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RECONSTITUTION OF CHLOROPHYLLASE WITH MIXED PLANT LIPIDS IN THE PRESENCE AND ABSENCE OF Mg^{2+}

INFLUENCE OF SINGLE AND MIXED PLANT LIPIDS ON ENZYME STABILITY

J.W.J. LAMBERS [a], A.J. VERKLEIJ [b] and W. TERPSTRA [a]

[a] *Department of Biophysics* * *and* [b] *Institute of Molecular Biology, State University of Utrecht, Utrecht (The Netherlands)*

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Chlorophyllase (chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) can be reconstituted using a mixture of a galactolipid (preferably monogalactosyldiacylglycerol (MGDG)) and phosphatidylglycerol (PG), with or without β-carotene. The enzyme does not combine, or combines only slightly, with the individual lipids. Three different methods were used to check whether chlorophyllase has been reconstituted with plant membrane lipids: (a) 'Free' and 'lipid-bound' chlorophyllase could be distinguished by the increased heat-stability of the lipid-bound enzyme. (b) Density changes, resulting from chlorophyllase-lipid association, were measured on a sucrose gradient. (c) Structural changes caused by interaction of the protein with lipids could be visualized by freeze-fracture electron microscopy. Association of chlorophyllase with mixed MGDG and PG (with or without β-carotene), and also with mixed digalactosyldiacylglycerol (DGDG) and PG leads to highly increased heat-stability of the enzyme conformation. This effect is independent of the presence or absence of Mg^{2+}. Single lipids were shown to have a much lower or no stabilizing influence. Only with PG, in the absence of Mg^{2+}, was any notable enzyme stabilization observed. The composition of the enzyme-lipid association product depends on the presence or the absence of Mg^{2+}. In the presence of Mg^{2+}, hexagonal rods, formed upon sonication of a MGDG/PG/β-carotene mixture in an aqueous medium, are converted into small vesicles and particles upon the addition of chlorophyllase. From their average density it is concluded that these vesicles and particles have a relatively high lipid:protein ratio. Without Mg^{2+}, a sonicated MGDG/PG/β-carotene dispersion consists of bilayers; the lipids combine with chlorophyllase to form a product with a relatively low lipid:protein ratio. The relevance of these results is discussed in relation to our earlier suggestion that chlorophyllase is a component of chlorophyll-protein complexes in thylakoid membranes.

Introduction

Chlorophyllase (chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) is situated in the photosynthetic membranes of higher plants and algae [1–4]. The enzyme catalyzes the hydrolysis of chlorophyll into chlorophyllide and phytol. Since chlorophyllase is an intrinsic membrane protein, its conformation and its enzymic activity can be expected to depend on the lipid environment, as is the case with many membrane enzymes [5].

In earlier experiments we did indeed find evidence that (natural) lipids are required for chlorophyllase activity [6]. Several individual plant lipids

* Correspondence address: Physics Laboratory, Princetonplein 5, Postbus 80000, 3508 TA Utrecht, The Netherlands.

Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine.

(MGDG, DGDG, PG, PC and β-carotene), when mixed with purified chlorophyll *a* in acetone, were found to affect chlorophyllase-catalyzed chlorophyll hydrolysis, but it was only with MGDG and with β-carotene that some association with the enzyme could be demonstrated [6]. It has been shown that the enzyme reaction can also be influenced by the interaction of lipids with the substrate, chlorophyll [6,7] and with the reaction product, chlorophyllide [8,9]. This complicates the interpretation of the experimental results. Therefore, to elucidate the mechanism of chlorophyllase functioning in photosynthetic membranes, it is of importance to identify, first of all, the lipids that are directly involved in the interaction with the enzyme.

In the experiments reported here, the interaction of chlorophyllase with individual plant lipids and with mixtures of these lipids has been studied. Since cations (Mg^{2+}) are known to affect the enzyme reaction [6,10], the effect of Mg^{2+} on lipid-enzyme association has been investigated as well. The following methods were used to check the formation of protein-lipid combinants. (1) Determination of the heat-stability. (2) Determination of the buoyant density of the reconstituted products. (3) Visualization by means of freeze-fracture electron microscopy of structural changes caused by protein-lipid interaction.

Although chlorophyllase associated poorly with single lipids, it is shown that the enzyme can be reconstituted with mixtures of PG and a galactolipid, preferably MGDG.

Material and Methods

Lipids were obtained from Sigma (PG and β-carotene) or from Lipid Products, Nutfield Nurseries, Surrey, U.K. (MGDG and DGDG).

Chlorophyllase was isolated from *Phaeodactylum tricornutum,* following the method described in Ref. 6; no detergents were used during the isolation procedure, cf. Ref. 11. The purified enzyme contains some tightly-bound lipids. No contaminating proteins were observed in our preparations upon filtration over Sepharose CL-4B. Unless mentioned otherwise, enzyme activity was assayed as described in Ref. 6; 'unpurified chlorophyll' containing chloroplast lipids was used as a substrate.

Chlorophyllase was reconstituted with lipids in the following manner: a solution containing the required amount of lipids in $CHCl_3$ was dried with nitrogen to form a thin film on the bottom of a test tube. The lipid was suspended by vortexing in 20 mM Tris-HCl (pH 8.0) with or without 10 mM $MgCl_2$, and then sonicated for 30 min in a Bransonic bath. Chlorophyllase was added to the lipid dispersion and both vortexing and sonication were repeated.

Heat-stability of chlorophyllase in the presence of lipids was determined as follows: 10 μl of lipid-chlorophyllase dispersion, containing 6 μg lipid(s) and 0.7 μg chlorophyllase but no $MgCl_2$, were added to 490 μl of a pre-cooled assay mixture comprising 20 mM Tris-HCl (pH 8.0)/6.7 mM dithiothreitol. $MgCl_2$ (10 mM) was added before or after heating (0–60 min) in a 37°C water bath. Unheated controls were kept in the refrigerator. The heated assay mixtures were cooled, $MgCl_2$ concentrations were adjusted to 10 mM in all tubes, and then 50 μg 'unpurified chlorophyll' were added.

It was observed that when no lipids had been added some chlorophyllase preparations were extremely labile in a dilute solution. Therefore, care was taken that the unheated control was kept diluted for as short a time as possible and at a low temperature. After the addition of 'unpurified chlorophyll', which contains stabilizing lipids, the enzyme is more stable (see Refs. 6 and 7 and Results).

For sucrose gradient centrifugation, about 25 μg chlorophyllase were mixed with about 600 μg total lipid, which was dispersed in 1 ml buffer, with or without 10 mM $MgCl_2$. Sucrose gradient centrifugation was performed as described in Ref. 7; if required, 10 mM $MgCl_2$ was added to the gradient solutions. Figures show characteristic experiments.

For study by electron microscopy 600 μg lipids (MGDG/PG/β-carotene, 1 : 1 : 1 (w/w)), suspended in 1 ml buffer, were mixed with 90–650 μg chlorophyllase. The protein/lipid dispersions were centrifuged for 60–90 min at 226000 × *g* (tube bottom). Both the supernatants and the pellets were assayed for chlorophyllase activity. Freeze-

fracture electron microscope photographs of the pellets were made according to an established procedure [12]. 30% glycerol was added to prevent freeze damage.

Results and Conclusions

(1) Heat-stability of free and lipid-treated chlorophyllase

Chlorophyllase activity was found to decline rapidly whenever the enzyme was handled in the absence of lipids. This led us to investigate the effect that lipids have on enzyme stability.

Fig. 1 (circles) shows the influence of heating on chlorophyllase activity. After 30 min at 37°C, only a small residual activity is left; this means that the greatest part of the enzyme has denatured during this treatment.

When various lipids are sonicated with chlorophyllase in the absence of Mg^{2+}, the heat-stability of the chlorophyllase is affected in varying degrees. Table I shows that DGDG and β-carotene exert only a slight protective action on the enzyme; the stabilization is somewhat more effective with MGDG. With these lipids, the effect is independent of the presence or absence of Mg^{2+} during the heating procedure. With PG, however, Mg^{2+} does have a marked effect: PG stabilization is significantly higher in the absence of Mg^{2+}, but even in this case about 30% of the enzyme activity disappears in the course of 30 min heating at 37°C (Table I). The effect of Mg^{2+} may be due to the fact that the PG transition temperature rises above 37°C in the presence of Mg^{2+} at a relatively high concentration [13].

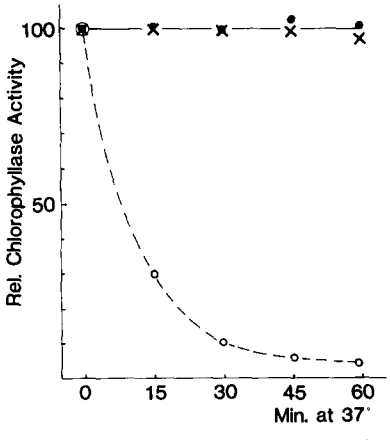

Fig. 1. Influence of heating on the activity of free and lipid-bound chlorophyllase. ○, free chlorophyllase; ×, chlorophyllase sonicated with MGDG/PG/β-carotene (1 : 1 : 1, w/w) in the presence of Mg^{2+}, ●, chlorophyllase sonicated with MGDG/PG/β-carotene in the absence of Mg^{2+}.

TABLE I

HEAT STABILITY OF CHLOROPHYLLASE UPON ITS SONICATION WITH VARIOUS SINGLE AND MIXED LIPIDS

Results for PG were as obtained in the presence ($+Mg^{2+}$) or absence ($-Mg^{2+}$) of Mg^{2+}. In all other cases Mg^{2+} had no significant effect during the heating period. See Material and Methods for details of the reaction mixtures. Results are means ± S.E. for the number of experiments shown in parentheses.

Lipids	Residual chlorophyllase activity (%) after heating for 30 min at 37°C
– (control)	7.0 ± 1.0(7)
MGDC	33.4 ± 1.5(7)
DGDG	10.1 ± 1.0(5)
PG ($+Mg^{2+}$)	19.4 ± 1.7(4)
PG ($-Mg^{2+}$)	68.7 ± 9.7(4)
β-Carotene	12.3 ± 0.3(2)
MGDG + PG	108.3 ± 3.1(9)
DGDG + PG	102.6 ± 3.4(7)
MGDG + DGDG	17.7 ± 3.2(2)

No significant inactivation is observed if the enzyme has been sonicated with mixtures of MGDG and PG, or DGDG and PG (Table I). In the former case there may even be a slight increase in enzyme activity upon heating. With these mixtures the effect is again independent of the presence or absence of Mg^{2+} during the heat treatment.

Addition of β-carotene to the MGDG/PG mixture does not significantly influence the results (Fig. 1).

Reconstitution of the enzyme with MGDG/PG with or without β-carotene in the presence of Mg^{2+} leads to a heat-stability similar to that found for the enzyme reconstituted in the absence of Mg^{2+} (Fig. 1, Table I); with and without Mg^{2+}, the lipids protect 90–100% of the enzyme activity from heat-denaturation. This is interpreted to mean that in both cases at least 90–100% of the enzyme has associated with the lipids (cf. sections 2 and 3).

It is concluded from these experiments that chlorophyllase denaturation is effectively prevented when the enzyme has been reconstituted

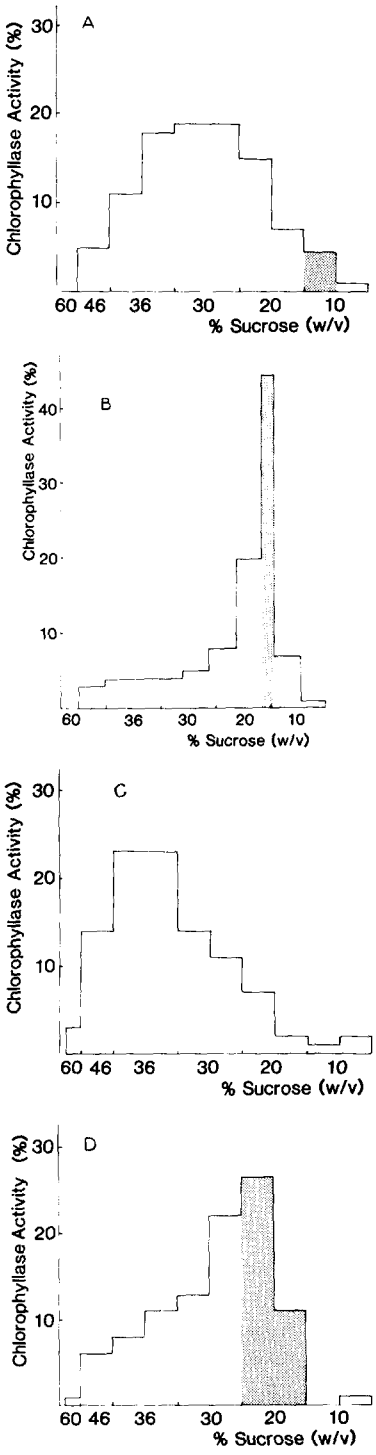

Fig. 2. Distribution of chlorophyllase in a sucrose gradient. (A) Chlorophyllase sonicated with MGDG/PG/β-carotene in the absence of Mg^{2+}; (B) chlorophyllase sonicated with MGDG/PG/β-carotene (1 : 1 : 1, w/w) in the presence of Mg^{2+}; (C) chlorophyllase without added lipids (control); (D) chlorophyllase sonicated with DGDG/PG/β-carotene in the presence of Mg^{2+}. In experiments (B) and (D) the sucrose solutions in the gradient contained 10 mM $MgCl_2$. Lipid/chlorophyllase ratio was about 24 : 1 (w/w). Chlorophyllase activity in the fractions is expressed as the sum of the activities of these fractions. The shaded areas indicate the lipid site.

with a combination of PG with a galactolipid, with or without β-carotene.

(2) Buoyant density changes of chlorophyllase upon its reconstitution with mixed plant lipids

Recently we found that chlorophyllase interacts with MGDG and with β-carotene [6]. However, only a very little amount of the enzyme could be combined with these lipids upon sonication in an aqueous medium. Similar, rather poor results were obtained for the heat-stabilizing action of single lipids, as described in the previous section. We therefore concentrated on the reconstitution of chlorophyllase with those mixed plant lipids which gave high heat-stability of the enzyme.

Fig. 2A,B illustrates the sucrose gradient flotation profiles obtained with lipid dispersions composed of MGDG, PG and β-carotene (1 : 1 : 1, w/w) which were sonified together with chlorophyllase in the absence or presence of Mg^{2+}. The site of the lipids on the gradient was visible as a more or less turbid yellow layer (the shaded area in the figures). In the absence of Mg^{2+}, chlorophyllase was located, predominantly, below the somewhat diffuse lipid band (Fig. 2A). In comparison with flotation profiles of chlorophyllase alone (Fig. 2C) a small upward migration of the enzyme to lower density was observed. In the presence of Mg^{2+}, however, most of the chlorophyllase activity was located at the site of the sharp lipid band, but appreciable activity was also measured immediately below this band (Fig. 2B). Replacement of MGDG by DGDG resulted in a relatively higher amount of high-density enzyme (Fig. 2D). Control experiments without β-carotene revealed that this lipid pigment does not significantly influence the results. However, it facilitates the visibility of the lipid layer.

The experimental results show that the buoyant density of chlorophyllase decreases upon reconstitution of the enzyme with a mixture of a galactolipid and PG. The extent of this decrease de-

pends on the presence or absence of Mg^{2+} and also on the identity of the galactolipid (MGDG or DGDG).

(3) The structure of MGDG/PG/β-carotene dispersions in the absence and presence of Mg^{2+}; influence of chlorophyllase association

In the absence of Mg^{2+}, an MGDG/PG/β-carotene (1 : 1 : 1) dispersion in 20 mM Tris-HCl (pH 8.0) looks clear, and hardly any precipitate is observed upon centrifugation for 60–90 min at 226 000 × *g* (tube bottom). Chlorophyllase, sonicated with such a dispersion remains in the supernatant (Table II).

In the presence of Mg^{2+}, a sonicated dispersion of MGDG/PG/β-carotene is turbid and precipitates upon centrifugation. After sonication with chlorophyllase, however, this turbid dispersion clarifies, but the lipids still precipitate upon centrifugation. Chlorophyllase now precipitates along with the lipids; with an excess of lipid only very low enzyme activity remains in the supernatant (Table II). Similar results were obtained in the absence of β-carotene. Replacement of MGDG by DGDG resulted in the precipitation of a much smaller part of the enzyme (Table II).

In order to characterize the submicroscopic structure of the pelleted preparations, we used freeze-fracture electron microscopy. Fig. 3A shows that, without Mg^{2+}, the pelletable lipid consists of vesicles of varying size, whereas in the presence of Mg^{2+} the pelleted lipid is in a hexagonal II phase, which consists of cylindrical arrays with the lipid headgroups facing inwards [14,15] (Fig. 3B). In the latter case, the formation of vesicles and of small particles can be seen after sonication with a relatively large amount of chlorophyllase (chlorophyllase/lipid ratio 1 : 1 (w/w) (Fig. 3C,D)). This means that the enzyme binds to the lipids. These results will be discussed in the following section.

TABLE II

CENTRIFUGATION OF CHLOROPHYLLASE SONICATED WITH VARIOUS LIPID MIXTURES IN THE PRESENCE OR ABSENCE OF Mg^{2+}

Centrifugation was for 60–90 min at 226 000 × *g*. For details of reaction mixtures see under Materials and Methods.

Lipids	Lipids/chlorophyllase (w/w)	Mg^{2+}	% Chlorophyllase in supernatant
MGDG + PG + β-carotene	6.5	+	3–5
MGDG + PG + β-carotene	1	+	~ 25
MGDG + PG + β-carotene	6.5	–	100
MGDG + PG	6.5	+	5–14
DGDG + PG + β-carotene	6.5	+	71–93

Discussion

Single lipids were shown to give only poor protection against the heat-denaturation of chlorophyllase. These results are in agreement with earlier experiments in which only a sligth association of chlorophyllase with some single lipids could be demonstrated [6].

Mixed lipids containing PG and a galactolipid are highly protective against heat-denaturation of the enzyme. It could be shown that nearly all protein molecules combine with these lipids.

Chlorophyllase and an MGDG/PG/β-carotene mixture were found to produce two different association products:

(a) With Mg^{2+} pelletable, low-density 'particles' are formed. The combination of these properties indicates that the diameter of these particles is relatively large; presumably they are small vesicles or particles (cf. also Fig. 3C,D) with a relatively large lipid/protein ratio.

(b) Without Mg^{2+}, non-pelletable, high-density 'particles' emerge. Consequently, the diameter of this chlorophyllase/lipid product will be much smaller than that of the vesicles formed with Mg^{2+}, whereas the lipid/protein ratio of the product is relatively low.

The formation of lipid-protein association products with different lipid/protein ratios has also been described for rhodopsin [16] and for lactalbumin [17].

Upon replacement of MGDG by DGDG a lipid-protein association occurred as well, as was deduced from the heat-stability of the complex. However, less precipitable low-density products were observed. Presumably, the vesicles formed are smaller in this case (Fig. 2D, Table II).

MGDG in an aqueous medium can form rod-like structures, consisting of a hexagonal II lipid/

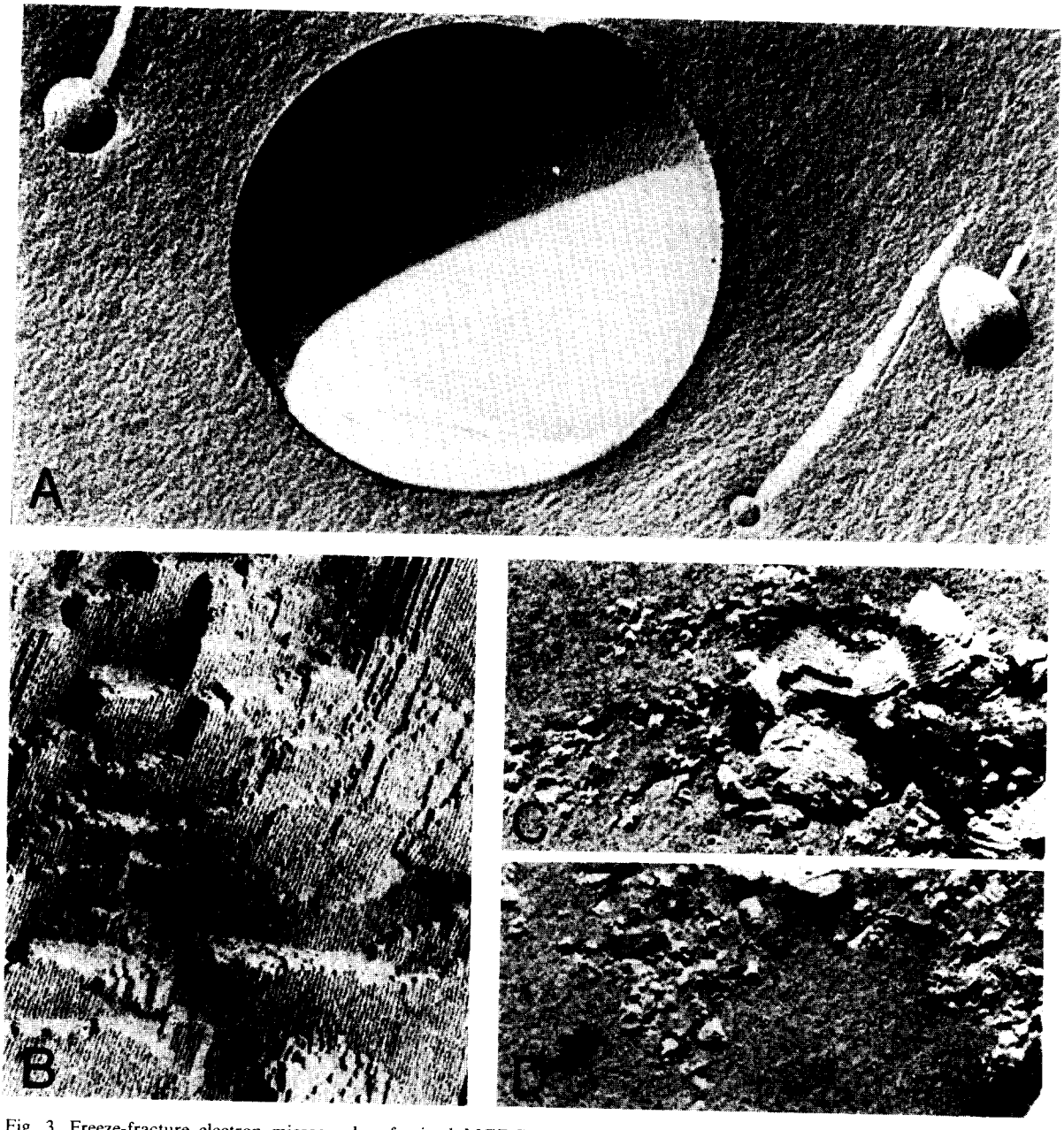

Fig. 3. Freeze-fracture electron micrographs of mixed MGDG/PG/β-carotene (1:1:1, w/w) dispersions, sonicated in 20 mM Tris-HCl buffer (pH 8.0). (A) Without Mg^{2+}; (B) with 10 mM Mg^{2+}; (C,D) with 10 mM Mg^{2+} and chlorophyllase (lipid/protein ratio, 1:1 (w/w)). Magnification, ×90000.

water phase [18–20]. In our case, similar structures were formed upon sonication of an MGDG/PG/β-carotene mixture in 20 mM Tris-HCl buffer, but only in the presence of Mg^{2+}. This triggering of hexagonal phase II formation by divalent cations was also found with phosphatidic acid [21] and with cardiolipin (disphosphatidylglycerol) [14,22,23]. Related structures were found to be formed in mixtures of bilayer- and hexagonal II-forming lipids (see the review by Verkleij and De Gier [15]) and in total polar lipid extracts of chloroplast membranes [14,24].

Upon sonication of chlorophyllase with an MGDG/PG/β-carotene dispersion the hexagonal

rods disappear and small vesicles and particles, probably protein-lipid micelles, are formed. A similar bilayer stabilization was found by Taraschi et al. [25] for glycophorin and cardiolipin in the presence of Ca^{2+}. Like glycophorin, chlorophyllase is a glycoprotein. MGDG, PG and β-carotene are natural lipid components of chlorophyllase-containing chloroplast thylakoids [26,27]; they were also shown to occur in chlorophyll-protein complexes [28–33]. In our preparations these lipids were often associated with the isolated enzyme (unpublished experiments). Our experiments may provide yet another example of the stabilizing action of a membrane (glyco)protein on the bilayer structure of its surrounding lipids.

Stabilization of protein conformation by lipids is found in the case of many membrane proteins [5,34–42]. Generally this is a more or less non-specific effect; Bohnenberger and Sandermann [43] suggest the importance of a definite hydrophylic-lipophylic balance in the lipid. One remarkable conclusion from our experiments with chlorophyllase is that effective stabilization requires the combination of two specific plant lipids, PG and a galactolipid. To our knowledge, there have been very few examples of similar observations with regard to other enzymes (Refs. 44, 45, see also Refs. 46, 47). We suggested earlier [3] that chlorophyllase is a component of chlorophyll-protein complexes in thylakoid (stroma) membranes. A preferential combination of MGDG and PG with chlorophyllase, or proteins from chlorophyll-protein complexes, would be in agreement with the suggestion of Quinn and Williams [19] that non-bilayer forming lipids may facilitate the incorporation of these proteins into lipid bilayers of the thylakoid membrane (see also Refs. 48–50).

In the experiments described association of chlorophyllase with a mixture of specific lipids was deduced from results of reactions which occur before enzyme assay. Consequently, interference with these results by interactions of the lipids with the enzyme substrate, chlorophyll, or with the reaction product, chlorophyllide [9] could be ruled out. It cannot simply be concluded that lipids which stabilize enzyme conformation will also directly affect the enzyme activity. Further investigations on this problem are now in progress.

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