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STRUCTURAL REORGANISATION OF CHLOROPLAST THYLAKOID MEMBRANES IN RESPONSE TO HEAT-STRESS

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Chloroplasts isolated from broad bean (*Vicia faba*) show major structural reorganisations on heating to temperatures above 35°C. Exposure to increasing temperatures in the range 35–45°C for 5 min, leads to a progressive destacking of the chloroplast membranes and the replacement of the normal granal arrangement by modified thylakoid attachment sites. An analysis of the size and packing densities of the freeze-fracture particles present in different membrane fracture-faces suggests that this rearrangement reflects the dissociation of the light-harvesting units of Photosystem II. The antennae complexes of Photosystem II appear to cluster together, maintaining regions of membrane adhesion, whilst excluding the core-complexes of Photosystem II and light-harvesting units of Photosystem I from these regions. If the chloroplasts are heated to higher temperatures, 45–55°C, phase-separated aggregates of non-bilayer-forming lipids are often observed. The release of these lipids from their normal constraints within the bilayer is consistent with the idea that they play a role in the packaging of the light-harvesting complexes within the thylakoid membrane.

Introduction

When leaves or isolated chloroplasts are gently heated, or exposed for short periods to higher temperatures, their photosynthetic apparatus shows characteristic temperature-dependent damage. Irreversible inhibitions of light-limited and light-saturated CO_2 fixation, photophosphorylation and PS II-mediated electron transport are observed [1]; at the same time, PS I-mediated electron transport rates tend to increase [2,3]. These changes which are accompanied by an increase in fluorescence yield of chlorophyll *a* associated with PS II [3–7], usually occur over the range 35–45 °C. The precise threshold temperatures for these effects, however, vary both with the plant species and growth conditions. Desert shrubs and other chilling-sensitive plants normally show appreciably higher threshold temperatures than plants from temperate habitats and chilling-resistant plants [1]. Adaptation of a given plant species to growth at higher temperatures also reduces its susceptibility to heat-induced damage [3,35].

Despite the many studies performed on the effect of heat treatment on the function of chloroplasts, little information is available regarding the structural changes associated with heat-induced damage. Armond et al. [8] have reported that chloroplasts isolated from leaves of *Nerium oleander* show reduced grana stacking on heating.

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Abbreviations: PS I, Photosystem I; PS II, Photosystem II; EF_s, EF_u, stacked and unstacked exoplasmic faces; PF_s, PF_u, stacked and unstacked protoplasmic faces; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

A progressive decrease in the average size of the large freeze-fracture particles observed in the exoplasmic fracture faces of the thylakoid membranes, with increasing pretreatment temperature over the range 40–55 °C, also occurs. We hve shown that similar changes in structure take place following the incubation of chloroplasts isolated from broad bean (*Vicia faba*) at temperatures between 35 and 45 °C [9]. A second reorganisation, involving a phase-separation of non-bilayer-forming lipids from the bulk phase often occurs in this latter system if the chloroplasts are incubated at temperatures above about 45–50 °C.

A number of studies [3,4,6] have demonstrated that the inhibition of PS II activity, and the accompanying increase in fluorescence yield, are associated with a functional dissociation of the chlorophyll *a/b* light-harvesting complex from the main reaction-centre complex of PS II. Grana stacking is believed to be mediated via the chlorophyll *a/b* light-harvesting protein [10.11]. The fact that losses in stacking and the reduction in size of the large freeze-fracture particles found in the exoplasmic fracture faces of stacked chloroplasts take place over very similar temperature ranges [8,9] strongly suggests that heat-stress leads to a direct physical dissociation of the light-harvesting apparatus of PS II. In this paper, we provide further details of this dissociation process and suggest how the observed changes in membrane organisation might be accounted for in terms of temperature-dependent changes in lipid-protein interactions within the thylakoid membrane.

Materials and Methods

Chloroplast isolation. Chloroplasts were isolated from freshly harvested leaf tissue of 4–5-week post-emergent broad beans (*V. faba* L. var. Express) according to the method of Stokes and Walker [12].

Heat treatment. Aliquots of 1 ml of chloroplasts (approx. 50 μg/ml chlorophyll) suspended in a medium containing 0.33 M sorbitol, 5 mM $MgCl_2$, buffered to pH 7.6 with 50 mM Hepes were incubated at different temperatures for 5 min. The samples were then cooled rapidly to 25 °C and prepared for electron microscopy.

Electron microscopy. Heat-treated chloroplast suspensions were examined by freeze-fracture and thin-section electron microscopy. Samples for freeze-fracture were first centrifuged to form pellets. These pellets were then resuspended in fresh medium containing 30% (w/w) glycerol. The samples were thermally quenched from 20 °C using a slurry of liquid and solid N_2 and fractured at −115 °C in a Polaron freeze-fracture device. Replicas were prepared by shadowing with platinum and carbon and cleaned by treatment with bleach. Samples for thin-sectioning were fixed in 4% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4). The sections were prepared in the usual way and stained with uranyl acetate and lead citrate. All samples were examined using a Philips EM 301G electron microscope.

Particle size and density estimations. Freeze-fracture particle sizes were estimated from high-magnification (180 000 ×) prints. In each case, 300 particles were measured. The diameter of the particles was measured at right angles to the shadowing direction and expressed as mean diameter plus or minus the standard deviation of the sample. Particle densities were estimated from similar prints. Ten or more separate areas, each corresponding to about 0.05–0.10 μm^2 were measured in the different fracture faces. The reported results represent the pooled measurements of three independent operators. The results obtained by the individual operators showed similar average values and standard deviations in all cases.

Results

Electron microscopy

Freeze-fracture studies

Incubation of isolated broad-bean chloroplasts at temperatures above 35 °C for 5 min, leads to marked changes in chloroplast membrane organisation. Two distinct stages in this reorganisation can be identified using freeze-fracture techniques. The first of these, occurring in the range 35–45 °C, involves a loss of grana stacking and the formation of modified thylakoid attachment sites. The second, occurring at temperatures above 45 °C, involves a general vesiculation of the thylakoid membranes. Phase-separation of non-bilayer-forming membrane lipids to form cylindrical inverted

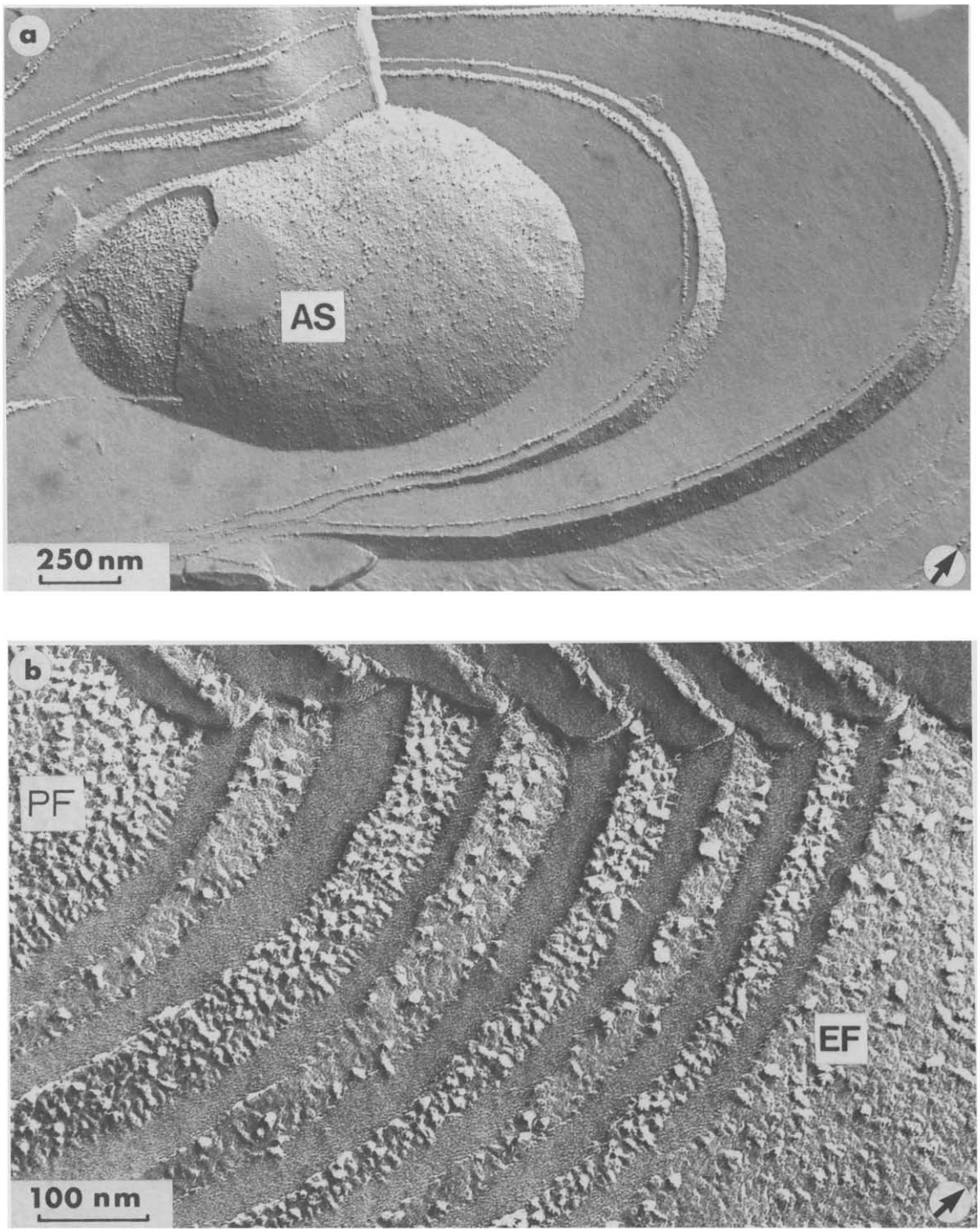

Fig. 1. Typical freeze-fracture electron micrographs showing the thylakoid organisation of chloroplasts incubated at 45 °C for 5 min and then thermally quenched from 20 °C. (a) Concentric arrangements of apposed thylakoid membranes and the attachment sites (AS) existing between the thylakoids. (b) A cross-fracture through a number of thylakoid membranes revealing alternating exoplasmic (EF) and protoplasmic (PF) fracture faces.

lipid micelles often occurs at this stage. Electron micrographs illustrating these changes are presented in Figs. 1, 2 and 5.

As the incubation temperature is raised between 35 and 45 °C, the chloroplast membranes show a rapidly increasing tendency to destack. Viewed in cross-section (Fig. 1a), they show a concentric appearance with the apposed membranes of the thylakoids arranged in pairs. Cross-fractures through these membranes (Fig. 1b) reveal alternate exoplasmic (EF) and protoplasmic (PF) faces. The two faces, as detailed below, are readily distinguished by the much greater density of freeze-fracture particles present in the PF faces. Although grana stacking is largely abolished on incubating the chloroplasts for 5 min at 45 °C or more, limited attachment sites between thylakoids still persist. These sites are much more widely dispersed throughout the chloroplasts than the original attachment sites associated with grana stacks. They are also appreciably smaller in area; typically 250–300 nm or less in diameter as opposed to 300–400 nm in stacked chloroplasts.

The size and distribution of the particles seen in the EF and PF faces of the heat-treated chloroplasts are very different from those of the corresponding faces of unheated controls (Table I). The most striking difference is the almost complete absence of intra membranous particles in the EF faces of the attachment sites of the heated samples (Fig. 2b). This contrasts strongly with the situation in the unheated controls where the stacked EF_s faces contain large numbers (approx. 1250 particles/μm^2) of intramembranous particles. Outside the attachment sites, the situation is reversed with the EF faces of the heated samples containing much higher numbers of particles than the unstacked EF_u faces of the controls (approx. 820 particles/μm^2 as opposed to about 350 particles/μm^2). The average diameter of the particles in both the EF faces of the heat-treated chloroplasts and the EF_u faces of the controls is about 9 nm. The larger particles, with an average diameter of about 11 nm, that were originally present in the EF_s faces of the controls are apparently excluded from the attachment sites of the heated chloroplasts and shift out into unattached regions of the membrane. At the same time, they appear to undergo a marked reduction in size. This decrease in particle size, as illustrated in the particle-size histograms presented in Fig. 3, reflects a sharp increase in the population of particles with diameters centred about 7–8 nm and a corresponding decrease in the number of particles with diameters around 10–15 nm. It is not clear, however, whether this is due to a genuine reduction in size of the intramembranous complexes giving rise to these particles or an apparent reduction reflecting differences in the way that these complexes are oriented within the membrane with respect to the different fracture faces.

In contrast to the EF faces, the PF faces of the attachment sites in the heat-treated samples contain high densities of particles (Fig. 2c). The small size and close packing of these particles make accurate measurement difficult but the average diameter of the intramembranous particles present in the region of the attachment sites appears to be about 7 nm, as opposed to about 9.5 nm in the remainder of the PF fracture face. Comparison of particle-size histograms for the PF_s faces of unheated controls and the PF_s faces of the attachment sites (Fig. 4) indicate an almost total loss of particles with diameters above about 9–10 nm from the attachment site regions. Again, it is not clear whether this reflects a transfer of larger-diameter particles to regions of the PF face outside the attachment sites, a change in particle size or merely a change in apparent particle diameter

TABLE I

AVERAGE DIAMETER AND PACKING DENSITIES OF PARTICLES ON THE FRACTURE FACES OF HEAT-TREATED BROAD-BEAN CHLOROPLASTS

Fracture face	Average diameter (nm) (±S.D.)	Particle density (particles/μm^2) (±S.D.)
Unheated controls (25 °C)		
EF_s	11.2±2.4	1238±163
EF_u	9.3±2.1	343± 59
PF_s	7.8±2.0	4583±322
PF_u	9.2±2.1	3580±263
Heated samples (45 °C)		
EF_s	–	–
EF_u	8.9±3.0	818± 38
PF_s	6.6±1.7	7290±258
PF_u	9.3±2.4	4346±478

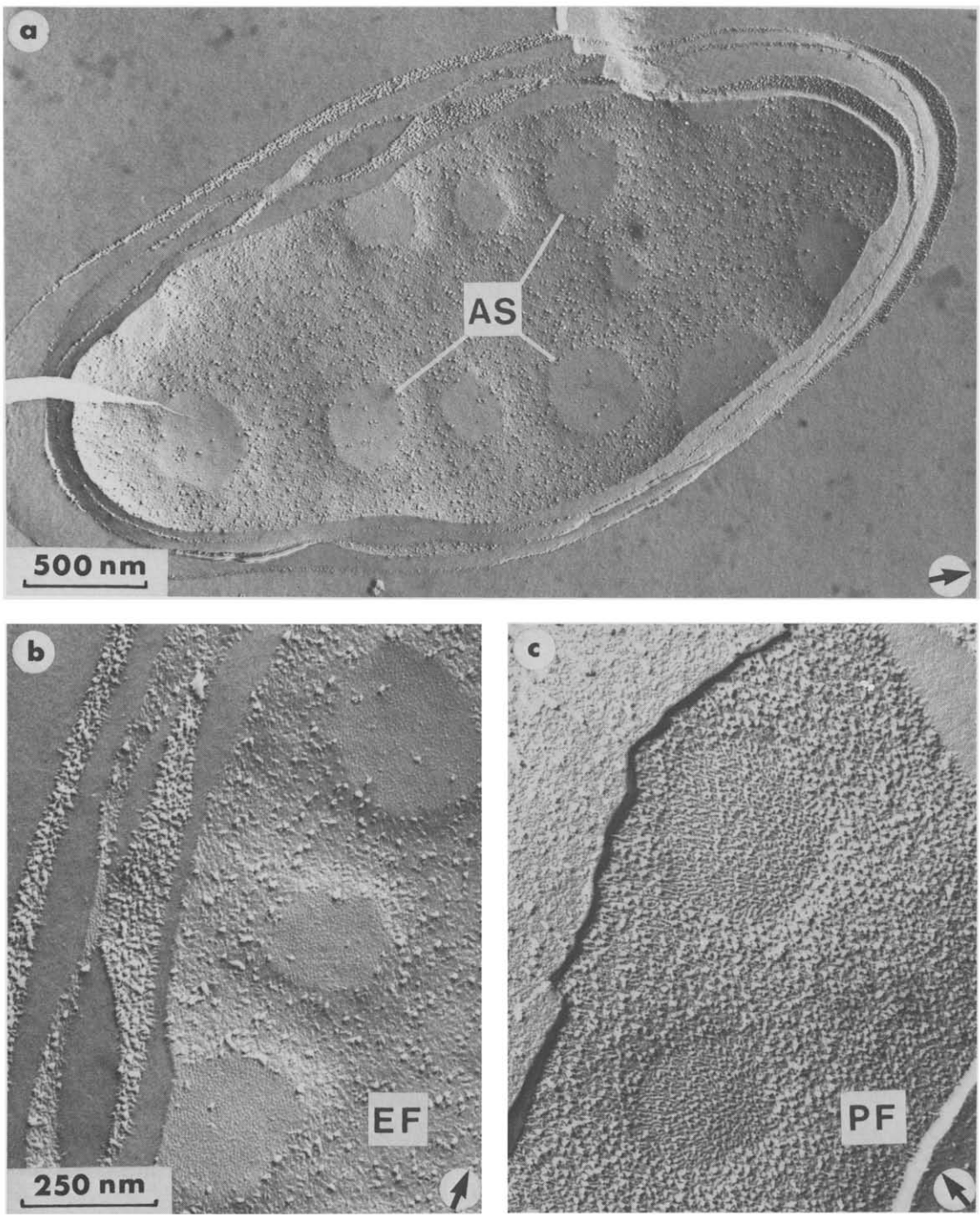

Fig. 2. (a) Freeze-fracture electron micrograph showing a number of the attalchment sites of the type existing in heat-treated chloroplasts (b) and (c) higher magnification views of the EF and PF faces of such sites. Chloroplasts were preincubated at 45 °C for 5 min and thermally quenched from 20 °C.

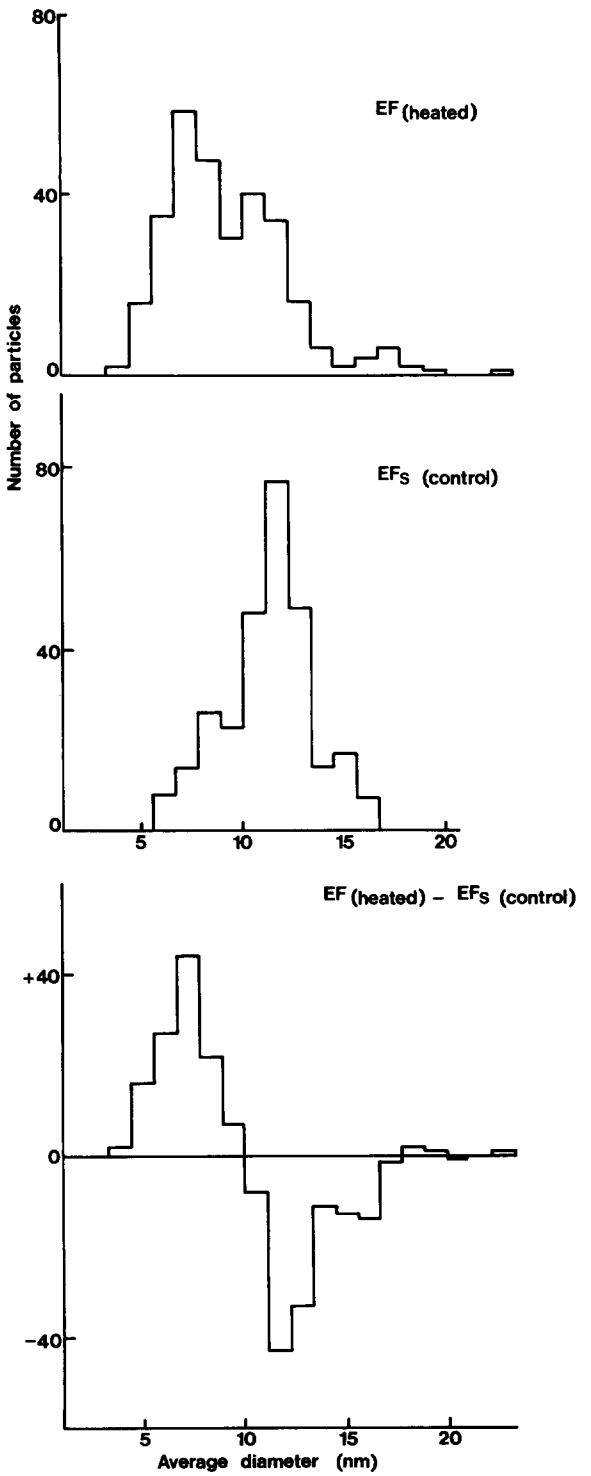

Fig. 3. Particle-size histograms showing the sizes of intramembranous particles found in the exoplasmic fracture faces of heat-treated and unheated control chloroplasts.

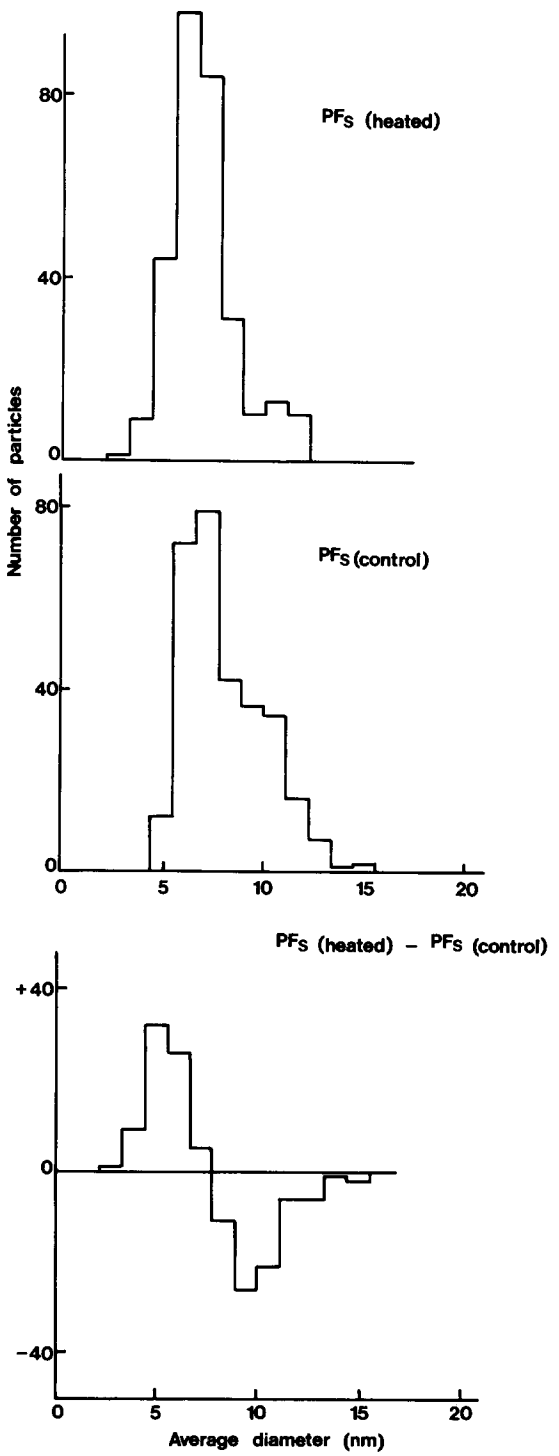

Fig. 4. Particle-size histograms showing the sizes of intramembranous particles found in the protoplasmic fracture faces of heat-treated and unheated control chloroplasts.

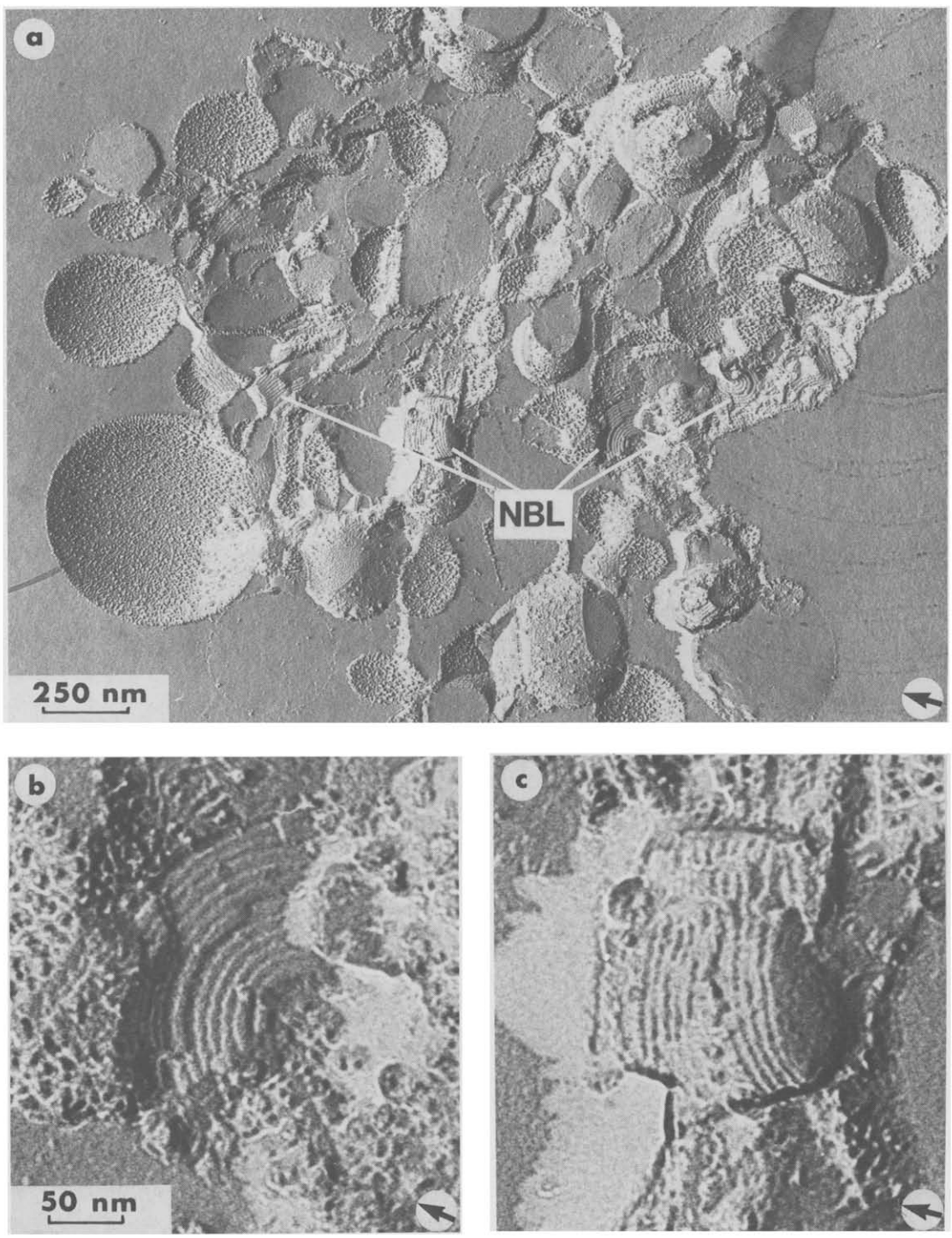

Fig. 5. Freeze-fracture electron micrographs showing (a) the general organisation of broad-bean chloroplasts incubated at 55 °C for 5 min before thermal quenching from 20 °C. Regions of non-bilayer lipid structures (NBL) are clearly visible. (b and c) Higher magnification views of NBL areas showing an aggregate of cylindrical inverted lipid micelles and a stacked bilayer structure in which the micelles are sandwiched between the individual bilayers, respectively.

with a reorientation of the individual particles within the membrane. Corresponding histograms for the unstacked and non-attached regions of the PF faces (not shown) show only minor differences in size distribution of the particles on heating. The relatively small area of the attachment sites with

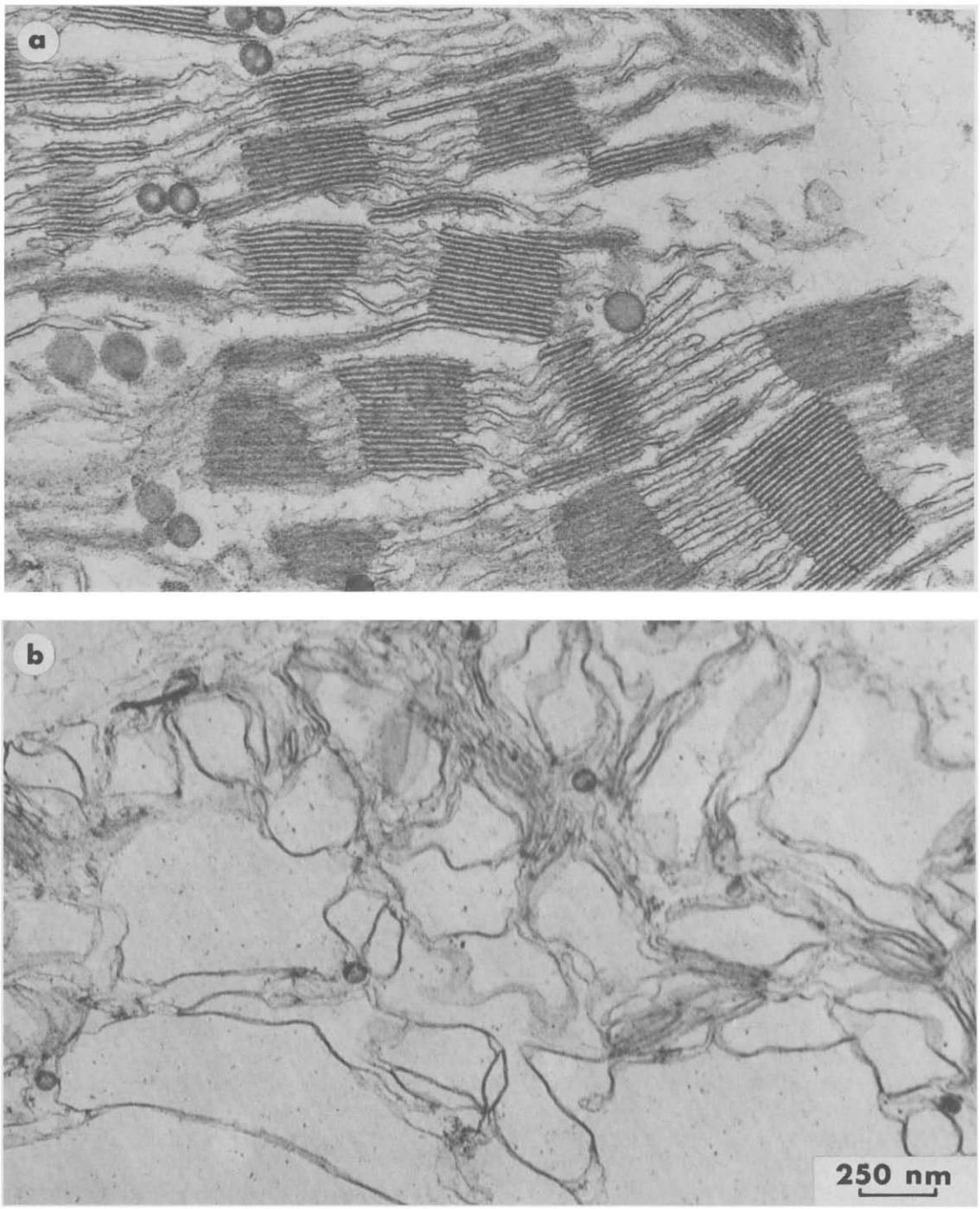

Fig. 6. Typical thin-section electron micrographs of broad-bean chloroplasts incubated at (a) 35 °C and (b) 45 °C for 5 min prior to fixation at 20 °C.

respect to the non-attached regions in the heated samples, and the small proportion (approx. 20%) of particles involved in a possible transfer between them, both mean that any perturbation of the size distribution of the particles in the non-attached regions due to such a transfer is likely to be small. A small increase in the proportion of particles with diameters above 10 nm was observed for the unattached faces on heating but its statistical significance is difficult to assess.

Chloroplasts heated to temperatures above 45 °C undergo further structural changes. The individual fracture faces are still identifiable as EF or PF faces on the basis of the size and densities of their freeze-fracture particles but the normal thylakoid structure is completely lost. The membranes become highly convoluted and appear to break down to form a mass of small fused vesicles (Fig. 5a). A common feature of this second rearrangement is the presence of phase-separated aggregates of cylindrical inverted micelles. These inverted micelles, which are approx. 10 nm in diameter, are very similar in appearance to those seen in freeze-fracture replicas prepared from aqueous dispersions of total polar lipid extracts of chloroplast membranes [13]. In most cases, they are present in the form of three-dimensional aggregates (Fig. 5b), and the individual micelles are clearly distinguishable. In some cases, however, the micelles are sandwiched within lipid bilayers (Fig. 5c) and the aggregates then take on an appearance of a stack of particularly thick bilayers.

Thin-section studies

Electron micrographs of positively stained thin-sections of heat-treated chloroplasts are presented in Fig. 6. Incubation at temperatures below 35 °C led to little or no observable changes in chloroplast ultrastructure (Fig. 6a). Above 35 °C, appreciable disorganisation of the chloroplast membranes takes place but grana stacking is still largely preserved up to about 45 °C. Samples incubated at temperatures greater than 45 °C, in contrast, show little or no evidence of grana stacking (Fig. 6b). The thylakoid membranes for the most part remain apposed but the individual thylakoids are greatly swollen. Incubation at 50–55 °C or above leads to a complete breakdown of the thylakoid membranes to form isolated vesicles. The unstacked but apposed membranes shown in Fig. 6b appear to be the direct counterparts of the concentric membrane systems seen in the freeze-fracture electron micrographs presented in Figs. 1 and 2.

Essentially identical results were obtained if whole leaves were heat-treated by immersion in a water-bath held at the desired temperature and thin sections prepared directly from the leaves or freeze-fracture replicas prepared from chloroplasts isolated from the heat-treated leaves.

Discussion

There is still considerable debate regarding the precise nature of the various intermembraneous particles observed in freeze-fracture replicas of thylakoid membranes. The particles found in the EF and PF faces were initially thought to be associated with PS II and PS I, respectively [14]. Biochemical studies of subchloroplast fractions derived from different thylakoid regions, however, suggest the existence of a lateral heterogeneity in the distribution of the chlorophyll-protein complexes of the two photosystems with PS II complexes preferentially located in the appressed grana partition regions and the PS I complexes restricted mainly to the non-appressed grana-end membranes and margins and the stromal membranes [15].

It is generally accepted that the large freeze-fracture particles found in the EF_s and EF_u fracture-faces reflect the presence of pigment-protein complexes of PS II [16,17]. The origin of the smaller particles seen in the PF faces is, however, less certain. Simpson [18], on the basis of careful analysis of the size and distribution of freeze-fracture particles found in the chloroplasts of wild type and a light-harvesting complex-II deficient barley mutant, has suggested that many of the particles in the PF_s face are associated with light-harvesting complex-II. Support for this view comes from a similar study of particle distribution performed using *Chlamydamonas reinhardii* [19] and from studies showing a movement of PF_s particles from appressed to non-appressed regions of the membrane following the phosphorylation of light harvesting complex-II [20,21]. Further support comes from a study of freeze-fracture particle dis-

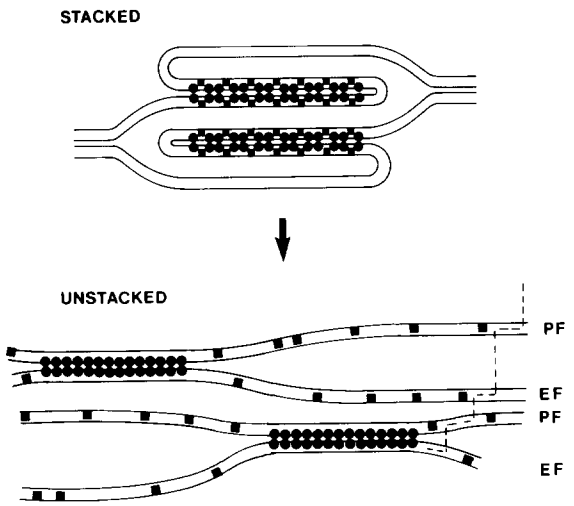

Fig. 7. Diagrammatic model illustrating the effect of physical dissociation of the light-harvesting apparatus on the organisation of heated chloroplasts. Dashed line indicates fracture plane. PS I units are omitted for clarity. (●) Chlorophyll *a/b* light-harvesting protein; (■) PS II core protein.

tribution in a maize mutant lacking PS I activity [22]. This latter study revealed a marked depletion of particles normally found in the PF_u face but little or no change in the size or packing densities of particles in the PF_s faces, suggesting that the particles in the appressed regions are mainly associated with PS II, whilst those associated with PS I complexes are largely restricted to the non-appressed regions of the membrane. In addition to the particles associated with the chlorophyll-protein complexes of PS I, the PF_u faces are thought to contain particles associated with the cytochrome f/b_b complex and the CF_0-CF_1 complex of the coupling factor, but an unequivocal assignment of specific groups of particles in the native membranes to these complexes has not, as yet, proved possible.

The general pattern of heat damage to the ultrastructure of isolated broad-bean chloroplasts resembles that observed by Armond et al. [8] for *N. oleander*. The more marked susceptibility to heat-stress of broad-bean chloroplasts, however, has allowed us to obtain a clearer picture of the individual steps involved in the structural reorganisations associated with this damage. As in the case of *N. oleander*, heating leads to a loss of grana stacking. This loss, which is initiated by exposure to a narrow range of temperature between about 35 and 40 °C, appears to be associated with a dissociation of the supramolecular chlorophyll-protein complexes normally located in the appressed membranes of the grana stacks. This is reflected in a loss of the large intramembranous particles normally observed in the EF_s fracture faces of the thylakoids and the appearance of a new population of somewhat smaller particles in the EF_u faces of the unstacked membranes. These changes are accompanied by a reduction in the contact area of the thylakoids and an increase in the packing density of the particles seen in the PF_s face.

Fluorescence studies [2,4,5] indicate that the ability of chlorophyll *a/b* light-harvesting protein to transfer excitation energy to the core complexes of PS II is lost over a similar temperature range to that associated with these ultrastructural changes. Our results suggest that heat treatment first leads to a physical dissociation of these complexes from the core complex of PS II along the lines proposed by Armond et al. [3]. The chlorophyll *a/b* complexes then appear to cluster together in the PF_s fracture faces maintaining adhesion between the thylakoid membranes at the attachment sites but excluding particles of different origin from both the EF and PF faces of the attachment regions. The spatial coherence of the two systems is thus lost and the membranes take on the appearance of destacked membranes with isolated points of attachment. This process, which is illustrated diagrammatically in Fig. 7, accounts for both the reduced diameter of these regions and the observed changes in particle size and distribution in the EF and PF faces of the heat-treated membranes listed in Table I. The changes occurring in the PF faces are, on this basis, simply reflections of the exclusion of particles other than those associated with the PS II-antennae complexes from the PF_s regions, and those in the EF faces are reflections of the transfer of PS II-core particles from the EF_s to EF_u faces. The reduction in particle size accompanying this latter transfer, it should be emphasised, does not necessarily reflect a change in size of the core complexes. It could equally well be accounted for by a change in the way that the particles are incorporated within the thylakoid membrane.

Membrane adhesion in stacked chloroplasts is believed to be mediated via the chlorophyll *a/b* light-harvesting complexes [23]. Our observation that the small intramembranous particles seen in the PF_s faces cluster into the attachment sites of heat-treated chloroplasts adds support to this idea. It is not clear whether the antennae complexes giving rise to these particles are clustered around the core complexes of PS II, which fracture with the EF_s face, in a random fashion or in a symmetrical arrangement along the lines reported for the light-harvesting unit of the photosynthetic bacterium *Rhodopseudomonas viridis* [24]. Any such symmetry, if it exists, is certainly disrupted by the fracture process. The fact that membrane adhesion is preserved whilst grana-stacking is lost suggests that the formation of the sac-like shape of the individual granum requires a higher degree of interaction between the membrane complexes than is needed for simple membrane adhesion. The nature of these higher-level interactions, which may well involve the core-particles situated in the EF_s faces, is however not yet known.

Functional changes are known to occur in both PS I and PS II activity over the temperature range that heat-induced unstacking occurs in the present study [2,3,9]. The threshold temperatures for both structural [8] and functional changes [3,5] have been reported to be modified by growth temperature and this has led to the suggestion that the lipid matrix of the thylakoid membrane may play some role in the stabilisation of the thylakoid membrane [25]. The present experiments give no direct evidence to support this hypothesis but it is, nevertheless, noteworthy that incubation of chloroplasts at temperatures in the range 45–55 °C lead to the phase-separation of non-bilayer lipids to form aggregates of cylindrical inverted lipid micelles (Fig. 5). If, as has been suggested [26,27], these lipids are normally involved in the packaging of the supra-molecular complexes of the light-harvesting apparatus within the thylakoid membrane, their release from this membrane under conditions in which these complexes dissociate may be of considerable significance.

Acknowledgement

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