Biochimica et Biophysica Acta, 722 (1983) 460–470
Elsevier Biomedical Press

BBA 41269

STUDIES ON WELL COUPLED PHOTOSYSTEM I-ENRICHED SUBCHLOROPLAST VESICLES

CONTENT AND REDOX PROPERTIES OF ELECTRON-TRANSFER COMPONENTS

FONS A.L.J. PETERS [a], JOHN E. VAN WIELINK [b], HARRO W. WONG FONG SANG [a], SIMON DE VRIES [c] and RUUD KRAAYENHOF [a]

[a] *Department of Plant Physiology and* [b] *Department of Microbiology, Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam and* [c] *Laboratory of Biochemistry, B.C.P. Jansen Institute, Universiteit van Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)*

(Received October 25th, 1982)

Key words: Photosystem I; Cytochrome; Electron transfer; Potentiometry; Spectrum deconvolution; (Spinach chloroplast vesicle)

Stable and well coupled Photosystem (PS) I-enriched vesicles, mainly derived from the chloroplast stroma lamellae, have been obtained by mild digitonin treatment of spinach chloroplasts. Optimal conditions for chloroplast solubilization are established at a digitonin/chlorophyll ratio of 1 (w/w) and a chlorophyll concentration of 0.2 mM, resulting in little loss of native components. In particular, plastocyanin is easily released at higher digitonin/chlorophyll ratios. On the basis of chlorophyll content, the vesicles show a 2-fold enrichment in ATPase, chlorophyll-protein Complex I, P-700, plastocyanin and ribulose-1,5-bisphosphate carboxylase as compared to chloroplasts, in line with the increased activities of cyclic photophosphorylation and PS I-associated electron transfer as shown previously (Peters, A.L.J., Dokter, P., Kooij, T. and Kraayenhof, R. (1981) in Photosynthesis I (Akoyunoglou, G., ed.), pp. 691–700, Balaban International Science Services, Philadelphia). The vesicles have a low content of the light-harvesting chlorophyll-protein complex and show no PS II-associated electron transfer. Characterization of cytochromes in PS I-enriched vesicles and chloroplasts at 25°C and 77 K is performed using an analytical method combining potentiometric analysis and spectrum deconvolution. In PS I-enriched vesicles three cytochromes are distinguished: *c*-554 ($E_0' = 335$ mV), *b*-559$_{LP}$ ($E_0' = 32$ mV) and *b*-563 ($E_0' = -123$ mV); no *b*-559$_{HP}$ is present (LP, low-potential; HP, high-potential). Comparative data from PS I vesicles and chloroplasts are consistent with an even distribution of the cytochrome *b*-563- cytochrome *c*-554 redox complex in the lateral plane of exposed and appressed thylakoid membranes, an exclusive location of plastocyanin in the exposed membranes and a dominant location of plastoquinone in the appressed membranes. The results are discussed in view of the lateral heterogeneity of redox components in chloroplast membranes.

Introduction

The elucidation of the fundamental principles of photosynthetic energy transduction is severely hampered by the complex physiological functions and membrane structures of which the primary coupling mechanism is only a part. Photosynthetic prokaryotes often used for these studies, such as

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CF_1, chloroplast ATPase; PS, photosystem; Chl, chlorophyll.

the purple bacteria [1,2] and more recently also the cyanobacteria [3,4], are complicated by the (partial) association of photosynthetic and respiratory functions, while chloroplasts consist of a complicated thylakoid structure with appressed and exposed regions showing a heterogeneous distribution of photosystem, redox and ATPase complexes [5–10]. Intact and broken chloroplast preparations often suffer from instability and poor reproducibility of measurements of sensitive functions due to proteinase and lipase action. Investigation of primary photosynthetic energy-transducing reactions would benefit greatly from the availability of relatively simple and stable membrane vesicles in which the native components and activitities are largely conserved.

An important line of investigation is the reconstitution of relatively pure photosynthetically active complexes such as the PS I reaction center and ATPase complex into lipid vesicles [11]. The isolation of an oxygen-evolving PS II particle [12] and a relatively pure cytochrome *b*-563–*c*-554 ('*b*-*f*') complex with a substantial plastoquinol-plastocyanin oxidoreductase activity [13] presents a promising progress in this field. Another line of research is the isolation of intact and well coupled PS I-enriched vesicle preparations which are of interest because of the apparent association of the ATPase complex with PS I. Almon and Böhme [14] introduced a gentle isolation procedure of the PS II-deficient heterocysts of the cyanobacterium *Nostoc muscorum*. The intactness of their preparation was confirmed by the presence of water-soluble cytochrome *c*-553, which seems analogous to plastocyanin in higher plant chloroplasts [15].

Following previous work initiated by Wessels [16] and continued by other groups [17–21] we decided to use well coupled PS I-enriched vesicles derived from spinach chloroplasts after mild digitonin fragmentation at high ionic and osmotic strength as a routine tool for studying photosynthetic energy transduction. We have earlier reported that such a preparation consists of a homogeneous population of unilamellar vesicles (diameter about 50 nm) and remains stable during prolonged storage in liquid nitrogen without additives like glycerol [22]. Such vesicles are commonly considered to represent resealed stroma lamellae thylakoids containing the redox components for native cyclic electron transfer except the released ferredoxin, as well as the ATPase complex.

In this paper we describe the optimal conditions for spinach chloroplast solubilization with digitonin that result in little loss of native components. For the characterization of the cytochromes present in these vesicles we have applied a recently described [23] computer-assisted spectrophotometric analysis, that combines redox potentiometric titrations with data from deconvolution of cytochrome absorption spectra. In this way we have quantitatively analyzed the presence and properties of the *b*- and *c*-type cytochromes in PS I-enriched vesicles as compared to chloroplasts. The consequences of the measured amounts of photosystems, electron-transfer components and ATPase are discussed in relation to the proposed heterogeneous lateral distribution of these components in the thylakoid membranes of higher plant chloroplasts [5–10].

Materials and Methods

Preparation of chloroplasts and PS I-enriched vesicles. Broken chloroplasts were prepared by homogenization of precut leaves of marked spinach (100 g fresh weight) in 150 ml of a medium containing 50 mM Tricine-KOH buffer (pH 7.8), 300 mM sorbitol, 25 mM NaCl, 25 mM KCl and 5 mM $MgCl_2$, in a Braun MX 32 mixer for 5 s at maximal speed. The slurry was filtered through four layers of perlon net (56 μm mesh width) followed by a 1.5 min centrifugation at $3000 \times g$. Broken chloroplasts were obtained by suspending the pellets in 30 ml of 10 mM $MgCl_2$ for 1.5 min, after which 30 ml were added of a mixture containing 40 mM Tes-KOH buffer (pH 7.8), 500 mM sorbitol, 50 mM KCl and 50 mM NaCl. After centrifugation at $3500 \times g$ for 1.5 min, the pellets were resuspended in 1–2 ml of 20 mM Tes-KOH buffer (pH 7.8), 250 mM sorbitol, 25 mM KCl, 25 mM NaCl and 5 mM $MgCl_2$.

PS I-enriched thylakoid vesicles were obtained by fractionation by digitonin (Merck, twice recrystallized from ethanol) in the latter medium, at a final digitonin concentration of 0.2% (w/v) and a digitonin/Chl (w/w) ratio of 1, unless stated otherwise. The mixture was stirred for 30 min in the dark at 4°C, followed by a 3-fold dilution with the latter medium. After two centrifugations of 30

min at 10000 × *g* and 30 min at 50000 × *g*, the PS I-enriched vesicles were isolated from the supernatant by centrifuging for 60 min at 129000 × *g* (D129 pellet). The D129 pellet was carefully resuspended with a pottering tube in a medium containing 5 mM Tes-KOH (pH 7.8), 2.5 mM KH_2PO_4, 20 mM NaCl, 20 mM KCl and 5 mM $MgCl_2$ at a chlorophyll concentration of 1–4 mg/ml. Of this suspension 0.1–0.2-ml aliquots were stored in 0.5-ml tubes and quickly frozen in liquid nitrogen. All operations were carried out at 0–4°C. The vesicles could be stored for 3–4 weeks without appreciable loss of phosphorylation activity [22].

Chlorophyll and Chl *a*/Chl *b* ratios were determined by the method of Bruinsma [24]. Protein determinations were carried out according to the method of Bradford [25] with crystalline bovine serum albumin as standard.

EPR techniques. For EPR measurements broken chloroplasts and PS I-enriched vesicles were resuspended in the homogenization medium. Samples (0.25 ml) were preincubated at 20°C with either 1 mM duroquinol or 1 mM potassium ferricyanide for 1 min in the dark prior to freezing to 77 K in calibrated (3 mm quartz) EPR tubes. Duroquinol solutions in methanol were made as described by White et al. [26]; the final concentration of methanol added to the chloroplast suspension never exceeded 1% (v/v). EPR spectra were recorded as described before [27]. The amounts of P-700 and plastocyanin were estimated from the differences of the duroquinol-reduced minus ferricyanide-oxidized signals at *g* 2.0026 (77 K, 0.5 mW microwave power) and *g* 2.05 (77 K, 50 mW microwave power), respectively. These values were related to spectrophotometric determinations of these components as previously described [22].

Electrophoretic analyses. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [28]. For characterization of chlorophyll-protein complexes, solubilization of thylakoid membranes for 30 min in the presence of 0.25% mercaptoethanol was carried out by the recently described method of Anderson [29]. Solubilized membranes were immediately applied to the gels at 4°C in the dark. Gels were stained with Coomassie brilliant blue G250 and destained in 25% methanol, 7.5% acetic acid. Molecular weights were estimated using phosphorylase (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000) and lactalbumin (14000) as marker proteins. The content of CF_1-ATPase was estimated from the relative intensities of the α- and β-bands on the gels supposing a molecular weight of 60000 and 57000 for the α- and β-subunits, respectively, and assuming the presence of two α- and two β-subunits per CF_1.

Redox potentiometry and data analysis. Redox titrations of cytochromes in thylakoid membranes (concentration 200 μg Chl/ml) were performed in a medium containing 100 mM Hepes-KOH buffer (pH 7.0), 15 mM $MgCl_2$ and 4 mM EDTA in the presence of 0.4 mM diaminodurene, 2.5 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 0.1 mM trimethylhydroquinone, 0.1 mM phenazine methosulfate, 0.1 mM phenazine ethosulfate, 0.4 mM 2-methyl-1,4-naphthoquinone, 1.2 mM tetramethyl-*p*-benzoquinone, 12.5 μM 2-hydroxyl-1,4-naphthoquinone, 12.5 μM riboflavin 5'-monophosphate, 12.5 μM anthraquinone-2-sulfonate and 1 μM benzyl viologen. Some mediators were dissolved in ethanol, but the final ethanol concentration never exceeded 1%. The final pH was 7.0. The sample was kept anaerobic by flushing continuously with ultrapure argon ($O_2 < 0.1$ ppm). The redox potential was measured with a platinum electrode against an Ag/AgCl reference electrode and monitored with a Philips digital mV meter, PW 9408. The electrode combination was calibrated before and after each titration by measuring the potential of a saturated solution of quinhydrone in 50 mM potassium hydrogen phthalate at 25°C. In oxidative titrations the potential was adjusted by additions of anaerobic aliquots of ferricyanide after full reduction of the cytochromes was accomplished by NADH and, at lower potentials, dithionite. Reductive titrations were performed by addition of NADH or, at lower potentials, dithionite. During the titrations at 25°C spectra were recorded in an Aminco DW-2a spectrophotometer equipped with a magnetic stirrer accessory. For recording of 77 K spectra the spectrophotometer was equipped with a low-temperature accessory (Aminco, J4-9603). Samples were poised at different electrode potentials at 25°C in a 35 ml glass vessel as described earlier [23] and were quickly transferred into special argon-flushed

1.0-ml cuvettes, light path 2 mm [23], and immediately frozen in liquid nitrogen. Concentrations of reduced cytochromes were correlated to peak areas underneath the α-band recorded in the dual-wavelength mode against a reference at 540 nm. After 5–10 min, when the redox mediators were in equilibrium with the adjusted potential, four to nine scans from 540 to 575 nm (minus 540 nm) of each sample were recorded at 1 nm/s and averaged by means of a PDP 11/03 microprocessor (Digital Equipment Co.) to improve the signal-to-noise ratio [23]. For data storage and retrieval, the microprocessor was connected on-line to a disc-based minicomputer (Hewlett-Packard, 21 M-E, with RTE IV-B).

For the determination of the best-fitting curve to a set of data points on the basis of a given function, a computer programme as described earlier [23] was used which optimizes the parameters of a given number of components by means of a nonlinear least-squares analysis. The number of fitted components was arbitrarily chosen as the minimum above which no substantial improvement of the fit was obvious. For interpretation of the potentiometric results the series of redox potential-dependent spectral areas were fitted to the Nernst equation, assuming one-electron transfer [23]; each component is characterized by the redox midpoint potential and the percentage of the peak area in the 540–575 nm range at full reduction. Spectrum deconvolution was based on the assumption that the shape of optical bands can be described by Gaussian functions, that are symmetrical on the energy scale. The integrated fit procedure, which combines potentiometric analysis with spectrum deconvolution of the whole set of spectra of thylakoid membranes at different redox potentials, was based on an equation in which the Nernstian and Gaussian functions are integrated as described earlier [23]. The cytochromes are then characterized by the redox midpoint potential and wavelength maximum, peak width and intensity of the α-bands.

Results

Broken chloroplasts were incubated with the detergent digitonin in the presence of ionic and osmotic agents so that conditions for stacking of the thylakoid membranes were established. Leakage of soluble components was minimized by a 3-fold dilution of the mixture immediately after incubation. For the quality of the derived vesicles, particularly concerning the maintenance of the original (stroma lamellae) composition, the used digitonin/Chl ratio was of crucial importance (as is also obvious from Refs. 21, 30 and 31). This is demonstrated by Fig. 1 and Table I where the composition of the PS I vesicles (D129 fraction, see Materials and Methods) derived at different digitonin/Chl ratios and that of chloroplasts are compared by SDS-polyacrylamide gel electrophoresis and quantitative determination of some important components.

From Fig. 1A, showing the electrophoretic polypeptide patterns of PS I vesicles and chloro-

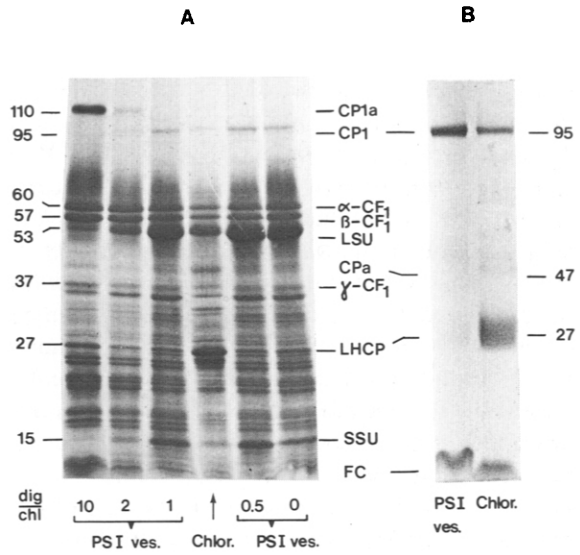

Fig. 1. Polypeptide composition and chlorophyll-protein complexes of PS I vesicles (PS I ves.) derived after chloroplast (Chlor.) solubilization at different digitonin/Chl (dig/Chl) ratios at a chlorophyll concentration of 0.2 mM and of chloroplasts. (A) Polypeptide pattern on an 8–20% SDS-polyacrylamide gradient gel. The same amount of chlorophyll (10 μg) was applied to each slot. (B) Chlorophyll-protein complexes of the PS I vesicles, isolated after solubilization at a digitonin/Chl ratio of 1, and chloroplasts, resolved on a 7.5–15% SDS-polyacrylamide gradient gel. The same amount of chlorophyll (15 μg) was applied to each slot. The relative distribution of chlorophyll among the complexes was for PS I vesicles 63% CP1, 3% LHCP and 34% free chlorophyll (FC) and for chloroplasts 25% CP1, 5% CPa, 41% LHCP and 29% FC. LSU/SSU, large/small subunit.

TABLE I

SOME COMPONENTS IN PS I VESICLES DERIVED AFTER SOLUBILIZATION OF CHLOROPLASTS AT DIFFERENT DIGITONIN/CHL RATIOS AND IN CHLOROPLASTS

P-700 and plastocyanin were determined by EPR spectroscopy; the amplitude of the EPR signal of P-700 (g 2.0026; line width 8 G) in chloroplasts was corrected for a 15% contribution by Signal II (g 2.0046; line width 20 G) which is assigned to PS II [56]. CF_1-ATPase was estimated from the subunit peak areas of the electrophoresis scans. n.d., not determined.

Preparation	Digitonin/Chl (w/w) for solubilization	Chl *a*/Chl *b* (w/w)	Protein/Chl (w/w)	Content of components (mol/10^3 mol Chl)		
				P-700	Plastocyanin	CF_1
PS I vesicles	0	3.9	6.7	n.d.	n.d.	2.2
	0.5	4.1	6.3	3.5	2.5	2.0
	1.0	4.5	6.0	3.8	3.0	2.4
	2.0	4.9	3.6	n.d.	n.d.	2.2
	10.0	4.2	4.6	4.5	0.6	2.6
Chloroplasts	–	2.5	3.9	1.6	1.4	1.2

plasts at equal sample chlorophyll concentrations, it is clear that the vesicles contain more protein when derived at the lower digitonin/Chl ratios or after incubation in the absence of digitonin. The chlorophyll-protein complex (CP 1) at 95 kDa associated with PS I is enriched in the vesicles, again at the lower digitonin/Chl ratios, whereas the content of the light-harvesting chlorophyll-protein complex (LHCP) at 24–27 kDa is relatively low in these vesicles. This is more clearly shown by the electrophoretogram in Fig. 1B that only shows the chlorophyll-containing proteins after a gentle treatment of chloroplasts and PS I vesicles (digitonin/Chl = 1) as recently introduced by Anderson [29]. The vesicles show a higher Chl *a*/Chl *b* ratio (Table I), in accordance with the lower content of the Chl *b*-containing LHCP complex [6]. Remarkably, the content of LHCP is increased when the vesicles are derived at a digitonin/Chl ratio of 10 (Fig. 1A), indicating a substantial cross-contamination with appressed membranes. From Fig. 1B it can also be seen that in chloroplasts but not in PS I vesicles a weak band at 47 kDa is detectable which is considered to be the reaction center complex of PS II (CPa) [6,31]. However, a more refined electrophoretic analysis would be required to make clear statements, because in particular the labile CPa complex was partly dissociated in the chloroplast gel resulting in a relatively high free chlorophyll band.

The amounts of P-700 and plastocyanin were determined by EPR spectroscopy (Table I). A loss of various electron-transfer components by digitonin treatment was observed previously [20,21, 32], in particular, the loosely bound plastocyanin, although this protein seems to be located at the internal thylakoid surface [33]. Table I shows that P-700 is not released by the digitonin treatments; the P-700/Chl ratio at all digitonin/Chl ratios is increased about 2-fold as compared to chloroplasts. Plastocyanin is drastically set free by digitonin, but only at higher digitonin/Chl ratios. Remarkably, the plastocyanin loss is very low at a digitonin/Chl ratio of 1.

An important constituent of the vesicles for our aim is the ATPase complex. As expected from earlier observations by others [34,35], CF_1 is enriched in the PS I vesicles consisting of originally exposed thylakoids. Fig. 1A reveals an enrichment of the α-, β- and γ-subunits of CF_1 in PS I vesicles in comparison to chloroplasts and on a chlorophyll basis. Table I shows an approx. 2-fold increase in CF_1/Chl in vesicles. However, it is also clear (Fig. 1A and Table I) that the used digitonin/Chl ratio hardly influences the CF_1/Chl ratio. This is in agreement with results of ATP hydrolysis measurements on digitonin-derived PS I vesicles as reported by Hauska and Sane [34]. On a protein basis the CF_1 content increases at higher digitonin/Chl ratios, due to the loss of other pro-

teins: the loosely bound ribulose-1,5-bisphosphate carboxylase, consisting of the large subunit at 53 kDa and the small subunit at 15 kDa [12], is gradually lowered at increasing digitonin/Chl ratios (Fig. 1A).

On the basis of the results presented so far (see also Ref. 22), a digitonin/Chl ratio (w/w) of 1 at a chlorophyll concentration of 0.2 mM is routinely used in preparing the PS I vesicles for further experiments.

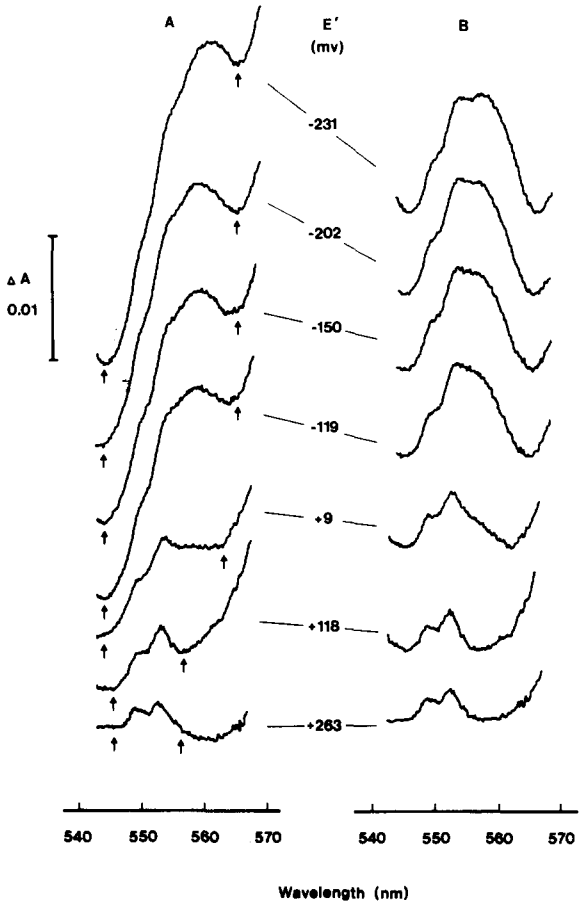

Fig. 2. Redox titration and baseline corrections of cytochromes in PS I vesicles at 77 K. Nine scans of each spectrum were recorded and averaged by means of a microprocessor. Subsequently, each spectrum was corrected by subtraction with the spectrum where all cytochromes were fully oxidized, which was controlled by extra additions of ferricyanide at a potential of +390 to +420 mV (column A). After that, a straight line was constructed between the estimated boundaries (indicated by the arrows) of the relevant spectrum and subtracted from the spectrum (column B). The chlorophyll concentration was 200 μg/ml.

For characterization of the *b*- and *c*-type cytochromes in PS I vesicles compared to chloroplasts, suspensions of thylakoid membranes were submitted to potentiometric titrations at 25°C and 77 K. The results of such an experiment on PS I vesicles at 77 K are presented in Fig. 2A. In order to separate the spectral absorption of cytochromes from irrelevant absorbances a straight line was drawn between the estimated boundaries of the relevant spectrum. After this the straight line was subtracted from the spectrum, resulting in the spectra of Fig. 2B.

In order to distinguish the cytochromes with respect to their redox midpoint potentials, spectral peak areas underneath the α-band were measured at different redox potentials. Fig. 3 shows typical plots of the redox titrations of cytochromes in PS I vesicles and chloroplasts at 25°C. On the basis of fitting to the Nernst equation and assuming one-electron transfer by all cytochromes in both fractions, a three-component fit in each case seems quite justified. Table II (left column) shows the redox midpoint potentials in PS I vesicles and chloroplasts and the fractional contributions of the three components. Apparently, in PS I vesicles the midpoint potential of the major cytochrome component is shifted to a lower value as compared to chloroplasts.

To analyze more precisely the characteristics of the cytochromes a method was used which combines the potentiometric analysis with spectrum deconvolution of the whole set of spectra from

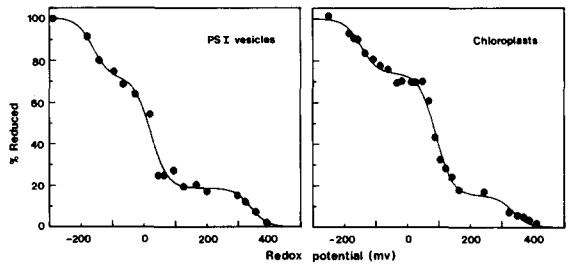

Fig. 3. Potentiometric titration of cytochromes in PS I vesicles and chloroplasts. The percentage of the peak area in the 540–575 nm range at full reduction is plotted vs. the redox potential. The solid lines represent the fit analysis for three components (see also Table II). The chlorophyll concentration was 200 μg/ml. Digitonin/Chl ratio for solubilization of chloroplasts was 1 (w/w).

TABLE II

BEST-FITTING PARAMETERS OF POTENTIOMETRIC AND COMBINED ANALYSES FOR CYTOCHROMES IN PS I VESICLES AND CHLOROPLASTS

The potentiometric analyses refer to the series of spectra recorded at 25°C as described in Fig. 3. The combined analyses refer to the sets of spectra, recorded at 25°C and 77 K, as described in Fig. 4. The percentage expresses the fractional contribution of a component to the total area of the α-band at full reduction, λ_m represents the wavelength of its absorbance maximum, ω is the band width at half-height and E_0' represents the midpoint potential at pH 7.0 in mV.

Preparation	Potentiometric analysis		Combined analysis at 25°C				Combined analysis at 77 K			
	%	E_0' (mV)	%	λ_m (nm)	ω (nm)	E_o' (mV)	%	λ_m (nm)	ω (nm)	E_o' (mV)
PS I vesicles	19	341	20	554.3	8.2	334	5	547.6	2.4	309
							16	551.6	5.0	319
	51	22	46	559.1	11.3	32	47	555.7	8.4	30
	30	−162	34	562.9	11.6	−123	21	557.9	7.0	−129
							11	561.2	5.1	−131
Chloroplasts	16	336	17	553.5	9.0	335	6	548.1	2.7	329
							11	552.0	3.2	334
	57	91	31	558.9	11.0	148	29	556.2	6.3	123
			24	559.3	11.0	40	26	554.7	8.3	21
	27	−147	28	562.8	10.0	−147	18	557.7	7.2	−113
							10	561.3	5.3	−128

which the midpoint potential, wavelength maximum, peak width and intensity of the α-bands are resolved [23]. Table II gives the computer-fitted parameters of such combined analyses of cytochromes in PS I vesicles and chloroplasts at 25°C and 77 K. Fig. 4 illustrates the corresponding three-dimensional plots. For PS I vesicles at 25°C a three-component fit with peaks at 554.3 nm (cytochrome *c*-554), 559.1 nm (cytochrome *c*-559_{LP}) and 562.9 nm (cytochrome *b*-563) with midpoint potentials of +334, +32 and −123 mV, respectively, represents the cytochrome composition of PS I vesicles and confirms the results of the potentiometric fit (Fig. 3). In chloroplasts at 25°C, however, four components can be distinguished. While cytochromes *c*-554 and *b*-563 were found to have midpoint potentials similar to those in PS I vesicles, the combined fit in chloroplasts clearly distinguished the low- and high-potential forms of cytochrome *b*-559 with midpoint potentials of +40 and +148 mV, respectively. The latter value corresponds to the converted form of cytochrome *b*-559_{HP} after partial deterioration of the chloroplasts, due to the 4 h duration of the titration, according to Cramer et al. [36], Okayama and Butler [37] and Rich and Bendall [38]. A titration

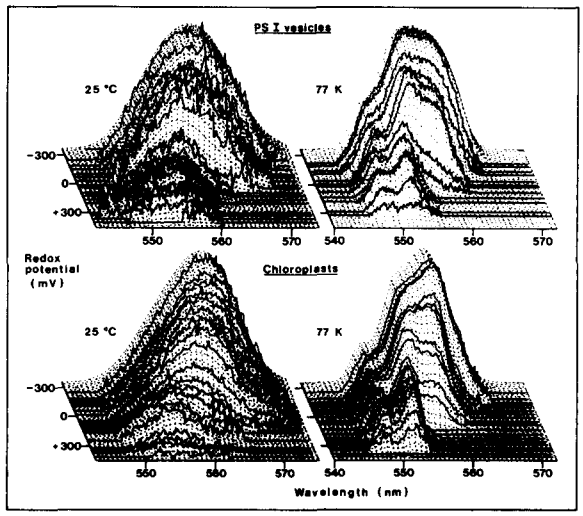

Fig. 4. Three-dimensional plots of combined analysis of cytochrome spectra and redox potentials of PS I vesicles and chloroplasts at 25°C and 77 K. The absorbance is plotted perpendicular to the plane determined by the wavelength and potential axes. Measured spectra are represented by the solid line and the best multicomponent fits (see also Table II) by the broken lines. The absorbance at full reduction for PS I vesicles at the peak maxima at 558 nm (25°C) and 553 nm (77 K) was 0.009 and 0.013, respectively, and for chloroplasts in the peak maxima at 560 nm (25°C) and 557 nm (77 K) 0.013 and 0.023, respectively. The chlorophyll concentration in each titration was 200 μg Chl/ml.

with chloroplasts carried out within 2 h, mainly in the positive potential range, showed that cytochrome b-559$_{HP}$ is present at a higher midpoint potential of about +340 mV (results not shown).

At 77 K, splitting of the α-band of several cytochrome components appears (Table II). The best-fit solution of the combined analysis at 77 K resulted in six components in chloroplasts and five components in PS I vesicles (Table II). At 77 K, a nonsymmetrical splitting occurs of the α-band of cytochrome c-554 into two bands with maxima at about 552 and 548 nm, as was also previously described by Boardman and Anderson [39]. Also a splitting of the α-band of cytochrome b-563 occurred giving peaks at about 558 and 561 nm which corresponds to earlier results with isolated cytochrome b-563 [40]. The apparent absence of cytochrome b-559$_{HP}$ in PS I vesicles at 77 K is confirmed and is obvious from the difference of spectral shapes at complete reduction of PS I vesicles and chloroplasts (Fig. 4).

Table III summarizes the data found for the concentrations of redox components and CF_1-ATPase expressed on the basis of total chlorophyll and P-700, in PS I vesicles and chloroplasts. It must be noted that calculations of the cytochrome concentrations are less satisfactory due to the observation that the total spectral area was rather variable by baseline changes of as yet unclear origin. Therefore, an under- or overestimation of the cytochrome concentrations is easily made. This could cause the large discrepancies in chloroplast cytochrome concentrations reported in the literature [7,12,38,39,41,42]. From the data in Table III, we tentatively conclude that on a chlorophyll basis PS I vesicles are about 2-fold enriched in P-700, plastocyanin and CF_1-ATPase; the concentration of plastoquinone is significantly lower but the concentrations of cytochromes, except cytochrome b-559$_{HP}$, are about the same as those in chloroplasts. Since the PS I vesicles are derived from exposed thylakoids with a composition that is not representative of the whole chloroplast, it is more relevant to compare the concentrations on the basis of P-700. On this basis the concentrations of plastocyanin and CF_1-ATPase are very similar in PS I vesicles and chloroplasts; the concentrations of plastoquinone, cytochromes b-559$_{LP}$, b-563 and c-554 in PS I vesicles are about 18, 57, 30 and 33%, respectively, of those in chloroplasts. Significant is the constant ratio of cytochrome b-563/c-554 (1.5–1.6) in PS I vesicles and chloroplasts, in agreement with the proposed existence of a native cytochrome b-c complex [13,43]. The ratio cytochrome b-559$_{LP}$/c-554 however, is different: 2.0 in PS I vesicles and 1.2 in chloroplasts. The ratio cytochrome b-559$_{HP}$/b-559$_{LP}$ in chloroplasts is about 1.4, which agrees with some previous reports [7,12] but is much lower than ratios of about 2.1 found by others [38,41].

TABLE III

CONCENTRATIONS OF REDOX COMPONENTS AND ATPase IN PS I VESICLES AND CHLOROPLASTS

The contents of cytochromes are calculated from the peak heights at maximal absorption, adopting the following millimolar extinction coefficients: $\varepsilon = 20$ for cytochromes b-563, b-559$_{LP}$ and b-559$_{HP}$ and $\varepsilon = 22$ for cytochrome c-554 [41].

Component	Component/Chl (mol/10^3 mol)		Component/P-700 (mol/mol)	
	PS I vesicles	Chloroplasts	PS I vesicles	Chloroplasts
Plastoquinone	40	100	11	62
Cytochrome b-559$_{LP}$	1.6	1.2	0.4	0.7
Cytochrome b-559$_{HP}$	0	1.6	0	1.0
Cytochrome b-563	1.2	1.6	0.3	1.0
Cytochrome c-554	0.8	1.0	0.2	0.6
Plastocyanin	3.0	1.4	0.8	0.9
P-700	3.8	1.6	1.0	1.0
CF_1-ATPase	2.4	1.2	0.6	0.7

Discussion

For the purpose of studying primary energy-transducing events we have optimized the preparation of stable well coupled PS I-enriched vesicles prepared from spinach chloroplasts by mild digitonin treatment to minimize the loss of natural components. These vesicles show a 2-fold enrichment in ATPase and PS I-associated components like the CP1 complex, P-700, plastocyanin and also ribulose-1,5-bisphosphate carboxylase, which fits with the increased activities of cyclic photophosphorylation and PS I-associated electron transfer as shown before [22]. While only about 2% of the original chloroplast material was recovered in the preparation, the PS I vesicles showed a remarkable stability of photochemical activities for weeks to months when stored in liquid nitrogen without requirement for additives like glycerol [22]. When no digitonin was used during fragmentation the yield was about 30% of that at digitonin/Chl = 1 (results not shown) but the membrane composition was essentially the same (Fig. 1A and Table I), suggesting that the mechanical treatment during the vesicles preparation in the absence of detergent also primarily affects the stroma lamellae.

PS I particles had been previously prepared by solubilization at a digitonin/Chl ratio of 10 [17]. These particles were isolated in high yield (about 10%) but had a limited capacity of photochemical activities. We have shown here that under these conditions the important electron-transfer component plastocyanin is largely released, which may be the principal cause for the decreased activities, as concluded earlier by Robinson and Wiskich [21]. Remarkably, the ATPase was not released by all tested digitonin concentrations. Although the PS I vesicles still contain some PS II-associated chlorophyll-protein complexes (LHCP, see Fig. 1A and B), they have less than 0.1% of the PS II electron-flow activity of broken chloroplasts [22]. It is very likely that any remaining PS II activity is inactivated by the isolation procedure and digitonin treatment; digitonin is known to inhibit electron flow at the water-splitting site of PS II [44]. Therefore, we cannot decide from these results whether the observed low content of LHCP and CPa in PS I vesicles represents their original presence in stroma lamellae or a contamination with some grana material during preparation. If the presence of these PS II-associated complexes is real, it would confirm the suggestions of Andersson and Anderson [6] and Armond and Arntzen [45] that a minor portion of PS II is present in the stroma lamellae, but would contradict the model of Park and Sane [8]. However, the relatively high PS II content (10–20% of total) and chlorophyll recovery (7%) in the Y-100 preparation of Andersson and Anderson [6] isolated after Yeda press treatment suggests to us that some contamination with appressed membrane material has occurred here. This is supported by the presence in this preparation of Signal II [46], an EPR component (g value 2.0046) used as a marker for PS II, which was absent in the PS I vesicles (results not shown).

The new method of combining potentiometric analysis with spectrum deconvolution provides a high resolution, greatly improving the analysis of cytochromes in thylakoid membranes. In former redox titrations [32,38,47] the midpoint potentials of cytochromes were calculated by plotting the absorbance change at an appropriate wavelength pair against the adjusted potential; the relative cytochrome contributions to overlapping spectra had to be estimated to get reliable data. The combined method offers an improvement in the discrimination of overlapping cytochrome species, because it takes into account both spectral and potentiometric data. Moreover the signal-to-noise ratios of the spectra can be improved by averaging of repeated scans.

Our results confirm observations by Cramer [48] and Rich and Bendall [38] that two different species of cytochrome b-559 are present in chloroplasts: the high-potential cytochrome b-559 ($E_0' \simeq +340$ mV) which can be converted into a lower potential form ($E_0' \simeq +130$ mV) under less coupled conditions (i.e., by ageing during the titrations), and the low-potential cytochrome b-559_{LP} with a midpoint potential of about +30 mV which is clearly different from the converted cytochrome b-559_{HP}. Cytochrome b-559_{LP} was found earlier in different PS I-enriched vesicles [32,39]. Its absence in other PS I preparations was also accompanied by the absence of plastocyanin [42]. In our PS I vesicles cytochrome b-559_{LP} is somewhat enriched when related to chlorophyll, which confirms recent

results reported by Henry and Møller [12]. This may indicate a possible role for this cytochrome in cyclic electron transfer around PS I in vivo, as suggested earlier by Knaff and Malkin [32]. The apparent absence of cytochrome b-559$_{HP}$ in our PS I vesicle preparation is in harmony with the suggestion that this cytochrome is associated with PS II [48–51].

The midpoint potentials of cytochromes c-554 (still often called cytochrome f) and b-563 are very similar in PS I vesicles and in chloroplasts (Table II). The value of cytochrome c-554 is about +330 mV which is in the lower range generally found by others, i.e., +330 to +380 mV [32,43,48], and corresponds to a functional site near P-700. The midpoint potential of cytochrome b-563 varied between −110 and −165 mV, in line with previously reported values. Other workers [47,48] reported rather different values for the midpoint potential of cytochrome b-563 that apparently depend on the state of the membrane being titrated.

Cox and Andersson [7] and Anderson [41] suggested a homogeneous distribution of cytochrome b-563 and cytochrome c-554 in the lateral plane of the thylakoid membrane. We found that the molar ratios of cytochrome b-563/P-700 and cytochrome c-554/P-700 in PS I vesicles are about 40% of those in chloroplasts, corresponding roughly to the fraction of exposed membranes of the total thylakoid membrane area [5], thus supporting the proposed even distribution of the cytochrome b-563-c-554 complex.

The similar molar ratios of plastocyanin/P-700 in both PS I vesicles and chloroplasts indicate an exclusive or at least preferential location of plastocyanin in the exposed membranes, which is not in harmony with the proposal [7,41] that plastocyanin acts as a lateral shuttle of reducing equivalents between the spatially separated photosystems in appressed and exposed membranes. Our results rather suggest a role of plastocyanin as a possible mobile electron carrier associated with P-700 as proposed by Haehnel et al. [52]. On the other hand, the apparent lack of coincidence between plastocyanin and the cytochrome b-563-c-554 complex suggests that these components do not form a supramolecular complex, as suggested earlier [53].

Plastoquinone seems to be present in both exposed and appressed thylakoids but the majority is in the appressed membranes, confirming results obtained with other PS I-enriched vesicles [9,42,54] and heterocysts of the cyanobacterium *N. muscorum* [14].

In a subsequent paper, we will present the use of the PS I vesicles in studies on photophosphorylation and electrical potential generation by ferredoxin-mediated cyclic electron flow, preliminary results of which are reported elsewhere [55].

Acknowledgements

The authors are grateful to Professors A. Trebst and W.A. Cramer for discussion of some of the results, to Dr. L.F. Oltmann for critically reading the manuscript, to Dr. F.J. Leeuwerik for his continuous help with the use of the computer facilities and to Dr. J.T. de Hey for his help with the electrophoresis experiments. This work is supported in part by the Foundation for Biophysics with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

1 Keister, D.L. (1978) in Photosynthetic Bacteria (Clayton, R.K. and sistrom, W., eds.), pp. 849–856, Plenum Press, New York
2 Zannoni, D. (1982) Biochim. Biophys. Acta 680, 1–7
3 Stanier, R.Y. and Cohen-Bazire, G. (1977) Annu. Rev. Microbiol. 31, 225–274
4 Matthijs, H.C.P., Scholts, M.J.C. and Schreurs, H. (1981) in Photosynthesis II (Akoyunoglou, G., ed.), pp. 269–278, Balaban International Science Services, Philadelphia
5 Anderson, J.M. (1981) FEBS Lett. 124, 1–9
6 Andersson, B. and Anderson, J.M. (1980) Biochim. Biophys. Acta 593, 427–440
7 Cox, R.P. and Andersson, B. (1981) Biochem. Biophys. Res. Commun. 103, 1336–1342
8 Park, R.B. and Sane, P.V. (1971) Annu. Rev. Plant Physiol. 22, 395–430
9 Melis, A. and Brown, J.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4712–4716
10 Arnon, D.I., Tsujimoto, H.Y. and Tang, G.M.-S. (1981) in Photosynthesis II (Akoyunoglou, G., ed.), pp. 7–18, Balaban International Science Services, Philadelphia
11 Hauska, G., Samoray, D., Orlich, G. and Nelson, N. (1980) Eur. J. Biochem. 111, 535–543
12 Henry, L.E.A. and Møller, B.L. (1981) Carlsberg Res. Commun. 46, 227–242
13 Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599

14 Almon, H. and Böhme, H. (1980) Biochim. Biophys. Acta 592, 113–120
15 Sandmann, G. and Böger, P. (1980) Plant Sci. Lett. 17, 417–424
16 Wessels, J.S.C. (1963) Proc. R. Soc. B 157, 345–355
17 Anderson, J.M. and Boardman, N.K. (1966) Biochim. Biophys. Acta 112, 403–421
18 Arnon, D.I., Tsujimoto, H.Y., McSwain, B.D. and Chain, R.K. (1968) in Comparative Biochemistry and Biophysics of Photosynthesis (Shibata, K., ed.), pp. 113–132, University of Tokyo Press, Tokyo
19 Nelson, N., Drechsel, Z. and Neumann, J. (1970) J. Biol. Chem. 245, 143–151
20 Sane, P.V. and Hauska, G.A. (1972) Z. Naturforsch. 27b, 932–938
21 Robinson, S.P. and Wiskich, J.T. (1975) Plant Physiol. 56, 535–539
22 Peters, A.L.J., Dokter, P., Kooij, T. and Kraayenhof, R. (1981) in Photosynthesis I (Akoyunoglou, G., ed.), pp. 691–700, Balaban International Science Services, Philadelphia
23 Van Wielink, J.E., Oltmann, L.F., Leeuwerik, F.J., De Hollander, J.A. and Stouthamer, A.H. (1982) Biochim. Biophys. Acta 681, 177–190
24 Bruinsma, J. (1961) Biochim. Biophys. Acta 52, 576–578
25 Bradford, M. (1976) Anal. Biochem. 72, 248–254
26 White, C.C., Chain, R.K. and Malkin, R. (1978) Biochim. Biophys. Acta 502, 127–137
27 De Vries, S., Albracht, S.P.J. and Leeuwerik, F.J. (1979) Biochim. Biophys. Acta 546, 316–333
28 Laemmli, U.K. (1970) Nature 227, 680–685
29 Anderson, J.M. (1980) Biochim. Biophys. Acta 591, 113–126
30 Wessels, J.S.C. (1977) in Encyclopedia of Plant Physiology, New Series (Trebst, A. and Avron, M., eds.), Vol. 5, pp. 563–573, Springer-Verlag, Berlin
31 Wessels, J.S.C. and Borchert, M.T. (1978) Biochim. Biophys. Acta 503, 78–93
32 Knaff, D.B. and Malkin, R. (1973) Arch. Biochem. Biophys. 159, 555–562
33 Trebst, A. (1980) Methods Enzymol. 69, 675–715
34 Hauska, G.A. and Sane, P.V. (1972) Z. Naturforsch. 27b, 938–942
35 Berzborn, R.J., Müller, D., Roos, P. and Andersson, B. (1981) in Photosynthesis III (Akayunoglou, G., ed.), pp. 107–120, Balaban International Science Services, Philadelphia
36 Cramer, W.A., Fan, H.W. and Böhme, H.V. (1971) J. Bioenerg. 2, 289–303
37 Okayama, S. and Butler, W.L. (1972) Plant Physiol. 49, 769–774
38 Rich, P.R. and Bendall, D.S. (1980) Biochim. Biophys. Acta 591, 153–161
39 Boardman, N.K. and Anderson, J.M. (1967) Biochim. Biophys. Acta 143, 187–203
40 Stuart, A.L. and Wasserman, A.R. (1973) Biochim. Biophys. Acta 314, 284–297
41 Anderson, J.M. (1982) FEBS Lett. 138, 62–66
42 Böhme, H. (1976) Z. Naturforsch. 31c, 68–77
43 Nelson, N. and Neumann, J. (1972) J. Biol. Chem. 247, 1817–1824
44 Robinson, S.P. and Wiskich, J.T. (1975) Plant Physiol. 55, 163–167
45 Armond, P.A. and Arntzen, C.J. (1977) Plant Physiol. 59, 398–404
46 Anderson, J.M. and Malkin, R. (1982) FEBS Lett. 148, 293–296
47 Böhme, H. and Cramer, W.A. (1973) Biochim. Biophys. Acta 325, 275–283
48 Cramer, W.A. (1977) in Encyclopedia of Plant Physiology, New Series (Trebst, A. and Avron, M., eds.), Vol. 5, pp. 227–237, Springer-Verlag, Berlin
49 Whitmarsh, J. and Cramer, W.A. (1978) Biochim. Biophys. Acta 501, 83–93
50 Heber, U., Kirk, M.R. and Boardman, N.K. (1979) Biochim. Biophys. Acta 546, 292–306
51 Horton, P. and Croze, E. (1977) Biochim. Biophys. Acta 462, 86–101
52 Haehnel, W., Pröpper, A. and Krause, H. (1980) Biochim. Biophys. Acta 593, 384–399
53 Haehnel, W. and Krause, H. (1982) in Short Reports, 2nd European Bioenergetics Conference, Vol. 2 (LBTM-CNRS edition), pp. 217–218, Lyon, France
54 Vernon, L.P. and Shaw, E.R. (1971) Methods Enzymol. 23, 277–289
55 Kraayenhof, R., Peters, A.L.J. and Van der Pal, R.H.M. (1982) in Short Reports, 2nd European Bioenergics Conference, Vol. 2 (LBTM-CNRS edition), pp. 335–336, Lyon, France
56 Blankenship, R.E., Babcock, G.T., Warden, J.T. and Sauer, K. (1975) FEBS Lett. 51, 287–293