STUDIES ON THE FRACTIONATION BY DIGITONIN OF CHLOROPLAST MEMBRANES

Robert C. JENNINGS, Paolo D. GEROLA, Flavio M. GARLASCHI and Giorgio FORTI
Centro di Studio del CNR per la Biologia Cellulare e Molecolare delle Piante, Istituto di Scienze Botaniche, Via Giuseppe Colombo 60, 20133 Milan, Italy

Received 14 April 1980

1. Introduction

Digitonin fractionated thylakoid membranes into light particles enriched in photosystem I and heavier particles enriched in photosystem II [1]. The basic action of digitonin was to separate the grana thylakoids from the intergrana thylakoids [2]. Thus the heavy fraction (10 000 × *g* pellet, enriched in photosytem II) consisted mostly of grana, while the light fraction (10 000 × *g* supernatant, enriched in photosystem I) consisted mostly of intergrana membranes. This method has been used as a quick and easy method to quantitate grana formation [3–5]. Grana formation and a normal digitonin fractionation pattern have been closely correlated [6] but have in the presence of 0.1 M ammonium acetate no membrane stacking to be observed with the electron microscope, although a normal digitonin fractionation pattern was observed [6]. This article reexamines the digitonin fractionation method to ascertain whether it is a reliable method for grana quantification. Our data show that, at least under certain conditions, factors other than the amount of grana strongly influence this fractionation procedure.

2. Materials and methods

Chloroplasts were extracted from freshly harvested spinach leaves in a tricine buffer (30 mM, pH 8.0) containing NaCl (10 mM) and sucrose (0.4 M) and, where indicated, $MgCl_2$ (3 mM). They were subsequently washed once and resuspended in the same buffer, unless otherwise stated. The reaction solution was the same medium minus sucrose, unless otherwise stated.

Chloroplast membranes were fractionated with digitonin as in [1]. Prior to the fractionation step the chloroplasts were washed with a solution containing tricine (30 mM, pH 7.4), NaCl (10 mM) and the appropriate [$MgCl_2$]. Chloroplasts were then suspended in the same medium at 300 μg chl/ml and digitonin (2%, w/v) was added to 0.5% final conc. Treatment with digitonin was done with agitation at 0°C for 30 min when the chloroplast membrane-detergent mixture was diluted with the same buffer, maintaining the [$MgCl_2$] unchanged. Separation of the heavy and light membrane fractions was achieved by centrifugation at 10 000 × *g* for 30 min. Chlorophyll was extracted with 80% acetone and *a*/*b* ratios were determined according to the equations of [7].

Chlorophyll fluorescence was excited at 440 nm and measured at 683 nm, at 4 μg chl/ml, in a Perkin-Elmer MPF-3 spectrofluorimeter. Sample preparation for electron microscopy was performed as in [8]. For the estimation of thylakoid membrane stacking, 14–20 randomly chosen chloroplasts were analyzed for every sample. The chloroplast micrographs were projected on a screen covered by randomly distributed points. The number of points lying on the membranes was counted. The degree of stacking (% stacked membranes) was determined by the ratio: $[2a/(2a + b)] \times 100$, where a is the number of points on appressed membranes and b is the number of points on single membranes.

Trypsin (type I) and soybean trypsin inhibitor (type I-S) were purchased from Sigma Chemical Co.

3. Results

Fig.1 shows the effect of pH on digitonin fractionation of chloroplast membranes. Data are presented for the light digitonin fraction, since changes

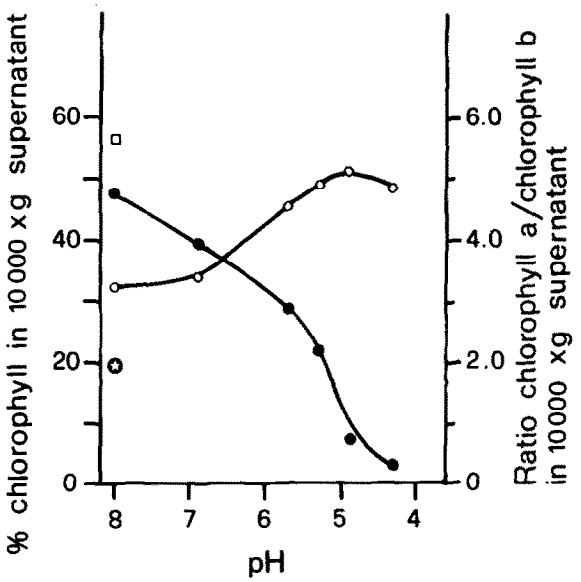

Fig.1. The effect of pH on fractionation by digitonin of chloroplast membranes. Chloroplasts were prepared in the absence of $MgCl_2$, as in section 2 and incubated for 30 min in the washing buffer. After a subsequent centrifugation they were directly resuspended in the various buffers at different pH values for 6 min before digitonin addition (see section 2). The different buffers used were: tricine (20 mM, pH 8.0), NaCl (10 mM); morpholine ethane sulfonic acid (MES) (20 mM, pH 6.9), NaCl (17.5 mM); MES (20 mM, pH 5.7), NaCl (27 mM); MES (20 mM, pH 5.3), NaCl (28 mM); succinate (20 mM, pH 4.9), NaCl (6.2 mM); succinate (20 mM, pH 4.3), NaCl (16 mM). The final $[Na^+]$ was 30 mN in all cases. (•) The yield of the light digitonin fraction; (○) the chl *a*/*b* ratio of the light fraction; (☆) the yield of the light fraction after the addition of $MgCl_2$ (5 mM); (□) the chl *a*/*b* ratio of the light fraction in the presence of $MgCl_2$ (5 mM).

in chl *a*/*b* ratios are more easily detected in this fraction. Clearly, changes in yield of the heavy fraction are directly related in an inverse fashion. Lowering from pH 8–4.3 resulted in a massive decrease of the light digitonin fraction, which at pH 4.3 was almost eliminated. The effect of 5 mM $MgCl_2$, added at pH 8.0, is also presented. Mg^{2+} at 5 mM is sufficient to cause maximal grana formation (unpublished) and it is also saturating for the production of the heavy digitonin fraction [4]. In fig.1 the yield at pH 5.3 of the light digitonin fraction was approximately equal to that produced by 5 mM $MgCl_2$ at pH 8.0, whereas pH <5.0 had a much more pronounced effect on fractionation yield. Decreasing the pH also resulted in a marked increase in the chl *a*/*b* ratio of the light digitonin fraction. This effect became saturated near pH 5.0, at values close to those induced by 5 mM $MgCl_2$ added at pH 8.0.

On the basis of electron microscope studies, we have shown that lowering the pH causes grana formation which seemed to become maximal near pH 5.4 [8]. Fig.2 shows a pH titration of grana formation in which it can be seen that pH 5.4 is indeed optimal for grana formation. The value attained at this pH is equal to that caused by 5 mM $MgCl_2$ added at pH 8.0 and is in the range usually reported [9,10].

These experiments indicate that under certain conditions the amount of chlorophyll in the digitonin fractions need not be an accurate indication of grana formation. To further investigate this we utilized the fact that a brief treatment of chloroplast membranes with trypsin, sufficient to eliminate the effect of saturating concentrations of cations on the chlorophyll fluorescence yield (3–5 mM $MgCl_2$), also eliminates the grana elicited by the same cation concentrations [11,12]. The data in table 1 show that even when the grana have been eliminated in this manner, the addition of 5 mM $MgCl_2$ is still capable of greatly decreasing the light digitonin fraction and increasing the heavy fraction, though the effect of $MgCl_2$ is smaller after trypsin treatment. However, it should be noted that this effect is not accompanied by

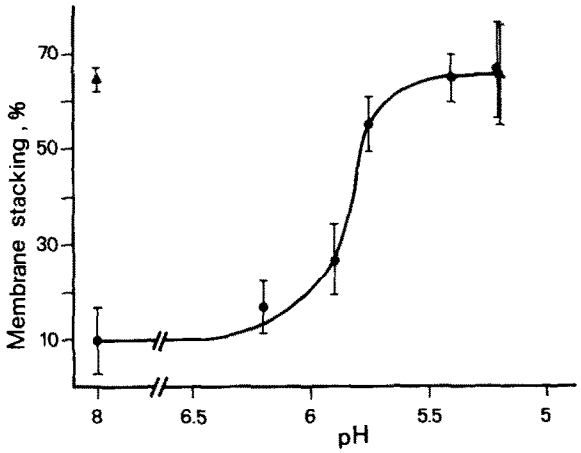

Fig.2. The effect of pH on thylakoid stacking. Chloroplasts were prepared in the absence of $MgCl_2$. They were subsequently incubated at 20 μg chl/ml for 60 min at room temperature at the various pH values. The different buffers used were: at pH 8.0, 20 mM tricine; at the other pH values 20 mM MES; NaCl, added to give a final $[Na^+]$ 30 mN, was 19.3 mM at pH 8.0, 18.7 mM at pH 6.2, 22.3 mM at pH 5.9, 25.2 mM at pH 5.75, 26.5 mM at pH 5.4, and 27.6 mM at pH 5.2. (•) $-MgCl_2$; (▲) +5 mM $MgCl_2$. Bars indicate the 95% confidence limits for the mean.

Table 1
The effect of addition of different [$MgCl_2$] on fractionation of chloroplast membranes by digitonin before and after treatment of membranes with trypsin

	$MgCl_2$ concentration (mM)			
	0	5	50	100
Chlorophyll fluorescence				
–Trypsin	29	61	61	54
+Trypsin	22	22	34	37
Digitonin fractions				
–Trypsin				
Light fraction				
Yield (%)	60	16	18	26
Ratio *a/b*	2.9	4.3	3.9	3.8
Heavy fraction				
Yield (%)	40	84	82	74
Ratio *a/b*	2.7	2.6	2.5	2.4
+Trypsin				
Light fraction				
Yield (%)	81	49	31	42
Ratio *a/b*	2.7	2.5	3.2	3.8
Heavy fraction				
Yield (%)	19	51	69	58
Ratio *a/b*	2.7	2.8	2.5	2.0

Chloroplasts prepared with $MgCl_2$ were treated at 20 μg chl/ml with trypsin (1.2 μg/ml, 5 min) in the presence of $MgCl_2$ (3 mM). The reaction was terminated by addition of a 50-fold excess of trypsin inhibitor. Control chloroplasts (not treated with trypsin) were resuspended for 5 min at room temperature in the absence of $MgCl_2$. Thus both control and trypsin-treated chloroplasts were unstacked. Subsequently, $MgCl_2$ at various concentrations was added, or not, and the chloroplasts were incubated in ice for 20 min, when they were centrifuged down and washed once in the buffer used for digitonin fractionation either in the presence or absence of the appropriate [$MgCl_2$]. 'Ratio' indicates the chl *a/b* ratio, and 'yield' indicates the % of total *a* + *b* chl found in that particular fraction

enrichment of the light fraction of chl *a*, as occurs with membranes not subjected to trypsin treatment. We would emphasize that electron microscope inspection of chloroplasts after this trypsin treatment, in the presence of 5 mM $MgCl_2$, indicated the complete absence of grana (not shown).

In table 1 it can also be seen that increasing $MgCl_2$ to >5 mM, after tryptic digestion of the membranes, leads to further decreases in the light digitonin fraction. This did not occur with control, non-trypsin-treated membranes. It can also be seen that these relatively small changes in yield measured in the presence of 50 and 100 mM $MgCl_2$ (in trypsin-treated membranes) are accompanied by quite large increases in the chl *a/b* ratio in the light fraction. This experiment thus demonstrates that changes in yield and in the chl *a/b* ratio in the digitonin fractions can occur independently of one another.

Table 2 shows that this effect of $MgCl_2$ on the digitonin fractionation pattern of chloroplast membranes is in fact a cation effect, which can also be elicited by the trivalent cation, tris(ethylenediamine)cobalt (III) (TEC), at very low concentrations, and by monovalent cations at high concentrations.

4. Discussion

These data demonstrate that estimates of chlorophyll membrane yield in the heavy digitonin fraction

Table 2
Effect of various salts on the yield of chlorophyll in the light digitonin fraction after trypsin treatment

Additions	None	$MgCl_2$ (0.5 mM)	$MgCl_2$ (5 mM)	$Mg(CH_3COO)_2$ (5 mM)	NaCl (20 mM)	NaCl (150 mM)	TEC (50 μM)
Chlorophyll fluorescence							
–Trypsin	24	–	53	–	–	–	–
+Trypsin	20	18	21	19	20	18	21
Light digitonin fraction after trypsin treatment							
Yield (%)	79	69	48	44	77	67	48
Ratio	2.9	2.8	2.8	2.7	2.9	2.9	2.8

Data are also presented for the chlorophyll fluorescence yield. For details of experimental procedure see the legend of table 1

cannot always be assumed to indicate the extent of grana formation in a chloroplast preparation. This conclusion is based essentially on two observations:

(1) Direct quantification by electron microscopy of grana formation induced by protons shows that this phenomenon is complete at pH 5.3–5.4, though digitonin fractionation studies indicate that membrane yield in heavy fraction is maximal not at these pH values, but at or below pH 4.3.

(2) Trypsin treatment of chloroplast membranes sufficient to eliminate all grana in the presence of 5 mM $MgCl_2$ does not eliminate the effect of 5 mM Mg^{2+} on increasing the heavy digitonin fraction at the expense of the light fraction.

In arriving at this conclusion we do not intend to detract from the conclusion that the heavy fraction is grana enriched and the light fraction is enriched in stroma lamellae [2]. Under 'normal' conditions at ~5 mM divalent cation, we find the same result.

The importance of the electrostatic potential at and near the surface of chloroplast membranes in controlling various cation-mediated effects on chloroplast membranes, including membrane stacking has been emphasized [5,13–15]. We have obtained data which indicate that membrane unstacking elicited by trypsin is also mediated by an increase of the negative surface potential (R. C. J. et al., submitted). These considerations suggest the possibility that the digitonin fractionation of chloroplast membranes is controlled by similar factors. The following data support this idea. The isoelectric point of chloroplast membranes, as measured by particle electrophoresis, is near pH 4.3 [16], and we observe that production by digitonin of the light membrane fraction decreases on lowering the medium pH to 4.3, at which ~97% of the membranes are 'heavy' under our experimental conditions. Monovalent, divalent and trivalent cations, in an increasing order of efficiency, are all capable of influencing the yield of membrane fractionation, in a manner which does not involve grana formation (i.e., after trypsin treatment of membranes). This order of cation effectiveness is predicted by the hypothesis in [5,17]. Thus we envisage that cations (by screening membrane surface charges) and protons (by neutralizing membrane surface charges) influence membrane organization in such a way as to bring about changes in yield of the digitonin fractions.

References

[1] Boardman, N. K., Thorne, S. W. and Anderson, J. M. (1966) Proc. Natl. Acad. Sci. USA 56, 586–593.
[2] Goodchild, D. J. and Park, R. B. (1971) Biochim. Biophys. Acta 226, 393–399.
[3] Argyroudi-Akoyunoglou, J. H. (1976) Arch. Biochem. Biophys. 176, 267–274.
[4] Argyroudi-Akoyunoglou, J. H. and Akoyunoglou, G. (1977) Arch. Biochem. Biophys. 179, 370–377.
[5] Barber, J. and Chow, W. S. (1979) FEBS Lett. 105, 5–10.
[6] Ohki, R., Kunieda, R. and Takamiya, A. (1971) Biochim. Biophys. Acta 226, 144–153.
[7] Mackinney, G. (1941) J. Biol. Chem. 140, 315–322.
[8] Gerola, P. D., Jennings, R. C., Forti, G. and Garlaschi, F. M. (1979) Plant Sci. Lett. 16, 249–254.
[9] Anderson, J. M., Goodchild, D. J. and Boardman, N. K. (1973) Biochim. Biophys. Acta 325, 573–585.
[10] Miller, K. R. and Staehlin, L. A. (1976) J. Cell. Biol. 68, 30–47.
[11] Jennings, R. C. and Forti, G. (1978) in: The proton and calcium pumps (Azzone, G. F. et al. eds) pp. 93–104, Elsevier/North-Holland, Amsterdam, New York.
[12] Jennings, R. C., Forti, G., Gerola, P. D. and Garlaschi, F. M. (1978) Plant Physiol. 62, 879–884.
[13] Barber, J. and Mills, J. (1976) FEBS Lett. 68, 288–292.
[14] Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174–181.
[15] Barber, J. and Searle, G. F. W. (1979) FEBS Lett. 103, 241–254.
[16] Nakatani, H. Y., Barber, J. and Forrester, J. A. (1978) Biochim. Biophys. Acta 504, 215–225.
[17] Barber, J. and Searle, G. F. W. (1978) FEBS Lett. 92, 5–8.