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FURTHER PURIFICATION OF "TRITON SUBCHLOROPLAST FRACTION I" (TSF-I PARTICLES). ISOLATION OF A CYTOCHROME-FREE HIGH-*P*-700 PARTICLE AND A COMPLEX CONTAINING CYTOCHROMES *f* AND b_6, PLASTOCYANIN AND IRON-SULFUR PROTEIN(S)*

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SUMMARY

The "Triton Subchloroplast Fraction I" or "TSF-I particles" can be further fractionated into a cytochrome fraction and a *P*-700-containing fraction essentially free of cytochromes. The cytochrome complex contains cytochromes *f* and b_6 in approx. equimolar amounts, and, in addition, also plastocyanin and one iron-sulfur protein, all in the bound state. Bound plastocyanin was characterized by EPR spectroscopy. The EPR spectrum of the bound iron-sulfur protein resembles that previously detected in Phostosystem I particles under highly reducing conditions at lower than −560 mV.

The redox potential of *P*-700 in the cytochrome-free high-*P*-700 particles was measured to be +468 mV; those of cytochromes *f* and b_6 are +345 and −140 mV, respectively.

Among the four components present in the complex, only cytochrome *f* can be coupled to a Photosystem I particle and undergoes photooxidation. This coupled photooxidation is totally inhibited by KCN and only partially inhibited by $HgCl_2$.

The similarity of the complex containing cytochromes *f* and b_6, plastocyanin, and an iron-sulfur protein to complexes III and IV of the mitochondrial respiratory redox chain and a possible involvement of the complex in cyclic photophosphorylation are noted and discussed.

INTRODUCTION

Spinach chloroplasts can be treated with detergents [1] or by mechanical disruption [2] to yield subchloroplast fractions corresponding to the two photosystems [3]. The non-ionic detergent, Triton X-100, has been extensively used for this purpose [4–6]. Subchloroplasts representing the two photosystems called "Triton subchloro-

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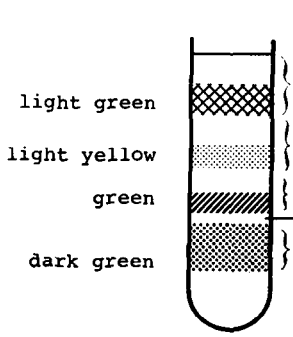
plast fractions I and II'' (TSF-I and -II particles) have been characterized [4–12]. The TSF-I particle contains approximately one each of *P*-700, cytochromes *f* and b_6 per 100–200 chlorophyll molecules. It was reported earlier that the cytochromes are not an integral part of the Photosystem-I particle but rather they co-sediment with the particles during the centrifugation step [8]. In the Photosystem-I particles prepared by digitonin treatment [13], the cytochromes are also partly solubilized. Wessels and Voorn [14] showed that digitonin-treated chloroplasts, when centrifuged in a sucrose density gradient, yield separate bands representing the Photosystem-I fragments and cytochromes. No definitive evidence has been reported thus far for coupled reactions involving the cytochromes in detergent-fractionated Photosystem-I particles, which is also consistent with the notion that the cytochromes are solubilized in most of these preparations. More recently, Nelson and Neumann [15] reported that cytochromes *f* and b_6 isolated from the system I particles by digitonin treatment of lettuce chloroplasts are a part of a single complex.

This paper reports the fractionation of the usually-prepared TSF-I particles from spinach into more purified Photosystem I particles essentially free of cytochromes and a separate cytochrome fraction by sucrose density gradient centrifugation. A routine method developed for preparing cytochrome-free high-*P*-700 particles will be discussed. It was further found that cytochromes *f* and b_6 isolated from the Triton-fractionated TSF-I particles are present as a complex, and that the complex also contains plastocyanin and iron-sulfur protein(s) in the bound state.

METHODS

Further fractionation of TSF-I particles

When the usually-prepared TSF-I particles (such as those used in ref. 5, or described in ref. 18) were further fractionated by centrifugation on a sucrose-density gradient, the band and material distributions appeared as shown in Fig. 1. The top light-green band contained mostly solubilized chlorophyll; the next two (light-yellow and green) bands contained mostly cytochromes plus some chlorophyll,



	volume, ml	cyt *f*	cyt b_6	*P*-700	Chl
		concn, nM			
light green	7.2	0.1	0.1	--	63
light yellow	5.2	2.3	3.1	--	57
green	3.7	2.3	3.4	0.6	107
	3.2	0.1	0.1	0.7	136
dark green	8.6	0.1	0.1	4.3	640

Fig. 1. Band and material distribution after further fractionation of a TSF-1 particles preparation by sucrose density gradient ultracentrifugation. Centrifugation was carried out in Polyallomer tubes (38.5 ml capacity) in a Spinco SW27 rotor at 131 000 × *g* for approx. 75 h. One pellet of TSF-I particles obtained by the usual procedure [18] was homogenized to give a chlorophyll concentration of 1.3 mg/ml, and was layered on the top of a continuous sucrose gradient (5–55 %).

but relatively little *P*-700; the bottom dark-green band consisted of predominantly *P*-700 and bulk chlorophyll, but few cytochromes. It is noted that the *P*-700/chlorophyll ratio of the purified photosystem I fraction, namely, the bottom dark-green band, was increased by approx. 20 % over the starting material.

Preparative procedures for cytochrome-free high-P-700 particles and a cytochrome complex

Photosystem I particles highly enriched in *P*-700 have been prepared by Triton treatment of photosynthetic membranes which are devoid of carotenoids [16, 17]. Carotenoids have been removed either by solvent extraction [16], as for spinach chloroplasts, or by using membrane fragments of *Anabaena variablis* grown in the presence of diphenylamine to suppress carotenoid formation [17], or by using a mutant strain of *Scenedesmus* which lacks carotenoids [17]. The high-*P*-700 particles prepared in this manner are enriched from the usual one *P*-700 per 100–200 chlorophyll molecules to approx. one in 30–40, while they still contain the same complement of cytochromes (typically one each of cytochromes *f* and b_6 per 100 chlorophylls). The high-*P*-700 particles apparently contain a fully active Photosystem I reaction center, since the absorption change associated with light-induced *P*-700 photooxidation is proportionally greater. However, while the high-*P*-700 particles prepared from *Anabaena* grown in diphenylamine and from the *Scenedesmus* mutant both have normal NADP-reduction activity, those prepared from solvent-extracted spinach chloroplasts have only negligible activity [16].

As a result of the fractionation study on TSF-I particles shown in Fig. 1, we subsequently explored a further modification of the basic routine procedure used in preparing TSF-I particles [18] for obtaining high-*P*-700 particles and the cytochrome complex. These procedures provide the greater amount of material that is necessary for use in characterization or studying photochemical reactions. The cytochromes absorb strongly in the Soret region, which overlaps not only with the Soret bands of chlorophylls and *P*-700, but the major band of *P*-430 [19] as well. Thus, a high-*P*-700 particle free of cytochromes would be extremely valuable for performing spectroscopic studies of Photosystem I in the Soret region.

Routine isolation of high-P-700 particles

Although the analytical procedure described in Fig. 1 may be performed on a larger scale to yield a greater quantity of the components, the procedure was modified for preparing the *P*-700-enriched fraction. First, a sufficient number of TSF-I particles pellets were resuspended in a 0.025 % Triton solution and recentrifuged at $50\,000 \times g$ for 10 h. The sediment and the upper half of the supernatant were discarded; the lower half of the supernatant was then recentrifuged. The lower 1/3 of the supernatant from this centrifugation was washed, concentrated, and layered on a 5–55 % sucrose gradient and recentrifuged at $131\,000 \times g$ for 65 h in a Spinco SW27 rotor. Four bands, namely, solubilized chlorophyll, a cytochrome complex, high-*P*-700 particles and a waxy, crusty material appeared at the end of this centrifugation.

In the following we present an extension of the basic fractionation scheme described in ref. 18 (p. 278) as an alternative procedure for the isolation of the high-*P*-700 particles. The original procedure [18] was first followed to obtain TSF-I particles. The bottom half of the supernatant was then diluted 1.5 times by adding

water and recentrifuged at 100 000 $\times g$ for 6–10 h. The sediment (designated as TSF-Ia) particles from this centrifugation was usually very active in NADP photoreduction. The high-*P*-700/low-cytochrome particles were usually located in the bottom 5 ml of the supernatant. This supernatant was washed on an Amicon XM100A ultrafilter to remove sucrose and then concentrated to a smaller volume. The concentrated suspension was layered on a 5–55 % sucrose gradient and centrifuged at 131 000 $\times g$ for 80–90 h. The major band containing the high-*P*-700/low-cytochrome particles may be purified once more by repeating the concentration and centrifugation steps as above. The particles so obtained contained one *P*-700 per 35$\pm$5 chlorophyll molecules, and had a chlorophyll/cytochrome ratio of 260 (once centrifuged) to 860 (twice centrifuged) or more.

Isolation of a cytochrome complex

This isolation procedure is also an extension of the usual fractionation scheme for TSF-I particles [18]. In this case, the upper 1/3 of the supernatant was removed and stored. The remaining supernatant was diluted 1.5 times with 0.05 M Tris buffer, pH 9 (hereafter designated as buffer, unless specified otherwise) and recentrifuged. The dilution-centrifugation step was repeated until very little sediment resulted. The combined supernatant was diluted twice with buffer and concentrated with an Amicon XM100A ultrafilter to remove Triton X-100, sucrose and small protein molecules. The concentrate was centrifuged at 105 000 $\times g$ for 4 h to remove any large particles. The supernatant was diluted 3 times with pH 9, 0.05 M Tris buffer and placed on a DEAE column which was pre-equilibrated with the same buffer. The column was washed with 1–2 l of buffer containing 0.1 % Triton, and then with buffer containing 0.2 % Triton until the deep green eluate ceased to appear. The column was then eluted with buffer containing 1 % deoxycholate until the brown band was completely eluted. In some cases, the yield was low or the eluate had a high cytochrome-f/b_6 ratio. If this happened, the column was finally eluted very slowly with a 0.05 M phosphate buffer, pH 9, containing 1 % deoxycholate. The combined fractions were washed and concentrated on the ultrafilter to remove deoxycholate, and the concentrate was rechromatographed on the DEAE column as before.

RESULTS AND DISCUSSION

Chemical composition; absorption and EPR spectra

As mentioned earlier, the low-cytochrome, high-*P*-700 particles prepared by the procedure described above contain one *P*-700 per 35$\pm$5 total chlorophyll molecules. For reasons to be discussed in the Redox Potentials section below, the *P*-700 content in these particles was estimated from light-induced absorbance changes in a reaction mixture containing methyl viologen as the electron acceptor and ferrocyanide as the donor [19].

Cytochromes *f* and b_6 are usually present in the complex in equimolar amounts. However, different preparations sometimes contained a different proportion of the two cytochromes; extreme deviations from the unity ratio usually lie within $\pm$25 %. The complex contains a small amount of chlorophyll but no detectable *P*-700; the absorption spectrum also suggests the presence of carotenoids (cf. ref. 15). The residual chlorophyll could not be removed by repeated column fractionation. The com-

TABLE I

CHEMICAL COMPOSITION OF THE CYTOCHROME COMPLEX

The molar concentrations of all components are shown for those in one particular suspension. The spectrophotometric methods for cytochrome determinations are described in the text and in the legend of Fig. 2. The EPR methods for measuring the iron-sulfur protein and plastocyanin are described in the legends of Figs 3 and 4. Total iron, non-heme iron and copper were estimated by the procedures described in ref. 42, and protein by the biuret reaction of Lowry et al. [43]. *P*-700 was estimated by the amount of light-induced absorbance change [19], using the molar extinction coefficient of 64 $mequiv^{-1} \cdot cm^{-1}$ at 700 nm [44].

Cytochrome *f*	35 μM
Cytochrome b_6	34
Non-heme iron	73
Acid-labile sulfur	60
Iron-sulfur protein (estimated by EPR)	10–15
Copper	30
Plastocyanin (estimated by EPR)	21
Chlorophyll	5
P-700	0
Protein	5 mg/ml

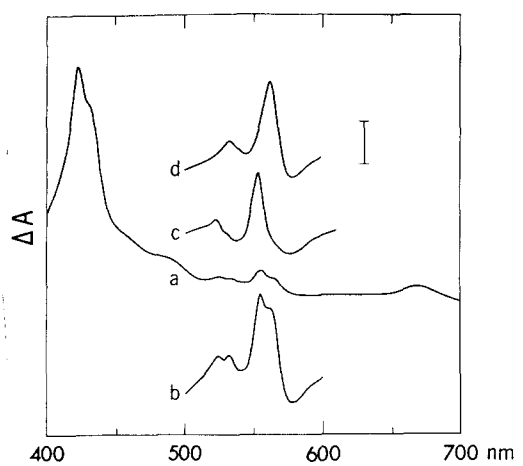


Fig. 2. Reduced-minus-oxidized difference spectra of the cytochrome complex (all spectra were measured with aliquots taken from a single suspension). Curve a, dithionite-minus-hypochlorite; curve b, dithionite-minus-ferricyanide; curve c, ascorbate-minus-ferricyanide; curve d, dithionite-minus-ascorbate. Bar length represents ΔA of 0.1 absorbance units for curve a and 0.01 absorbance units for curves b, c and d. The band maxima of cytochromes *f* and b_6 are at 554 and 563 nm, respectively.

position in protein, chlorophyll and metals of a typical preparation of the complex is presented in Table I.

The cytochrome contents were established spectrophotometrically by measuring the reduced-minus-oxidized difference spectra, as shown in Fig. 2. The dithionite-reduced vs. hypochlorite ($\approx$ 1 mM)-oxidized difference spectrum (curve a) covers the 400–700 nm region. Curve b is the dithionite-minus-ferricyanide difference in a

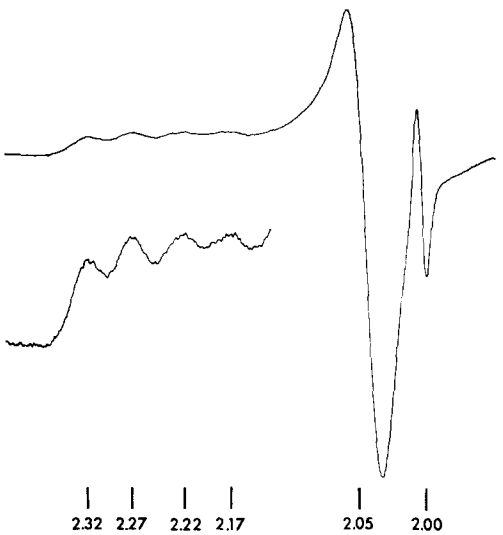


Fig. 3. EPR spectrum of the complex. Cytochrome concentrations of the EPR sample were 35 μM in cytochrome *f* and 34 μM incytochrome b_6; the sample also contained 10 mM ferricyanide (the sample containing no ferricyanide yielded the same spectrum with approx. 75 % of the magnitude). The EPR spectrum was measured in a modified Varian spectrometer [23] under the following conditions: sample temperature, 13.35 $\pm$ 0.15 °K; microwave frequency, 9.213 GHz; microwave power, 270 μW; modulation frequency, 100 kHz; amplitude, 10 G; time constant, 0.5 s. The field positions of the prominent features are presented on the *g*-factor scale. The hyperfine structure is also presented separately at a 5 times higher sensitivity.

solution of the complex at the same concentration as in curve a, but covering only the α- and β-band region, and presented at a 10 $\times$ higher sensitivity on the absorbance scale. Curve c shows the ascorbate-minus-ferricyanide difference, and thus represents cytochrome *f* only. Curve d shows the dithionite-minus-ascorbate difference, and represents cytochrome b_6. Using 20.6 and 24 as the millimolar differential extinction coefficients for cytochromes *f* and b_6 [15], respectively, the cytochrome f/b_6 ratio in this complex is near unity.

The presence of bound plastocyanin and iron-sulfur protein(s) can be most conveniently detected by EPR spectroscopy. Fig. 3 shows the EPR spectrum of the complex with 1 mM ferricyanide added, and the spectrum is characteristic of plastocyanin. The EPR spectrum consists of a major resonance line at $g = 2.05$ and four hyperfine lines in the low-field region (with *g* values as indicated in Fig. 3; $A_{//}$ equals 0.006 cm^{-1}). These characteristics are in good agreement with those previously reported for plastocyanin [21, 22]. Fig. 4 shows the EPR spectrum of the complex reduced by dithionite in the absence or in the presence of a trace amount of methyl viologen, prior to freezing the sample. This spectrum does not resemble that of soluble ferredoxin, since it shows very little of the resonance line at a *g* value of 1.94. Instead, the spectrum shows resonance lines at 2.05, 1.92 and 1.89 (the 1.86 resonance line is totally absent), which would correlate it with the iron-sulfur protein which we found earlier when the redox potential imposed onto the Photosystem-I particles was more negative than -560 mV [23]. Within experimental error, the plastocyanin and iron-sulfur-protein contents estimated from the EPR spectra are in reasonable agree-

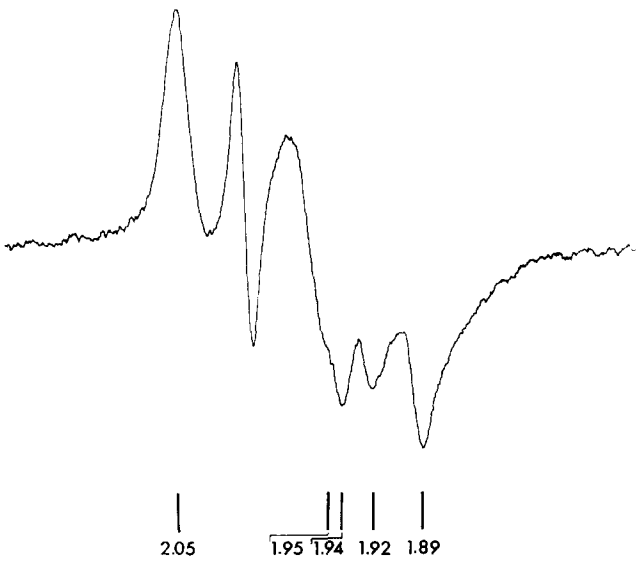


Fig. 4. EPR spectrum of the complex. The sample was the same as that used in Fig. 3, except it was pre-reduced with 10 μmol of dithionite and 50 nmol of methyl viologen under anaerobic condition [23]. Other conditions were the same as those used in Fig. 3, except the microwave power was 3 mW.

ment with the metal analysis. The accuracy of analysis only permits one to estimate that there are four or more iron atoms in the iron-sulfur protein molecules.

Redox potentials of P-700 and the cytochromes

Because of the enrichment and the special complex state of these electron-transport components, their electrochemical behavior is of interest. The redox potentials of *P*-700, cytochrome *f* and cytochrome b_6 were measured by potentiometric titration, using a potentiostatically controlled syringe drive for delivering the titrant together with the titration apparatus described recently [23–25]. Other details of the titration are included in the legend of Fig. 5. Fig. 5 presents the original traces of the continuous titrations; they are transposed onto the hydrogen scale after the Ag/AgCl reference electrode was calibrated with saturated quinhydrone solution at the end of the titrations.

Titration curve a is that for *P*-700; the first half of the titration appears normal and is consistent with a one-electron change. As the imposed potential was made more oxidizing, the slope of the titration curve became slightly greater, which suggests that a chlorophyll component other than *P*-700 was being oxidized and was responsible for part of the additional absorption change. In fact, at the end point of the titration shown in curve a, about 90 % of *P*-700 was electrochemically oxidized, as illumination at this point could still induce a reversible *P*-700 absorbance change which corresponded to about 10 % of the total change expected of *P*-700. The total absorption change of *P*-700 was separately determined using a fresh sample of identical *P*-700 concentration, and also containing methyl viologen as the electron acceptor and potassium ferrocyanide as the donor. The light-induced absorbance change

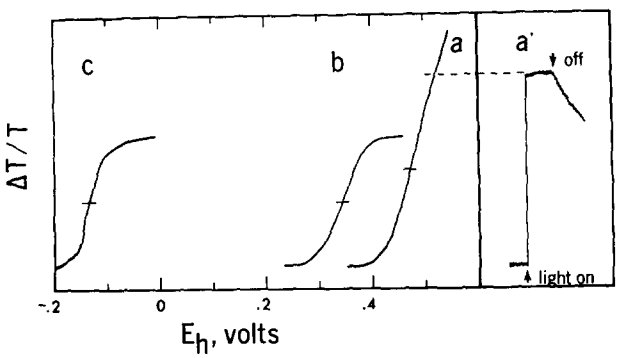


Fig. 5. Redox titration curves. Curve a, *P*-700 in the cytochrome-free high-*P*-700 particles (10 μg Chl/ml; the sample also contained 100 μM ferrocyanide initially) titrated oxidatively with 0.1 M ferricyanide and monitored at 700 vs 725 nm. Curve b, cytochrome *f* in the cytochrome complex titrated oxidatively as above and monitored at 552 vs 540 nm. Curve c, cytochrome b_6 in the cytochrome complex titrated reductively with 0.1 M dithionite solution; same cytochrome complex sample was used as above; the sample also contained the following redox mediators at 25 μM: 2,5-dimethylbenzoquinone, 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, anthraquinone-β-sulfonate. All samples were suspended in 0.02 M Tricine buffer at pH 7.8, and all titrations were carried out under anaerobic conditions. Curve a', estimation of the *P*-700 content from light-induced absorbance change at 700 vs 725 nm. The cytochrome-free high-*P*-700 sample was identical to that used for curve a, except it was suspended in 0.02 M phosphate buffer at pH 7 and it contained 100 μM ferrocyanide and 20 μM methyl viologen. See text for further discussions.

is shown by trace a' in Fig. 5. Taking these data together, the midpoint potential of *P*-700 in the high-*P*-700 particles prepared in this work was estimated to be + 468 mV. Separate titrations of *P*-700 in the digitonin-fractionated Photosystem I (D144) particles (not shown) yielded a midpoint potential of +460 mV. The titration curve for D144 was consistent with a one-electron change and was free of the extra increase such as that found in Fig. 5, curve a. It should be noted that while this redox-potential value is more positive than the value (+430 mV) first reported for *P*-700 by Kok [26], it is nearly the same as that reported by Yamamoto and Vernon [16], but substantially less positive than the value (+530 mV) reported more recently by Knaff and Malkin [27].

Curve b in Fig. 5 is the redox-titration curve for cytochrome *f*; it is consistent with a one-electron change and has a midpoint potential of +345 mV. Curve c in Fig. 5 is the redox-titration curve for cytochrome b_6, with an apparent midpoint potential of −140 mV. This potential value is rather close to that reported earlier by Fan and Cramer [28] for cytochrome b_6 in uncoupled chloroplasts. The spectral peak position of cytochrome b_6 (Fig. 2) and the more positive redox potential found earlier [11] for cytochrome b_{559} led us to conclude that this titration curve is likely to be contributed by cytochrome b_6 only. However, the titration course cannot be ascribed to either a one- or a two-electron change. Furthermore, the slope of the lower half of the curve is greater than that of the upper half. Since the oxidative titration was carried out very slowly (each titration took approx. 1 h), such irregularities in the titration course were not likely to be caused by insufficient equilibration in the titrated system, but more likely attributable to a heterogeneity of the redox state of the cytochrome. More recently, Böhme and Cramer [29] found that cytochrome

b_6 in coupled chloroplasts has a moidpoint potential near 0 V, and that the potential of at least half of the cytochrome b_6 complement undergoes a negative shift of 100–150 mV in the presence of uncouplers, which presumably caused structural changes in the membrane, and that the resulting two non-equivalent portions of the titration curve have different slopes.

Photochemical activity

It was shown previously that soluble *c*-type cytochromes (e.g. c_{552} from *Euglena*) and plastocyanin (from spinach) can be coupled to the Photosystem I reaction center and oxidized by photooxidized *P*-700 [30, 9]. More recently, it has been shown that purified cytochrome *f* can also be oxidized by Photosystem I [31]. Furthermore, the rate of cytochrome *f* oxidation was enhanced by the addition of plastocyanin.

As all the electron carriers contained in the complex are known to be associated with Photosystem I, we examined whether one or more of them might function as secondary electron carriers for the Photosystem I reaction center. Fig. 6 shows the

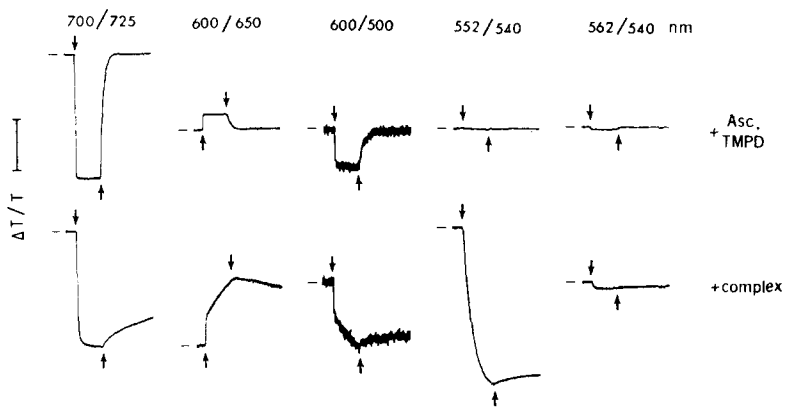


Fig. 6. Photochemical activity of the cytochrome complex determined by light-induced absorbance changes at various wavelengths indicated (the dual-wavelength spectrophotometer was described previously in ref. 12). The sample contained the high-*P*-700 particles (10 μg Chl/ml) plus either 1 mM ascorbate and 50 μM *N,N,N',N'*-tetramethylphenylenediamine in 0.04 M Tricine buffer at pH 8 (top row) or 0.7 μM cytochrome complex (bottom row). The $\Delta T/T$ bar preresents 0.01 for all traces except 0.005 for the traces measured at 600 vs 500 nm. See text for further discussions.

light-induced absorbance changes at several wavelengths for the Photosystem I particles in the presence of either the ascorbate-TMPD couple (top row) or the complex (bottom row). The light-induced absorbance change at 552 nm indicates that cytochrome *f* is photooxidized. Absorbance changes at 562 nm show no evidence that cytochrome b_6 in the complex is coupled with the Photosystem I reaction center. While the *P*-700 signal in the top row shows that *P*-700$^+$ recombines with the reduced primary acceptor, *P*-430$^-$, at a rapid rate, in the presence of the complex, the dark decay of *P*-700$^+$ becomes much slower, which suggests that *P*-700$^+$ is now coupled to a secondary donor present in the complex.

At first sight, the slow absorbance increase at 600 nm appears to indicate plastocyanin oxidation. However, this increase could have resulted from an absor-

bance decrease at the reference wavelength, 650 nm. Furthermore, the slow absorbance increase occurred at a steady rate and did not saturate for a long period of time. When 500 nm was used as the reference wavelength, only an absorbance decrease occurred. To further clarify this point, we measured the absolute difference spectrum of the same reaction mixture in the Cary model 14 spectrophotometer. The two light-minus-dark difference spectra shown in Fig. 7 were taken after the reaction mixture was illuminated for 1 and 3 min, respectively. The sample was illuminated while the difference spectrum was being recorded. The difference spectra show that, in addition to *P*-700 and cytochrome *f* oxidations, a chlorophyll band at 669 nm representing photobleaching appeared. The magnitude of the chlorophyll absorbance decrease is proportional to illumination time. Separate experiments (not shown here) showed that the chlorophyll being photobleached belonged to the complex and was not due to the bulk chlorophyll present in the TSF-1 particles.

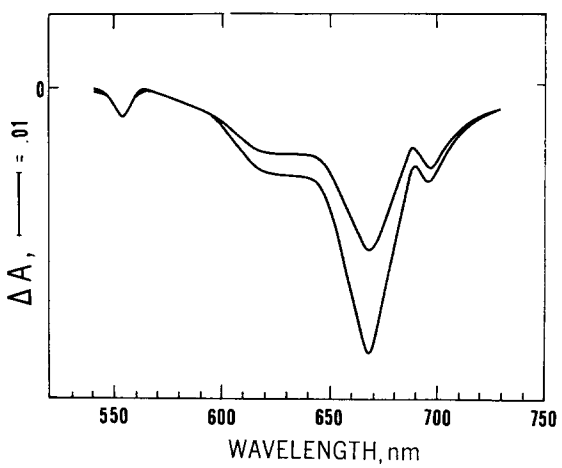


Fig. 7. Light-minus-dark difference spectra of the sample used in the bottom row of Fig. 6 in the Cary model 14 recording spectrophotometer. The sample was illuminated with light between 400 and 460 nm at 10^5 ergs · cm^{-2} · s^{-1} for 1 min (the upper curve) and 3 min (the lower curve), respectively, before recording was commenced. An identical sample placed in the reference compartment was always kept in the dark. See text for further discussions.

Effect of inhibitors on cytochrome f oxidation

A number of important inhibitors for blocking electron transport at or near Photosystem I have recently been introduced. Kimimura and Katoh reported that incubation of chloroplasts in $HgCl_2$ can inhibit electron flow at plastocyanin [32]. Izawa and coworkers [33, 34] demonstrated that treatment of chloroplasts with high concentrations of KCN inhibits reactions which involve Photosystem I. Photooxidation of cytochrome *f* by far-red light in KCN-treated chloroplasts was markedly slowed down. *P*-700 photooxidation was unaffected by KCN, but its reduction by Photosystem II was inhibited. The authors concluded from these observations that KCN blocks electron transport between cytochrome *f* and *P*-700, i.e. a reaction step presumably mediated by plastocyanin.

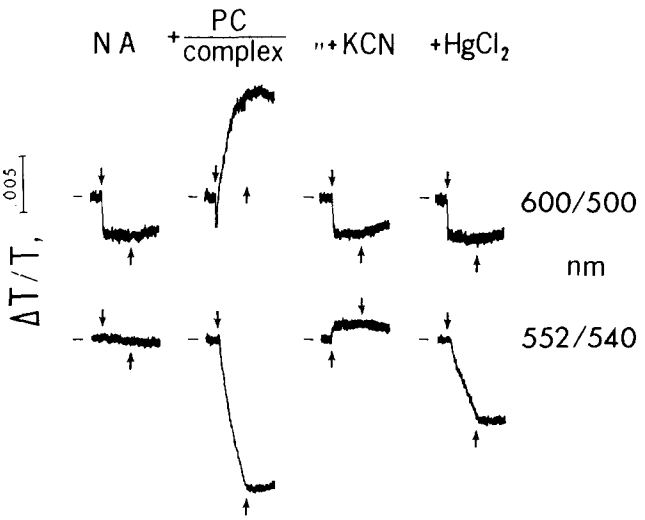


Fig. 8. Inhibitory effects of KCN or $HgCl_2$ on the photooxidation of soluble plastocyanin (top row; monitored at 600 vs 500 nm) or cytochrome *f* (in the complex) (bottom row; monitored at 552 vs 540 nm) coupled to Photosystem I. Samples contained the high-*P*-700 particles (10 μg chlorophyll/ml) plus 0.8 μM soluble plastocyanin (top row) or the cytochrome complex (0.7 μM in each of the two cytochromes) suspended in 0.02 M Tricine buffer, pH 7.8. KCN was used at 10 mM and $HgCl_2$ at 10 μM. Blue light as described above was used for illumination.

Fig. 8 shows the effect of KCN or $HgCl_2$ on the absorption changes associated with the oxidation of cytochrome *f* or soluble plastocyanin coupled to Photosystem I. The light-induced absorption changes in the bottom row of Fig. 8 show that cytochrome *f* oxidation coupled to photosystem I was completely inhibited by incubating the TSF-I particles in 10 mM KCN for 20 min, whereas 20 μM $HgCl_2$ inhibited approx. 40 % of the cytochrome *f* reaction. On the contrary, oxidation of soluble plastocyanin (top row) coupled to Photosystem I was completely abolished when the reaction mixture was incubated with either KCN or $HgCl_2$ for a comparable amount of time.

CONCLUSION

Nelson and Neumann [15] first fractionated from the digitonin Photosystem I particles of lettuce a complex containing cytochromes *f* and b_6 as well as trichloroacetic acid-extractable (non-heme) iron, and they noted the resemblance of this cytochrome complex to "complex III" of the mitochondrial respiratory redox chain. Our finding of a protein complex from the Triton Photosystem I particles with enlarged composition retains and amplifies the resemblance between chloroplasts and mitochondria. The presence of a plant copper protein in the complex suggests an equivalence to the mitochondrial complexes III and IV collectively. Mitochondrial complex IV is a heme-copper protein.

Of greater interest is perhaps a possible role of the complex associated with cyclic phosphorylation. Cytochromes *f* and b_6, plastocyanin, and the iron-sulfur protein are topographically related to Photosystem I. Their possible involvements in cyclic photophosphorylation have been proposed. Although the location of cyto-

chrome b_6 in the photosynthetic electron transport chain has not yet been definitively established, and the physiological function of the iron-sulfur protein with resonance at $g = 2.05$, 1.92 and 1.89 is also unknown, their possible roles in cyclic phosphorylation have suggested or speculated on [23 35, 36,]. A possible reduction of plastocyanin by a cyclic electron transfer around Photosystem I has also been suggested recently from EPR studies [37].

Although plastocyanin was previously thought to be a soluble protein, readily released from the chloroplast thylakoid, more recent studies showed that a substantial amount of plastocyanin remains in bound form in broken chloroplasts [22, 40] as well as subchloroplast fragments prepared by detergent [39, 40] and by French-press treatment [39-40]. It is not known whether the plastocyanin in some of these chloroplast fragments is present in the complex form as isolated here. The presence of the bound plastocyanin, in any event, was presumably responsible for the Photosystem I activity of the light particles obtained by French-press disruption [38]. On the other hand, it has been shown that the reactivity of bound plastocyanin with the chloroplast surface is not always correlated with the plastocyanin content [39]. The TSF-I particle usually prepared by the procedure of ref. 18 would presumably contain the cytochrome-plastocyanin-iron sulfur protein complex. However, it is evident that the plastocyanin present in the co-sedimented complex does not function to link the artifical electron donor towards NADP photoreduction, as we have shown previously that such a reaction has an absolute requirement for externally added soluble plastocyanin [9]. Our observations, shown in Fig. 6 above, that plastocyanin in the complex does not couple to the photooxidized *P*-700 in a Photosystem I particle is also consistent with this notion. Interestingly, the cytochrome *f* in the complex can be oxidized by the Photosystem I particle upon illumination. However, the NADP-reduction activity was negligible when the complex was used in place of soluble plastocyanin in a standard assay for Photosystem I.

The question of the location of plastocyanin in the photosynthetic electron-transport pathway has remained controversial for some time (see refs 32 and 41 for a recent review of the question). The availability of the isolated complex, unfortunately, does not offer an experimental avenue for elucidating this question. First, the plastocyanin present in the complex apparently does not interact with the Triton Photosystem I fragments. Second, the interaction of cytochrome *f* in the complex with the subchloroplasts is apparently not site-specific. When soluble plastocyanin was added to the reaction mixture containing the Photosystem I particle and the cytochrome complex, the rate of cytochrome *f* oxidation was slightly diminished. Similarly, the rate of photooxidation of soluble plastocyanin was also slightly diminished upon the addition of the cytochrome complex.

The direct interaction of cytochrome *f* present in the complex with the Photosystem I reaction center is also supported by the inhibitor data shown in Fig. 8. It was shown by Kimimura and Katoh [32] that $HgCl_2$ inhibits cytochrome *f* photooxidation by Photosystem I through inactivation of plastocyanin by replacing copper in the protein. The reduction of cytochrome *f* by Photosystem II was affected only slightly by $HgCl_2$.Fig. 8 shows that while photoooxidation of soluble plastocyanin by the Photosystem I particle was totally inhibited by $HgCl_2$, that of cytochrome *f* in the complex was inhibited by only about 40 %.

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