Esterification of Chlorophyllide in Prolamellar Body (PLB) and Prothylakoid (PT) Fractions from *Avena sativa* Etioplasts

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Etioplast membranes were fractionated into enriched prothylakoid (PT) and prolamellar body fraction (PLB) by known procedures [2]. The photoconversion of Protochlide to Chlide was 77% in the PT fraction but only 65% in the PLB fraction with optimum NADPH concentrations. The subsequent esterification reaction proceeds in both fractions indicating that the enzyme chlorophyll synthetase is present in both fractions. In the PLB fraction, 10–15% more Chl_P is formed than in the PT fraction. It is concluded that the concentration of the endogenous phytol precursor is higher in the membranes still present in the PLB fraction than in the PT fraction.

Introduction

In connection with chloroplast development during deetiolation of higher plants, much attention has been paid to the prolamellar body (PLB) of etioplasts. Recently, the concept was developed that tubules of PLB consist of saponins as the main building units; the prothylakoid membranes proper (PT) are in part tightly connected with this tubular system and in part more extruded into the stroma [1–5]. A PLB fraction which contained most of the tubular material but still considerable amounts of membranes was separated from a PT fraction which was only slightly contaminated with tubular material [2, 6, 7]. Investigation of these fractions led to the assumption that Protochl(ide) and – after irradiation – Chl(ide) are present in the membranes of both fractions but not in the tubules [5]. This was confirmed by Lütz and Männing [8]. The PLBs proper contain between 10–15%, the PTs 85–90% of total protochl(ide). However, it was not clear whether the status of these pigments in the membranes of PLB and PT fractions is the same or whether physiological differences of these fractions [9, 10] also influence biochemical reactions of these pigments.

Typical biochemical reactions of these pigments in intact etiolated *Avena* seedlings are the photoconversion of Pchlide to Chlide and the subsequent esterification to Chl_P via Chl_{GG} [11]. The reactions can also be studied *in vitro* in broken etioplasts either with exogenous substrates (*e.g.* GGPP) or with endogenous substrates of the membranes [12–14]. We describe here a comparative study of these reactions in PLB and PT fractions of *Avena sativa* etioplasts.

Abbreviations: PLB, prolamellar body fraction; PT, prothylakoid fraction; Chl_{GG}, Chl_{H_2GG}, Chl_{H_4GG}, Chl_P, chlorophyll a esterified with geranylgeraniol, 6,7-dihydrogeranylgeraniol, 6,7,10,11-tetrahydrogeranylgeraniol or phytol, respectively; Phe_{GG}, Phe_{H_2GG}, Phe_{H_4GG}, Phe_P, pheophytin a esterified with the respective alcohol; GGPP, geranylgeranylpyrophosphate; Protochl, protochlorophyll; Chlide, chlorophyllide a; Protochlide, protochlorophyllide.

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Materials and Methods

Etioplasts were isolated from 7 days old etiolated oat seedlings (*Avena sativa* L. var. Parsival, Baywa, München) by a previously described procedure [6]. PLB and PT fractions were obtained from these etioplasts by density gradient centrifugation in a Sorvall SV 288 vertical rotor after osmotic lysis [2]. Only one separation step in the density gradient was used here because repeated density gradient centrifugation led to a decrease of yield.

Electron microscopy previously had shown that even after 3 gradients a crosscontamination of tubules and membranes in isolated fractions exists. Therefore we developed methods to assay crosscontamination as follows:

a) assay for PLB-tubules: As was described earlier, two saponins (Avenacosid A and B) are integral parts only of PLB-tubules. Thus we determined these saponins in acetone extracts of our samples after TLC separation and scanning the developed plates with a densitometer. This is described in detail by Lütz [7].

b) assay for PT-membranes: It has been worked out [8] that the PLB-tubules proper contain 10–15% of the PChl(ide) and the PTs 85–90% of these pigments. Knowing the PLB-distribution (via saponins) and the PChl(ide) distribution in the recovered fractions a simple calculation gives the content of prothylakoid membranes in a fraction. The PLB or PT fractions, respectively, were removed from the gradient. To each fraction which contained 7–15 µg (10–20 nmol) Protochlide, 3.3 mg (4 mmol) NADPH and, in one series of experiments, 120 – 200 µg (250–400 nmol) GGPP were added. The mixture was then completed to a total volume of 3.0 ml with 50 mM potassium phosphate buffer (pH 7.1) and illuminated with white fluorescence light for 3 min. This caused saturating photoconversion of Protochlide to Chlide. The samples were then incubated at 28 °C for 60 min in the dark. Esterification was stopped with acetone and the pigments extracted and determined as previously described [14].

Results and Discussion

Characterization of PLB and PT fractions is summarized in Table 1. The distribution of saponins (based on recovery in both PLB and PT fraction = 100%) is 85.5% in PLB fraction and 14.5% in PT fraction. The values are somewhat different from the value found by Lütz and Klein [5] (94% in PLB and 6% in PT) and Lütz and Männing [8] (95.6% in PLB and 4.4% in PT). This is due to the different separation procedure (see Materials and Methods). The distribution of pigments was determined in our experiments at the end of *in vitro* incubation. Therefore we deal with unesterifed (Protochlide + Chlide) and esterified pigments (Protochl + Chl). Because the separation of PLB and PT fractions had been performed before illumination, the distribution of total pigments should be identical with the distribution of Protochl(ide) originally present in the preparations.

Comparing the mean values it is evident that the distribution of chlorophyll pigments between tubules and membranes in the region of 50% each is close to the PT-membrane distribution and obviously different from the distribution of PLB-tubules in the fractions (PLBs: 85.5%, PTs: 14.5%). These findings are in good agreement with the values determined by Lütz and Männing [8]: pigments in PLB-fraction: c. 55%, in PT-fraction: c. 45%.

The photoconversion is high in membrane fractions only if NADPH is added before illumination [15, 16]. We incubated our preparation with a saturating concentration of NADPH (molar ratio NADPH/Protochl(ide) = 200) to obtain optimum photoconversion. It can be seen (Table II) that under these conditions, photoconversion is higher in

Table I. Characterization of prolamellar body fraction (PLB) and prothylakoid fraction (PT). Percentage of PLB-tubules (determined via saponins), membranes (calculation see Materials and Methods) and total chlorophyll pigments (Chl(ide) + Protochl(ide)) in both fractions (respective compound in PT + PLB = 100%).

Experiment No.	PLB-tubules		PT-membranes		Chl(ide) + Protochl(ide)	
	PLB	PT	PLB	PT	PLB	PT
1	87.1	12.9	48.7	51.2	50.2	49.8
2	86.3	13.7	32	67.8	35.0	65.0
3	86.6	13.4	57	43	62.0	38.0
4*	81.8	18.2	51	49	56.5	43.5
mean	85.5	14.5	47.2	52.8	50.9	49.1

* Experiment 4: Protochl(ide) enriched by ALA treatment.

Table II. Photoconversion (% Chl(ide) of Chl(ide) + Protochl(ide)).

Experiment No.	Broken etioplast fraction	PT fraction	PLB fraction
1 a	60	45	18
2 a	82	80	70
3 a	79	75	71
3 b	79	80	72
4 a	88	89	82
4 b	87	89	84
5 a	66	74	57
5 b	66	79	63
mean	77	76.5	64.5

All experiments labelled a: without exogenous substrate. All experiments labelled b: with exogenous GGPP (~ 10 nmol/nmol pigment).

the PT fraction (77%) than in the PLB fraction (65%). The most simple explanation would be that the percentage of non-photoconvertible Pchl(ide) is higher in the PLB than in the PT fraction. Indications are given by *in vivo* spectra of fractions, which show for the PLB-fraction a selective enrichment of the (non-photoconvertible) pigment absorbing at 635 nm, while in the PT-fraction the 650 nm form (photoconvertible) is predominant (C. Lütz, M. Alhadeff, S. Klein, and J. A. Schiff, in preparation).

Esterification of Chlide is catalyzed by the particulate enzyme chlorophyll synthetase [14]. This enzymic reaction can be studied *in vitro* either with exogenous substrate (*e.g.* GGPP) or without exogenous substrate. In the latter case, the reaction proceeds with endogenous substrate present in the etioplast membranes [12]. Endogenous substrate yields Chl_P as the major and Chl_{GG} as a minor product besides some Chl_{H_2GG} and Chl_{H_4GG} [12]. Endogenous substrate must be present in the PLB and in the PT fraction because esterification of Chlide is found in both fractions without exogenous substrate. Under these conditions 10–20% of Chlide initially present in the membranes is esterified after the incubation. This corresponds to the values reported for broken etioplast membranes without exogenous substrate [12]. Whereas the percentage of esterification is about the same in the PT and PLB fractions, the distribution of the different products (analyzed after demetallation of Chl to Phe) is different (Table III). The percentage of Phe_P is about 10% higher and that of Phe_{GG} 8–10% lower in PLB than in PT. This means that the endogenous phytyl compound which leads to Phe_P is found predominantly in membranes of the PLB fraction whereas membranes of the PT fraction contain relatively more of the corresponding geranylgeranyl compound. The percentage (not given in Table III) of Phe_{H_2GG} is about 10% and that of Phe_{H_4GG} about 20% in all experiments in the PT as well as in the PLB fraction.

Addition of GGPP leads to esterification of 70–80% of Chlide initially present in the membranes of the PT as well as of the PLB fraction. This corresponds to values in broken etioplasts [12, 14]. Thus the enzyme chlorophyll synthetase must be present in membranes of both the PT and the PLB fraction. The difference of product distribution due to endogenous precursors is also found in these experiments (Table III). The percentage of Phe_P is again 10–15% higher and that of Phe_{GG} about 15% lower in PLB than in PT. The percentage of Phe_{H_2GG} (~ 15%) and Phe_{H_4GG} (2–3%) is again the same in PLB and PT. In summary, the presence of chlorophyll synthetase in PLB- and PT-fractions has been demonstrated. The localization of enzyme activity may be approached by comparing the product – distribution of the enzyme with the distribution of tubules resp. membranes (Table IV). As was already shown for PChl(ide), the esterified Chl is found in a distribution similar to the PT-membranes and dif-

Table III. Percentage of Phe_{GG} and Phe_P, respectively (esterified pheophytin = 100%) in PLB and PT fraction. Percentage of Phe_{H_2GG} and Phe_{H_4GG} is small and nearly constant in both fractions of all experiments.

Experiment No.	PLB fraction		PT fraction	
	Phe_{GG}	Phe_P	Phe_{GG}	Phe_P
1 a	12	61	20	50
2 a	17	52	26	39
3 a	30	47	n. d.	n. d.
4 a	18	50	30	37
5 a	20	48	31	40
mean 1 a–5 a	19.4	51.6	26.8	41.5
3 b	50	30	75	12
4 b	76	6	79	4
5 b	58	26	76	6
mean 3 b–5 b	61.3	20.7	76.7	7.3

All experiments labelled a: without exogenous substrate. All experiments labelled b: with exogenous GGPP (~ 10 nmol/nmol pigment).

Table IV. Distribution of chlorophyll formed by the synthetase reaction in relation to content of tubules and membranes in isolated fractions.

	% Tubules	% Membranes	% Chlorophyll esterified
Experiment A			
PLB-fraction	86.6	57	56.5
PT-fraction	13.4	43	43.5
Experiment B			
PLB-fraction	41.5	28.2	34
PT-fraction	58.5	71.8	66

Experiment A: Plants for this isolation were grown for 7 days in the dark.
Experiment B: Isolation was performed with 5 days old plants. In this stage more tubules remain connected to PT membranes, but again product distribution follows membrane distribution between fractions.

ferent to PLBs. This holds also for experiments including exogenous precursor (+ GGPP) as well as for fractions isolated from plants in a younger stage of development (experim. "B"). Thus we may assume that most or all of the chlorophyll synthetase activity is bound to the PT membranes and not to the PLB tubules. This agrees also with the distribution of the substrate PChlide (see above) and with data showing that the enzyme (protochlorophyllide reductase) is active in PTs only (C. Lütz, U. Röper, and W. T. Griffiths, in preparation).

Distinct differences between PLBs and PTs have been found on the substrate level. The percentage of non-photoconvertible PChl(ide) and of endogenous phytol precursor is higher in the PLB than in the PT fraction. This may be due to differences in the status of the membranes in both fractions. Differences between PLB and PT fractions in the PSI [9] and ATPase activity [10] have also been reported but only in greening plants (etiochloroplasts).

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