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EVIDENCE THAT A CHLOROPLAST MEMBRANE PROTEIN IS LOCATED IN THE MITOCHONDRIA OF PHOTOSYNTHETIC AND NON-PHOTOSYNTHETIC EUGLENOIDS

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A mis padres, Duccio y Ana

A Lucas y Stephen con cariño

TABLE OF CONTENTS

Chapter 2. MATERIAL AND METHODS

A detailed description of material and methods used for this study to first localize PS I and PS II in the chloroplast of E. gracilis and second to localize the CP47 in the mitochondria of euglenoids.

LIST OF FIGURES

LIST OF TABLES

Chapter 3.

Chapter 4.

LIST OF ABBREVIATIONS

List of abbreviations used in the figures

c	Mitochondrial cristae
C	Chloroplast
ch	Chromosome
Cmb	Chloroplast membrane
Cyt	Cytoplasm
G	Golgi apparatus
M	Mitochondria
n	Nucleolus
N	Nucleus
Nmb	Nuclear membrane
p	Pellicle
r	Ribosome
s	Chloroplast stroma
th	Thylakoid
V	Vacuole

ABSTRACT

1. Distribution of the two photosystems (PS I and PS II) in the thylakoid membranes of the alga *Euglena gracilis*

The distribution of photosystem I and II (PS I and PS II) in the alga *Euglena gracilis* Z strain was studied by electron microscopic immunocytochemistry. In this alga, the thylakoids are not organized in grana structures, as they are in higher plants. Two different antibodies were used to identify PS I. One is directed against particles of PS I from maize and the other against the 60 and 62 kDa PS I reaction centre proteins of the cyanobacterium *Synechococcus elongatus*. Both antibodies demonstrated the presence of PS I in the two types of thylakoid membranes, appressed (AM) and non-appressed (NAM). Quantitative analysis showed that 60-74% of PS I is in the AM and 26-40% is in the NAM, and since about 80-90% of the membranes are AM, that PS I is more concentrated in the NAM. An antibody directed against the CP47 protein of PS II also revealed labelling in both types of thylakoid membranes (54% in AM and 46% in NAM). PS II is again more concentrated in the NAM. I demonstrated by the photo-oxidation of 3,3'-diaminobenzidine that there is PS I activity in the two types of membranes and, moreover, that there are changes in this activity during the light cycle of the cell. My results indicate that the distribution of PS I and PS II in *Euglena gracilis* Z strain is different from that of higher plants and is similar to that seen in green algae. The possible evolutionary significance of our observations are discussed.

2. Localization of the protein CP47 (plastid protein) in the mitochondria of euglenoids

The localization of the CP47 protein to the mitochondria of euglenoids was studied by electron microscopic immunocytochemistry. My results demonstrate that this protein, which is coded by chloroplast DNA in all algae and plants, is present in whole or in part in the mitochondria of *Euglena gracilis* and related euglenoids. I used two different antibodies against the protein CP47 (anti-CP47 from *Chlamydomonas reinhardtii* and *S. elongatus*) to test wild-type, light-grown, cells of *Euglena*. Both antibodies selectively labelled the mitochondria. These results furthermore suggest that this labelling is particularly associated with mitochondrial cristae. Anti-CP47 from *S. elongatus* also labelled the mitochondria of other euglenoids, such as dark-grown cells of *Euglena gracilis*, the mutant Y9Z1NaL, and *Astasia longa*. Since the CP47 protein is present in dark-grown cells and in the mutant Y9Z1NaL, which are organisms that do not have an active *psbB* gene, I suggest that a gene transfer has occurred from the plastid to the mitochondria during evolution. Because our results show the presence of CP47 in the mitochondria of *Astasia longa*, I postulate that the transfer occurred before the branching of *Astasia* from *Euglena*.

RESUME

1. Distribution des deux photosystèmes (PS I et PS II) dans les membranes thylacoïdales de l'algue *Euglena gracilis*

La distribution des photosystèmes I at II (PS I et PS II) dans l'algue *Euglena gracilis* de type Z a été étudiée par une combinaison d'immunocytochimie et de microscopie éléctronique. Dans cette algue, les thylacoïdes ne sont pas organisés en grana, comme ils le sont dans les plantes supérieures. Deux anticorps différents ont été utilisés pour identifier le PS I de cette algue: le premier est dirigé contre une des particules de PS I du maïs et l'autre contre les protéines de 60 et 62 kDa du centre de reaction du PS I de la cyanobactérie *Synechococcus elongatus*. Les deux anticorps ont révélé la présence de PS I dans les deux types de membranes thylacoïdales: les juxtaposées (AM) et sur les non-juxtaposées (NAM). Une analyse quantitative de l'immunocytochimie a démontré que 60-74% du PS I se trouve sur les AM et 26-40% sur les NAM. Puisque les AM répresentent 80-90% de la totalité des membranes, la concentration de PS I est donc plus grande sur les membranes non-juxtaposées. De même, un anticorps dirigé contre la protéine CP47 du PS II a révélé la presence de ce photosystème sur les deux types de membranes thylacoïdales (54% sur les AM et 46% sur les NAM). Il en résulte que PS II est aussi en plus grande concentration dans les membranes non-juxtaposées. L'activité de PS I dans les deux types de membranes thylacoïdales a été démontrée par la photo-oxidation du 3,3'-diaminobenzidine. Cette méthode a aussi permis de révéler des changements dans l'activité de PS I au cours du cycle de lumière de la cellule. L'ensemble de ces résultats indique que la distribution de PS I et PS II dans l'algue *Euglena gracilis* de type Z est différente de celle des plantes supérieures et plus rapprochée de celle des algues vertes. L'importance possible de ces observations pour la compréhension de l'évolution des euglenoids est discutée.

2. Localization de la protéine CP47 (protéine du chloroplaste) dans les mitochondries des euglenoïdes

La présence de la protéine CP47 sur les mitochondries des euglenoïdes a été étudiée par une combinaison d'immunocytochimie et de la microscopie électronique. Mes résultats démontrent que cette protéine, qui est encodée par l'ADN du chloroplaste dans toutes les algues et les plantes, est présente en entier ou en partie dans les mitochondries d' *Euglena gracilis* et de certains autres euglenoïdes apparentés. Pour cette étude, j'ai fait l'utilisation de deux anticorps différents contre la protéine CP47 (un anti-CP47 de *Chlamydomonas reindhartii* et un anti-CP47 de *S. elongatus*) pour tester des cellules de type sauvage d' *Euglena* cultivées à la lumière. Chacun des anticorps a marqué les mitochondries d'*Euglena* de manière selective. De plus, le marquage est particulièrement associé aux crêtes mitochondriales. L'anticorps anti-CP47 de *S. elongatus* a aussi marqué les mitochondries d'autres euglenoïdes tels: les cellules de type sauvage d' *Euglena* cultivées dans l'obscurité, la mutante Y9Z1NaL et *Astasia longa*. Puisque la protéine semble être présente dans les cellules cultivées dans l'obscurité ainsi que dans la mutante Y9Z1NaL, qui sont tous des organismes qui ne possèdent pas de gène *psbB* actif, je propose qu'un tranfert de ce gène, du chloroplaste vers la mitochondrie, s'est produit au cours de l'évolution. Finallement, puisque mes résultats démontrent aussi un marquage sur les cellules *d'Astasia longa*, je suggère que le transfert s'est produit avant l'embranchement d'*Astasia* de la lignée d'*Euglena*.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

The following constitute original contributions to understanding:

1. The organisation of the photosynthetic proteins (PS I and PS II complexes) in the thylakoid membranes of the chloroplast of the unicellular alga *Euglena gracilis*. Chapter 3 describes in detail how:

1.1 Using immunoelectron cytochemistry techniques, I revealed for the first time that:

1.1.i. Photosystem I is localized on the appressed and non-appressed thylakoid membranes of *Euglena gracilis* Z strain. Quantitative analysis shows that the labelling density in non-appressed membranes is higher (four fold with anti-PS I from maize and six fold with anti-cp1-e from *Synechococcus elongatus)* than in the appressed membranes of the thylakoids.

1.1.ii Photosystem II is localized on both the non-appressed and appressed thylakoid membranes of *Euglena gracilis* Z strain. Quantitative analysis shows that the labelling density in non-appressed membranes is four times higher than in the appressed membranes of the thylakoids.

1.2 Using cytochemical techniques (photooxidation of DAB), I showed for the first time that the Photosystem I activity was also detected in both types of membranes of *E. gracilis*. Moreover, the location of this activity changes during the dark-light cycle. At the end of the dark-cycle, the activity is restricted to the outer non-appressed thylakoids; however, if the cells were tested during the light-cycle the activity becomes apparent in all the thylakoids of a band.

2. As well as describing for the first time that a plastid protein, namely CP47 a core protein of Photosystem II, is localized in the mitochondria of euglenoids, Chapter *4* demonstrates:

   2.1 The localization of CP47 in the mitochondria of *Euglena gracilis* Z strain by two different antibodies (anti-CP47 from *S. elongatus* and anti-CP47 from C. *reinhardtii).* The detection of a plastid protein in the mitochondria by two different antibodies (raised against the same protein from two different types of algae) makes the recognition of a random similar epitope unlikely. I then tested for the presence of the protein in the mitochondria where the chloroplast was not actively translating CP47:

   2.2 Dark-grown cells of *Euglena gracilis* Z strain (do not contain functional chloroplasts but proplastids) were labelled with anti-CP47 from *S. elongatus*; quantitative analysis makes evident that the mitochondria are labelled in a significant manner. This suggests that the *psbB* gene, which codes for the chloroplast CP47 protein (or part of it), was transferred to the mitochondria (or nucleus) during evolution.

   2.3 The mutant of *Euglena gracilis* Y9Z1NaL, which does not express the CP47 protein, was labelled as well with the antisera against the CP47 from *S. elongatus* and its mitochondria were significantly labelled. I then decided to study if the transfer of the *psbB* gene ocurred in euglenoids before or after the colourless species *Astasia longa* evolved from *E. gracilis.*

   2.4 Immunolabelling of *Astasia longa* with antisera against CP47 of *S. elongatus* shows that the mitochondria of that organism are significantly labelled, suggesting that the transfer of the *psbB* (or a significant part of it) to the

mitochondria or nucleus from the chloroplast of *E. gracilis* took place before the branching of *Astasia longa* from *E. gracilis*.

3. Data from Table 7 give preliminary observations suggesting that the CP47 protein is associated with the cristae of the mitochondria of euglenoids.

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To Lucas and Stephen: "Well kids, now Mamá has finally finished her thesis..." (¡gracias por aguantar!)

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

An overview of the localization of Photosystem I and II in plants and algae and a brief description of promiscuous DNA in the mitochondria of plants and algae.

Preamble

Chapter 1 will be divided into two different parts. The first will give an overview of what is known of the distribution of Photosystem I (PS I) and Photosystem II (PS II) in the thylakoid membranes of the different types of algae. This will be followed by some ideas on how the morphology of the chloroplasts can be used to clarify some evolutionary relationships between algae. The second part will give an overview on what is currently known on interorganellar DNA movement in plants and algae. This will be followed by a brief description of the preliminary results that led to this study.

A) Localization of PS I and PS II in *Euglena gracilis*

The oxygenic part of the photosynthetic process is important for life on earth. In eukaryotic organisms, this process occurs in the chloroplasts. Knowing the structural morphology of these organelles, or more specifically of the membranes where the proteins involved in the light reactions of photosynthesis are located, gives an insight on how the physical and chemical steps occur between the trapping of the energy of sunlight to the conversion of this energy into chemical forms.

1. Morphology of chloroplasts

Chloroplasts are organelles located in the cells of plants and algae and are surrounded by an envelope, which is composed of two (most commonly), three or even four membranes. The number of chloroplasts varies greatly between different types of cells, *i.e.*, one single plastid (in *Chlorella*) or up to sixty (in cells of higher plants). Two regions can be distinguished in the chloroplasts: the stroma (soluble portion) and the internal membranous structures known as thylakoids. The thylakoid membranes and their lumens are the place where the light reactions of photosynthesis occur.

2. Organization of the thylakoids

The organization of the thylakoids (*i.e.*, the number and the manner of stacking together), as well as the presence or absence of various light-harvesting systems is unique to each type of algae[1] and higher plants. This organization will be detailed in the following sections, but the main characteristic which is important to understand the subsequent chapters is that the thylakoid membranes are differentiated into appressed (when they abut other thylakoids) and non-appressed (when they are in contact with the chloroplast's

stroma) membranes. Some photosynthetic organisms have their thylakoids arranged such that they run singly, while others exhibit a stacked arrangement, or a combination of both (Illustration 1). The grana and stromal thylakoids of higher plants have the most complex arrangement. It is well accepted that the thylakoid membranes and their lumens are the place where the light reactions of photosynthesis occur. Therefore, it is important to know in detail the organization of the light-harvesting and reaction center proteins of these specialized membranes.

3. Organization of the Photosystems in the thylakoid membranes

The distribution of the two different photosystem (PS) complexes and other proteins involved in photosynthesis in the thylakoid membranes of chloroplasts of photosynthetic organisms is of importance because it may give a clue to the evolution of the photosynthetic process, as well as provide additional information on the evolution of algae and higher plants.

Four membrane-bound multiprotein complexes (Photosystem I (PS I), Photosystem II (PS II), cytochrome b_6/f (cyt b_6/f), and the ATP synthase), plus other electron carrier proteins, as well as light-harvesting proteins and the chlorophyll antenna complexes, are organized in a specific manner in the thylakoid membranes. The so-called Z scheme (Illustration 2) explains how the light energy is harvested and used to power the transfer of electrons from H_2O to NADP. Photons are absorbed by the double bonds of the porphyrin ring of the two main pigments, chlorophyll *a* and chlorophyll *b*, and by the accessory pigments of PS II, thereby elevating them to an excited state. This excitation energy is then transferred through the antenna complex to P680, one of the special pair of chlorophyll *a* molecules in PS II, complex converting it into its excited state, $P680^*$. $P680^*$ donates an electron to pheophytin *a*, producing a positive charge called an "electron hole." This hole will then be filled by the photolysis of H_2O (effectuated by the water-

[1] In the following literature review, we will discuss the cyanobacteria (blue-green algae), under the algae

Illustration 1. Schematic representation of appressed and non-appressed thylakoid

Illustration 1 shows the different arrangements of the thylakoids. In the algae, thylakoids tend in their majority to form bands. In the higher plants the thylakoids are organized in grana. The drawing shows an enlargement of thylakoids forming grana. (a combination of Figure1-7 in Lee, 1980 and Figure 8.15 in Taiz and Zeiger, 1991

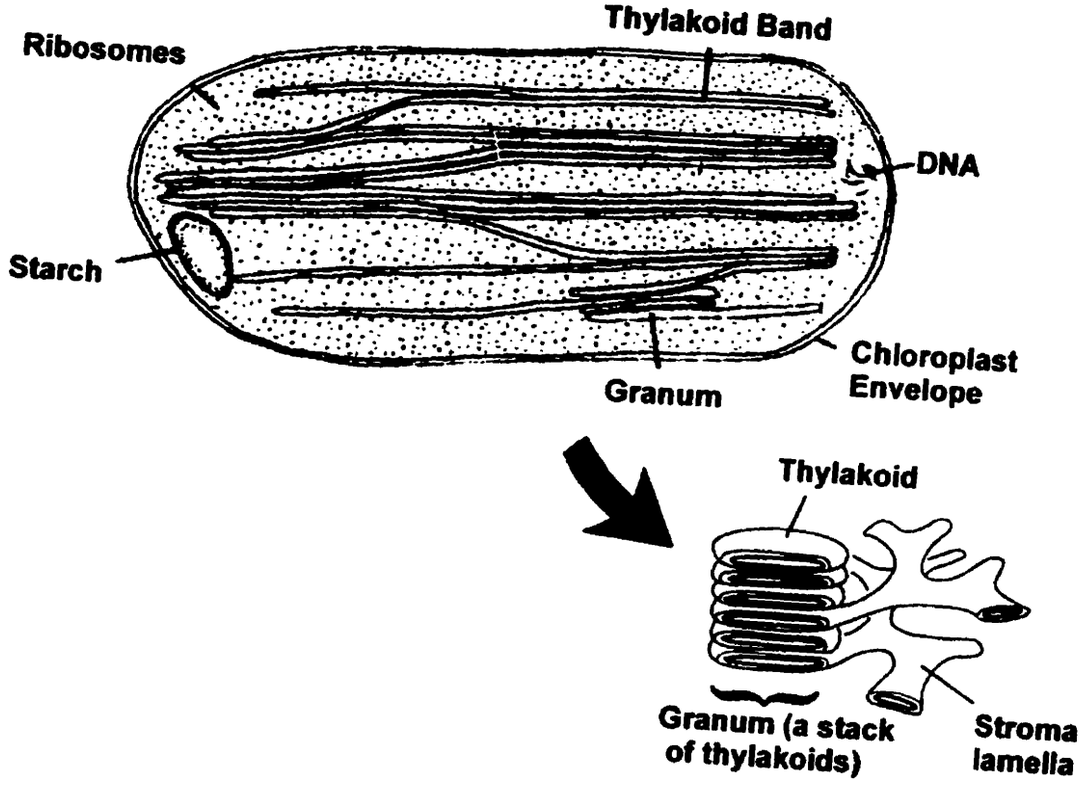
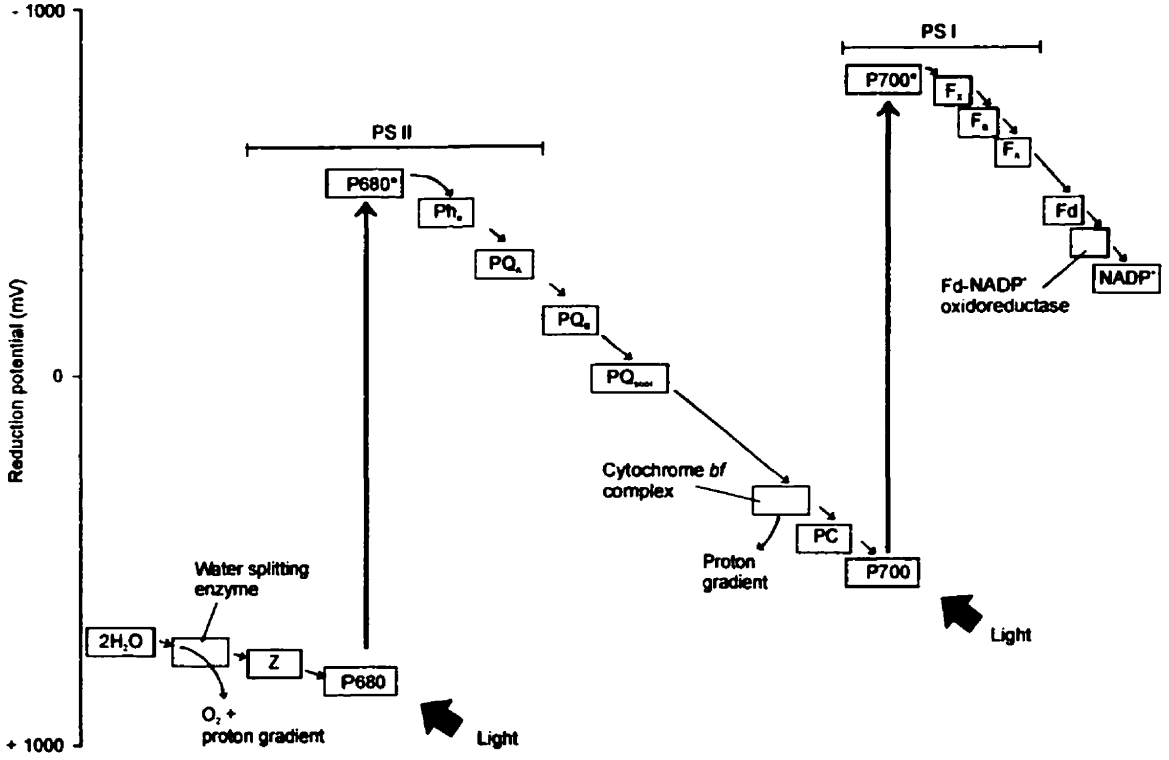

Thylakoid Band
Ribosomes
DNA
Starch
Chloroplast Envelope
Granum
Thylakoid
Granum (a stack of thylakoids)
Stroma lamella

Illustration 2. Diagram of the Z scheme

Illustration 2 shows the pathway followed by the electrons during photosynthesis. (Figure 18.9 in Rawn, 1989)


- 1000
0
+ 1000
Reduction potential (mV)
PS II
P680*
Ph
PQ
PQ
PQ
Cytochrome bf complex
PC
P700
Proton gradient
PS I
P700*
F
F
F
Fd
Fd-NADP+ oxidoreductase
NADP+
Water splitting enzyme
2H2O
Z
P680
O2 + proton gradient
Light
Light

splitting enzyme that is associated with PS II) that produces O_2 and 4 electrons and 4 protons. The electron-chain transfer begins as pheophytin *a* transfers its acquired electron to a pool of different plastoquinone molecules, then to cyt b_6/f which passes it to plastocyanin and finally to the "electron hole" of the PS I reaction center. Light excites the P700 of PS I to $P700^*$ and the electrons are transferred through a series of iron-sulfur proteins of PS I and finally to ferrodoxin. The Fe-$NADP^+$ oxidoreductase converts $NADP^+$ into NADPH in the chloroplast stroma.

The organization of the different protein complexes present in the two regions of the thylakoid membranes, appressed (AM) and non-appressed (NAM), has been studied in order to better understand the process of photosynthesis. It is accepted that these two regions have different composition and functional properties (Ojakian and Satir, 1974; Anderson, 1981).

4. Model of lateral heterogeneity

It is now widely accepted that PS I and PS II are segregated in different parts of the thylakoid membranes of chloroplasts. This model was first proposed by Andersson and Anderson.

In 1980, Andersson and Anderson reported a lateral heterogeneity in the distribution of the chlorophyll-protein complexes of the thylakoid membranes of chloroplasts in spinach. Chloroplasts were isolated and the grana separated from the stroma thylakoids using differential centrifugation and phase partition. SDS-PAGE was then used to measure chlorophyll-protein complexes in the two fractions. The amounts of chlorophyll protein found in the fractions derived from appressed (grana) and non-appressed (stroma) thylakoids were different. The stroma thylakoids had a high PS I content and much less PS II and light-harvesting chlorophyll-protein complex. The grana regions showed almost no PS I reaction center proteins, but high levels of PS II and light-harvesting proteins. Their data suggests also that the light-harvesting proteins were mainly

associated with PS II in the appressed thylakoid membranes. They concluded that there is a lateral heterogeneity in the distribution of the thylakoid proteins. PS II and light-harvesting proteins are located mainly in the grana regions, and PS I is located mainly in the non-appressed regions with ferrodoxin-NADP and the chloroplast ATP synthase.

This organization is thought to be important because it helps to explain some physiological consequences, *i.e.*, the need of mobile electron carriers between PS II and PS I, and prevention of "spillover" as was proposed as a hypothesis (Anderson, 1981; Anderson and Andersson, 1982).

This model was established mainly by experiments in higher plants, but was thought to be the general organization of thylakoid membrane proteins in all organisms that contain chloroplasts. Studies performed in algae, however, have shown that this model does not reflect the distribution of the photosynthetic proteins in their thylakoid membranes. We will review in the next section studies made on the distribution of PS I and PS II in different type of algae and briefly on higher plants.

5. Localization of PS I and PS II in algae and higher plants

I) Localization of the PS in the Cyanophyta

Blue-green algae, the Cyanophyta, are prokaryotic algae which perform oxygenic photosynthesis and contain chlorophyll *a* and phycobiliproteins as light-harvesting complexes. The thylakoids of these organisms are not organized in stacks as they are in higher plants and most algae. *Synechococcus*, for instance, has 3 or 4 single thylakoids arranged concentrically around the periphery of the cell. *Synechocystis*, on the other hand, has thylakoids that protrude radially into the cytoplasm from two or three poles located along the cytoplasmic membrane. These thylakoids all have phycobilisomes attached to their outer surfaces, which rules out the possibility of thylakoid stacking.

Few studies have been done on the localization of PS I and PS II in this group of algae. A discontinuous sucrose density gradient centrifugation was used in 1993 to isolate

four different membrane fractions from *Synechocystis* (Hinterstoisser *et al.*, 1993). These fractions were subsequently analyzed by biochemical methods, and it was concluded that there was a possible interaction between the plasma membrane and thylakoid membranes through the thylakoid centers (sites of contact between the thylakoid membranes and the cell membrane) for the synthesis and final distribution of chlorophyll in cyanobacteria. There is, therefore, a continuity between thylakoid and plasma membranes (Hinterstoisser *et al.*, 1993). In the same year, the regulation of the stoichiometry of PS I and PS II was studied in response to light in the same species (Murakami and Fujita, 1993). These authors concluded an inhibitor caused a decrease in the PS I/PS II ratio when stimulated with wavelengths of light that specifically activates either PS I or PS II (700 and 680 nm, respectively). The new observation was that the inhibitor suppresses the normal stimulation of PS I formation under PS II light. The model proposed was that the formation of the PS I complex can be controlled by the regulation of the synthesis of apoproteins and/or groups like chlorophyll *a*. In 1994, Sherman *et al.* published an elegant study on the localization of PS in the cyanobacterium *Synechococcus*. Cells were freeze-substituted, sectioned, labelled with antibodies and viewed using a transmission electron microscope. Proteins D1, D2, CP43, and CP47 (all part of the PS II complex) were clearly localized in an even manner throughout the thylakoids. The proteins PsaA and PsaB of the PS I complex and the ß-subunit of ATP synthase were found mostly on the outermost thylakoid and on the cytoplasmic membrane. They also reported (without showing data) that in two other blue-green algae, *Cyanothece* and *Synechocystis* which have thylakoids which protrude radially into the cytoplasm, PS I and PS II are evenly distributed on all thylakoids.

The PS I complex from *Synechococcus* has been isolated by a one step purification using a recombinant antibody Fv fragment containing an affinity tag (Tsiotis *et al.*, 1995). The gene products of *psaA*, *psaB*, *psaC*, *psaD*, *psaE*, *psaF* and *psaL* were identified by gel electrophoresis. The structure of the PS I was determined to be trimeric. A preferential distribution of PS I in the stromal side of the thylakoids of *Synechococcus*, in an even

pattern, was confirmed using immunocytochemical EM techniques, corroborating the work of Sherman *et al.* (1994).

The distribution of the PS in the thylakoids of cyanobacteria is therefore asymmetrical with ATP synthase and PS I preferentially located on the outermost thylakoids and PS II distributed equally on all thylakoids. It thus appears that all cyanobacteria where thylakoids are arranged concentrically have asymmetrical distribution of PS and ATP synthetase. In species that have their thylakoids radiating towards the center, however, the distribution appears to be uniform.

II) Localization of the PS in the Chromophyta

In the chromophyte algae, the majority of which are marine algae, the chloroplasts contain chlorophyll *a* and *c*, and a variety of carotenoids and xanthophylls. In the case of the Cryptophyceae, they also have phycobiliproteins. In this group, the thylakoid membranes are arranged such that they form bands crossing the chloroplast, but no real grana organization is observed. In most chromophyte algae, the bands are formed of three thylakoids, but unlike those in the other groups, these are not tightly appressed. The exception to this are Cryptophyceae which have lamellae of two thylakoids running through the chloroplast.

Only few immunolabelling studies have been done on the chromophytes. Immunocytochemical studies have been performed in the Cryptophyceae to localize the light-harvesting complexes associated with PS I and PS II and this gives an indirect localization of both types of PS. In 1989, Rhiel *et al.* showed that in *Cryptomonas maculata* embedded in Lowicryl resin and labelled with antisera against the chlorophyll *a/c* LHC associated with PS II, protein-A gold particles were distributed in an even manner along the paired thylakoids. Nitrogen (N) deprivation indicated that the labelling density was similar in presence or absence of N (39 ± 12 gold particles/μm^2 and 39 ± 28 gold particles/μm^2 respectively). Earlier freeze fracture studies (Lichtlé *et al.*, 1986) have

shown that it was possible to distinguish between the two faces of appressed and non-appressed thylakoid membranes. To further elucidate this, Lichtlé *et al.* (1992) investigated the distribution of PS I and the chlorophyll *a/c* LHC protein in *Cryptomonas rufescens*. In cells fixed in glutaraldehyde and embedded in LR White labelled with anti-PS I from spinach, the gold particles were present in both types of membranes of the thylakoids, but were more concentrated in the non-appressed membranes (external of the pair) than in the appressed (internal) (56.5% and 43.5% respectively). Whereas when cells of *C. rufescens* were labelled with antibodies against the 19 kDa chl *a/c* LHC of PS II, 48.2% was on the non-appressed (NAM) and 54.2% on the appressed membranes (AM). Since the pairs of thylakoids have equal lengths of appressed and non-appressed membranes, the data of Lichtlé *et al.* (1992) indicated that PS I was slightly more concentrated on the NAM and PS II in the AM, but the asymmetry was small.

In the Phaeophyceae (brown algae), Lichtlé *et al.* (1992) demonstrated in *Fucus serratus* by immunogold labelling techniques that LHC is distributed equally on the appressed and non-appressed membranes (17% and 16%, respectively) . However PS I was found to be localized differently between the two types of membranes (24% on each non-appressed and 13% on each appressed membrane). Therefore, the non-appressed membranes of this type of algae are enriched on PS I particles.

In the Bacillariophyceae, in a study on the diatom *Phaeodactylum tricornutum*, two antibodies against PS I and one against the 19 kDa protein of the fucoxanthin chl *a/c* LHC were used (Pyszniak and Gibbs, 1992). PS I was localized by labelling Lowicryl sections of cell with anti-PS I from maize in both types of membranes: appressed and non-appressed, but the concentration of PS I was higher in the two outer thylakoid membranes, namely non-appressed ones. This result was also corroborated with the photooxidation of DAB: the two outer thylakoids of each band were heavily stained while the middle one had some stain but not as intense. PS II was indirectly localized by the use of an antiserum raised against the 19 kDa fucoxanthin-chlorophyll *a/c* LHC of *P. tricornutum*. The labelling showed that this complex is equally distributed in both types of

thylakoid membranes (Pyszniak and Gibbs, 1992). However, it appears likely now that this LHC also feeds excitation energy to PS I, so this study needs to be repeated using an antibody against a specific PS II protein.

In summary, in this group of algae when the thylakoid are arranged in pairs, the dispersal of the photosystems is close to equal in both types of thylakoid membranes. When the thylakoid are organized in bands of three, PS I and PS II are present in both appressed and non-appressed membranes, but the former is more concentrated in the non-appressed thylakoid membranes.

III) Localization of the PS in the Rhodophyta

In the rhodophytes (red algae), which contain chlorophyll *a* and phycobilisomes, the thylakoids are not stacked in grana, but rather run singly in the chloroplast. In these organisms, thylakoids may be described as stromal (which run in the stroma of the chloroplast) and pyrenoidal (which run through the pyrenoid). The major light-harvesting proteins in red algae are the phycobiliproteins, which aggregate to form the phycobilisomes (PB). The PB are water-soluble and are known to be attached to the external surface of the photosynthetic thylakoids. The energy transfer therefore is directional and runs from the PB to the PS II.

Studies utilizing freeze-fracture techniques have shown that putative PS II particles in the exoplasmic face (EF) of the thylakoid membrane coincide with the particles present on the outer surface of the thylakoid membrane thought to correspond to the PB (Morschel and Muhlethaler, 1983; Giddings *et al.*, 1983). These data led to the conclusion that PB are in close association with PS II. This structural observation was reiterated in 1985 by Chereskin *et al.*. This group was able to purify a PS II-PB particle from thylakoids of the red alga *Porphyridium cruentum* by solubilization with detergent in a sucrose high ionic medium. This preparation, which lacked PS I and had low chlorophyll *a* content, was functionally active because it was able to evolve O_2. Once the association of

the PB with PS II was clearly established, scientists tried to investigate the stoichiometry of PS I and PS II. There was a two-fold increase in the ratio of PS II to PS I in *P. cruentum* grown under red light compared to those grown under orange light, whereas the ratio of PS II to PB was unaltered (Fujita *et al.*, 1985; Ohki *et al.*, 1987). It was found later that in *P. cruentum* grown under four different densities of white light (6 to 280 ($\mu E.m^{-2}.s^{-1}$), the ratios of PS II to PS I (varying from 0.43 to 0.54) remained relatively constant and three PS II centers for each PB were present under all conditions (Cunningham *et al.*, 1989). Cunningham *et al.* (1990) examined the numbers of PS I, PS II, and PB of *P. cruentum* grown under light absorbed primarily by PS I or PS II. They concluded that the ratio of PS II to PB is not fixed and varies according to the wavelength of light; ratios of PS II to PS I increased five-fold from 0.26 in cells grown in green light to 1.21 in cells grown in red light. The number of PS II per PB also changed from 1.6 in green light to 4.2 in red light. The total PS/PB ratio, however, remained constant under the different conditions of light. In summary, it is now accepted that up to four, but at least two, PS II complexes may be attached to each PB (two being the minimum). The distribution of PSI was not determined by this method.

Although biochemical studies give a good idea about the presence of PS in the thylakoid, the distribution of PB on the stromal versus the pyrenoidal thylakoids remained controversial. The localization of PS I and PS II was elucidated by McKay and Gibbs (1990) using immunological and cytochemical techniques. When cells of *P. cruentum* were labelled with antisera of phycoerythrin, gold particles were not associated with the intrapyrenoidal thylakoids, but were present on the stromal thylakoids, usually on their outer surface (87.2±3.0 particles/μm^2). The photooxidation of DAB was used to demonstrate the PS I activity was located on all types of thylakoids in the chloroplast of this red alga. Dense deposits were found in both stromal and pyrenoidal thylakoids. The photoreduction of DS-NBT was used to detect PS II activity in the lumen of stromal thylakoids, but PS II activity was absent from intrapyrenoidal thylakoids (McKay and Gibbs, 1990). The authors suggested that further studies were necessary to determine if

the absence of PS II activity in the pyrenoid thylakoids was due to a lack of PS II complexes or to a lack of PS II LH antenna pigments. Later that year Mustardy *et al.* (1990) made similar observations on the distribution of the photosynthetic proteins, after localizing seven chloroplast proteins. The major apoproteins of PS I (56 and 64 kDa) were found in both stromal and pyrenoidal thylakoids, while the core proteins of PS II (D2 and 45 kDa Chl-binding protein), the phycobilisomes (the 91 kDa PB-thylakoid linker protein and allophycocyanin) and the β subunit of the ATP synthase were present in the stromal thylakoids but not the pyrenoidal ones. In contrast, the 60 kDa core Chl-antenna proteins of PS I were found on both pyrenoidal thylakoids as well as those in the stroma (Cunningham *et al.*, 1991). When no distinction between the stromal and pyrenoidal thylakoids is made, the distribution of PS I and PS II is uniform in the thylakoids of *P. cruentum* (Mustardy *et al.*, 1992)

In summary, there is a structural and functional heterogeneity in the two types of thylakoid membranes of red algae between the pyrenoidal and stromal thylakoids, but when one considers only the stromal thylakoids, the distribution is uniform.

IV) Localization of the PS in the Prochlorophyta

The Prochlorophyta are prokaryotic organisms that contain chlorophyll *a*, *b* and in some species also *c*, but do not posses phycobiliproteins as do cyanobacteria. The organization of the thylakoid membranes present in the cell are different for each species, but the thylakoids are always tightly appressed.

The thylakoid organization of *Plochlorothrix hollandica* was studied by van der Staay and Staehelin (1994). After obtaining a grana-enriched fraction, they observed that the fraction did not present any PS I. Their conclusion was that PS II and chlorophyll *a/b* antenna proteins were highly enriched in the grana stacks, whereas PS I was segregated to the unstacked stromal thylakoids. These results, which are similar to the one observed in higher plants, are in disagreement with other studies done in the same group of algae

(Bullejahn and Post, 1993; Lichtlé *et al.*, 1995). An immunocytochemical study of two strains of *Prochlorococcus* showed that two different antibodies directed against PS II (anti-CP43 and anti-D2) labelled all the thylakoid membranes, both appressed and non-appressed. Antibodies directed against PS I resulted in a similar distribution. Dual labelling with anti-PS I and anti-D2 confirmed that PS I and PS II are not segregated on different thylakoid membranes (Lichtlé *et al.*, 1995).

Data seems to support the idea that in Prochlorophyta, PS I and PS II are distributed in both types of membranes: stacked and unstacked.

V) Localization of the PS in the Chlorophyta

Green algae, the Chlorophyta, are organisms that contain chlorophyll *a* and *b*. Their thylakoid membranes are usually organized in bands of two to six appressed membranes, but larger stacks can be found. There are some single thylakoids connecting the bands, and some that detach themselves from the stacks and run singly before fusing with a band again. Only the Charophyceae, the group most closely related to higher plants, have definite grana stacks connected by single thylakoids as in higher plants.

The distribution of PS I and PS II has been best studied in the green alga *Chlamydomonas reinhardtii*, a member of the Chlorophyceae. Freeze-fracture studies show that the distribution of particles on the different faces is not homogenous. The PS I complex is restricted to non-appressed membranes (Pfu face) (Pendland and Aldrich, 1973; Olive *et al.*, 1983), while the PS II particles appear to be present only in the appressed membranes (Efs face) (Pendland and Aldrich, 1973; Olive *et al.*, 1979; Wollman *et al.*, 1980). There is, however, some evidence that in *Chlorella sorokiana*, PS II could be also present in the appressed regions (Lacambra *et al.*, 1984). These studies agree with the model of lateral heterogeneity established for higher plants. Other efforts have been made to approach the same question employing different techniques and convincing data has been obtained with immunocytochemistry (Vallon *et al.*, 1985). Cells of *C. reinhardtii*

were labelled with four different antibodies against PS II. In each case, most of the labelling was over the appressed thylakoid membranes (90% of the labelling), the density of labelling being 3.0 to 3.5 times higher in the appressed thylakoids than in the non-appressed. A subsequent study tested two other antibodies against PS II (D1 and D2 proteins) as well as antibodies against PS I (apoprotein). Again 90% of the labelling was found on the appressed membranes for the antibodies recognizing PS II (density three to four times higher than in non-appressed membranes), whereas the labelling with the anti-PS I was localized exclusively on the non-appressed membranes (Vallon *et al.*, 1986). Olive and Vallon (1991) concluded that the distribution of PS I and PS II in *C. reinhardtii* is the same as in higher plants and follows the lateral heterogeneity model.

The distribution of the two photosystems in the thylakoid membranes of the primitive green alga *Tetraselmis subcordiformis* (Prasinophyceae) was studied by cytochemical detection and immunolabelling (Song and Gibbs, 1995). Both methods detected PS I and PS II in both types of membranes. Three different antisera were used (two against PS I and one anti-PS II), and all were approximately equally concentrated on all the thylakoid membranes (76% to 78% of gold particles were on the appressed membranes). The activities of PS I and PS II were detected by the photooxidation of DAB and the photoreduction of DS-NBT, respectively, in both types of membranes. The authors concluded that PS I and PS II are not segregated in this primitive green algae as they are in higher plants, but rather are uniformly distributed as in other types of non-green algae (Song and Gibbs, 1995).

Up to 1998, one could have accepted that the localization of the PS was different in primitive green algae from the one in the Chlorophyceae, but another study of *C. reinhardtii* was published by Gibbs' group. The distribution of PS I and PS II was studied by immunocytochemistry using three different antibodies (one against PS I and two against PS II). All the antisera labelled both types of membranes at similar densities, being slightly more concentrated in the non-appressed membranes (Bertos and Gibbs, 1998). The authors concluded that the distribution of the PS is similar to the ones reported for other

types of algae and different from the one proposed by Andersson and Anderson (1980). Their study is convincing since all of their distribution data (of different fixations and growing conditions) show similar labelling when analyzed. It is difficult to believe that *Tetraselmis* and *Chlamydomonas*, which both belong to the Chlorophyta and which have the same organization of thylakoids, would have evolved a different distribution of their photosynthetic proteins. Bertos and Gibbs (1998) also reproduced the growing conditions of Vallon and coworkers, and the discrepancy between their results and those of Vallon was explained to be due to the possible disruption of thylakoid organization under Vallon's conditions of specimen preparation; *i.e.*, Vallon used cells broken by Yeda press and their fixative had a very low osmolarity. These explanations are satisfactory. The micrographs shown by Bertos and Gibbs of cells of *C. reinhardtii* grown under the same conditions as Vallon's group did add a clear visual evidence since they show very clear thylakoid bands not distorted at all.

In summary, in the green algae, photosystems I and II are distributed in a uniform pattern along the two types of thylakoid membranes: appressed and non-appressed.

VI) Distribution of the PS in the higher plants

There is a consensus in the literature that PS I in higher plants is almost completely restricted to non-appressed membranes (Staehelin, 1986; Andersson and Anderson, 1980). This conclusion is a result of several types of experiments using distinct techniques such as immunogold electron microscopy (Goodchild *et al.*, 1985; Vallon *et al.*, 1986), biochemical techniques (Andersson and Anderson, 1980; Anderson and Melis, 1983) and the measurement of particle sizes after treatment by freeze-fracture and freeze-etch techniques (Simpson *et al.*,1983; Simpson *et al.*, 1989). Two types of PS I are present in the thylakoid membranes; PS Iα is located in the end membranes of the stacks of the thylakoids and PS Iβ on the stromal thylakoids (Andreasson *et al.*, 1988; Andreasson and Albertsson, 1993). Although most experiments show that PS I is located only on the

non-appressed membranes of the thylakoids of higher plants, there are some cytochemical results that contradict this theory (Nir and Pease, 1973; Wrischer, 1978).

The localization of PS II has been studied by the same means as that of PS I, and 70 to 80% of the PS II is localized in the stacked regions of the grana thylakoids. As in the case of PS I, there are also two distinct populations of PS II (Melis and Homann, 1978): PS IIα and PS IIβ, which are present in the appressed and non-appressed regions of the thylakoids respectively (Anderson and Melis, 1983).

The distribution of the two photosystems has been extensively studied in higher plants and less so in the algae, but there is, nonetheless, a pattern in their distribution. It seems that in algae, that have a less complex organization of their thylakoids than in higher plants, there is as well an almost uniform distribution of PS I and PS II in the thylakoid membranes. To summarize briefly, in the red algae *Porphyridium cruentum*, which has single thylakoids, both photosystems are homogeneously distributed in the thylakoid membranes (Mustardy *et al.*, 1992). In the cryptomonads, which have thylakoids stacked in pairs (Lichtlé *et al.*, 1992), in the brown alga *Fucus serratus* (Lichtlé *et al.*, 1992) and in the diatom *Phaeodactylum tricornutum* (Pyszniak and Gibbs, 1992), both of which have thylakoids arranged in band of three, PS I and the light-harvesting chlorophyll *a/c* protein, associated with PS II, were found to be equally concentrated or nearly equally concentrated in the appressed and non-appressed membranes. In the green algae also both photosystems were found in appressed and non-appressed membranes (Song and Gibbs, 1995; Bertos and Gibbs, 1998).

These results suggest that since the different types of algae have different organizations of thylakoid membranes as well as different types of chlorophylls, the separation of the two photosystems may have evolved gradually. In an unpublished thesis Kerr (1997) has shown that in the Charophycean alga *Coleochaete scutata*, many thylakoid are arranged in grana-like structures and PS II is 0.6 times more concentrated on the NAM than on the AM and PS I is 2.6 times more concentrated on the NAM. This

suggests that the segregation of photosystems has started to occur in this immediate ancestor of green plants. Studies on the localization of PS I and PS II could therefore be used to elucidate evolutionary relationships between algae and higher plants.

In order to explore the evolutionary relationship between green algae and euglenoids, we have studied the molecular organization of the thylakoid membranes of *Euglena gracilis* Z strain. *Euglena* is an interesting alga to study because its chloroplasts are believed to have been derived from a double endosymbiotic event. We have studied the distribution of the PS in *Euglena gracilis* to investigate if their distribution is similar to the green algae (organism that was engulfed by a non-photosynthetic euglenoid during evolution giving rise to the *Euglena* chloroplast).

6. Origin of the chloroplast of *Euglena gracilis* (Euglenophyta)

The chloroplasts of euglenoids, as well as dinoflagellates, are surrounded by three membranes (chloroplast envelope). This is in contrast to chloroplasts of higher plants, green and red algae that are enveloped by two membranes, and to other groups of the algae which have four membranes around their plastids. In the latter case two membranes are known to be the two of chloroplast envelope and the outermost two have been named chloroplast endoplasmic reticulum (ER) membranes, because ribosomes are present on the cytoplasmic surface of the outer membrane. The three membranes in *Euglena* are equally spaced, the outermost one does not have any ribosomes attached to it, there is no contact between chloroplast envelope and nucleus and/or ER, and lastly there are no vesicles between the plastid envelope and the third membrane (Gibbs, 1978). These characteristic led Gibbs (1978) to conclude that in *Euglena*, the two inner membranes are the chloroplast envelope and the outer one is derived from the plasmalemma of a green algal symbiont. It now well accepted that the chloroplast of *Euglena* is derived from a double endosymbiotic event. The innermost membrane of *Euglena* chloroplast consist of the cell membrane of

the original prokaryote, the second is likely the phagocytic vacuole of the intermediate eukaryote and the third is believed to be derived from the cell membrane of the eukaryote.

7. Thylakoid organization in *Euglena*.

The chloroplasts of euglenoids contain chlorophyll *a* and *b* like those of green algae and higher plants. The thylakoids are arranged in bands that can present different numbers of thylakoids, although bands of three are common. The thylakoids forming the bands are tightly appressed, giving two different populations of thylakoid membranes: appressed (in contact with the stroma, *i.e.*, the two thylakoids at the edge of the band) and non-appressed (the membranes located in the band).

To our knowledge there is no immunoelectron microscopy data published for the distribution of PS I and PS II in euglenoids; however, a few studies have dealt with the subject in a different manner. In 1989, Brant and Keiz studied the distribution of PS I and PS II in thylakoids of *Euglena* using biochemical techniques. The thylakoid membranes (unappressed and appressed) were separated by an aqueous two-phase-partitioning. The distribution of CCI (a PS I component) and LHCII (a component of PS II) was determined densiometrically from SDS-PAGE. The ratios of CCI/LHCII are different during the light phase of the cell cycle, but during the dark phase they are the same. The activity of PS II that was measured spectrophotometrically has the same behaviour and is not restricted to one type of membrane. Therefore, PS I and PS II are present in both types of thylakoid membranes; in addition both types of membranes were able to evolve O_2.

The LHCP II apoprotein of *Euglena* was localized by immunogold cytochemistry in the Golgi apparatus, as well as in thylakoid membranes (Osafune *et al.*, 1990). Even though the authors did not make a distinction between appressed and non-appressed thylakoids, the labelling (in the micrographs shown) seem to be present in the same

concentration all over the thylakoids. These data corroborate the studies of Brant and Keiz (1989).

In a review Murakami (1991) reported that activity of PS I was found in stroma (band of two thylakoids running along the chloroplast) and pyrenoidal thylakoids by photooxidation of DAB. Labelling with anti-LHCII was reported to be found on both types of membranes as well. Stromal thylakoids were more labelled than the pyrenoidal ones (60% to 70% was found on the stroma). Murakami concluded that in *Euglena*, PS II is predominant on stromal thylakoids whereas PS I in localized in both types of membranes. Unfortunately there were no pictures shown and no experimental details were given.

We have used *Euglena gracilis* Z strain cells, which belongs to the Euglenophyta, to study the distribution of PS I and PS II in its thylakoid membranes. Two different antibodies raised against PS I (one anti-CP1-e from a cyanobacterium, the other against the PS I complex from maize) and one raised against the CP47 protein from PS II of cyanobacteria were used. Our results show that both PS I and PS II are located on both appressed and non-appressed membranes. These results are closer to the distribution found in the green algae rather than the one found in higher plants.

B) Localization of CP47 in the mitochondria of euglenoids

This section will be divided into two main parts. First, a general description of cases where pieces of foreign DNA are found in genome-containing organelles will be given in order to put into context our findings, using immunocytochemical techniques, of the presence of a plastid protein (CP47) in the mitochondria of *e*uglenoids. Second, we will describe briefly the different type of organisms used in our studies and the reasons why we chose them will be stated.

1. Promiscuous DNA

In higher plants and algae, the total genome of the organisms is composed of three pools of DNA; the one present in the nucleus (nDNA) and the other two present in the membrane-bound organelles, namely the chloroplasts (ctDNA), and mitochondria (mtDNA). Each of these genomes is considered to be different at the ultrastuctural, biochemical, and molecular levels (Lonsdale *et al.*, 1983). Mitochondria and chloroplasts are considered to be independent organelles since they possess their own genetic material, have their own machinery to synthesize proteins and are able to replicate autonomously. But even though mitochondria and chloroplasts are autogenous organelles, they still require proteins coded by the nuclear genes and synthesized in the cytoplasm to be fully functional. The proteins that reside in the plastids and mitochondria and are coded by nuclear genes are translated as precursors in the cytoplasm and are then incorporated into the organelles (or their inner parts, *i.e.*, cristae and thylakoid membranes) by means of a transit peptide that orients them to their final destination. In algae which have chloroplasts surrounded by three or four membranes, nuclear-coded plastid proteins have an N-terminal signal peptide preceding the transit sequence, which directs them to the cytoplasmic endoplasmic reticulum (ER) and then to the plastid via the Golgi in euglenoids and dinoflagellates or to the chloroplast ER in the chromophyte algae (Schwartzbach *et al.*, 1998). The traffic of proteins in the cytosol is believed to be vectorial and unidirectional (from nucleus to organelles and not *vice versa*) since there are no reports in the literature about organelle-synthesized proteins directed to the cytoplasm.

While the movement or traffic of proteins between organelles across the cytoplasm is known and well accepted, in the last twenty years, reports of mobile genes migrating between the organelles of eukaryotic cells, and homologies between DNA sequences of the mitochondria, plastids, and the nucleus have challenged the idea that these three cellular compartments are genetically completely different and independent.

In 1982, Ellis proposed that this phenomenon be termed "intracellular promiscuity" and defined promiscuous DNA as "a nucleotide sequence which occurs in more than one of the three membrane-bound organellar genetic systems of eukaryotic cells and should be distinguished from the DNA of promiscuous plasmids, which can be transferred to a wide range of prokaryotic cells."

Evidence for transfer of complete genes or pieces of genomic sequences began to be reported in the 1980s. For example, van den Boogart *et al.* (1982) found in *Neurospora crassa* that the gene encoding the DCCD-binding protein, a component of the ATPase multi-complex, was present in the mitochondrial genome. This was a striking discovery because until then it had been thought that the DCCD-binding protein was coded by the mitochondrial genome in the yeast *Saccharomyces cervisiae*, but only by the nuclear genome of *N. crassa*. In the same year, Stern and Lonsdale (1982) published a report demonstrating homology between sequences of the mitochondria and chloroplast genomes in maize. This homologous sequence was found to be 12 kilobases (kb) long and contained a chloroplast 16S rRNA gene plus the coding sequences for the $tRNA^{Ile}$ and $tRNA^{Val}$. The next year, Farrelly and Butow (1983) reported that contiguous DNA sequences of the nuclear genome possessed homology with non-contiguous parts of the mitochondrial genome in yeast.

Since then, there have been more reports of other DNA movement between organelles in animals (Hadler *et al.*, 1983; Gellissen *et al.*, 1983; Corral *et al.*, 1989), humans (Tsuzuki *et al.*, 1983; Nomiyama *et al.*, 1984) and plants (Nugent and Palmer, 1991; Maréchal-Drouart and Guillemaut, 1988; Kemble *et al.*, 1983). There are also examples of movement of genomic material between different organisms during evolution (Martin and Fridovich, 1981; Bannister and Parker, 1985), but for the purpose of this thesis we will concentrate on the movement of DNA in one organism.

I) Movement of DNA between organelles in higher plants and algae.

Higher plants and algae are unique organisms since they possess an extra organelle not found in other organisms, namely the chloroplast. This characteristic gives photosynthetic organisms a greater possibility for exchange or transfer of pieces of genomes between their cytoplamic organelles and the nucleus.

a) Transfer of DNA between organelles

The distinct types of transfer of genetic material that have been reported in the literature for higher plants and algae are movements from organelles (plastid and mitochondria) to nucleus, movements between chloroplast and mitochondria (lateral movement) and what Schuster and Brennicke (1988) call "return route", that is, movement from nucleus to mitochondria. There is no evidence, to our knowledge, of escape of genomic material from the nucleus to the chloroplast (see Table 1 in Schuster and Brennicke, 1988). For the purpose of this thesis, we will emphasize the examples of transfer of genomic material between the cytoplasmic organelles (*i.e.*, lateral movement from chloroplast to mitochondria).

b) Mitochondrial DNA

The role of mtDNA is to code for large and small subunit ribosomal RNAs and a few proteins that take part in the constitution and metabolism of the organelle. In land plants, some of the mitochondrial ribosomal proteins as well as 5S rRNA are coded by the mtDNA. Although mitochondria have the same function in all organisms, their DNA varies in size, sequence, gene content, and expression (for a review see Gray *et al.* , 1999). The large size of mitochondrial genomes of higher plants, which ranges between 218-367 kilobase pairs, (Stern and Palmer, 1984; Gray *et al.*, 1999) when compared to their counterparts in other eukaryotic organisms (Lonsdale and Grienenberger, 1992) is explained by 1) the presence of DNA sequences that have been transferred from the chloroplast or nuclear genomes (insertion of foreign DNA) and 2) a higher rate of inter- and/or intra-molecular recombination (Palmer, 1992; Lonsdale and Grienenberger, 1992).

II) Evidence of plastid DNA sequences in mitochondria.

In 1982, Stern and Lonsdale, using hybridization techniques, reported for the first time an homology (>90%) between a region of the mitochondrial and chloroplast genomes of maize. The size of the mitochondrial piece was 12 kb long and it coded for the chloroplast 16S rRNA gene and the associated tRNAs for the amino acids Ile and Val (a). This finding was then echoed in 1983, when Timmis and Scott showed that there is region of homology between the ctDNA and mtDNA of spinach. This observation was made while testing for homology between nuclear and chloroplast genomes, indicating that it is possible that that particular sequence (derived from restriction enzymes cuts) is present in the three genomes of spinach (Timmis and Scott, 1983).

The presence of ctDNA in the mitochondria of angiosperms seems to be a general phenomenon. Stern *et al.* (1983) found that in 11 species the ctDNA probes hybridized with mtDNA. A specific gene from the chloroplast (ribulose-1,5-bisphosphate carboxylase large subunit) was found in its entire coding sequence as well as the 5' and 3' flanking sequences in the maize mtDNA (Lonsdale *et al.*, 1983). In 1984, Stern and Palmer, using hybridization techniques, studied homologies between mt and ctDNA of corn, mung bean, spinach and pea. In all the cases tested, the ctDNA reacted with one or more of the restriction fragments of mtDNA. It was also found that known chloroplast genes such as *rbcL* recognized the mtDNA of corn and the β subunit of ATPase gene sequence hybridized to mung bean mtDNA. The authors concluded that "inter-organellar DNA transfer is a general phenomenon in plants" (Stern and Palmer, 1984). Another example of homology between the three genomic pools of plants is the case of a PS I chloroplast gene (P_{700} chlorophyll *a* apoprotein product) in spinach. From the digestion restriction enzyme map, it was deduced that only part of the gene is located in the mitochondria and therefore it is unlikely to be functional (Whisson and Scott, 1985). In 1993, a Japanese group directed by Hirai found that the mtDNA of rice contained several regions (sixteen sets totaling 22 kb) that were homologous to the sequences in their chloroplasts (Nakazono

and Hirai, 1993). Since this appeared to be a common phenomenon the same group decided to survey the mtDNA of several Gramineae (rice, maize, sorghum, wheat and Italian rye grass) by looking at the chloroplast *rps19* gene. This sequence is present in the mitochondria of all the plants examined except for wheat and a common chloroplast-like sequence was found at one terminus. The other terminus is unique for each case and it does not show any homologies, letting the authors infer that the genes were not functional (Watanabe *et al.*, 1994). In 1997, Zheng *et al.* sequenced a 7.5 kb region of the maize (T cytoplasm) mtDNA and found that it contained a chloroplast-like *trnI* as well as several short sequences (17-187 base pairs) homologous to ctDNA.

Two interesting cases are noteworthy, and we will mention them even though they do not occur in higher plants. First, is the presence of an unusual group I intron in the rRNA gene region of the mitochondrial DNA of the non-photosynthetic protozoan *Acanthamoeba castellanii*, because it shows that this type of transfer could have occurred well back in the evolutionary lines of organisms. While analyzing the introns in the large subunit rRNA of the mitochondria of A. *castellanii*, Lonergan and Gray (1994) found that two of them belong to a subgroup that is only found in chloroplasts. One of them is similar in sequence to the group I intron found in the chloroplast *rnl* gene of *Chlamydomonas reinhardtii*. It also has identical insertion sites to those in green algae. Possible explanations of when and how it may have occurred are discussed in the paper (Lonergan and Gray, 1994). Second, two plastid genes (*psbG*, a subunit of PS II, and ORF400) have been found in the mitochondrial genome of *Paramecium aurelia* (Pritchard *et al.*, 1989).

All the examples described above (except the two latter ones) are from higher plants. Because algae also contain chloroplasts and are ancestors of the former, one would think that the same phenomenon would apply to the mitochondria of algae. Nonetheless, complete sequences of the mitochondrial genome of several algae are now known and no homologies with plastid genes have been found. This is the case for the green algae

Chlamydomonas reinhardtii (Ma *et al.*, 1990; Michaelis *et al*, 1990; Boer and Gray, 1991; Vahrenholz *et al.*, 1993), and *Prototheca wickerhamii* (Wolff *et al.*, 1994), and also the red alga *Chondrus crispus* (Leblanc et al., 1995). If this is true, then the phenomena of transfer of genes would represent a mechanism that occurred in evolution after the appearance of higher plants and not before.

III) Mechanisms of genomic transfer.

Since there is convincing evidence of foreign DNA sequences in host organelles of different organisms, it is now believed that the transfer of genomic sequences is a phenomenon that has occurred during evolution and still occurs in a very large scale (Schuster and Brennicke, 1988). The translocation and integration of the genomic material does not seem to be integrated according to the function of the gene (or part of it) -since most of them do not even have a physiological function in the mitochondria- but rather occurs randomly (Stern and Palmer, 1984). Because the mitochondrial DNA seems to be more flexible (*i.e.*, it accepts more insertions without its function being affected) than the genomes in chloroplasts, it has been postulated that the higher likelihood of the direction of the translocation is from plastid to mitochodrion rather than *vice versa*. The mechanisms of how these movements occur are not well understood. Two main hypotheses have been considered, and it is likely that both contribute to the phenomenon.

a) Direct method

The transfer of DNA may occur when organelles come together in contact, when damaged/broken organelles allow escape of DNA and when the breakdown of membranes allow for temporary connections between organelles (Stern and Palmer, 1984).

b) Transfer through a vector

The fact that in some organisms there is a need to import RNA (tRNA in plants) for the proper functioning of the mitochondria leads to speculation that one possible way of transfer is through the RNA (Stern and Palmer, 1984; Thorsness and Weber, 1996).

In 1995, Cerruti and Jagendorf published an experimental method that induced the entry of plasmid DNA into isolated, intact pea chloroplasts (organelles treated with hypotonic solutions, high concentrations of Mg2+, or by heat shock at 42° C). The authors speculated that during the life time of an organism (and during evolution) plant cells endure conditions that are stressful, such as osmotic pressure, and this may cause the morphological changes in the membrane of their organelles allowing the escape of genomic material. These authors concluded that these conditions - in an evolutionary context- could represent a mechanism for the transfer of DNA sequences between different organelles in a cell (Cerruti and Jagendorf, 1995).

IV) Is the plastid promiscuous DNA expressed in its host mitochondria?

Although there are many convincing examples of inter-organellar movement and integration of DNA, there are only a few examples of the expression of the transferred genomic sequences in their "new host" organelle. Since it is known that the different organelles have somewhat different genetic codes and tRNAs, it may be that even genes that have been transferred and successfully integrated into a different organelle would be unsuitable for expression. When the chloroplast-like transferred sequences are found in the mitochondria of higher plants, the transferred genes usually are either truncated or have mutations that disable their translation into functional proteins (Ellis, 1982; Stern and Lonsdale, 1982; Stern and Lonsdale, 1982; Lonsdale *et al.*, 1984; Iams *et al.*, 1985). A remarkable exception seems to be the tRNA sequences.

However, in 1985, Lacoste-Royal and Gibbs reported electron microscope studies showing that the mitochondria of *Ochromonas danica* were labelled by antiserum raised

against the small subunit of RuBisCo of *C. reinhardtii*, and proposed that the labelling was evidence for the expression in the mitochondria of a promiscuous gene from the chloroplast (Lacoste-Royal and Gibbs, 1985).

We will describe in detail the transcription and expression of plastid tRNA genes in mitochondria of higher plants since it seems to be one of the few genes that are successfully integrated in the mitochondria.

In order to be totally functional and translate all the necessary proteins, the mitochondria of higher plants have to import some tRNAs encoded by the nucleus. The rest of the tRNA pool is present in the mtDNA. However, there are two different sub-pools of tRNAs in the mitochondrial genome of the organelle: "native" tRNAs (encoded by genes derived from the prokaryotic ancestor of mitochondria) and "chloroplast-like' tRNAs (encoded by plastid sequences that were integrated into the mitochondrial genome during evolution) (Maréchal-Drouard *et al.* , 1993). In this section we will describe the latter.

In the mitochondria of maize, $tRNA^{Trp}$ (Maréchal *et al.*, 1987) and $tRNA^{Cys}$ (Wintz *et al.* , 1988) were found to be of plastid origin and were found to be transcribed in the mitochondria. In maize, also, a truncated $tRNA^{Arg}$ was found to be able to code for 14 amino acids of a mitochondrial protein (Dewey *et al.*, 1986). This implies that some of the integrated DNA, even if not in its usual and complete form, could be adapted and used by the host organelle.

A chloroplast $tRNA^{Met}$ was found in the mitochondria of two higher plants: soybean and *Arabidopsis thaliana*. Their 5' flanking regions, as well as the gene itself, were highly homologous and therefore believed to be expressed in the mitochondria (Wintz *et al.*, 1988). Another support for this observation was the report of an isolated chloroplast $tRNA^{Met}$ from bean mitochondria (Maréchal *et al.*, 1985). Moreover in 1989, Sangare *et al.* reported that in maize mitochondria $tRNA^{Met}$ showed very high homology with the same gene present in the chloroplast and that this was probable evidence for an

earlier transfer of genomic material from the chloroplast that was transcribed in the mitochondria.

In wheat mitochondria, some of chloroplast-like tRNA genes are expressed. This is the case for $tRNA^{Ser}$, $tRNA^{Phe}$, and $tRNA^{Cys}$ that were found in their mature form. Also, chloroplast-like elongator Met, Asp and Try tRNAs are present in the organelles and are transcribed (Joyce and Gray, 1989).

In sugar beet (*Beta vulgaris*) and in two related wild species, a plastid gene cluster (*trnP-trnW-petG*) was identified in the mtDNA, but only one of the genes *trn W* was shown to be transcribed (Kubo *et al.*, 1995).

The transcription and editing of RNA of a promiscuous DNA fragment was studied in rice. The *rpoB* sequence was chosen since it was a chloroplast-derived sequence and because it contained several editing sites. The authors determined that the four sites of editing remained unmodified in the rice mitochondria, showing that the promiscuous chloroplast *rpoB* sequence is transcribed in the mitochondria, but that its RNA is not edited (Zeltz *et al.*, 1996).

The gene coding for $tRNA^{His}$ is another example of a chloroplast-derived gene that is expressed in the mitochondrial genome of the Gramineae. By using molecular techniques such as RT-PCR and Northern hybridization, Kanno *et al.* (1997) showed that the $tRNA^{His}$ was transcribed from a promoter located in the mitochondrial sequence (Kanno *et al.*, 1997)

In potato, Fey *et al.* (1997) reported that two identical genes coding for $tRNA^{Asn}$ existed in the mtDNA. Both genes are of plastid origin and are transcribed in the mitochondria. They also showed that the same gene (using Northern hybridization) was expressed in maize mitochondria.

In 1998 Miyata *et al.* demonstrated by Northern-blot hybridization analysis that seven of the nine plastid-derived tRNAs of the mitochondria of rice were transcribed, suggesting that they could be used by the mitochondrial-protein machinery. The tRNAs

Cys, Phe, His, Met, Asn, Ser, and Trp were transcribed and processed normally (Miyata *et al.*, 1998).

It seems, therefore, that the pieces of ctDNA that were transferred to the mitochondria and were integrated in its genome, at least in the case of the chloroplast-derived genes encoding for tRNAs, are transcribed and form part of the biosynthetic machinery for the production of proteins in the mitochondria of some higher plants.

Using EM immunocytochemical techniques we have located a plastid protein in the mitochondria of *Euglena gracilis* and related euglenoids. The protein (CP47) is the product of the chloroplast gene *psbB*. We will discuss these findings and put them in the context of the reviewed literature.

2. Brief description of the organisms used in the study

I) *Euglena gracilis*

a) General description

Euglena gracilis is a difficult organism to classify. Biologists argued, for a long time, about its animal-like and plant-like characteristics and taxonomists were reluctant to classify it in a definite group. In fact, the great diversity of organisms that today make up the Euglenophyta explains the difficulty. Euglenoids occupy a diversity of ecological niches, including even parasitic forms (Johnson, 1968). About one third of the population is photosynthetic (Knoll, 1992) and the other two thirds are colorless. The size, shape, and number of chloroplasts that they contain is variable as is the presence of pyrenoids. *Euglena* has two flagella, a reservoir where the contractile vacuole empties, a stigma or eyespot, a spherical nucleus and numerous mitochondria (Johnson, 1968; Buetow, 1968). A special characteristic of euglenoids is that its reserve carbohydrate, paramylon, is a β-1,3-

glycan localized in the cytoplasm (Leedale, 1982). The mode of insertion of the flagella into an anterior invagination of the cell and the construction and helical symmetry of the pellicle are also unique to euglenoids (Leedale, 1982; Buetow, 1968). Studies on the sequences of the small subunit of ribosomal RNA have shown that euglenoids are an ancient group of organisms closely related to trypanosomes (Sogin *et al.*, 1986; Delihas *et al.*, 1981; Knoll, 1992).

b) Mitochondrial DNA

To our knowledge the mitochondrial genome of *Euglena gracilis* has not been sequenced. Earlier physical and chemical studies demonstrated that its G + C content is 25%, its buoyant density in CsCl density = 1.690 g/cm3, and its melting temperature is 77°C in standard citrate saline. These parameters led the authors to conclude that this genome is basically similarly organized as the yeast one (Fonty *et al.*, 1975). In 1997, Yasuhira and Simpson reported for the first time a sequenced piece of the mitochondrial DNA of *E.. gracilis*. This region contained the gene coding for the cytochrome oxidase subunit 1 (CO1). The main points of the report are that first, the mtDNA of *E. gracilis* is believed to be 60kb and is isolated as short linear fragments. Second, the presence of the codon TGG encoding for tryptophan instead of TGA used in the mitochondrial genomes of animals, fungi and in the closely related trypanosomes is a sign that mitochondria of *E. gracilis* utilize the universal codon for tryptophan. Therefore, it is likely that *Euglena* mitochondria use the universal genetic code.

c) Origin of chloroplasts of *Euglena*.

In 1978, Gibbs proposed that the chloroplast of *Euglena* arose by two sequential symbioses. It is widely accepted that chloroplasts of green algae evolved from symbiotic cyanobacteria (Gray and Doolittle, 1982). *Euglena* chloroplasts are similar to those of green algae in that they possess both chlorophyll *a* and *b*, but they differ in that they are surrounded by three membranes. The presence of this third membrane led Gibbs to hypothesize that a colorless phagotrophic euglenoid

engulfed a green alga and over time reduced it to a chloroplast surrounded by the plasmalemma of the green alga. Lefort-Tran (1981) proposed from studies based on the freeze-etch technique that the third membrane surrounding the *Euglena* chloroplast was like an endoplasmic reticulum membrane, not the plasmalemma. Cavalier-Smith (1982) suggested that the three membranes are simply the inner and outer cell membrane of a Gram negative bacteria and the third one the phagocytic vacuole membrane of the host. However, Gibbs' hypothesis is now widely accepted because the sequences of a number of chloroplast DNA genes of *E. gracilis* are more similar to those of green algae than they are to their cyanobacterial counterparts (Morden *et al.*, 1992).

d) Plastid DNA.

In general the ctDNA of *E. gracilis* is very similar to the one in higher plants. The size, gene content and the order within operons is comparable (Hallick and Buetow, 1989; Palmer 1991). The plastid DNA represents 3-5% of total cellular DNA of *Euglena* (Schmidt and Lyman, 1976).

In 1993 Hallick *et al.* published the complete sequence of the chloroplast DNA of *Euglena gracilis*. The chloroplast DNA is a circular molecule 143,170 bp long, and has a 26.1% G + C content. Its genes code for: rRNAs, tRNAs, transcription/translation proteins, photosynthetic proteins (PS I, PS II, cyt b_6, large subunit of Rubisco, ATPase, proteins of chlorophyll biosynthesis), ORFs with similarity to other plastid ORF, and other unknown ORFs.

e) *psbB* gene of *Euglena gracilis*.

The *psbB* gene of *Euglena gracilis* encodes for the 51 kDa chlorophyll *a* apoprotein of PS II. The entire sequence of the gene was published in 1989 by Keller *et al.*. This gene is 3269 bp long, and starts 60 bp downstream from the 3' end of $tRNA^{Gly}$ gene. It is organized into 5 exons separated by 4 introns. The exons code for a protein with a predicted molecular weight is 58.5 kDa.

II) Dark-grown *Euglena gracilis*

When wild type cells of *Euglena gracilis* are grown in the dark for several generations, they become colorless. The reason for this is that cells do not synthesize chlorophyll in the dark nor do they synthesize any of the photosynthetic proteins. The chloroplast in dark grown cells regresses to a small body containing few internal membranes, the proplastid. The proplastids when exposed to light proceed to differentiate into normal functioning plastids, biosynthesizing *de novo* all the macromolecules that are required for a mature chloroplast.

III) Mutant Y9ZNa1L

Y9ZNa1L is a yellow mutant of *E.. gracilis* (Z strain) that does not contain any chlorophyll or protochlorophyll and lacks the red-blue photomorphogenic system, but still does possess the blue photomorphogenic system (Russell *et al.*, 1978). It was isolated following treatments of wild type cells with nalidixic acid (inhibitor of chloroplast formation). Once the mutation has occurred it does not genetically revert to the wild genotype (Russel *et al.*, 1978). When cells are observed under the EM, the Y9ZNa1L mutant has a prolamellar body but is unable to form thylakoids (Russel and Draffan, 1978). In this mutant, it has been shown that the 1.6 kb monocistronic *psbB* messenger RNA was not detected by Northern hybridization, indicating that the *psbB-psbT* pre-mRNA is not processed or that the processed RNA does not accumulate. This could prevent the translation of *psbB* and *psbT* as well as result in a defective PS II (Hong *et al.*, 1995).

IV) *Astasia longa*

Astasia longa is a unicellular, colorless, and non-photosynthetic organism. It is phylogenetically closely related to *Euglena gracilis*. Blum *et al.* (1965) found that there were biochemical and ultrastructural differences between *Euglena* and *Astasia*. Those differences plus the evident absence of chloroplasts in *Astasia* are the reasons which support the separation of the two organisms into two different taxa. In 1989, Siemeister and Hachtel isolated a 73 kb piece of DNA that had strong similarities with the ctDNA of *Euglena* as well as some differences. The genes that were found to be present in this circular DNA were the ones coding for 16S and 23S rRNAs, elongation factor Tu, and the large subunit of ribulose-1,5-biphosphate carboxylase. Even though some photosynthetic genes (*psaA*, *psbA*, *psbD* and, *psbE*) were tested for, these were not detected. Therefore, the authors concluded that it is safe to assume that the chloroplast-like DNA of *Astasia* does not possess any genes that are responsible for the coding of PS I or PS II complexes. More reports were published by the same group on identification of genes in the 73 kb DNA of *Astasia* (half the size of the plastid DNA of *Euglena*) showing 1) the presence of $tRNA^{Ile}$, $tRNA^{Phe}$, $tRNA^{Cys}$, and some ribosomal proteins that are usually coded by the plastid (Siemeister et al., 1990; Gockel et al., 1994), and 2) that the arrangement of the genes is similar to the one in *Euglena* . In 1994, Gockel *et al.* reported that in the circular DNA of *Astasia* no genes coding for photosynthetic proteins have been found (except for *rbc*L) and that the genes involved in the translation mechanism have a high homology, have the same arrangement, and have introns in the same positions as in the ones present in *Euglena* chloroplast DNA.

V) Reasons for choosing the different euglenoids

After observing immunolabelling of the mitochondria of wild type *Euglena gracilis* by two different antisera raised against the CP47 plastid protein, we decided to test if this was a shared characteristic in the euglenoids. We chose to test three different organisms:

a) dark grown cells of *Euglena gracilis*, which contain the chloroplast gene for the CP47 but does not express it in the absence of light.

b) a chlorophyll-less mutant of *Euglena gracilis* (Y9ZNa1L) which does not synthesize messenger RNA for CP47 and probably lacks the *psbB* gene in its chloroplast DNA and

c) *Astasia longa*, since the *psbB* is not present in its chloroplast-like DNA. *Astasia longa* branched off *Euglena gracilis* in the evolutionary tree and will allow us to infer evolutionary relations on when the transfer of DNA occurred.

CHAPTER 2

MATERIAL AND METHODS

A detailed description of material and methods used for this study to first localize PS I and PS II in the chloroplast of E. gracilis and second to localize the CP47 in the mitochondria of euglenoids.

Preamble

Chapter 2 will describe in detail the sources where we obtained the organisms as well as their particular conditions of growth. The methods used for the electron microscopic immunocytochemistry and molecular biology will follow. The explanation on the quantitative analysis will be described for both the chloroplasts and mitochondria studies.

1. Cells

The unicellular algae, *Euglena gracilis* Z strain (UTEX 753) and *Astasia longa* (UTEX 512), were obtained from the University of Texas Culture Collection of Algae. The mutant strain of *E. gracilis* designated Y9Z1Na1 was kindly provided by Dr. H. Lyman of the Department of Biochemistry and Cell Biology, New York State University at Stony Brook.

2. Conditions of growth

The organisms were grown in 250 ml Erlenmeyer flasks containing 100 ml of the appropriate media under continuous agitation (rotary shaker at 100 rpm). *Euglena gracilis* Z strain was grown in the autotrophic medium of Cramer and Myers (1952). For the dark-grown cells and *Astasia longa*, the same medium was used, but with the addition of 0.061 M and 0.02 M acetate, respectively. The Y9Z1Na1 mutant was grown in Beale's medium (Beale *et al.*, 1981). Cells were inoculated into 100 ml of medium (an amount of liquid that provided a good surface for air exchange) and grown in an incubator at 25°C in a 12/12 hour dark-light cycle. Illumination was provided by cool-white fluorescent lamps at a photon fluence rate of 38 to 64 $\mu E \cdot m^{-2} \cdot sec^{-1}$. Cells were harvested in the logarithmic or stationary phase of growth in the second or third hour of the light period (unless specified otherwise). Although the experiments to localize the Photosystem II protein, CP47, in the mitochondria of the different organisms did not require special conditions of light, they were grown under the same conditions for convenience.

Cultures of dark-grown cells were obtained by inoculating photosynthetic cells of *E. gracilis* in Cramer and Myers' autotrophic medium plus 0.061 M Na acetate and placing the Erlenmeyer flasks in a black box. The box was placed on the rotary shaker in the same incubator. The first three transfers, which were one week apart, and the

harvesting of the cells were done in a dark room in the presence of green light only. This was to ensure that the cells used for the experiments were fully adapted to the dark.

In the immunological experiments, cells of *Astasia longa* were fixed immediately upon arrival due to their high propensity for contamination.

3. Electron microscopy

Cells were harvested by centrifugation (3500 rpm) at 4°C for 15 minutes (min). For the structural studies, cells were fixed at 4°C in 2% glutaraldehyde (glut) in 0.1 M phosphate buffer (PB) for one to two hours, rinsed in buffer and post-fixed in 1% osmium tetroxide in PB for one to two hours. For most of the immunolabelling studies, the cells were fixed in 1% glutaraldehyde in 0.1 M Na cacodylate buffer for one to two hours at room temperature. Some cells used for immunolabelling were fixed by a simultaneous method (*see* Appendix 2). Cells were fixed in 2% glut in 0.1 M PB for two to 5 minutes, then 0.5% osmium tetroxide was added to the fixing solution, and fixation allowed to continue at 4°C for another 30 minutes. All the solutions for the fixation steps were at pH 7.0 to 7.4.

The fixation step was followed by three successive washes with buffer and dehydration by a graded series of ethanol (25%, 50%, 75%, 95% and 100%). The cells were then embedded in Spurr's epoxy resin, Epon or Lowicryl K4M resin (all purchased at J. B. EM Services, Montreal, Quebec, Canada) (*see* Appendix 2) and cured at either 65°C (Spurr's epoxy resin and Epon) or at –20°C under UV lamps (Lowicryl K4M). The polymerization was done on BEEM capsules for Spurr's and Epon resins and gelatin capsules for Lowicryl. The blocks were then sectioned to a thickness of 800 Å (golden colour under the reflection of light) and mounted on formvar-coated grids. On each grid, an average of 8 to 10 sections were mounted. The grids were stained, or treated for immunocytochemistry and then stained. The staining procedure consisted of incubations of 15 min on droplets of 2% (w/v) aqueous solution of uranyl acetate, followed by 15 min

incubation in Reynolds' lead citrate (Reynolds, 1963). Between the two incubations and at the end of the procedure, the grids were washed for 2 min with ddH_2O. Precautions were taken at these two steps. Since uranyl acetate is light sensitive, the incubations were performed in the dark. The incubation with lead citrate was done in the presence of NaOH pellets to catch the CO_2 present in the air in order to avoid dark precipitates that would make observation under the electron microscope (EM) difficult. The grids were observed on a Philips EM 410 microscope at 80 kV. Photographs were taken on Kodak 35 mm fine grain release positive film and developed for further analysis (*see* below).

4. Cytochemical detection of PS I

To determine the activity of PSI, the photooxidation of 3,3'-diaminobenzidine.4HCl (DAB) method was used (McKay and Gibbs, 1990). Cells of *E. gracilis* were harvested at the end of the dark period and at hour 6 of the light period of the cycle. Cells were then fixed on ice for 20 min with 2% paraformaldehyde (v/v) in 0.1 M phosphate buffer containing 0.2 M sucrose at pH 7.4. Cells were then rinsed with a decreasing gradient of sucrose solutions and incubated for one hour in a solution of 1 mg/ml DAB in 0.1 M PB, pH 7.4, at 25°C under cool-white fluorescent lamps at 58 $\mu E \cdot m^{-2} \cdot sec^{-1}$. For the control, this last incubation was performed in the dark. Cells were rinsed and post-fixed for an hour with 1% OsO_4 (w/v) in 0.1 M Na cacodylate buffer, pH 7.4, in the dark. They were then washed, dehydrated with an ethanol gradient and embedded in Spurr's resin. Gold sections (800 Å) were mounted on formvar-coated copper grids, stained, viewed and photographed under the EM.

5. Immunocytochemistry

I) Antisera and reagents

The antisera used to localize the PS I reaction center were anti-PS I and anti-CP1-e. Anti-PS I is a polyclonal antibody raised in rabbits against sucrose gradient-purified PS I particles from maize and was generously donated by A. Barkan (Institute of Molecular Biology, University of Oregon, Eugene, OR). Anti-CP1-e was raised against the two reaction center proteins (60 and 62 kDa) of PS I from the cyanobacterium *Synechococcus elongatus* (Kashino *et al.*, 1990) and was kindly donated by Y. Kashino (Department of Life Science, Himeji Institute of Technology, Hyogo, Japan).

Two rabbit polyclonal antisera were used to localize the 47 kDa core antenna protein of the PS II reaction center. The first was raised against CP47 purified from *S. elongatus* (donated by Y. Kashino, *see* above) and the second was raised against the polypeptide 5 from *Chlamydomonas reinhardtii*, cordially given by G. Schmidt (Department of Botany, University of Georgia, Athens, GA). The latter was only used for the mitochondrial localization of the CP47 protein.

Pre-immune serum (PIS) from rabbit was bought from Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada. Protein A-gold (10 nm) was purchased from EY Laboratories, San Mateo, CA, USA.

II) Method

For the immunolabelling studies, freshly cut sections were mounted on formvar-coated nickel grids. A special step was performed on the samples that contained OsO_4 to increase the antigenicity of the exposed proteins. The grids were first incubated for 30 to 45 min in a 12% Na-metaperiodate (w/v) solution and rinsed with phosphate-buffered saline (PBS). They were then incubated in 1% bovine serum albumin (BSA) (w/v) for 45 min to 1 hour. This was followed by an incubation of 45 to 70 min with the antibody diluted in PBS (*see* Appendix 3), 3 washes with PBS of 4 min each, and a 45 to 60 min incubation in protein A-gold diluted 1:9 in PBS. The grids were then washed 4 times for 3 min and a final rinse with ddH_2O for 2 min was performed. Grids were then stained and viewed.

6. Quantification of gold labelling

To analyze our results, the number of gold particles attached to the membranes had to be determined as an indication of the presence of the studied protein in the organelle.

When the plane of section is perpendicular to the membranes sectioned, the membranes in the organelles can clearly be seen. Depending on whether or not OsO_4 was present in the fixative (see Appendix 2), the membranes appear in different colours. In the presence of osmium tetroxide, they appear dark black while in cells fixed in glutaraldehyde only, they are white. In the latter case, the zone of appression between the membranes of two adjacent thylakoids displays a grey colour.

To quantify the labelling of the thylakoids and the mitochondria, between 339 and 1821 gold particles were counted for a single experiment (see Appendix 3). The number of micrographs examined for each experiment is also given in Appendix 3. The areas of the organelles analyzed and the total lengths of each thylakoid membrane was determined on a digitizer tablet using the Sigma-Scan program, version 3.92 (SPSS, Chicago, IL).

I) Chloroplast studies

Two lines perpendicular to the thylakoid bands were drawn, the lengths of the appressed (AM) and non-appressed (NAM) membranes in the delimited area measured, and the total number of gold particles on each type of membrane were counted. Every particle lying on the outermost membrane and half of the particles residing in the first lumen (on each side of a band of thylakoids) were considered to be attached to the NAM and every particle that was located on the appressed membranes or over the lumens of the inner thylakoids, plus half of the particles in the first lumens, were assigned to the AM category.

The labelling density expressed as gold particles/μm of membrane was calculated for each micrograph by dividing the total number of gold particles on each type of membrane by the total length of each type of membrane. The mean value of the labelling was determined for both types of membranes and the ratio of labelling of NAM and AM was obtained. The statistical significance of the difference between the labelling density on AM and NAM regions was tested by a paired Student's t-test.

To measure the background, we compared the means of the labelling density over the chloroplast with that over the remaining area of the cell.

II) Mitochondria studies

In each micrograph, the area of all clearly identified mitochondria was measured. The labelling on the mitochondria was calculated. In this context "labelling on" means that the gold particles were either directly over the organelle or within 20 nm of the outermost mitochondrial membrane. The labelling density in the mitochondria was expressed in this case as gold particles/μm^2. The mean value of the labelling density over the mitochondria was calculated for each organism and compared by a paired Student's t-test to the labelling density over the background cytoplasm.

Although not all sections were cut perpendicular to the cristae, gold particles attached to the cristae membranes were counted when possible. The total length of all cristae membrane sectioned perpendicularly was measured to give the number of gold particles per μm. The percentage of mitochondrial gold particles attached to the cristae was calculated to give a rough estimate of the attachment of the antibody to such membranes.

To measure background labelling, we did counts over the total cytoplasm including paramylon and vacuoles.

7. Molecular biology

I) Isolation of chloroplasts

To test the specificity of the CP47 antibody in *E. gracilis*, the chloroplasts of its cells (harvested during the light period of the cycle) were isolated following the method described by Ortiz *et al.* (1980). *E. gracilis* cells were digested with an enzyme to separate the pellicular strips from each other and form spheroplasts: six grams of wet weight of cells (kept at 4°C overnight) were incubated at 0°C in 10 ml of buffer (50 mM K phosphate pH 7.0, 30 mM sorbitol, and 50 mg trypsin) for one hour. The solution was then diluted two fold with the buffered-sorbitol and centrifuged at 500 rpm for 5 minutes. The pellet was transferred to another tube containing a solution of 0.25 M sorbitol, 20 mM Hepes (pH 7.4), and 0.4 mM EDTA to which soy bean trypsin inhibitor was added to a final concentration of 1 mg/ml. These spheroplasts were then disrupted by vigorously stirring with a magnetic bar for 10 minutes. The chloroplasts were pelleted by a double centrifugation: 1000 rpm (270 g_{max}) and 3000 rpm (240 g_{max}). The pellet recovered consists of the "crude chloroplast extract". This pellet was then used to isolate the proteins.

II) Isolation of proteins

The chloroplast proteins were extracted using the phenolic extraction method described by Monroy and Schwartzbach (1983). All procedures were performed in a cold room (4°C). The "crude chloroplast" extract was ground in 3 ml of phenol per gram of sample, kept on ice. One volume of extraction buffer (0.7 M sucrose, 0.05 M Tris, 0.02 M EDTA (pH 8.0), 0.5 M NaCl and 0.5% β-mercapto-ethanol) was added and let stand for 1 hour. The solution was then transferred to a tube and centrifuged at 8 000 rpm for 5 minutes. The phenolic phase was transferred to a tube and 4 volumes of precipitation

solution (0.1 M NH_4 acetate in methanol) added. The solution was mixed well and incubated overnight. A centrifugation at 8 000 rpm to discard the supernatant was performed twice. The pellet was washed with 2 ml of acetone per gram of pellet and an ethanol rinse was used to wash out the remaining chlorophyll. The pellets were air-dried and used for the Western Blot experiments (if needed, the pellet was stored at –70°C until further use).

III) Western blots

The protocol of Laemmli (1970) for SDS-PAGE (10%) was performed to separate the proteins. The resolved proteins were electroblotted onto a Polyscreen polyvinylidene fluoride (PVDF) transfer membrane (NEN-DuPont, Boston, MA) in transfer buffer (192 mM glycine, 25 mM Tris, pH 8.3, Towbin *et al.*, 1979) using a Bio-Rad Trans-Blot apparatus (Bio Rad Laboratories, Mississauga, Ont). The efficiency of the transfer was tested by staining the membrane with 0.1% (w/v) Ponceau S in 1% acetic acid solution. The staining was then removed by rinsing with several washes of dH_2O. Nonspecific binding sites on the transfer membrane were blocked by a 1 hour incubation in PBS plus 0.2% Tween, then probed for 60 min at 37°C with the primary antibody (anti-CP47 from *S. elongatus* diluted 1:500 in PBS buffer). Unbound antibodies were removed by four consecutive washes with PBS plus 0.05% Tween at room temperature. The membranes were incubated with a horse-radish peroxidase-linked (HRP) goat anti-rabbit immunoglobulin G secondary antibody (Transduction Laboratories, Lexington, MA) diluted in blocking buffer (1:3000) for 45 min at 37°C followed by four washes in PBS plus Tween as described above. Antigen-antibody complexes were localized by addition of the chromogenic substrate DAB.

CHAPTER 3

IMMUNOLOGICAL AND CYTOCHEMICAL LOCALIZATION OF PS I AND II IN *EUGLENA GRACILIS*

Results of our study on the localization of the two photosystems in the thylakoid membranes of Euglena gracilis.

Preamble

The purpose of the present study was to determine whether Photosystem I and Photosystem II, the two reaction centers involved in the photosynthetic process, are differentially located on appressed and non-appressed thylakoid membranes in autotrophically grown cells of *Euglena gracilis* Z strain. To determine if the activity of PS I was restricted to specific thylakoids or present in all thylakoids of a band, the photooxidation of 3-3' diaminobenzidine (DAB) was employed. To directly localize the proteins of PS I and PS II in the plastid's thylakoids immunoelectron microscopy techniques were used.

1. Morphology of the cell

The ultrastructure of autotrophic logarithmic-phase cells of *Euglena gracilis* is illustrated in Figures 1 to 4. This unicellular alga has a large spherical nucleus with a prominent nucleolus and permanently condensed chromosomes (Figure 1). The nuclear envelope consists of a double membrane that is interrupted by nuclear pores (Figures 1 and 3).

Several conspicuous chloroplasts that, like those in green algae, contain chlorophylls *a* and *b* are present (Figure 1). Two regions are distinguishable in the chloroplasts: the thylakoids that transverse the chloroplasts in numerous extended bands (also called lamellae or stacks) and the stroma (which lies between the bands of appressed thylakoids) (Figures 1 and 2). The granularity observed in the stroma of the plastids (Figure 1) is due to the presence of abundant 70S chloroplast ribosomes. Scattered plastoglobuli are also visible in the stroma of the chloroplasts (Figures 1 and 2). Although the thylakoids are organized in stacks, these are clearly different from the grana stacks seen in chloroplasts of higher plants (Illustration 1, Chapter 1). The number of thylakoids per band varies in different regions of the chloroplast, or even within a single band, as bands join each other or separate along their length (Figure 2). Even though bands of three appressed thylakoids are most common (Figure 2, thin arrow) in the cell, bands of two thylakoids are frequently observed especially crossing the pyrenoid (data not shown), and stacks of six or more appressed thylakoids are not uncommon. Figure 2 shows a stack of ten thylakoids at the thick arrow. Usually bands of two thylakoids cross the pyrenoid and, on very few occasions, bands consisting of a single thylakoid can be seen traversing it (data not shown). In the stacks, the thylakoids that form the bands have two different types of membranes: appressed membranes (AM) and non-appressed membranes (NAM). The AM are the ones that are found in the inner part of the band, and they are tightly appressed as they are in the grana of higher plants (*i.e.*, it is not possible to distinguish a space between the two adjacent membranes). The NAM are the two

Morphology of *E. gracilis*

FIGURE # 1.

Ultrastructure of *Euglena gracilis*. A cross section of a cell of *E. gracilis Z* strain showing that the nucleus (N) is surrounded by a double membrane (Nmb) and contains a nucleolus (n) and condensed chromosomes (ch). Visible in the cytoplasm of the cell are ribosomes (r), vacuoles (V), Golgi apparatus (G), mitochondria (M), and chloroplasts (C). These last two organelles have inner structures; the mitochondria have flattened cristae (c) and the chloroplasts have bands of thylakoids (th) surrounded by stroma (s). (X36,200)


M
N
V

FIGURE # 2.

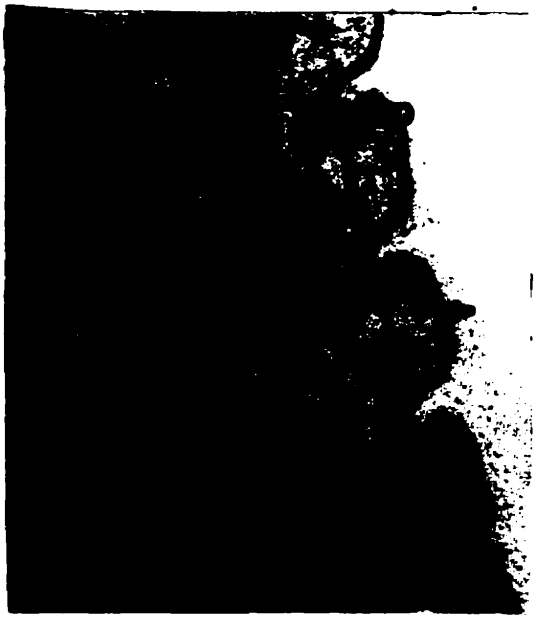
Chloroplast ultrastucture. The chloroplast (C) in *E. gracilis* contains bands of thylakoids (th) (indicated by thin short arrow) running through the stroma (s). These bands correspond to the grana of higher plants. The number of thylakoids per band is variable: the thin long arrow indicates a band of 3 thylakoids and the thick arrow a band of 10 appressed thylakoids. We will refer to the thylakoid membranes that are in contact with the stroma as non-appressed thylakoid membranes (NAM) and the ones found inside of the thylakoid band as appressed (AM). (X40,400)

FIGURE # 3.

A chloroplast of *E. gracilis* at higher magnification showing that the plastid is surrounded by 3 membranes (Cmb; short arrow). The thin long arrow points to a nuclear pore in the nuclear membrane (Nmb) of adjacent nucleus. (X101,000)

FIGURE 4.

Mitochondrial ultrastructure. The mitochondria (M) of *E. gracilis* have plate-like cristae (c) which are invaginations of the inner membrane of the mitochondrial envelope (shown by the long thin arrows). Microtubules (short arrow) are closely associated with the ridge-shaped pellicle (p) which surrounds the cell. (X78,000)



membranes that are exposed to the stroma of the chloroplast. A distinctive feature of *Euglena* chloroplasts, shared only with dinoflagellate chloroplasts, is that they are enclosed by a three-membraned envelope (Figure 3).

In Figure 1, the cytoplasm can be seen to be filled with 80S ribosomes, only a few of which are associated with membranes of endoplasmic reticulum (ER). A prominent vacuole and a single Golgi body (dictyosome) consisting on four or five stacked cisternae and associated transport vesicles are among the visible organelles. Numerous mitochondrial sections show the flattened cristae, which result from infoldings of the inner membrane of the mitochondrial envelope (Figure 4, arrows).

Figure 4 shows the ridges and grooves that form the pellicle complex of *Euglena gracilis*. Overlapping pellicular strips lie just beneath the plasmalemma. In this figure the morphology of the plasmalemma is very clear showing the dark-light-dark appearance of a unit membrane. The pellicular strips appear as a thin finely granular layer attached to the inner surface of the plasmalemma. Associated with the ridges are a set of four microtubules at the base of the groove and on the ridge (Figure 4, arrow). Deeper in the cytoplasm, a system of ER elements is also associated with each ridge.

2. Localization of the photosystems

I) Photosystem I

a) Immunolabelling studies

We have employed two different antisera against photosystem I (PS I) to localize this complex in the thylakoid membranes of *E. gracilis*. The first, anti-PS I, was raised against whole PS I particles purified from maize and had the advantage of reacting with antigens in sections of cells fixed simultaneously in glutaraldehyde and osmium tetroxide followed by embedding Epon resin. Figures 5 and 6 show both very clear morphology and good labelling. Note that both the appressed membranes (curved arrows) and non-appressed membranes (straight arrows) are

Localization of Photosystem I (PS I)

FIGURE 5.

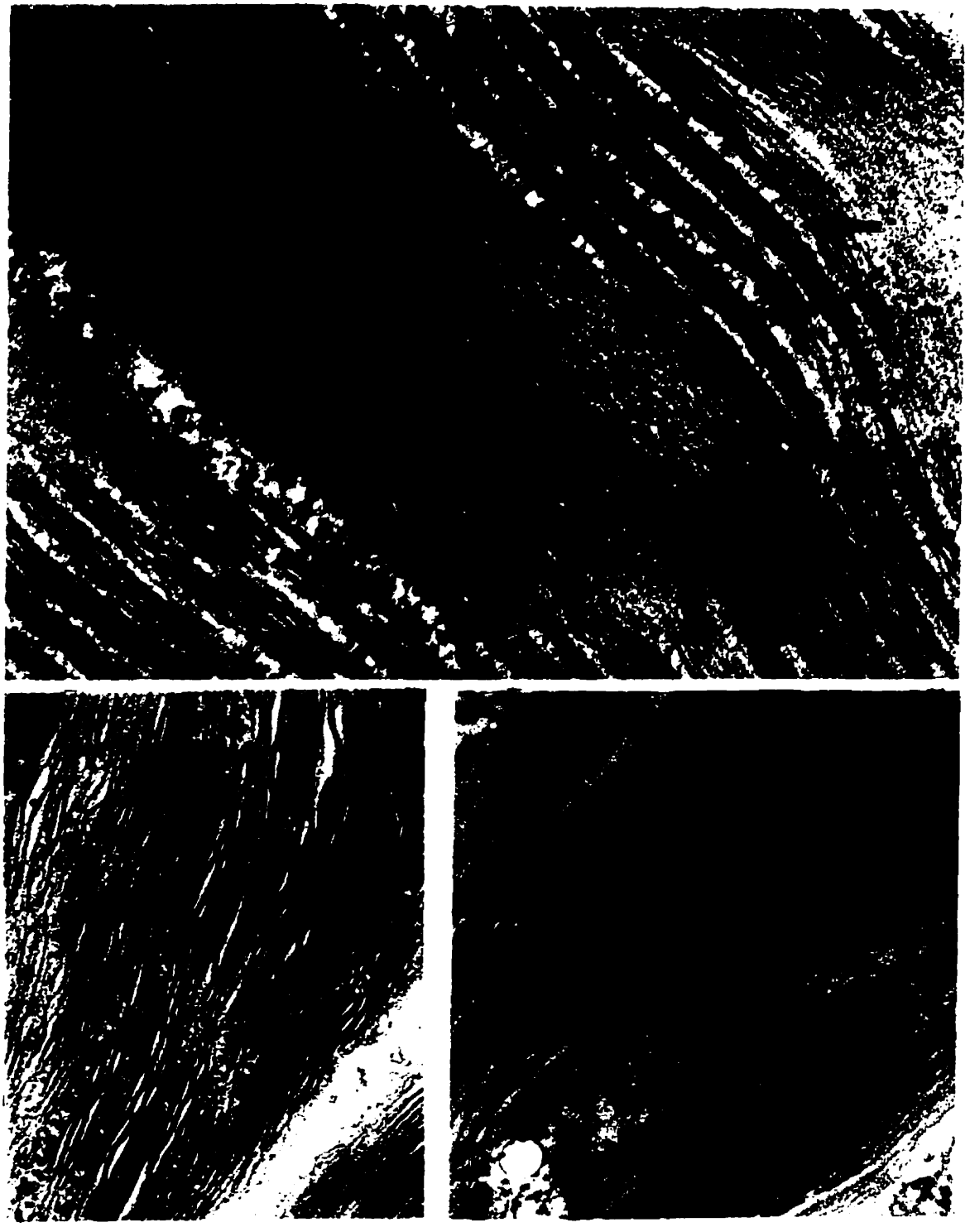
Immunolocalization of PS I. A section of a chloroplast embedded in Epon resin labelled with anti-PSI antiserum (1/200 dilution) from maize followed by protein-A gold (10 nm). Gold particles are situated on both the appressed (curved arrows) and non-appressed (straight arrows) thylakoid membranes whereas the chloroplast stroma (s) is largely unlabelled. (X96,400)

FIGURE 6.

Detail of a chloroplast section showing the localization of the specific labelling. Gold particles are located on the thylakoid membranes and not the stroma region. The arrow indicates the granular appearance of the chloroplast stroma due to the presence of chloroplast ribosomes. (X78,500)

FIGURE 7.

Control cells were treated as were the experimental group except that the antibody incubation was replaced by an incubation with a 1/50 dilution of pre-immune serum from rabbit. Very few or no particles were present in either the stroma (s) or thylakoid (th) region. (X66,500)



labelled. Nearly no background was observed in the stroma of the chloroplast (Figure 5) or any other organelle in the cell and labelling was very specific to the thylakoid membranes. Quantitative analysis of the labelling showed that the labelling density is 3.6 higher on the NAM (1.57 ± 0.19 gold particles/μm of membrane) than on the AM (0.44 ± 0.19 gold particles/μm of membrane) (Table 1). The difference is statistically significant ($p < 0.001$).

Figure 7 shows a control experiment where cells labelled with pre-immune serum from rabbit display no labelling; in addition, when the incubation with the antibody was omitted very few or no gold particles were observed (data not shown). Another test of the antibody specificity and the relative absence of non-specific background performed was to compare the number of gold particles/μm^2 of chloroplast sectioned to the number of gold particles/μm^2 of cytoplasm in the labelled sections. Table 2 shows that with anti-PS I, chloroplast labelling is 6.0 times higher than background labelling.

Since the anti-PS I from maize was made against the whole particle of PS I, a more specific antibody was used to corroborate these results, namely anti-CP1-e. This polyclonal antiserum was raised against the 60 and 62 kDa reaction center proteins of PS I from *Synechococcus elongatus*. Because this antibody did not label cells which had been exposed to osmium tetroxide, cells of *E. gracilis* were fixed in glutaraldehyde in Na cacodylate buffer and embedded in Lowicryl resin (see Appendix 2). Figures 8 and 9 show sections embedded by this technique and labelled with anti-cp1-e. In this preparation, the thylakoid bands appear as white bands (of varying width) separated by dark stromal regions. When sectioned perpendicularly, the number of thylakoids per band and hence the number and length of AM and NAM in a marked area can readily be determined. As with the previous antisera gold particles are located on both the AM (curved arrows) and the NAM (straight arrows). Both micrographs give the visual impression that anti-cp1-e labelling is more concentrated on the NAM; the quantitative analysis

Table1. Labelling of appressed (AM) and non-appressed (NAM) thylakoid membranes of *Euglena gracilis* with antibodies against PSI and PSII proteins.

Antibody	Membrane length	AM	Label on AM	Label on NAM	Labelling density		Density ratio NAM/AM
					AM	NAM	
	μm	%	%	%	gold particles/μm ± SEM		
Anti-PSI	1118.4	91.0	73.9	26.1	0.44 ± 0.19	1.57 ± 0.19	3.6
Anti-cp1-e	1450.7	85.0	60.0	40.0	0.40 ± 0.11	2.30 ± 1.07	5.8
Anti-CP47	717.0	81.7	54.0	46.0	0.63 ± 0.07	2.47 ± 0.40	4.0

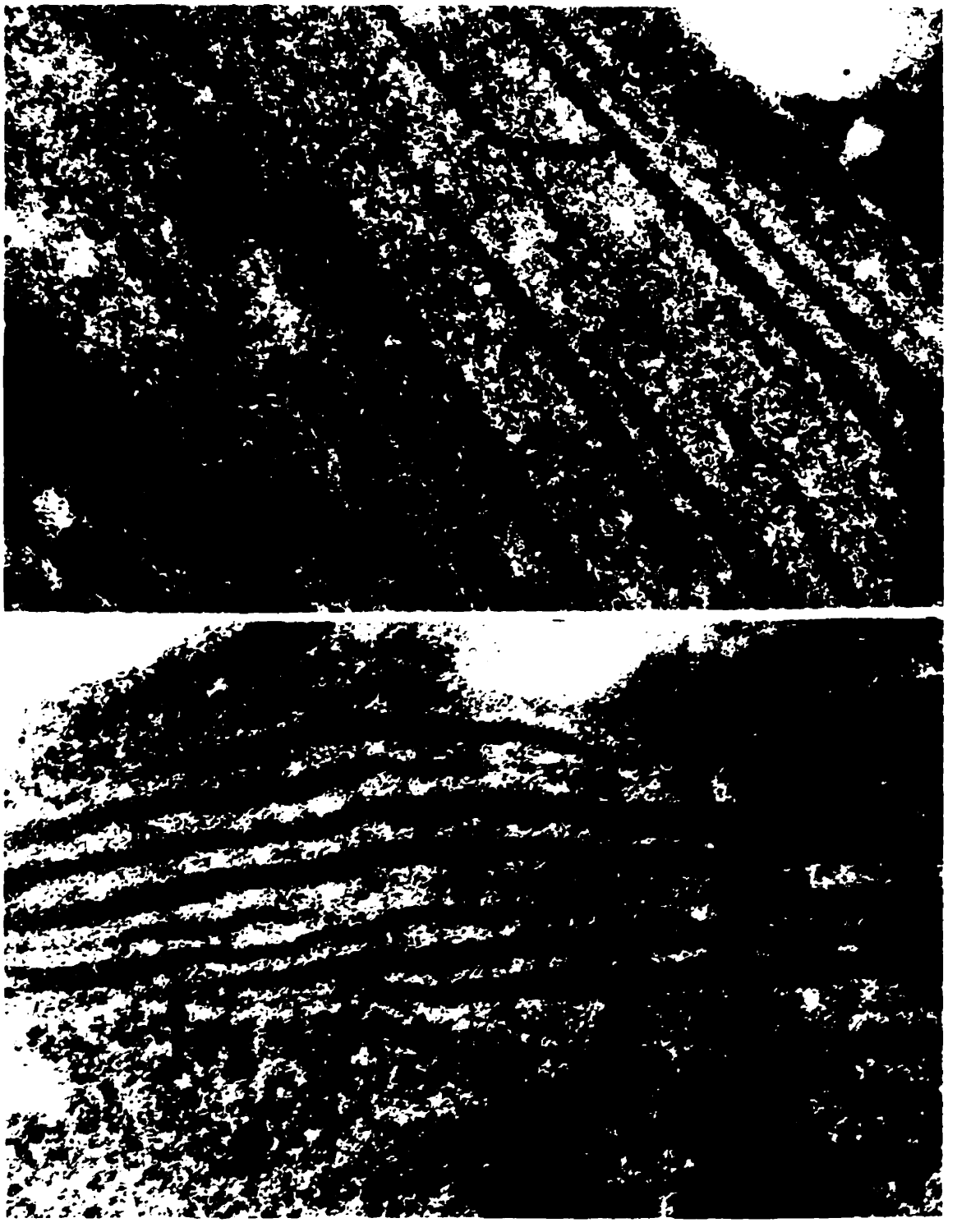
Table 2. Labelling density over chloroplast and cytoplasmic areas in *Euglena gracilis*.

Antibody	Labelling density		Density ratio
	Chloroplast	Cytoplasm	Chloroplast/cytoplasm
	gold particles/μm^2 ± SEM		
Anti-PSI	26.4 ± 5.23	4.4 ± 1.62	6.0
Anti-cp1-e	46.4 ± 3.04	15.4 ± 1.30	3.0
Anti-CP47	59.1 ± 5.44	17.4 ± 1.84	3.4
Pre-immune serum[1]	0.02 ± 0.001	1.0 ± 0.26	NA

[1]On glutaraldehyde – fixed, Lowicryl – embedded cells

FIGURES 8 and 9.

Sections of chloroplasts embedded in Lowicryl resin labelled with a different PS I antibody, anti-CP1-e (1/50 dilution) from S. *elongatus*, followed by an incubation with protein-A gold (10 nm). Gold particles are situated on both the appressed and non-appressed thylakoid membranes. In Lowicryl resin, the stroma region (s) appears dark and the thylakoid (th) region is lighter. The straight arrows show the labelling in the NAM regions and the curved arrows show labelling in the AM regions. (X93,600)



summarized in Table 1 confirms this observation. Gold particles are 5.8 times more concentrated on the NAM (2.30 ± 1.07 particles/μm of membrane) than on the AM (0.40 ± 0.11 particles/μm of membrane). The difference is statistically significant ($p < 0.001$). On chloroplast sections labelled with pre-immune serum (Figure 17), virtually no gold particles are observed on the thylakoid membranes, nor is there any labelling over other parts of the cell (Table 2). Table 2 also shows that chloroplast labelling by anti-cp1-e is 3.0 times higher than background cytoplasmic labelling.

b) Cytochemical localization of PSI activity

The photooxidation of DAB was used to detect the activity of PS I. The photooxidation of DAB by components of the thylakoid membranes can be distinguished as electron dense deposits in the thylakoids. If there is activity of the PS I, dark deposits are seen in the thylakoids of the chloroplast. The mitochondrial cristae are also stained due to their cytochrome oxidase activity (data not shown). When cells are harvested at the end of the dark period, PS I activity was restricted to the outer non-appressed thylakoids of each band. This is easily observed in Figures 10 and 11. No or very little electron dense material was seen in the appressed thylakoids. Figure 12 shows a control cell, which was incubated with DAB in the dark. No dense deposits were present over the thylakoids. Although the chloroplast envelope appears to contain some dense deposit, the third membrane surrounding the chloroplast was unlabelled. However, if cells were harvested during the light period of the cycle, PS I activity was detected both in the appressed and non-appressed regions of the thylakoids. The thick arrows in Figure 13 show that dense reaction product is present across the entire thylakoid band of four appressed thylakoids. As in Figures 10 and 11, the electron dense deposits are not continuous along the thylakoids, but instead are interrupted by clear regions. In Figure 13, the dense deposits in one thylakoid are aligned with the dense deposits in all the other thylakoids constituting the

FIGURES 10.

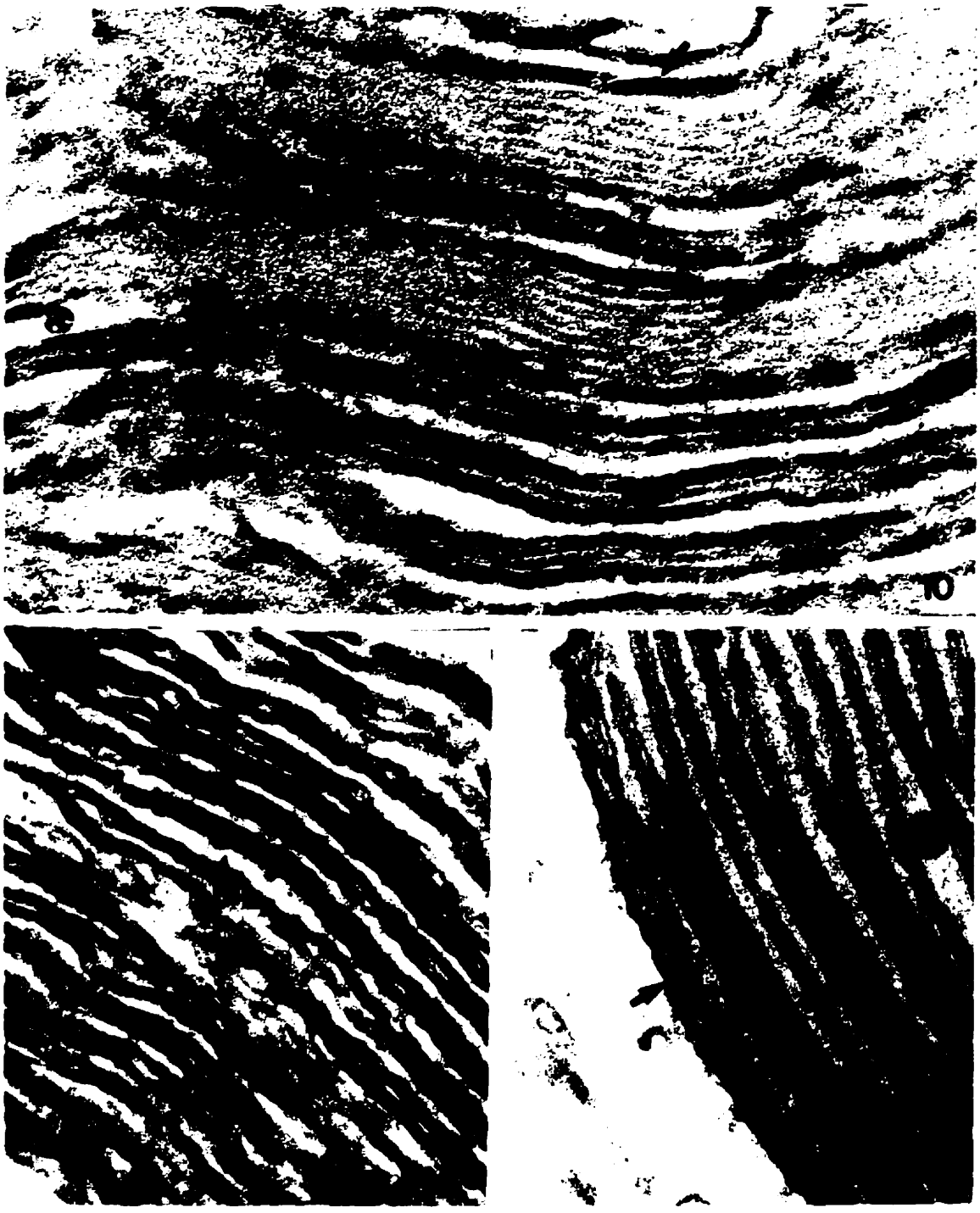
Cytochemical localization of PS I. Photosystem I activity was determined by the photooxidation of 3-3'-diaminobenzidine (DAB). In sections of cells harvested at the end of the dark period of growth, the dense dark reaction product (arrows) is concentrated in the thylakoids that abut the stroma. (X138,600)

FIGURE 11.

In this chloroplast section, most bands consist of three appressed thylakoids. Note that the outermost thylakoids contain dense reaction product, whereas the central thylakoids do not. The arrow indicates a two thylakoid band. In this case each thylakoid contains dense precipitate. Note that in most stained thylakoids, the staining is irregular with darker sections alternating with more lightly stained areas. This can also be seen in FIGURE 10. (X74,700)

FIGURE 12.

Control cells were prepared in the same manner but were incubated in the dark. No reaction product was evident in the thylakoids. The arrow shows dark deposits on the inner two membranes of the chloroplast envelope. (X90,500)



band. In control cells incubated with DAB in the dark, no dense material is seen in the thylakoids (Figure 14), but again the chloroplast envelope appears stained, showing presence of oxidative reactions. This method that successfully localizes the PS I activity corroborates our immunological results.

II) Photosystem II

a) Immunological studies

Figures 15 and 16 show sections through the chloroplast of *E. gracilis* labelled with antiserum to CP47, raised against the 47 kDa protein of the PS II complex of *Synechococcus elongatus*. Although the morphological structure of membranes is not as well preserved after glutaraldehyde fixations as it is with fixations that include osmium tetroxide, almost all the thylakoid bands in the two micrographs have been cut perpendicular to the plane of the thylakoid membranes, allowing one to determine the number of thylakoids in each band. Gold particles are present on both the non-appressed (long thin arrows) membranes and on the appressed membranes (short arrows). Note that no or few gold particles are present in the stroma of the chloroplast. The density of labelling on the non-appressed membranes (2.47 ± 0.4 gold particles/μm) is significantly higher ($p < 0.001$) than that on appressed ones (0.63 ± 0.07 gold particles/μm), the ratio of labelling NAM/AM being 3.9 (Table 1). Control experiments, where the incubation with the antibody was replaced by an incubation with pre-immune serum (1/50 dilution) from rabbit, show no labelling in the thylakoid or stromal regions of the chloroplast (Figure 17). Table 2 shows that chloroplast labelling (gold particles/μm^2) is 3.4 times higher than cytoplasmic labelling.

FIGURE 13.

In cells harvested at hour 6 of the light period, the DAB staining shows that the activity of Photosystem I is localized across the thylakoid band. Both outer and inner thylakoids have dense deposits (arrows). The long thin arrow indicates a single thylakoid which also contains reaction product. (X67,400)

FIGURE 14.

Control for the same experiment. No dark deposits are found in the thylakoids of the chloroplast. Arrow shows reaction in the chloroplast envelope. (X79,600)

Localization of Photosystem II (PS II)

FIGURE 15.

Immunolocalization of PS II. Section of a chloroplast labelled with anti-CP47 antibody (1/50 dilution) from *S. elongatus* followed by an incubation with protein-A gold (10 nm). Gold particles are localized on the appressed (short arrows) and non-appressed (long thin arrows) thylakoid (th) membranes. There were very few gold particles present on the stroma (s). (X93,600)

FIGURE 16.

PSII is located on the AM and NAM. Long thin arrows show gold particles attached to outermost thylakoid membranes. The thicker arrows point to particles of gold that are lying on the appressed thylakoid membranes. (X93,600)

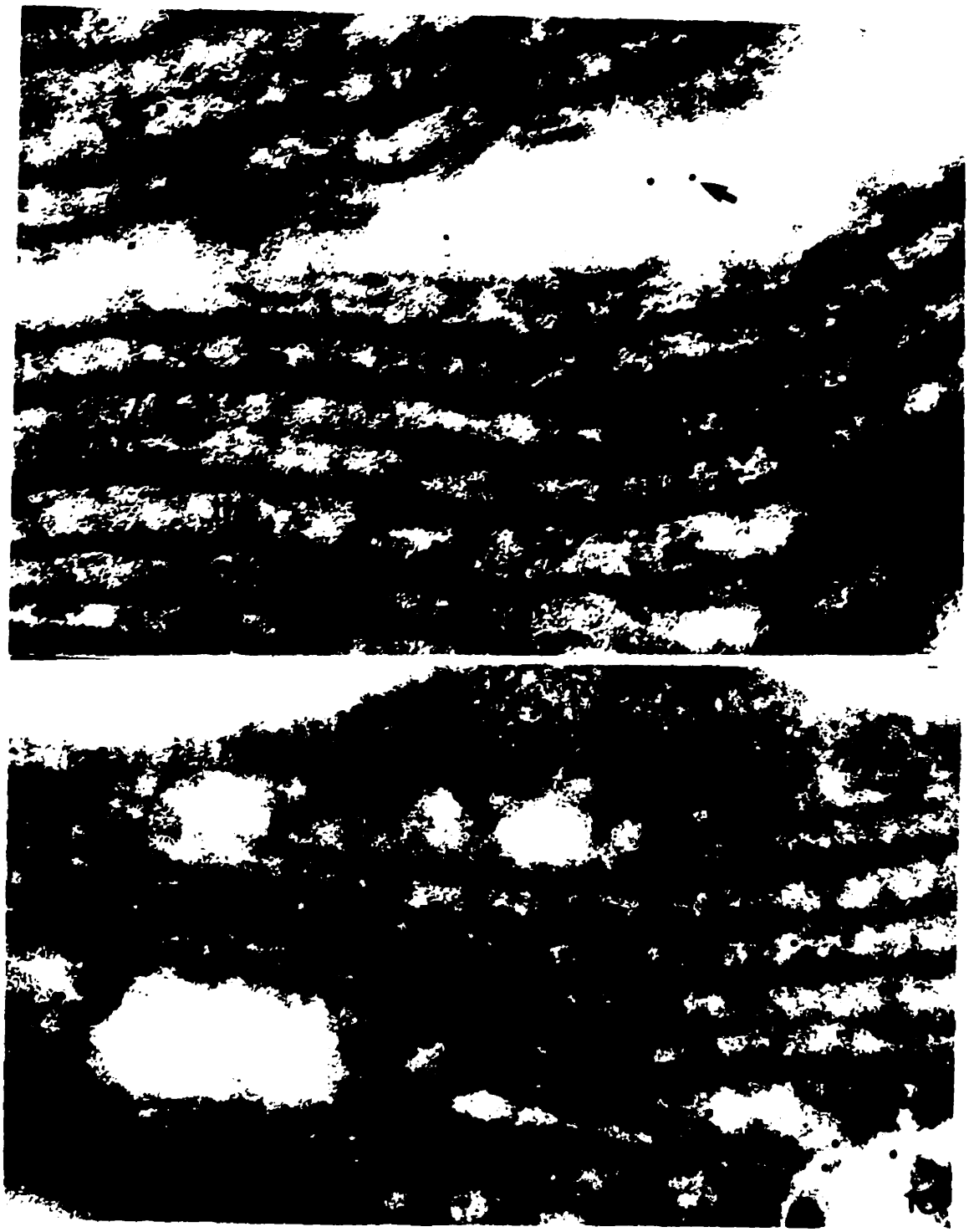
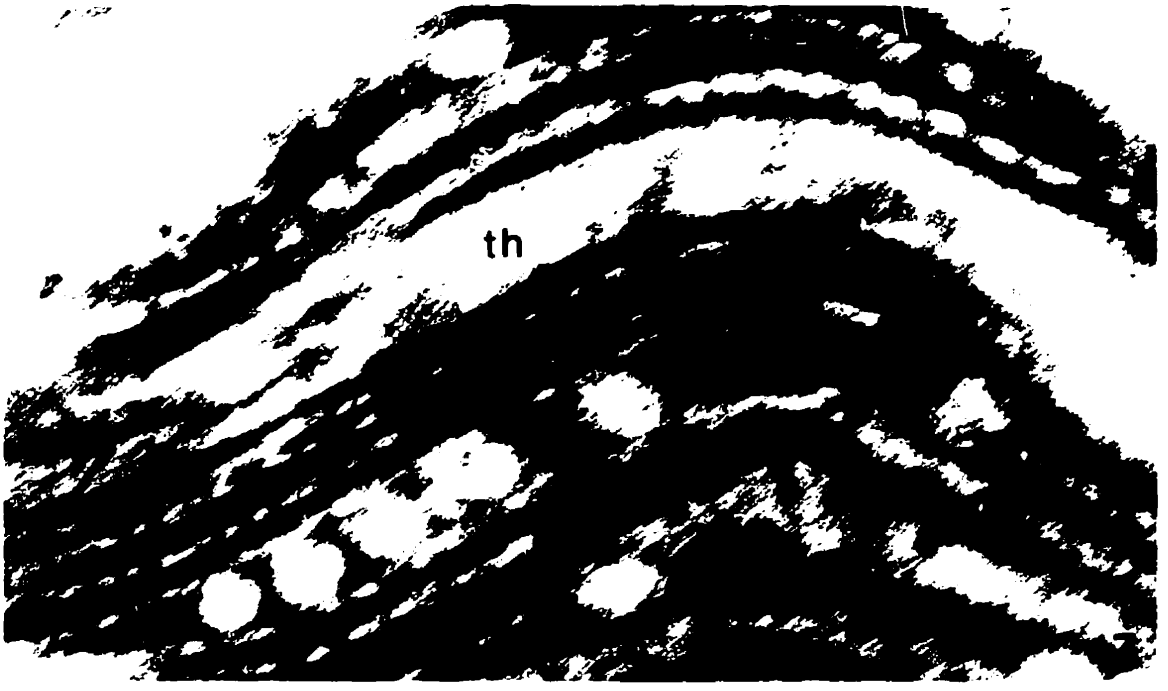


FIGURE 17.

Control experiment for the localization of PS I (cp1-e protein) and PS II (CP47 protein). Cells were treated as were the experimental group except that the antibody incubation was replaced by an incubation with a 1/50 dilution of pre-immune serum from rabbit. Very little labelling was observed on either the thylakoids (th) or the stroma (s). (X104,000)


th

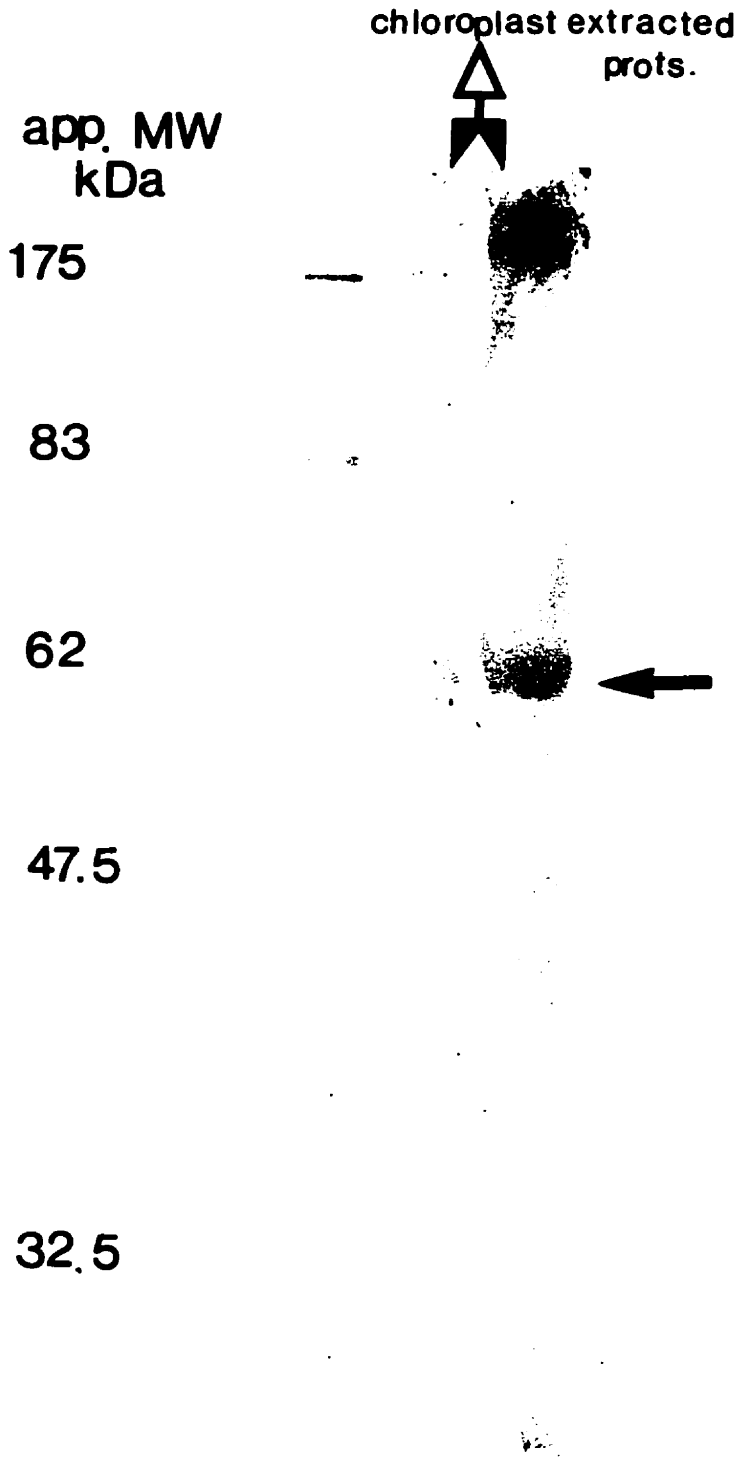
b) Specificity of the CP47 antibody

To test the specificity of the antiserum raised against CP47 of *S. elongatus* for the corresponding PS II core antenna protein, *i.e.*, the *psbB* chloroplast gene product of *E. gracilis*, a crude chloroplast extract was prepared and their proteins extracted. An SDS-PAGE gel was run and the proteins were transferred to a PVDF-transfer membrane incubated with CP47 antibody and detected with HRP-DAB. The results in Figure 18 shows that anti-CP47 recognizes a single band of ≈ 60 kDa (which is very close to the size already reported in the literature for the *psbB* gene product in *Euglena gracilis*: 58.5 kDa). Thus, this indicates that the CP47 antibody from *S. elongatus* is recognizing in a specific manner the corresponding PS II core antenna protein of *E. gracilis*.

Specificity of the anti-CP47 antisera

FIGURE 18.

Western Blot. Proteins isolated from a "crude chloroplast" fraction from *E. gracilis* were run on an SDS-PAGE gel, transferred to a PVDFT-transfer membrane and probed with the CP47 antibody from *S. elongatus*. The results show one single band of approximately 60 kDa (arrow).


chloroplast extracted
prots.
app. MW
kDa
175
83
62
47.5
32.5

CHAPTER 4

LOCALIZATION OF THE CP47 PROTEIN IN THE MITOCHONDRIA OF *EUGLENA GRACILIS* AND RELATED ORGANISMS

Results of our study on the localization in the mitochondria of the protein encoded by the psbB gene of the chloroplast in different euglenoids

The purpose of the present study was to determine whether the CP47 protein (core antenna of the PS II complex), product of the chloroplast gene *psbB*, was present in the mitochondria of *Euglena gracilis* Z strain and related organisms such as: dark grown cells of *E. gracilis*, the mutant Y9Z1NaL, and *Astasia longa*. To localize the CP47 protein in the mitochondria of those organisms, immunoelectron microscopy techniques were employed and the data analyzed. Since CP47 is a thylakoid membrane protein expressed in plastids, the main criteria used in selecting the organisms related to *Euglena gracilis* was that they should not be expressing the CP47 protein or did not have the plastid gene for it.

1. Preliminary observations

During the course of the study on localization of Photosystem I and II in autotrophically grown cells of *Euglena gracilis*, it was fortuitously observed that antiserum against the core antenna protein CP47 of PS II of *S. elongatus* also labelled the mitochondria of the cells and, moreover, that many of the gold particles were associated with their cristae. Since the labelling was clearly higher than the background over the cell, in order to corroborate and extend this observation, light-grown cells of *Euglena gracilis* were labelled with a different antibody raised against CP47 but this time from the green alga, *Chlamydomonas reinhardtii.* In addition, organisms that do not express the CP47 protein, *i.e.* dark-grown cells of *Euglena gracilis* and cells of the non-photosynthetic mutant Y9Z1NaL were labelled with the antisera. A third euglenoid A*stasia longa* was also probed. The results of this last experiments helped elucidate if a transfer of the chloroplast gene *psbB* to the mitochondria (or nucleus) had occurred before or after the branching of this colorless euglenoid from *Euglena gracilis.*

2. Immunolabelling of the mitochondria of autotrophic *Euglena gracilis* with antisera against CP47 of PS II

Figure 19 shows a cross section of an autotrophically grown cell of *E. gracilis* (through a chloroplast, the pellicle and numerous mitochondria) fixed in glutaraldehyde and embedded in Lowicryl. The section has been labelled with an antibody raised against CP47 from *S. elongatus* (1/100 dilution). As shown in Chapter 3, the light thylakoid bands are heavily labelled (large empty arrow), whereas the dark regions of the stroma are virtually unlabelled. The numerous mitochondria that appear in the micrograph also display heavy labelling. The mitochondrial labelling was calculated to be 47.9 ± 6.06 gold particles/μm^2 (Table 3). This labelling is almost as high as that observed over the chloroplast (59.1 ± 5.44 gold particles /μm^2) (Table 2). Furthermore, a number of gold

Detection of CP47 in the mitochondria of *E. gracilis*

FIGURE 19.

Localization of CP47 in *E. gracilis*. A cross section of *E. gracilis* embedded in Lowicryl resin and labelled with anit-CP47 from *S. elongatus* showing the localization of CP47 in both the thylakoid membranes of the chloroplast (C) and the mitochondria (M). White arrow points to gold particles on the thylakoid membranes as expected, whereas the black arrow indicates labelling in the mitochondria, and even more specifically, the long thin arrow indicates a gold particle that is lying on the mitochondrial cristae (c). (X63,100)

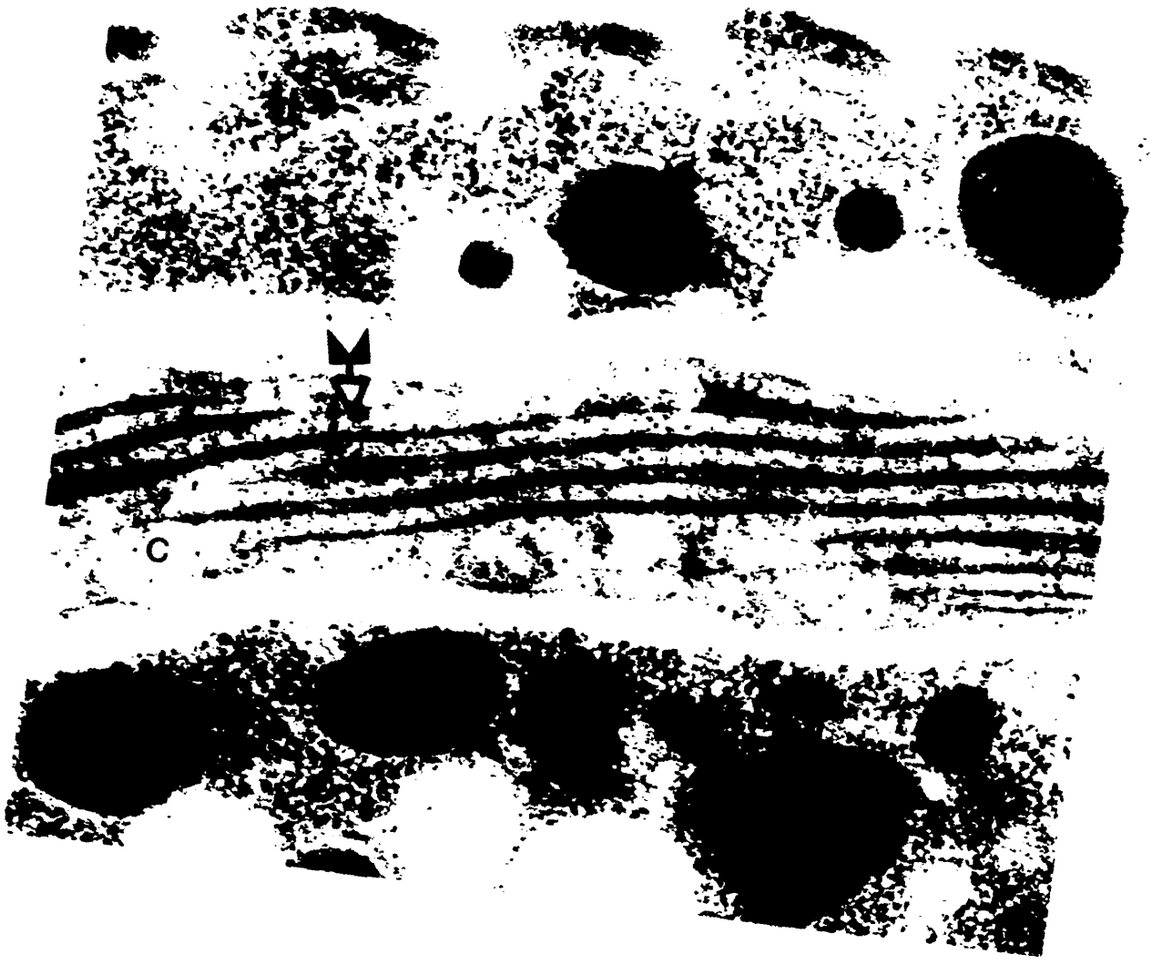

C

Table 3. Labelling of the mitochondria of autotrophic cells of *Euglena gracilis* with two different antibodies against CP47.

Source of antibody	Labelling density		Density ratio
	Mitochondria	Cytoplasm	Mitochondria/cytoplasm
	gold particles/μm^2 ± SEM		
S. elongatus	47.9± 6.06	17.4 ± 1.84	2.8
C. reinhardtii	23.3± 2.79	6.1 ± 0.48	3.8
Pre-immune serum	3.10± 1.13	1.0 ± 0.26	NA

particles are observed to lie over the cristae (Figure 19, long thin arrow; Figure 20, top arrow).

In control experiments, where the antibody incubation was replaced by one with pre-immune serum (1/50 dilution) from rabbit, no or very little labelling was found (Figure 21 and Table 3). In the labelled cell sections, mitochondrial labelling was 2.8 times higher than the background (Table 3).

To verify the presence of this protein in the mitochondria, we used a different antibody raised against the CP47 protein of another organism. Figure 22 shows a cross section of a cell treated with anti-CP47 from *Chlamydomonas reinhardtii* (1/50 dilution). Again numerous gold particles were localized on the mitochondria, and more specifically on their cristae (Figure 22, arrows). Table 3 shows that the mitochondria labelling was 23.3 ± 2.79 gold particles/μm^2 of organelle sectioned and that the mitochondrial labelling was 3.8 times heavier than that found in the cytoplasm.

3. Immunolabelling of the mitochondria of dark-grown cells of *Euglena gracilis* with antisera against CP47 of PS II

To test if the mitochondria were labelled when the chloroplast was not making any CP47 protein, we worked with cells grown in the dark (for several generations) whose chloroplasts are reduced to small proplastids and are not functional. Results show that when cell sections are incubated with 1/100 dilution of CP47 from *S. elongatus* there is labelling in the mitochondria (Figure 23, arrows) and specifically on their cristae (Figure 24, thin long arrows). Note that the cytoplasm of the cell displays very little background labelling.

Quantitative analysis (Table 4) shows that the mitochondria of dark grown cells, though much larger than those in light-grown *Euglena*, had 26.0 ± 1.34 gold particles/μm^2. This labelling was 3.8 times higher than the 6.9 ± 0.68 gold particles/μm^2 observed over the cytoplasm.

FIGURE 20.

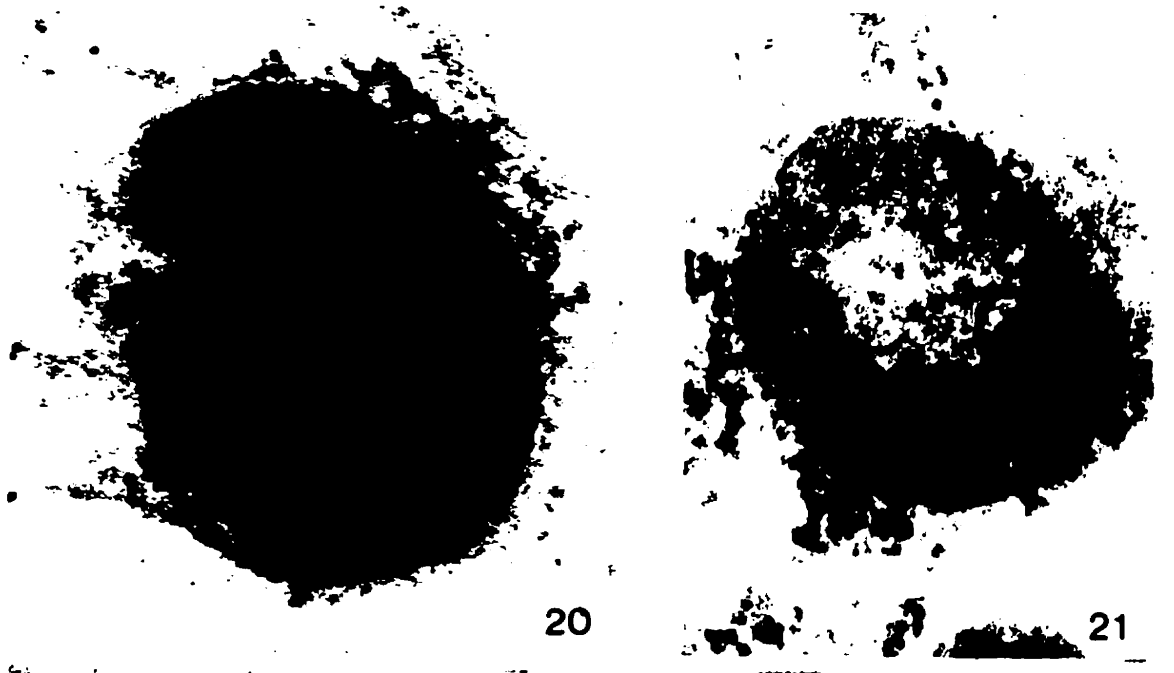
Localization of CP47 in a mitochondrion of wild type cell of *E. gracilis*. Immunolabelling of wild type cells with CP47 antibody from *S. elongatus* (1/100 dilution) shows binding in the mitochondria of the cell. Arrows show gold particles attached to the membranous parts of the mitochondrion, both the mitochondrial envelope and cristae (c). (X145,500)

FIGURE 21.

Control experiments (for FIGURES 19, 20 and 22) in which the incubation with the antibody was exchanged by an incubation with pre-immune serum of rabbit. No or very little labelling was observed over either the mitochondria or surrounding cytoplasm. (X86,100)

FIGURE 22.

Immunolabelling of wild type cells using a different antibody (1/50 dilution) against the CP47 protein of PSII. This antibody was raised against what was then designated chloroplast Protein 5 of *C. reinhardtii*. This antibody also shows conspicuous binding in the mitochondria (M) of the cell. Arrows show gold particles attached to the mitochondrial cristae. Only a few background gold particles are present over the cytoplasm (cyt). (X93,300)



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21

Detection of CP47 in the mitochondria of dark grown cells of *E. gracilis*

FIGURE 23.

Localization of CP47 in mitochondria of wild type cells of *E. gracilis* grown in the dark. Immunolabelling of dark-grown wild type cells grown in the dark with CP47 antibody from *S. elongatus* (1/100 dilution) shows binding in the mitochondria (M) of the cell (large empty arrows); while the cytoplasm (cyt) is almost unlabelled. (X61,600)

FIGURE 24.

A cross section showing the cristae (c) of the mitochondria of dark grown cells with gold particles attached to them. (X76,500)

FIGURE 25.

In the control experiments the antibody incubation was replaced by an incubation with a 1/50 dilution of PIS from rabbit. No labelling was seen in the mitochondria or more specifically in their cristae. (X76,500)

23

24

25

Table 4. Labelling of the mitochondria of dark grown cells of *Euglena gracilis* with antisera against CP47.

Antibody	Labelling density		Density ratio
	Mitochondria	Cytoplasm	Mitochondria/cytoplasm
	gold particles/μm^2 ± SEM		
Anti-CP47	26.0 ± 1.34	6.9 ± 0.68	3.8
Pre-immune serum	0.21 ± 0.14	0.66±0.26	NA

On control sections incubated with pre-immune serum (1/50 dilution) from rabbit, virtually no gold particles were present in the mitochondria or their cristae (Figure 25 and Table 4).

4. Immunolabelling of the mitochondria in *Euglena gracilis* mutant strain Y9Z1NaL with antisera against CP47 of PS II

Y9Z1NaL, a mutant strain of *Euglena*, is an organism where the CP47 protein cannot be transcribed and translated. Nonetheless, in Lowicryl sections of the mutant labelled with anti-CP47 from *S. elongatus* (1/100 dilution), the mitochondria show heavy labelling in the mitochondria (Figure 26) with many gold particles associated with the cristae (Figure 26, long thin arrow).

Quantitative analysis (Table 5) shows that the density of gold particles/μm^2 over the mitochondria is $30.5 \pm 2.08/\mu m^2$ after labelling with anti-CP47, 2.4 times the density of labelling over the cytoplasm.

Control experiments shown in Figure 27 indicate that there is essentially no labelling when cells are incubated with pre-immune serum (1/50 dilution) from rabbit (see also Table 5).

5. Immunolabelling of the mitochondria of *Astasia longa* with antisera against CP47 of PS II

As a last effort to corroborate the fact that a chloroplast protein involved in photosynthesis is present in the mitochondria of some euglenoids, we decided to test *Astasia longa*. *Astasia longa* is a non-photosynthetic euglenoid which has evolved from *Euglena gracilis* . This colorless euglenoid possesses only a part of the chloroplast DNA and no *psbB* gene (responsible for coding the CP47 protein) has been found in the reduced plastid genome. Figure 28 shows a section of mitochondria of *A. longa* labelled with a

Detection of CP47 in the mitochondria of the mutant strain Y9Z1NaL

FIGURE 26.

Localization of CP47 in mitochondria of the Y9Z1NaL mutant. Immunolabelling of mutant cells with CP47 antibody from *S. elongatus* (1/100 dilution) shows binding on the cristae (c) of the mitochondria of the cell (long thin arrows); while the cytoplasm remains almost unlabelled. (X74,900)

FIGURE 27.

In the control experiments, the antibody incubation was replaced by an incubation with a 1/50 dilution of PIS from rabbit. No or very few gold particles could be detected in the mitochondria (M) of the mutant strain. (X76,800)

26

27

Table 5. Labelling of the mitochondria of cells of *Euglena gracilis* mutant Y9Z1NaL with antisera against CP47.

Antibody	Labelling density		Density ratio
	Mitochondria	Cytoplasm	Mitochondria/cytoplasm
	gold particles/$\mu m^2 \pm$ SEM		
Anti-CP47	30.5 ± 2.08	13.0 ± 1.66	2.4
Pre-immune serum	3.00 ± 1.23	0.32 ± 0.20	N/A

1/50 dilution of anti-CP47 *of S. elongatus*. The mitochondria are heavily labelled (67.5 ± 4.66 gold particles/μm^2), with many gold particles labelling the cristae (Fig. 28, arrows). The mitochondrial labelling is 3.9 higher than the background cytoplasmic labelling (Table 6).

Figure 29 shows a section of *A. longa* labelled with pre-immune serum. No gold particles are present over either the mitochondria or cytoplasm. Table 6 confirms the low labelling obtained with pre-immune serum.

6. Quantification of cristae labelling by antisera against CP47 of PS II

Even though, usually, it is difficult to visualize the cristae of the mitochondria unless they are cut perpendicularly to their membranes, we have made an attempt to quantify the labelling related to the cristae. In every mitochondrial profile analyzed, the total length of visible cristae membrane was measured. As well, the number of gold particles on or touching the cristae membrane were counted. The total area of each mitochondrial profile and the total gold particles were calculated as well. Table 7 gives the labelling density (gold particles/µm of cristae membrane) and the percentage of mitochondrial label on the cristae. This latter figure which ranges from 18.2% in light-grown cells of *Euglena gracilis* to 56.1% in cells of *Astasia longa* is without doubt an underestimation. Also, the more conspicuous the cristae are in glutaraldehyde-fixed mitochondria, the higher the percentage of gold particles localized in the mitochondria.

Detection of CP47 in the mitochondria of *Astasia longa*

FIGURE 28.

Localization of CP47 in mitochondria of wild type cells of *Astasia longa*. Cells of *Astasia longa* labelled with anti-CP47 from *S. elongatus* (1/50 dilution). Numerous gold particles are present over the mitochondria (M), many attached to the cristae (c) (long thin arrows). The cytoplasm remains almost unla-belled. (X65,500)

FIGURE 29.

A cross section of a control cell, where the antibody incubation was replaced by an incubation with 1/50 PIS from rabbit, shows no labelling. (X73,000)

Table 6. Labelling of the mitochondria of cells of *Astasia longa* with antisera against CP47.

Antibody	Labelling density		Density ratio
	Mitochondria	Cytoplasm	Mitochondria/cytoplasm
	gold particles/μm^2 ± SEM		
Anti-CP47	67.5 ± 4.66	17.2 ± 1.65	3.9
Pre-immune serum	1.50 ± 0.42	3.8 ± 1.10	NA

Table 7. Labelling of the mitochondrial cristae by antibody against the chloroplast protein, CP47.

Organism	Labelling density in the cristae [1]	Percent of mitochondrial label on cristae
	gold particles/μm ± SEM	%
Euglena gracilis light grown	0.4 ± 0.08	18.2
Euglena gracilis dark grown	0.7 ± 0.13	25.9
Euglena gracilis mutant Y9Z1NaL	2.9 ± 0.26	45.4
Astasia longa	7.0 ± 0.65	56.1

[1]The length of all the cristae cut perpendicularly was measured.

CHAPTER 5

DISCUSSION

Preamble

Chapter 5 will be divided in two parts: A) Discussion of the localization of PS I and PS II in *Euglena gracilis*, followed by B) a discussion of the localization of the CP47 protein in the mitochondria of *Euglena gracilis* and related euglenoids.

A) Localization of PS I and PS II in *Euglena gracilis*

In order to have a broad picture of evolution, scientists have used structural and molecular similarities among organisms to group them together or differences to demonstrate their separations (*i.e.*, branchings) in the evolutionary tree. This task has been straightforward for some organisms but for others, even with the biological information presently existing, it is difficult to give definite answers.

The information coming from molecular biological studies can elucidate greatly some of the still existing puzzles in evolutionary lineages, but still morphological and structural characteristics will be important tools to support the inferences of those studies.

We believe that the study of the ultrastructural morphology of chloroplasts of algae will give an insight into the evolution of algae and land plants. Algae and higher plants have different types of chloroplasts. The number of membranes (two, three or four membranes) surrounding the chloroplast, the types of chlorophyll present, the organization of the thylakoid membranes in the plastid's stroma and the distribution of the two different photosystems in these latter membranes are among the morphological traits that can help elucidate evolutionary relationships. Moreover, it is possible that the structural organization of the thylakoid membranes in algae is related to the segregation of the two types of photosystems in them.

Euglena gracilis is an interesting organism to study since its chloroplast is surrounded by three membranes. This feature led Gibbs (1978) to hypothesize that the chloroplast of *Euglena* is derived from a double endosymbiotic event, where a non-photosynthetic euglenoid may have engulfed a primitive green alga, which itself was the product of another endosymbiotic event involving a photosynthetic prokaryote and a non-photosynthetic eukaryote.

We have studied the localization of PS I and PS II in the thylakoid membranes of the plastid of *E. gracilis* employing immunocytochemical techniques. The resolution of the immunocytochemical techniques has been shown to be sufficient for the study of localization

of photosystems in algae (Song and Gibbs, 1995; Bertos and Gibbs, 1998). We have also been very careful to count in a consistent manner and analyze for background (see Chapter 2. Material and Methods). The antibodies used for this study were specific for the proteins that they were raised against. Kashino *et al.* (1990) have shown using Westerns blots that anti-CP1-e raised against the 60 and 62 kDa PS I reaction center proteins of the cyanobacterium *Synechococcus elongatus* cross reacted with isolated thylakoid membrane proteins of the same molecular weight from three different green algae. The authors have also shown that anti-CP47 labels a 47 kDa protein in Western blots of thylakoid membrane proteins of the same green algae. We are confident that the specificity seen for green algae is also present in *Euglena*. Moreover, we tested for the specificity of the antiserum raised against the CP47 of *S. elongatus*, and the result in Figure 18 shows that anti-CP47 recognizes a single band of *circa* 60 kDa (which is close to the size reported for *E. gracilis* in the literature: 58.5 kDa).

PS I was localized by two different techniques. First, EM immunogold cytochemistry, using two different antibodies raised against either whole particles or specific proteins of the reaction center, located PS I in the thylakoid membranes. Second, PS I activity was demonstrated by the photo-oxidation of DAB. In both cases our results indicate that the PS I reaction centers in the thylakoid membranes of *E. gracilis* are located on both types of membranes: appressed and non-appressed. This is in contrast with the accepted organization of PS I in higher plants where PS I is mostly located on the non-appressed membranes (Anderson, 1982).

This obvious segregation of photosystems observed in higher plants does not occur in *Euglena*. Both antibodies (one raised in higher plants: maize, and the other in cyanobacteria: *S. elongatus*) have a similar labelling density for the same type of membrane (0.44-0.40 and 1.57-2.30 gold particles/μm in AM and NAM respectively). Both results show that 60-74% of the labelling was localized on appressed membranes, a marked discrepancy with the land plants results. Our findings are closer to the PS I labelling results found in the primitive green alga *Tetraselmis subcordiformis* where 76-78% of the

labelling was localized in the appressed membranes (Song and Gibbs, 1995) and the one in the green alga *Chlamydomonas reinhardtii* where 50-62% of the labelling was found on appressed membranes (Bertos and Gibbs, 1998). Even though there are two conflicting results in the literature for *C. reinhardtii* (Vallon, 1986; Bertos and Gibbs, 1998), we believe that the conditions in the method of fixation used by Bertos and Gibbs (1998) and the fact that they did not break the cells are more suitable for obtaining accurate results. We thus suggest that our results are closer to those found in green algae than those found in higher plants.

When the activity of PS I was analyzed by a cytochemical method, we found that both types of membranes display the dense deposits that are a sign of activity. These results confirm that PS I is located in both types of membranes in the alga *Euglena gracilis* Z strain. With this method our observations are different depending on the time of harvesting the cells. If the cells are harvested at the end of the dark cycle, PS I activity is mostly present in the lumens of the outer thylakoid membranes, but when the cells are harvested at hour six of the light cycle then the activity is found throughout the thylakoid band. In the latter case, the internal thylakoid lumens are stained as intensely as the lumens of the outermost thylakoids. This is an interesting observation since this could imply a physiological mechanism. It is possible that the photosystems that are closer to the stroma, and therefore closer to the source of light, are the ones that start functioning first and hence the presence of the dark electron dense deposits in the lumen of the non-appressed thylakoid (and not in the inner thylakoid of the band) when the cells are harvested at the beginning of the light cycle.

Our results also show that PS II is located in both types of membranes in *Euglena gracilis* (with labelling density of 0.63 and 2.47 gold particles per μm for AM and NAM respectively). Forty-six percent of the labelling is present on NAM. These results are in agreement with the results for the green algae. There is a consensus that PS II is present in both types of membranes (Vallon *et al.*, 1985; Song and Gibbs, 1995; Bertos and Gibbs, 1998).

Our results show that both photosystems are more concentrated in the NAM than in the AM. This has previously been reported by Song and Gibbs (1995) and Bertos and Gibbs (1998); it seems to be a consistent trait among green alga chloroplasts. A plausible explanation is that this happens either firstly, because the proteins studied are coded by plastid genes and translated in polysomes in the stroma. These proteins are inserted first in the outer membranes of the thylakoids and have to "travel" to their final destination in the inner membranes which are appressed. Or secondly, because the outer membranes of the thylakoids are the ones exposed to stroma and, therefore are closer to the source of light, and this higher concentration of both types of photosystems would enhance the efficiency of the photosynthetic process. If one assumes that the higher concentration of PS I and II in the outer membranes is not genuine, one could argue that in the appressed membranes there is a higher possibility for steric hindrance that will make recognition of the antigen by the antibody more difficult. Stys (1995) has suggested that the dense material observed between pairs of appressed thylakoids membranes consists of a network of projecting proteins, such as PS II complexes, LHCII and PS I complexes.

The most important difference between the chloroplasts of algae and those of higher plants, aside from the number of membranes surrounding them as well as the pigment that they contain, is the organization of their thylakoid membranes. The chloroplasts of land plants have their thylakoids organized in a very well arranged and distinct manner known as grana. These structures are made of stacks of short thylakoids that are connected by long ones to other neighbouring stacks. The long thylakoids that connect the well-packed stacks are known as stroma thylakoids. The non-appressed membranes in higher plants consist mostly of the membranes of this type of thylakoids, that are exposed to the stroma, and of the top, bottom, and edges membranes of the grana thylakoids. It is this peculiar arrangement that supports the physiology of the photosynthetic process. Light is absorbed by the light harvesting proteins of PS II and the energy is transferred to PS I by the intermediary of a LHC II which is phosphorylated in the AM and then moves to the NAM stromal thylakoids membranes where it donates the

energy to PS I (Staehelin and Arntzen, 1983). This phenomenon is known as state transition and is in part responsible for the stacking and unstacking of thylakoids. In the green algae, the thylakoids are not yet organized in true grana, but they are associated in bands that transverse the stroma; single thylakoids are rare. In these algae the NAM are made up of the top and bottom (stroma exposed) membranes of the band. The organization of the thylakoids in the chloroplast's stroma of *Euglena gracilis* are much closer to those of green algae than those of higher plants. It has been shown that the distribution of the photosynthetic reaction centers (PS I and PS II) is different in organisms that have different thylakoidal organization. For example, in the red algae, whose chloroplasts have evolved from cyanobacteria and have single thylakoids, PS I and PS II are uniformly distributed along the thylakoid membrane (Mustardy *et al.*, 1992). Cryptomonads, algae whose chloroplasts are closely related to red algae and cyanobacteria since they contain phycobiliproteins, also have the PS I and the chlorophyll *a/c* LHC of PS II distributed in both the appressed and non-appressed thylakoids membranes (Rhiel *et al.*, 1999; Lichtlé *et al.*, 1992). The brown algae and the diatoms have bands of three thylakoids in the stroma of their chloroplasts and contain chlorophylls *a* and *c*. Immunogold labelling showed that in those organisms PS I and PS II were distributed on both types of membranes (Lichtlé *et al.*, 1992; Pyszniak and Gibbs, 1992). In a charophyte alga, *Coleochate scutata* which has a chloroplast with some thylakoids in extended bands, but many in typical grana, PS I has started to become more concentrated on the NAM and PS II more concentrated on the AM (ratio of NAM/AM of 2.6 for PS I and 0.6 for PS II) (Kerr, 1997) thylakoids.

Our results show that in *Euglena gracilis*, which contains chlorophyll *a* and *b* and has thylakoids arranged in extended bands along the stroma of the chloroplast, PS I and PS II are localized in the two types of thylakoid membranes. Moreover, both PS are more concentrated in the NAM. This distribution correlates with the fact than in all the groups

of algae that have the same organization of thylakoids, both photosystems are present in AM and NAM membranes. Thus, the distribution of PS I and PS II appears to be closely related to the particular organization of the thylakoids, and to have evolved gradually. Only when the grana structures are present is the heterogeneous distribution of the PS observed, as it is for higher plants.

B) Localization of the CP47 protein in *Euglena gracilis* and related euglenoids.

Movement of genetic material between DNA-carrying organelles has been observed in a variety of animals and plants. The biological significance of these occurrences is still unclear, since only rarely is the integrated DNA sequence expressed in its new host. Even though the meaning of these events is not well understood, their study is of importance since it can help illuminate phylogenetic relationships between organisms. In the past two decades more and more evidence of such transfers have been identified (see Chapter 1, Introduction). The pieces that have been transferred, during evolution, from one organelle to another were either entire genes, cluster of them or truncated pieces. The sequencing of organellar genomes from different species will undoubtedly uncover more examples. The three DNA carrying organelles, the nucleus, mitochondrion, and chloroplast, are supposed to have genomes that are largely distinct. The presence of chloroplasts in land plants and algae provides a greater opportunity for organellar DNA translocation when compared with non-photosynthetic organisms. The mechanism by which DNA escapes from organelles and its meaning is not well understood. The escape of DNA from the nucleus is likely to occur through the nuclear pores, but the mechanism of escape from the membrane-bound organelles seems to be more complicated. Mitochondria are surrounded by two membranes, as are the chloroplasts of higher plants. However, some chloroplasts in algae are surrounded by up to four membranes. It is not easy to envision precisely how DNA pieces exit and enter these organelles. It is believed that the presence of promiscuous DNA in the different organelles is explained by the escape of genomic material that occurs under special circumstances - stress, organelle degradation, and close contact between organelles - allowing the pieces of DNA to leave one organelle and enter the other. These pieces are then inserted into the genome of the host organelle.

During evolution some genes have moved from chloroplasts to mitochondria. Good examples of successful integration are the tRNAs of plastid origin found in

mitochondria of land plants since they are expressed in the mitochondria. In contrast, very few genes (or sequences) that code for proteins of the photosynthetic reaction centers (PS II and I) have been found in the mitochondria of organisms. One that is highly unusual is the *psbG* gene that codes for the PS II protein G in the plastid of plants. This sequence was found in the mitochondria of *Paramecium aurelia* (Pritchard *et al.* , 1989). Also part of the *psbA* gene that codes for the quinone binding protein of PS II was found in the mitochondria of maize (Sederoff *et al.*, 1986). Another ctDNA gene involved in photosynthesis which has been found in the mitochondria of organisms is the large subunit of Rubisco (Stern and Palmer, 1984; Lonsdale *et al.*, 1983).

We have localized by EM immunocytochemistry a plastid-coded protein (or part of it) in the mitochondria of four euglenoids. This protein (CP47) forms part of the photosynthetic multi-complex reaction center of PS II. In the chloroplast of higher plants and algae, this peptide is coded by the *psbB* gene. To dismiss the possibility of having either background binding or random cross-reaction, we used two different antibodies raised against the CP47 protein of two different algae, a cyanobacterium (*S. elongatus*) and a green alga (*C. reinhardtii*). In both cases we found similar binding. We therefore believe that the antibody binding is genuine and that the CP47 is localized in the mitochondria of euglenoids (see chapter 4). Moreover, our results show that the CP47 is associated with the mitochondrial cristae (this is observed when sections are cut perpendicular to the cristae membranes). This distribution is consistent with the fact that the peptide is normally located in the thylakoid membranes of the chloroplast.

The only other chloroplast protein that has been found in algal mitochondria is the small subunit of Rubisco, coded by the gene *rbcS* (Lacoste-Royal and Gibbs, 1985). Their immunocytochemical data showed that the *rbcS* gene product was present either in its entire form or in a truncated form in light- and dark-grown cells of the crysophyte alga *Ochromonas danica*. Since then the entire mtDNA of *O. danica* has been sequenced and the *rbcS* gene was not found (Gray *et al.*, 1998). In *Euglena*, green algae, and higher plants, *rbcS* is located in the nucleus, but in *Cyanophora*, the diatom *Odontella* and the red

alga *Porphyra* as it is in *Olisthodiscus luteus*, *rbcS* is located in the plastid genome (Martin *et al.*, 1998; Reith and Cattolico, 1986). *Olisthodiscus* is an heterokont alga like *Ochromonas* and the diatoms also are closely related to *Ochromonas* which would suggest that the *rbcS* gene could be located as well in the chloroplast genome of *Ochromonas*. However, Cattolico's group (Lee and Cattolico, 1992; Shivji *et al.*, 1992) were unable to find the *rbcS* gene in the chloroplast DNA of *Ochromonas danica* suggesting that in this organism the gene for the small subunit of Rubisco may be found in the nucleus, and the presence of SSU in the mitochondria of *Ochromonas danica* may be the result of mistargeting of a nuclear coded protein.

The *psbB* gene is always found in the sequenced chloroplast genome of photosynthetic organisms (Martin *et al.*, 1998). Among these organisms are glaucocystophytes, rhodophytes, diatoms, dinoflagellates (Zhang *et al.*, 1999), euglenophytes and land plants. To our knowledge this gene has never been found in the nuclear genome of any organism. Thus, we consider it very unlikely that the presence of the CP47 in the mitochondria of wild *Euglena gracilis*, dark-grown cells of the wild type, mutant Y9Z1NaL, and related euglenoid *Astasia longa* could be the result of mistargeting of a nuclear-coded protein into the mitochondria.

There are two possible mistargeting scenarios. The CP47 protein could be coded by a nuclear *psbB* gene, translated in the cytoplasm, and transported to the mitochondria or it could be coded by a plastid *psbB* gene, translated in the chloroplast, and transported mistakenly to the mitochondria. As discussed above the former event is unlikely since the gene has not been found in the nucleus of other photosynthetic organisms. Organisms that do not produce the plastid-CP47 protein were used to address the latter possibility. Wild type dark-grown cells of *E. gracilis* do not possess functional chloroplasts, but rather organelles known as proplastids. These proplastids do not differentiate into chloroplasts until they are exposed to light, and therefore do not transcribe and translate photosynthetic proteins. We also tested the mutant Y9Z1NaL that does not accumulate transcripts of *psbB*. In both types of organisms, we found that there was labelling in their mitochondria

when tested with anti-CP47 antiserum. Moreover, when the micrographs were analyzed, we did not observe any labelling that could be considered an indication of proteins being transported through the cytosol to the mitochondria. The background analysis also shows that there is no significant labelling of the cytoplasm and its organelles.

We therefore propose that the presence of the CP47 protein in the mitochondria of *E. gracilis* is due to a DNA translocation from the plastid to the mitochondria that occurred during the evolution of euglenoids.

Since our results cannot be explained by protein mistargeting mechanisms, and because they occurred consistently in wild type, dark-grown cells and a mutant, it could be that the gene or part of the gene for CP47 was transferred during the evolution of euglenoids from the chloroplast to the mitochondria. Since our immunocytochemical results show that the CP47 protein is localized in the cristae of the mitochondria, and because we have used two different antisera to localize it in wild type cells we believe that at least part of the protein must be expressed. Since the Y9Z1NaL mutant was isolated from cultures of wild type *Euglena* grown in presence of a chloroplast inhibitor, the fact that we found labelling in its mitochondria could suggest that the transfer had already occurred.

To situate when the transfer of the *psbB* gene from plastid to mitochondria occurred in the evolutionary scale, we tested *Astasia longa*, an organism that branched off the *Euglena* lineage. *Astasia* is a non-photosynthetic euglenoid which has lost its chloroplasts as well as the *psbB* gene, which is no longer present in its remnant plastid-like DNA. The results show that there is labelling for the plastid-coded protein in the mitochondria of this organism. This indicates that the transfer of the *psbB* sequence occurred before the branching of these two euglenoids.

A corollary of the observations on *Astasia longa* is that a euglenoid which never possessed chloroplasts could not have a chloroplast protein in its mitochondria. If the mitochondria of a non-photosynthetic euglenoid which had never possessed a chloroplast were labelled by antiserum against CP47, this would mean that the two antibodies we used

were both recognizing a prominent epitope shared by CP47 and a mitochondrial protein. Recently A. Jenks in Dr. Gibbs' laboratory has performed this control experiment. *Peranema trichophorum*, a phagotrophic euglenoid, was chosen since ribosomal RNA data indicate that it is closely related to green euglenoids (Priesfeld *et al.*, 2000). When cells of *P. trichophorum* were fixed, embedded and labelled with anti-CP47 from *S. elongatus*, they displayed no mitochondrial labelling. No cell structure was specifically labelled. Sections of *E. gracilis* fixed for this study labelled with anti-CP47 at the same time displayed both chloroplast and mitochondrial labelling (S. Gibbs, personal communication).

There are many reports on expression of chloroplast-derived tRNA genes in mitochondria of organisms (Maréchal *et al.*, 1987; Joyce and Gray, 1989; Kanno *et al.*, 1997); also some photosynthetic genes have been found integrated into the mtDNA of plants (Stern and Lonsdale, 1982; Stein *et al.*, 1983). Is it then possible that the *psbB* (or a substantial part of it) would be integrated and expressed in the mitochondria of *E. gracilis* and the related euglenoids studied?

In 1997, Tessier *et al.* sequenced the first mitochondrial gene of *E. gracilis*: the *cox1* gene. They discovered that this gene had marked differences from nuclear DNA (*i.e.*, A+T content is higher and it lacks introns). Also, it was found that the amino-acid sequence of the COX1 protein was similar to other COX1 proteins from other eukaryotics (*i.e.*, protists, plants, fungi and animals). The authors suggested that the universal genetic code is used in the mtDNA of *Euglena* (Tessier *et al.*, 1997). It would then be possible that a foreign sequence could be integrated and become functional in the mitochondria. In the chloroplast DNA of *Euglena gracilis* the *psbB* gene forms part of a 2.4 kb cluster that also contains the *psbT*, *psbH*, and *psbN* genes. This genes are co-transcribed as pairs: *psbB-psbT* and *psbH-psbN* in opposite directions. When the nucleotide sequence of the *psbB* was published in 1989, it was reported that this gene is made of five exons and four introns. The exons code for a protein of approximately 58.5 kDa (Keller *et al.*, 1989). Our Western blot results (see Figure 18) shows that anti-CP47 recognizes a protein of the

correct molecular weight. We were not able to isolate the mitochondria and perform a Western blot on that fraction. However, the specificity of the antibody is tested by our results. Also, Keller *et al.* (1989) reported that the *psbB* spans a 3269 bp region and that it starts 60 bp downstream from the 3' end of the $tRNA^{Gly}$. Because there are so many examples of functional integration and expression of plastid tRNAs in the mitochondria in the literature, we believe it could have been possible that these two genes or part of them moved and integrated into the mitochondria of euglenoids from the plastid.

The other two possible explanations for the presence of the CP47 plastid protein in the cristae of euglenoids are: - first, that the gene was transferred from the chloroplast to the nucleus and then, a second migration to the mitochondria, or - second that once in the nucleus, it acquired a transit sequence that targets the product to the mitochondria. Even though there is evidence of migration a) from chloroplast to nucleus, and b) from nucleus to mitochondria (Schuster and Brennicke, 1988), we believe that the odds for the *psbB* gene to have migrated and integrated twice in a foreign genome and still be able to be expressed are very low. As for the second scenario as we stated earlier, in our results there are no indications that the protein is made in the cytoplasm and then enters the mitochondria.

Nonetheless, we are aware that we have not proven that the binding is genuine. It is difficult to dismiss without performing a number of molecular biological experiments the possibility that the labelling represents a cross-reaction with a mitochondrial protein that shares a common epitope with the CP47 plastid protein. Even though we checked in genome and protein data banks and could not find any shared sequences between the *psb*B gene and or the CP47 protein and known genes or proteins in the mitochondria, we are aware that we have used polyclonal antibodies and that these increase the chance of non-specific cross-reactions. Future experiments to determine if the labelling is genuine would require either the isolation of mtDNA of *E. gracilis* and its sequencing or *in situ* hybridization at the EM level with a DNA probe against the *psb*B gene.

We conclude that the *psbB* gene –a photosynthetic plastid gene– could be integrated, stable and properly transcribed and translated in the mitochondria of *Euglena gracilis* grown under two different conditions: light and dark, and also in the mitochondria of the mutant Y9Z1NaL and Astasia *longa*.

CHAPTER 6

CONCLUSIONS

1) Immunolabelling (with two different antibodies: anti-PS I from maize and anti-cp1-e from *Synechococcus elongatus*) and cytochemical techniques (photo-oxidation of DAB) demonstrated that Photosystem I is localized in both types of thylakoid (appressed and non-appressed) membranes in *Euglena gracilis* Z strain. Quantitative analysis shows that Photosystem I is more concentrated in the non-appressed membranes.

2) Photosystem II was also localized in both types of thylakoid membranes in *Euglena gracilis* Z strain by immunolabelling techniques. Similar to PS I, Photosystem II is also more concentrated in the non-appressed membranes.

3) Immunolabelling with two different antibodies raised against CP47 (anti-CP47 from *Chlamydomonas reinhardtii* and from *Synechococcus elongatus*) show that the CP47 protein of PS II is present in the mitochondria of light-grown cells of *Euglena gracilis*.

4) Immunolabelling of dark-grown cells of *Euglena gracilis* Z strain with anti-CP47 shows this PS II protein is present in the mitochondria of dark-grown cells.

5) Immunolabelling of the *Euglena gracilis* mutant Y9Z1NaL with anti-CP47 shows that the CP47 protein of PS II is present in its mitochondria.

6) Immunolabelling of the colorless *Astasia longa* with antisera against CP47 of PS II shows that this chloroplast protein is present in the mitochondria.

7) The labelling with anti-CP47 present on the mitochondria of light-grown cells of *Euglena gracilis* , of dark-grown cells of *Euglena gracilis*, of the mutant Y9Z1NaL, and of *Astasia longa* appears to be associated with the cristae of their mitochondria.

CHAPTER 7

APPENDIXES

Appendix 1

Organism	Media	Reference
Euglena gracilis Z strain	Autotrophic medium: Cramer & Myers	M. Cramer and J. Myers. Arch. Mikrobiol. 17,384-402 (1952)
Euglena gracilis Z strain dark grown	Cramer & Myers + 0.061 M Na acetate	
Astasia longa	Cramer & Myers + 0.02M Na acetate	D.E. Buetow and G.M. Padilla. J. Protozool 10,121-123(1963)
Y9Z1Nal mutant	Heterotrophic medium: Beale's	S. Beale , T. Folley, and V. Dzelzkanlns Proc. Natl. Acad. Sci. USA 78:1666-1669 (1981)

Appendix 2

Figure	Organism	Cell counts cells/ml	Fixation conditions	Resin
1	*E. gracilis*	$1.0x10^5$	2%glut, 1%OsO_4, 0.1M PB	Spurr
2	*E. gracilis*	$1.0x10^5$	2%glut, 1%OsO_4, 0.1M PB	Spurr
3,4	*E. gracilis*	$8.8x10^5$	2%glut, 1%OsO_4, 0.1M PB	Spurr
5,6,7	*E. gracilis*	$4.0x10^4$	2%glut+0.5%OsO_4, 0.1M PB simultaneously	Epon
8,9	*E. gracilis*	ND	1%glut, 0.1M NaCacodylate	Lowicryl
10,11,12	*E. gracilis*	ND	2%paraformaldehyde, 0.1M PB, 0.2M sucrose, 1%OsO_4 in 0.1M Cacodylate	Spurr
13,14	*E. gracilis*	ND	2%paraformaldehyde, 0.1M PB, 10.2M sucrose, 1%OsO_4 in 0.1M Cacodylate	Spurr
15,16	*E. gracilis*	$3.9x10^5$	1%glut, 0.1M NaCacodylate	Lowicryl
17	*E. gracilis*	$3.9x10^5$	1%glut, 0.1M NaCacodylate	Lowicryl
19	*E. gracilis*	$4.0x10^5$	1%glut, 0.1M NaCacodylate	Lowicryl
20,21	*E. gracilis*	$4.0x10^5$	1%glut, 0.1M NaCacodylate	Lowicryl
22	*E. gracilis*	$2.3x10^5$	1%glut, 0.1M NaCacodylate	Lowicryl
23,24,25	*E. gracilis* dark grown	$2.2x10^6$	1%glut, 0.1M NaCacodylate	Lowicryl
26,27	Y9Z1Nal mutant	ND	1%glut, 0.1M NaCacodylate	Lowicryl
28,29	*A. longa*	ND	1%glut, 0.1M NaCacodylate	Lowicryl

Appendix 3

Study	Organism	Antibody concentration	# of photo	# of gold part counted
Localization of PS I	*E. gracilis*	1/200 *Z. mais*	31	610
Localization of PS I	*E. gracilis*	1/50 *S. elongatus*	26	764
Localization of PS II	*E. gracilis*	1/100 *S. elongatus*	39	542
Localization of CP47 in mito	*E. gracilis*	1/100 *S. elongatus*	19	1088
Localization of CP47 in mito	*E. gracilis*	1/50 *C. reindhartii*	11	339
Localization of CP47 in mito	*E. gracilis* dark grown	1/100 *S. elongatus*	20	710
Localization of CP47 in mito	*A. longa*	1/50 *S. elongatus*	21	1821
Localization of CP47 in mito	Mutant Y9Z1NaL	1/100 *S. elongatus*	21	1061
Controls	All organisms	1/50 PIS* rabbit	10 each	ND

* As pre-immune serum, we used a normal rabbit serum from rabbit that we acquired commercially (see Chapter 2).

Abbreviations:

photo = photographs
part = particles
mito = mitochondria

CHAPTER 8

LITERATURE CITED

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