Table I, reduced DPIP and reduced DAD sustain only low rates of reduction of MV after fixation. Still, these PSI

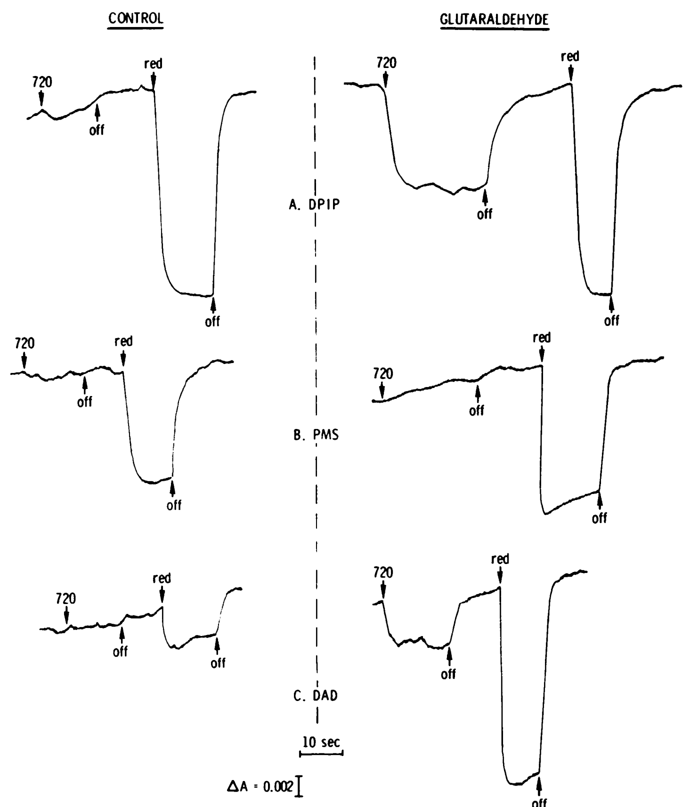

FIG. 3. P700 absorption changes in control and in GA-fixed chloroplasts in presence of cofactors of cyclic electron transport. Optical path length 3 mm. Chl concentration of control chloroplasts 147 μg/ml, and of the fixed 136 μg/ml. Concentration of DPIP 0.2 mM; PMS 0.2 mM; DAD 0.6 mM.

reactions are 3 to 10 times less inhibited than the over-all reaction $H_2O \rightarrow$ MV. We can explain these data by assuming that GA inactivates the endogenous donor to $P700^+$ which, in normal chloroplasts, is reduced rapidly by DADH or DPIPH. Reduced PMS can react rapidly with $P700^+$ itself but only slowly with DPIPH and DADH.

Cytochrome *f* Absorption Changes. Observations of light-induced absorption changes of Cyt *f* in fixed and unfixed chloroplasts are shown in Figure 4. Figure 4A shows the results obtained when DCMU, MV, and reduced DPIP were present so that photochemical activity was restricted to PSI. In the control, the behavior of Cyt *f* resembled that of P700: a strong red beam (but not 720 light) causes rapid oxidation and DPIPH causes rapid reduction in subsequent darkness. After GA treatment, the photooxidation by the red beam is severely inhibited. Since the photooxidation of P700 is not affected (Fig. 2), it appears that the electron transfer from Cyt *f* to P700 is inhibited, which points to plastocyanin as the site inhibition. In the experiment summarized by Fig. 4B, only MV was added so that the entire electron transport chain was operative. In the control, the events resemble those seen with P700 (Fig. 2 [17]). The 720 light, given after a long dark period, causes a slow oxidation, characterized by an initial lag, reflecting the oxidation of the pool of PSII acceptors (17). After the 720 light is turned off, however, the Cyt *f* remains oxidized whereas P700 slowly becomes reduced. The subsequent application of a weak (650 nm) actinic beam activates PSII and reduces most of the Cyt *f* at approximately one-third the rate it reduces P700.

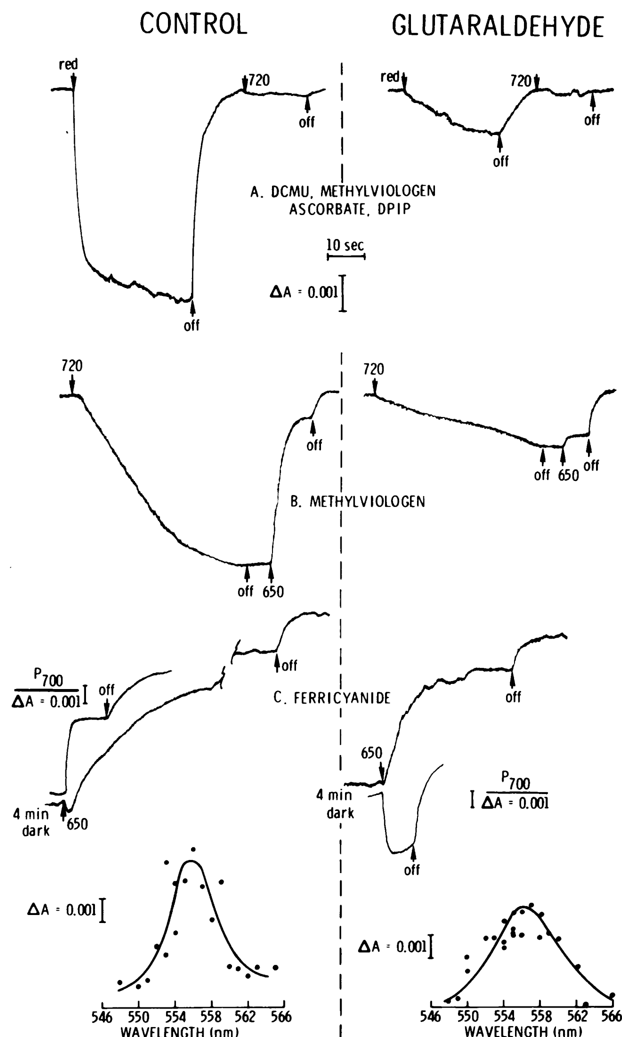

FIG. 4. Cyt *f* absorption changes in control and in GA-fixed chloroplasts. Optical path length 3 mm. Chl concentration (μg/ml) in A 136; in B 160; in C 300 for Cyt *f* and 125 for P700. MV 100 μM; DCMU 10 μM; ascorbate 5 mM; DPIP 100 μM; FeCn 0.4 mM. Measuring and reference beams for Cyt *f* were 554 and 540 mM, respectively. Actinic filters were as described in Figure 2. Absorption spectra refer to the total (650 nm and dark) reduction of Cyt *f*, after 4-min dark incubation in FeCn. Slit width was 1 mm and spectral dispersion 3.3 nm/mm.

In fixed chloroplasts, the behavior of Cyt *f* is very different from that of P700 (Fig. 2B). The oxidation of Cyt *f* by 720 light is slow and partial (30%), whereas the oxidation of P700 is rapid and nearly complete. A subsequent exposure to 650 light (which causes either no change or a further oxidation of P700) induces only a small reduction of Cyt *f* which is completed in about 1 sec during the following dark period. The spectra of light-driven oxidation and of light-induced reduction agree well with the absorption spectrum of Cyt *f* (1, 9) (data not shown).

Figure 4C shows the absorption changes in the presence of 0.4 mM ferricyanide. Since this agent oxidizes Cyt *f* very slowly, the chloroplasts were incubated 4 min in the dark (11). In the control, application of the weak 650 beam causes a small transitory oxidation (1), followed by a slow biphasic reduction of ~80% of Cyt *f*. This reduction contains an initial phase ($t_{0.5}$, 5-8 sec) and a secondary smaller, very slow component ($t_{0.5}$, 30-40 sec). The remaining 20% of Cyt *f* is reduced in the subsequent dark period. As in Figure 4B, this reduction, presumably by reducing equivalents generated by PSII, is rather slow ($t_{0.5}$, ~2 sec).

In GA-fixed chloroplasts following a 4-min incubation with ferricyanide, Cyt *f* was largely oxidized. The 650 light did not induce the initial fast oxidation dip and caused a partial (~80%) reduction which was monotonous and faster than in the control ($t_{0.5}$, 6-8 sec). Again, in the subsequent dark period, there was a further reduction (20%). Using equal Chl concentrations, we found the total absorption change due to Cyt *f* in GA-treated material to be about 75% of that in the control and its spectrum was broader, as seen in Figure 4C, bottom. Figure 4C also includes the absorption changes of P700 observed after a 4-min incubation in ferricyanide, which causes the oxidation of only a small fraction (15%) of P700. In control chloroplasts, the 650 light rapidly reduces most of P700 (see Fig. 2). In the fixed ones, it causes a further oxidation; in the subsequent dark period, an initial reduction ($t_{0.5}$ ~3 sec) precedes the very slow, partial reoxidation by ferricyanide. Thus, in the fixed chlorplasts, 650 light influences P700 and Cyt *f* in opposite directions, while in the dark, the PSII-generated reducing power still has slow access to both.

Effect of GA on Cytochrome and on Plastocyanin *in Vitro*. In a few cursory experiments, we compared the sensitivity of isolated Cyt *c* (assumed to be an analogue of Cyt *f*) to 5% GA and of plastocyanin isolated from leaves. Upon adding GA, Cyt *c* remained in solution. Its reduced form (obtained with either ascorbate or dithionite) showed no shift in the position of the α- and β-bands and a 10 nm blue shift of the γ-band relative to the reduced untreated protein. The amplitude of these absorption changes was 30 to 40% smaller than in the untreated protein. The original spectrum returned upon reoxidation with ferricyanide. In contrast, adding GA to a solution of plastocyanin produced precipitation within 1 to 2 min and prevented observation of oxidation or reduction.

DISCUSSION

The similarity between the effects of KCN, $HgCl_2$, polycations, and GA (Table I) probably reflects a common site of inhibition. The pattern of O_2 yields induced by flashes (Fig. 1), showed that neither the traps of PSII nor the O_2 evolving apparatus is much affected. The proton exchange data confirmed that

GA fixation causes a severe rate limitation in the electron transport chain but does not greatly affect proton translocation as such.

The fluorescence induction data revealed that the large pool of PSII electron acceptors is still operative. It is reoxidized in the dark, but cannot be oxidized by PSI. GA has the same effect on the fluorescence induction as $HgCl_2$, which is presumably due to inactivation of the plastocyanin (11, 12). However, since there has been no clear demonstration that $HgCl_2$ inhibition can be bypassed with PSII lipophilic acceptors (12) (and our unpublished results), this agent may act at more than one site.

The observations of P700 (Figs. 2 and 3) and of the PMS-mediated proton uptake showed that PSI was unaffected by GA. However, a severe inhibition on its donor side was evident. In the fixed chloroplasts the oxidation of Cyt *f* via PSI is slow and incomplete (Fig. 4). The behavior of P700 and Cyt *f* in fixed chloroplasts shows a resemblance to their behavior in chloroplasts inhibited by KCN (9). The conclusion with KCN is that it inhibits before P700 but after Cyt *f*, so that reduced DPIP and DAD were poor donors to MV. Most of our observations are readily explained if we assume that GA inactivates plastocyanin and that this agent functions as the immediate electron donor of P700 and an electron acceptor of Cyt *f*. Supporting evidence is the *in vitro* observation that plastocyanin is much more sensitive to GA than Cyt *c*. This higher sensitivity might be due to its high content of the reactive (6) glycine and/or possibly aspartic and glutamic acid, which causes the acidic nature of plastocyanin (10) as opposed to the basic nature of Cyt *c* (16).

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