Low pH and phospholipase A_2 treatment induce the phase-separation of non-bilayer lipids within pea chloroplast membranes

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Exposure of chloroplasts to pH < 4.5, or incubation in the presence of phospholipase A_2, leads to membrane lipid phase separations and the irreversible formation of non-bilayer lipid structures. Freeze-fracture replicas of the thylakoid membranes of treated chloroplasts are characterized by the presence of aggregates of cylindrical inverted lipid micelles. These structural changes are accompanied by an inhibition of photosystem II-mediated electron transport and a stimulation of photosystem I-mediated transport. These data have important implications both with respect to the factors governing the stability of thylakoid membranes and the use of lipases as probes of chloroplast structure.

Membrane lipid *Hexagonal phase* *Freeze-fracture* *Chloroplast*

1. INTRODUCTION

One of the characteristic features of biological membranes is that they nearly all contain polar lipids that when dispersed alone in aqueous media form hexagonal-II structures. The thylakoid membrane of higher plant chloroplasts is particularly rich in such lipids; the non-bilayer forming lipid, MGDG, representing about half of the total polar lipid fraction (see, e.g., [1]).

When total polar lipid extracts of chloroplast membranes are dispersed in dilute salt solutions extensive phase separations of the non-bilayer forming lipids from the bilayer forming lipids are observed [2]. The non-bilayer lipids usually form spherical or cylindrical inverted micelles which are either sandwiched between leaflets of the membrane bilayer or separate out as 3-dimensional aggregates [3]. Phase separation is favoured by elevated temperatures and/or the presence of high concentrations of cryoprotectants [4,5], low pH or high concentrations of metal cations [6], and high degrees of unsaturation of the acyl chains of the non-bilayer forming lipid component [7].

Under normal conditions, non-bilayer structures are not found in chloroplast, or other biological membranes. They are, however, observed when such membranes are subjected to stress. Non-bilayer structures are formed in the membranes of heat-stressed chloroplasts [8] and chloroplast thylakoids treated with 6 M guanidine thiocyanate [9]. Similar structures have also been observed in mitochondria following the addition of high concentrations of Mn^{2+} [10] and dehydrated retinal rod outer segment disk membranes [11,12] and sarcoplasmic reticulum [13].

Here, we report further examples of such lipid phase separations in chloroplast membranes associated with changes in pH and the action of

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Abbreviations: Chl, chlorophyll; PS, photosystem; DCPIP (H_2), dichlorophenolindophenol; MV, methyl viologen; CIM, cylindrical inverted micelles; MGDG, monogalactosyldiacylglycerol

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phospholipase A_2 and discuss their significance in terms of chloroplast stability and the use of lipases as probes of chloroplast structure.

2. MATERIALS AND METHODS

Chloroplasts were isolated from leaf tissue of 2–3 week old pea seedlings (*Pisum sativum* L. var. Feltham First) by the procedure of Stokes and Walker [14] and resuspended either in normal assay medium (330 mM sorbitol, 2.5 mM EDTA, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes (pH 7.6)) or destacking medium (330 mM sorbitol, 2.5 mM EDTA, 15 mM KCl, 50 mM Hepes (pH 7.6)). Total chlorophyll concentration was determined by the method of Arnon [15].

The pH treatments were performed by incubating chloroplasts (20 μg chl · ml^{-1}) in assay medium of defined pH for 5 min at 25°C. The chloroplasts were then harvested by centrifugation and resuspended in appropriate media for further structural and functional studies. Alternatively, chloroplasts (0.5 mg chl · ml^{-1}) were incubated at 25°C with phospholipase A_2 (phosphatide 2-acylhydrolase; EC 3.1.1.4) from bee venom at a concentration of 10 Sigma units · mg chl^{-1} in the dark; no exogenous Ca^{2+} was added. Aliquots of the incubation mixture were taken at various times, washed and resuspended in assay medium for further study.

Electron transport rates through PS I, PS II and PS II to PS I were estimated from rates of O_2 up-

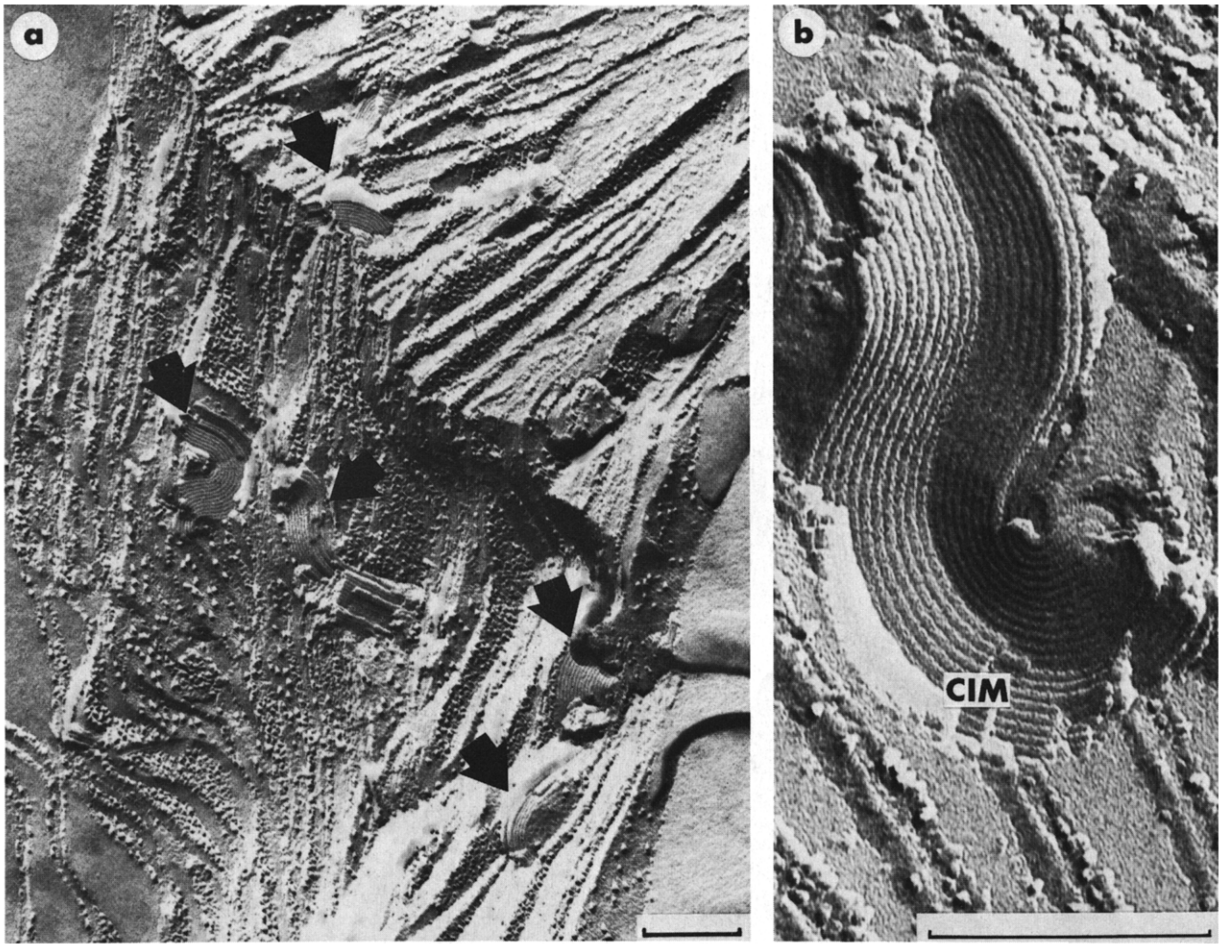

Fig.1. Electron micrographs of freeze-fracture replicas of pea chloroplasts suspended in assay medium at pH 4.6 for 5 min and then restored to pH 7.6. Note the presence of large aggregates of CIM indicated by arrows. Bar, 200 nm.

take/evolution associated with electron flow from $DCPIPH_2 \longrightarrow MV$, $H_2O \longrightarrow DCPIP$ and $H_2O \longrightarrow MV$, respectively. Details of reagent concentrations are given in the appropriate figure legends. All measurements were made at 25°C under light-saturating conditions using a Hansatech oxygen electrode assembly. Samples were prepared for freeze-fracture electron microscopy, as described elsewhere [5], and examined using a Philips EM 301 G electron microscope.

3. RESULTS

3.1. *Structural studies*

Electron micrographs showing typical examples of non-bilayer structures formed in chloroplast preparations suspended in assay medium at pH 4.6 are presented in fig.1a,b. The lower magnification view, fig.1a, shows examples of 3-dimensional aggregates of CIM protruding from the thylakoid membranes. The higher magnification view shows a single aggregate in greater detail. These aggregates, which closely resemble those seen in chloroplasts subjected to heat-stress [8], usually take the form of closed whorls consisting of several layers of inverted lipid micelles. The diameter of the individual micelles is normally about 12 nm. Phase-separated non-lamellar lipid structures were common in chloroplasts incubated at pH < 5.0. Few such structures, however, were seen in

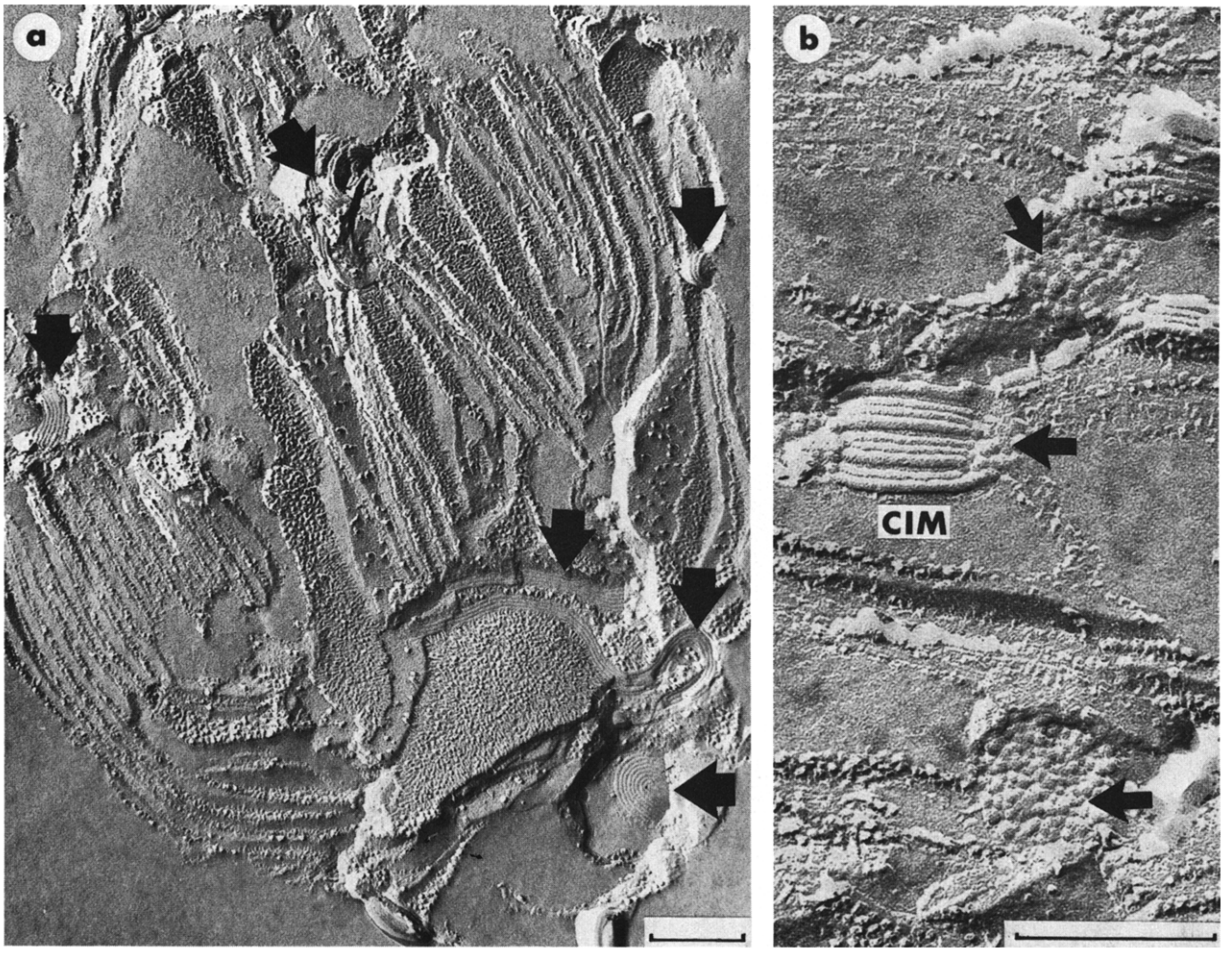

Fig.2. Electron micrographs of freeze-fracture replicas of pea chloroplasts treated with bee venom phospholipase A_2 (10 Sigma units · mg chl^{-1}) for 30 min in the dark at 25°C showing aggregates of CIM (large arrows) and associated particulate structures (small arrows). Bar, 200 nm.

chloroplasts suspended in media of pH 6 and none were seen in control preparations at pH 7.6. Little or no difference was observed between preparations suspended at a given pH for 5 min and then restored to pH 7.6 prior to thermal quenching and preparations thermally quenched directly from the test pH, indicating that the formation of these non-bilayer structures was irreversible.

The effect of exposure of isolated chloroplasts to phospholipase A_2 was examined in a second series of experiments. Incubation of the chloroplasts for 30 min under the conditions outlined above again resulted in the phase separation of non-bilayer lipids. Typical examples are shown in fig.2a. Whilst most of the phase-separated domains resembled those seen in the pH-treated chloroplasts, other non-bilayer structures were also observed. Examples of these are presented in fig.2b. One type of structure appeared to consist of bundles of curved cylindrical inverted micelles of about 18 nm diameter. Tightly packed particle-like structures of similar diameter were also observed. It is not clear, however, whether these corresponded to spherical inverted micelles or cross-fractures of the cylindrical micelles. In contrast to heat-stress treatment, neither the pH nor the lipase treatment led to destacking of the chloroplast thylakoids. No lipid phase separations were seen on lipase treatment of chloroplasts suspended in destacking medium. However, similar results were obtained for pH-treated chloroplasts suspended in normal and destacking media. It should be noted that at low pH chloroplasts show stacking, albeit of a different form to that seen in normal chloroplasts, even in destacking media [16–18].

3.2. *Functional studies*

The relative rates of whole chain and PS I-mediated electron transport by chloroplasts incubated in media of different pH, prior to assay at pH 7.6, are shown in fig.3. Corresponding plots for phospholipase A_2 treated chloroplasts are presented in fig.4. Both treatments lead to an inhibition of whole chain electron transport and a stimulation of PS I activity. Measurements of PS II-mediated electron transport using DCPIP as a terminal electron acceptor and diphenylcarbazide as an electron donor in place of H_2O yielded essentially identical results to those obtained for

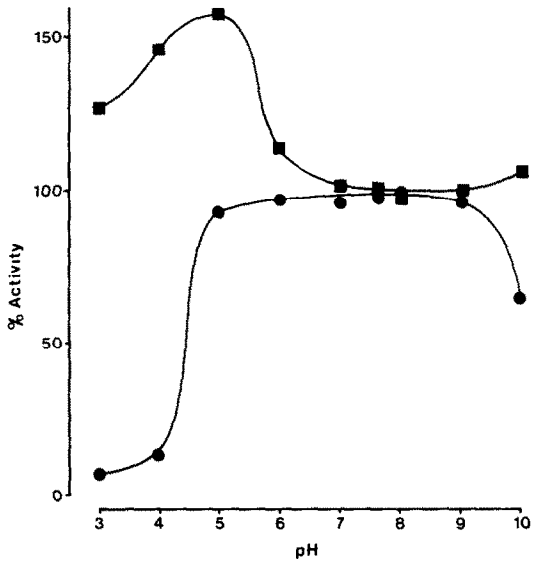

Fig.3. Relative rates of whole chain (●) and PS I-mediated (■) electron transport by isolated pea chloroplasts assayed at 25°C and pH 7.6 after 5 min exposure to media of varying pH. The reaction mixture for whole chain studies ($H_2O \longrightarrow MV$) contained 0.4 mM MV and 0.8 mM NaN_3 and that for PS I-mediated electron transport studies ($DCPIPH_2 \longrightarrow MV$) 0.2 mM DCPIP, 2 mM ascorbate, 0.4 mM MV, 0.8 mM NaN_3 and 15 μl DCMU. Samples were uncoupled using 10 mM NH_4Cl.

transport from H_2O to MV indicating that the site of inhibition of PS II activity, as under heat-stress conditions [8], lies close to the reaction centre of PS II rather than at the level of the oxygen evolution system.

3.3. *Linolenic acid studies*

Incubation of chloroplasts in the presence of phospholipase A_2 leads to the formation of lysophosphatides and the release of free fatty acids. Addition of free fatty acids, particularly linolenic acid, to isolated chloroplasts is known to lead to the inhibition of PS II activity [19]. In order to test whether or not the structural changes observed in our lipase-treated samples were associated with free fatty acid release, we investigated the effect of added linolenic acid ($10\ \mu g – 10\ mg \cdot mg\ chl^{-1}$) on chloroplast structure. Whilst addition of increasing concentrations of linolenic acid led to a progressive destacking and finally a vesiculation of the thylakoid membranes,

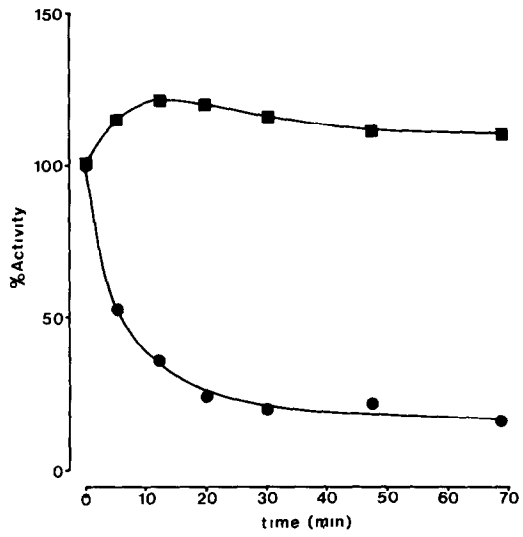

Fig.4. Relative rates of whole chain (●) and PS I-mediated (■) electron transport by isolated pea chloroplasts assayed at 25°C after exposure for an increasing length of time to bee venom phospholipase A_2 (10 Sigma units · mg chl^{-1}). Reaction media as described in the caption to fig.2.

no non-bilayer lipid phase separations were observed, suggesting that the structural changes seen in treatment with phospholipase A_2 are unlikely to be a direct consequence of free fatty acid release.

4. DISCUSSION

Non-bilayer lipid phase separations do not normally occur in native chloroplast membranes, presumably because of interactions of the non-bilayer forming lipids with other membrane components that keep them in a bilayer configuration. Exposure of thylakoid membranes to conditions known to favour non-bilayer phase separations in model systems might be expected to overcome these constraints and indeed there is evidence for this in the case of heat-stressed chloroplasts [8]. We show here that lowering the pH of the chloroplast suspension medium has a similar effect (fig.1a,b) although it is not clear at this stage whether this is due to the neutralisation of acidic lipids, as is the case in model systems [7], or charge-shielding effects on membrane proteins. Note that the isoelectric point of thylakoid membranes is reported to be about pH 4.5 [20–24], which corresponds closely to the upper limit of the range in which phase separations are observed. The factors governing the phase separation seen in the thylakoid membranes of phospholipase A_2-treated chloroplasts (fig.2a,b) are also not fully clear. Control experiments indicate that it is unlikely to be associated with the free fatty acid release. Analogy with model systems [7] would suggest that it is probably the loss of the negatively charged phosphatidylglycerol molecules that is of importance.

It is clear from the present study that thylakoid stacking plays a major role in the formation of the large non-bilayer aggregates seen in stressed membranes. Their formation is particularly favoured in chloroplasts suspended in low pH media conditions, where stacking will occur even at low concentrations of divalent and monovalent cations [16,17], whilst no non-bilayer lipid aggregates are found in chloroplasts treated with phospholipase A_2 under conditions in which stacking is prevented. This suggests that the formation of such structures is dependent on the existence of two or more bilayers containing 'destabilised' non-bilayer lipids in close proximity to each other. Steponkus et al. [25], working with freeze-stressed protoplasts, have come to a similar conclusion. Failure to observe such aggregates, it should be emphasised, does not necessarily imply the absence of any phase separation of non-bilayer lipids in unstacked membranes. It is quite possible that phase separations are occurring which involve smaller, more transient, non-bilayer structures.

An interesting feature of the results presented here is that the treatments that induce non-bilayer lipid separations also result in a loss of PS II activity. A similar correlation was observed for heat-stressed chloroplasts [8]. Measurements using diphenylcarbazide as an alternative electron donor indicate that this lesion occurs close to the PS II reaction centre. In the case of heat treatment there is clear evidence of a physical dissociation of the light-harvesting antennae and core complexes of PS II [26]; the possibility that similar rearrangements are occurring following exposure to pH and phospholipase digestion is currently being investigated.

Selective attack by lipases has been widely employed as a method of investigating both the

disposition and specific roles of phospholipids and galactolipids in the thylakoid membrane [27–37]. The assumptions underlying these studies are that (i) membrane bilayer integrity remains intact, (ii) the lipases do not have ready access to substrates on the inner leaflet of closed vesicles and (iii) changes in function associated with the hydrolysis of a particular lipid class implies a direct functional requirement for that lipid. Our results clearly demonstrate that changes in one lipid class can result in a massive reorganisation of membrane constituents not directly affected by the original perturbing agent. This casts considerable doubt on the validity of some, or all, of these assumptions. A re-appraisal of the significance of many of these studies may, therefore, be necessary.

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