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Visualization of contact sites between outer and inner envelope membranes in isolated chloroplasts

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Contact sites between outer and inner envelope membranes of isolated chloroplasts have been studied at the ultrastructural level by two different electron microscopical methods, i.e., freeze fracturing and freeze substitution followed by ultrathin sectioning. Both methods demonstrate that approx. 10% of the chloroplast population exhibits blister-like structures. Cross fractures and ultrathin sections of freeze-substituted chloroplasts reveal that the blisters originated from a separation of the outer and inner envelope membranes. Exposure of isolated chloroplasts to hypertonic conditions results in an almost complete separation of the two envelope membranes, except for small regions in which the two membranes are in close contact. These contact sites are clearly recognized in freeze-fracture replicas by ridges of a high density of intramembrane particles. In addition, cross fractures and thin sections of freeze-substituted chloroplasts demonstrate the presence of small vesicles associated with the outer envelope membrane. As indicated by the opacity of the vesicle and demonstrated by immuno-gold labeling, these vesicles, which originate from the inner envelope membrane, contain stroma-derived proteins.

Introduction

Chloroplasts, as well as mitochondria, are cellular organelles surrounded by two envelope membranes. The two envelope membranes are usually in close proximity, exhibiting specific sites of contact [1,2], which are thought to play an important role in the transport of proteins from the cellular cytoplasm into the chloroplast or mitochondria. Most chloroplast proteins are encoded in the nuclear genome and are synthesized in the cytoplasm as precursor proteins which are post-translationally taken up by chloroplasts (Ref. 3; for reviews, see Refs. 4 and 5). The actual translocation mechanism of precursor proteins across the two envelope membranes is unknown. Each membrane can be crossed separately or, alternatively, translocation can occur at contact sites between the outer and inner membrane. In the mitochondrial system evidence for the role of such contact sites in protein translocation has been presented. Schleyer and Neupert [6] have shown that after binding of precursor proteins at low temperatures processing could take place until the mature size is reached, while a large part of the protein molecule still protruded from the outer membrane. From this result they concluded that import occurs via regions where the outer and

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; PF, protoplasmic fracture; EF, exoplasmic fracture; IMP, intramembrane particle.

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inner membrane are very close to each other. Using immunocytochemical methods, Schwaiger et al. [7] recently showed that these partly translocated precursor proteins are indeed located in areas where both membranes are in close proximity, indicating the involvement of contact sites in protein transport.

The morphology of the chloroplast envelope membranes has already been investigated by Cline et al. [1] using freeze-fracture electron microscopy and they have shown that under hypertonic conditions chloroplast outer and inner membranes are separated except in specific regions which are thought to represent contact sites.

The development of a number of electron microscopical methods in combination with immuno-gold labeling allows, in principle, the visualization and localization of bound precursor proteins to contact sites [7]. Therefore, in this paper we have applied freeze fracture and freeze substitution followed by ultrathin sectioning to improve the visualization of contact sites in chloroplasts, since both methods have been demonstrated to be suitable for coupling with immunogold labeling [8,9] in order to permit a more detailed study of these contact sites in the future.

After isolation, intact pea chloroplasts were ultrarapidly frozen without the use of cryoprotectants or chemical fixatives in order to avoid structural artifacts. Subsequently, chloroplasts were freeze fractured and replicated or alternatively subjected to freeze substitution. The results reported here show that both electron microscopical techniques allow the visualization of contact sites between outer and inner membranes. Furthermore, under hypertonic conditions the existence of inner membrane vesicles associated to the outer membrane was clearly demonstrated. The implications of this for the observation of chloroplast contact sites is discussed.

Materials and Methods

Isolation of chloroplasts. Intact pea chloroplasts were isolated from 9–11-day-old pea seedlings by Percoll (Sigma) gradient centrifugation according to the method of Cline et al. [10]. The isolated chloroplasts were washed and suspended in either an isotonic buffer (50 mM Hepes-KOH/330 mM sorbitol, pH 7.5) or a hypertonic buffer (10 mM Tris-HCl/2 mM EDTA/0.6 M sucrose, pH 7.5) and ultrarapidly frozen in the absence of cryoprotectants in liquid propane using the Reichert KF 80.

Electron microscopy. Frozen chloroplasts were freeze fractured in a Balzers BAF 300 according to standard procedures. The samples were fractured at $-105\,^{\circ}C$ and shadowed with platinum-carbon. The replicas were cleaned by floating them on nitric acid and bleach and were examined in a Philips EM301 electron microscope. Alternatively, frozen chloroplasts were transferred (under liquid nitrogen) into the Cs-auto freeze-substitution unit (Reichert-Jung). Freeze substitution took place at $-90\,^{\circ}C$ in 0.5% glutaraldehyde and 0.5% uranylacetate in methanol for 8 h [11]. After raising the temperature to $-35\,^{\circ}C$ (at a rate of $4\,^{\circ}C$/h) the samples were infiltrated with lowicryl K_4M and polymerized by ultraviolet light at $-35\,^{\circ}C$ for 2 days. After further polymerization at room temperature (2–4 days) ultrathin sections were cut and examined. Sections were immunolabeled with a rabbit antibody against ribulose biphosphate carboxylase (RuBisCo) and protein A-gold complexes as described by Van Bergen en Henegouwen and Leunissen [12]. The sections were stained for 5 min with 6% uranyl acetate and for 1 min with 1% Millonig [13] lead acetate.

Results and Discussion

As shown in Fig. 1a, freeze-fracture replicas of the chloroplast outer membrane, in particular the protoplasmic fracture face (PF face) which is characterized by a relatively low density of intramembrane particles (IMPs), reveal the presence of blister-like structures. These blisters appear in approximately 10% of the chloroplast population and originate from the separation of outer and inner envelopes as described by Cline et al. [1]. It is of interest to mention that the density of IMPs on blister-like structures is significantly lower than that observed in the membrane areas adjacent to the blisters (Fig. 1a), indicating a redistribution of IMPs during blister formation. A cross fracture of a chloroplast shows regions where the inner and outer membranes have been separated. Contact points between both membranes can be seen in

areas where the outer and inner membranes are in close proximity (Fig. 1b and inset).

The presence of contact sites and the appearance of blisters in chloroplasts has been studied in more detail by resuspending the isolated chlrooplasts in hypertonic buffer. Under these conditions the outer and inner membranes are physically separated on account of the permeability properties of the outer membrane for low molecular weight components as described previously [14,15]. As shown in Fig. 2a, the PF face of the outer membrane is composed almost entirely of blister-like structures. Furthermore, the density of IMPs in these blisters is low, comparable to those in blisters occurring in chloroplasts under normal isotonic conditions (Fig. 1a). The blisters, as shown in Fig. 2a, are separated by ridges characterized by a high density of IMP's.

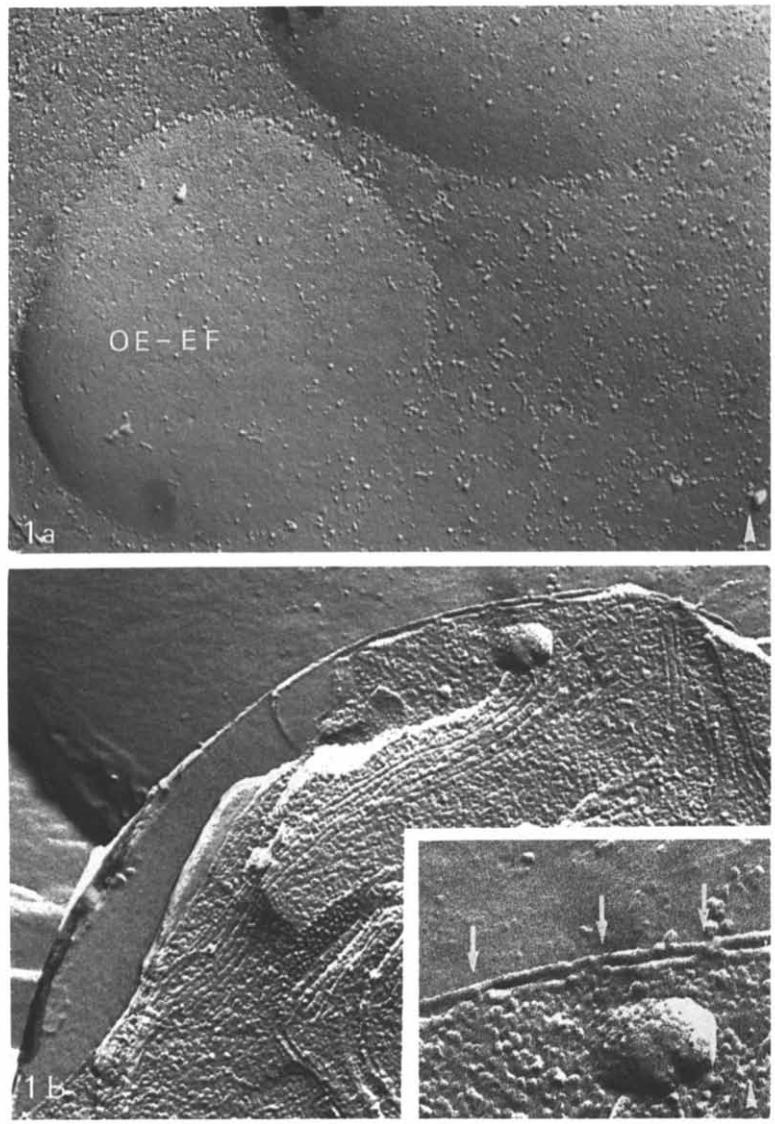

Fig. 1. Electron micrographs of freeze-fracture replicas of ultrarapidly frozen intact pea chloroplasts resuspended in an isotonic buffer. (a) EF-face view of the outer envelope membrane. A blister-like structure is shown (×80000; after reduction: ×56000). (b) A cross fracture of a chloroplast (×80000; after reduction: ×56000). In the inset contact points between the outer and inner membrane are visible.

These ridges most likely represent lines of contact between the outer and inner membrane. In contrast to the PF face of the outer membrane, the exoplasmic fracture (EF) face of the inner membrane appears rather smooth, containing a relatively high density of IMPs, while no blisters or ridges (or grooves) can be identified (Fig. 2a).

Of particular interest is the observation that a cross fracture of regions in which the outer and inner membranes are separated, forming blister-like structures, reveals the presence of small membrane vesicles situated between the two envelope membranes (Fig. 2b). These vesicles apparently have no contact with the inner membrane, but instead are almost exclusively associated to the outer membrane (Fig. 2b). Moreover, the contact

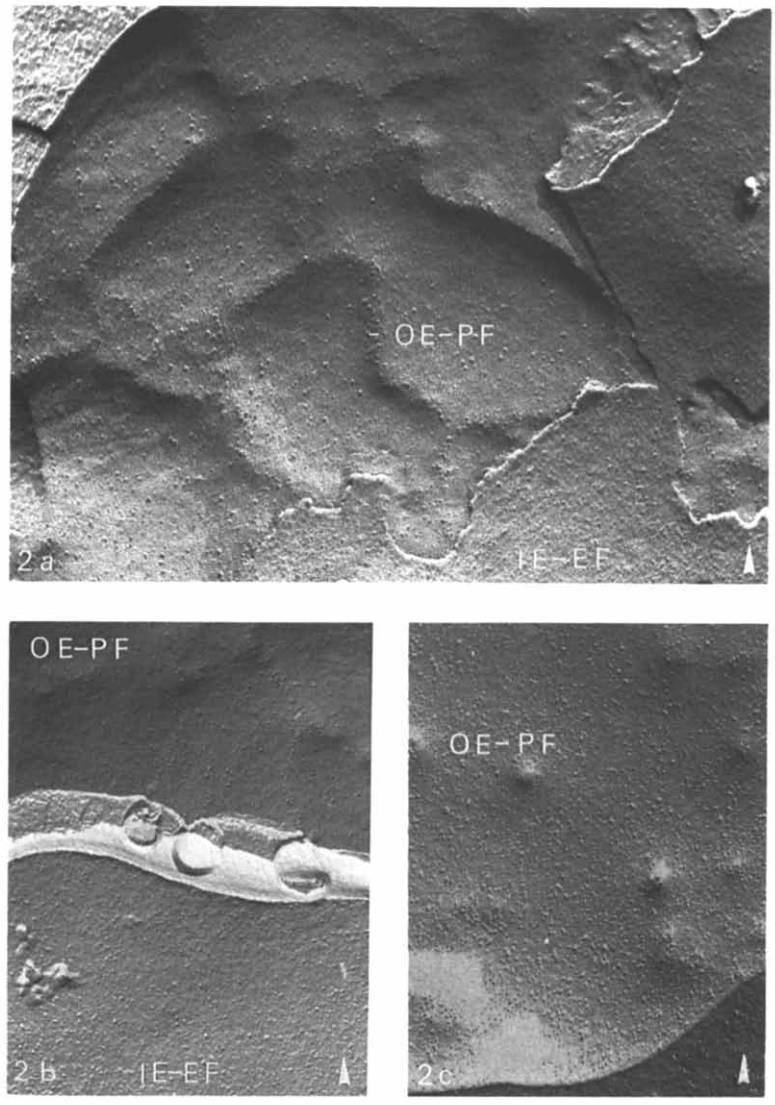

Fig. 2. Freeze-fracture replicas of chloroplasts resuspended in a hypertonic buffer. (a) PF-face view of the outer membrane and EF-face of the inner membrane. On the PF face of the outer membrane, contact lines between the two membranes are visible as grooves. (b) The same faces as in (a). Vesicle-like structures are seen in the space between the two membranes. Vesicles are in contact with the outer membrane. (c) The PF-face view of the outer membrane. Contact points between outer membrane and inner membrane vesicles are visible as pits. (a, b and c: ×80000; after reduction: ×56000).

site between the outer membrane and the membrane vesicle is characterized by a row or ridge of IMPs, extending clearly into the PF face of the outer membrane (Fig. 2b). These observations indicate that the rows or ridges of IMPs in the PF face of the outer membrane probably represent contact lines between this membrane and either vesicles or the inner membrane. In addition to chloroplasts in which the PF face of the outer membrane contains a high density of IMPs organized into lines or ridges, chloroplasts have been observed in which these ridges are not apparent. Instead of ridges, the PF face of the outer membrane of these chloroplasts contains numerous particles and pits (Fig. 2c). These pits or lumps most likely represent contact points between the outer membrane and vesicle-like structures as described above.

In order to obtain a better insight into the origin and nature of the vesicle-like structures and the appearance of contact sites or contact lines that are visualized after freeze fracturing, chloroplasts have been subjected to freeze substitution as described under Materials and Methods. Ultrathin sections of freeze-substituted chloroplasts exhibit a morphology that is similar to that deduced from freeze-fracture studies. Under isotonic conditions most of the chloroplasts demonstrate a morphology where the outer and inner membranes are apparently in close contact (data not shown), since no clear space can be observed between the inner and outer membranes. In accordance with the freeze-fracture resutls, a small population of freeze-substituted chloroplasts contains blister-like structures (Fig. 3a). These observations clearly demonstrate that the blisters originate from a separation of the outer and inner membranes. Freeze substitution of intact pea leaves shows no blister-like structures on the chloroplasts, in spite of ice crystal damage outside the chloroplast (data not shown), suggesting that the blister-like structures seen in isolated chloroplasts are the result of isolation procedures.

Hypertonic treatment of chloroplasts results in a separation of outer and inner membranes along almost the entire surface of the chloroplast (Fig. 3b). These thin sections demonstrate the presence of contact sites between the outer and inner membranes of hypertonically treated chloroplasts. These sites of contact are most likely the ridges with a high IMP density observed in the PF face of the outer membrane (Fig. 2a) using freeze-fracture techniques. In addition, thin sections of hypertonically treated chloroplasts demonstrate the presence of small membrane vesicles that are associated with the outer membrane, but not with the inner membrane (Fig. 3c), in accordance with the freeze-fracture observations. The diameter of the vesicles appears to vary between 0.1 and 0.3 μm and corresponds well with that observed in freeze-fracture replicas.

As clearly demonstrated in Fig. 3c, the density of the membrane vesicles is comparable to that of the stroma of the chloroplast, suggesting that the vesicle content originates from the stroma and, correspondingly, the vesicle membrane will be largely composed of the inner chloroplast membrane. The nature of the vesicle contents has been identified by immunocytochemical methods. After sectioning of freeze-substituted chloroplasts, the sections were incubated with an antibody directed against rubisco and protein A-gold complex as described under Materials and Methods. As shown in Fig. 4, the stroma of the chloroplast is heavily labeled with colloidal gold particles, demonstrating the presence of rubisco in the stroma. In addition, the contents of the vesicles are also densily labeled, demonstrating that the vesicles indeed contain stroma. Treatment of freeze substitution sections with protein A-gold complex alone, without anti-rubisco antibody, caused no labeling either at the stroma or at the inner membrane vesicles (data not shown).

From these observations it can be concluded that despite the forced separation of the two chloroplast envelopes, caused by the shrinking of the inner envelope under hypertonic conditions, the contact points between the two membranes remain intact. When the stress upon the inner envelope membrane becomes excessive fragments of the membrane sheer off, giving rise to the observed stroma-containing vesicles. This would certainly explain the difficulty in obtaining a pure inner envelope membrane fraction after physical disruption and density gradient purification [16–18]. It seems likely that, during biochemical isolation, double membrane vesicles are formed from outer envelope membranes wrapping around

these inner envelope vesicles and, because of the high protein density in the inner membrane, these vesicles co-migrate with inner envelope fractions during centrifugation. This agrees well with the freeze-fracture observations of Cline et al. [1]. Such contamination during the purification of paired membranes is also seen in mitochondrial and Gram-negative bacteria [19,20]; furthermore, for mitochondria it is possible to isolate the contact sites after osmotic lysis and density gradient centrifugation [21].

Little is known about the nature of the contact points between the two chloroplast envelope membranes. The morphology of the particles observed on freeze-fracture replicas would suggest that these IMPs are probably proteins. Such a protein could

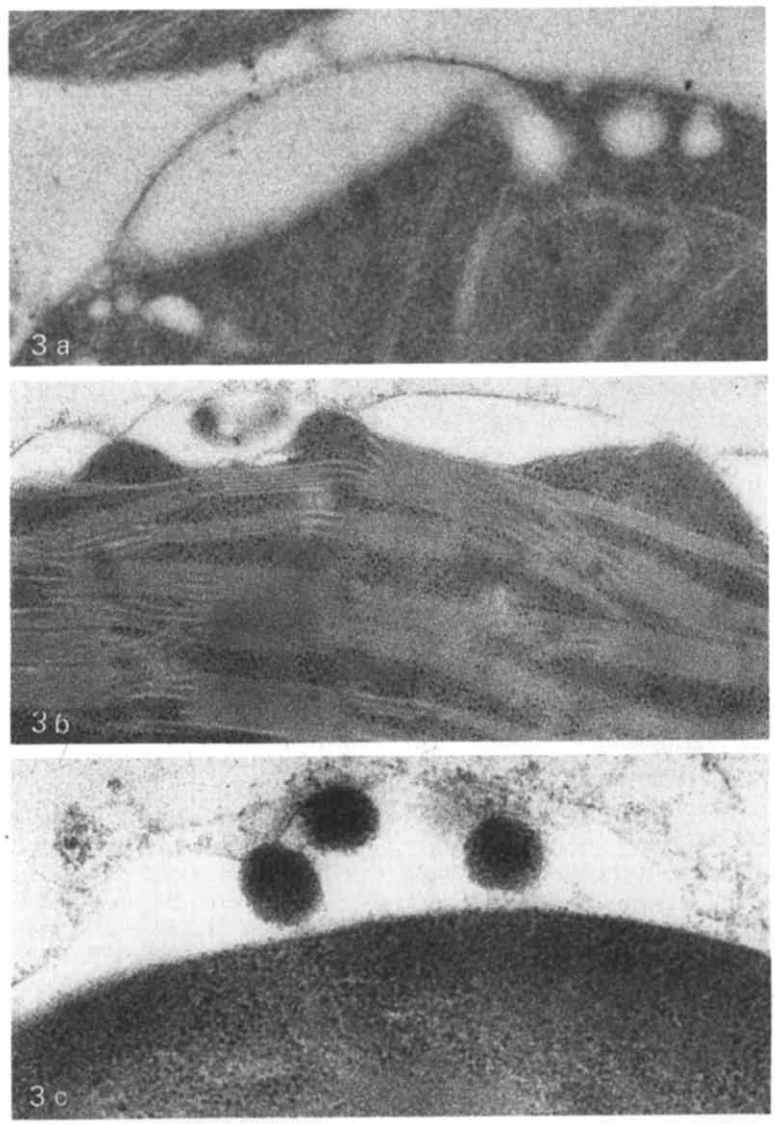

Fig. 3. Ultrathin sections of freeze-substituted chloroplasts. (a) Under isotonic conditions some chloroplats show blister-like structures (×60000; after reduction: ×42000). (b) Under hypertonic conditions the two membranes are separated over practically the whole surface except on the contact sites (×50000; after reduction: ×35000). (c) Under hypertonic conditions inner membrane vesicles are formed on the contact sites. These vesicles are in direct contact with the outer membrane (×75000; after reduction: ×52500).

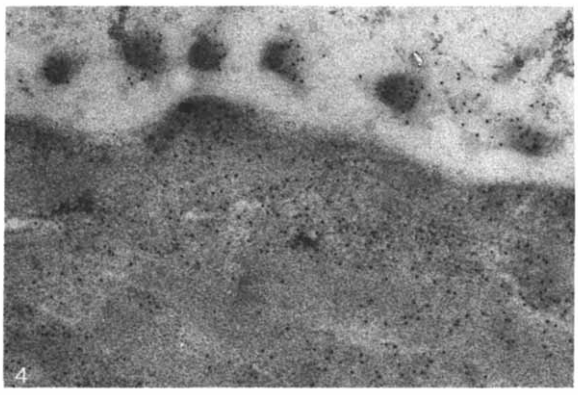

Fig. 4. Freeze-substitution sections of hypertonically treated chloroplasts are labeled with an antibody against rubisco and protein A-gold complexes. The inner membrane vesicles show the same labeling as that found in the stroma (×80000; after reduction: ×43000).

be composed of a single complex spanning both envelope membranes or of two protein molecules, one in each envelope membrane, firmly bound together. For mitochondria it has been suggested that the membranes in the contact points are fused or connected by hydrophobic materials [2]. An interpretation like that has been presented by Van Venetië and Verkleij [22], who proposed a semifusion model for the contact sites in which non-bilayer lipids are involved.

In conclusion, we have demonstrated the presence of contact sites between inner and outer envelope membranes in chloroplasts using two different electron microscopical methods, i.e., freeze fracture and freeze substitution followed by ultrathin sectioning. The combination of these two electron microscopical techniques has provided a better insight into the nature of these sites and has allowed us to explain the reason why it is difficult to obtain pure fractions of chloroplast inner envelope membranes. Central to this is the permanence of these contact sites between the two chloroplast envelopes.

Acknowledgements

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