

Partition of Phylloquinone K_1 between Digitonin Particles and Chlorophyll-Proteins of Chloroplast Membranes from *Nicotiana tabacum*

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The partition of phylloquinone (vitamin K_1), of chlorophylls a and b and of the two main carotenoids, β -carotene and lutein, in subthylakoid particles (digitonin treatment) and chlorophyll protein complexes (sodium dodecylsulfate polyamide-gel electrophoresis) isolated from tobacco chloroplasts (*Nicotiana tabacum* L.) is described.

1. The "light particle" fractions (S 90000, S 150000) of digitonin fragmented chloroplasts are enriched in CP I and contain a higher proportion of phylloquinone, chlorophyll a and β -carotene as compared to whole chloroplasts. This is visualized by high values for the ratio a/b (6–8) and for β -carotene/lutein (1.7) as well as about 3 mol of K_1 per 100 mol of total chlorophyll.

The "heavy digitonin particle" fraction (10000 $\times g$ sediment), in turn, contains a higher proportion of chlorophyll b and lutein, but a lower level of phylloquinone than whole chloroplasts.

2. The chlorophyll a-protein CP I of pigmentsystem I, isolated by preparative gel electrophoresis using 0.5% and 4% SDS, is characterized by a stable level of phylloquinone (1 mol K_1 per 100 mol of total chlorophyll), high chlorophyll a/b ratios (7–10) and high values for β -carotene/lutein (~ 6.0).

The light-harvesting chlorophyll a/b-protein LHCP of photosystem II (chlorophyll a/b = 1.1 – 1.5, β -carotene/lutein = < 0.1) contains either low amounts of phylloquinone (0.5% SDS) or only trace amounts of K_1 (4% SDS).

The free pigment fraction (FP) contains at 0.5% SDS 57% of the total phylloquinone of thylakoid membranes. At 4% SDS the K_1 amount in the free pigment fraction increases to 84%.

3. The phylloquinone partition studies in digitonin particles and SDS chlorophyll proteins indicate that there exist at least two localization sites for phylloquinone K_1 in the photosynthetic membrane. The CP I complex and a second site, presumably near photosystem II (CPa?).

Introduction

Phylloquinone, also known as vitamin K_1 , is a 2-methyl, 3-phytyl, 1,4-naphthoquinone. In plants it is bound to plastids and present in green plant tissues in a much higher concentration than in white, etiolated or chromoplast-bearing tissues [1–3]. As a genuine component of chloroplasts [4] it is bound together with chlorophylls, carotenoids and other prenylquinones to the photochemically active thylakoids [5, 6]. Since it contains the phytyl side as the chlorophylls, it had already been postulated by Dam and Glavind 1938 that it is associated with the

chlorophylls in the photosynthetic apparatus in form of a functional unit [7].

Within the chloroplasts phylloquinone is not exclusively bound to the thylakoids but also appears in the osmiophilic chlorophyll-free plastoglobuli of the plastid stroma [8, 9], which function as storage site for excess chloroplast lipids [10]. A very recent investigation shows that phylloquinone is also located together with plastoquinone and α -tocopherol in the chloroplast envelope [11]. Though there are three different localization sites for phylloquinone in the chloroplast, it is evident that the major part of K_1 is bound to the thylakoids [11, 12].

Phylloquinone is a potential photosynthetic electron carrier; its functional site is, however, not known. Since synthetic naphthoquinones stimulate cyclic electron flow in isolated chloroplasts, it was postulated that the endogenous naphthoquinone K_1 may be the *in vivo*-carrier [12]. This hypothesis was further stressed by the observation that the "light particle" fraction (enriched in photosystem I) of

Abbreviations: a/b, ratio chlorophyll a/b; c/l, ratio β -carotene/lutein; CPI, chlorophyll protein I; CPa, chlorophyll protein a; DCPIP, 2,4-dichlorophenolindophenol; FP, free pigments; K_1 , phylloquinone, vitamin K_1 ; LHCP, light harvesting chlorophyll a/b protein; PAGE, polyacrylamide-gel electrophoresis; SDS, sodium dodecylsulfate.

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digitonin-treated spinach chloroplasts contained very high amounts of phylloquinone while the "heavy particle" fraction (with mainly photosystem II activity) contained much less [12, 13]. From the results of more recent inhibitor studies with halogenated naphthoquinones it is concluded that phylloquinone may have a double function 1) as a quencher of excitation energy and 2) as an electron carrier in cyclic and non-cyclic electron transport [14]. The functional site of K_1 in the photosynthetic electron transport chain appears to be before the larger plastoquinone pool [14, 15] at or near the binding site B for DCMU and other photosynthesis herbicides [16]. This position may also represent the possible re-entry point for cyclic electrons into the main chain [15]. On the other hand, in a quencher function one would expect phylloquinone to be part of the reaction centers of the two photosystems.

Polyacrylamide-gel electrophoresis of sodium dodecylsulfate digested chloroplasts (SDS-PAGE) revealed two main chlorophyll proteins, the light harvesting chlorophyll a/b protein (LHCP) and the chlorophyll protein of the pigment system I (CPI), which exhibits different chlorophyll and carotenoid composition [17–19]. Besides these several other minor chlorophyll proteins have been found more recently [20, 21].

In order to get more information on the function and distribution of phylloquinone within the photosynthetic membrane, we studied in *Nicotiana* chloroplasts whether the phytylnaphthoquinone K_1 is associated with the two main chlorophyll proteins LHCP and CPI. The results of this SDS-PAGE analysis are compared with that obtained by digitonin fragmentation of *Nicotiana* chloroplasts.

Materials and Methods

Green tobacco plants (*Nicotiana tabacum* L.) were cultivated in the greenhouse (23 °C; 50% rel. humidity). Chloroplasts were isolated from fully developed green leaves of 3 to 4 months old plants at 4 °C in saccharose (0.5 M), phosphate (0.1 M) buffer (pH 7.5) by modifying the method of Park and Pon [22]. The chloroplasts were washed two times in the isolation buffer. Chlorophyll determination was carried out after the method of Arnon [23].

Digitonin fragmentation

The purified chloroplasts were treated (1 h, 4 °C) in an incubation buffer (0.01 M phosphate, 0.05 M

KCl, pH 7.2) with 1.3% digitonin (chlorophyll: digitonin 1:10, w/w) and then centrifugated at $10\,000 \times g$ (20 min), $50\,000 \times g$ (30 min), $90\,000 \times g$ (40 min), and $150\,000 \times g$ (60 min) resulting the sediments S10, S50, S90, S150, respectively. The latter and the final supernatant were analyzed for their chlorophyll [24], carotenoid [25], and phylloquinone content [26, 27]. The Hill-activity with dichlorophenolindophenol and methylviologen was measured according to [28].

SDS-Polyacrylamide gel electrophoresis

The purified chloroplasts were osmotically broken (suspension in distilled water for 30 min), centrifuged at $2000 \times g$ (30 min) and the broken membrane fraction sedimented at $100\,000 \times g$ (30 min). These membranes were suspended in a Tris-glycine-buffer (0.005 M; pH 8.3), frozen in liquid nitrogen and stored in a deep freeze. Before gel electrophoresis the membrane fraction was dissolved by adding 0.5% SDS in the 0.005 M Tris-glycine-buffer, pH 8.3 (molar ratio chlorophyll to SDS 1:17.5 or 1:140), which also contained 20% saccharose. At 0.5% SDS a sedimentation step ($20\,000 \times g$, 30 min) was applied to remove the very small amounts of undissolved membrane material.

Disk electrophoresis was carried out in gel cylinders (length 45 mm, diameter 6 mm). The preparative separation (length 50 mm, diameter 22 mm) was performed in a WTW-apparatus (Weilheim) with an automatic elution program. A spacer gel was not necessary. The separation gel (9%; pH 8.9) and the electrode buffer (0.05 M Tris-glycine buffer pH 8.3) contained 0.1% SDS (gel system I of [29]).

The SDS-solubilized membranes contained 1 mg chlorophyll and 5 mg protein per ml; applied were 0.1 or 0.2 ml on the analytical tube and 1 ml in the preparative tube. The separation was performed at a constant current of 2 mA (analytical tube) or 32 mA (preparative column). The relative amounts of the different chlorophyll protein bands were determined by densitometer scanning at 670 nm. The protein bands were visualized by fixation in a methanolic acetic acid solution of amido black. The proteins were eluted from the preparative column in a 0.1 M Tris-HCl buffer (pH 8.1), registered in a flow photometer at 280 nm and collected in a fraction collector.

The level of proteins in the SDS-membrane extracts and in the column-eluates was determined

after Lowry [30]. In order to exclude interference with the light absorbance of chlorophyll the extinction was read at 750 nm. Since 0.1% SDS results in a 20% decrease of the extinction, the probes were diluted before the protein determination to a 0.05% SDS level.

For the determination of pigments and prenylquinones the different column-eluates were extracted with acetone-light petrol. The chlorophylls a and b were determined as pheophytin a (667 nm) and b (655 nm) in diethyl ether using the extinction coefficients of [31]. The transformation of chlorophylls to pheophytin was performed with 25% HCl (0.1 ml per 1 ml of the diethyl ether chlorophyll solution) at room temperature and was completed after 5 min.

Carotenoids were separated by thin layer chromatography [25] and estimated using a Camag-T-Scanner (blue filter, transmission 400–500 nm); the values given for β -carotene and lutein are based on 4 parallel measurements for each determination.

Phylloquinone and the other plastid prenylquinones were separated by thin layer chromatography on silica gel plates [26]. K_1 was quantitatively determined fluorometrically [26], from the extinction difference before and after reduction with buffered borohydride solution [26], and also by the quantitative HPLC method [27].

The results reported here are based on at least 4 separate experiments of independent growth and chloroplast isolation. In the digitonin and SDS fragmentation work two typical experiments are reported.

Results

Digitonin particles

Fractionation of *Nicotiana* chloroplast preparations by digitonin treatment and subsequent centrifugation yields thylakoid particles which are different in their chlorophyll, carotenoid and phylloquinone content. The “heavy particle” fraction S 10, which mainly exhibits photosystem II activity (DCPIP reduction) contains a higher proportion of chlorophyll b, lutein and a lower level of phylloquinone than the chloroplasts. Correspondingly the ratios chlorophyll a/b and β -carotene/lutein (c/l) are lower and the ratio chlorophylls to phylloquinone is higher than in the chloroplast fraction (Table I).

The sediments S 90 and S 150 obtained at 90 000 and 150 000 $\times g$, in turn, represent the “light particle” fraction, which exhibits photosystem I activity (donor: ascorbate/DCPIP, acceptor: methyl viologen). They are enriched in chlorophyll a (high values for a/b), in β -carotene (higher values for c/l) and also contain high amounts of phylloquinone K_1 (Table I). The level of K_1 reaches values of about 13 to 17 μg per mg chlorophyll and is about 3 times higher than in the unfractionated chloroplasts. The latter contain 1 mol of phylloquinone and the “light digitonin particles” about 3 mol per 100 mol of total chlorophyll.

The sediment S 50, obtained at 50 000 $\times g$, represents a transition fraction, which contains components of the “heavy” and of the “light particle” fraction. The final supernatant is also a mixed fraction, as

		Chloroplasts	S 10	S 50	S 90	S 150	Supernatant
Chlorophyll a/b	I	2.9	2.5	3.7	5.2	8.4	3.8
	II	2.8	2.6	3.8	6.2	5.8	3.7
β -carotene/ lutein (c/l)	I	0.9	0.6	0.8	1.4	1.7	0.9
	II	0.8	0.6	1.1	1.7	1.5	0.8
phylloquinone/ chlorophyll ($\mu g/mg$)	I	4.6	4.0	3.6	5.3	12.9	6.2
	II	5.4	3.3	10.3	16.6	7.8	11.6
chlorophylls/ phylloquinone (a + b/ K_1)	I	216	250	267	187	77	160
	II	184	302	98	60	128	86
% Chlorophyll (in the fractions)	I	100	40	31	12	4	13
	II	100	59	8	10	9	14
% phylloquinone (in the fractions)	I	100	33	24	14	12	17
	II	100	29	13	24	10	24

Table I. Pigment-composition and phylloquinone content of *Nicotiana* chloroplasts and of different thylakoid fractions obtained by digitonin fragmentation and subsequent differential centrifugation. I and II = experiment I and II.

can be seen from the a/b and c/l ratios. This is confirmed by SDS-gel electrophoresis of the two fractions, which revealed both chlorophyll-proteins, the LHCP and the CPI.

The S10 fraction, in turn, mainly contained the light-harvesting chlorophyll a/b-protein LHCP, but also traces of CPI, indicating a light contamination by photosystem I particles. The S90 to S150 fractions mainly contain the chlorophyll protein CPI of photosystem I, but also exhibit a small but distinct LHCP band. These data indicate that the S10 and the S90/S150 fractions are enriched in pigmentsystem II and pigmentsystem I particles, respectively, but are not pure particles.

The pattern of particle distribution in the different sediments may vary to some extent in each experiment depending upon the disintegration degree, the centrifugation time etc. This is the reason, why the "light digitonin particle" fraction is either found in the S150 or S90 fraction.

The differential fractionation of chlorophylls and phylloquinone can also be seen from the % distribution in the different particle fractions. The "heavy particle" fraction S10 contains a higher percentage of chlorophylls than phylloquinone, the S90 to S150 sediments, in turn, a higher % proportion of K_1 than chlorophyll (Table I). There is also a higher proportion of K_1 than chlorophyll in the supernatant, which could be due to plastoglobuli, which contain some phylloquinone and which routinely show up in the supernatant during centrifugation of chloroplast fragments [32]. In digitonin-treated chloroplasts the plastoglobuli are, however, no more visible, they get dissolved by digitonin [13].

Disc-electrophoresis

As in other plants [17–21, 33] SDS-polyacrylamide-gel electrophoresis of purified *Nicotiana* chloroplast membranes reveals three major chlorophyll containing fractions: the chlorophyll protein (CPI), the light-harvesting chlorophyll a/b-protein LHCP, and the free pigment zone (FP) (Fig. 1). The latter contains free, non protein-bound chlorophylls and carotenoids. At lower SDS-levels (0.5%; molar ratio chlorophyll/SDS 1:17.5) an additional chlorophyll-protein band (CPa) is found just before the LHCP. At a higher SDS concentration (4%, molar ratio chlorophyll/SDS 1:140) there is less of the chlorophyll in the CPI and LHCP fraction and an

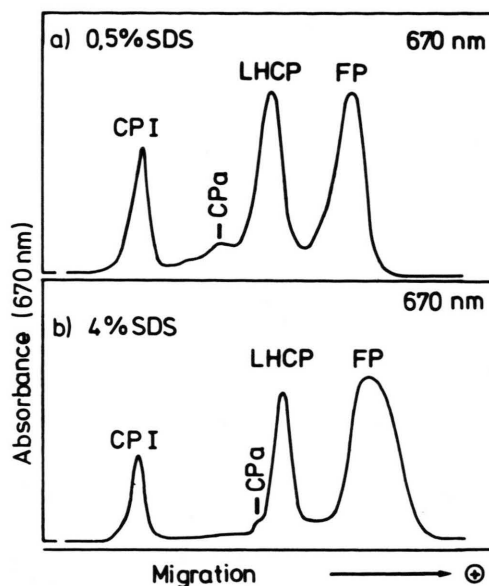


Fig. 1. Densitometer scans of the chlorophyll-proteins separated by gel electrophoresis. a) 0.5% SDS; chlorophyll/SDS 1:17.5 M/M and; b) 4% SDS; chlorophyll/SDS 1:140 M/M.

increasing proportion in the free pigment zone (Fig. 1), the CPa band has almost disappeared.

The two major chlorophyll proteins LHCP and CPI are very different in their chlorophyll and carotenoid composition, which can already be seen from the absorption spectra (Fig. 2). The isolated LHCP fraction, which contains both chlorophylls (a/b ratio 1.1–1.6), exhibits in the red region a second absorption peak at 653 nm and also an increased absorbance between 430 and 500 nm, which is mainly due to chlorophyll b. This second absorption peak is missing in the isolated CPI, which primarily contains chlorophyll a (a/b ratios 7–10). The free pigment zone (FP) contains at low SDS levels (0.5%) predominantly chlorophyll a (Fig. 2). Using 4% SDS, however, a shoulder indicating also chlorophyll b shows up in the spectrum; this is paralleled by a decreasing chlorophyll a/b ratio (5 → 2.6; Table II).

The CPI of *Nicotiana* chloroplasts contains β -carotene as main carotenoid (ratio c/l 6.0) as has been reported for the CPI of other plants [18, 19, 34, 35]. The LHCP-fraction, in turn, predominantly contains lutein and other xanthophylls and only trace amounts of β -carotene. Correspondingly the ratio c/l of the LHCP is very low (0.1).

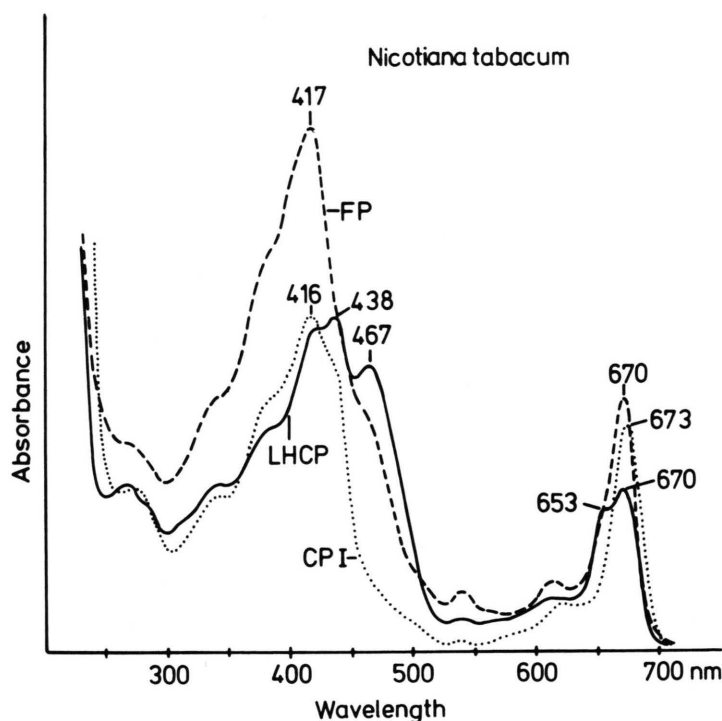


Fig. 2. Absorption spectra of the two chlorophyll proteins LHCP and CPI and of the free pigment fraction isolated by SDS-gel electrophoresis from SDS-digested *Nicotiana* chloroplasts (0.5% SDS; chlorophyll/SDS 1:17.5 M/M).

CPI and LHCP not only differ in their chlorophyll and carotenoid composition, but also in their phylloquinone content. Using 0.5% SDS the CPI contains on a protein basis double the amount, the LHCP, however, half the amount of K_1 as compared to the unfragmented thylakoid membrane (Table II). This preferential association of phylloquinone K_1 with the

CPI is maintained also at higher SDS concentrations (4%). In both cases there are about 1 mol of K_1 per 100 mol of total chlorophyll as compared to about 0.8 mol K_1 per 100 mol chlorophyll in the unfragmented membranes. In the preparative polyacrylamide-gel electrophoresis, applied here, it was not possible to separate the CPa complex from the

Table II. Prenyllipid ratios and phylloquinone content (chlorophyll a and protein basis) of a thylakoid fraction (untreated membrane) and of three pigment fractions obtained by preparative SDS-PAGE of SDS-digested *Nicotiana* chloroplasts a) 0.5% SDS, molar ratio chlorophyll:SDS = 1:17.5 and b) 4% SDS, ratio chlorophyll:SDS = 1:140.

	a/b	c/l	a + b		K_1		$\mu\text{g } K_1 \text{ per}$	$\mu\text{g } K_1 \text{ per}$	a + b
			$[\mu\text{g}]$	$[\%]$	$[\mu\text{g}]$	$[\%]$	1 mg protein	1 mg a + b	K_1
a) 0.5% SDS									
CPI	7	—	117	21	0.53	24	0.8	4.5	221
LHCP	1.1	—	240	42	0.43	19	0.2	1.8	570
free pigments	5	—	213	37	1.25	57	—	5.9	171
untreated membrane	2.6	—	570	100	2.2	100	0.4	3.9	260
b) 4% SDS									
CPI	9.5	6.0	56	9	0.30	11	0.6	5.3	187
LHCP	1.5	< 0.05	180	30	0.13	5	0.1	0.7	1385
free pigments	2.6	0.5	375	61	2.25	84	—	5.9	167
untreated membrane	2.6	0.9	611	100	2.7	100	0.4	4.4	230

a/b = ratio chlorophyll a/b, a + b = chlorophylls a and b, c/l = β -carotene/lutein, K_1 = phylloquinone (vitamin K_1).

LHCP, as shown in Fig. 1 for the analytic procedure. Thus during elution both chlorophyll proteins (LHCP, CPa) appeared in the same eluate fraction. At 0.5% SDS this LHCP/CPa fraction exhibits an a/b ratio of 1.1 and contains a distinct phylloquinone amount (0.2 µg/mg protein). At higher SDS levels (4%), which represent more rigorous disintegration conditions, the CPa has almost disappeared. The a/b ratio of the LHCP-fraction increases to 1.5 and contains only trace amounts of K_1 (< 0.05 µg/mg protein). This indicates that it is primarily the CPa chlorophyll protein that contains the K_1 amounts found in the 0.5% SDS LHCP+CPa fraction. The increase in the a/b ratio cannot be explained by the disappearance of the CPa, which mainly contains chlorophyll a. It may be caused by a more easier extraction of chlorophyll b than chlorophyll a from the LHCP at higher SDS levels.

The highest proportion of K_1 (57%) is not found in the two chlorophyll proteins LHCP and CPI, but in the free pigment fraction already at the milder disintegration condition of 0.5% SDS. At higher SDS-levels the K_1 percentage increases to 84%, while that for chlorophylls increases only from 37 to 61% (Table II). This shows that the major part of the phylloquinone is not bound to the SDS extractable chlorophyll proteins CPI and LHCP.

The chlorophyll proteins CPI, LHCP and the free pigment (FP) of *Nicotiana* chloroplasts were also analysed qualitatively for the presence of other chloroplast prenylquinones. The benzoquinone derivative plastoquinone-9 is associated to some extent with the two chlorophyll proteins, but the major part of it is found in the FP fraction. This also applies for the chromanol α -tocopherol.

Discussion

That the "light particle" fraction of digitonin fragmented spinach chloroplasts is enriched in chlorophyll a, β -carotene and in phylloquinone K_1 had been shown before [12, 13] and is confirmed in this investigation for tobacco chloroplasts. There is also an agreement between spinach and tobacco that the heavy digitonin particles are enriched in chlorophyll b and lutein, but contain very little phylloquinone.

The "light particle" fraction, which only shows photosystem I activity, contains 70–80 mol (spinach) and 30–40 mol (tobacco) of total chlorophyll per 1 mol of phylloquinone K_1 as compared to

130–150 mol (spinach) and 90–110 mol of total chlorophyll in the whole chloroplasts, respectively. On a chlorophyll basis, the enrichment of K_1 in the photosystem I digitonin particles is twofold for spinach and threefold for tobacco as compared to whole chloroplasts or isolated thylakoids. From these data it appears that the naphthoquinone K_1 is an integral functional component of photosynthetic pigment system I. In spinach there are 2 mol K_1 per 1 mol of P 700 [12, 13]. Whether phylloquinone is associated with P 700 in the reaction center of photosystem I, as it is assumed for β -carotene [35–37], needs further research.

That the phylloquinone content of the light digitonin particles of tobacco is higher than that of spinach may partly be due to genetic differences and to the slightly varying isolation procedures, but also to differences in the growth conditions. It is known that the K_1 level (per chlorophyll or leaf area basis) in sun leaves and in high-light plants is always higher than in shade leaves and in low-light plants [15, 38, 39]. The average leaves of the solitary grown tobacco plants used in this investigation, received more light than the more densely grown spinach of the former experiment [12, 13].

In contrast to digitonin treatment which yields thylakoid particles, SDS fragmentation of *Nicotiana* chloroplast membranes results in the formation of smaller membrane units, the chlorophyll proteins CPI, LHCP and CPa. The increasing formation of free pigments with increasing SDS concentration shows that this agent is more rigorous than desintegration with the saponine digitonin. Since at low SDS levels (0.5%) the free pigment fraction contains predominantly chlorophyll a, it appears that under this condition mainly the CPI is sensitive to chlorophyll extraction. The decreasing a/b ratio of the free pigment zone at higher SDS levels indicates that under this condition also the LHCP is broken down.

Phylloquinone is enriched to some extent in the CPI fraction while the LHCP contains little K_1 as compared to the whole thylakoids. The level of phylloquinone per chlorophyll remains the same at low and at high SDS levels showing that a certain amount of K_1 is rather tightly bound to the CPI. The amount of 0.8 µg K_1 per mg protein in the CPI (0.5% SDS) is only slightly decreased at 4% SDS (Table II).

The isolated CPI has many characteristics of the "light digitonin particles", e.g. higher chlorophyll

α -, β -carotene- and K_1 levels than the thylakoids. The K_1 amounts per chlorophyll found in the CPI are, however, lower as compared to the "light digitonin particles". This difference can only partly be explained by a different growth of the tobacco plants. The chloroplasts of the plants used for the SDS disintegration experiments had in fact a somewhat lower K_1 level per total chlorophyll than the plants used for the digitonin fragmentation. The main reason for this difference between CPI and light digitonin particles seems to be the different disintegration agent. Under the milder digitonin fragmentation more K_1 sticks to the larger pigment protein units (particles), while with the stronger SDS agent smaller pigment proteins are formed, which contain a distinct, but lower amount of K_1 per chlorophyll. There appear to be two pools of K_1 in the "light digitonin particles", one which is associated with the chlorophylls in the CPI and a second pool that consists of more loosely bound K_1 (reaction center of photosystem I?).

The observation that the LHCP and CPa fraction contains some K_1 (0.5% SDS), but that the almost CPa-free LHCP (4% SDS) exhibits only trace amounts, indicates that some K_1 is associated with the CPa protein, which is thought to be the reaction center of photosystem II [20, 21, 35]. Synthetic naphthoquinones as well as the endogenous K_1 are

quenchers of chlorophyll fluorescence *in vitro* and chloroplasts [14]. Therefore it is possible that phylloquinone functions as a quencher of excitation energy in both the CPI and the CPa. In a Triton-prepared CPI complex one mol of K_1 was found per one mole of P 700 [35].

The major part of K_1 and of plastoquinone-9 are found in the free pigment fraction. The percentage of prenylquinones in this pigment fraction is always higher than that of chlorophylls, no matter whether mild or more rigorous disintegration procedures are applied. This observation may mean that the larger part of K_1 and plastoquinone are not bound to the different chlorophyll proteins, but are in a close association with the structural lipids of the thylakoid bilayer. The K_1 and plastoquinone molecules found in this localization site may function as electron carriers. Though the data reported here are not yet fully conclusive they indicate, that K_1 and other prenylquinones are bound to different structural and functional pools within the thylakoid membrane.

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