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Immunocytochemical Localization of Photosystems I and II in the Green Alga *Tetraselmis subcordiformis*

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Canada

Localization of Photosystems I and II in a green alga

Abstract

The distribution of photosystem I (PS I) and photosystem II (PS II) in a primitive green alga *Tetraselmis subcordiformis*, which belongs to Prasinophyceae and does not have grana in its chloroplast, was studied by immunoelectron microscopy. Two PS I antibodies were used: one against a PS I component of maize, the other against the 60 and 62 KDa PS I reaction centre proteins of the cyanobacterium *Synechococcus elongatus*. Both antibodies showed that 76-78% of the labelling is on the appressed thylakoid membranes and only 22-24% is located on the unappressed membranes. Use of antiserum against cp-47 of PS II from *S. elongatus* also gives 76% of the labelling on appressed thylakoid membranes and 24% on unappressed thylakoid membranes. Cytochemical detection of PS I activity by the photooxidation of 3,3'-diaminobenzidine and of PS II activity by the photoreduction of distyryl nitroblue tetrazolium chloride also revealed that PS I and PS II activities exist on both types of thylakoid membranes. Therefore, our results indicate that the distribution of PS I and PS II in green algae may differ from that in higher plants.

Résumé

La distribution des photosystèmes I et II (PS I, PS II) dans l'algue verte primitive *Tetraselmis subcordiformis* a été étudiée par une méthode immunologique de detection couplée à de la microscopie électronique. Dans cette algue, une prasinophyte, les thylacoïdes ne sont pas organisés en grana.

Deux anticorps différents ont été utilisés pour identifier le photosystème I de cette algue: un dirigé contre une des particules de PS I de maïs et l'autre contre les protéines de 60 et 62 KDa de la cyanobactérie *Synechococcus elongatus*. Les deux anticorps ont montré une distribution similaire, 76-78% du marquage est présent dans les membranes juxtaposées des thylacoïdes et seulement 22-24% dans les non-juxtaposées.

De même, en utilisant un anticorps dirigé contre la protéine cp47 du PS II, le marquage est aussi de 76% dans les membranes juxtaposées et 24% dans les non-juxtaposées.

La présence de l'activité de PS I et PS II dans les deux différents types de membranes thylacoïdales a aussi été démontrée par la photo-oxidation du 3,3'-diaminobenzidine et la photoréduction du distyryl nitrobleu chlorure de tétrazolium respectivement.

Ainsi, nos résultats indiquent que la distribution de PS I et PS II peut être différente dans les algues vertes et les plantes supérieures.

Contributions to original knowledge

This investigation has demonstrated for the first time that photosystem I and photosystem II in the primitive green alga *Tetraselmis subcordiformis* are located on both appressed and unappressed thylakoid membranes. These results may indicate that the heterogeneous distribution of PS I and PS II seen in higher plants may not have evolved in primitive green algae which do not possess grana in their chloroplasts. Thus from an evolutionary point of view, one question has arisen; when did the heterogeneous distribution of PS I and PS II seen in higher plants develop? Or, in other words, what kind of thylakoid structure supports this heterogeneous distribution?

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My thanks firstly and especially go to my supervisor Sarah P. Gibbs, not only for her patient and strict guidance during this investigation and during the writing of this thesis, but also for her great encouragement which I appreciate more than I can express. When I first arrived in Canada, English was my big problem, and I needed to learn it very quickly; five months after I arrived in her lab, I became pregnant and had complications which forced me to lie in bed for at least two months; later, my son was born, who took a lot of time to care for. It is Dr. Gibbs who patiently encouraged me to overcome these three challenges. Without her, I might not have finished this study.

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appressed membrane

membranes of adjacent thylakoids adhering together

BSA bovine serum albumin

cp1-e 60-62 KDa PS I reaction centre protein

cp29 PS II reaction centre protein

cp47 47 KDa PS II reaction centre protein

cytochrome b_6/f complex

a complex molecules in electron chain between PS I and PS II

DAB 3.3'-diaminobenzidine, an artificial electron donor in PS I chain

DS-NBT distyryl nitroblue tetrazolium chloride, a PS II activity inhibitor

EFs stacked thylakoid membrane bordering the lumen

EFu unstacked thylakoid membrane bordering the lumen

LHC-I light harvesting complex I

LHC-II light harvesting complex II

P680 a special pair of chlorophyll molecules in PS II reaction centre

P700 a special pair of chlorophyll molecules in PS I reaction centre

PBS phosphate buffered saline (PH 7.2-7.4)

PFs stacked thylakoid membrane bordering the adjacent membrane

PFu unstacked thylakoid membrane bordering the chloroplast stroma

unappressed membrane

membranes including stroma-exposed thylakoid membrane, outer most membrane of grana and the edges of grana

Chapter I Literature Review

In higher plants and green algae, there are four supramolecular complexes which are involved in photosynthesis (Staehelin 1986). These four complexes are: 1. photosystem I (PS I), which includes the PS I reaction centre containing two molecules of P700, a light-harvesting complex I containing chlorophyll *a/b* proteins (LHC I) and electron transfer intermediates which transfer electrons from PSI to $NADP^+$ to form NADPH; 2. photosystem II (PS II), which includes the PS II reaction centre containing two molecules of P680, a light-harvesting complex II containing chlorophyll *a/b* proteins (LHC II) and several intermediates in the electron transfer chain (electrons from the PSII reaction centre pass through a series of intermediates and then go to the PSI reaction centre); 3. the cytochrome b_6/f complex, which is the main intermediate in electron flow between PS II and PS I; 4. ATP synthetase, which forms ATP from ADP using the energy gained by discharge of the H^+-gradient formed across the thylakoid membrane by the electron transfer chain or by the cyclic pathway which involves PS I.

The pathway of electron flow in photosynthesis is called the Z scheme (see Fig.1). The water-splitting enzyme, which lies under the PS II reaction centre and projects into the thylakoid lumen, catalyses the photolysis of water into oxygen, H^+ ions and electrons. The resulting electrons fill the "electron holes" in the PS II reaction centre chlorophylls, that occur when these are converted to $P680^+$. Light energy, *i.e.* photons absorbed by LHC II, causes excitation of chlorophyll in the LHC II antenna complex, this excitation is subsequently transferred to a special pair of chlorophyll molecules, called P680, in the

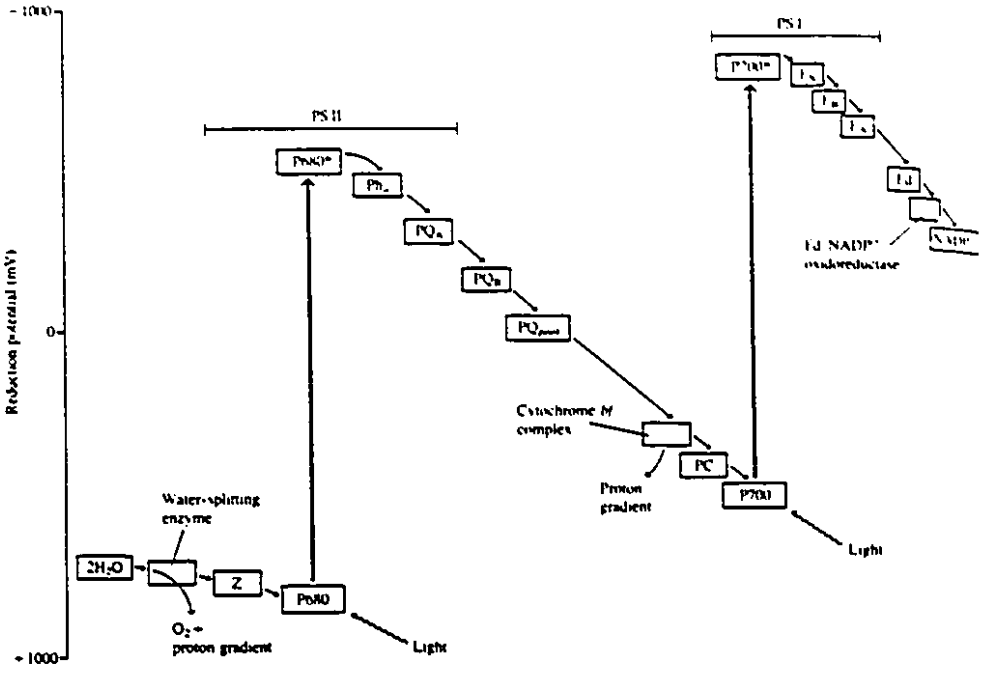


Fig. 1. Z scheme. The water-splitting enzyme catalyses the photolysis of water into oxygen, H^+ ions, and electrons which are transferred to $P680^+$. The two P680 molecules in the PS II reaction centre are changed to an excited state by the absorption of photons and donate an electron to Ph_a, this electron will then be passed through the electron chain, PQ_A, PQ_B, PQ pool, Cytochrome b_6/f complex, PC, and a special pair of chlorophyll molecules in PS I reaction centre which have each lost an electron and are called $P700^+$. P700 is also changed to an excited state in the same way as P680 and passes an electron to F_X, F_B, F_A, Fd and $NADP^+$. Pha, pheophytin; PQ_A and PQ_B, plastoquinone molecules; PQ pool, mobile plastoquinone molecules; PC, plastocyanin; F_X, F_B, and F_A, Fe-S electron transport proteins; Fd, ferredoxin. (Fig. 18.9 in Rawn 1989).

PS II reaction centre. As a result, P680 is changed to an excited state ($P680^*$), which donates an electron to pheophytin a and becomes $P680^+$. The excited electron in pheophytin *a* passes through a series of intermediates to the cytochrome b_6/f complex, plastocyanin and ultimately to the electron holes in the PS I reaction centre. Photons also raise electrons in the chlorophyll molecules in PS I (P700) to the excited state; these electrons then pass through a series of intermediates to NADP+ to form NADPH.

All four of these supramolecular complexes are located on the thylakoid membranes of the chloroplast. In higher plants and in some green algae, the thylakoid membranes are composed of stroma-exposed thylakoid membranes and grana thylakoid membranes, which are formed by adjacent thylakoid membranes stacking together. Grana thylakoid membranes consist of the end region (outermost membrane of grana), the margin region (edges of grana thylakoids), and the partition region (membranes of adjacent thylakoids adhering together). The partition regions are also called appressed membranes, whereas the end and margin regions belong to unappressed membranes. Therefore, grana thylakoid membranes contain both appressed membranes and unappressed membranes. However, stroma-exposed thylakoid membranes contain only unappressed membranes. The concept of unappressed and appressed membranes is very important for the localization of the photosynthetic complexes, PS I and PS II, which is the main topic in the following discussion.

1.1 Photosystem I

Over the past twenty to thirty years, the localization of PS I has been studied using a variety of research methods. However, most research has been done on higher plants, while very few studies have been performed on green algae. Only *Chlamydomonas* has been studied by immunogold electron microscopy. The currently favoured model for the localization of PS I, proposed by Anderson and Andersson (1982), is that PS I is located exclusively on unappressed membranes, although there are some studies which do not agree with this hypothesis.

The evidence which supports this model first came from biochemical studies. Åkerlund and his co-workers (1976) developed a technique in which Yeda press treatment of spinach thylakoids was used, followed by aqueous polymer two phase partition, to separate thylakoid membranes into appressed membrane vesicle and unappressed membrane vesicle fractions. With this method, Anderson and Melis (1983) observed that P700 photoxidation was seen mainly in the unappressed membrane fraction, while only about 20% was found in the grana fraction. They explained this result by hypothesizing that the 20% of the PS I activity observed in the appressed membrane fraction was due to contamination from unappressed membranes which did not separate from the grana fraction, since there is a similar amount of ATPase coupling factor to be found in grana fractions (Berzborn et al. 1981). ATP synthetase theoretically must be located on unappressed membranes because it needs the H^+-gradient to catalyse its ATP synthesizing function. Appressed membranes cannot provide this H^+-gradient. Therefore, they concluded that PS I resides exclusively on unappressed membranes.

Further support for this model of PS I localization is derived from measurement of particle sizes in freeze-fractured and freeze-etched thylakoid membranes. After freezing, thylakoid membranes can be fractured into four faces (PFs, PFu, EFs, and EFu). In the chloroplast, the EF half of the thylakoid membrane is that bordering the lumen, while the PF half of the membrane borders the chloroplast stroma or in the case of appressed grana membrane, the adjacent membrane. The subscripts s and u refer to stacked and unstacked, respectively. Studies using mutants deficient in various photosynthetic complexes have revealed that PS I particles are correlated with the PFu face. Freeze-fractured PS I particles isolated from thylakoids of pea seedlings and incorporated into phosphatidylcholine vesicles are 10.6 nm in diameter (Mullet et al. 1980). In a barley mutant, *viridis-zb*^{63}, which has completely lost one of the PS I reaction centre proteins, results showed that the density of the large particles (10-13 nm) on the PFu face was greatly reduced, while the density of the small particles on the PFu face and that of the particles on EFs and PFs faces were almost unchanged (Simpson 1983). The maize mutant 1481, which lacks the PS I core protein, and the *C. reinhardtii* mutant F14, which has lost both the PSI primary electron donor P700 and the PSI reaction centre antenna protein, both exhibited similar results (Miller 1980; Olive et al. 1983). These PFu face-associated large particles not only contain PS I core and antenna proteins, but also contain LHC I, since these particles are decreased on the presumed PFu face of the thylakoids of *C. reinhardtii* mutant AC40, which is deficient in part of the LHC I complex (called CPO), while the particles on the EF face and the presumed PFs face showed no significant change (Olive et al. 1983).

Immunogold labelling gives the most direct evidence for Anderson and Andersson's model. The protein in question can be detected by incubation with its antibody followed by incubation with protein A, which has an antibody-binding site and has been conjugated to colloidal gold particles. Vallon et al. (1986) used an antibody against the PSI reaction centre protein CPI to label spinach and *Chlamydomonas reinhardtii* thylakoid membranes, and found that about 84% of labelling was on the unappressed membrane in *C. reinhardtii* and 96% in spinach. Since use of an antibody against the α and ß subunits of CFI (an ATPase coupling factor which should only reside on unappressed membranes) gave a similar labelling pattern, they considered the small amount of labelling on appressed membranes to be nonspecific background. Therefore, they concluded that PSI is located entirely on unappressed membranes. A study of the barley mutant *viridis*-^{115}, which totally lacks PSII activity, using antibodies raised against the PSI reaction centre protein, also showed that PSI was completely located on unappressed thylakoid membranes (with 4.9% of labelling background on appressed membranes) (Simpson et al. 1989). However, the results from the *Chlamydomonas* studies (Vallon et al. 1986) should be considered with caution. One reason is that the authors did not show any micrograph of PS I labelling, although they gave a statistical result. The other reason is that the thylakoid structure of *Chlamydomonas* seen in the figures of that paper is very swollen, when compared with the normal thylakoid structure of *Chlamydomonas* shown in Trémolières's paper (1991). This swelling may have been induced by not balancing the osmolarity during the fixation, or it is sometimes seem in

very old cells (at stationary phase). Whatever the reasons, the swollen thylakoids may change their membrane properties and protein distribution.

A recent biochemical study made by Andreasson and her colleagues (1988) using spinach is a extremely important one for PS I localization. They used sonication to break thylakoids already separated in an aqueous two phase system. They then obtained unappressed membranes and appressed grana membranes by countercurrent distribution or by a batch procedure. Two types of PS I were found, differing in their antenna sizes and in different localizations. These two types of PS I are termed PS I_α (located in grana membranes), and PS I_β (located in unappressed membranes). PS I_α has a larger antenna complex than PS I_β. Most of the PS I_α is associated with LHC I, while small amounts are associated with LHC II. Since grana membranes consist of both appressed and unappressed membranes, further work should be done to determine the origin of PS I_α. Andreasson and Albertsson (1993) separated the margin region from the grana fraction and found that the components of LHC II from the margin region are very similar to those of LHC II associated with isolated PS I_α, but differ from LHC II particles from the appressed grana membrane fraction (with the margin region removed). Their results indicate that PS I_α resides on the margin regions, which consist of unappressed membranes, as well as on the end membranes. Thus, it was confirmed in spinach that PS I distribution is heterogeneous and all PS I particles are exclusively located on unappressed membranes.

Data derived from cytochemical studies using 3,3'-diaminobenzidine (DAB) as a marker for the localization of PS I disagree with Anderson and Andersson's model.

DAB, as an artificial electron donor, can be photooxidized to form dark deposits in the thylakoid lumens if PS I activity exists on their surrounding membranes (Chua 1972). On the DAB-labelled barley thylakoids (Vaughn et al. 1983), dark DAB deposits could be seen on both unappressed and appressed membranes. This result suggested that PS I of barley may reside on both types of membranes. Similar results were also seen in studies from spinach (Nir and Pease 1973) and *Coleus blumei* (Marty 1977). However, since these cytochemical studies alone can not give quantitative measurements, a combination of both cytochemical and immunological studies may offer more conclusive results. Callahan and co-workers (1989) used both cytochemical and immunological measurement to detect the location of PS I activity in *Spirodela*. Cytochemical results showed that very strong dark DAB deposits occurred on unappressed membranes, but grana appressed membranes showed a relatively weaker DAB deposition. Meanwhile, densitometric scanning of immunoblots revealed that ca. 13% of PS I was associated with appressed membranes (17% for subunit I and 9% for subunit II, so 13% is the average of both), and 87% with unappressed membranes (Callahan et al. 1989). In research done on maize (Wrischer 1989) and greening bean leaf chloroplast (Wrischer 1978), it was also found that DAB reaction deposits were present on both membranes, but it was relatively weak in the grana membranes, except in the top and bottom thylakoid of each granum. Since the color of the deposits on appressed membranes is evenly gray, there must be very few, if any, PS I particles located on appressed membranes. According to my observations, only strong dark deposits can be counted as sites of DAB photooxidation reaction, while the evenly gray color may only reflect experimental background.

From the studies mentioned above, Anderson and Andersson's model is quite acceptable; nevertheless, some of the cytochemical studies do not agree with it. Since very few species of green algae have been used for the location of PS I, I do not think that this model should considered true for green algae before a variety of green algae have been studied for this purpose. Actually the thylakoid structure of green algae is varied. In many species (for example, in the primitive green algae, *Tetraselmis subcordiformis*), chloroplasts do not have grana; instead, they are traversed by extended bands of appressed thylakoids. The number of thylakoids in each band varies from 1 to 20. Unlike higher plants in which unappressed membranes are made up mostly of stroma-exposed thylakoids (or single thylakoids), these green algae only have a very small amount of single thylakoids; therefore, the unappressed membranes are mostly located on the end regions of stacked thylakoids. In other green algae, such as *Nitella flexilis* (Hilgenheger and Menke 1965) and *Chaetosphaeridium globosum* (Moestrup 1974), both members of the Charophyceae, chloroplasts have well-developed grana. From the evolutionary point of view, grana are a highly developed form of thylakoid arrangement. Blue-green algae and red algae only have single thylakoids and most chromophyte algae have thylakoids arranged in bands of three (Gibbs 1970). Therefore, the heterogeneous distribution of PS I in higher plants may also have proceeded in evolutionary steps. Since higher plants evolved from green algae, studying the distribution of PS I in a variety of green algae may let us elucidate when this distinct distribution of PSI evolved.

1.2 Light Harvesting Complex I (LHC I)

LHC I is the accessory light-harvesting antenna complex of PS I; proteins associated with LHC I migrate in the 20-25 KDa range, and are immunologically different from those of LHC II (Melis 1991). There are two types of LHC I: one is LHC I-680 which has a short fluorescence wavelength; the other is LHC I-730 which has a longer fluorescence wavelength. Reconstitution studies revealed that the presence of LHC I-680 is necessary for the transfer of excitation energy from LHC II to PS I (Bassi and Simpson 1987). Immunological study has revealed that LHC I is located on unappressed membranes (Di Paolo et al. 1990).

1.3 Photosystem II

The localization of PS II is quite certain. Almost all the studies for this purpose agree with the model suggested by Anderson and Andersson (1982). In this model, nearly 80% of PSII reaction centres reside on appressed grana membranes, the remaining 20% being located on stroma-exposed unappressed membranes.

Evidence for this comes from biochemical studies. Åkerlund and co-workers (1976) used the Yeda press to homogenize spinach cells with low salt-destacked grana, followed by aqueous polymer two-phase partitioning to separate thylakoid membranes into two fractions. They found that about 75% of the total PS II activity is derived from the grana region. Andersson and Anderson (1980) used the same techniques to derive two fractions and then ran both fractions on discontinuous SDS polyacrylamide gels. The fluorescence absorbance of each component also revealed that 80-90% of PS II reaction

centre complexes are restricted to the appressed membranes, while 10-20% can be localized to the unappressed stromal thylakoid membranes.

Freeze-fracture studies also support this model of PS II localization. Several researchers have found that PSII activity is correlated with the density of large particles (about 16 nm) on the EFs face. Freeze-fracture micrographs of a barley mutant which has lost all PS II activity revealed that particles on the EFs face were reduced by 96%, while the density of the particles on the PFs and PFu faces exhibited no significant change (Simpson et al. 1989). The correlation is even clearer in two mutants of *Chlamydomonas reinhardtii*. In one mutant, F34, which is totally lacking in PS II reaction centres, 16 nm sized particles (which represent intact PS II - LHC II complexes) on the EFs face are totally absent; in another mutant, F34SU3, which only lacks half of the PSII reaction centres, the density of 16 nm particles on the EFs face is half of that seen in wild type cells (Wollman et al. 1980). Other freeze-fracture studies on *Tradescantia* (Kereszte et al. 1976), *Chlorella* (Lacambra et al. 1984) and *Chlamydomonas* mutant FUD 34 (Olive and Vallon 1991) all produced similar results. It has also been confirmed that these 16 nm size particles on the EF face in higher plants and green algae represent intact PS II - LHC II complexes. A chl *b*-less barley mutant (Miller et al. 1976), chl *b*-deficient *Chlamydomonas* (Goodenough and Staehelin 1971) and chl *b*-deficient soybean mutants (Keck et al. 1970) have their PS II reaction centres intact, but lack LHC II; on the EF face of their thylakoids, the density of particles did not change, but the size of particles was reduced.

More evidence for this scheme of PS II localization is derived from cytochemical studies. Photoreduction of tetrazolium salts can be used for this purpose, since these salts form insoluble deposits in the thylakoid lumen if PS II activity is present. Photoreduction of tetrazolium salts can be inhibited by a PS II inhibitor, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU). When the tetrazolium salt thiocarbamyl nitroblue tetrazolium (TCNBT) was used to detect PSII activity in barley (Vaughn et al. 1983), bean etioplasts (Wrischer 1988), *Coleus blumei* (Marty 1977) and maize (Wrischer 1989), the results showed that the strong dark deposits were predominantly localized on the grana, while only a small amount of deposition was seen on the stroma-exposed unappressed membrane. Immunogold labelling at the electron microscopic level gives a great deal of direct evidence for this model. When antibodies against spinach PS II-core proteins D1 and D2 were used to label thylakoids of spinach and *Chlamydomonas*, nearly 88% and 90% respectively of the labelling appeared over the grana thylakoids (Vallon et al. 1986). Use of other antibodies raised against PS II proteins of *Chlamydomonas* to label the thylakoids of spinach and *Chlamydomonas* with 10 nm gold particles has confirmed these results (Vallon et al. 1985). Antibodies against barley PS II herbicide receptor protein D1, and PS II reaction centre antenna proteins, CP47, and CP43, also gave similar results (Simpson et al. 1989). Therefore, it is not surprising that three different antibodies against oxygen-evolving proteins which are associated with the PS II reaction centre complex also showed 84% of labelling on grana membranes (Goodchild et al. 1985; Simpson et al. 1989).

However, as I have mentioned before, grana membranes are composed of both unappressed membranes (end region and margin region) and appressed membranes, thus it can be questioned whether the 80% of PSII found on grana membranes accurately reflects the percentage of PSII on appressed membranes. When Webber and his co-workers (1988) used polyoxyethylene sorbitan monolaurate (Tween 20) to separate the margin areas from the grana, they found that there are no PS II proteins in this region. End regions only occupy a small amount of the total grana membranes. Thus, it can be concluded that most PS II complexes (70-80%) are located on appressed membranes.

The PSII population associated with grana appressed membrane is different from that associated with unappressed membranes in both light-harvesting antenna size (Melis and Homann 1975) and in the midpoint redox potential of plastoquinone (Horton and Croze 1979). Two populations of PS II were first found by Armond and Arntzen (1977); they observed that in pea chloroplasts, about 20-25% of the total PS II activity is derived from the stroma thylakoids and that this activity is regulated by light less efficiently than PS II activity derived from grana thylakoids. Biphasic fluorescence kinetics have shown a fast and sigmoidal phase (termed PS II_α) followed by a slower exponential phase (PS II_β) (Melis and Homann 1975). Destacked thylakoids created by the absence of Mg^{++} show the properties of PS II_β, while restacked thylakoids created by incubating destacked thylakoids in a cation solution reveal PS II_α properties; thus it was proposed that PS II_α units are located in the grana appressed membranes, while PS II_β resides in unappressed membranes (Melis and Homann 1978). Analysis of fractionated spinach thylakoid using aqueous polymer two-phase partitioning to fractionate the thylakoid membrane into

appressed and unappressed membrane populations has directly revealed that appressed membrane are enriched in PS II_{α}, and unappressed membranes in PS II_{β} (Anderson and Melis 1983).

1.4 Light Harvesting Complex II

There are two types of LHC II complexes: bound LHC II, which is tightly associated with PS II reaction centres, and mobile LHC II, which is loosely associated with bound LHC II and can move between appressed and unappressed membranes. Freeze-fracture studies have revealed that LHC II particles are 8 nm in diameter (Kyle et al. 1983; Mullet and Arntzen 1980). Most LHC II complexes are associated with the PSII reaction centres, and a small amount of them are associated with the PS I reaction centres (Andreasson and Albertsson 1993). Therefore, it can be estimated that the localization of LHC II is similar to that of the PS II reaction centres. This has been experimentally demonstrated; antibodies against two different denatured LHC II apoproteins gave 77%-81% of the labelling on grana membranes (Callahan et al. 1989). CP29 which has been found to be a PS II reaction centre protein (Camm and Green 1989), CP26 and CP24 are three proteins of the LHC II complex, and immunological investigation with antibodies raised against these three proteins have shown that they are confined to grana appressed membranes (Di Paolo et al. 1990). Immunogold labelling studies with antibodies against LHC II complexes on chloroplast thin sections and antibodies against the LHC II amino terminus on freeze-etched stacked membrane surfaces also gave similar results (Vallon et al. 1986; Simpson et al. 1987; Hinshaw and

Miller 1989). Di Paolo and his colleagues (1990), using immunological studies, not only found that most of the LHC II is located on grana thylakoid membranes, but they also found that LHC II has a mobile character. They used polyclonal antibodies against LHC II, PS II, CP29, the PS I - LHC I complex, an oxygen evolving enhancer, and the ATPase coupling factor, CF1, and incubated them with stroma membranes which were derived from dark-adapted and high light-adapted maize leaves. The results showed that dark-adapted stroma membranes have very little labelling; however, the high light-adapted stroma membranes have heavy LHC II labelling, while the concentration of all the other components (PS II, CP29, PSI-LHCI, the oxygen-evolving enhancer, and ATPase coupling factor CF1) did not change in this state transition. These results strongly support the state I - state II transition hypothesis. In this hypothesis, when PS II produces electrons faster than PSI can absorb them, there is an excess of reduced plastoquinone. This reduced plastoquinone activates a membrane-bound kinase that phosphorylates one or two threonine residues on LHC II. The phosphorylated LHC II becames charged and migrates from the appressed membranes in the grana (state I) to the unappressed stroma membranes (state II), where it donates energy to PSI. During state II -state I transitions, the reverse happens. Mobile LHC II is dephosphorylated, migrates back to the appressed grana membranes and associates with PS II again (Staehelin and Arntzen 1983). The mobile LHC II has the function of stacking membranes when it is in state I. This hypothesis also has been supported by a freeze-fracture study, which shows that 23% of total appressed membranes become unappressed due to light-induced phosphorylation of the LHC II (Kyle et al. 1983).

Chapter II Introduction

In higher plants and green algae, four supramolecular complexes are involved in photosynthesis. They are: 1. Photosystem I and its light-harvesting complex (LHC I); 2. Photosystem II and its light-harvesting complex (LHC II); 3. Cytochrome b_6/f complexes; 4. ATP synthetase. The localization of these complexes in thylakoids has been studied for more than 20 years. The currently most favoured hypothesis for these localization states that PS I is exclusively located on unappressed membranes, PS II mostly (about 80-85%) resides on appressed membranes; cytochrome b_6/f complexes are almost equally distributed on both types of thylakoids and ATP synthetase is only located on unappressed membranes. However, this model was based mainly on investigations performed using higher plants; very few green algae (probably only *Chlamydomonas* and *Chlorella*) have been studied for this purpose. In fact, the chloroplast structure of higher plants and green algae are very different. In some green algae, the thylakoid arrangement is similar to that seen in higher plants which have single thylakoids and grana in their chloroplasts. But in other green algae, like *Tetraselmis subcordiformis*, chloroplasts are traversed by extended thylakoid bands which consist of ca. 2 - 20 tightly appressed thylakoids. There are no grana and very few single thylakoids in the chloroplast of this alga. The transfer of energy between PS II and PS I is mediated by the mobile LHC II by means of state transitions, in which light determines the state of thylakoid membrane protein phosphorylation, in higher plants. However, some studies have found that in *Chlamydomonas*, the presence of light does not obviously change thylakoid membrane

protein phosphorylation (Owens and Ohad 1982). In addition, the molecules of LHC II in green algae like *C. reinhardii* may be quite different from those in higher plants (i.e. pea, barley, and fern), since monoclonal antisera against pea LHC II can label the thylakoid membranes of pea, barley and fern, but not those of *C. reinhardii* (Thaler 1986). Therefore, it is possible that the photosynthetic supramolecular complexes in green algae may be arranged in a different fashion from those in higher plants. Green algae evolved from cyanobacteria, the distribution of whose photosynthetic supramolecular complexes is still unknown. At what point the heterogeneous distribution of these complexes evolved in green algae or higher plants is an interesting question. Thus, it is necessary to study the localization of photosynthetic complexes in the chloroplasts of primitive green algae.

In this study, we used a primitive green alga, *Tetraselmis subcordiformis*, which belongs to the Prasinophyceae, to study the distribution of PS I and PS II. Two antibodies raised against PS I components (one against cp1-e from cyanobacteria, *Synechococcus elongatus*, the other against a PS I component from maize) and one antibody raised against PS II (cp-47 from *S. elongatus*) were used. Our results showed that both PS I and PS II are located on both appressed and unappressed membranes, which is quite different from the current models of its distribution in higher plants.

Chapter III Materials and methods

3.1 Cell culture

Tetraselmis subcordiformis (UTEX 171) was obtained from the University of Texas Culture Collection of Algae and grown at 25°C without shaking in 250 ml Erlenmeyer flasks containing 75 ml of Guillard's f/2 sea water medium (McLachlan 1973) under a bank of cool-white fluorescent lamps (approximately 450 ft-c) on a 12 h light/12 h dark cycle.

3.2 Antisera and reagents

Antiserum against sucrose gradient-purified maize PS I protein particles was kindly donated by A. Barkan (Institute of Molecular Biology, Univ. of Oregon, Eugene OR). Antisera against the 60 and 62 KDa PS I reaction centre proteins (designated cp1-c) and the 47 KDa PS II reaction centre protein (cp-47) from *Synechococcus elongatus* (preparation and characterization of these two antisera are described in Kashino at al. 1990) were generously donated by Y. Kashino (Dept. of Life Sciences, Himeji Institute of Technology, Harima Science Park City, Hyogo, Japan). Protein A-gold (10 nm) was bought from EY Laboratories, San Mateo, CA.

3.3 Electron microscopy

Cells of *T. subcordiformis* were harvested at late logarithmic phase of growth 30 min before the end of the dark period, and were then fixed in 2% glutaraldehyde with 0.6 M sucrose in 0.1 M phosphate buffer (pH 7.2), for 40 min at 4°C. After being washed with buffers containing decreasing concentrations of sucrose three times for 10 min each,

the cells were postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.2) for 30 min at 4°C. The cells were washed again and then dehydrated through a graded ethanol series and embedded in Spurr's epoxy resin (Spurr 1969). Pale gold sections were mounted on formvar-coated copper grids, and stained with 2% uranyl acetate followed by Reynold's lead citrate.

3.4 Immunogold localization of PS I on Epon sections

Cells of *T. subcordiformis* were harvested during the late logarithmic phase of growth 30 min after the beginning of the light period, and collected by centrifugation. The cells were resuspended in Guillard's f/2 sea water medium containing 2% glutaraldehyde and fixed for 2 min at room temperature, osmium tetroxide was then added to the fixative plus cells to give a final concentration of 0.1%, and the cells were left in fixative for another 28 min at room temperature. The cells were centrifuged and washed three times in fresh sea water medium for 10 min each, then dehydrated through a graded ethanol series. Following 5 min immersion in 100% propylene oxide, the cells were infiltrated and embedded in Epon resin. Yellow-gold sections were collected on formvar-coated nickel grids, and placed on drops of the following solutions: 0.58 M (saturated) sodium meta-periodate, 30 min; distilled water 3 min, 4 times; 1% bovine serum albumin (BSA) in PBS, 30 min; anti-PS I antiserum from maize, diluted 1:50 in PBS, 45 min; protein A-gold, diluted 1:10 in PBS, 30 min; PBS, 3 min, 4 times; distilled water, 2 min. The sections were stained as described above.

3.5 Immunogold localization of PS I (cp1-e) and PSII (cp-47) on Lowicryl sections

The cells were harvested at the late logarithmic phase, 30 min before the end of dark period, and fixed in 2% glutaraldehyde with 0.3 M sucrose in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature, and then washed with buffers containing decreasing concentrations of sucrose. The cells were dehydrated in 25% ethanol at 4°C, 50%, 75%, and 95% ethanol at -18°C, and this was followed by infiltration and embedding in Lowicryl K4M resin at -18°C: 95% ethanol:Lowicryl, 1:1, overnight; 95% ethanol:Lowicryl, 1:2, 2 h twice; pure Lowicryl overnight; pure Lowicryl, 2 hours twice. The cells in Lowicryl were put into BEEM or gelatin capsules, concentrated at the bottom of capsules by centrifugation at 3500 rpm, and polymerized at -18°C under ultraviolet light at 360 nm for 24 h, then another 48 h under UV light at room temperature. Yellow-gold sections were collected on formvar-coated nickel grids and incubated in the following solution: 1% BSA in PBS, 30 min; anti-cp1-e or anti cp-47, diluted 1:50 in PBS, 1.5 h; PBS, 3 min, 4 times; protein A-gold, diluted 1:10 in PBS, 30 min; PBS, 3 min, 4 times and distilled water, 2 min. After staining with uranyl acetate and lead citrate, all sections were viewed under a Philips EM 410 electron microscope at 80 KV.

3.6 Cytochemical detection of PS I and PS II

The photooxidation of 3,3'-diaminobenzidine (DAB) and the photoreduction of distyryl nitroblue tetrazolium chloride (DS-NBT) were used to determine PS I and PS II activity respectively, as described by McKay and Gibbs (1990). Cells of *T. subcordiformis* were harvested at the end of the logarithmic phase of growth 30 min

before the end of the dark period, spun at 3,500 rpm, and fixed in 2% paraformaldehyde in 0.1 M phosphate buffer (containing 0.2 M sucrose), pH 7.4, for 20 min on ice in the dark. Cells were rinsed in buffer containing decreasing concentrations of sucrose and then incubated in a freshly prepared solution containing 1 mg/ml DAB in 0.1 M phosphate buffer (pH 7.4) for 1 h or 1 mg/ml DS-NBT in 0.1 M phosphate buffer (pH 7.4) for 45 min at 25°C under cool-white fluorescent lamps at 450 ft-c. The control was performed in the dark at room temperature. Cells were then washed in buffer again, and postfixed in 1% OsO_4 (for cells incubated in DAB) or 1.5% OsO_4 (for cells incubated in DS-NBT) in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4°C in the dark, then washed in buffer, dehydrated in a graded ethanol series, and embedded in Spurr resin (Spurr, 1969). Yellow gold sections were mounted on formvar-coated copper grids and viewed unstained in a Philips EM410 electron microscope at 80 kV.

3.7 Quantification of gold label

3.7.1 Anti-PS I from maize

In these sections, the thylakoid membranes were black and their lumens white since 0.1% osmium tetroxide was present in the fixative, so it was possible to make a detailed analysis. A total of 109 micrographs printed at a magnification of x 102,500 were analyzed. Whenever the bands of thylakoids were cut perpendicular to the plane of section, two lines were drawn perpendicular to the band and all the gold particles in the enclosed area were counted and assigned to the following categories: a particle was assigned to the outermost unappressed membrane if it was localized on the membrane,

or in the stroma touching the membrane or almost touching the membrane (no more than 5 nm away from the membrane); particles were assigned to the lumen (lumen 1) of the outermost thylakoid of a band if they lay squarely over the lumen or no more than 1/4 of the 10 nm gold particle overlapped a membrane; particles localized on the two appressed membranes separating the outermost thylakoid from the next thylakoid were counted as one category (termed membrane 2) as were those of the next lumen (termed lumen 2). Counts were made in this fashion for each pair of appressed membranes and each lumen, assigned by its number from the outermost thylakoid up to the category of lumen 6 (*i.e.*, in a band of 11 or 12 thylakoids). The total length of all the membranes present in each marked area was measured on a computerized tablet by the Sigma-Scan program, version 3.92 (Jandel Scientific, San Rafael, CA).

For our analysis, all the gold particles on the appressed membranes and in the interior lumens were considered as particles on appressed membranes. One-half of the particles in the outermost lumen was assigned to the appressed membranes, and one half was added to the counts on the outermost membrane. A total of 942 gold particles were counted. The statistical significance of the observed distributions was determined by Student's t-test. The total counts from each micrograph was counted as one variable, except when the length of unappressed membrane measured in the micrograph was less than 10 cm (=0.98 μm). To obtain this length, 2 micrographs were grouped together in 14 cases, and in one case, the data from 3 micrographs were grouped together.

3.7.2 Anti-cp1-e and anti-cp47 from Synechococcus elongatus

For both these antibodies, cells fixed in glutaraldehyde and embedded in Lowicryl had to be used. In these micrographs, the space between two appressed membranes appears as a solid dark line, while the lipid layer of the thylakoid membranes and lumens are white. In the middle of each lumen there is an interrupted faint dark line. Between the outermost thylakoid membrane and stroma, there is an irregular dark line which may reflect extrinsic proteins on outermost membranes or dense stroma contents. For CP1-e, 19 micrographs were analyzed, and for CP-47, 24 micrographs, all at a magnification of x 102,500. Areas where the thylakoids were cut perpendicularly to the section were marked as before, and every gold particle in the enclosed areas counted. In these counts, three categories were distinguished. All gold particles localized on the irregular dark line (*i.e.*, the outermost unappressed membrane) or lying within 5 nm outside the irregular dark line were classified as labelling on the outermost unappressed thylakoid membrane. The second category consisted of gold particles which lay squarely over the space between the outer irregular dark line and the solid dark line or had no more than 1/4 of their 10 nm width overlapping one of these two lines. One half of particles in this category was assigned to the unappressed membrane, and one half was assigned to appressed membranes. All gold particles lying over the outermost solid dark lines, *i.e.* over the zone of appression between the first and second thylakoids, and all gold particles interior to the outermost solid dark lines, were assigned to the appressed membrane. The length of every membrane within the areas analyzed was measured by Sigma-Scan. Statistical significance was determined by Student's t-test, with the total counts from one

micrographs serving as one variable. Labelling on Lowicryl sections was so heavy that no grouping of micrograph had to be performed. For anti-cp1-e, a total of 2569 gold particles were counted; for anti-cp-47, 1436 particles were counted.

Chapter IV. Results

4.1 Ultrastructural observations on Tetraselmis subcordiformis

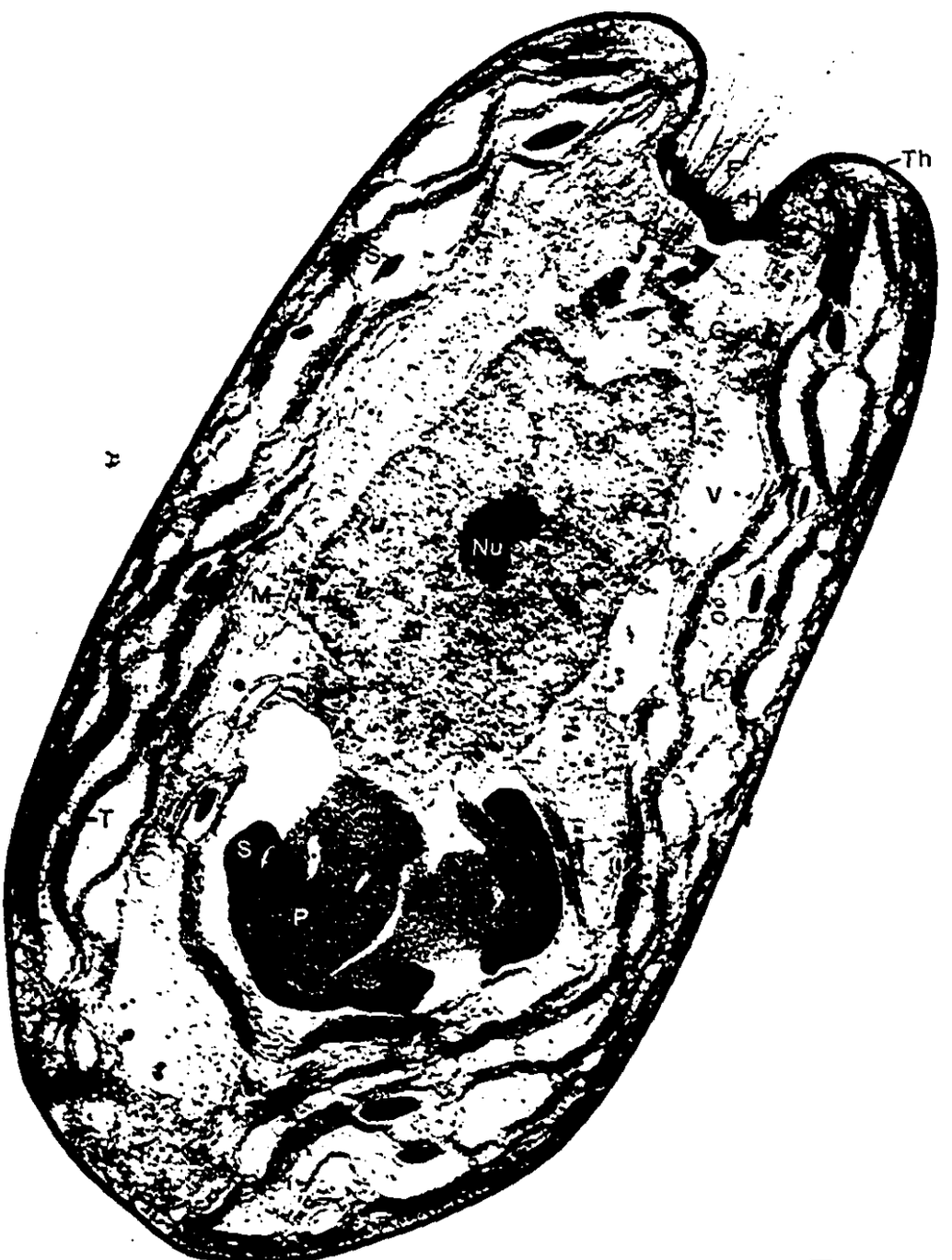
Cell Cells of *T. subcordiformis* have a pear shape and are approximately 10 - 11 μm long and 5 - 6 μm wide (Fig. 1).

Theca The theca encloses the cell. It is the most electron-dense material of the cell and is about 20-50 nm thick. It lies close to the cell membrane, except that in the area where the cell's shape is irregular, the theca and cell membrane are separated (Figs.1,2,9,10,11).

Flagella Each cell of *T. subcordiformis* has four flagella. On the flagellar pits, dense, hair-like material can be observed (Fig.1,4,10). Four electron dense lines (called half-desmosomes, Norris 1980) can be found under the flagellar pit (Fig.3). There are two dense lines which connect the half-desmosomes to the basal bodies (Fig.3). Two large striated rootlets extend from the basal bodies to the nucleus (Fig.6).

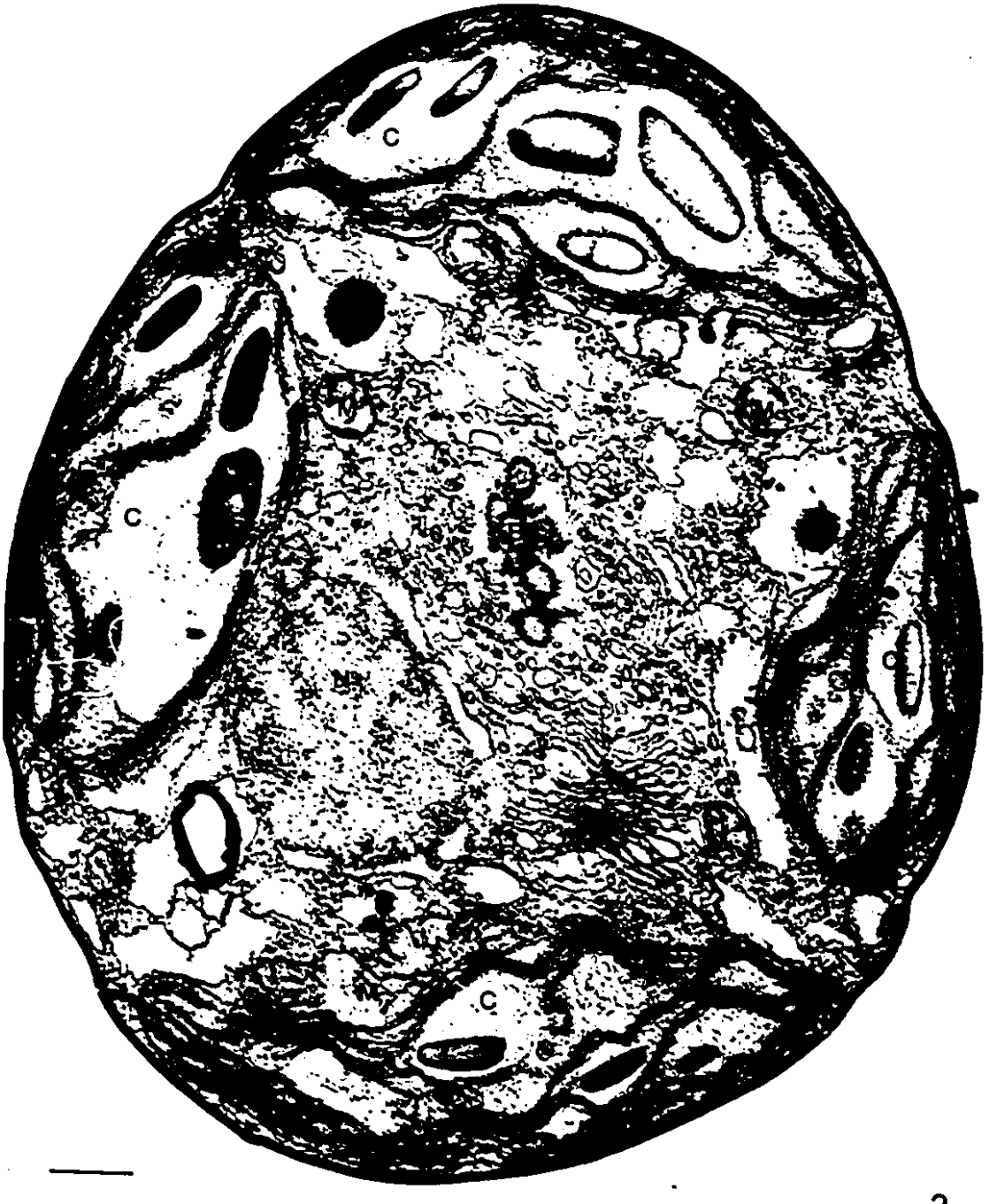
Basal bodies There are four basal bodies which extend into the cell from the flagellar pit for 0.77 - 0.98 μm in depth and have a zig-zag arrangement (Figs. 2,4). They are located close to each other and end at the same level, beneath which there is a narrow electron dense line (Fig.4). The basal bodies are composed of 9 microtubule triplets (Fig.2).

Fig. 1. Longitudinal section of a cell of *T. subcordiformis* showing a cup-shaped chloroplast (*C*) with anterior lobes, a nucleus (*N*) in the middle of the cell, Golgi apparatus (*G*), rootlets (*R*) and basal bodies (*B*) in the region anterior to the nucleus, and a pyrenoid (*P*) which is posterior to the nucleus and surrounded by starch (*S*). *Nu* nucleolus; *V* vacuole; *M* mitochondrion; *T* thylakoids; *Th* theca; *F* flagella; *L* lipid droplets; *H* hair. x 18 900; Bar=0.5 μm



1

Fig. 2. A cross section through the anterior part of a cell showing four basal bodies (*B*), four chloroplast (*C*) lobes, Golgi apparatus (*G*), nucleus (*N*) and mitochondria (*M*). x 26 800; Bar=0.5 μm



2

Fig. 3. Longitudinal section of a cell showing basal bodies (*B*), half-desmosomes (*D*) lying under the flagellar pit, and one of the four flagella (*F*). *M* mitochondrion; *N* nucleus. x 25 400; Bar=0.5 μm

Fig. 4. A longitudinally cut cell showing the four basal bodies (*B*) under the flagellar pit. *N* nucleus. x 33 800; Bar=0.3 μm

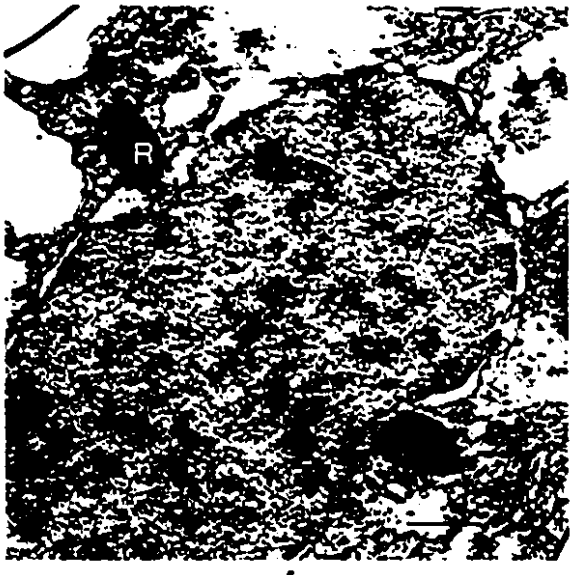
Figs. 5, 6. Two sections which show that the large striated rootlets (*R*) connect with the outer membrane of the nucleus (*N*). x 21 500, x 25 400; Bar=0.5 μm

Fig. 7. The eyespot (E) of the chloroplast. The double-membraned chloroplast envelope is indicated by the *arrow*. x 53 200; Bar=0.2 μm

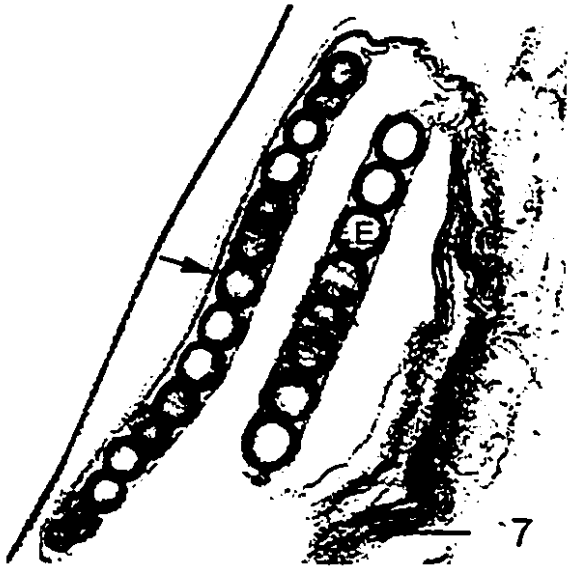
Fig. 8. Thylakoids (*T*) invaginated (*white arrow*) and thylakoid ends (*black arrow*). x 53 200; Bar=0.2 μm


F
M
D
B
N


B
N
4


R


R
6


B
7


T
8

Chloroplasts The chloroplast occupies up to 1/3 to 1/2 of the total cell volume (Fig.1); it is cup-shaped with four anterior lobes which terminate at or near the most anterior part of the cell (Figs.1,2). The two-layered membrane of the chloroplast envelope encloses the chloroplast (Fig.7). There are some starch granules and lipid droplets in the stroma. The electron density of starch granules varies: newly formed daughter cells have starch granules which are not electron-dense except at their margins (Fig. 11), whereas cells harvested at log-phase have electron-dense starch granules (Fig.1,2,9,10). The chloroplasts are traversed by extended bands of thylakoids containing from two to twenty thylakoids per band. Single thylakoids can be observed projecting out from the end of a band or crossing over from one band to another (Fig.12,13). The thylakoids in the bands are tightly appressed (Fig.12,13,14). The total width of a thylakoid is about 15 nm, with a membrane width of 3.75 nm and a lumen width of 7.5 nm. The space between two appressed membranes is about 1.8 nm. Bands of thylakoids may split in two or join neighbouring bands, therefore the length of the thylakoids and the number of thylakoids in a band varies. The unappressed membranes of thylakoids consist mostly of the outermost membranes in each band. Invagination of thylakoids also can be observed (Fig.8). There are no grana in the chloroplasts of *T. subcordiformis*.

Pyrenoid In each chloroplast there is a pyrenoid which resides in a middle to posterior position. In the cell it is located posterior to the nucleus (Fig.1,11). The electron-dense pyrenoid matrix is surrounded by curved starch grains. The pyrenoid of *T. subcordiformis* is unusual in that fingers of cytoplasm extend into the pyrenoid where the pyrenoid abuts

Fig. 9. A cross section of a cell showing a pyrenoid (*P*) with projections of low density (*arrows*). *E* eyespot; *M* mitochondria; *C* chloroplast; *S* starch grain. x 27 400; Bar=0.5 µm

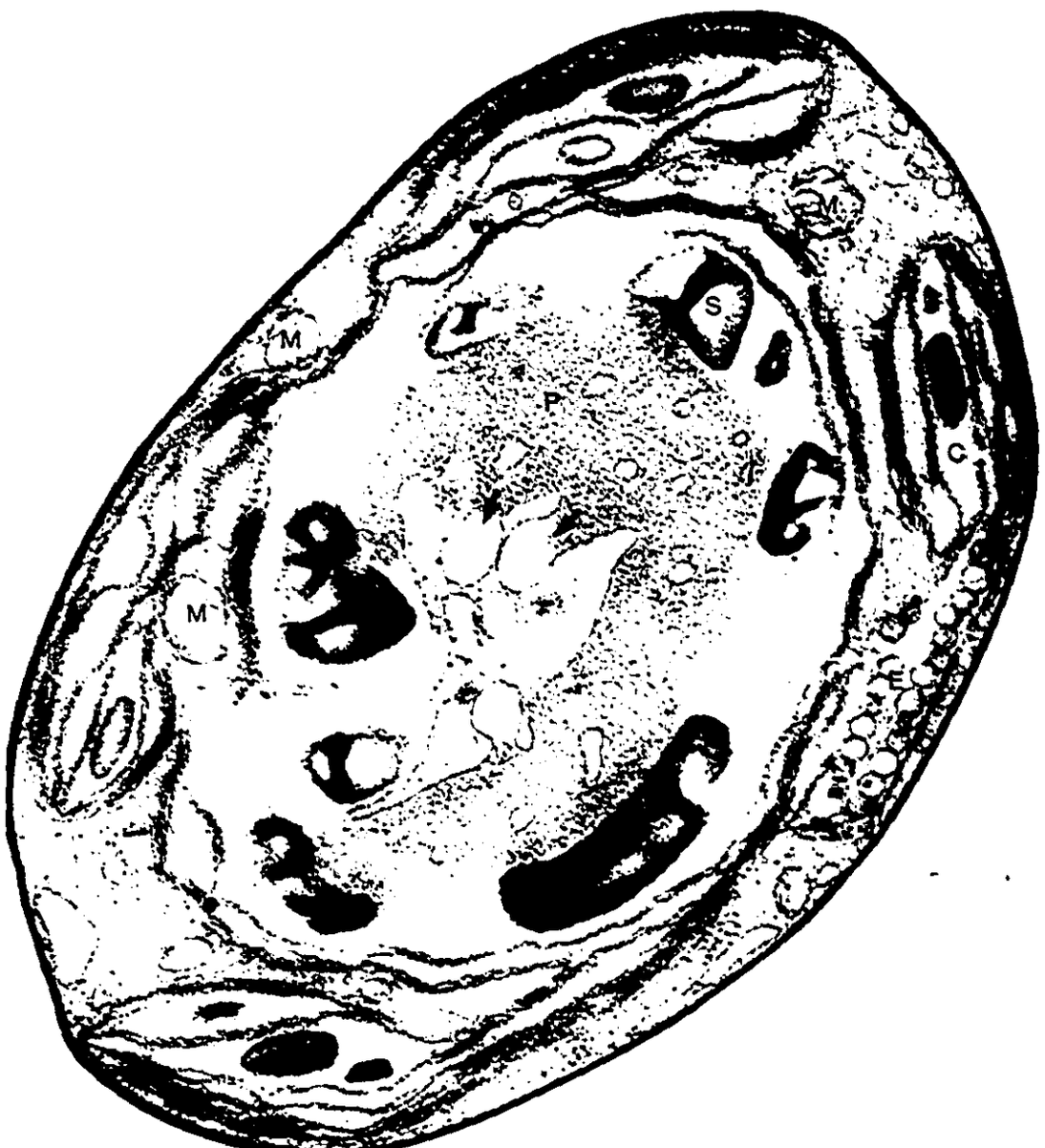

M
S
P
C
M

Fig. 10. Nucleus (*N*) whose outer membrane projects as rough ER into the spaces which separate each Golgi apparatus (*G*) from the chloroplast (*C*) as well as into the cytoplasm (*arrowheads*). *M* mitochondria; *S* starch; *Nu* nucleolus. x 25 900; Bar=0.5 μm

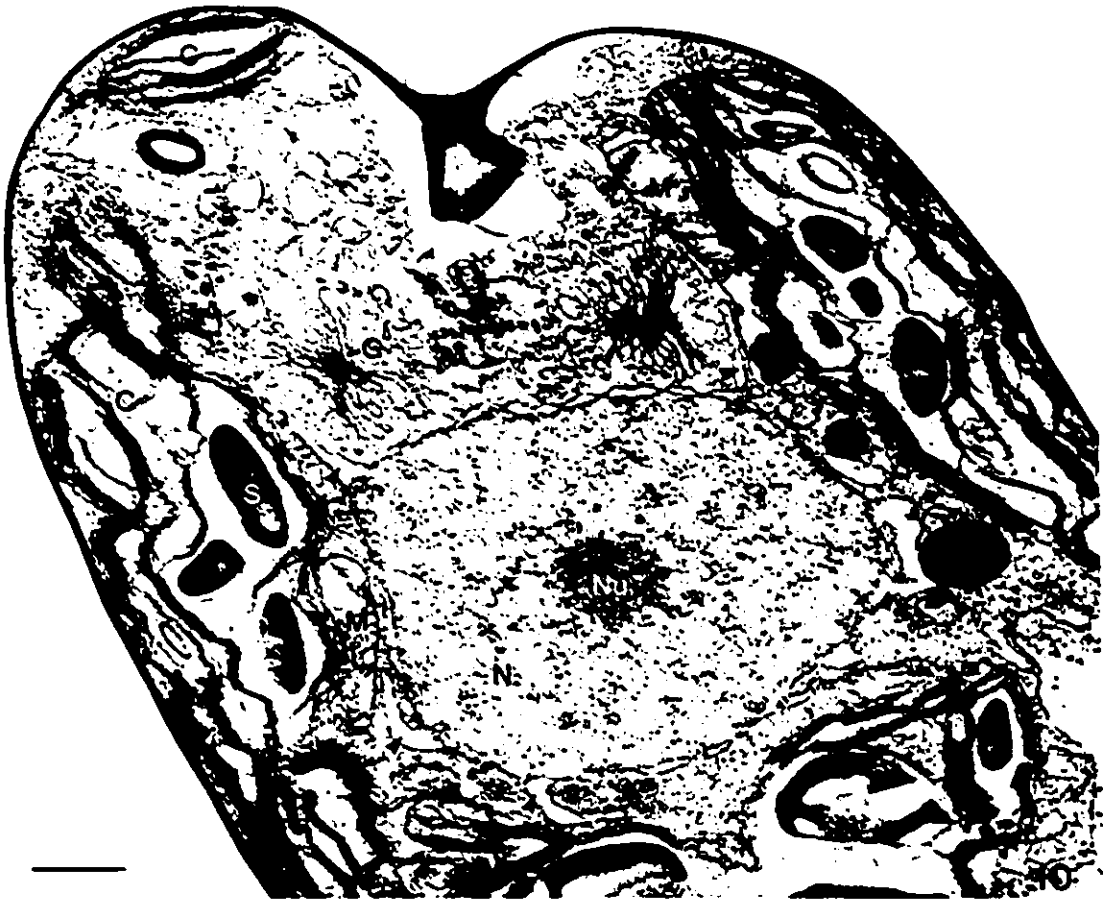
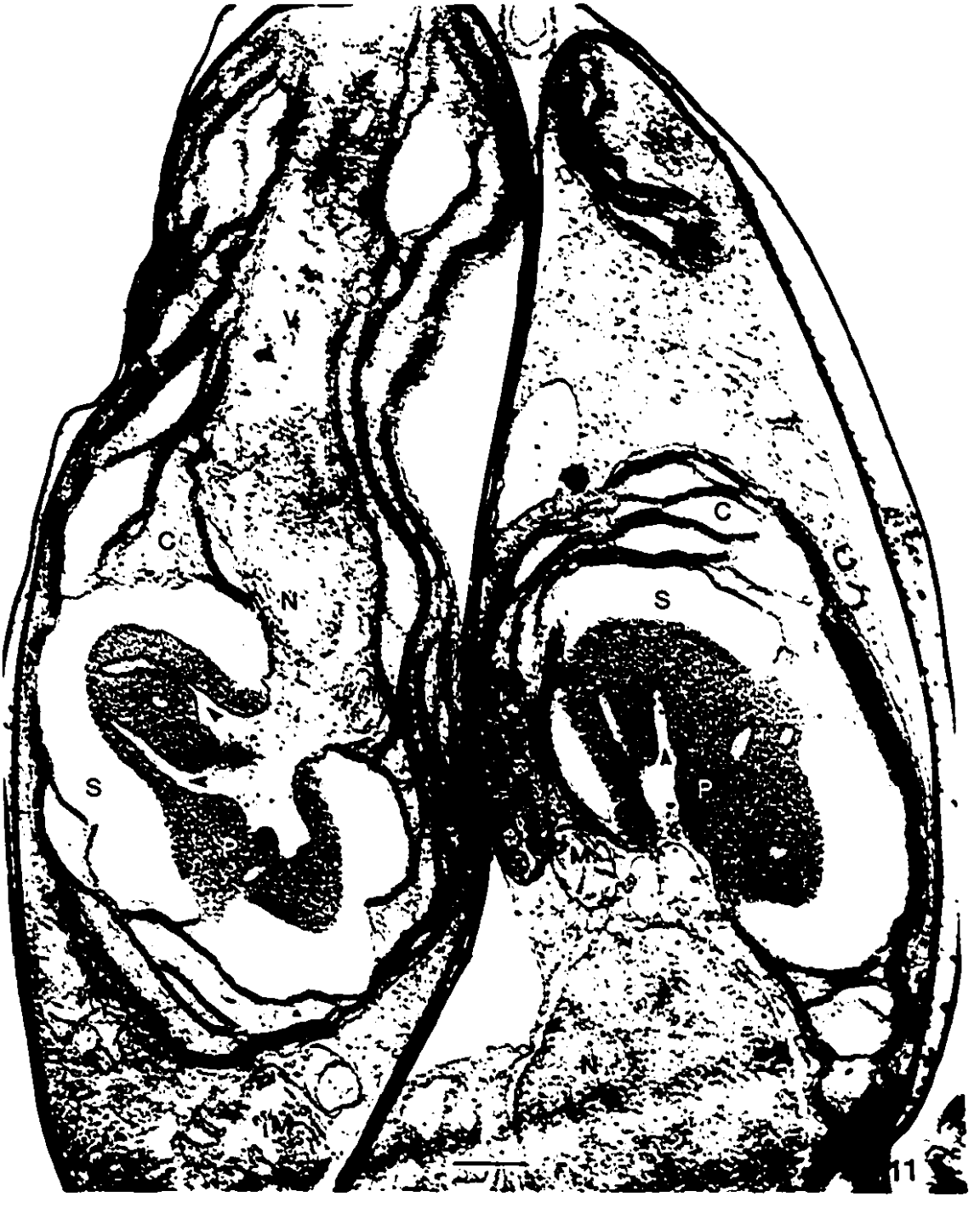

C
O
G
S
N
M
10

Fig. 11. Two oppositely oriented daughter cells in a parent theca. The pyrenoids (*P*) are invaded by fingers of cytoplasm (*arrowheads*). *S* starch grains; *N* nucleus; *V* vacuole; *C* chloroplast; *E* eyespot; *M* mitochondria. x 24 200; Bar=0.5 μm


C
N
A
S
P
C
S
A
P
N

the nucleus (Figs. 1,11,15). The double-membraned chloroplast envelope separates the pyrenoid matrix from the fingers of cytoplasm (Figs. 9,11). The projecting fingers of cytoplasm in the pyrenoid have very low electron density and do not appear to contain ribosomes (Figs.9,11). At times, projections of the pyrenoid which have lost their dense material extend into the cytoplasm (Fig.9).

Eyespots Eyespots reside in the chloroplast and are located in a posterior position, very close to the chloroplast envelope (Fig.7,9,11). There are two layers of lipid granules in each eyespot, each layer being separated by a single thylakoid which may be very wide (Fig.7). In most cases, eyespots are adjacent to the chloroplast envelope, although occasionally one thylakoid is observed between the eyespot and the chloroplast envelope.

Nucleus The nucleus is located in the middle to anterior part of cell (Fig.1). There is only one nucleolus in each nucleus. The outer nuclear membrane is made up of rough endoplasmic reticulum which projects into the area beneath the forming face of the Golgi apparatus, and also projects into the other regions of the cytoplasm (Fig.10).

Golgi apparatus and other organelles The Golgi apparatus is always situated anterior to the nucleus, with the cis face close to the chloroplast and the trans face facing the central axis of the cell. The dark electron-dense material that forms the theca can be seen in the Golgi lumen (Fig.10). Mitochondria are often found very close to the chloroplast

envelope. Most mitochondria are round and about 0.3 - 0.8 μm in diameter (Fig.1,2,9). There are also vacuoles and lysosomes in the cytoplasm.

4.2 Immunocytochemical studies and cytochemical studies

Figures 12 and 13 show a chloroplast from a cell simultaneously fixed with glutaraldehyde and osmium tetroxide and labelled with anti-PS I from maize. The labelling is very specific. Almost all of the gold particles are on the thylakoid bands; only a few are seen in the stroma or over the cytoplasm. Gold particles are localized on the internal appressed membrane (*white arrows*) as well as on the outer unappressed membranes (*arrowheads*) at the edges of the bands. Statistical analysis of the results (Table 1) indicates that there is no significant difference in labelling density between appressed membranes (1.28 ± 0.89 gold particles/μm of appressed membrane length) and unappressed membranes (1.32 ± 0.43 gold particles/μm of unappressed membrane length). However, since appressed membranes comprised 78% of the total membrane length, the gold particles on appressed membrane comprise 76% of total gold particles. Therefore, there are three times as many gold particles on appressed membranes as on unappressed membranes. The control cell (Fig.14), which was incubated with pre-immune serum instead of antiserum, has a labelling density of 0.02 gold particles/μm of membrane length.

Figs. 12-14. Immunogold localization of PS I on Epon thin sections of cells fixed simultaneously with glutaraldehyde and osmium.

Figs. 12,13. Sections incubated with antibody from maize, followed by protein A-gold (10 nm). Gold particles are located on both appressed (*white arrows*) and unappressed (*arrowheads*) thylakoid membranes. Very few gold particles are present in the stroma (St) or outside the chloroplast. At the *black arrows*, a single thylakoids crosses from one band to another. M mitochondrion; St stroma; T thylakoid band. x 94 800; Bar=0.1 µm

Fig. 14. In a control section incubated with pre-immune serum, no gold particles are present. T band of three appressed thylakoids; St stroma. x 94 800; Bar=0.1 µm


St
12
St
M
13
St
14

Table I. Labelling of appressed (AM) and unappressed membranes (UAM) with antibodies against PS I and PS II in *Tetraselmis subcordiformis*

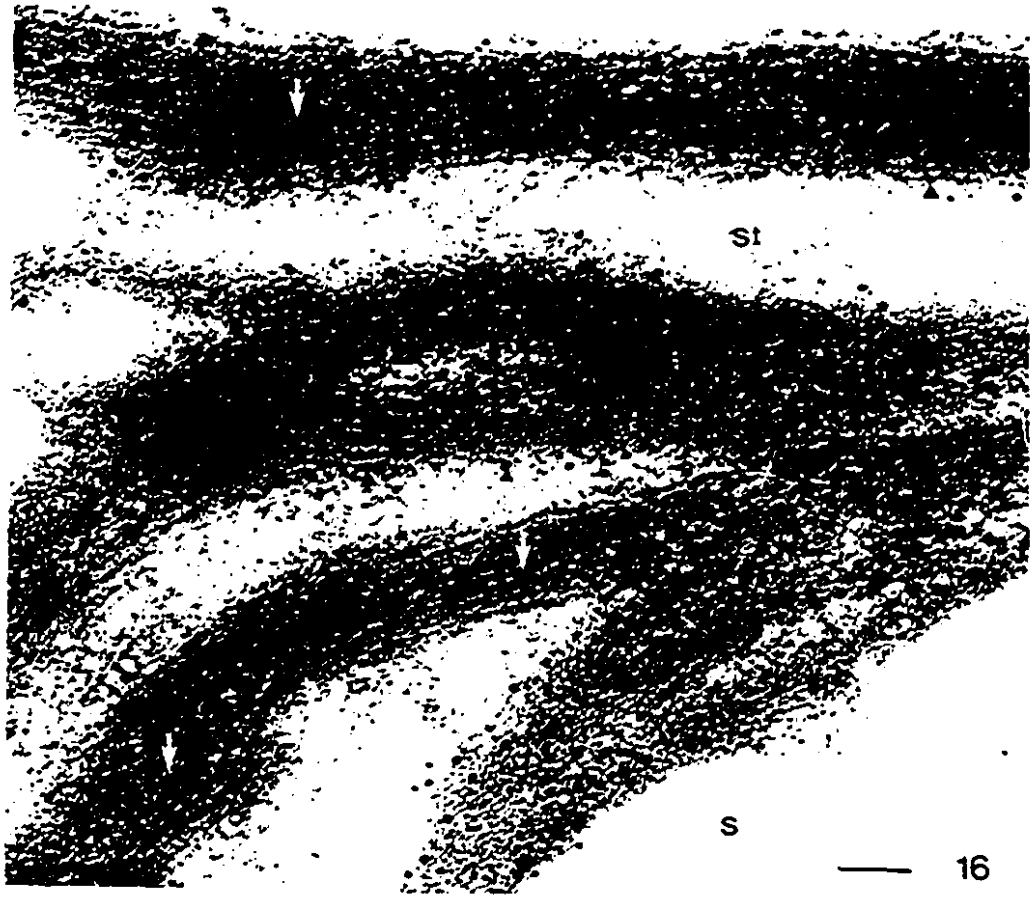
antibodies	membrane length (μm)	%AM	% gold particles on AM	labelling density (particles/μm)		density ratio (UAM/AM)
				AM	UAM	
PSI (maize)	772	78	76	1.28±0.89	1.32±0.43	1.03
cp1-e	775	86	78	3.25±0.74	5.25±1.97	1.62
cp-47	1045	86	76	1.22±0.24	2.17±1.16	1.78
pre-immune	130	ND	0.02	ND		ND

In another immunogold labelling of PS I, we used antiserum raised against cp1-e from a cyanobacterium, *Synechococcus elongatus*. cp1-e is the PS I reaction centre complex that contains the 60 and 62 KDa apoproteins of PS I (Kashino 1990), and has 83-84% sequence homology with the corresponding proteins of *Chlamydomonas* and 78-81% with those of maize (Kashino 1990). Immunoblot studies showed that this antiserum crossreacts with the 60-62 KDa thylakoid membrane proteins of the green algae *Dunaliella, Chlorella, Chlamydomonas* and the higher plants, *Oryza and Spinacia*. (Kashino 1990). This antiserum did not label cells of *T. subcordiformis* fixed in glutaraldehyde and osmium tetroxide and embedded in Epon. Therefore, we used the Lowicryl embedding method and fixed cells only with 2% glutaraldehyde, and in this case the antigenicity of cp1-e was well preserved. Although the ultrastructure of thylakoids in the Lowicryl sections is not as clear as in the Epon sections, we can still clearly distinguish the chloroplast stroma, which is white, from thylakoids which are dark in the micrographs (Figs. 15,16). Immunogold labelling with this antibody is very specific: 96% of gold labelling is on the thylakoid bands, and only 4% of the gold labelling is on the stroma and other cell structures. It should be noted that although the labelling on thylakoid bands is very dense, there is no labelling at all on mitochondria, eyespots and nucleus (Fig. 15). The single thylakoid between the two layers of lipid droplets of the eyespot is also specifically labelled. The pyrenoid has a low level of background labelling (Fig. 15). The density of labelling on unappressed membranes (5.25 ± 1.97 gold

Fig. 15. Immunogold localization of PS I on Lowicryl sections of a cell fixed with 2% glutaraldehyde. The section was incubated with antibody against PS I (cp1-e) from *Synechococcus elongatus*. Abundant gold particles are present on both appressed and unappressed membranes, whereas very few gold particles exist in the stroma (*St*) or on starch grains (S). Note that there are no gold particles on the mitochondria (*M*), lipid granules of the eyespot (*E*) or the nucleus (*N*). The single thylakoid (*arrow*) located between the two layers of lipid granules in the eyespot is also specifically labelled. A low level of background labelling is present on the pyrenoid (*P*). x 43 600; Bar=0.3 μm

15

Fig. 16. High magnification of part of Fig. 15. Gold particles on both appressed membranes (*white arrows*) and unappressed membranes (*arrowheads*) can be observed clearly. Very few gold particles are found on the stroma (*St*) or starch grains (*S*). x 109 800; Bar=0.1 μm


St
S
16

particles/μm of unappressed membrane length) is significantly more than that seen on appressed membranes (3.25 ± 0.74 gold particles/μm of appressed membrane length) ($P<0.05$). However, since the appressed membranes occupy 86% of the total thylakoid membrane, the total amount of gold particles on appressed membrane is 78% of the total. This indicates that the gold particles on appressed membranes are nearly four times as numerous as those on unappressed membranes. Very little labelling was present in sections incubated with pre-immune serum or in PBS alone (not shown).

Since DAB has been successfully used to localize the PS I activity, we used it for further indication of the site of PS I localization. Fig. 17 shows that the DAB reaction deposits are located in all the thylakoids, both internal and external, of the chloroplast. Mitochondria also show a DAB-positive reaction because of their cytochrome oxidase activity. At high magnification (Fig. 19), the pattern of the DAB reaction deposits can be seen to have a punctate appearance and the density of DAB reaction deposits in internal and external thylakoids seems almost identical. Control cells (Fig. 18), which were incubated with DAB in the dark, show heavy DAB reaction deposits in the mitochondria, but none at all in the thylakoids.

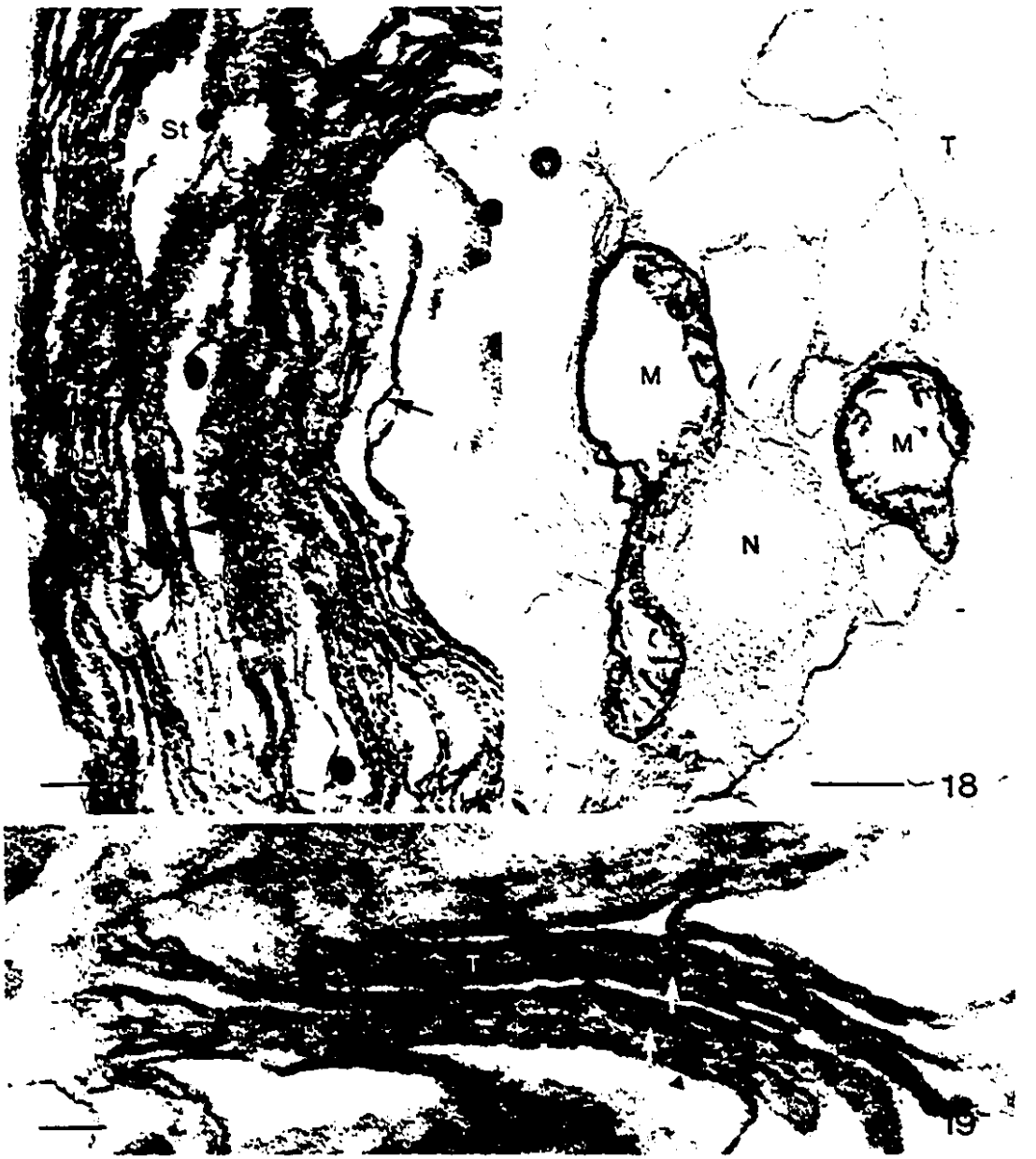
Antiserum against the cp-47 protein from *Synechococcus elongatus* was used to localize the distribution of PS II on thylakoid membranes of *T. subcordiformis*. The 47 KDa protein of the green algae *Dunalliella, Chlorella, and Chlamydomonas* as well as the

Figs. 17-19. Cytochemical detection of PS I activity in log-phase cells.

Fig. 17. Cell incubated with 3,3'-diaminobenzidine (DAB) in bright light. Oxidized DAB deposit appears in both appressed (*white arrows*) and unappressed membranes (*arrows*) of the thylakoids (*T*). x 27 900; Bar=0.5 μm

Fig. 18. Control cell incubated with DAB in the dark. Oxidized deposits of DAB appear in the mitochondria (*M*), but not on thylakoid membranes (*T*). *N* nucleus. x 27 900; Bar=0.5 μm

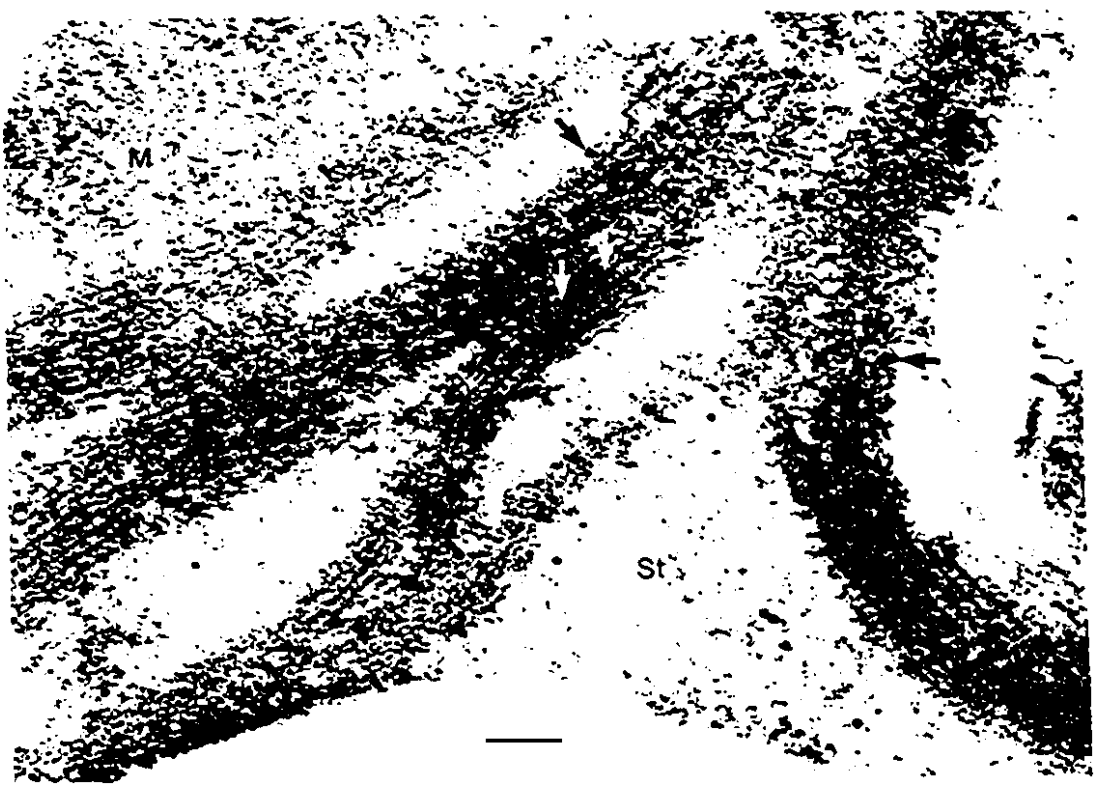
Fig. 19. High magnification of thylakoids (*T*) stained with DAB. The dense deposits have a punctate appearance and appear to be located in both inner (*white arrows*) and outer (*arrowheads*) thylakoids. x 103 800; Bar=0.1 μm


St
T
M
M'
N
18
T
19

corresponding protein of the higher plants, *Oryza* and *Spinacia*, have been shown in Western blots to cross-react, but not in tensely, with this antiserum (Kashino 1990). Fig. 20 shows that the labelling is very specific. Most of the gold particles are on the thylakoid bands and very few are on the stroma. Surprisingly analysis of the results shows that the labelling densities of gold particles per μm of appressed membranes (2.17 ± 1.16) is significantly more than that of gold particles per μm of unappressed membranes (1.22 ± 0.24). Still, since appressed membranes take up 86% of total membranes, 83% of total gold particles are located on appressed membranes. Therefore, there are four times as many cp-47 antigens on appressed membranes as on unappressed membranes.

To further confirm the immunolabelling results of PS II localization, DS-NBT was used to localize the PS II activity. Fig. 21 shows that DS-NBT reaction deposits are visible over the entire thylakoid band. Single thylakoid also show DS-NBT reaction (arrow). Control cells (Fig.22), incubated with DS-NBT in the dark, have no DS-NBT reaction deposits. Therefore, both immunolabelling and cytochemical results have revealed that PS II resides on both appressed and unappressed membranes.

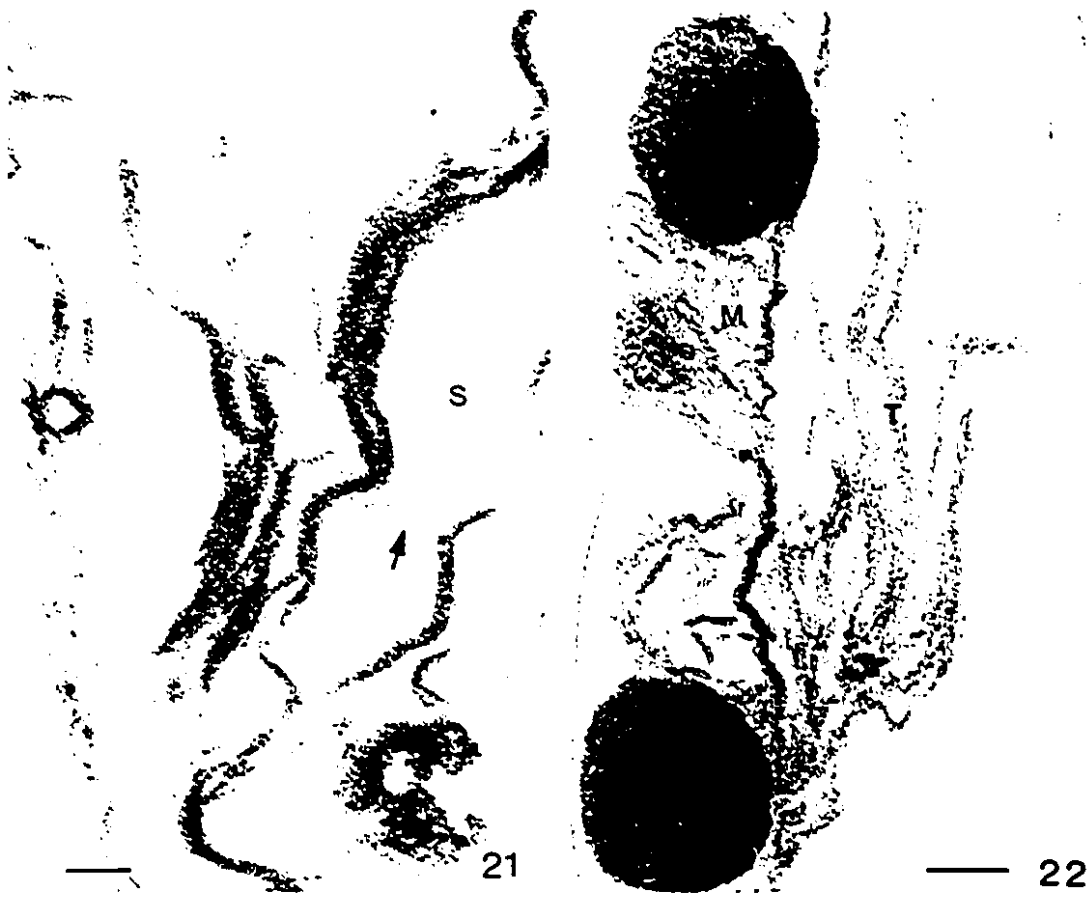
Fig. 20. Immunogold localization of PS II on a Lowicryl section of a cell fixed with 2% glutaraldehyde. Section were incubated with antibody against the 47 KDa protein of PS II of *S. elongatus*. Note that gold particles are located on both appressed (*white arrows*) and unappressed (*arrows*) thylakoid membranes, whereas very few gold particles are present on the chloroplast stroma (*St*) or on the mitochondrion (*M*). x 103 800; Bar=0.1 µm


M
St

Figs. 21-22. Cytochemical detection of PS II activity.

Fig. 21. Cell incubated with DS-NBT in the presence of light. Reduced deposits are evenly distributed on the thylakoid bands (*T*). A single thylakoid (*arrow*) also has DS-NBT reaction product. x 92 200; Bar=0.1 μm

Fig. 22. Control cell incubated with DS-NBT in the dark. Note both thylakoids (*T*) and mitochondria (*M*) do not have reduced deposits.
x 55 200; Bar=0.2 μm



21 22

Chapter V Discussion

Our data indicate that the PS I reaction centres in the thylakoids of *T. subcordiformis* reside on both unappressed and appressed membranes. This result is surprising, since the model of thylakoid organization suggested by Anderson and Andersson (1982) for higher plants and green algae, in which PS I is largely distributed on unappressed thylakoid membranes and PS II is predominantly located on appressed thylakoid membranes, is widely accepted. To confirm our results, we used two PS I reaction centre protein antibodies: one from a higher plant, maize, the other from the cyanobacterium *Synechococcus elongatus*. Both results show that 77-82% of the labelling is localized on appressed thylakoid membranes. A cytochemical method in which DAB was used as a sensitive probe to localize PS I activity also showed that PSI activity exists on both appressed and unappressed thylakoid membranes, since DAB reaction deposits strongly stain all thylakoid lumens, those of internal thylakoids being as intensely stained as the lumens of the outermost thylakoids.

Immunogold labelling is a very good technique for studying the localization of membrane proteins; however, there are limitations in resolution. The length of the probe from the centre of the gold particle to the antigen-binding site is 5 nm (the half diameter of the gold) plus 5 nm (the length of protein A (Roth 1982)), plus 10 nm (the average diameter of an immunoglobulin G molecule). Therefore, the distance from the labelled antigen to the centre of a gold particle may be as much as 20 nm. As was mentioned in the Results section, the total width of a thylakoid is approximately 15 nm with two 3.75

nm membranes and a 7.5 nm lumen, and the narrow space between appressed membranes is 1.8 nm. Although previous results from our laboratory indicate that the number of gold particles labelling a structure falls off with the distance from the structure (Fig. 4 in Ludwig and Gibbs 1989), it is possible that a gold particle lying over the inner appressed membrane of an outermost thylakoid could be labelling a PSI antigen in the outer unappressed membrane of this thylakoid, since a thylakoid is only 15 nm wide. In fact, since a gold particle may be as much as 20 nm from the labelled antigen, a particle lying over the outermost membrane of the second thylakoid of a band could also be labelling PS I in the outermost thylakoid membrane. If we make the assumption that all gold particles over the outermost membrane of the outer thylakoid, plus all particles over its lumen, plus all the particles over the first pair of appressed membranes are in fact labelling PS I complexes in the outermost unappressed membrane, then we can calculate that there are 3.15 PS I antigens/μm on the outermost unappressed membranes and only 0.23 PS I antigens/μm on the first pair of appressed membranes. When we compare these theoretical results with the uniform distribution of PS I labelling observed over each of the membranes of the thylakoid stack (Fig. 23), we can see that they are extremely unlikely. It is, of course, just as likely that a gold particle lying over the outer membrane of an outer thylakoid is labelling an antigen over the inner appressed membrane as visa versa. The consistency of PS I concentration on each thylakoid membrane (Fig. 23) is excellent evidence that errors in labelling due to the gold particle being at its maximum distance from the labelled antigen have cancelled each other out.

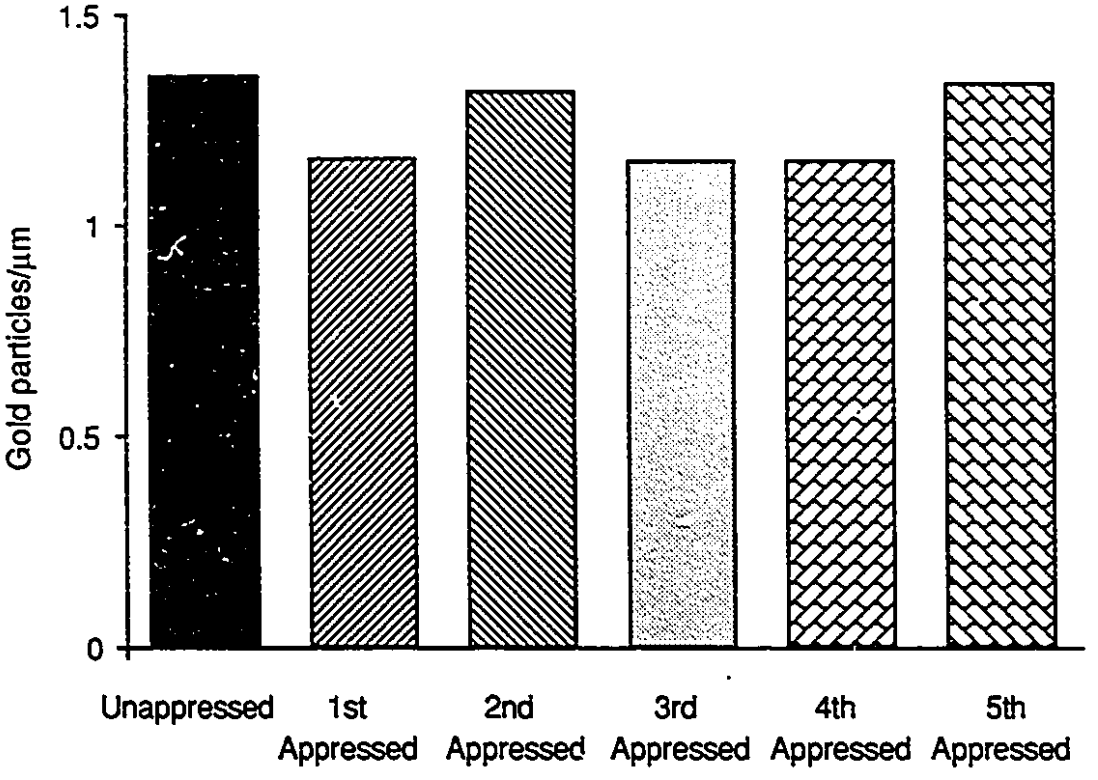


Fig. 23 The labelling densities of gold particles over varoius thylakoid membranes in cells fixed simultaneously with glutaraldehyde-osmium tetroxide. For the gold particles lying over thylakoid lumens, half are assigned to the membrane on one side of the lumen and half to the membrane on the other side.

Our experimental results may arise from some characteristics of green algal chloroplasts. First, the organization of thylakoid membranes in higher plants is different from that in *T. subcordiformis* and most other green algae. Higher plant chloroplasts have distinct grana. The grana consist of stacks of thylakoids, in which short thylakoids alternate with long thylakoids, which often extend across the chloroplast stroma to a neighbouring granum. These single thylakoids which extend out from the border of the granum are called stroma thylakoids. Their limiting membranes make up most of the unappressed membrane in the plastid. The top and bottom membranes of the grana and the edges of the grana thylakoids make up the rest. This structural arrangement of thylakoids supports state transitions, in which the energy absorbed by PS II is transferred to PS I by mobile LHC II which is phosphorylated in appressed membranes, and then moves to the unappressed stromal thylakoid membranes where it donates energy to PS I (Staehelin and Arntzen 1983). In contrast, in a primitive green alga like *Tetraselmis subcordiformis*, the chloroplast is transvered by extended thylakoid bands with each band consisting of from 2 to 20 thylakoids. Bands of 3, 4, 5, or 6 thylakoids are most common. There are no grana and single thylakoids are rare. Therefore, unappressed membranes are mostly made up of the stroma-exposed thylakoid membranes at the top and bottom of each band. Research on other groups of algae has already revealed that different thylakoid membrane organizations are associated with different distribution patterns of the photosynthetic complexes. Red algae, whose chloroplasts evolved directly from cyanobacteria, have only single thylakoids to which phycobilisomes are attached. An immunolabelling study showed that both PS I and PS II in the red alga *Porphyridium*

cruentum are almost uniformly distributed throughout the thylakoid membrane (Mustardy et al. 1992). Cryptomonads, whose phycobiliproteins are evolutionarily closely related with those of red algae and cyanobacteria, also have PS I and the chl *a/c* LHC associated with PS II distributed on both unappressed and appressed thylakoid membranes (Rhiel et al. 1989, Lichtlé et al. 1992). Brown algae and diatoms have thylakoids associated into bands of three and contain both chlorophyll *a* and *c*. Immunogold labelling showed that PSI and the fucoxanthin chl a/c LHC of PS II were distributed on both unappressed and appressed thylakoid membranes of *Fucus serratus* (Lichtlé et al. 1992) and *Phaeodactylum tricornutum* (Pyszniak and Gibbs 1992). *Euglena gracilis*, which contains chl *a* and *b*, also has PS I and PS II on both types of membranes (Brandt and Keiz 1989). Therefore, the heterogeneous distribution of PS I and PS II in higher plants must have evolved gradually. When this distribution began and how it evolved are two very interesting questions. Secondly, light-dependent thylakoid membrane phosphorylation may be different between higher plants and green algae. In higher plants, the transfer of energy between PS II and PS I is mediated by the mobile LHC II by way of state transitions in which light determines the state of thylakoid membrane protein phosphorylation (Staehelin and Arntzen 1983). However, it has been found that in *Chlamydomonas reinhardtii*, the presence of light does not noticeably alter thylakoid membrane protein phosphorylation (Owens and Ohad 1982). In the scaly green alga *Mazzoniella squamata*, which also belongs to the Prasinophyceae and has thylakoids in long bands of 2, 3, or 4 (Krämer 1988), the light energy absorbed by chl *a/b/c* LHC is transferred to both PS I and PS II without any preference and state transitions do not

occur (Wilhelm 1989). Thirdly, the molecular structure of LHC II of green algae may be quite different from that of LHC II from higher plants, since monoclonal antisera against pea LHC II can label the thylakoid membranes of pea, barley and fern by the immunogold method, but not those of *C. reinhardii* (Thaler 1986). Therefore, it is not too surprising that PS I and PS II are distributed on both types of membranes in *T. subcordiformis*, and it may indicate that the heterogenous distribution of PS I and PS II has not evolved in any primitive green algae.

Although some studies, which are mostly based on freeze fracture analysis, showed that in green algae PS I is located on unappressed membrane and PS II is mostly located on appressed membrane (Olive et al. 1983; Lacambra et al. 1984), very few green algae have been used for studying the distribution of PS I and PS II. Only *Chlamydomonas reinhardtii* has been used for immunogold labelling investigation (Vallon et al. 1985; Vallon et al. 1986) and the chloroplast ultrastructure of *Chlamydomonas* cells illustrated in these two papers seems different from that of the wild type, since the thylakoids are very swollen. Whether or not the swollen thylakoids still retain their normal physiological state is questionable. In addition, there are no micrographs in these papers for PS I labelling and the gold particles labelling CF1 (a subunit of ATPase) were very few. The preliminary immunogold labelling results from our laboratory show that PS I of *C. reinhardtii* is distributed on both unappressed and appressed membranes (N. Bertos, unpublished data). Therefore, the conclusions made in previous studies on the distribution of PSI in green algae should be reconsidered.

Our study shows that most PS II is located on appressed membranes and only a small amount of PS II resides on unappressed thylakoid membranes of *T. subcordiformis*. As we mentioned above, the immunogold labelling method has limitations in resolution. The gold particles labelling unappressed membranes or the external lumen may have come from the binding with an antigen located on adjacent appressed membranes. However, the cytochemical study using DS-NBT revealed that both appressed and unappressed membrane are positively stained. Thus, it can be concluded that PS II resides on both types of membranes.

Chapter VI Conclusion

The distributions of PS I and PS II were determined by using immunogold labelling on the thylakoid membranes of a primitive green alga *Tetraselmis subcordiformis*, a member of the Prasinophyceae which does not have grana in its chloroplast. The results of this study showed that both PS I and PS II in the chloroplast of this alga mostly reside on both appressed and unappressed thylakoid membranes in approximately equal concentrations. It may be possible that the heterogeneous distribution of PS I and PS II seen in higher plants has not evolved in the primitive green algae, which do not have grana in their chloroplasts.

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