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THE CYANELLE AND THE CYANELLE GENOME OF CYANOPHORA PARADOXA

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THE CYANELLE AND THE CYANELLE GENOME OF *CYANOPHORA PARADOXA*

BY

Catherine Clair Wasmann

A DISSERTATION

Submitted to
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in partial fulfillment of the requirements
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Department of Botany and Plant Pathology

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ABSTRACT

THE CYANELLE AND THE CYANELLE GENOME

OF *CYANOPHORA PARADOXA*

By

Catherine Clair Wasmann

The photosynthetic organelle, called a cyanelle, of *Cyanophora paradoxa* has characteristics of both cyanobacteria and chloroplasts. In an attempt clarify the evolutionary position of the cyanelle an investigation has been carried out to determine the sites of synthesis of, and the locations of the genes for, the cyanelle proteins. Particular emphasis has been placed on the large and small subunits of ribulose-1,5-bisphosphate carboxylase and on the β subunit of ATP synthase.

Cyanelle proteins were labeled *in vivo* in the presence of inhibitors specific for cyanelle or cytoplasmic protein synthesis. The results suggest that cyanelle proteins are synthesized both in the cyanelle and in the cytoplasm. Among those polypeptides which may be synthesized in the cyanelle are the large subunit of RUBISCO, the α and β subunits of the ATP synthase and the 32,000 dalton membrane protein.

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The genes for the large (*rbc*L) and small (*rbc*S) subunits of RUBISCO and the β subunit (*atp*B) of the ATP synthase have been localized in the cyanelle DNA from *Cyanophora*. The nucleotide sequences of the *rbc*L and the *rbc*S genes and approximately 210 nucleotides of the coding sequence of the *atp*B gene have been determined. Based on the DNA sequence, the cyanelle *rbc*S gene is located on the same DNA strand as the *rbc*L gene 108 basepairs from the termination codon of *rbc*L. The coding sequences of the *rbc*L and *atp*B genes are separated by 481 basepairs and are transcribed divergently.

The recognition sites of the restriction endonucleases BglII, XhoI, PstI, BamHI, and SalI have been localized in the cyanelle DNA and a map constructed. The size of a monomer of cyanelle DNA is approximately 128 kilo-basepairs (kb). The restriction map of the cyanelle DNA is circular. A 10-kb segment of the cyanelle DNA is duplicated. The duplicated segments are arranged in inverted orientation and are separated by DNA segments of unequal size that are unique in sequence. The cyanelle *rbc*L, *rbc*S, and *atp*B genes are located in the larger single copy region of the cyanelle DNA.

DEDICATION

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I would like to thank all of the faculty, graduate students, post docs, and staff of the Plant Research Lab for contributing to the atmosphere that makes the PRL such a pleasant and efficient place to "do science".

Special thanks to my advisor Peter Wolk and to the members of my guidance committee, Barry Chelm, Norm Good, Lee McIntosh, Ken Poff, and Harold Sadoff, for their thoughtful advice.

I would also like to thank Trek Bicycle Corporation for manufacturing the TREK 970 without which my sanity would have been in serious jeopardy.

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Chapter

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TABLE OF CONTENTS

LIST OF TABLES

Table

11. DNA frag
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Table | Page

LIST OF FIGURES

Figure

9. Physica
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10. Nuclec
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11. Compar
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CHAPTER I

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CHAPTER I

INTRODUCTION AND SURVEY OF THE LITERATURE

Introduction

According to the "endosymbiont hypothesis" of eukaryotic cell evolution (114,171), eukaryotic cells are chimeras, their various organelles derived from once free-living prokaryotes that were engulfed by heterotrophic cells. The strong similarities between the photosynthetic apparatus and mechanisms of cyanobacteria and photosynthetic eukaryotes (82,184) led to the generally accepted view that chloroplasts evolved from oxygen-producing photosynthetic prokaryotes resembling cyanobacteria and Prochloron (114).

Within the context of the endosymbiont hypothesis, the transition from endosymbiont to chloroplast is a process of increasing the degree of integration between the endosymbiont and its host (114). The distinction then between an endosymbiont and a chloroplast is primarily a matter of degree: the cell-plastid symbiosis is more highly integrated (114). At one end of the spectrum of integration is the chloroplast, at the other end are the cyanobacteria, one of the presumed ancestors of the plastid (114). The

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photosynthetic inclusion, or cyanelle, of Cyanophora paradoxa has characteristics of both chloroplasts and cyanobacteria. It has been considered a chloroplast (17,153), an endosymbiotic cyanobacterium (70,89,145,174), and a "bridge between cyanobacteria and chloroplasts" (1).

The chloroplast and other components of the green plant cell are highly integrated. One example of the multilevel integration that unites the chloroplast and the cytoplasm is CO_2 fixation. Whereas the metabolic function of the chloroplast is to fix CO_2, much of the utilization of fixed carbon occurs in the cytoplasm. In leaves, substrates for CO_2 fixation, CO_2, and inorganic phosphate (Pi), move from the cytoplasm to the chloroplast stroma and the transfer of the product of CO_2 fixation, triose phosphate, moves in the reverse direction. The exchange of Pi for triose phosphate is mediated by a specific protein, the phosphate translocator, localized in the inner membrane of the chloroplast envelope (75). The phosphate translocator is an example of a protein serving to integrate the functions of the chloroplast and the cytoplasm, a protein that would be useless, even detrimental, in the prokaryotic ancestor of the chloroplast.

Nitrate assimilation is also a cooperative process involving both the chloroplast and the cytoplasm. The second step in this process, nitrite reduction, occurs in the chloroplast (102). The incorporation of the product of

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nitrite reduction, NH_3, into glutamine, glutamate, aspartate, and alanine, occurs in the chloroplast. The carbon compounds, oxaloacetate, α-ketoglutarate, and pyruvate, which serve as the acceptors of reduced nitrogen in the chloroplast, are synthesized in the cytosol and mitochondria from the triose phosphate exported by the chloroplast (69,102).

The proteinaceous constituents of the chloroplast itself are encoded by both the chloroplast and nuclear genomes. Some chloroplast proteins are synthesized inside the chloroplast, whereas many others are synthesized in the cytoplasm (25,51). Even the chloroplast ribosome is a hybrid in that the ribosomal RNA is synthesized in the chloroplast, whereas at least some ribosomal proteins are synthesized in the cytoplasm. Although the Calvin cycle operates in the chloroplast, many of its enzymes are synthesized in the cytoplasm. The first enzyme of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase (RUBISCO), about which more will be said in the paragraphs that follow, consists of large subunits synthesized in the chloroplast and of small subunits that, in higher plants and eukaryotic green algae, are synthesized in the cytoplasm.

The general goal of the work presented in this thesis was to investigate the degree to which the cyanelle and nuclear genomes of Cyanophora have become integrated. Because the biosynthesis of chloroplast proteins expresses

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well the cooperation between the chloroplast and nuclear genomes, I thought that an investigation into the locations of the genes for and the sites of synthesis of the large and small subunits of RUBISCO might help to clarify the evolutionary position of the cyanelle. RUBISCO was chosen as the focus of this study for the following reasons:

1. RUBISCO catalyzes the initial reaction in photosynthetic carbon assimilation by the reductive pentose cycle (Calvin cycle) and is, therefore, presumed to be metabolically important to any organism having it, regardless of whether the enzyme is located in an organelle or in an endosymbiont;

2. RUBISCO is present in Cyanophora and both its quaternary structure and the molecular weights of its subunits are very similar to that of chloroplasts and of cyanobacteria;

3. RUBISCO is perhaps the best studied example of an enzyme whose biosynthesis, in all photosynthetic eukaryotes examined to date, involves both chloroplast and nuclear genomes;

4. The biosynthesis of RUBISCO is a good paradigm of plastid-nuclear integration;

5. The cyanelle has characteristics similar to cyanobacteria, in particular, the presence of peptidoglycan. It was therefore possible that the cyanelle DNA encodes, and the cyanelle synthesizes, both subunits of RUBISCO.

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Survey of the Literature

The sites of synthesis of, and the locations and the structures of the genes for, the large and small subunits of RUBISCO

The development and maintenance of a photosynthetically competent chloroplast entails interactions between the nuclear and plastid genomes of the cells of higher plants (25,51). The cooperation between the genomes is exemplified by the biosynthesis of RUBISCO. This key enzyme in photosynthetic carbon assimilation commonly consists of eight catalytic large subunits and eight small subunits of unknown function (120). In plants and the eukaryotic green algae, the large subunit of carboxylase is encoded, transcribed, and translated in the chloroplast (2,10,27,31,34,41,44,63,65,108,113,121,142,181,182), whereas the small subunit of RUBISCO is encoded in the nuclear DNA and translated in the cytosol on free ribosomes as a precursor polypeptide (31,33,39,44,46,81,88,113,152).

Genetic evidence indicating that the nuclear DNA of *Nicotiana* contains the gene for the small subunit of RUBISCO was obtained by Kawashima and Wildman (88). They showed that specific peptides produced by digestion of the small subunit with trypsin were inherited in a Mendelian fashion in reciprocal F1 hybrids between *N.tabaccum* and *N. glutinosa* and between *N.tabaccum* and *N.tabaccum* and *N.glauca*. Genetic evidence suggesting that the large subunit is encoded in the

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chloroplast DNA came from the work of Chan and Wildman (34). They showed that peptide maps of the large subunit of three Australian species of Nicotiana (N.gossei, N.excelsior, and N.suaveolens) were identical to one another and to five American species (N.tabaccum, N.glauca, N.glutinosa, N.sylvestris, N.rustica) with the exception that the Australian species contained an additional tryptic peptide. The peptide maps of the large subunit from F1 hybrids from reciprocal crosses between N.gossei and N.tabaccum demonstrated that the extra tryptic peptide was inherited maternally.

The results of investigations into the sites of synthesis of the large and small subunits of RUBISCO parallel the results of the genetic studies. Criddle et al. (44), working with intact leaves of barley, first suggested that the chloroplast and cytoplasmic protein-synthesizing systems are involved in the formation of the two subunits of RUBISCO. In a double labeling experiment they found that cycloheximide, an inhibitor of cytoplasmic protein synthesis, blocked synthesis of the small subunit, whereas chloramphenicol, an inhibitor of organelle protein synthesis, affected the formation of the large subunit. Subsequent in vivo inhibitor studies with Chlamydomonas (113) and pea (31) have yielded similar results.

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Blair and Ellis (10) first demonstrated that the large, but not the small, subunit is a major product, in terms of the amount of [^{35}S]methionine incorporated, of protein synthesis by isolated intact pea chloroplasts. The *in vitro* labeled product was shown to be identical, on the basis of tryptic peptide maps, to the native large subunit. The large subunit is also a major product of protein synthesis by isolated chloroplasts of spinach (27,121), barley (2), and *Euglena* (178).

Gray and Kekwick (65) have described results complementary to those of Blair and Ellis (10), using an *in vitro* protein-synthesizing system. Using 80S polysomes from French bean they demonstrated that [^{14}C]amino acids were incorporated into a protein that was precipitated by antisera to the small subunit. Gooding *et al.* (63) made antibodies to the large and small subunits of RUBISCO from wheat. [^{3}H]puromycin was used both to label and to release nascent polypeptides from 80S and 70S ribosome, which were separated by density gradient centrifugation. The 70S ribosomes were found to react only with antisera against the large subunit, whereas 80S ribosomes reacted with antisera against either subunit. Gooding *et al.* (63) suggested that the association of both large and small subunits with 80S ribosomes reflected the complexing of completed large subunits with the nascent chains of small subunit and that it did not mean that large subunits were made in both the

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chloroplast and cytoplasm. The *in vitro* translation of messenger RNA for the large subunit of RUBISCO was obtained first by Hartley *et al.* (72) who translated total chloroplast RNA using a cell-free extract of *E.coli*. They found that a polypeptide of 52,000 daltons was synthesized. The 52,000 dalton polypeptide was ca. 1,500 daltons smaller than the large subunit that was synthesized by isolated spinach chloroplasts. Analysis of the 52,000 dalton protein synthesized by the *E.coli* system showed that it contained only seven of the nine chymotryptic peptides of the chloroplast-synthesized large subunit. A polypeptide of 35,000 daltons was also synthesized from total chloroplast RNA by the *E.coli* cell-free system (72). Wheeler and Hartley (181) separated spinach chloroplast RNA into polyadenylated RNA [poly(A)$^+$RNA] and non-polyadenylated RNA [poly(A)$^-$ RNA], and then translated those fractions *in vitro* using the *E.coli* cell-free system. They found that only the poly(A)$^-$ RNA programmed the synthesis of the 52,000 dalton protein. Sagher *et al.* (142) translated poly(A)$^+$ and poly(A)$^-$ RNA from *Euglena* chloroplasts using a cell-free protein-synthesizing system from wheat germ. They found that messenger RNA for the large subunit was present only in the poly(A)$^-$ RNA fraction. Moreover, the product made by the wheat germ system was identical, on the basis of two-dimensional polyacrylamide gel electrophoresis, to the native large subunit. They also found that the poly(A)$^-$RNA from a mutant, W_3BUL, that lacks chloroplast DNA, does not

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Coen et al. (42) presented the first direct physical evidence that the large subunit of RUBISCO is encoded by the chloroplast DNA. They used an in vitro linked transcription-translation system to show that a cloned fragment of maize chloroplast DNA could program the synthesis of a polypeptide that corresponds in its size, serological properties, and papain and chymotryptic peptides to native RUBISCO from maize. Similar results have been obtained with cloned chloroplast DNA from Chlamydomonas (108) and spinach (182) and with total chloroplast DNA from spinach (26).

Experiments complementary to those of Wheeler and Hartley (181) and Sagher et al. (142) were performed by Dobberstein et al. (46). They translated poly(A)$^+$RNA from Chlamydomonas in a cell-free system from wheat germ. Upon immunoprecipitation with antibody against authentic small subunit (16,500 daltons), a single polypeptide of ca. 20,000 daltons was obtained. Because the immunoprecipitated protein had a larger molecular weight than the small subunit, Dobberstein et al. (46) tentatively identified it as a precursor (pS) to the small subunit. Treatment of pS with a specific soluble endoprotease found in polysomal supernatant fractions from Chlamydomonas, resulted in its cleavage to a polypeptide of the same size as authentic small subunit. Because only the 16,500 dalton form of the

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small subunit is found in the chloroplast, Dobberstein et al. (46) suggested that the small polypeptide that is removed is involved in the transport of the small subunit into the chloroplast. Though the synthesis of the small subunit as a higher molecular weight precursor that is processed to yield the mature protein is reminiscent of the synthesis of some secretory proteins (11,12), Dobberstein et al. (46) noted that several features of the synthesis of the small subunit differ from the synthesis of secretory proteins. In the case of secretory proteins, Blobel and Dobberstein (11,12) had proposed that the amino terminal chain extension ("signal sequence") found in presecretory proteins serves to facilitate the attachment of the ribosomes to the microsomal membrane. Whereas processing of the presecretory protein, i.e., removal of the signal sequence, is accomplished by a membrane-associated enzyme before translation of the polypeptide is complete, processing of the small subunit is post-translational. In addition, cytoplasmic ribosomes do not appear, in electron micrographs, to be attached to the outer envelope of the chloroplast (36,46). Dobberstein et al. (46) suggested that the mechanism of transport of the small subunit may be analogous to that of diphtheria toxin. This toxin is synthesized as a single polypeptide that is subsequently cleaved to yield α and β chains that remain linked by a disulfide bridge (130). The precise mechanism of transfer of the toxin across the plasma membrane is uncertain, but it

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involves prior binding of the β subunit to a receptor located in the plasma membrane. Dobberstein et al. (46) proposed that the small peptide that is cleaved from pS is functionally equivalent to the β subunit of diphtheria toxin. Schmidt et al. (152) proposed the term "transit sequence" for the leader sequences of transported chloroplast proteins. The results of Dobberstein et al. (46) with Chlamydomonas have been confirmed in spinach (152) and pea (33,39,81,152), so that the mechanism proposed by Dobberstein et al. (46) may be more generally applicable.

In the cyanobacteria Anacystis 6301 (162), also called Synechococcus (139), and Anabaena 7120 (127), the gene (rbcS) for the small subunit of carboxylase is located 3' (mRNA sense) from the gene (rbcL) for the large subunit. The coding sequences of the rbcL and rbcS genes are separated by 93 basepairs (bp) in Anacystis (162) and by 545 bp in Anabaena (127).

Most genes coding for the large (50,000-55,000 dalton) subunit of carboxylase consist of single open reading frames coding for 472-477 amino acids (45,47,116,125,137,161,163,190). An exception is the large subunit gene of the eukaryotic alga, Euglena (93), which although it is chloroplast encoded, contains nine intervening sequences. The rbcL gene is present in one copy per monomer of chloroplast DNA but in 15-30 copies per chloroplast in immature leaves of spinach (158). Because a

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leaf cell may contain several hundred chloroplasts (158), the copy number of the rbcL gene is thought to greatly exceed that of the rbcS gene. Because the large and small subunits are present in RUBISCO in equimolar amounts, the apparent discrepancy between the reiteration frequencies of the rbcL and rbcS genes in higher plants raises interesting, and as yet unsolved questions, about the regulation of the synthesis of RUBISCO. Whether there is a lower rate of transcription and/or translation of the copies of the large subunit gene than for the copies of the small subunit gene or whether the rbcS gene is amplified is not known. In the cyanobacteria Anabaena 7120 and Anacystis, the synthesis of stoichiometric amounts of the two subunits of RUBISCO may be achieved in part by cotranscription of the closely linked genes for RUBISCO. The serial arrangement of the rbcL and rbcS genes together with the absence of a sequence with homology to promoter sequences in the spacer between these genes led Shinosaki and Sugiura (162) to suggest that these genes are cotranscribed in Anacystis. Recently, Nierzwicki-Bauer et al. (127) have demonstrated that in Anabaena the rbcL and rbcS genes are cotranscribed.

The genes for the small subunit of RUBISCO in soybean (8), wheat (29), and pea (32,42) occur in the nucleus as small multigene families. One of the soybean rbcS genes has been sequenced and consists of three open reading frames separated by two intervening sequences (8). The rbcS gene of

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wheat contains a single intron (29). In Anacystis (162) and Anabaena (127), the small subunit genes consist of single open reading frames of 333 bp and 327 bp, respectively. The coding sequence of the transit peptide common to the nuclear encoded small subunit genes of plants (8,29), is not present in the rbcS genes of Anacystis (162) and Anabaena (127).

Cyanophora paradoxa

Cyanophora paradoxa is a small photosynthetic eukaryote first described in 1924 by Korschikoff (95). In his description, Korschikoff noted small intracellular bodies resembling unicellular cyanobacteria. Pascher (131) had described, ten years earlier, the general phenomenon of a cyanobacterium living in a symbiotic association with a colorless protist, a symbiosis to which he referred as a Syncyanose. Korschikoff (95) emphasized the cyanophycean nature of the blue-green inclusion and believed the cyanelle to be an organism sui generis. In 1929, Pascher (132) in response to a growing number of reports on symbiotic cyanobacteria, some occurring intracellularly, distinguished between "Ectocyanosen" and "Endocyanosen". It is Pascher who first used the term cyanelle (Cyanellen) to refer to the cyanobacteria-like bodies (132).

As its name suggests, Cyanophora paradoxa has proven difficult to classify taxonomically (57,133). Until quite recently, the view was widely held that it is in fact two

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organisms, a colorless biflagellated protist and an endosymbiotic cyanobacterium (70,89,174). Attempts to classify the flagellate have been largely unsatisfactory. One conception is that it is related to the cryptomonad algae (174). However, a careful electronmicroscopic study of the protist led Mignot et al. (119) to conclude that it is not related to the cryptomonads, but rather to primitive dinoflagellates. Dinoflagellates possess a distinctive and unusual spindle (98,101,168). The chromosomes appear to be attached to the nuclear envelope, which remains intact throughout mitosis. Microtubules penetrate the mitotic nucleus through membrane-lined channels but do not make direct contact with the dinoflagellate chromosomes. In an attempt to clarify the nature and taxonomic affinities of the host, Pickett-Heaps (133) studied cell division in the flagellate. He found that in contrast to dinoflagellates, Cyanophora has a typical mitotic spindle. Pickett-Heaps (133) concluded that Cyanophora is not related to the dinoflagellates, but agreed with Mignot et al. (119) that the basal body of the flagellum of Cyanophora was unlike that of any alga which had been examined previously. The unique structure of the flagellar roots was also noted by Kies (89) who compared the the morphology and ultrastructure of Cyanophora paradoxa, Gloeochaete wittrockiana and Glaucocystis nostochinearum, three algae containing cyanelles. Kies (89) found that all three of these algae have four flagellar roots, but that the flagellar roots of

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Cyanophora are much simpler than those of Gloeochaete and Glaucocystis. Each of the flagellar roots of Glaucocystis and Gloeochaete consists of approximately 20-50 microtubules, whereas the flagellar roots of Cyanophora are present in pairs, one pair having approximately 3 microtubules per root and the other pair having approximately 10 microtubules per root. Kies (89) pointed out that except for the unique structure of the flagellar roots of Cyanophora, the three organisms shared several structurally unique characters, including two flagella at an angle of 120°-180° to each other, cytoplasmic starch granules, a parabasal dictysome, flagella lacking the star-shaped pattern of microtubules in the transition zone, and a system of lacunae (intracellular flattened membrane vesicles located next to the plasma membrane). He discounted the importance of cyanelles as a taxonomic feature because they also occur in zoological organisms. He suggested that Gloeochaete, Glaucocystis, and Cyanophora be assigned to a separate algal class, Glaucophyceae, as proposed earlier by Skuja (165).

Over the years, a considerable body of evidence has accumulated that is consistent with the hypothesis that the cyanelle is an endosymbiotic cyanobacterium. The division of the cyanelles is not synchronous with that of the nucleus resulting in unequal numbers of cyanelles in the daughter cells (70,133,174). Whether this is truly a sign of the

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cyanelle's autonomy is questionable because the possibility that division of the cyanelles is controlled by the nucleus has not been excluded.

Hall and Claus (70) conducted a detailed ultrastructural study of the cyanelles of C.paradoxa. They found that the cyanelle lacks the double-layered cell wall of cyanobacteria, but is surrounded by a thin protoplasmic membrane. The cyanelle protoplasm is divided into a lamellar chromatoplasm containing the photosynthetic pigments, and a non-lamellar centroplasm with a large centrally located electron-opaque body. Between the lamellar region and the central body is a fibril-containing halo. They found, within the lamellar region, two types of inclusions, one of which resembles the polyphosphate granules found in cyanobacteria. The other type of inclusion resembles oil droplets, a feature not found in cyanobacteria. The binary fission of the cyanelle was found to be very similar to the division of cyanobacteria. Although Hall and Claus (70) noted that several of the features of the cyanelle, namely the absence of a cell wall, the large electron-opaque central body and the inclusions tentatively identified as oil droplets, are atypical of cyanobacteria, they considered that the similarities to the cyanobacteria were sufficient to merit classification of the cyanelle as a new cyanobacterium, Cyanocyta korschikoffiana, belonging to the order Chroococcales. Because of the

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structural peculiarities of the cyanelle they found it necessary to create a new family, Cyanocytaceae, to include it.

Because chloroplasts lack cell walls, whereas algal endosymbionts generally retain some wall structure, the presence or absence of a typical cyanobacterial cell wall is an important character in determining the evolutionary status of the cyanelle. Conclusions drawn from ultrastructural studies have varied. Hall and Claus (70) found that the cyanelle possessed a thin limiting membrane directly apposed to a protoplasmic membrane of the host. Pickett-Heaps (133) noted the presence of wall-like material located in the division furrow of the cyanelle. Trench et al. (174) found that cyanelles have a thin layer of material external to their cytoplasmic membrane. They suggested that this layer is homologous to the peptidoglycan of cyanobacteria. Giddings et al. (60) subjected isolated cyanelles to freeze fracture. They found, external and adjacent to the plasma membrane of the cyanelle, a 5- to 7-nanometer layer that seems to correspond to the peptidoglycan layer of cyanobacteria. External to this layer they observed a layer that exhibited freeze fracture faces similar to those of the lipopolysaccharide layer of gram negative bacteria. They concluded that the cyanelle has a wall that differs only slightly from that of free-living cyanobacteria. The presence of a peptidoglycan

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similar to that of cyanobacteria has been demonstrated biochemically. Aitken and Stanier (1) purified and analyzed peptidoglycan from cyanelles. The results of the chemical analysis indicated that N-acetylmuramic acid, N-acetylglucosamine, alanine, glutamic acid and diaminopimelic acid are present in a molar ratio of 1:1:1.6:1:1. Analysis of peptidoglycan from cyanobacteria has yielded similar results. The results of Aitken and Stanier (1) are consistent with the earlier observations of Schenk (146) who showed that cyanelles, which are normally osmotically stable, can be rendered osmotically sensitive by treatment with lysozyme, suggesting that they are bounded by a peptidoglycan wall. Despite the evolutionary significance of the presence of peptidoglycan in the cyanelle, the biosynthesis of the peptidoglycan has not been studied nor have the genes for it been localized. Now that recent work (described below) has shown that the cyanelle has a chloroplast-like genome, the major distinction between the cyanelle and a chloroplast is the presence of peptidoglycan in the cyanelle.

The photosynthetic pigment system of the cyanelle resembles closely that of cyanobacteria (35,174). C-phycocyanin and allophycocyanin, chlorophyll *a*, β carotene, zeaxanthin, and two xanthophylls found in many cyanobacteria (35,62), echinenone and myxoxanthophyll, are present in the cyanelle. Chlorophyll *c*, normally present in

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the cryptomonad algae, is not present, indicating that Cyanophora is not a cryptomonad (35). The biliproteins C-phycocyanin and allophycocyanin have been purified from cyanelles by Trench and Ronzio (174). C-phycocyanin was found to consist of two subunits with apparent molecular masses, determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), of 13,200 daltons and 14,500 daltons. Allophycocyanin was found to consist of two subunits of 12,600 daltons. Trench and Ronzio (174) noted that the molecular masses of the subunits are lower than have been observed for the biliproteins from cyanobacteria. They suggested that the differences result from differences in the methods used, in particular, the system of SDS-PAGE employed. In order to estimate the dimeric molecular masses of allophycocyanin and C-phycocyanin, Trench and Ronzio (174) treated electrophoretically pure preparations of each biliprotein with the bifunctional reagent dimethylsuberimidate (DMSI), and then subjected the DMSI-crosslinked proteins to SDS-PAGE. The dimeric molecular masses, 28,000 daltons and 31,000 daltons, of allophycocyanin and C-phycocyanin determined in this manner, are within the range of values reported for the corresponding proteins from cyanobacteria (13,59,186).

Recently, the cyanelle DNA has become a focus of attention with the result that a number of similarities to chloroplast DNA have been found. From measurements of

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kinetic complexity, Herdman and Stanier (80) deduced that the size of the cyanelle DNA was equivalent to 177 kilobase pairs (kb). Estimates of the size derived from restriction endonuclease digestion of cyanelle DNA are smaller, approximately 127 kb (14,16,17,123, and results presented in this thesis). Although too small for that of a free-living cyanobacterium (79), the cyanelle genome is well within the size range of chloroplast genomes (15). Cyanelle DNA is a circular molecule containing a 10-kb repeated unit in inverted orientation separated by two single copy regions of unequal size (16,17,123). The 16S and 23S ribosomal RNA (rRNA) genes are located in the inverted repeat regions (16,17,123). Perhaps due to intramolecular recombination within the inverted repeat regions, the cyanelle DNA exists in two forms that appear to differ only in the orientation of the single copy segments (16). The recognition sites of the restriction endonucleases BamHI, SalI, and SmaI have been localized in the cyanelle DNA (17).

Sufficient homology exists between cyanelle DNA and chloroplast DNA to permit the use of cloned fragments of chloroplast DNA as probes in Southern (167) hybridizations. Heinhorst and Shively (77) demonstrated that DNA fragments containing the coding sequence of the large subunit of RUBISCO from maize and Chlamydomonas hybridize with cyanelle DNA. Bohnert and coworkers (17) reported that fragments of spinach DNA containing portions of the genes for the large

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subunit of RUBISCO, the α , β , and ε subunits of chloroplast ATP synthase, the dicyclohexylcarbodiimide-binding protein, the 32,000 dalton protein (*psbA* gene product), cytochrome b_6, and subunit 4 of the cytochrome b_6f complex all hybridize with cyanelle DNA under stringent conditions of hybridization. Under conditions of low hybridization stringency, the nuclear-encoded gene for the small subunit of RUBISCO from pea hybridizes with cyanelle DNA (17,77).

There appear to be at least two distinct strains of *Cyanophora* in common use (14,17). The DNA of one of these strains, the "Pringsheim" strain (UTEX LB555, from the culture collection of the University of Texas at Austin), has a buoyant density in cesium chloride of 1.692 to 1.695 g/cm^3 (14,17,80) and a molecular weight of 126.5 ± 0.5 kb (17). The other strain, the "Kies" strain, has a buoyant density of 1.692 g/cm^3 and a molecular weight of 138 kb (17). Preliminary experiments indicate that the cyanelle DNA of the Kies strain contains a repeated unit of the same size as the inverted repeat found in the cyanelle DNA from the Pringsheim strain (17). Jaynes *et al.* (87) have reported that the cyanelle DNA from the Pringsheim strain has a buoyant density of 1.716 g/cm^3. The reason for the discrepancy between the value of the buoyant density of the cyanelle DNA reported by Herdman and Stanier (80) and Bohnert *et al.* (14,17) and that reported by Jaynes *et al.*

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(87) is unclear. It was suggested (87,90) that the strain examined by Jaynes *et al.* may have been not the Pringsheim strain but a third strain.

Whereas the cytological and morphological properties of *Cyanophora* have been extensively investigated, relatively little experimentation has been directed towards the physiological and biochemical features of the organism. Most of the attention in this area has focused on aspects of photosynthesis and carbon metabolism. Schenk (147) found that oxygen evolution by *Cyanophora*, 240-290μmol O_2/(mg chl·hr) is comparable to that by other plants. Dark CO_2 fixation is negligible (97). Trench *et al.* (174) measured the rate of $^{14}CO_2$ fixation in isolated cyanelles; the highest rate attained, determined on the basis of mg of chlorophyll, was 12 % of the rate by intact cells. Floener and Bothe (53) measured $NaHCO_3$ dependent oxygen evolution by isolated cyanelles. The rate obtained, extrapolated to one hour, was 30 μmoles O_2 evolved/(mg chl·hr). Although this rate is equivalent to ca. 33% of the rate by intact cells, the isolated cyanelles continued to fix CO_2 for only 3 min. The pattern of products labeled by $NaH^{14}CO_3$ is consistent with the operation of the reductive pentose phosphate (Calvin) cycle (97); phosphate esters, especially 3-phosphoglycerate, predominate among the initial products of CO_2 assimilation (97).

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The enzyme catalysing the initial step in the Calvin cycle, ribulose 1,5-bisphosphate carboxylase, has been purified from Cyanophora (40) and from isolated cyanelles (30). RUBISCO appears to be located within the cyanelle of Cyanophora (40). Codd and Stewart (40) lysed the "host" cells by osmotic shock and collected the cyanelles by centrifugation at 20,000xg for 5 min. Both the pellet, consisting of intact cyanelles and cell wall debris, and the supernatant, consisting of "host" cell cytoplasm were assayed for carboxylase activity. Before being assayed for enzyme activity, the cyanelles were disrupted by passage through a French press at 16,000 lb/in^2. The disrupted cyanelles catalyzed ribulose bisphosphate-dependent $^{14}CO_2$ fixation with a specific activity of 0.55 μmol $^{14}CO_2$ fixed/(mg protein·hr). No enzymatic activity was detected in the 20,000xg supernatant fluid at up to 6 mg protein/assay suggesting that RUBISCO is located in the cyanelle of Cyanophora. Codd and Stewart (40) isolated RUBISCO from Cyanophora and determined, by electrophoresis in polyacrylamide gels, that the molecular weight of the purified enzyme is 525,000 daltons. Electrophoresis of the purified enzyme on sodium dodecyl sulfate-containing polyacrylamide gels (10 % acrylamide) showed that RUBISCO from Cyanophora consists of two kinds of subunits with molecular weights of 15,000 daltons (small subunit) and 51,000 daltons (large subunit). From these results Codd and

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Stewart (40) concluded that the native enzyme is composed of eight small and eight large subunits. Burnap and Trench (30) have purified RUBISCO from isolated cyanelles. The molecular weights, 56,000 daltons and 12,200 daltons, of the subunits of RUBISCO were determined by SDS-PAGE on a 12.5 % acrylamide gel using a stacking gel of 5% acrylamide. The disparity between the molecular weights of the subunits reported by Codd and Stewart (40) and by Burnap and Trench (30) may be due to the different polyacrylamide gel systems employed by these groups. The molecular weights deduced from the DNA sequences (results presented in chapter 3) are 12,400 daltons and 52,800 daltons. The specific activity of the purified enzyme, 0.48 μmoles CO_2 fixed/(mg protein·min), reported by Burnap and Trench (30) was much higher than that reported by Codd and Stewart (40). However, the value of the specific activity reported by Codd and Stewart (40) was determined on a crude cyanelle lysate. The specific activity of purified RUBISCO was not reported by Codd and Stewart (40).

The transfer of fixed carbon from the cyanelle to the cytoplasm has been studied by two groups of investigators. Trench et al. (174) labeled cyanelles in vitro with $NaH^{14}CO_3$ and then subjected extracts of the cyanelles and of the labeling medium to thin layer chromatography. They found that the cyanelles released principally two organic compounds, glucose and a disaccharide, into the medium.

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They did not identify the disaccharide, but suggested that it was probably sucrose. Kremer and coworkers (97) were unable to label isolated cyanelles. Instead, they examined the distribution of assimilates by labeling intact cells of Cyanophora with $H^{14}CO_3$ and then by isolating the cyanelles. Glucose and maltose were the predominant sugars. They found no evidence for sucrose among the assimilates. Most of the labeled glucose, but very little of the maltose, was associated with the cyanelles. Organic acids, e.g, malate, fumarate, citrate and other intermediates of the tricarboxylic acid cycle, were barely detectably labeled in either cells or cyanelles.

Information on the assimilation and metabolism of compounds other than CO_2 is scanty. Bothe and Floener (23) found that cultures of Cyanophora grown on nitrate either aerobically or anaerobically for 3-6 days in the absence of combined nitrogen were unable to reduce acetylene. The anaerobic cultures had been flushed continuously with a mixture of either 20% H_2/ 75% N_2/ 5% CO_2 or 95% N_2/ 5% CO_2; 3,4-dichlorophenyl-N,N-dimethylurea (DCMU) had not included in the medium. From this result and the failure of Cyanophora to grow in the absence of nitrate under either aerobic or anaerobic conditions Bothe and Floener (23) concluded that Cyanophora is "unable to synthesize nitrogenase". However, because DCMU, which inhibits photosynthetic O_2 evolution, was not added to cultures grown

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under "anaerobic" conditions, those conditions may be more accurately described as microaerobic. The extreme sensitivity of nitrogenase to O_2 together with the uncertainty regarding the concentration of O_2 in cultures grown microaerobically, suggest that the results of Bothe and Floener (23) should not be regarded as definitive proof that Cyanophora does not synthesize nitrogenase. Cyanophora grown anaerobically does synthesize hydrogenase (23). Hydrogen consumption by cells grown anaerobically in the presence of molecular H_2 (20% H_2/ 75% N_2/ 5% CO_2) was almost nine times greater than in cultures grown anaerobically in the absence of H_2 (95% N_2/ 5% CO_2) suggesting that hydrogenase is inducible. Whereas the formation of hydrogenase appeared to be completely inhibited by O_2, O_2 was required for activity of the hydrogenase (23). The experiments of Bothe and Floener (23) do not exclude the possibility that the absence of hydrogenase activity in aerobically grown cultures of Cyanophora reflects a requirement for induction by H_2 rather than an inhibition of the synthesis of hydrogenase by O_2.

Cyanophora is capable of growth on NO_3^- as sole source of nitrogen implying that it is able to form assimilatory nitrate and nitrite reductases. Bothe and Floener (23) found that the rate of nitrate reduction was two fold greater in the light than in the dark. In contrast to nitrate, nitrite reduction did not occur at all in the dark

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(23). More recently, Floener et al. (54) and Böttcher et al. (24) have shown that a NADH-dependent nitrate reductase is located exclusively in the cytoplasm. Other aspects of nitrate assimilation are controversial. Floener et al. (54) reported that nitrite reductase is ferredoxin-dependent and is bound to the thylakoid membranes. Glutamine synthetase and ferredoxin-dependent glutamate synthase were found in both the cyanelles and the cytoplasm. They proposed that the pathway of assimilatory nitrate reduction in Cyanophora is similar to that of photosynthetic eukaryotes and unlike that of cyanobacteria. In contrast to the results of Floener et al. (54), Böttcher et al. (24) found that most (75%) of the nitrite reductase activity was associated with the cytoplasm and suggested that the cyanelle does not play a major role in nitrate assimilation.

In plants, $SO_4^=$ incorporation occurs in the chloroplast (157). The more than 50 species of higher plants and the eukaryotic algae, other than Cyanophora, that have been examined, have only adenosine-5'-phosphosulfate (APS) sulfotransferases, whereas both APS and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) sulfotransferases are found in cyanobacteria (149,150,157,177). Schmidt and Christen (151) have partially purified a PAPS sulfotransferase from cyanelles. The enzyme is inhibited by 5'-AMP, 5'-ADP, and especially by APS.

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Although the activity of this enzyme is stimulated ca. 1.6-fold by thioredoxin, thioredoxin is not required for its activity (151). It is not certain that the cyanelle sulfotransferase characterized by Schmidt and Christen (151) is the only sulfotransferase in Cyanophora and it is therefore not certain what role the cyanelle may play in assimilatory sulfate reduction.

Prospectus

This thesis contains the results of an investigation into the sites of synthesis and the locations of the genes for the large and small subunits of ribulose 1,5-bisphosphate carboxylase. Chapter 2 describes the initial approach, the labeling of cyanelle proteins in vivo in the absence or in the presence of inhibitors of cyanelle (chloramphenicol) or cytoplasmic (cycloheximide) protein synthesis. The preliminary results suggested that the large subunit of RUBISCO, and several other proteins, may be synthesized in the cyanelle. The results also suggest that many cyanelle proteins are synthesized in the cytoplasm.

Although it had been known since 1977 (80) that the cyanelle genome was substantially smaller than that of free-living cyanobacteria, it was not known what genes are encoded in the cyanelle DNA. A reasonable approach to the study of the evolutionary status of the cyanelle is to elucidate the structure of its genome and to identify the

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genes that it contains. I began to look for specific genes in the cyanelle DNA by using cloned genes from maize chloroplast DNA as probes in heterologous hybridizations. The results of those hybridization experiments, shown in Appendix A, suggested that the 5' ends of the large subunit of RUBISCO and the β subunit of the ATP synthase are located near each other in the cyanelle DNA. If true, then the relative locations of these genes in the cyanelle DNA is strikingly similar to their relative locations in the chloroplast DNAs of several plant species (28,55,96,160,180). In order to verify the results of the hybridization experiments, fragments of cyanelle DNA containing the large subunit of RUBISCO and the β subunit of the ATP synthase were cloned, and their DNA sequences determined. The results of the DNA sequencing confirmed the hybridization results and also showed that a sequence located 108 basepairs 3' from the coding sequence of the large subunit is an open reading frame of 321 basepairs. Because the deduced amino acid sequence of this open reading frame closely matched the amino acid sequence of the small subunit from Anacystis (162), that open reading frame was identified as the structural gene for small subunit of RUBISCO. Chapter 3 contains the results of the DNA sequencing of the genes for the large and small subunits of RUBISCO and of their flanking regions.

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In order to gain insight into the overall organization of the cyanelle DNA, the recognition sites of the restriction endonucleases BamHI, SalI, XhoI, BglII and PstI were localized in the cyanelle DNA. The results of those experiments are presented in chapter 4 and show that the cyanelle genome is similar to the genomes of plants and eukaryotic green algae.

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CHAPTER II

LABELING OF CYANELLE PROTEINS *IN VIVO*

Introduction

The endosymbiont hypothesis (114) views modern plastids as having developed from an ancestral endosymbiont by a progressive integration of metabolic function with the host cell. Chloroplast proteins, for example, are biosynthesized collaboratively by the nuclear and chloroplast genomes. In the previous section it was suggested that the biosynthesis of one particular protein, RUBISCO, is a good paradigm for the interactions of the chloroplast and nuclear genomes.

The cyanelle of *Cyanophora* has the photosynthetic pigments (35,175), structural appearance (60,70,174), and peptidoglycan of the cyanobacteria (1). However, the cyanelle genome is approximately 10- to 20-fold smaller (16,17,80) than the genome of free-living cyanobacteria (78). In both size (16,17,80) and the presence of an inverted repeat segment (16,17) the cyanelle DNA resembles that of the chloroplast (14). Whereas the presence of peptidoglycan suggests that the cyanelle is an endosymbiont, the structure of its genome suggests that it is a

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chloroplast. Aitken and Stanier (1) proposed that it is a bridge between the cyanobacteria and chloroplasts.

Because in higher plants and eukaryotic green algae protein synthesis in general, and the synthesis of RUBISCO in particular, illustrates well the cooperation between the chloroplast and nuclear genomes, I thought that the evolutionary position of the cyanelle might be clarified by studying the synthesis of its proteins. A demonstration that some cyanelle proteins, for example the small subunit of RUBISCO, are synthesized in the cytoplasm would be strong evidence that the cyanelle is, in essence, a chloroplast. On the other hand, if the cyanelle synthesizes all of its proteins, and depends upon the host only for an array of substrates (an array sufficiently large as to allow for the observed reduction in genome size), it should be thought of as an endosymbiont.

Although protein synthesis has not been studied previously in Cyanophora, Trench and Siebens (176) have examined the effect of the protein synthesis inhibitors, cycloheximide and chlormphenicol, on the synthesis of ribosomal RNA (rRNA) and chlorophyll a in Cyanophora and its cyanelles. They found that cycloheximide inhibited the synthesis of cytoplasmic but not cyanelle rRNA, whereas chloramphenicol inhibited cyanelle but not cytoplasmic rRNA synthesis. Cycloheximide markedly inhibited the synthesis

of chlorophyll *a* whereas chloramphenicol was significantly less inhibitory.

Trench *et al.* (174) examined the uptake of dissolved organic carbon compounds. They incubated intact cells in the light in growth medium containing radioactively labeled compounds. They found that acetate and cyanocobalamin were taken up, whereas glucose, sucrose, ribose, an amino acid mixture, and (in contrast to results described below) leucine were not taken up.

Three major approaches have been used to determine the sites of synthesis of chloroplast proteins, *viz.*, *in vitro*, *in situ* (i.e., in isolated chloroplasts) and *in vivo*. For *in vitro* determinations, there are in turn, two sub-approaches, namely, ribosome run-off and use of heterologous protein-synthesizing systems. As will be described below, each of these approaches has certain advantages and certain disadvantages.

In a ribosome "run off" system polysomes are isolated and peptide chains already initiated are allowed to elongate and terminate. Alternatively, heterologous protein synthesizing systems can be utilized to synthesize chloroplast proteins *in vitro* by translation of isolated messenger RNA. Cell-free *in vitro* translation systems prepared from *E.coli* and from rabbit reticulocytes have been used successfully to translate chloroplast mRNAs. Extacts

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of wheat germ have been used to translate cytoplasmic mRNAs. Polysomal "run off" and heterologous systems for protein synthesis *in vitro* allow the study of chloroplast proteins synthesized in the cytoplasm and, separately, of such proteins synthesized in the chloroplast. Because cytoplasmic mRNA and polysomes synthesize a great number of proteins, a prerequisite for study of polysomal "run off" and of heterologous systems using total cytoplasmic mRNA is a method of selecting the product of interest. Polysomal "run off" has been used successfully to identify the site of synthesis of the small subunit of RUBISCO in French bean (65) and wheat (63,141). One difficulty that has been encountered is the apparent lack of specificity of the antibodies used to select the polysomes. As already mentioned (page 8), Gooding *et al*. (63) found that polysomes from the cytoplasm reacted with antisera against both the large and the small subunit and suggested that the phenomenon was due to the complexing of completed large subunits with the nascent chains of the small subunit. Heterologous systems differ from polysomal "run off" synthesis in that the synthesis occurs in partly purified cell-free systems from heterologous sources. Some of the chloroplast proteins which have been synthesized using heterologous systems for *in vitro* protein synthesis are the large subunit of RUBISCO from total spinach chloroplast RNA (72), and from non-polyadenylated RNA from the chloroplast of spinach (181), *Euglena* (142), and *Chlamydomonas*

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(108,143); the small subunit of RUBISCO from polyadenylated RNA of spinach (33), Chlamydomonas (46), and pea (81); and the psbA gene product from chloroplast RNA from spinach (72) and maize (6).

The second way to demonstrate that a protein is synthesized in the chloroplast is to label intact, isolated chloroplasts. This approach has the advantage that chloroplast protein synthesis can be examined in the (virtually) complete absence of cytoplasmic protein synthesis. An in vitro approach has a number of disadvantages. Chloroplast protein synthesis may be "crippled" in the absence of the cytoplasm, and it may include synthesis from nuclear encoded mRNAs which may move into the chloroplast [although there is, in fact, no evidence for the movement of mRNA across the chloroplast envelope (51)]. Another disadvantage of an in vitro approach is that it does not provide positive information about polypeptides synthesized in the cytoplasm. In addition, this method appears to be effective primarily for those proteins which either occur in great abundance in the chloroplast, have high rates of turnover, or do not require the presence of cytoplasmically synthesized proteins for their assembly, for example into membranes (38). Nonetheless, this approach has been widely used since Blair and Ellis (10) demonstrated that isolated intact chloroplasts of pea synthesize the large, but not the small,

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subunit of RUBISCO. Similar results have been obtained in spinach (27,121), Hordeum vulgare (2), and Euglena (178). Some of the proteins which have been identified as products of protein synthesis in isolated chloroplasts are the α, β, γ, and ε subunits of ATP synthase (126) and the P-700-chlorophyll a-binding proteins of spinach (188), the α and β subunits of ATP synthase of maize (68), and the 32,000 dalton membrane protein (atpB gene product) of spinach (27), maize (precursor) (68), pea (48), and Euglena (178).

The third major approach that has been used to determine the sites of synthesis of chloroplast proteins is to label chloroplast proteins in vivo. This method relies on the differential sensitivity of the ribosomes of the cytoplasm and the chloroplast to inhibitors of protein synthesis. An in vivo approach has the advantage over the in vitro approaches that duplicate samples of cells may be treated with different inhibitors during the same experiment. Information can thereby be gained regarding the sites of synthesis of proteins both in the cytoplasm and in the chloroplast. A disadvantage of this approach is uncertainty regarding the specificity of the inhibitors used. Hoober and Blobel (86) examined the effect of chloramphenicol and cycloheximide on the ribosomes of greening cells of Chlamydomonas. In their experiments the "inhibitory" effect of the antibiotics was measured as the

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disappearance of free ribosomes and the concurrent appearance of polysomes. Hoober and Blobel (86) found that cycloheximide severely decreased the amount of free 80S but not 70S ribosomes, whereas chloramphenicol markedly decreased the number of free 70S but not 80S ribosomes. The results of Hoober and Blobel (86) were confirmed by Avadhani and Buetow (4). They isolated intact polysomes from the cytoplasm, chloroplasts, and mitochondria of Euglena. All three preparations incorporated labeled amino acids into protein. The incorporation of amino acids into protein by cytoplasmic ribosomes was inhibited 78% by cycloheximide but only 9% by chloramphenicol. Incorporation of amino acids by chloroplast ribosomes was inhibited 52% by chloramphenicol, but only 7% by cycloheximide. Mitochondrial ribosomes were inhibited 53% by chloramphenicol and 10% by cycloheximide.

Results from labeling intact leaves of barley (44) in vivo provided the first evidence suggesting that both cytoplasmic and chloroplast ribosomes were involved in the synthesis of RUBISCO. Criddle et al. (44) immunoprecipitated RUBISCO labeled in vivo in the absence and in the presence of cycloheximide and chloramphenicol. They found that cycloheximide preferentially inhibited the synthesis of the small subunit, whereas chloramphenicol specifically inhibited the synthesis of the large subunit. Cashmore (31) labeled green, i.e., not etiolated, pea seedlings with [^{35}S]methionine and then autoradiographed the

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polypeptides following their separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). He found that total protein synthesis was decreased significantly by cycloheximide and that the decrease was most pronounced for soluble leaf proteins. The large subunit of RUBISCO was unique among the soluble proteins in that its synthesis was inhibited by chloramphenicol. The synthesis of the small subunit, as well as the other soluble proteins, and the major lamellar protein associated with photosystem II were affected only by cycloheximide. Many of the lamellar proteins were sensitive to chloramphenicol suggesting that they are synthesized on chloroplast ribosomes. Chua and Gillham (38) labeled intact cells of Chlamydomonas in the absence and in the presence of inhibitors specific for chloroplast (chloramphenicol, spectinomycin) or cytoplasmic (anisomycin) protein synthesis. The labeled membrane polypeptides were fractionated by SDS-PAGE. Of the 33 polypeptides resolved, at least 9 were made on chloroplast ribosomes, that is, their synthesis was inhibited by chloramphenicol or spectinomycin, but not by anisomycin. Two of the polypeptides synthesized on chloroplast ribosomes are associated with the reaction centers of photosystems I and II. The sites of protein synthesis in vivo of a large number of chloroplast proteins, in addition to RUBISCO and thylakoid membrane proteins, have been studied using inhibitors. These proteins include RNA polymerase,

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ferredoxin, several of the enzymes of the Calvin cycle, and fatty acid synthetase. A more complete list is presented in ref. 25.

The inhibitors most frequently used to study the synthesis of chloroplast proteins *in vivo* are D-threo-chloramphenicol, lincomycin, spectinomycin, or streptomycin to inhibit the chloroplast protein-synthesizing system and cycloheximide to block protein synthesis in the cytosol. The results of Hoober and Blobel (86) and Avadhani and Buetow (4), described above, suggest that chloramphenicol and cycloheximide are quite specific with respect to the class of ribosome affected *in vitro*, however, those results do not exclude the possibility that cellular processes other than protein synthesis are affected. In fact cycloheximide and chloramphenicol do inhibit, both *in vivo* and *in vitro*, processes other than protein synthesis (50,58). Chloramphenicol has been found to inhibit respiration, sulfate uptake, oxidative phosphorylation (*in vitro*), and mitochondrial electron transport (*in vitro*); cycloheximide inhibits asparagine synthesis, malate oxidation by mitochondria (*in vitro*), and chloride uptake (50). Despite these shortcomings, chloramphenicol and cycloheximide have been used frequently and successfully to study the synthesis of chloroplast proteins and were chosen for use in this study.

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Because information regarding proteins synthesized both in an organelle and in the cytoplasm can be gained from an *in vivo* approach, I chose such an approach for the study of the sites of synthesis of cyanelle proteins. I labeled intact cells of *Cyanophora* in the absence or in the presence of inhibitors of protein synthesis and then isolated the cyanelles from those labeled cells. The labeled polypeptides were separated by SDS-PAGE. Fluorographs were made of the stained gels to establish the resulting patterns of inhibition.

Materials and Methods

Organism and growth

Cyanophora paradoxa UTEX LB 555 was obtained from the Culture Collection, University of Texas, Austin, Texas. Axenic cultures derived from this bacterized culture (see below) were grown in 250-ml cotton stoppered Erlenmeyer flasks, under continuous fluorescent illumination at 26°C on a gyrorotatory shaker at ca. 110 rpm. Incident illumination, measured with a Kettering Radiometer Model 68 (Laboratory Data Control, Riviera Beach, FL) was approximately 5000 ergs/cm^2/sec. The medium, designated CYB, was medium CYII (174) with the following modifications. 5 mM N,N-bis(2-hydroxyethyl)glycine (Bicine) was substituted

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for tris(hydroxymethyl)aminomethane (Tris). Three mM, each, of $NaNO_3$ and KNO_3 were added. The pH was adjusted to 7.8 rather than to 7.6. The composition of CYB is shown in Appendix B.

Purification of Cyanophora

In order to limit the growth of foreign organisms, contaminated cultures of Cyanophora were maintained on Bold's 3N Bristols medium (169, Appendix B), referred to as B3N, a medium with a low content of fixed carbon. Removal of contaminating organisms was accomplished as follows. Cells were sedimented by centrifugation at 220xg in a clinical centrifuge. The pelleted cells were resuspended in B3N to a density of ca. $8x10^5$ cells/ml and incubated in the dark for 45 minutes to reduce the growth of Cyanophora. Filter-sterilized fructose, final concentration 5 mM, and sterile ampicillin, final concentration 100 µg/ml, were added and the cells returned to the dark. After 4 hours the cells were diluted 10-fold, 100-fold, 1000-fold, and 10^4-fold. One ml of each dilution was added to 1 ml of 0.5% agarose and overlayed onto B3N containing 1% agarose in 60x15mm Petri dishes. To prevent killing the cells, the 0.5% agarose was cooled to near the gelling point. The edges of the Petri dishes were sealed with strips of Parafilm (American Can Co., Greenwich, CT.) and the Petri dishes were kept at room temperature (23-24°C) under

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fluorescent lamps. After 6 days, small groups of cells that appeared to be free of contaminating organisms were transferred, using a sterile Pasteur pipette, to 50-ml Erlenmeyer flasks containing 10 ml of B3N. The resulting cultures were checked for purity by microscopic examination following growth on a variety of media. No contaminants were found. Cultures were susequently transferred to and maintained on CYB.

Conditions for labeling Cyanophora

Axenic cultures of Cyanophora were harvested at 477xg and resuspended to a density corresponding to 10-25 µg chlorophyll a/ml. Cells that were to be labeled with amino acids were resuspended in CYB, cells that were to be labeled with acetate were resuspended in CYB-Ac, and cells that were to be labeled with sulfate were resuspended in CYB-S. CYB-Ac is CYB modified by substituting $(NH_4)_2SO_4$ for NH_4 acetate; CYB-S is CYB modified by substituting Cl^- for $SO_4^=$. The cell suspension was added to flasks containing one of the following compounds dried on the bottom of the flask: [^{35}S]methionine (>500 mCi/mmole), [^{35}S]cysteine (792 mCi/mmole), $Na_2{}^{35}SO_4$ (1028 mCi/mmole), [^{14}C]leucine (342 mCi/mmole) or [^{14}C]acetate (57 mCi/mmole). 1-2 µCi of radioactivity were added for each ml of cell suspension. The cells were incubated at 26°C on a gyrorotatory shaker. Illumination was provided by cool white fluorescent bulbs

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(General Electric). Dark controls were wrapped in 2 layers of aluminum foil. Incubations were terminated by one of two methods. The first method was used when taking samples only for determination of radioactivity, as, for example, during a time course experiment. Duplicate samples of the cell suspension were spotted onto 2.3-mm circles of Whatman 3MM paper which were placed immediately into a 4°C solution of 10% trichloracetic acid (TCA) containing 100 mM of the stable form of the substrate (viz., acetate, leucine, methionine, cysteine, or sulfate) used to label the cells. The filters were processed as described below. When further manipulations of the labeled cells were required (viz., when cyanelles were to be isolated for analysis of their proteins) the cells were collected at 477xg and resuspended in 4°C CYB containing 100 mM of the stable form of the substrate used to label the cells. The centrifugation and resuspension were repeated once.

Isolation of cyanelles from radioactively labeled Cyanophora

After the initial wash described above, cells were sedimented by centrifugation in 1.5-ml Eppendorf microcentrifuge tubes at 477xg for 3 min. The pellets of cells were resuspended in CYB containing 0.5 M sucrose and kept at room temperature for 10 min. The cells were then sedimented by centrifugation at 1740xg for 5 min. The cells, but not the cyanelles, were lysed by resuspending them in

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CYB containing 0.1 M sucrose. After 5 min at room temperature cyanelles, that had been released were sedimented at 880xg for 5 min. The cyanelles were washed twice by centrifugation and resuspension in CYB. The first centrifugation was performed at 477xg for 5 min and, the second at 288xg for 5 min. All centrifugations were performed in a clinical centrifuge.

Determination of incorporation of radioactively labeled compounds into protein by Cyanophora.

The following process was used to determine the amount of radioactivity present in protein, duplicate samples of either intact cells or isolated cyanelles. Samples were spotted onto 2.3-cm Whatman 3MM filters which were then transferred to a 4°C solution of 10% TCA containing 100 mM of the stable form of the substrate (viz., acetate, leucine, methionine, cysteine, or sulfate) used to label the cells. Following the procedure of Mans and Novelli (112), the filters were kept in the solution of 10% TCA described above, for at least 1 hr at 4°. The solution was decanted and the filters were resuspended in 5% TCA for 15 min. The solution was decanted and the filters were washed once with and then resuspended in 5% TCA, and incubated at 90°C for 30min. The filters were then washed once with 5% TCA, resuspended in ether/ethanol (1:1, v/v) and incubated at 37°C for 30 to 60 min. The filters were then washed 2 times with ether and dried in air. Radioactivity was measured in

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5 ml of ACS (Aqueous Counting Scintillant, Amersham Corporation) with a model LS100C Liquid Scintillation Counter (Beckman Instruments).

SDS polyacrylamide gel electrophoresis of radioactively labeled Cyanophora

Radioactively labeled intact cells, or cyanelles isolated from them, were first extracted with 100% methanol and then extracted three times with chloroform-methanol (2:1, v/v). After extraction the pellets were dried in air, suspended in the SDS lysis buffer of Laemmli (99), and boiled for 5 min. Immediately thereafter, electrophoresis was performed in SDS-containing polyacrylamide gels composed of a 5% acrylamide stacking gel and a separating gel of 10%, 12.5% or 15% acrylamide (99). Equal amounts of cyanelles, equivalent to 1.3-1.6 µg of chlorophyll *a*, were loaded in each lane. Gels were prepared for fluorography according to the procedure of Bonner and Laskey (22) modified by adding 5% (v/v) glycerol to the final wash to prevent cracking of the gels during drying. Fluorographs were prepared using KODAK XR-5 film exposed at -40°C.

Determination of protein and chlorophyll a.

Protein was determined according to the method of Lowry *et al*. (106). The amount of chlorophyll *a* was estimated by measuring the absorbance at 665 nm of a methanol extract of

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Cyanophora or of cyanelles. The value, 74.5, of the absorption coefficient used to calculate the concentration of chlorophyll a was obtained from Mackinney (107). Absorbance was measured with a DB-G grating spectrophotometer (Beckman Instruments).

Results

Characteristics of the incorporation of labeled substrates into protein by intact cells of Cyanophora paradoxa

Two prerequisites of an in vivo approach to the study of the sites of synthesis of cyanelle proteins are the ability to label cyanelle proteins with exogenously supplied compounds and the ability to differentiate, by using inhibitors of protein synthesis, between proteins made on cyanelle ribosomes and proteins made on cytoplasmic ribosomes.

Because protein synthesis had not been studied previously in Cyanophora, it was not known which substances would most effectively label proteins in that organism. Therefore, several different substances were tried: [^{14}C]acetate, [^{35}S]methionine and cysteine, [^{14}C]leucine, and $^{35}SO_4^{=}$. Moreover, because the effects of cycloheximide and chloramphenicol on protein synthesis in Cyanophora had not been determined, I examined the effects of those

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inhibitors. Concentrations that might specifically inhibit protein synthesis had to be determined.

The results described below show that all of the compounds tested are taken up and incorporated into protein by Cyanophora and that protein synthesis by intact cells and cyanelles is affected by cycloheximide and chloramphenicol. Moreover,the synthesis of the cycloheximide-sensitive proteins of the cyanelle is not detectably inhibited by chloramphenicol. Conversely, the synthesis of the chloramphenicol-sensitive proteins of the cyanelle is not detectably inhibited by cycloheximide.

i. Incorporation of $Na_2[^{14}C]$acetate

Intact cells of Cyanophora were labeled with $Na_2[^{14}C]$acetate at a concentration of 35 µM, 175 µM, 438 µM, 700 µM, or 1mM. The time course for this experiment is shown in Figure 1. The results show that Cyanophora is capable of incorporating $[^{14}C]$acetate into protein. Increasing the concentration of $[^{14}C]$acetate resulted in an increase in the rate and magnitude of incorporation of acetate into protein. For all concentrations of acetate used the rate of incorporation increased with time. Figure 2 shows that the incorporation of acetate was lower in the dark than in the light.

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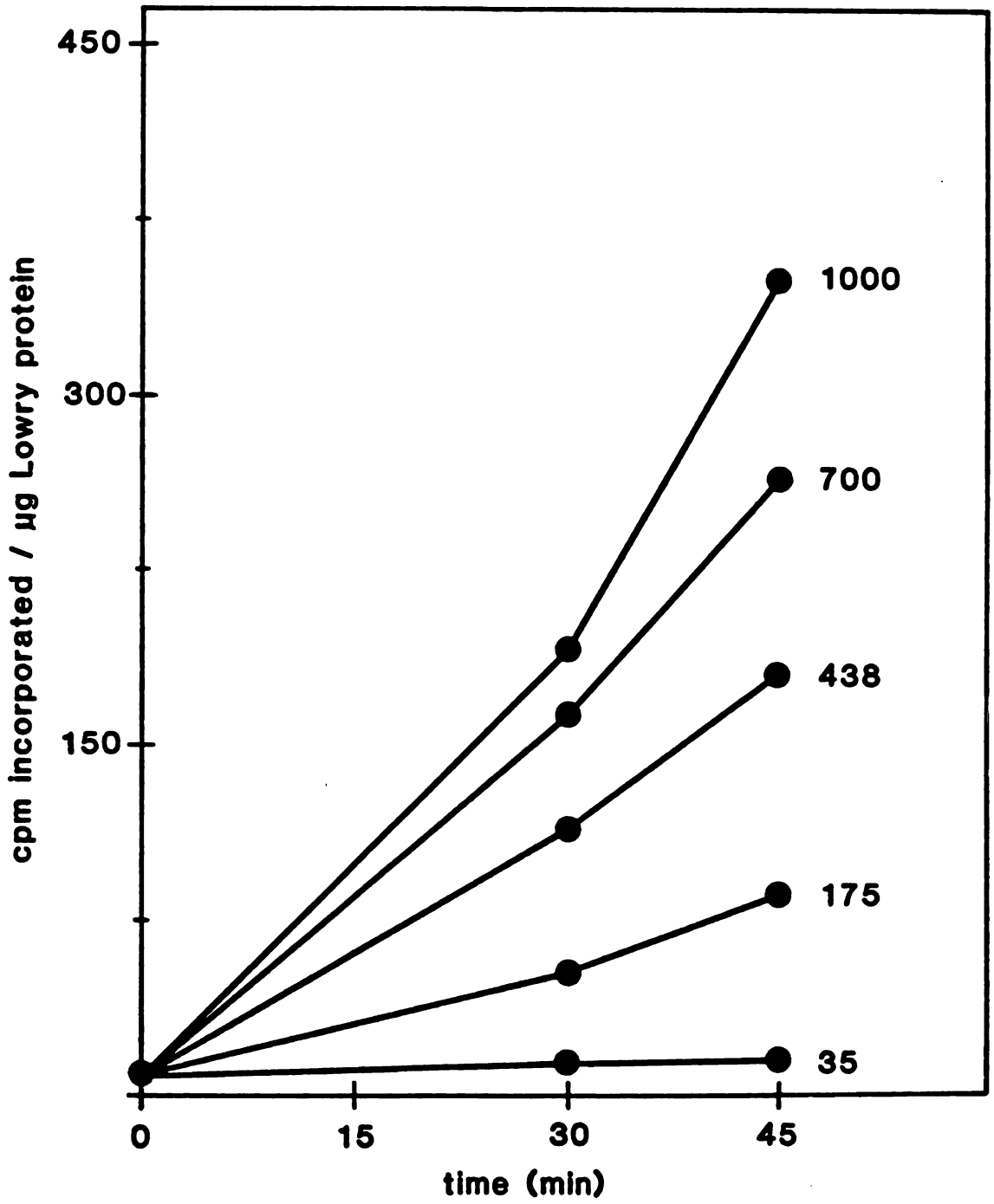

Figure 1. Time courses of the incorporation of various concentrations (µM) of [^{14}C]acetate into protein by intact cells of Cyanophora paradoxa.

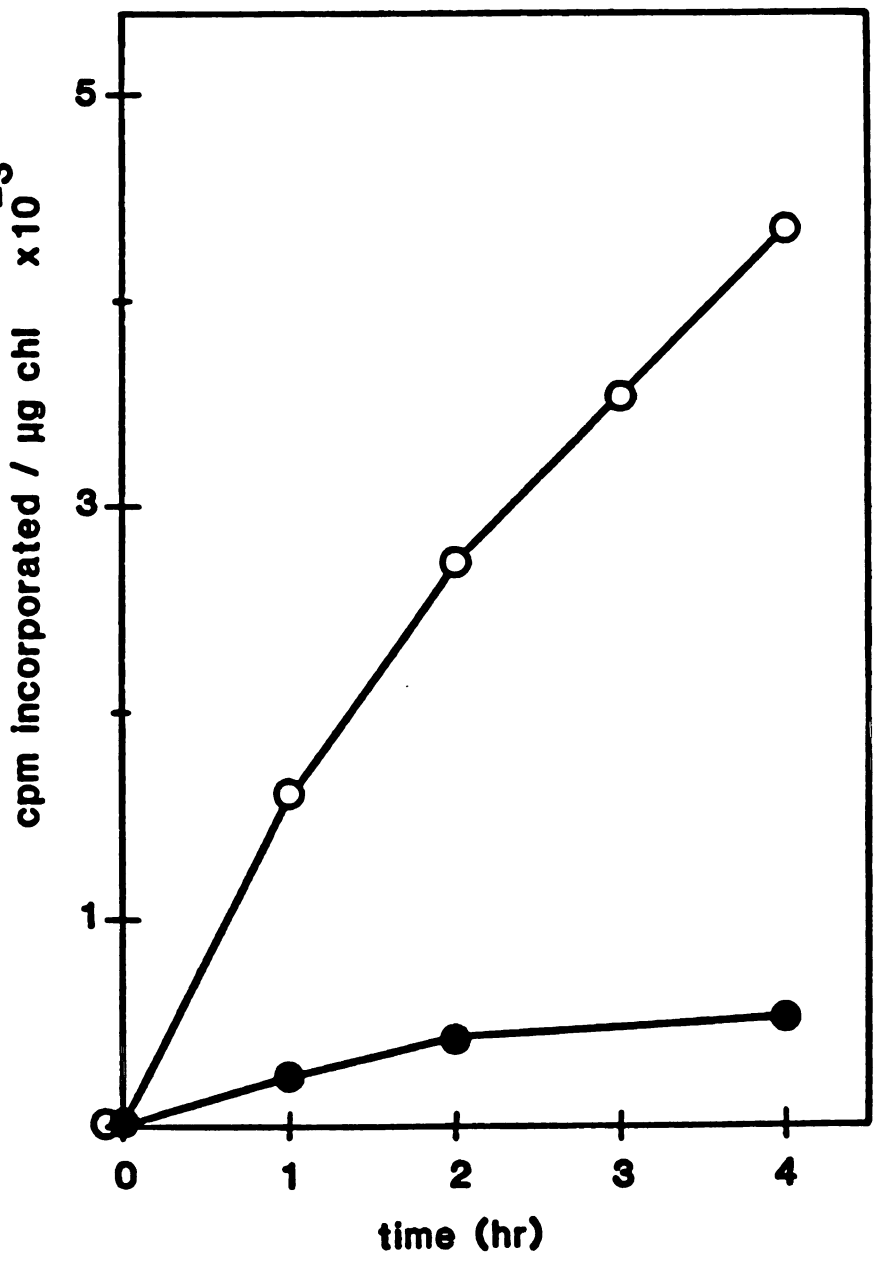

Figure 2. Time course of the incorporation of [^{14}C]acetate into protein by intact cells of Cyanophora paradoxa incubated in the light (O—O) and in the dark (●—●).

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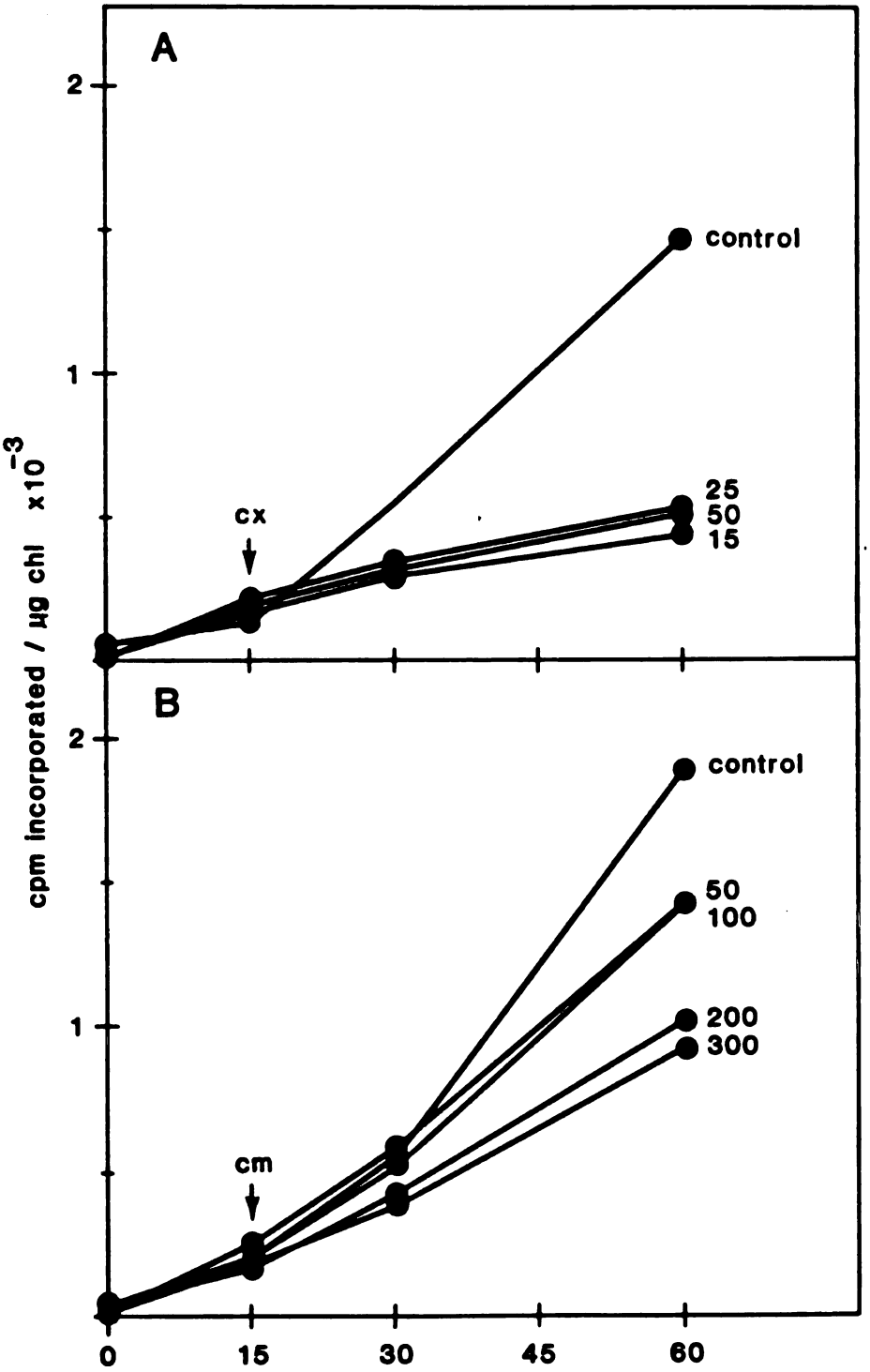
ii. The effect of D-cycloheximide and D-threo-chloramphenicol on the incorporation of acetate.

The appropriate concentrations of cycloheximide and chloramphenicol were determined by measuring the incorporation of acetate into protein by intact cells in the presence of various concentrations of these inhibitors. The cells were allowed to incorporate acetate for 15 min, a sample for the determination of radioactivity was taken, and the inhibitors were added. The incorporation was followed for a further 60 minutes. All concentrations of cycloheximide used (15-50 µg/ml) inhibited incorporation approximately 65% (Figure 3). Inhibition by chloramphenicol increased with increasing concentration, reaching 45% at 300 µg/ml (Figure 3). On the basis of these results, concentrations of 25 µg cycloheximide/ml and 300 µg chloramphenicol/ml were chosen for use in subsequent experiments.

iii. Incorporation of amino acids by Cyanophora

In contrast to the findings of Trench et al. (174), I found that Cyanophora does take up and incorporate amino acids into protein. Time courses for the incorporation of L-[^{35}S]methionine, L-[^{35}S]cysteine, and L-[^{14}C]leucine are shown in figures 4, 5, and 6. In contrast to the rates of incorporation of acetate (see above) and sulfate (see below), the rates of incorporation of amino acids did not

Figure 3. The effect of various concentrations of D-cycloheximide (A) and D-threo-chloramphenicol (B) on the incorporation of [^{14}C]acetate into protein by intact cells of Cyanophora paradoxa. Chloramphenicol (cm) or cycloheximide was added at 15 min. The concentrations shown are µg/ml.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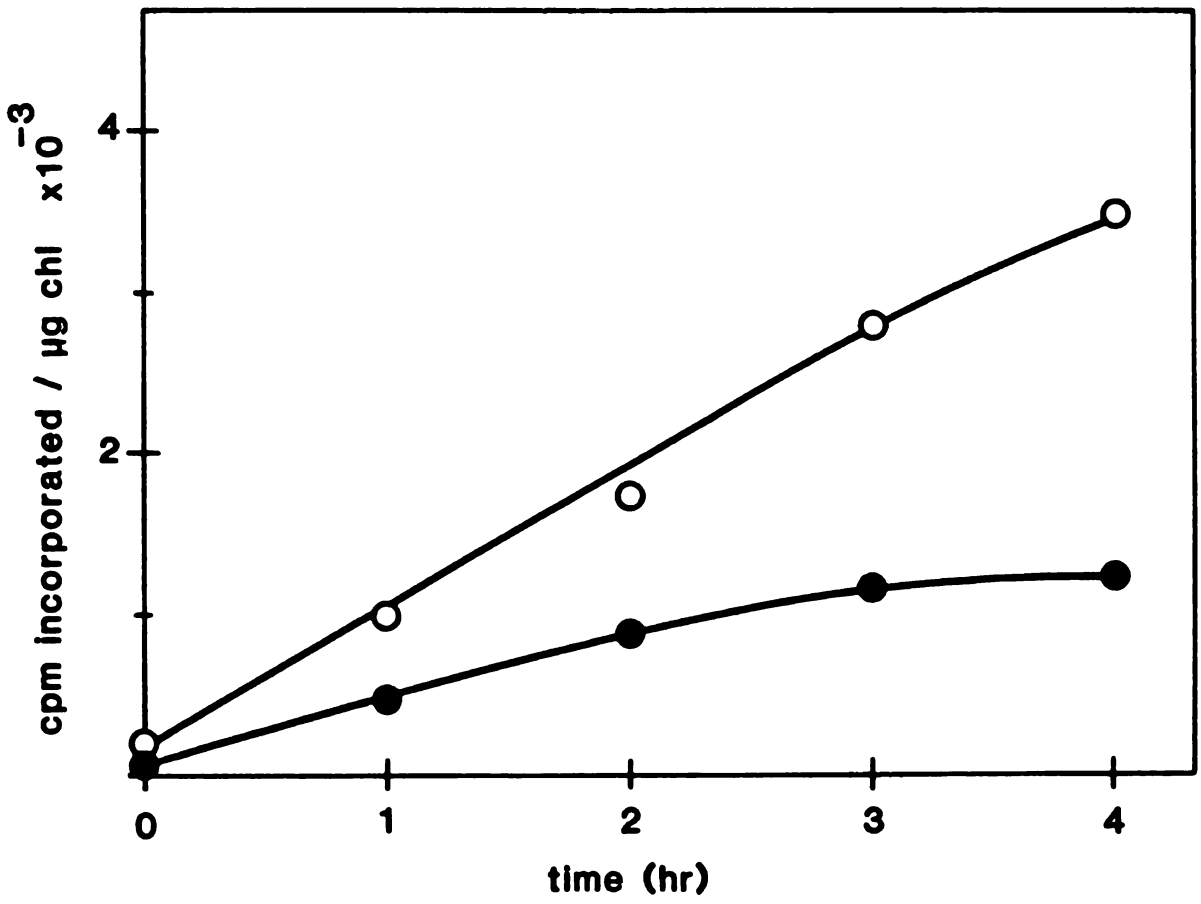


Figure 4. Time course of the incorporation of [^{35}S]methionine into protein by intact cells of Cyanophora paradoxa incubated in the light (O—O) or in the dark (●—●).

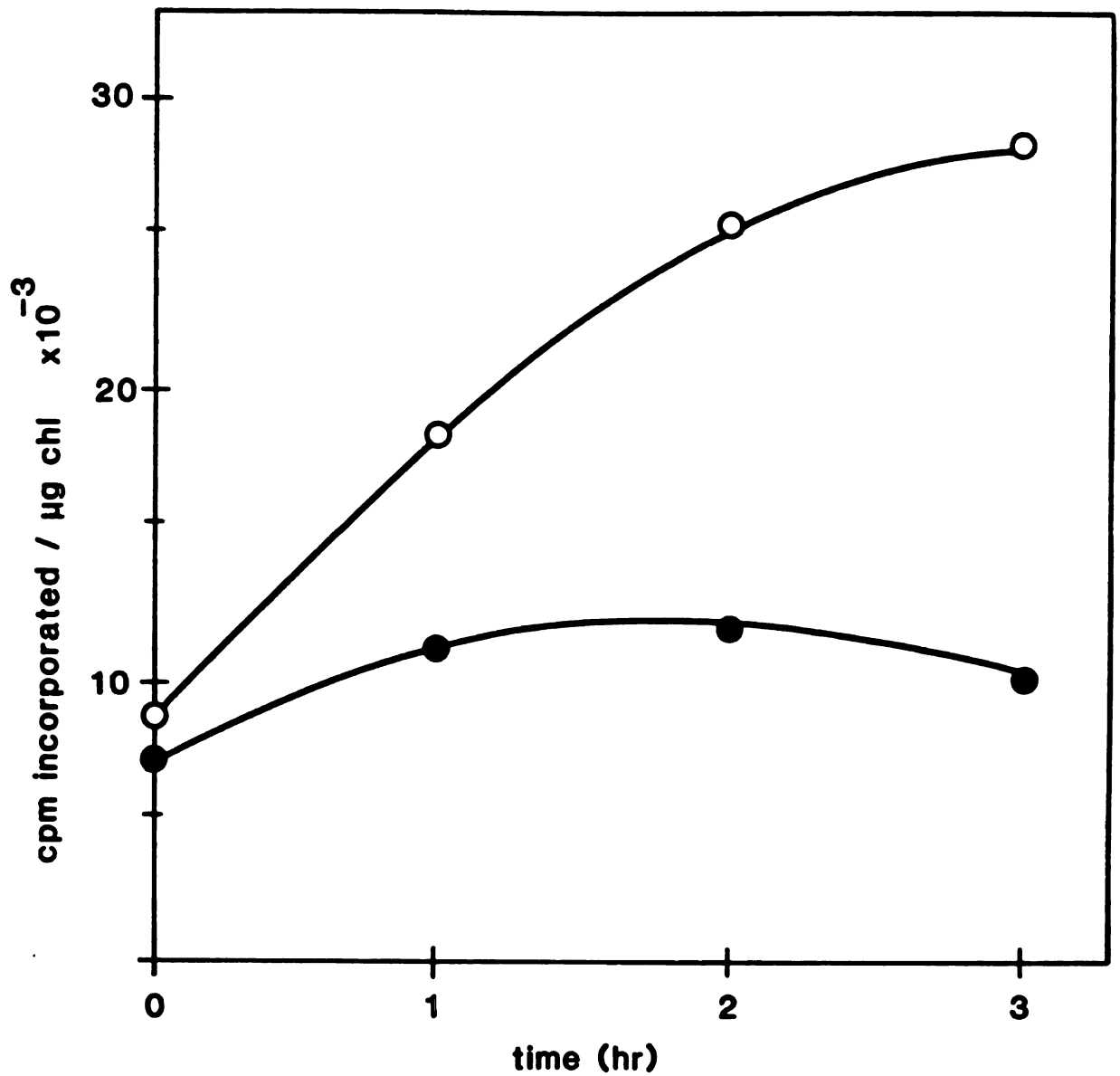
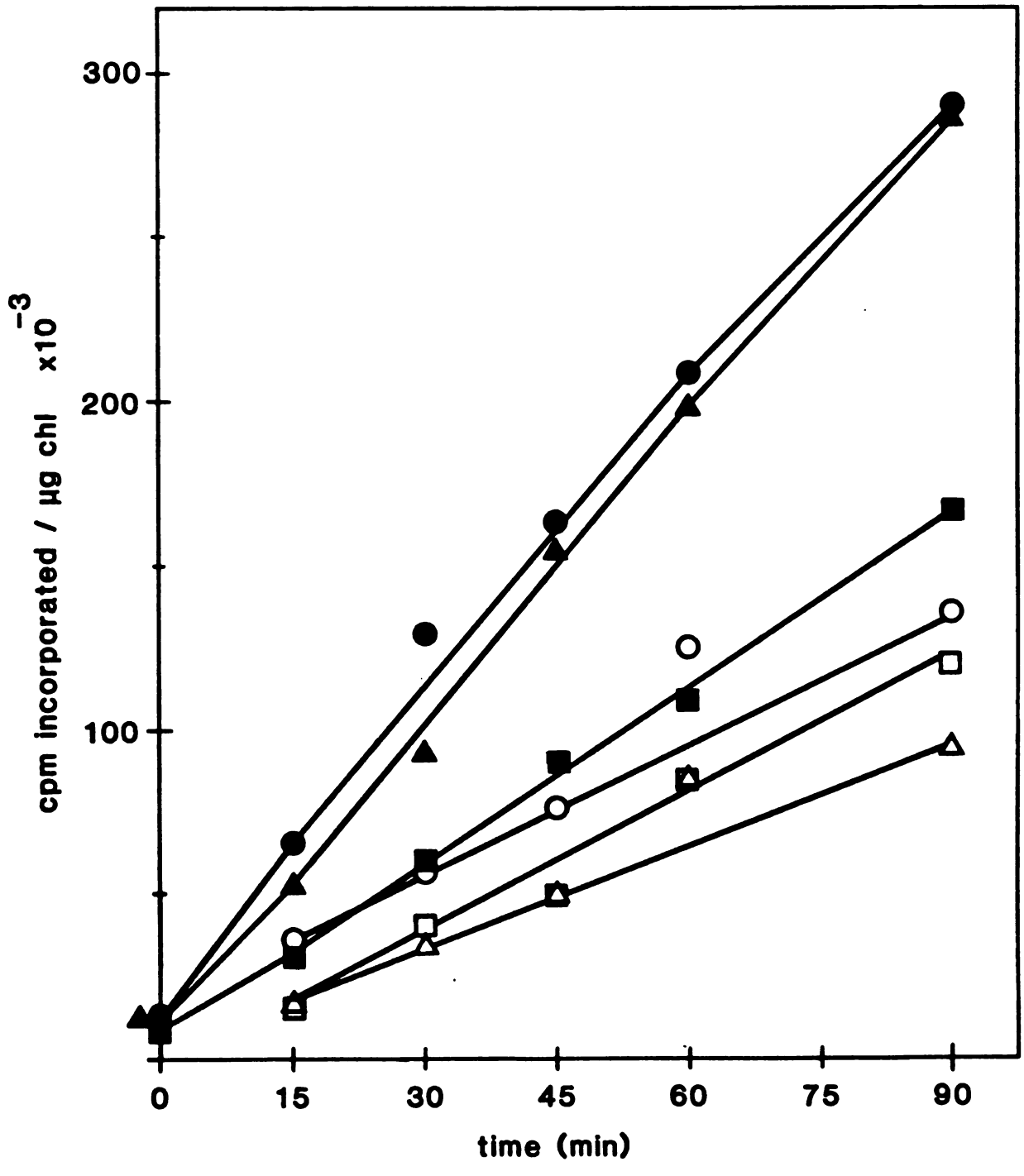


Figure 5. Time course of the incorporation of [^{35}S]cysteine into protein by intact cells of Cyanophora paradoxa incubated in the light (O—O) or in the dark (●—●).

Figure 6. Time course of the incorporation of [^{14}C]leucine into protein by intact cells and cyanelles of Cyanophora paradoxa upon incubation of intact cells in the absence or in the presence of D-cycloheximide or D-threo-chloramphenicol. At the times shown, aliquots of cells were taken, the cyanelles were isolated, and the radioactivity in protein both in the intact cells and the isolated cyanelles was determined. Intact cells were incubated in the light without inhibitor (●—●), or plus 25 µg cycloheximide/ml (■—■), or plus 300 µg chloramphenicol/ml (▲—▲). (○—○) Cyanelles isolated from cells incubated in the light without inhibitor or (□—□) plus 25 µg cycloheximide/ml or (△—△) plus 300 µg chloramphenicol/ml. Each time point is the average of two samples.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Cells labeled with cysteine (Figure 5) showed an anomalous result: very high levels of apparent incorporation, 9,000-10,000 cpm/μg chl*a* at the zero-time point. The initial rate of incorporation of cysteine, although relatively high (approximately 170cpm/ μg chl*a*·min), was not high enough to account for the label accumulated during the few minutes required to take the zero-time sample. Unlike cells labeled with other compounds, methionine-labeled cells showed a relatively high rate of dark incorporation, 35% of the rate in the light.

The results described above show that *Cyanophora* can incorporate exogenous organic substances into protein and that their incorporation into protein is reduced by cycloheximide and chloramphenicol. In order to determine whether cyanelle proteins are labeled and their synthesis affected by the inhibitors, a time course experiment was performed in which intact cells were labeled with [^{14}C]leucine, in the absence of inhibitors or in the presence of 25 μg cycloheximide/ml or 300 μg chloramphenicol/ml. Cyanelles were isolated at 15 minute intervals to 1 hour, and at 1.5 hours, and the amount of radioactivity incorporated into protein determined (Figure 6). The rate and magnitude of incorporation observed for each of the treatments are presented in Table 1 and Table 2, respectively. In this experiment, unlike that of

Table 1. Rates and relative rates of incorporation of [^{14}C]leucine into protein by intact cells and cyanelles of *Cyanophora paradoxa*.

	Rate cpm/(μg chl·hr)	Relative rate %[1]	Relative rate %[2]
Intact Cells	192	100	
Cyanelles	80	42	100
Intact Cells + Cm	196	102	
Cyanelles + Cm	60	31	75
Intact Cells + Cx	108	56	
Cyanelles + Cx	86	45	108

1 Rate relative to intact cells incubated in the absence of inhibitor.
2 Rate relative to cyanelles isolated from cells incubated in the absence of inhibitor.

Table 2. The amount of radioactivity present in protein of cells and cyanelles after labeling intact cells of *Cyanophora paradoxa* with [^{14}C]-leucine for 90 minutes.

	cpm/µg·chl*a*	% incorporation 1	% incorporation 2
Intact cells	289	100	
Cyanelles	136	47	100
Intact cells + Cm	290	100	
Cyanelles +Cm	95	33	69
Intact cells +C	167	58	
Cyanelles +Cx	120	42	88

1 Incorporation relative to intact cells incubated in the absence of inhibitor.
2 Incorporation relative to cyanelles isolated from intact cells incubated in the absence of inhibitor.

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Figure 3, in which incorporation of [^{14}C]acetate was followed, the rate of incorporation of [^{14}C]leucine by intact cells was not significantly reduced by 300 µg chloramphenicol/ml. However, compared to cyanelles isolated from control cells, the cyanelles isolated from cells treated with chloramphenicol showed a 25% reduction in the rate of incorporation. Cycloheximide decreased the rate of amino acid incorporation by whole cells 46% but had no significant effect on the rate of incorporation by cyanelles. The cyanelles accounted for approximately 40% of the total incorporation by intact cells.

iv. Incorporation of $Na_2{}^{35}SO_4$ by Cyanophora

The time course of incorporation of sulfate into protein by intact cells of Cyanophora (Figure 7) is similar to that of acetate (compare Figure 2). However, in contrast to the incorporation of acetate, which was not saturated at 1 mM, the incorporation of sulfate was saturated at the lowest concentration tested, ca. 50 µM. The steady state rate of incorporation of sulfate (50 µM) was ca. 0.1 nmoles/(µg chl·hr).

The incorporation of sulfate into protein was sensitive to inhibitors of protein synthesis. Intact cells were labeled with 50 µM $Na_2{}^{35}SO_4$ for 40 minutes in the light or in the dark in the absence, or in the light in the presence, of inhibitor. At the end of the labeling period, the cells

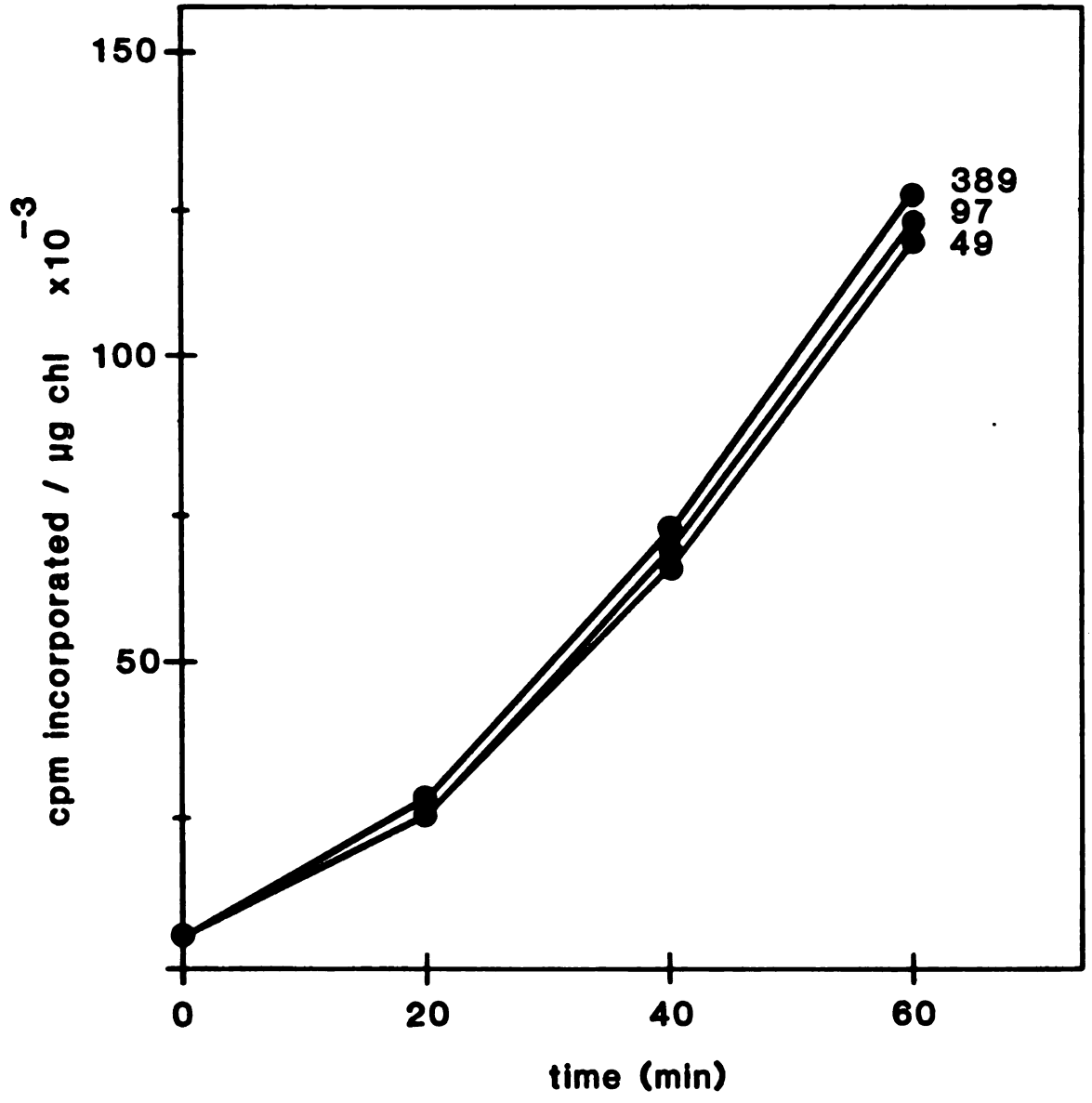

Figure 7. Time courses of the incorporation of 49 µM, 97 µM, and 389 µM $^{35}SO_4^=$ into protein by intact cells of Cyanophora paradoxa.

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from each treatment were divided into two aliquots. Cyanelles were isolated from one aliquot and the radioactivity present in protein both in the intact cells and in the isolated cyanelles was determined (Table 3).

Whereas cyanelles accounted for ca. 40% of the total incorporation of [^{14}C]leucine, they accounted for approximately 15% of the incorporation $^{35}SO_4^=$ into protein by intact cells. Chloramphenicol inhibited the incorporation of ^{35}S from $^{35}SO_4^=$ into total cell protein by 38% and into cyanelle protein by ca. 24%. Cycloheximide decreased the incorporation of ^{35}S from $^{35}SO_4^=$ into protein by intact cells 83%, a reduction nearly equal to reduction resulting from incubation of the cells in the dark. Whereas cycloheximide reduced the incorporation of leucine into cyanelle protein by only 12% (Table 2), it decreased the incorporation of sulfate into cyanelle protein by 59%.

v. SDS-PAGE of proteins from cyanelles labeled with $Na_2{}^{35}SO_4$ in vivo

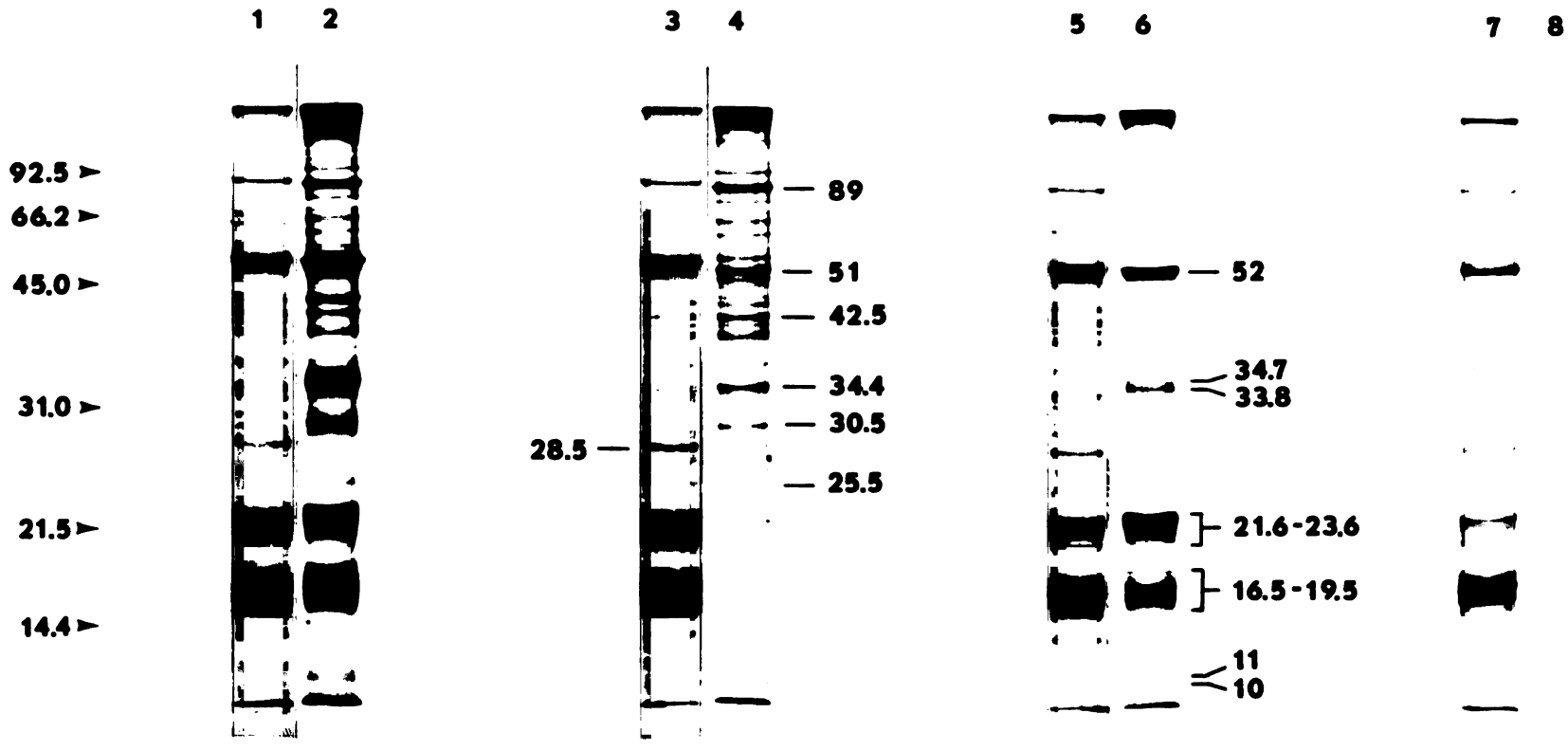
To identify which cyanelle polypeptides are synthesized in the cyanelle and which in the cytoplasm, the protein from isolated labeled cyanelles was electrophoresed on SDS-containing polyacrylamide gels. In Figure 8 the fluorograms resulting from cyanelles labeled in the absence of inhibitor or in the presence of 25 µg cycloheximide/ml, 300 µg chloramphenicol/ml or 25 µg cycloheximide/ml plus 300

Table 3. Radioactivity present in protein of intact cells and cyanelles of Cyanophora paradoxa labeled with $^{35}SO_4^=$.

Treatment	Intact Cells (cpm)	%1	Cyanelles (cpm)	%2
light (lt)	58,575	100	9,025	100
dark	7,981	14	1,262	14
lt + chloramphenicol	36,688	62	6,873	76
lt + cycloheximide	10,207	17	3,723	41
lt + chloramphenicol + cycloheximide	4,750	8	1,039	12

1 Rate relative to intact cells incubated in the light in the absence of inhibitor.
2 Rate relative to cyanelles isolated from cells incubated in the light in the absence of inhibitor.

Figure 8. Patterns of polypeptides from the cyanelles of Cyanophora paradoxa labeled in vivo with $^{35}SO_4^{=}$. Intact cells were labeled for 40 min in the light in the absence of inhibitor (lanes 1,2) or in the presence of 300 µg chloramphenicol/ml (lanes 3,4), 25 µg cycloheximide/ml (lanes 5,6), or 300 µg chloramphenicol/ml plus 25 µg cycloheximide/ml (lanes 7,8). The cyanelles were isolated and their proteins separated by SDS-PAGE. Fluorograms (lanes 2,4,6,8) were prepared from the Coomassie Blue-stained gels (1,3,5,7). Equal amounts of cyanelles, equivalent to 1.6 µg chlorophyll a were loaded in each lane.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µg chloramphenicol/ml have been aligned with the Coomassie Blue-stained polyacrylamide gel. A summary of the polypeptides labeled in the presence of cycloheximide and chloramphenicol is contained in Table 4. The apparent molecular weights presented in Table 4 are derived from a comparison of the fluorogram with the Coomassie Blue-stained gel. It proved helpful when making the comparisons to utilize photographic contact prints of the fluorograms. When making the contact prints several different exposures were made to compensate for the differences in the intensity of the bands in the fluorogram. This was especially useful for polypeptide bands in the molecular weight range of 16,000-24,000 daltons of cyanelles from cycloheximide-treated cells.

Comparison of the fluorograms of proteins from cycloheximide-treated (Figure 8 lane 6) and chloramphenicol-treated (Figure 8 lane 4) cyanelles with the fluorogram of proteins from untreated cyanelles (Figure 8 lane 2), shows that, generally, each polypeptide is affected by only one of the antibiotics. Two closely spaced polypeptides of ca. 28,500 daltons (Figure 8 lane 3) are exceptional in that their synthesis was significantly inhibited by both chloramphenicol and cycloheximide. A greater number of polypeptides was observed in cyanelles isolated form cells treated with chloramphenicol thn in cyanelles isolated from cycloheximide-treated cells. The

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Table 4. Polypeptides associated with cyanelles labeled *in vivo* with $^{35}SO_4^=$ in the presence of cycloheximide or chloramphenicol.

+ chloramphenicol M_r x10^{-3} daltons	+ cycloheximide M_r x10^{-3} daltons
89	89
80	67
55.4-53.	52
51	51
50.4	44
45.7	44
44	34.7
42.7-41.8	33.8
41.5	23.6
39.7-41.3	23.2
38	22.7
36.5-36	21.6
34.4	20.6
33.6	19.5
31.5	19.2
30.5	18.3
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number of proteins synthesized in the presence of chloramphenicol is not well reflected in the summary presented in Table 4 because many of the bands are composed of several closely spaced polypeptides which were considered together (see Figure 8). Whereas fewer polypeptides were labeled in the presence of cycloheximide, they appeared to be made in greater amounts. No radioactive polypeptides were visible in the fluorogram of cyanelles labeled in the presence of both inhibitors (Figure 8 lane 8).

Of those polypeptides synthesized in the presence of cycloheximide, a polypeptide (or polypeptides) having an apparent molecular weight of 52,000 daltons was the most extensively labeled. Polypeptides of sizes 34,700, 22,500, 17,600, 16,700, 16,500, and a diffuse band of ca. 33,800 daltons were also extensively labeled in the presence of cycloheximide. Two additional polypeptides of ca.10,000-11,000 daltons were synthesized in the presence of cycloheximide. The molecular weights of the 10,000-11,000 dalton proteins could not be accurately determined because no protein standard of molecular weight smaller than 14,400 daltons was included and because polypeptides of less than ca. 15,000 daltons behave anomalously on SDS-polyacrylamide gels (179).

The most highly labeled polypeptide in cyanelles isolated from chloramphenicol-treated cells had an apparent molecular weight of ca. 89,000 daltons. Polypeptides of

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51,000, 42,500, 34,400, 30,500, and 25,500 were also prominant in the fluorogram of chloramphenicol-treated cyanelles.

Discussion

The sites of synthesis of cyanelle proteins have not been studied previously. I undertook to investigate their sites of synthesis by labeling proteins *in vivo* in the presence of inhibitors specific for either cyanelle or cytoplasmic protein synthesis. The cyanelles were then isolated and the labeled polypeptides were separated by SDS-PAGE. It was first necessary to identify substrates that would label cyanelle proteins *in vivo*.

i. Characterization of the incorporation of radioactively labeled compounds into protein by Cyanophora paradoxa

Intact cells of *Cyanophora paradoxa* are capable of taking up and incorporating exogenous organic compounds and sulfate into protein. In contrast to Trench *et al.* (174), who observed no uptake of amino acids, I determined that methionine, cysteine and leucine were taken up and incorporated into protein by *Cyanophora*.

The kinetics of the incorporation varied depending upon the radioactive compound used. The rate of incorporation of ^{14}C from acetate or of ^{35}S from sulfate into protein by

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intact cells increased with time, whereas the rate of incorporation of leucine did not. The lower initial but increasing rate of incorporation from acetate and sulfate may be a consequence of the time for the synthesis of amino acids from these compounds. The absence of a lag phase in the incorporation of leucine may be an indication that intracellular pools of leucine are sufficiently small that they can be rapidly saturated even at the relatively low concentration, 3 μM, of leucine used.

Twenty-five μg cycloheximide/ml appears to be a saturating level of antibiotic because higher concentrations did not further reduce the incorporation of amino acids into protein. When cells were labeled with sulfate in the presence of both inhibitors the incorporation into protein was reduced 88% and no labeled polypeptides were visible in the fluorographs (Figure 8). This result suggests that the concentrations of the inhibitors used were saturating or that the presence of both inhibitors is rapidly lethal. With respect to the degree of inhibition, 300 μg chloramphenicol/ml may not have been saturating. Although the reduction in incorporation of ^{14}Cacetate at 300 μg chloramphenicol/ml (45%) is only slightly greater than the reduction in incorporation at 200 μg chloramphenicol/ml (38%) concentrations greater than 300 μg/ml might have had a further inhibitory effect. Higher concentrations were not used because of a concern that nonspecific inhibition

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The incorporation of radioactivity into protein was sensitive to the inhibitors of protein synthesis used in these experiments. The rate of incorporation of [^{14}C]leucine into cyanelle protein was reduced 25% by chloramphenicol, whereas the rate of incorporation of [^{14}C]leucine into total cell protein was not detectably affected by chloramphenicol. These results suggest that there is a compensation in the rate of cytoplasmic protein synthesis when a competing sink is shut off. Whereas the rate of incorporation of [^{14}C]leucine into cyanelle protein was not significantly affected by cycloheximide, the corresponding rate of incorporation into total cell protein was decreased 44% by cycloheximide. SDS-PAGE of cyanelle proteins labeled with $^{35}SO_4^=$ *in vivo* shows that the synthesis of many cyanelle proteins is sensitive to cycloheximide. However, the cycloheximide-insensitivity of leucine incorporation into cyanelle protein cannot mean that most of the labeled protein associated with the cyanelle is synthesized in the cyanelle. Instead, there appears to have been an increase in the rate of protein synthesis in the cyanelle in response to the inhibition of cytoplasmic protein synthesis. The chloramphenicol-insensitivity of incorporation into total cell protein and the cycloheximide-insensitivity of incorporation into cyanelle protein may, for example, result from changes in the sizes

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In contrast to the incorporation of [^{14}C]leucine, the incorporation of sulfate into total cell protein and into cyanelle protein was inhibited by both chloramphenicol and cycloheximide (refer to table 3). In this experiment, cyanelles accounted for approximately 15% of the incorporation into total cell protein. The incorporation of sulfate into cyanelle protein was reduced 34% by chloramphenicol. If chloramphenicol were inhibiting translation on cyanelle ribosomes only, then one would expect chloramphenicol to reduce the incorporation of sulfate into total cell protein ca. 5% (0.15x 0.34 = 0.05). Therefore, the 38% reduction in incorporation into total cell protein in the presence of chloramphenicol is not due solely to the inhibition of cyanelle protein synthesis. The incorporation of sulfate into cyanelle protein was reduced to a greater degree (59%) in the presence of cycloheximide but only 34% in the presence of chloramphenicol. These results seem to suggest that proteins of both the cyanelle and the cytoplasm are involved in the incorporation of sulfate into protein.

It is also possible that some secondary effects are observed when cells are labeled with sulfate (or other compounds) in the presence of inhibitors. That is, the inhibitor may perturb some cellular process which indirectly affects protein synthesis. Of particular interest here is

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the inhibition by chloramphenicol of sulfate uptake in beet, carrot and pea, oxidative phosphorylation in corn (*in vitro*), and photophosphorylation in spinach (*in vitro*) (50). Whereas inhibition of sulfate uptake would apply only to those experiments in which $^{35}SO_4^=$ was used to label cells, an inhibition of energy supply would affect the incorporation of all compounds.

Although I have not done so, it is possible to test whether chloramphenicol inhibits protein synthesis only directly, at the ribosome level, or also indirectly by affecting the uptake of precursors or the supply of energy. Chloramphenicol has two asymmetric carbon atoms, and therefore four stereoisomers. Protein synthesis by isolated ribosomes is inhibited only by the D-threo isomer of chloramphenicol, whereas all four stereoisomers inhibit phosphorylation, photophosphorylation and ion uptake in various tissues (52). If it can be shown that the inhibition is produced specifically by the D-threo isomer, then it can be concluded that the effect is upon protein synthesis.

ii. SDS-PAGE of intact cells and cyanelles labeled with $Na_2{}^{35}SO_4$

Intact cells were labeled with $Na_2{}^{35}SO_4$ in the absence or in the presence of chloramphenicol or cycloheximide or both, and the cyanelles then isolated. Cyanelle proteins were separated by SDS-PAGE and the labeled polypeptides

visualized by fluorography. The presence of chloramphenicol or cycloheximide during *in vivo* labeling of cyanelles resulted in different patterns of labeled polypeptides on fluorograms of SDS-polyacrylamide gels (see Figure 8). For the most part, the incorporation of radioactivity into individual polypeptides was sensitive to only one of the two inhibitors. In this discussion a polypeptide will be considered to be synthesized in the cyanelle if it is labeled in the presence of cycloheximide, but not in the presence of chloramphenicol. Conversely, proteins labeled when chloramphenicol, but not cycloheximide, is present will be considered to be synthesized in the cytosol. A summary of polypeptides labeled in the presence of 25 µg cycloheximide/ml or in the presence of 300 µg chloramphenicol/ml is presented in Table 4.

In the paragraphs that follow, tentative identities are suggested for some of the cyanelle polypeptides that are labeled in the presence of cycloheximide. The polypeptides that have been tentatively identified are the large subunit of RUBISCO, the α and β subunits of the ATP synthase and the 32,000 dalton membrane protein. The identification of these polypeptides is based on the following evidence. i. Their apparent molecular weights by SDS-PAGE are similar to those of cyanelle proteins [RUBISCO (30,40), ATP synthase (91)] that have been purified. ii. Sequences are present in the cyanelle DNA that hybridize at high stringency with

the genes for the large subunit of RUBISCO from maize (77, appendix A of this thesis), spinach (17), and Chlamydomonas (77); the 32,000 dalton membrane protein from spinach (17); the α subunit of ATP synthase from spinach (17); and the β subunit of ATP synthase from spinach (17) and maize (appendix A of this thesis).

Because the coding capacity of the cyanelle genome is approximately 10- to 20-fold less than that of free-living cyanobacteria (16,17,80,78), it is likely that some cyanelle proteins are synthesized in the cytosol. Although it would be of interest to know which cyanelle polypeptides are synthesized in the cytosol, the results presented in this chapter do not allow tentative identities to be assigned to the chloramphenicol-insensitive polypeptides. The reasons that no tentative identities are suggested for these polypeptides are the following. No cyanelle protein which is cytoplasmically synthesized in plants or eukaryotic green algae has been characterized. Thus, the identity of these polypeptides would be based solely on a comparison between the apparent molecular weights of the cyanelle polypeptides and the chloroplast polypeptides. The molecular weights for a given protein that are reported in the literature may vary significantly. The large subunit of RUBISCO from Cyanophora has been reported to have a molecular weight of 51,000 daltons (40) and 56,000 daltons (30) whereas the reported values for the small subunit are 15,000 (40) and 12,400

daltons (30). Moreover, the molecular weights of other Cyanophora proteins might vary from that of their plant or algal homologues. Such appears to be the case for the β subunit of the ATP synthase which has a molecular weight in spinach of 55,000 daltons (118,126), whereas the cyanelle β subunit is 52,000 daltons (91). Finally, the fact that a polypeptide is synthesized in the cytosol in plants or algae does not necessarily mean that such will be the case in Cyanophora. For example, some of the better characterized proteins that are synthesized in the cytoplasm in plants and eukaryotic green algae are the small subunit of RUBISCO and the polypeptides of the light harvesting chlorophyll a/b protein (25). While the site of synthesis of the small subunit has not been established in Cyanophora, the presence in the cyanelle DNA of a sequence for the small subunit (see chapter 3), is consistent with its being synthesized in the cyanelle. Cyanelles lack a chlorophyll a/b protein.

iii. The polypeptides synthesized in the cyanelle

A polypeptide, or polypeptides, with a molecular weight of ca. 52,000 daltons appears to be a major, i.e. highly labeled, product of cyanelle protein synthesis. For the following reasons, I believe that there are at least three polypeptides of ca. 52,000 daltons and that they are (or include) the large subunit of RUBISCO, and the α and β subunits of the ATP synthase. DNA sequences for the large

subunit of RUBISCO and the α and β subunits of the ATP synthase are present in the cyanelle DNA (17, see also chapter 3, and appendix A of this thesis). The molecular weight, 52,000 daltons, of this band is close to the molecular weight, 52,800 daltons, of the large subunit deduced from the DNA sequence (see chapter 3) of the large subunit of RUBISCO, and within the range of the reported molecular weights of 51,000 (40) and 56,000 (30) daltons for this protein. The molecular weights, 53,000 and 52,000 daltons, respectively, of the α and β subunits of the ATP synthase, as determined by Klein et al. (91) also closely match the size of this band. The large amount of radioactivity present in this band is consistent with the presence of more than a single polypeptide. It should be noted that the use of values of the molecular weights of the subunits of the ATP synthase complex determined by Klein et al. (91), may not be entirely valid because the strain of Cyanophora used by Klein et al. (91) may differ from the strain used in these studies (17,87,90).

A polypeptide present as a diffuse band with a molecular weight of 33,800 daltons and a peptide of 34,700 daltons do not correspond closely to bands in gels stained with Coomassie Blue. The most rapidly labeled product of protein synthesis by isolated spinach chloroplasts is a polypeptide of 36,000 daltons, that also fails to coincide with a stained band (27). Similar polypeptides have been

found among the products synthesized by isolated chloroplasts of maize (6,68), pea (10,164), Spirodela (138), and Euglena (178). This membrane protein, originally referred to as peak D (164), is now usually referred to as the 32,000 dalton protein or by the name of the gene, psbA, coding for it. It is characterized by its abundant synthesis in green tissue (164), a high rate of turnover (83), and synthesis as a slightly larger precursor protein [34,700 daltons, in maize (68) and 33,500 daltons in Spirodela (49)]. The psbA gene is present in the cyanelle DNA (17).

Two groups of polypeptides that were heavily labelled in the presence of cycloheximide had apparent molecular weights ranging from 21,600-23,600 daltons and from 16,500-19,500 daltons (see Figure 8). Several of these polypeptides (16,300, 16,500, 16,700, 17,600, 18,300, 19,200, and 19,500) have molecular weights similar to the molecular weights of the red algal and cyanobacterial pigments, C-phycocyanin and allo-phycocyanin (13,59,186). Trench and Ronzio (175) have determined the molecular weights of the two subunits of C-phycocyanin (14,500 and 13,200 daltons) and Allo-phycocyanin (12,600) of Cyanophora. As noted by Trench and Ronzio (175), the apparent molecular weights of the cyanelle biliproteins are considerably smaller (by 2,000-4,000 daltons) than the molecular weights of their homologues from cyanobacteria and red algae. No

polypeptides having molecular weights of 12,600, 13,200, or 14,500 were observed in these experiments. It is possible that some of the labeled polypeptides in the molecular weight range of 16,500-19,2000 dalton are the subunits of the biliproteins phycobilin and allophycocyanin but because of the uncertainty regarding their molecular weights, I have not identified specific polypeptides as biliproteins.

iv. Recommendations

It seems clear from these results that a means of selecting the product of interest, RUBISCO, from among the many proteins synthesized would have been advantageous. Immunoprecipitation of RUBISCO labeled in vivo in the presence of inhibitors followed by separation of the subunits by SDS-PAGE and quantitation of the radioactivity present in the subunits might have given more specific information on the sites of synthesis of the subunits. With the use of monoclonal antibodies highly specific antibodies can be made. Ideally, the antibodies should be made against the purified enzyme from Cyanophora. Although I could isolate RUBISCO from the cyanobacterium Anabaena 29413 by the method used by Codd and Stewart (40) to isolate RUBISCO from Cyanophora, I was unsuccessful, despite numerous attempts, in applying their method to cyanelles.

Another approach to identifying which proteins are encoded in the cyanelle is to study the cyanelle DNA

directly. The results of experiments in which the genes encoding the large and small subunits of RUBISCO and the β subunit of the ATP synthase were localized in the cyanelle DNA, the DNA encoding the large and small subunits sequenced, and a restriction endonuclease map of the cyanelle DNA constructed, are presented in the remainder of this thesis.

CHAPTER III

CHAPTER III

THE NUCLEOTIDE SEQUENCES OF THE GENES FOR THE LARGE AND SMALL SUBUNITS OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE

Introduction

Cyanophora paradoxa is a small biflagellated eukaryote of uncertain taxonomic affinities (57,133). The photosynthetic organelle, called a cyanelle, of Cyanophora has a peptidoglycan wall layer (1), phycobilin pigments (35,175), and ultrastructural resemblance to free-living cyanobacteria (70). The generally accepted viewpoint that the cyanelle is an endosymbiotic cyanobacterium was challenged in 1977 by the discovery that the kinetic complexity of the cyanelle DNA was approximately 177 kilobasepairs (kb) (80), a size 10- to 20- fold smaller than the genome of free-living cyanobacteria (78), but well within the range of values obtained for chloroplast genomes (15). The overall structure of a restriction endonuclease map of the cyanelle DNA and the localization of the 16S and 23S ribosomal RNA (rRNA) genes to a large inverted repeat on that map showed also that the organization of the cyanelle genome (16,17) is similar to that of the chloroplast (15).

These characteristics, when considered in light of the endosymbiont hypothesis of eukaryotic cell evolution (114), suggest that the cyanelle may represent an evolutionary stage which is intermediate between cyanobacteria, one of the presumed plastidal ancestors, and chloroplasts.

The development and maintenance of a photosynthetically competent chloroplast requires the interaction of the nuclear and chloroplast genomes of the plant. This intergenomic cooperation is exemplified by the biosynthesis of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO). This key enzyme in photosynthetic carbon assimilation commonly consists of eight catalytic large subunits and eight small subunits of unknown function (120). The carboxylase of Cyanophora has essentially the same subunit size and stoichiometry as the enzyme from plants, eukaryotic algae, and cyanobacteria (30,40). In plants and eukaryotic green algae, the large subunit of carboxylase is encoded, transcribed, and translated in the chloroplast (25). The small subunit is encoded in the nuclear DNA (88) and translated in the cytosol on free ribosomes as a precursor polypeptide (33,39,81). In vitro reconstitution experiments have demonstrated that movement of the precursor polypeptide into the chloroplast and processing of that polypeptide are post-translational events (39,81). In the cyanobacteria Anacystis 6301 (162), also called, Synechococcus (139), and

Anabaena 7120 (127), the gene (rbcS) for the small subunit of carboxylase is located 3' (mRNA sense) from the gene (rbcL) for the large subunit. The coding sequences of the rbcL and rbcS genes of Anacystis (162) and Anabaena (127) are separated by 93 bp and 545 bp, respectively.

Most genes encoding the large, 50- to 55-kilodaltons (kd), subunit of carboxylase consist of single open reading frames coding for 472-477 amino acids (45,47,116,125,137,161,163,190). An exception is the large subunit gene of the eukaryotic alga, Euglena (93), which although it is chloroplast encoded, contains nine intervening sequences. The rbcL gene is present in one copy per chloroplast DNA but in 15-30 copies per chloroplast in immature leaves of spinach (158). Because a leaf cell may contain several hundred chloroplasts (158), the copy number of the rbcL gene greatly exceeds that of the rbcS gene implying a reduction in transcription and/or translation of the rbcL gene or perhaps an amplification of the rbcS gene in order to maintain stoichiometric amounts of the two subunits.

The genes for the small subunit of RUBISCO in soybean (8) wheat (30), and pea (32,42) occur in the nucleus as small multigene families. The soybean rbcS gene that has been sequenced consists of three open reading frames separated by two intervening sequences (8). The rbcS gene of wheat contains a single intron (29). In Anacystis (162) and

Anabaena (127), the small subunit genes consist of single open reading frames of 333 bp and 327 bp, respectively. The coding sequence of the transit peptide common to the nuclear encoded small subunit genes of plants (7,8,29), is not present in the rbcS genes of Anacystis (162) and Anabaena (127).

Recently it has been reported that the genes for both the large and small subunits of carboxylase are located in the DNA of the cyanelle of C.paradoxa (17,77). However, conditions of low hybridization stringency were required to obtain hybridization between the cyanelle DNA and the probes, which were prepared from cDNA clones of the gene for the pea small subunit. No evidence was found for the presence of a small subunit gene in the nuclear DNA of Cyanophora (77). Based on the hybridization of a DNA fragment containing the 5' end of the rbcL coding sequence to the same 2.2 kb HindIII fragment, which hybridized with the cDNA clone of the pea small subunit, Heinhorst and Shively (77) suggested that the rbcS gene of the cyanelle may be located near and 5' from the rbcL gene. If true, and not simply the result of fortuitous hybridization due to the low stringency conditions employed, then Cyanophora is the first eukaryotic organism in which the genes for both of the subunits of carboxylase are located in the same cellular compartment.

In addition to reporting that the rbcL and rbcS genes

are located in the cyanelle DNA, Bohnert and co-workers (17) reported that the genes for the α, β, and ε, subunits and the dicyclohexylcarbodiimide-binding polypeptide of the ATP synthase complex, cytochrome b_6, and subunit 4 of the cytochrome b_6f complex are also located in the cyanelle DNA. The localization of the gene (atpB) for the β subunit of the ATP synthase in the cyanelle DNA is of particular interest because in all higher plants examined to date the atpB gene is located approximately 760-820 bp 5' (mRNA sense) from the rbcL gene (28,55,96,160,180), i,e., close to where Heinhorst and Shively (77) tentatively localized the rbcS gene.

In this chapter, I describe the organization of the 4-kb region (Appendix A and Chapter 4) of the cyanelle genome containing the genes for the large and small subunits and the β subunit of the cyanelle coupling factor. The arrangement of these genes in the cyanelle DNA of Cyanophora paradoxa strain UTEX 2344, the Pringsheim strain (17), is compared to the analogous regions in both chloroplasts and cyanobacteria. I also present the complete DNA sequences of the genes for the large and small subunits of RUBISCO. The DNA sequence of the region 5' from the large subunit, up to and including the first 210 nucleotides of the coding sequence of the atpB gene is included. The nucleotide sequences and the deduced amino acid sequences for the proteins are compared with the corresponding sequences from plants and cyanobacteria.

The small subunit sequence presented in this chapter is not a sequence of the region identified by Heinhorst and Shively (77) as encoding the the gene for the small subunit. Heinhorst and Shively (77) found that a 580 bp fragment from maize chloroplast DNA containing the active site region of the large subunit of RUBISCO hybridized to an 8.2 kb BglII fragment of cyanelle DNA. This same 8.2 kb BglII fragment and a 3.9 kb BglII fragment hybridized to a cloned DNA fragment containing the 3' two-thirds of the coding region of the rbcL gene from Chlamydomonas. Only the 8.2 kb BglII fragment hybridized with a 280 bp DNA fragment of the rbcS gene from pea. These results are consistent with the presence of both the small subunit gene and the 5'region of the large subunit on the 8.2 kb BglII fragment and with the presence of a BglII site in the 3'region of the coding sequence of the large subunit. My results from sequencing show a BglII site 350 bp from the termination codon of the large subunit confirming the results of Heinhorst and Shively (77). The small subunit which I have sequenced is located 108 bp 3' from the termination codon of the large subunit placing it in the 3.9 kb BglII fragment, a fragment which did not hybridize with the small subunit of pea (77). My results also show that the nearest gene 5' from the rbcL gene is the atpB gene. From these results it can be concluded that the putative small subunit, which Heinhorst and Shively (77) identified on the basis of its hybridization with the small subunit of pea, must be located

on the opposite side of the atpB gene from the rbcL gene.

Results and Discussion

i. Organization of the cyanelle rbcL, rbcS and atpB genes

A physical map of the region of the cyanelle genome containing the structural genes for the large and small subunits of RUBISCO and the β subunit of the cyanelle coupling factor, together with the strategy employed in sequencing those genes, is shown in Figure 9.

Based on the DNA sequence, the N-terminal methionine codons of the large subunit of RUBISCO and of the β subunit of the coupling factor are separated by 481 basepairs and are transcribed divergently. The relative locations and directions of transcription of these two genes are strikingly similar to those found in tobacco (55,160), petunia (28), maize (96), and spinach (189), in which the corresponding intergenic distances are 817 bp, 770 bp, 759 bp, and 785 bp, respectively. Because of the similarities found in the organization of these genes in several plant species and in Cyanophora it is of interest to know if these genes are arranged similarly in the cyanobacteria, the putative ancestors of both chloroplasts and cyanelles. Though little detailed information on the locations of the rbcL and atpB genes in the cyanobacteria is available, it appears that these genes are not located near

Figure 9. Physical map of the region of the cyanelle DNA containing the rbcL, rbcS, and atpB genes. The thick bars represent the locations of the rbcL, rbcS, and atpB genes, with the 5' and 3' ends indicated. The strategy used in the Maxam and Gilbert sequencing of the rbcL and rbcS genes and the 5' 210 nucleotides of the atpB gene is indicated in the lower portion of the figure.

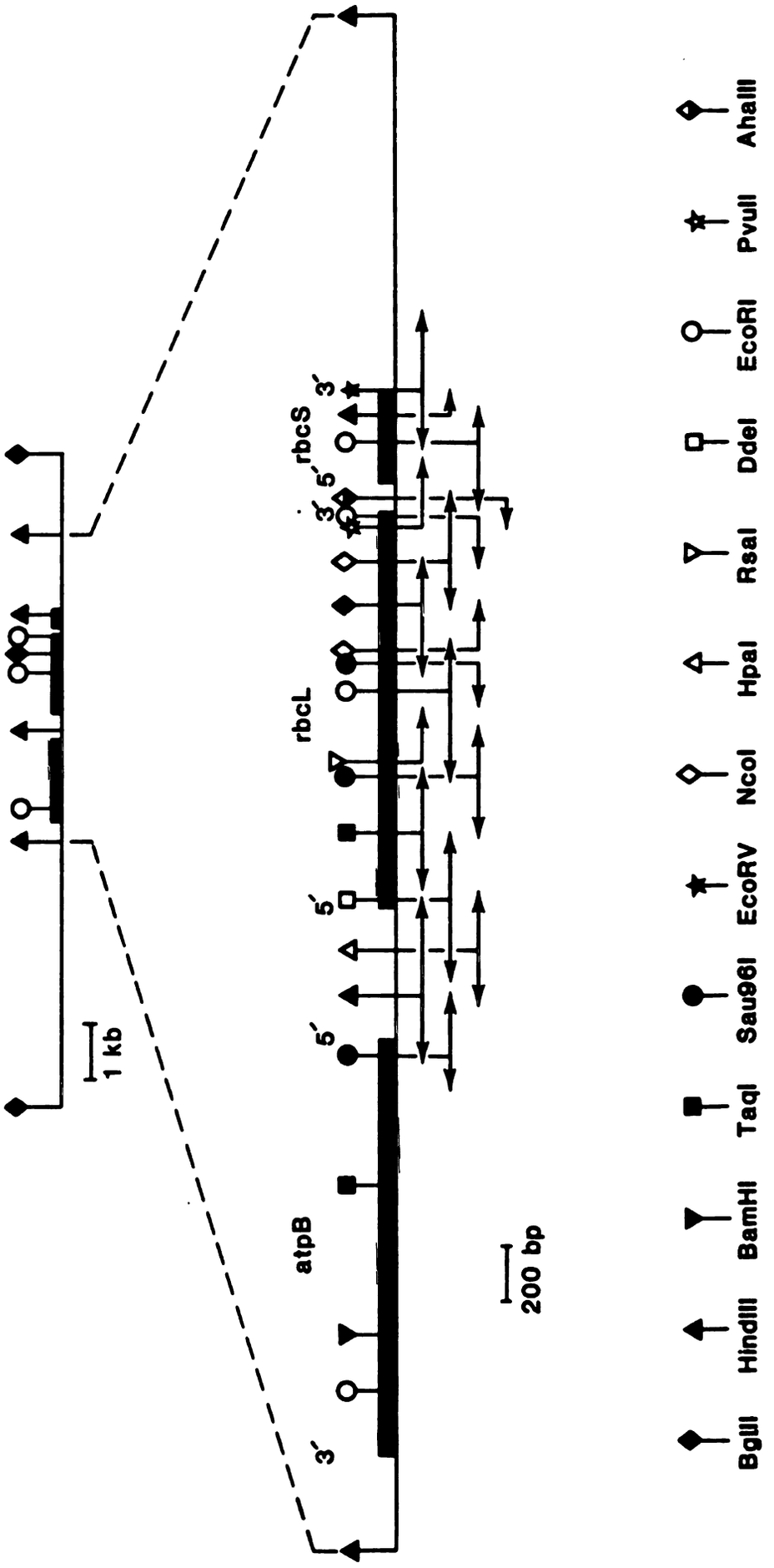

1 kb
atpB
rbcL
rbcS
5'
3'
200 bp
BglII
HindIII
BamHI
TaqI
Sau96I
EcoRV
NcoI
HpaI
RsaI
DdeI
EcoRI
PvuII
AhaIII

Figure 9

each other in Anabaena (45, C.C. Wasmann, unpublished data).

The organization of the genes for the large and small subunits of carboxylase is consistent with the hypothesis that the cyanelle is derived from a cyanobacterium. The coding region of the cyanelle small subunit is located on the same DNA strand 108 bp 3' from the termination codon of the large subunit. This arrangement is virtually identical to that found in Anacystis in which the intergenic distance is 93 bp (162). In Anabaena the corresponding distance is 545 bp and the genes are cotranscribed (127). The nature of the transcription of these genes in Cyanophora has not been determined.

ii. The coding regions of the cyanelle rbcL, rbcS, and atpB genes

The sequence of the noncoding DNA strand of an open reading frame of 1425 bp located in the cyanelle DNA is shown in Figure 10. I identify this sequence as that of the large subunit of RUBISCO because it hybridizes (Appendix A) with a maize chloroplast DNA fragments containing portions of the large subunit coding sequence, and because the amino acid sequence deduced from this open reading frame closely matches the amino acid sequences of other large subunits. The correct reading frame for the large subunit of RUBISCO was determined by comparison of the predicted translation

Figure 10. Nucleotide sequence of the rbcL gene of the cyanelle of Cyanophora. Numbering begins with the first A of the methionine codon. The first methionine codon of the atpB gene at position -481 and the first menthionine of the rbcS at position 1537 are shown. The inverted repeats at positions -291 to -219 and 1439-1486 are underlined.

```
                                Met
-484                            CATA TTTTTACCTC ATTGGGAAAA AGAAAAAATA TTTTTATAAT TTTAGTGTTA AAAACACTGA TATAAAAATT TTAATG

-404  TTCT AAAATTTTTG ATACTAACAA ATATTATACG CAAATTCATG AGTGTTTGAA TATGTAAAAA CAATTTTTAT AAAGCTTATA AATTCTAAAC TGTTAAG

-303  TTG TTTATCCTGT TATTTATATT TTAATAATAT AAATACTAAT AAATTTATTA AACATGTATT TAAAATATTT AATATAAATA AATACTTATA TTTTATAA

-202  TA AGTATTTTTA ATTTTTTAAA TAAAAAAAAA GAATATTTTA GATGTTAACT TAATGATAAC TTTATAAAAG AATTTTGTTT TAAAAACTCC AAAAAAGAG

-101  T TTTTCCGACT ATTACTAATT CATTAAATAT AATTTAAGCT ATTTGTTTAA ATTAATTTAA ATTAATGAAG TAATAGCATT TAAAAAGCAA GGAGAAATAC

                                        10                                                  20
ATG TCA TCA CAA GCT AGA ACT CAG ACA AGA GCA GGC TTC AAA GCA GGT GTA AAA GAT TAT CGT TTA ACT TAT TAT ACT CCT GAA    84
Met Ser Ser Gln Ala Arg Thr Gln Thr Arg Ala Gly Phe Lys Ala Gly Val Lys Asp Tyr Arg Leu Thr Tyr Tyr Thr Pro Glu

    30                                      40                                      50
TAT ACT CCA AAA GAA ACT GAC ATT CTA GCA GCT TTC AGA ATC ACT CCT CAA CCA GGA GTA CCT CCT GAA GAG TGT GCA GCA GCA   168
Tyr Thr Pro Lys Glu Thr Asp Ile Leu Ala Ala Phe Arg Met Thr Pro Gln Pro Gly Val Pro Pro Glu Glu Cys Ala Ala Ala

            60                                      70                                      80
GTA GCA GCA GAA TCT TCC ACT GGT ACT TGG ACA ACT GTA TGG ACT GAT GGT TTA ACC AGT CTT GAC CGT TAC AAA GGT AGA AGC   252
Val Ala Ala Glu Ser Ser Thr Gly Thr Trp Thr Thr Val Trp Thr Asp Gly Leu Thr Ser Leu Asp Arg Tyr Lys Gly Arg Ser

                    90                                      100                                     110
TAT GGT TTC GAA CCA GTT CAT GGT GAA GAA AAC CAA TAC ATT TGT TAC GTA GCA TAT CCT TTA GAT TTA TTT GAA GAA GGT TCT   336
Tyr Gly Phe Glu Pro Val His Gly Glu Glu Asn Gln Tyr Ile Cys Tyr Val Ala Tyr Pro Leu Asp Leu Phe Glu Glu Gly Ser

                        120                                     130                                     140
GTT ACT AAC ATG TTA ACT TCC ATC GTA GGT AAC GTA TTT GGT TTC AAA GCA TTA CGT GCA TTA CGT TTA GAA GAT TTA CGT ATT   420
Val Thr Asn Met Leu Thr Ser Ile Val Gly Asn Val Phe Gly Phe Lys Ala Leu Arg Ala Leu Arg Leu Glu Asp Leu Arg Ile

                                150                                     160
CCA GTT GGT TAT TCC AAA ACT TTC CAA GGT CCT CCA CAC GGT ATT ACT GTA GAG CGT GAC AAA TTA AAC AAA TAT GGT CGT GCT   504
Pro Val Gly Tyr Ser Lys Thr Phe Gln Gly Pro Pro His Gly Ile Thr Val Glu Arg Asp Lys Leu Asn Lys Tyr Gly Arg Ala

    170                             180                                     190
TTA TTA GGT TGT ACT ATT AAA CCA AAA TTA GGT TTA TCT GCT AAA AAC TAC GGT CGT GCA GTT TAT GAA TGT TTA CGT GGT GGT   588
Leu Leu Gly Cys Thr Ile Lys Pro Lys Leu Gly Leu Ser Ala Lys Asn Tyr Gly Arg Ala Val Tyr Glu Cys Leu Arg Gly Gly

            200                                     210                                     220
TTA GAC TTC ACT AAA GAT GAT GAA AAC GTA AAC TCT CAA CCG TTC ATG CGT TGG CGT GAT CGT TTC TTA TAT GTA ATG GAT GCA   672
Leu Asp Phe Thr Lys Asp Asp Glu Asn Val Asn Ser Gln Pro Phe Met Arg Trp Arg Asp Arg Phe Leu Tyr Val Met Asp Ala

                    230                                     240                                     250
ATT AAG AAA TCT CAA GCT GAA ACT GGT GAA ATT AAA GGT CAC TAC TTA AAT GCA ACA CCA CCT ACT TCT GAA GAA ATG ATC AAA   756
Ile Lys Lys Ser Gln Ala Glu Thr Gly Glu Ile Lys Gly His Tyr Leu Asn Ala Thr Pro Pro Thr Ser Glu Glu Met Ile Lys

                        260                                     270                                     280
CGT GCT GAA TTC GCA GCT GAA TTA GAT GCT CCG ATC ATC ATG CAT GAC TAC ATT ACT GCT GGT TTC ACA TCT AAC ACT ACA TTA   840
Arg Ala Glu Phe Ala Ala Glu Leu Asp Ala Pro Ile Ile Met His Asp Tyr Ile Thr Ala Gly Phe Thr Ser Asn Thr Thr Leu

                                290                                     300
GCT AGA TGG TGT CGT GAT AAT GGT CCT CTT TTA CAC ATT CAC CGA GCA ATG CAC GCG GTA ATT GAC CGT CAA AAG AAC CAT GGT   924
Ala Arg Trp Cys Arg Asp Asn Gly Pro Leu Leu His Ile His Arg Ala Met His Ala Val Ile Asp Arg Gln Lys Asn His Gly

    310                             320                                     330
ATT CAC TTC CGT GTA TTA GCT AAA ACA TTA AGA ATG TCT GGT GGT GAC CAC TTA CAT TCT GGT ACT GTT GTA GGT AAA TTA GAA  1008
Ile His Phe Arg Val Leu Ala Lys Thr Leu Arg Met Ser Gly Gly Asp His Leu His Ser Gly Thr Val Val Gly Lys Leu Glu

            340                                     350                                     360
GGT GAC CGT GCA GGT ACT TTA GGT TTC GTA GAC TTA ATG CGT GAC GAT CAT ATC GAA CAA GAT AGA TCT CGT GGT ATT TTC TTC  1092
Gly Asp Arg Ala Gly Thr Leu Gly Phe Val Asp Leu Met Arg Asp Asp His Ile Glu Gln Asp Arg Ser Arg Gly Ile Phe Phe

                    370                                     380                                     390
ACT CAA GAT TGG GCT TCC ATG CCT GGT GTT ATG CCA GTT GCT TCT GGT GGT ATT CAC ATT TGG CAC ATG CCT GCG TTA GTA GAC  1176
Thr Gln Asp Trp Ala Ser Met Pro Gly Val Met Pro Val Ala Ser Gly Gly Ile His Ile Trp His Met Pro Ala Leu Val Asp

                        400                                     410                                     420
ATT TTC GGT GAC GAT TCT TGT TTA CAA TTC GGT GGT GGT ACA TTA GGT CAC CCA TGG GGT AAC GCT CCA GGT GCT GTA GCT AAC  1260
Ile Phe Gly Asp Asp Ser Cys Leu Gln Phe Gly Gly Gly Thr Leu Gly His Pro Trp Gly Asn Ala Pro Gly Ala Val Ala Asn

                                430                                     440
CGT GTT GCT CTT GAA GCA TGT GTT CAA GCA CGT AAC GAA GGT CGT AAC TTA GCA CGT GAA GGT AAT GAA ATT ATC CGT GAA GCT  1344
Arg Val Ala Leu Glu Ala Cys Val Gln Ala Arg Asn Glu Gly Arg Asn Leu Ala Arg Glu Gly Asn Glu Ile Ile Arg Glu Ala

    450                             460                                     470
GCA CGT TTC AGT CCT GAA TTA GCA GCT GCA TGT GAA GTT TGG AAA GAG ATT AAG TTC GAA TTC GAA ACT ATT GAT ACT ATC TAA  1428
Ala Arg Phe Ser Pro Glu Leu Ala Ala Ala Cys Glu Val Trp Lys Glu Ile Lys Phe Glu Phe Glu Thr Ile Asp Thr Ile ochre

TTTCATTTAA TTTATTTAAT TATTTAGAGT TTAAAAAAAC TCTAAATAAT TAATCAAAAT GATATTACTT CAATCTATTT TTATCCTTAA AATTCGGAAT T  1529

ATAAATTAT G                                                                                                      1539
      Met
```

Figure 10

products with the known sequence of the large subunit of RUBISCO from maize (116). Based on the nucleotide sequence the large subunit is comprised of 475 amino acids and has a calculated molecular weight of 52.8 kd. This value agrees well with earlier determinations, 51 kd (40) and 56 kd (30), based on the electrophoretic mobility of the purified protein.

A comparison of the derived amino acid sequences of the large subunits of Cyanophora, spinach (190), maize (116), Anabaena (45), and Anacystis (Synechococcus) (137,163) is presented in Figure 11. The homology of the amino acid sequence of the Cyanophora largesubunit with the large subunit sequences from plants and cyanobacteria is greater than 80 %.

Inspection of Figure 11 shows that the amino acid changes in the large subunit are not distributed randomly throughout the coding region. The first 13 amino acids of the Cyanophora protein show differences in both the kind and the number of amino acids present when compared with the corresponding regions of the other species. The high degree of homology observed for the polypeptide as a whole begins with lysine 14 of the Cyanophora protein. In barley (135), direct determination of the amino acid sequence of the large subunit polypeptide indicated that the amino terminus of the mature protein is an alanine corresponding to the alanine at position 15 of the Cyanophora sequence. Later work

Figure 11. Comparison of amino acid sequences of the large subunits of RUBISCO from the cyanelle of Cyanophora (Cy), Anabaena 7120 (A7120), Anacystis (An), spinach chloroplasts (Sp), and maize chloroplasts (Zm). Residue numbering refers to the cyanelle sequence. Sequences other than that of the cyanelle were obtained from the following references: Anabaena (45), Anacystis (137,162), spinach (190), and maize (116). Boxes surround positions at which at least three of the proteins have the same amino acid. The lysine residue labeled CO_2 is the site of carbamate formation during the activation of the enzyme by CO_2.

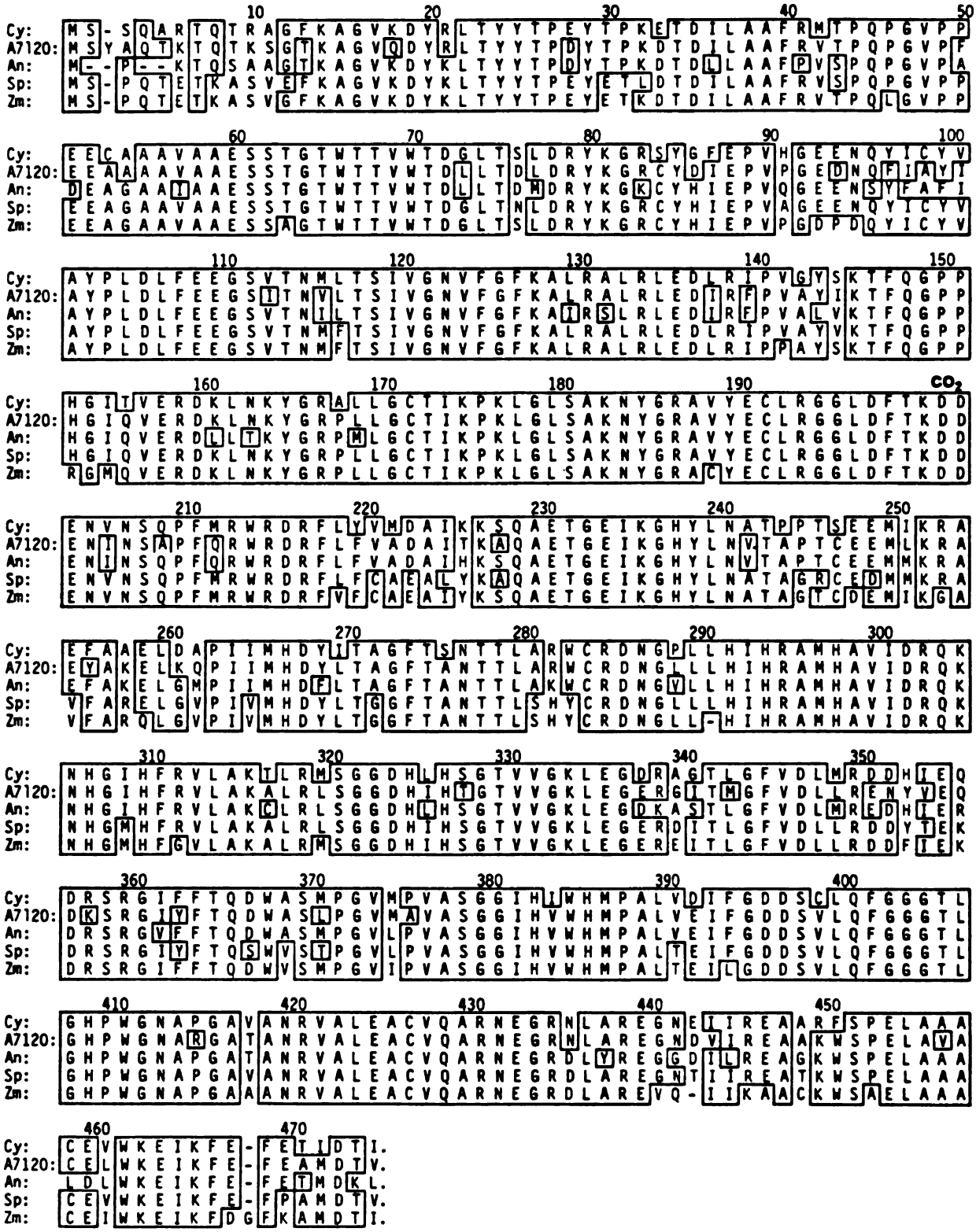


Figure 11

suggested that this result may have been an artifact resulting from the relatively slow techniques used for large scale preparation of the enzyme used in that study (116). Langridge (100) found that translation of spinach chloroplast RNA in an E.coli cell-free system resulted in a polypeptide 1-2 kd larger than that purified from chloroplasts. Subsequent treatment of the in vitro synthesized product with a soluble chloroplast extract converted it to a polypeptide indistinguishable from the in vivo synthesized large subunit (100). It is possible, as proposed by Zurawski et al. (190), that post-translational processing of the large subunit polypeptide occurs in some plant species. However, there is no direct evidence to suggest that processing occurs in either cyanobacteria or Cyanophora. In the absence of the relevant protein sequencing data, statements that the sequence heterogeneity observed in the amino terminal portion of the polypeptide is consistent with the removal of that portion of the protein during processing (163) seem premature.

The large subunit catalyses both the carboxylation and oxygenation of ribulose 1,5-bisphosphate (120). Hartman and co-workers (73,148) used active site-directed affinity labeling to identify two lysyl residues, positions 175 and 334, located within the domain of the active site. The epsilon amino group of lysine 201 reacts with CO_2, forming a carbamate, during activation of the enzyme by Mg^{++} and CO_2

(105). Lorimer (104) has suggested that the three acidic residues, Asp-Asp-Glu, immediately following lysine 201 may be involved in binding Mg^{++} and, that the bound Mg^{++} coordinates to and thus stabilizes the carbamate. Not unexpectedly, the parts of the protein containing these residues are well conserved. Indeed, amino acid residues 165 to 220 comprise one of the most conserved regions of the protein having substitutions at only 7 positions out of 56. The region surrounding lysine 334 is also well conserved. The residues surrounding cysteine 459 are less conserved and in Anacystis this cysteine is replaced by leucine, suggesting that it is not directly involved in catalysis.

The sequence of the noncoding DNA strand of an open reading frame of 321 bp located 3' from the large subunit coding sequence of Cyanophora is shown in the upper part of Figure 12. I identify this sequence in the cyanelle DNA as encoding the small subunit of RUBISCO because it closely matches the known sequence of the small subunits from Anacystis (162) and Anabaena (127). A polypeptide of 107 amino acids with a calculated molecular weight of 12.4 kd may be deduced from the 321 bp which comprise the coding sequence of the cyanelle small subunit.

The derived amino acid sequence of the small subunit of Cyanophora is compared with the sequences of Anabaena (127), Anacystis (162), pea (7), soybean (8), spinach (115), wheat (29), and tobacco (124) in the lower part of Figure

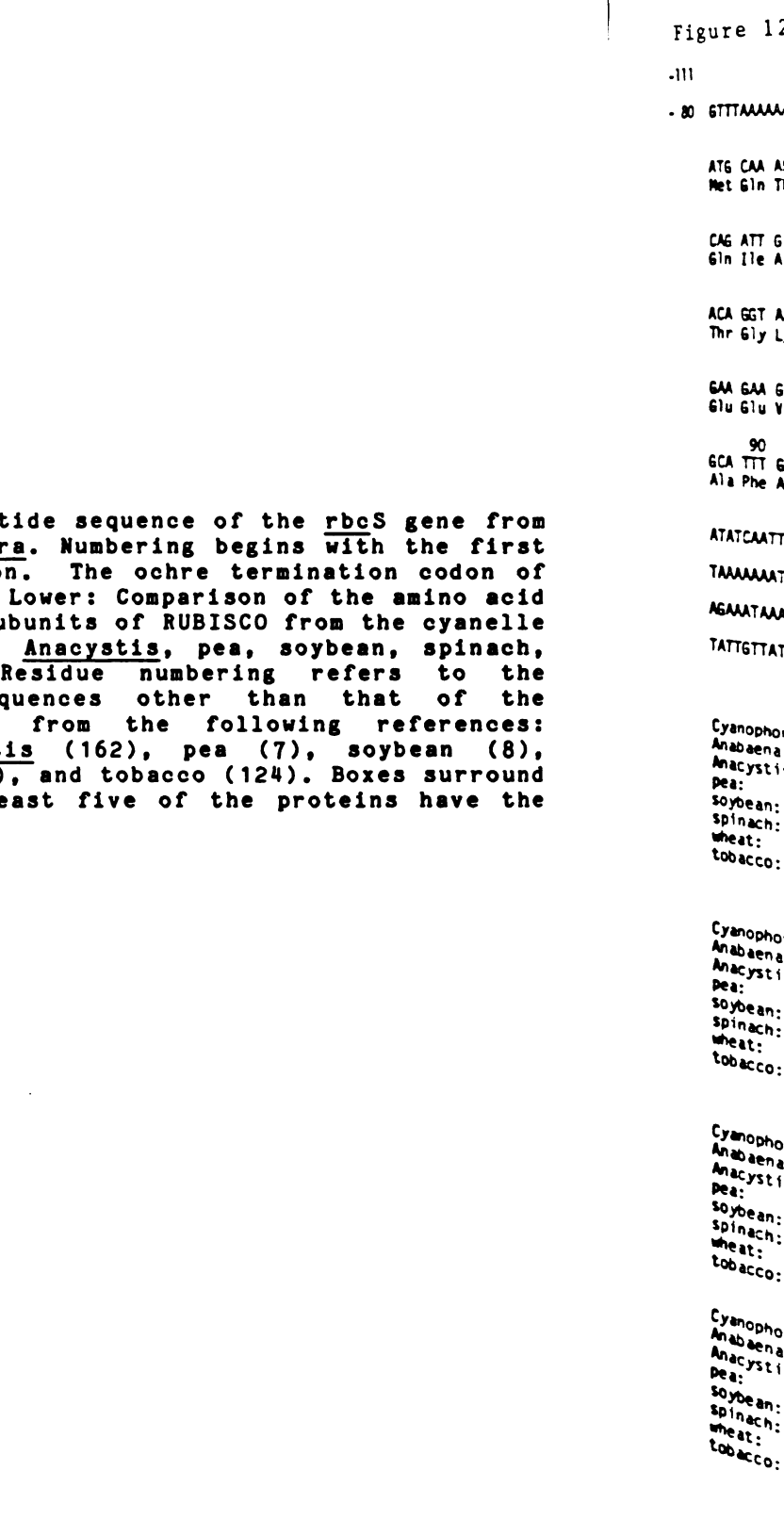
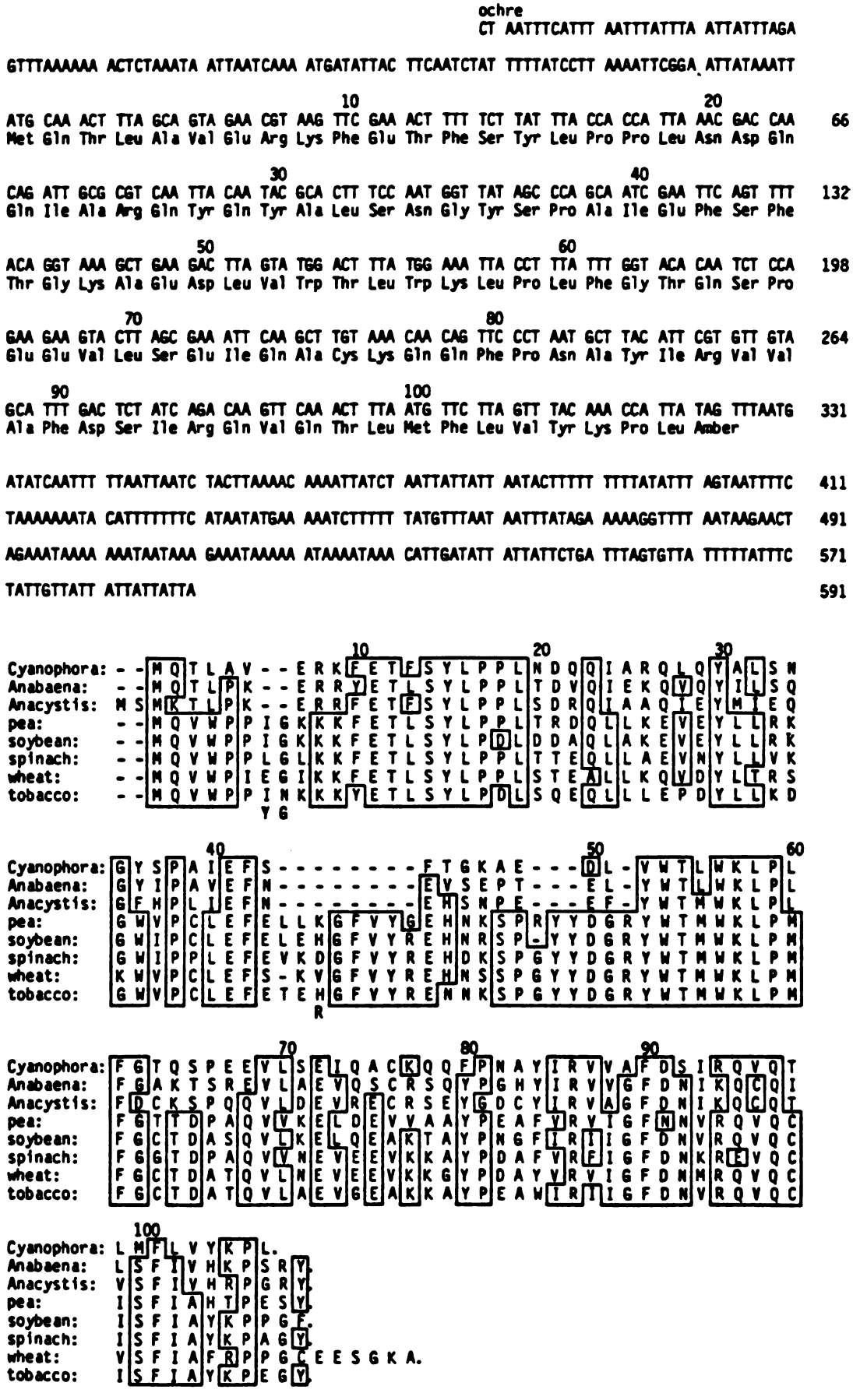


Figure 12. Upper: Nucleotide sequence of the rbcS gene from the cyanelle of Cyanophora. Numbering begins with the first A of the methionine codon. The ochre termination codon of the rbcL gene is shown. Lower: Comparison of the amino acid sequences of the small subunits of RUBISCO from the cyanelle of Cyanophora, Anabaena, Anacystis, pea, soybean, spinach, wheat, and tobacco. Residue numbering refers to the cyanelle sequence. Sequences other than that of the cyanelle were obtained from the following references: Anabaena (127), Anacystis (162), pea (7), soybean (8), spinach (115), wheat (29), and tobacco (124). Boxes surround positions at which at least five of the proteins have the same amino acid.

Figure 12


ochre
-111 CT AATTTCATTT AATTTATTTA ATTATTTAGA
- 80 GTTTAAAAAA ACTCTAAATA ATTAATCAAA ATGATATTAC TTCAATCTAT TTTTATCCTT AAAATTCGGA ATTATAAATT
ATG CAA ACT TTA GCA GTA GAA CGT AAG TTC GAA ACT TTT TCT TAT TTA CCA CCA TTA AAC GAC CAA 66
Met Gln Thr Leu Ala Val Glu Arg Lys Phe Glu Thr Phe Ser Tyr Leu Pro Pro Leu Asn Asp Gln
CAG ATT GCG CGT CAA TTA CAA TAC GCA CTT TCC AAT GGT TAT AGC CCA GCA ATC GAA TTC AGT TTT 132
Gln Ile Ala Arg Gln Tyr Gln Tyr Ala Leu Ser Asn Gly Tyr Ser Pro Ala Ile Glu Phe Ser Phe
ACA GGT AAA GCT GAA GAC TTA GTA TGG ACT TTA TGG AAA TTA CCT TTA TTT GGT ACA CAA TCT CCA 198
Thr Gly Lys Ala Glu Asp Leu Val Trp Thr Leu Trp Lys Leu Pro Leu Phe Gly Thr Gln Ser Pro
GAA GAA GTA CTT AGC GAA ATT CAA GCT TGT AAA CAA CAG TTC CCT AAT GCT TAC ATT CGT GTT GTA 264
Glu Glu Val Leu Ser Glu Ile Gln Ala Cys Lys Gln Gln Phe Pro Asn Ala Tyr Ile Arg Val Val
GCA TTT GAC TCT ATC AGA CAA GTT CAA ACT TTA ATG TTC TTA GTT TAC AAA CCA TTA TAG TTTAATG 331
Ala Phe Asp Ser Ile Arg Gln Val Gln Thr Leu Met Phe Leu Val Tyr Lys Pro Leu Amber
ATATCAATTT TTAATTAATC TACTTAAAAC AAAATTATCT AATTATTATT AATACTTTTT TTTTATATTT AGTAATTTTC 411
TAAAAAAATA CATTTTTTTC ATAATATGAA AAATCTTTTT TATGTTTAAT AATTTATAGA AAAAGGTTTT AATAAGAACT 491
AGAAATAAAA AAATAATAAA GAAATAAAAA ATAAAATAAA CATTGATATT ATTATTCTGA TTTAGTGTTA TTTTTATTTC 571
TATTGTTATT ATTATTATTA 591
Cyanophora:
Anabaena:
Anacystis:
pea:
soybean:
spinach:
wheat:
tobacco:

12. Compa
small sub
57% and 5
the Cyano
from 41%
conservat
for (117
Anacystis
76-77%, a
subunit (
53-62 of
the prote
amino ter
52 of the
of the pr
amino aci
the cyano

At t
Cyanophor
50% homol
unexpecte
cyanobact
experimen
sequence,
stringenc
homology

12. Compared with the amino acid sequence of the Cyanophora small subunit, the sequences of Anabaena and Anacystis are 57% and 50% homologous, respectively. The homology between the Cyanophora sequence and the sequences from plants ranges from 41% for pea and wheat, to 44% for soybean. If conservative and compensating amino acid changes are allowed for (117), the homologies are Anabaena/cyanelle 84%, and Anacystis/cyanelle 81%, (pea, soybean, and wheat)/cyanelle 76-77%, and spinach/cyanelle 79%. Two regions of the small subunit (see Figure 12) corresponding to residues 9-19 and 53-62 of the Cyanophora sequence are well conserved. All of the proteins except that from Anacystis have Met-Gln at the amino terminus. The region bounded by serine 43 and valine 52 of the Cyanophora sequence is variable. In this portion of the protein, the plant sequences have an insertion of 12 amino acids not found in the sequences from Cyanophora and the cyanobacteria.

At the nucleotide level the small subunit sequences of Cyanophora, Anabaena, and Anacystis are all, approximately 50% homologous, with the sequence of pea. This was somewhat unexpected because the pea sequence recognizes the cyanobacterial sequences in Southern hybridization experiments but fails to hybridize with the Cyanophora sequence, even under conditions of low hybridization stringency (17,77). An examination of the nucleotide homology between pea and Cyanophora suggests that the region

most likely to hybridize with the pea sequence is located between amino acids 9 and 19 of the Cyanophora sequence. Although the overall homology of this segment is 84% (27/32), the longest stretch of perfectly matched bases is 8. If optimal strands are chosen and G-T pairs accepted as matches, then the length of the closely matched region may be increased to 15 bases. This degree of homology together with the relatively low guanine plus cytosine (G+C) content of this region, 27% (32% for the entire coding sequence), is unlikely to allow hybridization under the conditions employed (128).

The sequence of the initial 210 nucleotides of the noncoding DNA strand of an open reading frame located in the cyanelle DNA 485 bp 5' from the coding sequence of the rbcL gene is presented in Figure 13. I identify this sequence as the coding sequence of the β subunit of the cyanelle ATP synthase because it hybridizes (Appendix A) with a maize chloroplast DNA fragment that contains a portion of the β subunit of the ATP synthase and because the amino acid sequence deduced from this open reading frame is homologous with the amino acid sequences of other β subunits. The sequence shown includes the region lying between the N-terminal methionine codons of the atpB and rbcL genes. The deduced amino acid sequences of cyanelle, E.coli (144), spinach chloroplast (189), and maize chloroplast (96) β subunits are aligned for comparison in the lower portion

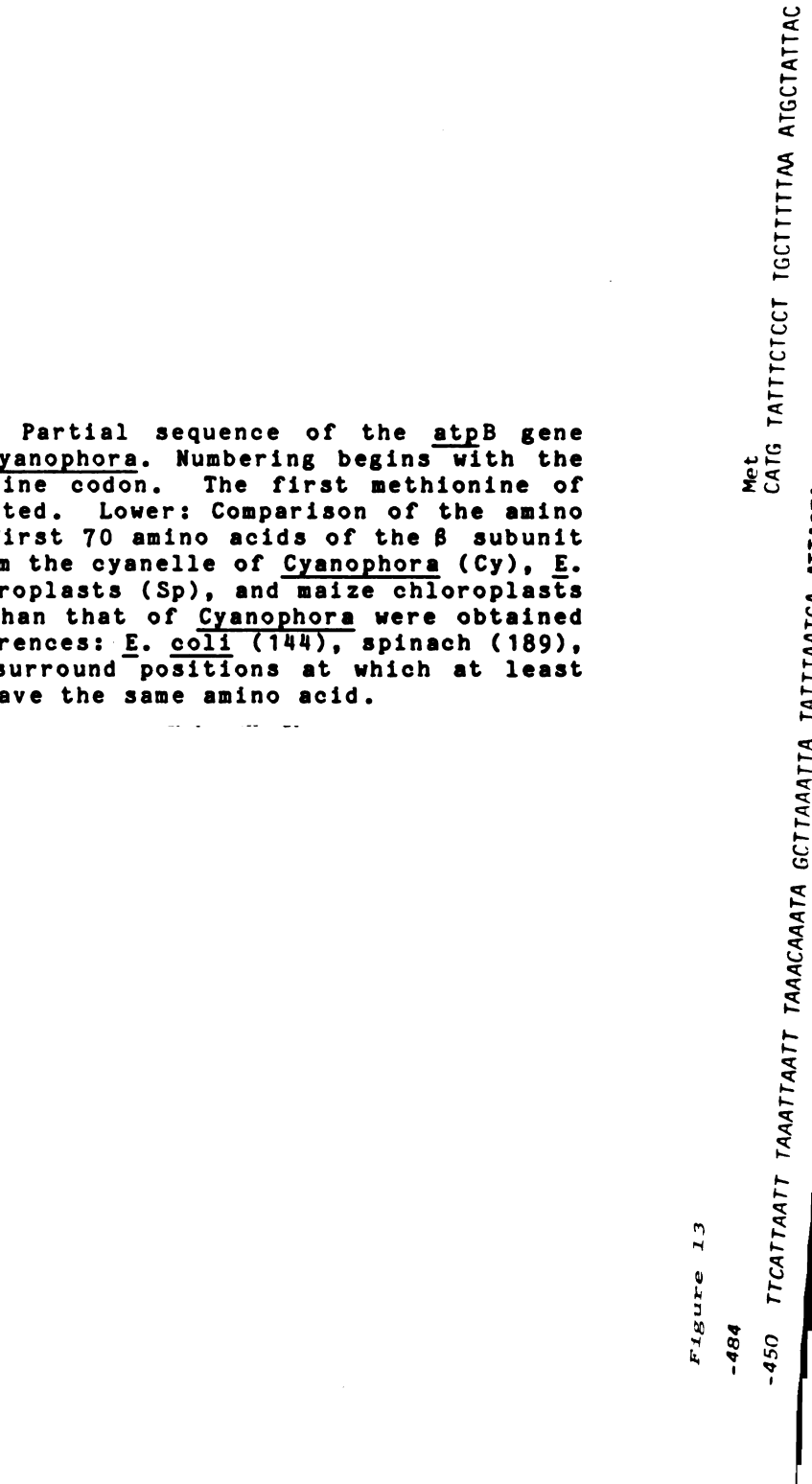


Figure 13

-484

Figure 13. Upper: Partial sequence of the atpB gene from the cyanelle of Cyanophora. Numbering begins with the first A of the methionine codon. The first methionine of the rbcL gene is indicated. Lower: Comparison of the amino acid sequences of the first 70 amino acids of the β subunit of the ATP synthase from the cyanelle of Cyanophora (Cy), E. coli (Ec), spinach chloroplasts (Sp), and maize chloroplasts (Zm). Sequences other than that of Cyanophora were obtained from the following references: E. coli (144), spinach (189), and maize (96). Boxes surround positions at which at least three of the proteins have the same amino acid.

Figure 13

```
                                                                  Met
-484                                                              CATG TATTTCTCCT TGCTTTTTAA ATGCTATTAC

-450  TTCATTAATT TAAATTAATT TAAACAAATA GCTTAAATTA TATTTAATGA ATTAGTAATA GTCGGAAAAA CTCTTTTTTG GAGTTTTTAA

-360  AACAAAATTC TTTTATAAAG TTATCATTAA GTTAACATCT AAAATATTCT TTTTTTTTAT TTAAAAAATT AAAAATACTT ATTATAAAAT

-270  ATAAGTATTT ATTTATATTA AATATTTTAA ATACATGTTT AATAAATTTA TTAGTATTTA TATTATTAAA ATATAAATAA CAGGATAAAC

-180  AACTTAACAG TTTAGAATTT ATAAGCTTTA TAAAAATTGT TTTTACATAT TCAAACACTC ATGAATTTGC GTATAATATT TGTTAGTATC

- 90  AAAAATTTTA GAACATTAAA ATTTTTATAT CAGTGTTTTT AACACTAAAA TTATAAAAAT ATTTTTTCTT TTTCCCAATG AGGTAAAAAT

                                        10                  20
ATG GCT ACT ACA ACA TCC AAA ACC AAT ACT GGC TAT GTT ACT CAA GTA ATT GGT CCA GTA TTA GAC GTT TCA TTT    75
Met Ala Thr Thr Thr Ser Lys Thr Asn Thr Gly Tyr Val Thr Gln Val Ile Gly Pro Val Leu Asp Val Ser Phe

                  30                                  40                                  50
CCT AAT GGT CAA TTA CCA AAA ATC TAT AAT GCG ATT ACC GTA AAA GGT AAA AAC GAA GCA GGT CAA GAT ATT ACC   150
Pro Asn Gly Gln Leu Pro Lys Ile Tyr Asn Ala Ile Thr Val Lys Gly Lys Asn Glu Ala Gly Gln Asp Ile Thr

                                        60                  70
GTA ACC TCT GAA GTA CAA CAG TTA CCT GGC GAT AAT CAA GTT CGT GCG GTA TCT ATG AGT . . .                210
Val Thr Ser Glu Val Gln Gln Leu Pro Gly Asp Asn Gln Val Arg Ala Val Ser Met Ser . . .
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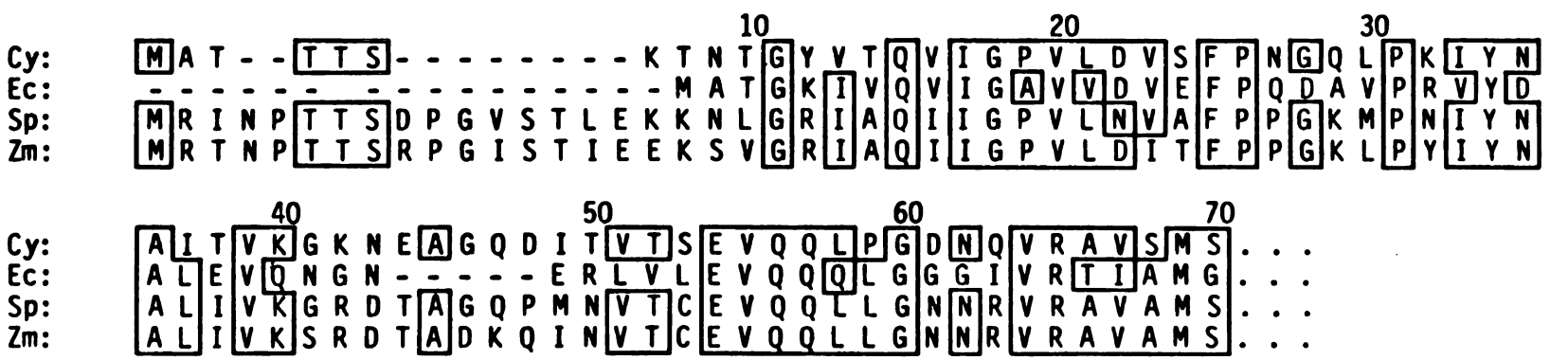

of Figure 13.

The amino acid sequences of the β subunits are ca. 50% (cyanelle/E.coli), and ca. 60% (chloroplast/cyanelle) homologous. If compensating and conservative amino acid changes are allowed (117), the homology is 80% or greater for all pairs. As with the small subunit sequences, the chloroplast β subunits have additional amino acids at the amino terminus relative to the sequences from E.coli and Cyanophora.

iii. Codon usage in the cyanelle of Cyanophora

The combined codon usage of the large and small subunits of RUBISCO and the partial sequence of the β subunit of the cyanelle coupling factor is presented in Table 5. Codon usage in Cyanophora displays many asymmetries. Although all 20 amino acids are found in these sequences, 14 codons are not found. All of the absent codons except ATA, isoleucine, have either a cytosine or a guanine residue in their third position. In the coding regions of the rbcL, rbcS, and atpB (partial sequence) genes there are 275 thymine, 231 adenine, 100 cytosine, and 46 guanine residues in the third position of the codon. Of the 46 codons ending in guanine residues, 27 are forced encoding either methionine, ATG, or tryptophan, TGG. Similar though less pronounced biases for adenine and thymine residues in

Table 5. Codon utilization in the cyanelle rbcL(L), rbs(S) and atpB[1] (B) genes from Cyanophora paradoxa.

Gene	L	S	B		L	S	B		L	S	B		L	S	B
TTT-Phe	2	4	1	TCT-Ser	12	3	2	TAT-Tyr	10	2	2	TGT-Cys	8	1	0
TTC-Phe	19	4	0	TCC-Ser	4	1	1	TAC-Tyr	6	3	0	TGC-Cys	0	0	0
TTA-Leu	32	11	3	TCA-Ser	2	0	1	TAA- *	1	0	0	TGA- *	0	0	0
TTG-Leu	0	0	0	TCG-Ser	0	0	0	TAG- *	0	1	0	TGG-Trp	8	2	0
CTT-Leu	3	2	0	CCT-Pro	11	2	2	CAT-His	5	0	0	CGT-Arg	25	3	1
CTC-Leu	0	0	0	CAC-Pro	0	0	0	CAC-His	10	0	0	CGC-Arg	0	0	0
CTA-Leu	1	0	0	CCA-Pro	10	5	2	CAA-Gln	11	9	5	CGA-Arg	1	0	0
CTG-Leu	0	0	0	CCG-Pro	2	0	0	CAG-Gln	1	2	1	CGG-Arg	0	0	0
ATT-Ile	18	3	3	ACT-Thr	25	4	3	AAT-Asn	3	2	4	AGT-Ser	2	1	1
ATC-Ile	7	2	1	ACC-Thr	1	0	4	AAC-Asn	13	1	1	AGC-Ser	1	2	0
ATA-Ile	0	0	0	ACA-Thr	7	2	2	AAA-Lys	18	4	3	AGA-Arg	7	1	1
ATG-Met	13	2	2	ACG-Thr	0	0	0	AAG-Lys	3	1	0	AGG-Arg	0	0	1
GTT-Val	10	3	2	GCT-Ala	19	3	1	GAT-Asp	15	0	2	GGT-Gly	43	3	4
GTC-Val	0	0	0	GAC-Ala	0	0	0	GAC-Asp	12	3	1	GGC-Gly	1	0	2
GTA-Val	16	4	6	GCA-Ala	23	4	1	GAA-Glu	29	7	2	GGA-Gly	1	0	0
GTG-Val	0	0	0	GCG-Ala	2	1	2	GAG-Glu	3	0	0	GGG-Gly	0	0	0

1) Codon usage in the atpB gene is for the initial 70 codons.

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the third position of the codon have been noted for both chloroplast (116,190) and cyanobacterial (45) genes. Further additions to the list of codons may be made as additional cyanelle gene sequences are obtained.

The reduced number of codons found in these gene sequences does not reduce the number of tRNAs required for their translation. Allowing the G-U base pairing invoked in the wobble hypothesis (43), I calculate that 31 tRNAs would be required to translate the mRNAs of these proteins. By isolating tRNAs from two-dimensional polyacrylamide gels and identifying them individually by amino acylation, a minimum of 19 different tRNA genes specific for 16 amino acids have been located in the cyanelle DNA (17). The discrepancy between the number of tRNA species identified and the codons present may reflect the technical difficulties with the isolation and identification of the individual tRNA species resulting in an underestimate of the number present (116). Among the tRNA species identified (17) were two isoaccepting tRNAs for valine and arginine and three isoacceptors for leucine. It is, therefore, unlikely that the cyanelle utilizes either the "two out of three" codon reading pattern (5,21) or the modification of it proposed by Heckman (76) for mitochondria of Neurospora. Both of these alternative codon recognition patterns require a smaller tRNA complement for translation (23-24 tRNAs) than predicted by wobble base pairing (43).

iv. Compar

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iv. Comparison of the 5' and 3' flanking regions

Examination of the 5' flanking sequences of the rbcL, rbcS, and atpB genes (see Figures 2, 4,and 5) shows three sequences, 5'-AGGAG at residues -7 to -11 from the rbcL gene coding sequence, 5'-GAGGT at residues -6 to -10 from the atpB coding sequence, and 5'-GGA at residues -11 to -13 from the small subunit coding sequence, that are complementary to the 3'terminal sequence, 5'-UCACCUCCUUU, of the 16s rRNA of the maize chloroplast (156) and Anacystis (173). Similar sequences are also found in E.coli (159) and have been shown to be a constituent of ribosome binding sites (for a review, see 61).

A search of the 5' flanking regions (mRNA sense strand) of the large and β subunits revealed a number of sequences with varying degrees of homology with known promoters (15,74). A comparison of the 5' flanking regions to each other showed that two sequences, one located -219 bp to -264 bp from the large subunit and the other located at -190 bp to -231 bp from the β subunit, are homologous. The two sequences overlap forming a large imperfect inverted repeat extending from -291 bp to -219 bp relative to the large subunit. The center of the inverted repeat is a palindrome of 14 nucleotides, TAATAAATTTATTA. Although these two sequences are reminiscent of some promoter sequences (15,74) their function, if any, in the cyanelle is unknown. Several

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other shorter palindromes, interrupted inverted repeats and direct repeats are also located in this spacer region. Some of these repeats and palindromes may result from the high AT content, 85%, of this region.

Examination of the spacer region separating the coding sequences of the large and small subunit genes did not reveal any homology with 5' flanking sequences of the large or β subunits nor any homology with known promoter sequences (15,74). Starting 11 residues past the termination codon of the large subunit and extending to residue 56 is an interrupted inverted repeat. Inverted repeats can form stem and loop structures in DNA and RNA and have been shown to be involved in rho-independent termination of transcription in bacterial systems (85,134,140). This inverted repeat is atypical of known rho-independent terminators both in having a low GC content and in lacking the run of consecutive thymidylate residues which follow terminators and are involved in the enzyme release (85,134,140). Despite the high AT content of this loop, it is quite stable having a calculated free energy (172) of -19.6 kcal. The presence of this loop may not be sufficient to prevent the cotranscription of the two subunit genes. In Anabaena (45,127) the rbcL and rbcS genes are co-transcribed despite the presence of a terminator-like structure in the intergenic spacer. It has not been demonstrated, but has been proposed (162), that the rbcL and rbcS genes are

co-transcribed in Anacystis; an inverted repeat sequence is located in the region separating the rbcL and rbcS genes of Anacystis (137,163).

Examination of the 3' flanking region of the small subunit of carboxylase shows two overlapping inverted repeats located 102 bp to 121 bp and 118 bp to 158 bp past the termination codon. Estimation of the free energy of the secondary structures predicted by these sequences (172) suggests that they would not be stable. No other sequences capable of forming the stable stem and loop structures characteristic of rho-independent terminators in E.coli (85,134,140) have been found. That a terminator-like sequence or sequences may be located more than 275 bp 3' from the rbcS coding sequence is not excluded.

It is also possible that in some instances termination in the cyanelle is factor dependent. A comparison of three rho-dependent termination sites (85,134) revealed that they all occurred in AT rich regions and had dyad symmetry which was weaker than that usually found at rho-independent terminators. The intergenic regions in the cyanelle are AT rich (85-91%) and contain regions of dyad symmetry. The low GC content of the inverted repeats located in the 3' flanking regions of the large and small subunit genes, and the unstable structures predicted for those 3' from rbcS, are typical of rho-dependent terminators (85,134).

v. The cyanelle and the endosymbiont hypothesis

The strong similarities (82,184) between the photosynthetic mechanisms in cyanobacteria and photosynthetic eukaryotes has led to the generally accepted view that chloroplasts evolved from oxygen producing photosynthetic prokaryotes resembling cyanobacteria and Prochloron. There are two alternative hypotheses concerning this evolution. According to one, the "autogenous origin" (171) or "direct filiation" (3) hypothesis, compartmentation of a single photosynthetic cell gave rise to the nuclear and organellar genomes, which diverged intracellularly during or after the formation of the first protoeukaryote. According to the second, the "endosymbiont hypothesis" (114), chloroplasts are derived from once free-living oxygenic photosynthetic prokaryotes the genomes of which were phylogenetically unrelated to that of the heterotrophic nuclear ancestor.

Data derived from comparisons of sequences of cytochromes c(f) (154,155) and ferredoxins (155), and from comparisons of plastid 16S ribosomal RNA (rRNA) and cytoplasmic 18S rRNA catalogues (19,20,183), have favored the endosymbiotic theory of the origin of plastids. In particular, comparison of 16S rRNA catalogues of Porphyridium cruentum, a red alga (19), Euglena (187), and Lemna (183), suggest that cyanobacteria and the Porphyridium

chloroplast diverged from each other more recently than either did from Euglena or Lemna (56,66). The 16S rRNA of Porphyridium is especially homologous to the 16S rRNA of cyanobacteria (19,20). No homology between the cloroplast 16S rRNA of Lemna (183) and Porphyridium (19,20) and their respective cytoplasmic counterpart, 18S rRNA, was found. Unless cyanobacteria are derived from plastids, the last common ancestor of the chloroplast of Porphyridium and cyanobacteria was not itself a plastid but an oxygenic photosynthetic prokaryote. Cyanobacteria antedate eukaryotes in the fossil record. Thus, the last common ancestor of the plastids of Porphyridium and Euglena must also have been a free-living prokaryote. Restated, the chloroplasts of Porphyridium and Euglena are of diphyletic origin.

Autogenous origin hypotheses cannot accommodate evidence for the polyphyletic origin of plastids unless it is also assumed that nuclei arose separately in each lineage (171). It is then necessary to invoke convergent evolution on an unprecedented scale to explain the many similarities between these nuclei (171). The lack of homology between nuclear and cytoplasmic rRNA componants within the same organism is also problematic for autogenous origin hypotheses unless it is assumed that the plastid rRNAs have diverged little since the intracellular divergence of the plastid and nucleus while their cytoplasmic

(nuclear-encoded) homologues have diverged greatly.

The close structural resemblances of the cyanelle to free-living cyanobacteria has been noted previously (70). In this chapter I have presented the DNA sequences of the large and small subunits of carboxylase and shown their organization in the cyanelle DNA. In the location of the gene for the small subunit of RUBISCO downstream from the gene for the large subunit and in the lack from the deduced amino acid sequence of the small subunit both of a transit peptide and of 12 amino acid residues common to nuclear encoded small subunits, the cyanelle resembles cyanobacteria. Based on the roughly spherical shape of the cyanelle and the 35.7 molar % GC content of its DNA (80) I suggest that its closest cyanobacterial relative may be a member of the low GC group (35 to 37 mol %GC) of the genus Synechocystis, e.g., Synechocystis 6808, and not Anacystis (Synechococcus) 6301 or Anabaena 7120, which have DNAs richer in GC content (55% and 42.5%, respectively) (139,79).

In general, phylogenetic relationships based on protein sequences are not completely satisfactory because there are few proteins with identifiable homologues in plastids, eukaryotic cytoplasms, and all prokaryotes (66). Phylogenetic relationships based on the small subunit of carboxylase are especially problematic because, though the small subunit functions in the chloroplast, it is encoded in the nuclear DNA. The influence of the nuclear genome on the

divergence of small subunit genes is unknown. Because of these shortcomings I regard the hypothesis that the cyanelle is derived from cyanobacteria as highly probable but not proven. Ribosomal and transfer RNAs do have identifiable homologues coded for by plastid, nuclear and cytoplasmic genomes. If in the future the sequence of the cyanelle 16S rRNA becomes available, it may be possible to assign the cyanelle to a phylogenetic position within the cyanobacteria, as has already been done for the chloroplast of the rhodophyte Porphyridium cruentum (19).

CHAPTER IV

CHAPTER IV

RESTRICTION ENDONUCLEASE MAPPING OF THE CYANELLE DNA

Introduction

The chloroplast genome of higher plants and eukaryotic algae is a circular DNA molecule ranging in size from 85 to 195 kb (15). In many higher plants and Chlamydomonas the chloroplast DNA contains two single copy regions of unequal size and two inverted repeat regions each of which contains one copy of the ribosomal RNA genes (15). Chloroplast DNAs of broadbean (92), pea (36), and Euglena gracilis strain Z-S (67) contain a single copy of the ribosomal RNA genes whereas the ribosomal RNA genes of E. gracilis strain Z-Ha (185) are arranged in a closely spaced triple tandem repeat. The genes for several chloroplast proteins including the genes for the large subunit of ribulose-1,5-bisphosphate carboxylase (rbcL), the 32,000 dalton protein (psbA), and three subunits of the ATP synthase F1 complex (atpA, atpB, and atpC) have been localized on the restriction endonuclease maps of chloroplast DNAs from several plant species (15).

The photosynthetic organelle of Cyanophora paradoxa,

called a cyanelle, is of interest because it has a peptidoglycan wall layer (1) and phycobilin pigments (35,175) typical of cyanobacteria, but its genome is chloroplast-like in size (14,103,123) and in some of the details of its organization (14,16,17,103,123). From measurements of kinetic complexity Herdman and Stanier (80) deduced that the size of the cyanelle DNA is equivalent to 177 kb. Bohnert and coworkers (14,103,123) digested the cyanelle DNA with restriction endonucleases and estimated that the cyanelle DNA is approximately 127 kb in size. Both estimates of the size of the cyanelle genome are 10-20-fold smaller than the genome of a free-living cyanobacterium (78) but are within the size range of chloroplast genomes (15).

The cyanelle DNA appears circular in electron micrographs (17). Its circularity was confirmed by restriction endonuclease mapping studies based on the recognition sites of BamHI, SalI, and SmaI (17). Data in support of the proposed map have not been presented. Like the chloroplast DNA from many plants, the cyanelle DNA contains a repeated region arranged in an inverted orientation and separated by two single copy regions (16,17). Each of the repetitive sequences contains one set (16S, 23S, 5S) of the ribosomal RNA genes (16,17,123). Due to intramolecular recombination within the inverted repeat elements, cyanelle DNA exists in two alternative conformations that differ only in the relative orientation

of the two single copy segments (14).

I have studied the region of the cyanelle genome containing the genes for the large and small subunits of carboxylase and the β subunit of the cyanelle ATP synthase and have completed the sequence of the rbcL and rbcS genes and their flanking regions (Chapter 3 and Appendix A. In order to understand better how these genes fit into the overall organization of the cyanelle genome, I have localized the recognition sites for several restriction endonucleases in the cyanelle DNA. In this chapter I present a map of the recognition sites for the enzymes BamHI, SalI, XhoI, and a partial map of the BglII and PstI sites. The locations of the rbcL, rbcS, and atpB genes on the restriction endonuclease map of the cyanelle DNA is shown.

Abbreviations: DTT - dithiothreitol; CIAP - calf intestinal alkaline phosphatase; EDTA - ethylenediaminetetraacetic acid (disodium salt); kb - kilobase pair; RUBISCO - ribulose 1,5,-bisphosphate carboxylase; SDS - sodium dodecyl sulfate; Tris - tris(hydroxymethyl)aminomethane; Bicine - N,N-bis[2-hydroxyethyl]glycine

Materials and Methods

Strains and culture conditions

Cyanophora paradoxa UTEX LB 555 purified of contaminating organisms was used in most experiments. Some experiments utilized *C.paradoxa* UTEX 2344. No differences between UTEX LB 555 and UTEX 2344 were observed. Cultures were grown axenically in a Microferm Laboratory Fermentor (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 26°C, under continuous illumination (General Electric cool white lamps). Cultures were sparged with filtered air. The culture medium (CYB) was medium CYII (174) modified as follows: 3 mM $NaNO_3$ and 3 mM KNO_3 were added, 5 mM Bicine was substituted for Tris and the pH of the medium was adjusted to pH 7.8 with KOH.

Cyanelle isolation

Approximately 8 liters of cells were harvested by centrifugation at 7000xg. The resulting pellet containing free cyanelles and whole cells was washed by resuspending it in ca. 10 volumes of CYB containing 0.5 M sucrose and centrifuging at 1700xg in a clinical centrifuge. The soft pellet was resuspended in 4 volumes of CYB 0.5 M sucrose and kept at 25°C for 10 minutes. Cell lysis was achieved by a stepwise dilution in sucrose concentration: 0.25 M, 0.125 M,

0.06 M. Cyanelles were washed three times by suspension in ca. 4 pellet volumes of CYB and centrifugation at 470xg in a clinical centrifuge. Contaminating cellular RNA and DNA were removed by digestion with 200 µg/ml DNAase I, 2 µg/ml RNAase A, and 250 units RNAase T1, in 10 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM $CaCl_2$ for 1 hr at 26°C. After digestion cyanelles were washed three times by resuspension in NET and centrifugation. NET is 0.05 M Tris, pH 9.0, 0.015 M NaCl, 0.1 M EDTA.

Isolation of cyanelle DNA

Isolated cyanelles were suspended in NET plus 25 % sucrose, centrifuged, and resuspended to 15 mls in NET plus 25 % sucrose; 4 mg lysozyme was added per ml and the cyanelle suspension was incubated at 25°C for 15 min followed by 1.5 hrs at 4°C. Proteinase K (20 mg/ml in 20 mM Tris, pH 7.6, 1 mM EDTA, predigested for 2 hrs at 37°C followed by 2 min at 80°C) was added to a final concentration of 500 µg/ml for 20 min at 4°C. The cyanelle suspension was warmed to 55°C. The cyanelles were lysed by the addition of 5 ml of 55°C NET plus 4 % Sarkosyl. The suspension was mixed by inverting several times and incubated at 55°C for 3 hr. The lysate was transferred to a 150 ml Corex (Corning Glass Works) centrifuge tube, extracted 4x with an equal volume of redistilled phenol (equilibrated with 1 M Tris, pH 8, and containing

1 g 8-hydroxyquinoline/L) and the upper phenol layer removed. One volume of NET was added, and the solution was extracted with an equal volume of phenol:chloroform (1:1, v/v), centrifuged and the upper, aqueous layer transferred with the wide end of a 10 ml sterile glass pipette to a clean 150 ml Corex tube. The cyanelle DNA was precipitated by the addition of 5 M NaCl to 0.1 M and 1 volume of isopropanol at 25°C. The precipitated DNA recovered by spooling or centrifugation was dissolved in TE-8 (10 mM Tris, pH 8, 1 mM EDTA) and purified further by equilibrium density centrifugation on CsCl-ethidium bromide density gradients in a VTi-50 rotor, for 20 hrs, at 45,000 rpm in a Model L8-55 Ultracentrifuge (Beckman Instruments, Palo Alto, CA).

Restriction endonuclease analysis

Digestions with restriction endonucleases were performed in Core Buffer (Bethesda Research Laboratories, Gaithersburg MD). Core Buffer is 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl. DNA fragments from endonuclease digestion were separated by electrophoresis in 0.2, 0.6, 1.5 % agarose gels or in 6 % polyacrylamide gels. Gels were prepared and subjected to electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). Molecular weight standards consisted of phage lambda DNA digested with HindIII, and pBR322 digested with HinfI.

Photographs of gels stained with 5 µg/ml ethidium bromide were taken with a Polaroid MP-4 camera with a Wratten 23A gelatin filter using Polaroid type 57 or type 55 film. Illumination at 300 nm was provided by a Fotodyne UV Transilluminator model no. 3-3000 (Fotodyne, Inc., New Berlin, WI).

Construction and isolation of recombinant plasmids containing cyanelle DNA

BamHI fragments were cloned as follows. Cyanelle DNA and plasmid pBR322 (18) were digested with BamHI. Following restriction, pBR322 DNA was precipitated with sodium acetate and ethanol, dried and then resuspended in 50 µl of 50 mM Tris, pH 9.0. CIAP, 0.01 unit/µgDNA, was added and the mixture was incubated at 37°C for 1 hr followed by 30 min at 65°C. Approximately 6 µg of restricted cyanelle DNA was mixed with 0.2 µg of dephosphorylated pBR322 and the DNA was immediately extracted once with phenol and 3 times with chloroform, and was then precipitated with sodium acetate and ethanol. The precipitated DNA was washed with 80 % ethanol, dried under vacuum (water aspirator), and resuspended in double distilled water. Ligation was performed in 66 mM Tris, pH 8.0, 6.6 mM $MgCl_2$, 10 mM DTT, 66 µM ATP. One unit of T4 DNA ligase was added/µg DNA and the mixture was incubated at 4°C for 48 hr. The ligated DNA was transformed into $CaCl_2$-treated *E.coli* strain HB101 (pro *lac*Y Sm^r *endo*I *rec*A^- r^- m^-) prepared according to Mandel and

Higa (109). Transformed cells were selected on 75 ug ampicillin/ml. PstI fragments of cyanelle DNA were cloned as follows. pBR322 (18) was digested with PstI and dephosphorylated with CIAP as described above. Cyanelle DNA was digested with PstI and the fragments separated by electrophoresis in an 0.4 % agarose gel. The gel, stained in TBE containing 5 µg ethidium bromide/ml, was examined under 300 nm light, and the individual DNA bands were excised. A trough was cut into a 1 % agarose gel and lined with dialysis membrane that had been boiled 2 times in 2.5 mM EDTA, rinsed with double distilled water and autoclaved in 0.25 mM EDTA. A gel slice containing a DNA band was placed into the trough and enough TBE buffer to cover the agarose slice was added. The DNA was eluted from the agarose slice by subjecting it to electrophoresis at 7-8 volts/cm. The eluted DNA was purified by extracting 3 times with phenol and 3 times with chloroform. 300-500 ng of purified cyanelle DNA fragment were combined with 50 ng of digested and dephosphorylated pBR322. The mixture was extracted once with phenol and 3 times with chloroform and precipitated with sodium acetate and ethanol. The precipitated DNA was washed with 80% ethanol and resuspended in double distilled water. Ligation and transformation were performed as described above. Transformed cells were selected on 12 µg tetracycline/ml. To clone BglII fragments of cyanelle DNA 2 µg of cyanelle DNA digested with BglII was combined with 0.2 µg of plasmid pKC7 (136) which had been

digested with BglII and dephosphorylated with CIAP. The mixture was extracted once with phenol and 3 times with chloroform and precipitated with sodium acetate and ethanol. The DNA was resuspended in double distilled water. Ligation was performed as described above. Frozen competent cells of E.coli HB101 were prepared according to the procedure of Hanahan (71) modified by omitting glycerol and cobaltic hexamine chloride from solution FSB (71). Transformation was performed by adding 2-5 µl of the ligation mixture to 210 µl of thawed competent cells and incubating the mixture for 30 min at 4°C followed by 2 min at 42°C. One ml of LB (Luria-Bertani) medium was added and the cells were incubated for 1 hr at 37°C. One liter of LB contains 5 g Bacto-yeast extract, 10 g Bacto-tryptone, and 5 g NaCl. The cells were concentrated by centrifugation and spread onto agar plates containing 60 µg ampicillin/ml.

Isolation of plasmid DNA

Plasmid DNA from individual selected colonies was prepared from 5 ml cultures by using the method of Holmes and Quigley (84) or the method of Birnboim and Doly (9) as modified by Ish-Horowicz (110, page 368). Large scale plasmid preparations were performed according to the method of Birnboim and Doly (9) as modified by Ish-Horowicz (110, page 90).

Nick translation and filter hybridization

DNA was labeled *in vitro* with α-^{32}P dCTP by nick translation (111). Millipore filter paper (Millipore HA, 0.45 µm) containing immobilized DNA to be hybridized with homologous DNA was first incubated for 1 hr in 5X SSPE plus 0.3 % SDS and 150 µg denatured, sheared salmon sperm DNA (ssDNA) per ml at 65°C. 1X SSPE is 0.18 M NaCl, 10 mM $NaPO_4$ (pH7.7), 1 mM EDTA. Hybridization was performed in 5X SSPE plus 0.3 % SDS and 100 µg ssDNA/ml at 65°C for 12-24 hr. Following hybridization the filters were first washed 3 times in 2X SSPE plus 0.1 % SDS at 50°C for 15min and then washed 2 times in 0.1X SSPE plus 0.1 % SDS at 25°C for 15 min. Hybridizations with heterologous DNA were performed as above with the following changes. 6X SSPE was used in place of 5X SSPE. Prehybridization and hybridization were performed at 60°C. The minimum time for hybridization was increased to 16 hrs. Filters were washed at 25°C. Autoradiographs were prepared using KODAK XAR-5 film exposed at either 25°C, without an intensifying screen, or at -80°C in the presence of an intensifying screen (Dupont Cronex Quanta III).

Sources of the enzymes

Bovine pancreatic Deoxyribonuclease I, Ribonuclease A and chicken egg white lysozyme were obtained from Sigma Chemical Company, St. Louis, MO. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim Corporation, New York, New York. Proteinase K was obtained from Anspec, Ann Arbor, MI. E.coli DNA polymerase I, T4 DNA ligase, BamHI, EcoRI, HindIII, HinfI, PstI, SalI, and XhoI were obtained from Bethesda Research Laboratories, Gaithersburg, MD. BglII was purchased from New England Biolabs, Beverly, MA.

Results

i. Restriction endonuclease mapping of the cyanelle genome

The cyanelle DNA was digested with one, two, and three restriction endonucleases and the resulting DNA fragments were separated by gel electrophoresis (Figure 14). Each DNA fragment is denoted by the first two letters of the restriction endonuclease used to generate the fragment and by a number. The largest fragment of each digest is denoted with the number "1". Two DNA fragments that differ in nucleotide sequence but are identical in size are distinguished by an alphabetical subscript. Because the DNA fragments produced by digestion of cyanelle DNA range in

Figure 14. Agarose gel electrophoresis of the cyanelle DNA of Cyanophora paradoxa restricted with various enzymes. 1: Cyanelle DNA digested with BglII. 2: Cyanelle DNA digested with PstI. 3: Cyanelle DNA digested with XhoI. 4: Cyanelle DNA digested with BamHI. 5: Cyanelle DNA digested with SalI. 6: Cyanelle DNA digested with BamHI+SalI. 7: Cyanelle DNA digested with BamHI+XhoI. 8: Cyanelle DNA digested with SalI+XhoI. 9: Cyanelle DNA digested with BamHI+SalI+XhoI.

size from ca. 41 kb to a few hundred base pairs, a range of agarose gel concentrations (0.2 -2 %) was necessary to determine the sizes of the restriction fragments. The molecular weights of the cyanelle DNA restriction fragments obtained from those electrophoretograms are summarized in Table 6.

The size of the cyanelle DNA was estimated by summing the molecular weights of the DNA fragments from each restriction digest. Two different estimates of the size of the cyanelle genome were obtained (Table 6), ca. 180 kb and ca. 128 kb. Bohnert and Loeffelhardt (16) found that the cyanelle DNA occurs in two different orientations due to recombination within the inverted repeat segments. Enzymes which cut within the repeat segments generate an identical set of restriction fragments from each orientation. Enzymes that do not have sites within the repeat units, e.g., BamHI and SalI, generate a set of orientation-specific restriction fragments from the region of the inverted repeats. The orientation-specific restriction fragments occur with a stoichiometry of 0.5 in restriction digests (16). Digests of cyanelle DNA with either BamHI or SalI contain eight restriction fragments. Four of these fragments have a stoichiometry of one and are present in both conformations of the cyanelle DNA (16,103). The remaining four fragments hybridize with probes for ribosomal RNA genes and have a stoichiometry of 0.5 in BamHI or SalI digests of cyanelle

Table 6. Sizes and stoichiometries of DNA fragments resulting from single, double, and triple digestions of cyanelle DNA from C. paradoxa

Band	BglII	PstI	XhoI	BamHI	SalI	BamHI+SalI	BamHI+XhoI	SalI+XhoI	BamHI+SalI+XhoI
1	16.5[a]	17.1	26.0	41.1	34.1	34.1	21.5	21.5	21.5
2	12.75	13.75	25.5	38.0	29.5	25.2	14.2	18.6	12.5
3	11.7	11.5	21.5	28.0	27.2	24.9	14.0	16 2	11.5
4	10.3[a]	10.25	20.2	26.8	23.0	23.0	12.5	14.9	9.5[a]
5	8.2	9.0	12.5	15.5	18.6	13.5	11 5	12 5	9.2
6	7.3[a]	8.25	9.2	13.8	18.5	12.8	10.0	9.2	8.4
7	6.1	7.6	4.5	10.0	18.2	9.5	9.5[a]	6.3	6.3
8	4.95	6.7	4.4	9.5	11.7	8.4	5.9	5.6	5.4
9	3.8	5.06	4.1			7.1	4.5	4.9	4.5[a]
10	3.65	4.6				5.4	4.4	4.5	4.4
11	3.6	4.0				4.7	4.1	4.4	4.0
12	2.3	3.65				4.6	3.2	4.0	3.2
13	2.1	2.5[b]				1.9	2.5	2.45	2.45
14	1.2	2.43				0.35	0.8	2.1	2.1
15	0.95	2.32[c]						1.9	1.9[a]
16	0.4	2.1							0.8
17		1.8							0.35
18		1.63							
19		1.33							
20		1.18							
21		1.15							
22		1.11							
23		0.99							
24		0.73							
25		0.53[c]							
26		0.52							
27		0.48							
Sum:	129.9	127.0[d]	127.9	182.7	180.8	175.45	128.1	129.05	123.9

a The stoichiometry of these DNA fragments is two and their nucleotide sequences are unique.
b The stoichiometry of these DNA fragments is two and their nucleotide sequences are identical.
c The stoichiometry of these DNA fragments is two; whether their nucleotide sequences are unique or identical has not been determined.
d The sizes and stoichiometries of PstI fragments smaller 0.48 kb have not been determined.

DNA (16,103). Because the sum of the molecular weights of the fragments from one orientation equals the sum of the molecular weights from the second orientation, it is possible to divide the eight fragments of the BamHI and the SalI digests into two sets of six restriction fragments, each set comprising one of the two conformations of the cyanelle DNA (16, and results presented in this thesis). Table 7 contains the BamHI and SalI fragments arranged according to the conformation of the cyanelle DNA. As can be seen in Table 7, the sum of the molecular weights of the fragments from orientation I is equal to the sum of the molecular weights of the fragments from orientation II and is in agreement with the size of the cyanelle DNA, ca. 128 kb, estimated from the restriction digests generated by the enzymes, e.g., XhoI, which cleave within the inverted repeat segments.

An examination of the pattern of fragments generated by digestion of cyanelle DNA with BglII (Figure 14) shows that three fragments, Bg1, Bg4, and Bg6, appear to have a stoichiometry of greater than one. Table 8 shows the redigestion of these bands and of Bg2 and Bg3 with BamHI, SalI, or XhoI. Digestion of Bg1, Bg4, or Bg6 with these enzymes generates fragments with molecular weights that add up to approximately twice the molecular weight of the original DNA band whereas the sum of the molecular weights of the fragments resulting from the digestion of Bg2 or Bg3

Table 7. BamHI and SalI restriction fragments grouped according to the orientation of the cyanelle DNA.

BamHI (kb)	Orientation		SalI (kb)	Orientation	
	I	II		I	II
41.1		41.1	34.1		34.1
38.0	38.0	38.0	29.5	29.5	
28.0	28.0		27.2	27.2	27.2
26.8	26.8		23.0	23.0	
15.5	15.5	15.5	18.6	18.6	18.6
13.8		13.8	18.5		18.5
10.0	10.0	10.0	18.2	18.2	18.2
9.5	9.5	9.5	11.7	11.7	11.7
Sum: 182.7	127.8	127.9	180.8	128.2	128.3

Table 8. Digestion of BglII fragments (Bg1, Bg2, Bg3, Bg4, Bg6) of cyanelle DNA with the restriction endonucleases BamHI, SalI or XhoI.

BglII fragment	Restriction Fragments (kb)		
	BamHI	SalI	XhoI
Bg1	16.5, 12.3, 4.3	16.5, 11.8, 4.85	14.5, 10.0, 6.6
Bg2	12.8	12.8	10.2, 2.8
Bg3	10.3, 1.7	6.5, 5.7	11.7
Bg4	10.3, 9.0	8.3, 8.2, 2.3, 2.0	8.3, 6.5, 3.7, 2.0
Bg6			7.4, 4.5, 2.7

approximates the molecular weight of the original DNA band. From these results I conclude that Bg1, Bg4, and Bg6 each consists of two different DNA fragments of identical size and that Bg2 and Bg3 each consists a single DNA fragment.

To construct a map of the cyanelle DNA, recombinant plasmids containing PstI (Ps1, Ps2, Ps3, Ps5, Ps7, Ps8) and BglII (Bg10 and Bg12) fragments and an isolated BamHI fragment, Ba5, were hybridized to nitrocellulose blots of cyanelle DNA restricted with various enzymes. The cloned PstI fragments and Ba5 do not overlap and together contain ca. 64 % of the cyanelle genome. A summary of the results of the hybridization experiments is presented in Table 9. Each cyanelle DNA fragment used as a probe was mapped with the enzymes BamHI, BglII, SalI, PstI, and XhoI. These restriction maps were used to align the cyanelle DNA fragments with the restriction maps of the cyanelle DNA and to verify the hybridization results. Two BglII fragments, Bg13 and Bg15, which were electrophoresed off of the gels used for the hybridizations, were localized during the mapping of Ps7 and Ps1.

The results of the hybridization experiments alone (Table 9) cannot be used to assign the DNA fragments that comprise the Bg1, Bg4 or Bg6 DNA bands to a position on the restriction map of the cyanelle DNA. To aid in the localization of these fragments and of Bg2 and Bg3, the results presented in Table 8 were augmented by isolating

Table 9. Summary of hybridization results

Recombinant plasmid	Restriction endonuclease fragment contained in plasmid	Cyanelle DNA fragments that hybridized to plasmids containing cyanelle DNA fragments								
		PstI	BglII	XhoI	BamHI	SalI	BamHI+SalI	BamHI+XhoI	SalI+XhoI	BamHI+SalI+XhoI
pCp002	Ps1	1	1,4	3,9	(1,2)††	(2 3)	(2,3)	1,10	1,15	1,15
pCp003	Ps2	2,3	1,2,3,9*	2,5	(1,2),3,4,6	1,(2,3),4,(6,7)	1,3,4,5,(11,12)	3,4,5	3,5,6	2,3,5,9
pCp004	Ps3	2,3	1,2,9	2,5	(1,2),3,4,6	1,(2,3),4,(6,7)	1,3,4,5	3,4,5	3,5,6	2,3,5
pCp005	Ps5	5	6,7,8	4	(1,2),7	(6,7)	6,10	6*,7	4	4,8
pCp006	Ps7	7	1,6	6,3	(1,2),4	(2 3)	2	1*,8,12	1*,9,12	1*,11,12,15
pCp007	Ps8	8	1,5	1	8	(5,6,7)	7	7	2	4
†	Ba5a	4,12	4,5,11	1	5	(5,6,7),8	8,9	2	2,7	6,7
pCp028	Bg10	6	10					(9,10)	(10,11)	(9,10)
pCp029	Bg12	3	12					3	6	5

†Ba5 has not been cloned. Isolated Ba5 was used as a hybridization probe.
††DNA fragments in parentheses did not resolve.
*Hybridization was very weak.

together the fragments Bg1, Bg2, Bg3, and Bg4 and by redigesting them with BamHI, SalI, XhoI, BamHI+XhoI, or SalI+XhoI (Table 10). From the results presented in Tables 8 and 10 I conclude that one of the Bg1 fragments, designated $Bg1_a$ has both a BamHI and a SalI site whereas the second Bg1 fragment, designated $Bg1_b$ has neither a BamHI nor a SalI site. Based on the results shown in Tables 8 and 10 the following designations were made for the Bg4 and Bg6 fragments. $Bg4_a$ is that Bg4 fragment which contains a BamHI site; $Bg4_b$ is that Bg4 fragment which does not have a BamHI site. $Bg6_a$ is that Bg6 fragment which lacks an XhoI site; $Bg6_b$ is that Bg6 fragment which contains an XhoI site.

ii. Ordering the restriction fragments

The restriction endonuclease map of the cyanelle genome is presented in a linear fashion in Figure 15 so that it may be read more easily. The 0 kb coordinate of this map has been positioned at the XhoI site of Xh1 that is located in $Bg1_a$. Cyanelle DNA occurs in two alternative orientations (16). Orientation I in which Xh5 is adjacent to Xh1 is shown in Figure 15. The restriction endonuclease maps of both orientations of that region of the cyanelle DNA containing the inverted repeats and the smaller single copy segment is shown in Figure 16. In the discussion which follows, "right" and "left" refer to the restriction map as presented in Figure 15.

Table 10. Sizes and stoichiometries resulting from single and double digestions of a mixture of BglI fragments (Bg1, Bg2, Bg3, Bg4) of cyanelle DNA from C. paradoxa.

	Restriction Endonuclease					
undigested	BamHI	SalI	BamHI+SalI	XhoI	BamHI+XhoI	SalI+XhoI
16.5[a]	16.5	16.5	16.5	14.3	14.3	14.3
12.8	12.8	12.8	12.8			
	12.3					
		11.8	11.8	11.7		
11.7						
10.5[a]	10.5				10.3	
	10.3			10.0[a]	10.0[a]	10.0[a]
	9.3				8.4	
		8.3	8.3	8.3		
		8.2				
			7.3			
				7.0		
				6.8	6.8	6.8
		6.6				6.6
		5.6	5.6			5.6
		4.8	4.8			4.8
			4.7			
	4.3		4.3		4.3	
				3.8	3.8	
				2.75		
				2.6		
		2.3				
		2.0		2.0		
	1.65					
	1.2					

a The stoichiometry of these DNA fragments is two.

Figure 15. Restriction endonuclease map of the cyanelle DNA of Cyanophora paradoxa The cyanelle DNA was mapped with the restriction endonucleases BamHI, SalI, PstI, and XhoI. The rbcL, rbcS, and atpB genes are shown as solid bars. The inverted repeat segments are shown as solid arrows. The fine vertical lines mark the borders of the fragments (Ps8, Ps5, Ps1, Ps7, Ps2, Ps3, Ba5, Bg12, Bg10) that were used as probes in the hybridization experiments.

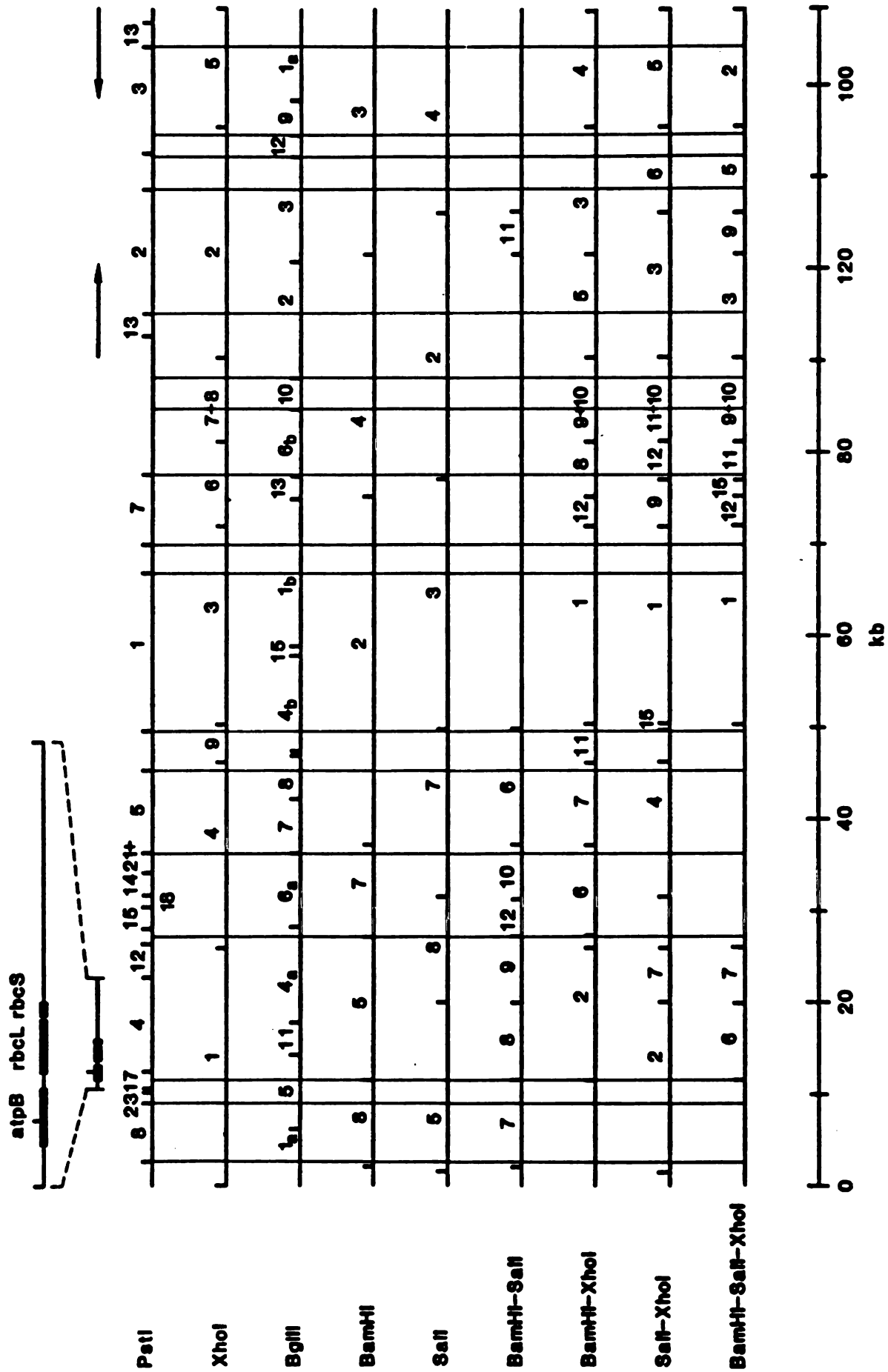
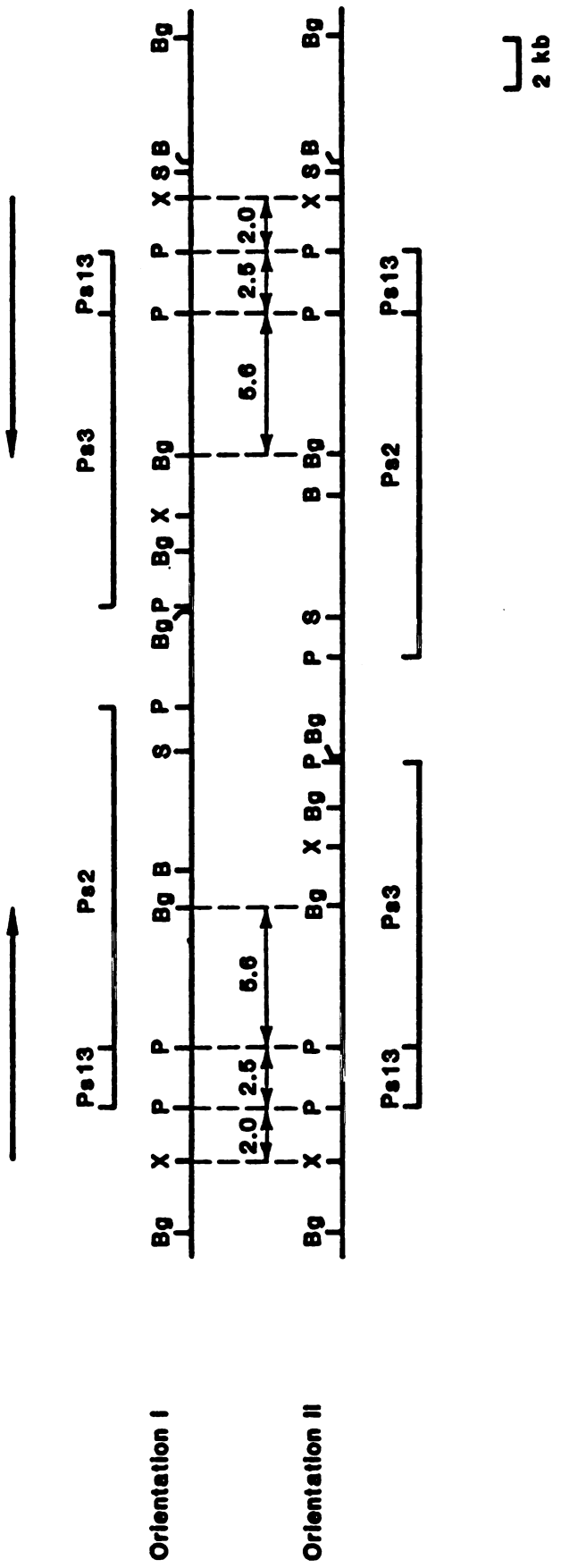

atpB rbcL rbcS
PstI
XhoI
BglII
BamHI
SalI
BamHI-SalI
BamHI-XhoI
SalI-XhoI
BamHI-SalI-XhoI
0
20
40
60
80
100
120
kb

Figure 16. Restriction endonuclease map of the repeated region of the cyanelle DNA from Cyanophora paradoxa showing both orientations. The inverted repeat segments are shown as the solid arrows at the top of the figure. The restriction fragments comprising the repeated segments are shown in kilo basepairs within the dotted lines. The PstI fragments (Ps2, Ps3, Ps13) are shown to aid in comparison with the map presented in Figure 2. Bg: BglII; X: XhoI; S: SalI; B: BamHI; P:PstI.


Orientation I
Orientation II
Bg
X
P
S
B
Ps13
Ps3
Ps2
2.0
2.5
5.6
2 kb

Ps8 (6.7 kb) hybridizes to $Bg1_a$ and/or $Bg1_b$ (16.5 kb), Bg5 (8.2 kb), and Ba8 (9.5 kb). From this result I conclude that $Bg1_a$ or $Bg1_b$ is adjacent to Bg5. Ba5 (15.5 kb) hybridizes to $Bg4_a$ and/or $Bg4_b$ (10.3 kb), Bg5 (8.2 kb), Bg11 (3.6 kb), BaSa8 (8.4 kb) and BaSa9 (7.1 kb). Mapping of Ba8 with SalI, PstI, and BglII showed that Ba8 does not have a SalI site, that Ps8 is contained within Ba8, and that Ba8 contains a single BglII site located within Ps8. From these data I conclude that Bg1 and Bg5 are overlapped by Ps8 and by Ba8. Because $Bg1_a$ contains a BamHI site whereas $Bg1_b$ does not, I conclude that the Bg1 fragment adjacent to Bg5 is $Bg1_a$. Digestion of Ba5 and BaSa8 with BglII showed that Bg11 is contained within Ba5 and BaSa8 and that Bg4 is not contained entirely within Ba5. Therefore, Bg11 is located between Bg4 and Bg5, and the Bg4 fragment that is overlapped by Ba5 is $Bg4_a$. $Bg1_a$ and Bg5 are adjacent; therefore, the order of the BglII fragments is $Bg1_a$-Bg5-Bg11-$Bg4_a$. Ba8 and Ba5 overlap Bg5; therefore, a BamHI fragment located between Ba8 and Ba5 must lie completely within Bg5. There is no BamHI fragment smaller than Bg5. I therefore conclude that Ba8 is adjacent to Ba5.

Because the sum of the molecular weights of BaSa8 (8.4 kb) and BaSa9 (7.1 kb) is equal to the molecular weight of Ba5 (15.5 kb), I conclude that Ba5 has a single SalI site and that BaSa8 and BaSa9 contain the complete sequence of Ba5. BaSa8 contains Bg11 (3.6 kb) and two BamHI-BglII

fragments, 2.6 kb and 2.2 kb. When Ba5 is digested with BglII the BamHI-BglII fragments that flank Bg11 are 2.6 kb and ca. 9.5 kb. Bg5 (8.2 kb) overlaps Ba5 and Ba8. Ba8 does not have a SalI site therefore BaSa7 (9.5 kb) is Ba8 (9.5 kb). Because Bg5 is adjacent to Bg11, smaller than 9.5 kb, and overlaps Ba5 and Ba8, I conclude that the 2.6 kb BamHI-BglII fragment is adjacent to Ba8. Therefore, BaSa8 is adjacent to BaSa7 (Ba8) and the order of the BamHI+SalI fragments in this region is BaSa7(Ba8)-BaSa8-BaSa9.

The XhoI site at the right end of Xh1 (26 kb) was localized by examining the results of the hybridization of Ba5 (15.5 kb) to double (BamHI+SalI, BamHI+XhoI, and XhoI+SalI) and triple (BamHI+SalI+XhoI) digests of cyanelle DNA. Ba5 hybridizes to BaXh2 (14.2 kb) (fragments <1.8 kb were not present on the gel). From this result I deduce that an XhoI site is present in Ba5 and that it is located ca. 1.3 kb from the end of Ba5. When cyanelle DNA is digested with BamHI+SalI, Ba5 hybridizes with BaSa8 (8.4 kb) and BaSa9 (7.1 b), whereas when cyanelle DNA is digested with BamHI+SalI+XhoI, Ba5 hybridizes with BaSaXh6 (8.4 kb) and BaSaXh7 (6.3 kb). From these results I conclude that the XhoI site located in Ba5 is located in BaSa9. Because both Ba5 and Ps8 hybridize with Xh1 (26 kb), the XhoI site in Ba5 is the right end of Xh1. From the size of Xh1, 26 kb, I deduce that the left end of Xh1 is located in $Bg1_a$ ca. 6.1 kb from the right end of $Bg1_a$ (16.5 kb). The sizes of

the fragments generated by digestion of the Bg1 DNA band with XhoI (Table 8), 10.0 kb and 6.5 kb, are close to the sizes predicted.

Ba5, but not Ps8, hybridizes with Sa8 (11.7 kb). If the SalI site in Ba5 is the right end of Sa8, then the left end of Sa8 would be located in Ba8, which does not have a SalI site. Therefore, the SalI site in Ba5 is not the right but the left end of Sa8, and Sa8 contains BaSa9 (7.1 kb). The remainder of Sa8, 4.6 kb, must consist of a second BamHI+SalI fragment because no BamHI fragment is small enough that Sa8 could contain BaSa9, a BamHI fragment, and a second BamHI+SalI fragment. Because BaSa12 is 4.6 kb it was placed adjacent to BaSa9.

Ba5 and Ps8 hybridize with SaXh2 (18.6 kb) and Ba5 hybridizes with SaXh7 (6.3 kb). SaXh7 is derived from BaSa9 by cleavage with XhoI (described above). Xh1 (26 kb) is large enough that its left end is ca. 2 kb to the left of SaXh2. From these results I conclude that SaXh2 is a SalI fragment and that its left end is located in Bg1 to the left of that portion of $Bg1_a$ that overlaps Ba8. Because Sa5 is 18.6 kb it was identified as the SalI fragment that is synonomous with SaXh2 and was placed adjacent to Sa8.

Ps5 hybridizes to Ba7 (10.0 kb), $Bg6_a$ and/or $Bg6_b$ (7.35 kb), Bg7 (6.1 kb), Bg8 (4.95 kb), and Xh4 (20.2 kb). Localization of the PstI, BamHI, SalI, and BglII sites in

the recombinant plasmids that contain Ba7 and Ps5 made it possible to align Ba7 and Ps5. Ba7 contains all of $Bg6_a$ or $Bg6_b$, 1.7 kb of Bg7, and Ps14, Ps15, Ps18, and Ps21. Ps5 contains Bg7 and ca. 0.3 kb of $Bg6_a$ or $Bg6_b$. From these results I conclude that $Bg6_a$ or $Bg6_b$ is adjacent to Bg7. Ba7 does not contain an XhoI site, therefore the Bg6 fragment contained by Ba7 is $Bg6_a$ and the order of the BglII fragments that hybridize with Ps5 is $Bg6_a$-Bg7-Bg8. Ba7 contains a single SalI site. Restriction of Ba7 with SalI generates BaSa10 and BaSa12 (4.6 kb). Ps5 hybridizes with BaSa10 and BaSa6 (12.8 kb). From these result I conclude that the order of the BamHI+SalI fragments is BaSa12-BaSa10-BaSa6 and that the SalI fragment which hybridizes with Ps5, Sa6 (18.5 kb) or Sa7 (18.2 kb) [Sa6 and Sa7 did not resolve on the gels probed with Ps5], contains BaSa10 and BaSa6. The sum of the molecular weights of BaSa10 and BaSa6, 18.2 kb, is close to the molecular weight of Sa7, 18.5 kb. Although Sa6 has a molecular weight (18.5) that is close close to the sum of the molecular weights of BaSa10 and BaSa6, it was not placed adjacent to Sa8 because it has a stoichiometry of 0.5 (16) implying that it is located in the repeated region. Ps5 does not hybridize with Ps2 or Ps3 or with any of the BglII, XhoI, BamHI+XhoI, SalI+XhoI, or BamHI+SalI+XhoI fragments which hybridize with Ps2 and Ps3 suggesting that it is not located in the repeated region of the cyanelle DNA. I therefore conclude that Sa6 is not adjacent to Sa8. Sa7 (discussed in above paragraphs) and Ba7

contain the same 4.6-kb BamHI-SalI fragment, a result of their overlap. Ba7 also overlaps Sa8 to produce BaSa10 (5.4 kb). Sa8 is adjacent to Sa7 and contains BaSa9, formed by its overlap with Ba5, and BaSa12, formed by its overlap with Ba7. From these data I conclude that Ba5 is adjacent to Ba7. The relative positions of the BglII, BamHI, SalI, and BamHI+SalI fragments that had been established on the basis of restriction maps of Ba7 and Ps5 are such that when Sa7 is adjacent to Sa8 and Ba5 is adjacent to Ba7, the BglII site at the right end of $Bg4_a$ and the BglII site at the left end of $Bg6_a$ coincide.

Ps5 hybridizes with Xh4 (20.2 kb) and SaXh4 (14.6 kb), Ba7(10.0 kb), BaXh6 (10.0 kb), and BaXh7 (9.5 kb). Ps5 does not have an XhoI or a SalI site. Ba7 has a SalI site but no XhoI site. Ba5, which contains an XhoI site located ca. 0.8-1.3 kb from its right end is adjacent to Ba7. From these results I conclude that SaXh4 extends from the SalI site in Ba7 to the XhoI site at the right end of Xh4 and that the left end of Xh4 is the XhoI site in Ba5. The localization of Xh1 adjacent to Xh4 is consistent with the results of the hybridization of Ps5 to BamHI and BamHI+XhoI digests. Because Ba7, does not have an XhoI site, I conclude that BaXh6 is a BamHI fragment, i.e., Ba7. From these results I deduce that Xh4 is comprised of a BamHI fragment (Ba7) flanked by two BamHI-XhoI fragments one of which is BaXh6 (9.5 kb). The sum, 19.5 kb, of the molecular weights of Ba7

(10.0 kb) and BaXh7 (9.5 kb) is 0.7 kb smaller than the molecular weight of Xh4 (20.2 kb). If Xh4 is adjacent to Xh1, then the BamHI-XhoI fragment of 0.8-1.3 kb that is formed by the overlap of Xh4 and Ba5 is the size expected, ca. 0.7 kb, for the second BamHI-XhoI fragment contained by Xh4. From these results I deduce that Xh1 and Xh4 are adjacent.

Ps1 hybridizes with $Bg1_a$ and/or $Bg1_b$, $Bg4_a$ and/or $Bg4_b$, Xh3, and Xh9. Ps7 hybridizes with $Bg1_a$ and/or $Bg1_b$, $Bg6_a$ and/or $Bg6_b$, Xh6, and Xh3. From these hybridization results I conclude that the order of the BglII and XhoI fragments that hybridize to Ps1 and Ps7 is [$Bg4_a$ or $Bg4_b$]-[$Bg1_a$ or $Bg1_b$]-[$Bg6_a$ or $Bg6_b$] and Xh9-Xh3-Xh6, respectively. Because $Bg6_a$ is overlapped completely by the PstI fragments Ps14, Ps15, Ps18, and Ps21, I conclude that the Bg6 fragment that hybridizes with Ps7 is $Bg6_b$. Digestion of Ps1 and Ps7 with BglII showed that Ps1 contains Bg15 (0.95 kb) and that Ps7 contains Bg13 (2.1 kb). [Bg15 and Bg13 were electrophoresed off of the gels probed with Ps1 and with Ps7 and therefore are not present in the hybridization results.] Because Bg15 is an internal fragment, it must lie between the BglII fragments $Bg1_a$ or $Bg1_b$ and $Bg4_a$ or $Bg4_b$ that hybridized with Ps1. Similarly, Bg13 must lie between $Bg1_a$ or $Bg1_b$ and $Bg6_b$. The revised order of the BglII fragments in this region is therefore [$Bg4_a$ or Bg_b]-Bg15-[$Bg1_a$ or $Bg1_b$]-Bg13-$Bg6_b$.

Ps1 and Ps7 were aligned relative to the BglII and XhoI

maps in the following manner. Ps7 contains a single site for BamHI, SalI, and XhoI, whereas Ps1 contains only an XhoI site. Ps7 hybridizes to Bg1, Bg6, Xh6 (9.2 kb), Xh3 (21.5 kb), BaXh1 (21.5 kb), BaXh8 (5.9 kb), BaXh12 (3.2 kb), SaXh1 (21.5 kb), SaXh9 (4.9 kb), SaXh12 (4.0 kb), BaSaXh1 (21.5 kb), BaSaXh11 (4.0 kb), BaSaXh12 (3.2 kb), and BaSaXh15 (1.9 kb). Because Ps7 hybridizes with a fragment the size of Xh3 (21.5 kb) in double (SalI+XhoI and BamHI+XhoI) and triple (BamHI+SalI+XhoI) digests of cyanelle DNA, I conclude that Xh3 does not have a BamHI or a SalI site. Therefore, the BamHI and the SalI site present in Ps7 are located in that portion of Ps7 that overlaps Xh6. Ps1 hyridizes with $Bg1_a$ and/or $Bg1_b$, $Bg4_a$ and/or $Bg4_b$, Xh1 (21.5 kb), Xh9 (4.1 kb), BaXh1 (21.5 kb), BaXh11 (4.1kb), SaXh1 (21.5 kb), SaXh15 (1.9 kb), BaSaXh1 (21.5 kb), and BaSaXh15 (1.9 kb). From these results I conclude that Xh9 contains a SalI site but no BamHI site and that Xh3 does not have a BamHI or a SalI site. This conclusion was also deduced from the results of the hybridization of Ps7 to XhoI, BamHI+XhoI, SalI+XhoI, and BamHI+SalI+XhoI digests. Ps1 does not contain a SalI site and, therefore, its left end must lie to the right of the SalI site located in Xh9.

It cannot be deduced from the hybridization data whether Ps1 and Ps7 recognize the same Bg1 fragment. However the identity of the Bg1 fragment can be deduced in the following manner. If Ps1 and Ps7 hybridize with a

different Bg1 fragment then the order of the BglII fragments that they overlap is Bg1-Bg15-Bg4-$Bg6_b$-Bg1'. This order leads to the prediction that digestion of Bg1 with XhoI should generate Xh9 (4.1 kb) and a fragment of ca. 4.9 kb. These predictions are inconsistent with the results presented in Tables 8 and 10 which show that digestion of Bg1 with XhoI produces fragments of ca. 14.3 kb, 10 kb, and 6.6 kb. If on the other hand, Ps1 and Ps7 hybridize with the same Bg1 fragment, then the the order of the BglII fragments is Bg4-Bg15-Bg1-Bg13-$Bg6_b$. This fragment order leads to the prediction that the Bg1 fragment at this location has neither a BamHI nor a SalI site (for reasons outlined above) and that digestion of this Bg1 fragment should produce a fragment of ca. 14.2 kb. These predictions are in agreement with the results presented in Tables 8 and 10. I therefore conclude that Ps1 and Ps7 hybridize with the same Bg1 fragment and that this fragment, which lacks both a BamHI and a SalI site, is $Bg1_b$. Moreover, because a fragment that hybridizes with $Bg1_b$ must also hybridize with Xh3, BaXh1, SaXh1, and BaSaXh1 and because Ps2, Ps3, and Ps8 do not hybridize with these fragments, I conclude that Ps2, Ps3, and Ps8 hybridize with $Bg1_a$.

Xh3 does not have a SalI site, therefore, the SalI site located in Xh9 and the SalI site in Xh6, are the left and right ends, respectively, of a single SalI fragment of ca. 27.1 kb. Ps1 hybridizes with Sa2 or Sa3, whereas Ps7

hybridizes with Sa1, Sa2 or Sa3, and Sa4. From these results I conclude that either Sa2 or Sa3 is adjacent to Sa7. If Sa2 (29.2 kb) is adjacent to Sa7 then a an additional fragment of ca. 2 kb would be required in order to align the SalI map with BglII and XhoI maps. There is no 2-kb XhoI fragment and a 2-kb BglII fragment would lie either in Ps1 or in Ps7 (Figure 8) and would have been observed, as were Bg15 (0.95 kb) and Bg13 (2.12 kb), when Ps1 and Ps7 were digested with BglII. Because the placement of Sa2 adjacent to Sa7 leads to predictions about the BglII, PstI, and XhoI maps that are inconsistent with observed results, I conclude that Sa2 is not adjacent to Sa7. Because Sa3 (27.2 kb) matches the size (27.1 kb) that was deduced from the locations of the SalI sites in Ps7, Xh6, and Xh9, and because placement of this fragment adjacent to Sa7 is consistent with the hybridization data, I conclude that Sa3 is adjacent to Sa7.

Neither Xh3 nor Xh9 has a BamHI site, therefore, the BamHI site located in Ps7 is the right end of a BamHI fragment of at least 29 kb. Ps1 hybridized with Ba1 (41.1 kb) and Ba2 (38 kb) and BaSa2 and BaSa3. Ps7 hybridized with Ba1, Ba2 and Ba4 (26.8 kb). Ps5 hybridized with Ba1 and Ba2, and with Ba7. [Ba1 and Ba2 and BaSa2 and BaSa3 did not resolve on the gels probed with Ps5, Ps1, and Ps7.] From these results it was deduced that BamHI fragment that contains Ps1, Xh3 and Xh9, and is adjacent to Ba7, is

either Ba1 or Ba2. If Ba1 (41.1 kb) is adjacent to Ba7 then a fragment of ca. 3.1 kb is required between Sa7 and Sa8 and between Xh9 and Xh4 in order to align the SalI and XhoI maps with the BamHI map. There is no 3.1 kb SalI or XhoI fragment and, therefore, it was concluded that Sa3 and Sa7 and Xh4 and Xh9 are adjacent. If Ba2 (38.0 kb) is adjacent to Ba7, then Sa7 and Sa8 and Xh4 and Xh9 are separated by ca. 0.5 kb. Because it is difficult to determine accurately the size of large fragments, e.g., Ba1, Ba2, Sa3, and Xh3, a mismatch of 0.5 kb was not considered unreasonably large and it was concluded that Ba2 is adjacent to Ba7, that Xh4 is adjacent to Xh9, and that, as deduced earlier, Sa7 is adjacent to Sa3. There is a BglII fragment of ca. 0.4 kb and it is possible that a fragment of this size is present between Bg8 and Bg4 and that Sa3 and Xh3 are slightly larger than measured. Because the Bg4 fragment that hybridizes with Ps1 is contained entirely within a BamHI fragment I conclude that this Bg4 fragment is $Bg4_b$. The alignment of Ps1 and Ps5 relative to the BglII fragments with which they hybridize is such that the right end of Bg8 and the left end of $Bg4_b$ coincide when the BamHI, SalI, XhoI maps are aligned with Ps1 and Ps5.

Ps2 (13.75 kb) and Ps3 (11.5 kb) hybridize not only to themselves but also to each other. In addition they each recognize a similar set of DNA fragments produced by other enzymes. For example, both recognize Xh2, Xh5, $Bg1_a$, Bg2,

BaXh3, BaXh4, BaXh5, SaXh3, SaXh5, SaXh6, BaSaXh2, BaSaXh3, and BaSaXh5. These data suggest the presence of a repeated DNA sequence on these fragments. Digestion of Ps2 and Ps3 by BglII and digestion of $Bg1_a$ and Bg2 by PstI generates a 5.6 kb BglII-PstI fragment. From these results together with the hybridization results (Table 9), I deduce that the 5.6kb BglII-PstI fragment present in Ps2, Ps3, $Bg1_a$, and Bg2 contains the repeated sequence which results in the cross hybridization of Ps2 and Ps3. The remaining portion of Ps2 that hybridizes to Bg3 and the remaining portion of Ps3 that hybridizes to Bg9 and Bg12, contain unique DNA sequences. Two conformations of the region containing Ps2 and Ps3 can be deduced from the data presented in Tables 6-10.

iii. Ordering the restriction fragments of orientation I

Ps3 hybridizes with $Bg1_a$, Bg2, and Bg9. Bg12 hybridizes with Ps3. (Bg12 was electrophoresed off of the gels probed with Ps3.) Digestion of Ps3 by BglII produces a 5.6 kb BglII-PstI fragment, an internal BglII fragment, Bg9 (3.8 kb) and a second BglII-PstI fragment of 1.8 kb. Digestion of $Bg1_a$ by PstI produces a fragment of 5.6 kb and a fragment of 2.5 kb that coincides with Ps13. From these results it was concluded that Ps3 and $Bg1_a$ overlap by 5.6 kb and that Bg9 is located between $Bg1_a$ and Bg12. $Bg1_a$ is adjacent to Bg5 (discussed above), therefore, the order of the BglII fragments is (left to right) Bg12-Bg9-$Bg1_a$-Bg5-Bg11-$Bg4_a$.

contains an XhoI site located within Bg9. $Bg1_a$ also contains an XhoI site. The XhoI site in Ps3 and the XhoI site in $Bg1_a$ are 12.5 kb apart, a size equal to that of Xh5. Ps3 hybridizes with Xh5 (12.5 kb) and Xh2 (25 kb). From these data I conclude that the order of XhoI fragments in this region of the cyanelle DNA is Xh2-Xh5-Xh1.

Ps2 hybridizes with $Bg1_a$, Bg2, Bg3, Xh2, Xh5 and BaSa11. Digestion of Ps2 and Bg3 with BamHI+SalI produces BaSa11 (4.7 kb). Digestion of Bg2 with PstI and digestion of Ps2 with BglII produces a fragment of 5.6 kb. From these results I conclude that Ps2 and Bg2 overlap by ca. 5.6 kb in the same manner as Ps3 and Bg1 and that the remaining portion of Ps2 overlaps Bg3. Bg2 contains a single XhoI site 2.7 kb from one end. Because Ps2 hybridizes to Xh2 but does not contain an XhoI site I conclude that Ps2 lies within Xh2 and that the XhoI site located in Bg2 is the left end of Xh2; the right end of Xh2 coincides with the XhoI site in Bg9.

The distance between the SalI site in $Bg1_a$ and the SalI site in Ps2 is 23 kb. Sa4 is 23 kb, hybridizes with Ps2 and Ps3, and has been placed in this position. Similarly, a BamHI fragment of ca. 28 kb is required to extend from the left end of Ba8 to the BamHI site located in Ps2. Ba3 is 28.1 kb, hybridizes to Ps2 and Ps3, and has been placed in this position.

iv. Ordering the restriction fragments of orientation II

The rationale for orientation II is similar to that for orientation I. Orientation I resulted when the two 5.6 kb BglII-PstI fragments were chosen as the overlap between $Bg1_a$ and Ps3 and between Bg2 and Ps2. Once this choice was made the order of the remaining BglII fragments and of the XhoI, BamHI, and SalI fragments was determined by locations in Ps2 and Ps3 of the sites for those enzymes. Ps2 and Ps3 hybridize with $Bg1_a$ and Bg2. Orientation II can be deduced if the 5.6 kb BglII-PstI repeated fragments are considered to result from the overlap between $Bg1_a$ and Ps2 and between Bg2 and Ps3. Whereas the position of $Bg1_a$ and Bg2 are not altered, the positions of Ps2 and Ps3 are inverted with respect to each other. Xh5 can not be adjacent to Xh1 as it is in orientation I because that would place an XhoI site in Ps2 which does not have an XhoI site. Therefore, the order of the XhoI fragments must be inverted as well and their order in orientation II is Xh5-Xh2-Xh1. The BglII fragments were aligned, as in orientation I by using the XhoI sites. The order of the BglII fragments of orientation II is Bg2-Bg9-Bg12-Bg3-$Bg1_a$-Bg5-Bg11-$Bg4_a$. In this orientation the BamHI and the SalI sites in Ps2 are closer to the BamHI and the SalI sites in $Bg1_a$ and a smaller BamHI, 13.8 kb, and SalI, 18.5 kb, fragment is predicted. Because Ba6 (13.8 kb) hybridizes to Ps2 and Ps3 and is the correct size it was

placed next to Ba8 in orientation II. This localization of Ba6 was verified by mapping Ba6 with BglII, PstI, XhoI, and SalI. Digestion of Ba6 with BglII+PstI generates a 5.6 kb BglII-PstI fragment as expected. The SalI fragment Sa6 has been placed adjacent to Sa5, because it is the correct size, 18.5 kb, and is present in SalI digests with a stoichiometry of 0.5 (16), and because its placement adjacent to Sa5 is in agreement with the hybridization results (Table 9).

Thus far Ba6 and Sa6 are associated with orientation II and Ba3 and Sa4 are associated with orientation I. The remaining BamHI and SalI fragments present in the two conformations were assigned in the following manner. Ps2 and Ps3 hybridize to Sa1, Sa2, Sa3, Ba1, Ba2, Ba4, and Ba6. Ps7 hybridizes to Sa1, Sa2, Sa3, Ba1, Ba2, and Ba3. [Ba1 and Ba2 and Sa2 and Sa3 did not resolve in the gels used in these experiments.] Ps1 hybridizes to Ba1, Ba2, Sa2, Sa3. Ba2 and Sa3 contain Ps1 and overlap Ps7. Ps7 contains a BamHI site located 1.9 kb from a SalI site. From these results I conclude that Ps7 overlaps Ba2, a fragment that is present in both orientations, and either Ba1 (41.1 kb) or Ba3 (28.5 kb), depending upon the orientation of the cyanelle DNA. Similarly, Ps7 overlaps Sa3, a fragment that is present in both orientations, and either Sa1 or Sa2, depending upon the orientation of the DNA. The sum of the molecular weights of the orientation-specific BamHI fragments of orientation I should equal the sum of the

molecular weights of the orientation-specific fragments of orientation II. The sum of the molecular weights of Ba6 and Ba1, 54.9 kb is close to the sum of the molecular weights of Ba3 and Ba4, 54.5 kb. The sum of the molecular weights of Sa4 and Sa2, 52.2 kb, is close to the sum of the molecular weights of Sa6 and Sa1, 52.6 kb.

Xh7 (4.5 kb), Xh8 (4.4 kb), Bg10 (3.65 kb), Bg14 (1.2 kb), and Bg16 (0.4 kb) did not hybridize with Ba5, nor with any of the PstI fragments used as probes in the hybridization experiments. Double digests (BamHI+XhoI and SalI+XhoI) of cyanelle DNA suggest (Table 6) that neither Xh7 not Xh8 has a BamHI or a SalI site. There is a gap of 8.9 kb located between Xh6 and Xh2 in the map of orientation I and between Xh6 and Xh 5 in the map of orientation II. Because the sum (8.9 kb) of the molecular weights of Xh7 and Xh8 matches the size of this space, these fragments have been placed adjacent to Xh6 (Figure 15). The order of Xh7 and Xh8 relative to Xh6 has not been determined. Similarly, a gap of ca. 3.7 kb is present between $Bg6_b$ and Bg2. Bg10 has been placed in this position (Figure 15) because its size matches closely that of the space between $Bg6_b$ and Bg2, and because it contains an XhoI site as is expected of a fragment located at this position. To verify that Xh7, Xh8 and Bg10 are located as described (Figure 15), Bg10 was hybridized to cyanelle DNA that had been digested with PstI, BglII, BamHI+XhoI, SalI+XhoI, and

BamHI+SalI+XhoI. Bg10 hybridized with fragments BaXh9 (4.5 kb), BaXh10 (4.4 kb), SaXh10 (4.5 kb), SaXh11 (4.4 kb), BaSaXh9 (4.5 kb), and BaSaXh10 (4.4 kb). [BaXh9 and BaXh10, SaXh10 and SaXh11, and BaSaXh9 and BaSaXh10 did not resolve in the gels used in these experiments.] From these hybridization results and from the presence, in Bg10, of an XhoI site, it was concluded that Xh7 (4.5 kb) and Xh8 (4.4 kb) do not have BamHI or SalI sites, are overlapped by Bg10, and that the locations of Bg10, Xh7 and Xh8 as shown in Figure 15 are correct.

v. Location of the rbcL, rbcS, and atpB genes on the map of the cyanelle DNA

The rbcL and rbcS genes of the Cyanophora paradoxa cyanelle have been identified by heterologous hybridization of the cloned rbcL and atpB genes of maize to restriction fragments of cyanelle DNA (see Appendix A). Restriction fragments Ba5 and Ps4 contain the entire coding sequence of the cyanelle rbcL gene and the 5' ca.two thirds of the atpB gene. The remainder (3' end) of the atpB gene is located in Ba8 (Append A and Chapter 3). The exact locations of the 5' ends of the rbcL and atpB genes were determined by nucleotide sequencing (Chapter 3). The rbcS gene of the cyanelle was identified by comparing the predicted amino acid sequence of an open reading frame of 321 bp located 108 bp 3' from the coding sequence of the rbcL gene with the amino acid sequences of the rbcS genes of Anacystis (162)

and Anabaena (127). The locations of the rbcL, rbcS, and atpB genes on the restriction map of the cyanelle DNA of Cyanophora are shown in Figure 15.

Discussion

The physical map of the cyanelle DNA which has been constructed with the restriction endonucleases BamHI, SalI, BglII, XhoI and PstI is circular, with a molecular size of approximately 128 kb. I found, in agreement with earlier reports (16,17), that the cyanelle DNA of C.paradoxa exists in two conformations that differ in the relative polarity of the two single copy regions. The cyanelle rbcL, rbcS, and atpB genes are located in the larger single copy region, 17 and 22 kb from one of the inverted repeat segments (Figure 15). The locations of the BamHI and SalI sites in the map that I have constructed appear to correspond to those in the map presented by Bohnert and coworkers (16,17).

There are some areas in which the restriction map of the cyanelle genome that I have constructed is not as well supported by the data as it might be. One area of the map which is especially tenuous is the localization of Bg8 adjacent to $Bg4_b$ and of Xh4 adjacent to Xh9. Hybridizing BaSa6 to digests of cyanelle DNA could serve to verify the locations of these fragments. The placement of Xh4 adjacent to Xh1 is also not supported by the hybridization data

despite the fact that, based on the restriction map, Ba5 should hybridize with both Xh1 and Xh4. The reason for the failure of Ba5 to hybridize is not clear. The positions of Xh1, and Xh4 as well as Bg4, Bg6, Ba5, Ba7, BaSa12 and Sa8 could be verified by using Sa8 as a hybridization probe. Based on the restriction map Sa8 should overlap Xh4 to a greater degree than does Ba5 and might therefore hybridize more strongly to Xh4. As already noted in the paragraphs above, the results of the hybridization experiments cannot distinguish the BglII fragments contained in Bg1, Bg4, and Bg6. In order to localize these fragments by hybridization, double digests with BglII and a second enzyme are necessary. The results presented in Tables 8 and 10 suggest that BglII+XhoI digests could be used for this purpose.

The cyanelle DNA, like the chloroplast DNA of several plant species and Chlamydomonas, contains a repeat sequence arranged in an inverted orientation (15). Consistent with the presence of a repeated sequence in the cyanelle DNA, two cloned PstI fragments, Ps2 and Ps3, were found to hybridize not only to themselves but to each other. In addition, both recognize a similar set of fragments generated by other restriction enzymes. Digestion of Ps2 or Ps3 with BglII generates a 5.6 kb DNA fragment. Digestion of Bg2 or $Bg1_a$, both of which hybridize with Ps2 and Ps3, with PstI also produces a 5.6 kb fragment. Because Ps3 hybridizes to Bg9 and Bg12, but not to Bg3, whereas Ps2 hybridizes to Bg3 but

very weakly to Bg9 and not at all to Bg12, it can be concluded that Bg3, Bg9, and Bg12 contain unique sequences. Therefore, the two 5.6 kb BglII-PstI fragments common to Ps2 and Ps3 (see Figure 16) must contain the repeated sequence that gives rise to their cross hybridization. An examination of Figure 16 shows that the two 5.6 kb BglII-PstI fragments and the two Ps13 fragments are arranged in an inverted orientation. Ps2 and Ps3 hybridize to BaXh3, BaXh4, BaXh5. From this result I deduce that one copy of the repeated sequence (orientation I) extends from the BglII site at the $Bg1_a$/Bg9 border to the XhoI site at the Xh5/Xh1 border and that the other copy extends from the BglII site at the Bg2/Bg3 border to the XhoI site at the Xh2/(Xh7 or Xh8). Thus a minimum estimate of the length of the repeated segment is ca. 10 kb. Based on the hybridization results, the maximum size of the repeated segment is ca. 12.8 kb because when Bg10, which is adjacent to Bg2, was hybridized with cyanelle DNA digested with BglII, it hybridized only with itself, and, therefore, must have a unique DNA sequence. However an examination of Figure 16 suggests that the repeated segment can extend only up to the SalI site located to the right of Ps3 in orientation I or to the right of Ps2 in orientation II, because a SalI site is not present in the analogous position on the left side of the restriction map. Based on the restriction map, the maximum size of the repeat segment is ca. 11 kb, i.e., the distance from the BglII site to the SalI site. The smaller single

copy region located between the inverted repeat segments is comprised of Bg3, Bg12, and Bg9 and is 16.8 kb in size. The conclusions which I have drawn regarding the size of the repeated DNA segments, the orientation of the repeated segments, and the size of the smaller single copy region are in close agreement with the results obtained by Bohnert and coworkers (16,17).

The presence of a large inverted repeat sequence is an almost universal feature of chloroplast genomes (15). Kolodner and Tewari (94) predicted that, as a result of intramolecular recombination occurring between the duplicated regions, molecules containing an inverted repeat segment might be expected to exhibit a reversal of polarity of the single copy sequence that is located between the repeated segments. As has been shown here, the polarity of the unique sequences can be determined from restriction fragment analysis. The analysis requires the use of an enzyme that does not cleave within the inverted repeat but that does cleave asymmetrically within the unique regions (64). To date only the chloroplast DNA of Phaseolus vulgaris (122,129) and the cyanelle DNA of Cyanophora (16,17), and results presented here) have been shown to occur in two orientations. The repeated sequence of plant chloroplasts is generally ca. 20 kb (15) or approximately twice the size of duplicated sequence of the cyanelle. The difficulty in determining the polarity of the single copy region in

chloroplasts may be a reflection of the larger size of the repeat region which increases the probability that an enzyme will cleave within the sequence.

The occurrence of the rbcL (17,77), rbcS (17,77), and atpB (17) genes in the cyanelle DNA has been reported previously, but the locations of these genes on the restriction map of the cyanelle have not been shown. The relative locations of the rbcL and atpB genes and their presence in the larger single copy region of the cyanelle DNA is very similar to the arrangement and locations of these genes in the chloroplast DNAs of higher plants. The presence of the rbcS gene in the cyanelle DNA is, thus far, unique among organelles. Two previous reports (17,77) have shown that under conditions of low hybridization stringency, the small subunit of pea hybridizes to Bg5 but not to Bg11. The rbcS gene, which I have located by DNA sequencing (Chapter 3), is located in Bg11 (Figure 15). Bg11 did not hybridize with the small subunit of pea (17,77) and I therefore believe that the small subunit that I have identified from its DNA sequence, is distinct from the sequence identified (17,77) on the basis of DNA hybridzation.

CHAPTER V

CHAPTER V

SUMMMARY AND GENERAL DISCUSSION

Summary

The genes for the large (rbcL) and small (rbcS) subunits of ribulose-1,5-bisphosphate carboxylase (RUBISCO) and the β subunit (atpB) of the ATP synthase F_1 complex have been localized in the cyanelle DNA from Cyanophora paradoxa. The nucleotide sequences of the rbcL and the rbcS genes, their flanking regions, and approximately 210 nucleotides of the coding sequence of the atpB gene have been determined (Chapter 3). Based on the DNA sequence, the coding sequence of the cyanelle small subunit is located on the same DNA strand as the large subunit 108 basepairs (bp) from its termination codon; the large subunit and the β subunit are separated by 481 bp and are transcribed divergently.

The large subunit of RUBISCO of Cyanophora is comprised of 475 amino acids and has a calculated molecular weight of 52,800 daltons. This value agrees well with estimates, 51,000 daltons (40) and 56,000 daltons (30), based on the electrophoretic mobility of the purified protein. The amino acid sequence of the large subunit is ca. 80% homologous

with the amino acid sequences of large subunits from eukaryotes (47,116,161,190) and cyanobacteria (45,137,163). In the region of the protein surrounding the residues involved in activation of the enzyme by CO_2 and in catalysis, the homology with other large subunits is nearly 100%.

The amino acid sequence of the cyanelle small subunit more nearly resembles the small subunits of cyanobacteria than the nuclear encoded small subunits of plants and eukaryotic green algae. The cyanelle small subunit is 57% homologous with the small subunit of Anabaena (127) and 50% homologous with the small subunit of Anacystis (162), whereas its homology with plant small subunits (7,8,29,115,124) is 41-44%. In that part of the polypeptide bounded by serine 43 and valine 52 of the Cyanophora sequence, the plant small subunits (7,9,27,115,124) have an insertion of 11 or 12 amino acids that are absent from the cyanelle and cyanobacterial small subunits. In addition, the cyanelle and cyanobacterial small subunits lack the transit sequence that is present in the nuclear-encoded small subunits of plants.

In plants and eukaryotic green algae the large subunit is encoded in the chloroplast DNA (34) and synthesized in the chloroplast (25,41), whereas the small subunit is encoded in the nuclear DNA (88) and synthesized in the cytoplasm (33,39,44,81). Cyanophora is the first eukaryote

in which sequences for both subunits of RUBISCO are present in the organelle DNA. The arrangement in the cyanelle DNA of the rbcL and rbcS genes is very similar to the arrangement of these genes in the cyanobacteria Anabaena (127) and Anacystis (162). In Anabaena (127), the rbcL and rbcS genes are separated by 545 bp, whereas in Anacystis (162), the intergenic distance is 93 bp. The rbcL and rbcS genes of Anabaena are cotranscribed (127); cotranscription of the rbcL and rbcS genes has been proposed (162), but not yet demonstrated, to occur in Anacystis.

In contrast to the cyanobacteria-like arrangement of the cyanelle rbcS and rbcL genes, the relative locations and directions of transcription of the cyanelle atpB and rbcL genes are similar to those of several plant chloroplasts [tobacco (55,160), petunia (28), spinach (180), and maize (96)] in which the distance between coding sequences of these genes ranges from 759 bp to 817 bp. The locations of the rbcL and atpB genes have been investigated in only a single cyanobacterium, Anabaena; the genes are not adjacent (45).

In general the chloroplast genomes of plants and eukaryotic green algae are circular, range in size from 85 to 195 kilobasepairs (kb), and contain two single copy regions of unequal size separated by two repeated DNA segments that are arranged in an inverted orientation (15). Each repeated segment contains one copy of the ribosomal RNA

genes (15). Known exceptions to this organization are the chloroplast genomes of broad bean (92), pea (37) and Euglena (67) which vary in the copy number and the orientation of the DNA segment which contains the ribosomal RNA genes. It has been suggested (94) that intramolecular recombination within the repeats might lead to a reversal of the polarity of the single copy segments and result in a population of chloroplast DNA molecules that differ in the relative orientation of the single copy regions. With the exception of the chloroplast DNA of Phaseolus vulgaris (122,129) which does exist in two conformations, the orientation of the single copy regions of chloroplast DNAs have not been determined.

The cyanelle DNA was digested with the restriction endonucleases BglII, XhoI, PstI, BamHI, and SalI and a restriction map was constructed (Chapter 4). The size of a monomer of cyanelle DNA, estimated by summing the molecular weights of the fragments produced by restriction of the cyanelle DNA, is approximately 128 kb. The restriction map of the cyanelle DNA is circular. A segment of the cyanelle DNA of approximately 10 kb is present twice in the cyanelle DNA. The duplicated segments are arranged in inverted orientation and are separated by DNA segments of unequal size that are unique in sequence. The cyanelle rbcL, rbcS, and atpB genes are located in the larger single copy region approximately 17 kb from one of the inverted repeats

(Appendix A, Chapter 4). The cyanelle DNA, like the chloroplast DNA of Phaseolus vulgaris (122,129), occurs in two conformations, which differ in the relative orientation of the single copy regions. These results are in agreement with those of Bohnert and coworkers (14,16,17,103,123).

In order to identify the sites of synthesis of cyanelle proteins, cyanelle proteins were labeled in vivo in the presence of inhibitors specific for cyanelle or cytoplasmic protein synthesis. Preliminary results suggest that cyanelle proteins are synthesized both in the cyanelle and in the cytoplasm. Among those polypeptides which may be synthesized in the cyanelle are the large subunit of RUBISCO, the α and β subunits of the ATP synthase and the 32,000 dalton membrane protein (psbA gene product).

General Discussion

The evolutionary status of the cyanelle has been problematic because the cyanelle has characteristics of both cyanobacteria and chloroplasts. In the introduction to this thesis it was suggested that the biosynthesis of chloroplast proteins, particularly RUBISCO, is a good paradigm for the integration of chloroplast and nuclear genome function and that an investigation into the location of the genes and the sites of synthesis of the subunits of RUBISCO might help to clarify the evolutionary position of the cyanelle. The results presented in this thesis do not prove that the large and small subunits of RUBISCO are synthesized in the cyanelle. However, in view of the presence in the cyanelle DNA of sequences for the large and small subunits of RUBISCO, the similarity of their arrangement to that of the cyanobacteria (the presumed ancestors of the cyanelle), and the synthesis by the cyanelle of a polypeptide having a molecular weight similar to that of the large subunit, it seems likely that RUBISCO is encoded in the cyanelle DNA and synthesized on cyanelle ribosomes.

If the location of the biosynthesis of the subunits of RUBISCO is a good measure of the integration of chloroplast and nuclear genome function, then the results presented in this thesis do not support the idea that the cyanelle is extensively integrated into the host. However, the biosynthesis of RUBISCO may not be such a measure. That is,

whereas the biosynthesis of this enzyme in other photosynthetic eukaryotes is an excellent example of cooperation by the chloroplast and nuclear genomes, the synthesis of both subunits by an organelle does not necessarily imply that the organelle is poorly integrated or semiautonomous.

All photosynthetic eukaryotes studied previously have separated the genes for the large and the small subunits of RUBISCO into different cellular compartments, i.e., the chloroplast and the nucleus. However only a relatively narrow spectrum of plant and algal species has been investigated. In particular, no chloroplast which contains biliproteins (e.g., the red algal chloroplast) has been studied. The results from comparisons of 16S ribosomal RNA (rRNA) catalogues have demonstrated that chloroplasts are polyphyletic (66), that is, chloroplasts are derived indepedently from distinct prokaryotes. The comparison of 16S rRNA catalogues of the red alga Porphyridium cruentum (19), Lemna (183), and Euglena (187) suggests that these chloroplasts have different prokaryotic ancestors (66). The chloroplast of Porphyridium was found to be especially closely related to cyanobacteria (19). Because the cyanelle has phycobilin pigments (35,174) and peptidoglycan (1), and is similar both in ultrastructure (60,70,174) and in the arrangement of the rbcL and rbcS genes (Chapter 3) to cyanobacteria, it seems almost certain to be derived from a

cyanobacterium. Although data for a quantitative comparison are not yet available, the red algal chloroplast and the cyanelle are probably more closely related to each other than either is to plant (and green algal) chloroplasts and their progenitors. Because the cyanelle and plant and green algal chloroplasts are likely polyphyletic and because there is no obvious reason why the pathway from endosymbiont to organelle should be the same for organelles derived from different prokaryotes, it is perhaps not surprising that the cyanelle differs from chloroplasts in synthesizing both subunits of RUBISCO. It is likely that as additional diverse photosynthetic eukaryotes are studied, choroplasts will be found, perhaps among the red algae, in which the chloroplast DNA contains the genes for both of the subunits of RUBISCO.

Can a conclusion be drawn regarding the evolutionary status of the cyanelle? In addition to the striking similarities between the organization of the cyanelle genome and the genome of chloroplasts, there is a growing body of evidence suggesting that the cyanelle is well integrated into the metabolism of Cyanophora. The coding capacity of the cyanelle genome is equivalent to that of plant and green algal chloroplasts implying that the number of cytoplasmically-synthesized cyanelle proteins may be comparable to the number of cytoplasmically-synthesized chloroplast proteins. Whereas the results from in vivo labeling of cyanelle proteins do not permit an accurate

estimate to be made of the number of cytoplasmically-synthesized cyanelle proteins, they do suggest that many cyanelle proteins are synthesized in the cytoplasm. Kremer et al. (97) and Trench et al. (174) found that carbohydrate is exported from the cyanelle to the cytoplasm. That a flow of carbon also takes place from the cytoplasm to the cyanelle is implied by the in vivo labeling of cyanelle proteins with exogenous carbon compounds. Floener et al. (54) and Böttcher et al. (24) found that nitrate reductase is localized in the cytoplasm. In addition, Floener et al. (54) found that nitrite reduction occurs in the cyanelle. On the basis of their results they suggested that the pathway of nitrate assimilation is partitioned between the cytoplasm and the cyanelle in the same manner as nitrogen assimilation is partitioned between the chloroplast and cytoplasm in plants. Thus, there is evidence suggesting that the cyanelle and the cytoplasm cooperate in some important metabolic processes. That is, despite the presence of the genes for both subunits of RUBISCO in the cyanelle DNA, I think that one must conclude that the cyanelle is a highly integrated organelle.

Whether or not the cyanelle is a chloroplast is a matter of choice. The major reason for not considering the cyanelle a chloroplast is the presence of peptidoglycan. Because peptidoglycan is a component of prokaryotic cell walls, it has been assumed that it is synthesized by the

cyanelle. In fact, the biosynthesis of the cyanelle peptidoglycan has not been studied. The transfer of genes from an endosymbiont to the host nucleus could be a means both of integrating the partners and of eliminating biosynthetic pathways of the endosymbiont which are no longer required. Compared to free-living cyanobacteria (78), the coding capacity of the cyanelle genome is much reduced (by ca. 90%) and it is quite possible that the biosynthesis of peptidoglycan is no longer solely a cyanelle function.

APPENDICES

APPENDIX A

APPENDIX A

MOLECULAR CLONING OF THE CYANELLE *rbcL* AND *rbcS* GENES AND THEIR FLANKING REGIONS

Introduction

This appendix describes the molecular cloning of the genes for the large (*rbcL*) and small (*rbcS*) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) and of a portion of the gene (*atpB*) for the β subunit of the ATP synthase from *Cyanophora paradoxa*. Fragments of cyanelle DNA that contain the large subunit and the β subunit genes were identified by heterologous hybridization with ^{32}P-labeled fragments of maize chloroplast DNA that contain the sequences of the *atpB* and *rbcL* genes. The small subunit was identified by comparing the deduced amino acid sequence of an open reading frame, located 108 bp 3' from the coding sequence of the large subunit, with the amino acid sequence of the small subunit of *Anacystis* (162).

Materials and Methods

The isolation of cyanelles, the isolation of cyanelle DNA, digestion of cyanelle and plasmid DNA with restriction endonucleases, and electrophoresis were performed as described in the Materials and Methods section of chapter 4.

DNA probes

Two cloned fragments of maize chloroplast DNA containing portions of the gene (rbcL) for the large subunit RUBISCO were used as probes in heterologous hybridizations. To localize the 5' end (mRNA sense) of the large subunit, pY440 (obtained from Dr. L. McIntosh, Michigan State University) was used. This plasmid carries a 440 basepair (bp) EcoRI fragment of maize chloroplast DNA (116) that encodes the first 140 amino acids of the large subunit protein. To identify cyanelle DNA fragments containing the 3' end of the large subunit coding sequence, the 840 bp PstI-BglII fragment (obtained from Dr. L. McIntosh) of maize chloroplast DNA (116) that encodes the 3' half of the large subunit protein and ca. 160 bp of 3' flanking sequence was used. To identify cyanelle DNA fragments that contain the β subunit of the ATP synthase, two recombinant plasmids, pZR48 (96) and pBS40, were used. pZR48 (obtained from Dr. L. McIntosh) carries the 3.8 kilobasepair (kb) EcoRI

fragment of maize chloroplast DNA that contains the entire sequence of the β and ε subunits of the ATP synthase. The plasmid pBS40 (obtained from J. Fitchen, Michigan State University) carries a 1040 bp HincII-BamHI fragment of maize chloroplast DNA (96) that encodes the first 233 amino acids plus 335 nucleotides of 5' flanking sequence of the β subunit of the ATP synthase.

Nick translation and filter hybridization

DNA was labeled in vitro with α-[^{32}P] CTP by nick translation (111). Millipore filter paper (HA,0.45 μm) containing immobilized DNA to be hybridized with heterologous DNA was first incubated for 1-2 hr in 6x SSPE plus 0.3 % sodium dodecyl sulfate (SDS) and 150 μg denatured sheared salmon sperm DNA (ssDNA) per ml at 60°C. 1x SSPE is 0.18 M NaCl, 10 mM $NaPO_4$ (pH 7.7), 1 mM ethylenediaminetetraacetic acid (EDTA). Hybridization was performed in 6x SSPE plus 0.3 % SDS and 100 μg ssDNA/ml at 60° for 16-24 hr. Following hybridization, the filters were first washed 3 times in 2x SSPE plus 0.1 % SDS at 25°C for 15 min and then washed 2 times in 0.1 % SSPE plus 0.1 % SDS at 25°C for 15 min. Autoradiographs were prepared using KODAK XAR-5 film exposed at either 25°C, without an intensifying screen, or at -80°C in the presence of an intensifying screen (Dupont Cronex Quanta III).

Nomenclature

Each fragment of cyanelle DNA is denoted by the first two letters of the restriction enzyme used to generate the fragment and by a number. The largest fragment of each digest is denoted with the number "1". Two DNA fragments that differ in sequence but are identical in size are distinguished by an alphabetical subscript.

Isolation of cyanelle DNA fragments

Cyanelle DNA was digested with EcoRI, BamHI, BamHI+SalI, or BglII+XhoI and the DNA fragments separated by subjecting them to electrophoresis in either 0.5 % agarose (BamHI and BamHI+SalI digests) or 0.8 % agarose (EcoRI and BglII+XhoI digests) gels. Because Bg10 and Bg11 do not resolve on preparative gels, a double digest with BglII and XhoI (XhoI cleaves Bg10 but not Bg11) was used when Bg11 was to be isolated. DNA fragments were recovered from agarose slices by electroelution into TBE buffer (89 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.3, 2.5 mM EDTA, 89 mM boric acid). An agarose slice containing the DNA band was placed in a trough cut into a 25 cm 1 % agarose slab gel and lined with a single thickness of dialysis membrane (prepared by boiling twice in 2.5 mM EDTA, rinsing in double distilled water, and autoclaving in

0.25 mM EDTA). TBE buffer sufficient to cover the agarose slice was added and electrophoresis was performed at 175-200 V until the DNA was eluted from the agarose slice. Isolated DNA fragments were purified by extracting 2 times with phenol, 3 times with phenol/chloroform (1:1, v/v), and 3 times with chloroform. The DNA was precipitated with sodium acetate and ethanol at -70°C. The precipitated DNA was washed 3 times with 80 % ethanol, dried under vacuum (water aspirator) and resuspended in 10 mM Tris, pH 8.0, 0.1 mM EDTA.

Molecular cloning

pCp001 was cloned as follows. The vector pBR328 (166) was digested with EcoRI and dephosphorylated by treatment with 0.01 unit of calf intestinal alkaline phosphatase (CIAP)/µgDNA for 1 hr at 37°C. Approximately 300 ng of EcoRI fragment was mixed with 25 ng of pBR328 and the solution was immediately extracted once with phenol and 3 times with chloroform. The DNA was precipitated with sodium acetate and ethanol. The precipitated DNA was washed 3 times with 80 % ethanol. Ligation was carried out in ligase reaction buffer (66 mM Tris, pH 8.0, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 66 µM ATP) with 1 unit T4-DNA ligase at 4°C for 20 hr. Cells of *E.coli* HB101 (pro *lac*Y Sm^r *endo*I *rec*A^- r^- m^-) were made competent for transformation according to the method of Mandel and Higa (109). To

transform E.coli the DNA in ligation buffer was added to 200 µl of cells at 4°C and incubated for 30 min at 4°C followed by either 2 min at 42°C or 10 min at 37°C. One ml of LB (Luria-Bertani) medium was added and the cells were incubated for 1 hr at 37°C with shaking. One liter of LB medium contains 5 g Bacto-yeast extract, 10 g Bacto-tryptone, and 5 g NaCl. The cells were concentrated by centrifugation and spread onto agar plates containing 75 µg ampicillin/ml. pCp024 was cloned as follows. The vector pBR322 (18) was digested with EcoRI in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl for 12 hr. One µl of 0.5 M Glycine-NaOH, pH 9.5 and 100 units of bacterial alkaline phosphatase (BAP) were added and the solution was incubated at 65°C for 1 hr. Insert DNA was prepared by digesting the BaSa8 fragment of cyanelle DNA with EcoRI. Approximately 450 ng of digested BaSa8 was combined with 250 ng of dephosphorylated pBR322 and the solution was extracted once with phenol and 3 times with chloroform. The DNA was precipitated with sodium acetate and ethanol. The precipitated DNA was washed three times with 80 % ethanol and dried under vacuum (water aspirator). The ligation was performed in ligase reaction buffer with 1 unit T4-DNA ligase at 4°C for 10.5 hr. The ligated DNA was transformed into frozen transformation-competent cells of E.coli HB101. Frozen transformable cells of E.coli HB101 and E.coli ED8654 were prepared according to the procedure of Hanahan (71) modified by omitting glycerol and cobaltic hexamine chloride

from the transformation buffer for frozen storage of competent cells (FSB). Transformation of frozen competent cells was performed by adding 2-5 µl of the ligation reaction mixture to 210 µl of cells that had been thawed and kept at 4°C. The mixture was incubated at 4°C for 30 min followed by 2 min at 42°C. One ml of LB medium was added and the cells were incubated for 1 hr at 37°C with shaking. The cells were concentrated by centrifugation and spread onto agar plates containing 75 µg ampicillin/ml. pCp025 was cloned as follows. The vector, pKC7 (136), was digested with BamHI+BglII and dephosphorylated with BAP as described above. Approximately 1.2 µg of the Ba5 fragment of cyanelle DNA was digested with BglII and combined with 0.38 µg of dephosphorylated pKC7. The solution was extracted once with phenol and 3 times with chloroform. The DNA was precipitated with sodium acetate and ethanol. The precipitated DNA was washed 3 times with 80 % ethanol and dried under vacuum (water aspirator). Ligation, transformation and selection of transformed cells were performed as for pCp024. pCp029 was cloned as follows. Approximately 400 ng of the Bg11 fragment of cyanelle DNA was digested with EcoRI+SphI and mixed with 80 ng of pBR322 which had been digested with EcoRI+SphI. The solution was extracted once with phenol, twice with phenol/chloroform (1:1, v/v), and three times with chloroform. The DNA was precipitated by adding 0.1 volume of 10 M LiCl and 2 volumes ethanol. The precipitated DNA was washed three times with

80 % ethanol and dried under vacuum (water aspirator). Ligation was performed in ligase reaction buffer with 1 unit of T4-DNA ligase at 4°C for 12 days. The ligated DNA was transformed into frozen competent cells of E.coli ED8654 (hsd r^- m^+ supE supF met^- gal^-) as described above. Transformants were selected on 75 µg ampicillin/ml. PstI and BamHI fragments of cyanelle DNA were cloned as described in the Materials and Methods section of chapter 4.

Sources of the enzymes

Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim Corporation, New York, NY. E.coli DNA polymerase I, T4 DNA ligase, bacterial alkaline phosphatase, BamHI, EcoRI, HindIII, HinfI, PstI, SalI, SphI, and XhoI were obtained from Bethesda Research Laboratories, Gaithersburg, MD. BglII was purchased from New England Biolabs, Beverly, MA.

Results

i. Location of the DNA fragments containing the large subunit of RUBISCO and the β subunit of ATP synthase

To determine which restriction fragments contain the genes for the large subunit of RUBISCO and the β subunit of the ATP synthase, nitrocellulose blots of cyanelle DNA digested with PstI, EcoRI, or BamHI were hybridized with

^{32}P-labeled pY440, the 840 bp PstI-BglII fragment, pZR48 or pBS40. pY440, a probe specific for the 5'end of the large subunit, hybridized with a 10.25 kb PstI fragment (Ps4), a 15.5 kb BamHI fragment (Ba5), and two EcoRI fragments 2.5 kb (Ec16) and 3.2 kb (Ec14) (Figures 17 and 18). When the 840 bp PstI-BglII fragment of maize chloroplast DNA containing the 3' half of the large subunit gene was hybridized with cyanelle DNA, Ps4 and Ba5 hybridized (Figure 19) but Ec16 did not hybridize (data not shown). pZR48, which contains the genes for the β and ε subunits of the maize chloroplast ATP synthase, hybridized (Figures 17 and 18) to fragments Ba5, Ps4, Ec14, Ec16, Ps17 (1.8 kb), and Ba8 (9.5 kb). pBS40, which contains the 5' half of the coding sequence of the β subunit from maize chloroplast DNA, hybridized (Figure 20) with Ps4, Ps17, Ec14 and Ec16.

Restriction endonuclease mapping of Ba5 and Ps4 (discussed below) together with the results of hybridization experiments, suggest that both Ps4 and Ba5 contain the entire sequence of the large subunit of RUBISCO. Ps4 and Ba5 also hybridized with probes for the β subunit and therefore contain a portion of the β subunit. The part of the β subunit not contained on Ps4 and Ba5 is contained on Ps17 and Ba8. Because probes for 5' ends of the coding sequences of the large subunit of RUBISCO and the β subunit of the ATP synthase hybridized with an EcoRI fragment, identified as Ec16, and because Ec16 is relatively small, 2.5 kb, it

Figure 17. Hybridization of pY440 and pZR48 to cyanelle DNA. Cyanelle DNA was digested with PstI or EcoRI and the DNA fragments were separated by electrophoresis in an agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized with pY440 or pZR48. 1: Cyanelle DNA digested with PstI. 2: Autoradiograph of cyanelle DNA digested with PstI and hybridized with pY440. 3: Autoradiograph of cyanelle DNA digested with PstI and hybridized with pZR48. 4: Cyanelle DNA digested with EcoRI. 5:Autoradiograph of cyanelle DNA digested with EcoRI and hybridized with pY440. 6: Autoradiograph of cyanelle DNA digested with EcoRI and hybridized with pZR48.

Figure 18. Hybridization of pY440 and pZR48 to cyanelle DNA. Cyanelle DNA was digested with BamHI and the DNA fragments were separated by electrophorsis in an agarose gel. The DNA fragments were tranferred to nitrocellulose and hybridized with pY440 or pZR48. 1 & 3: Cyanelle DNA digested with BamHI. 2: Autoradiograph of cyanelle DNA digested with BamHI and hybridized with pZR48. 4: Autoradiograph of cyanelle DNA digested with BamHI and hybridized with pY440.

Figure 19. Hybridization of cyanelle DNA with the 3' half of the large subunit of RUBISCO from maize. Cyanelle DNA was digested with PstI or BamHI and the DNA fragments were separated in an agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized with the large subunit. 1: Cyanelle DNA digested with PstI. 2: Autoradiograph of cyanelle DNA digested with PstI and hybridized with the large subunit. 3: Cyanelle DNA digested with BamHI. 4: Autoradiograph of cyanelle DNA digested with BamHI and hybridized with the large subunit.

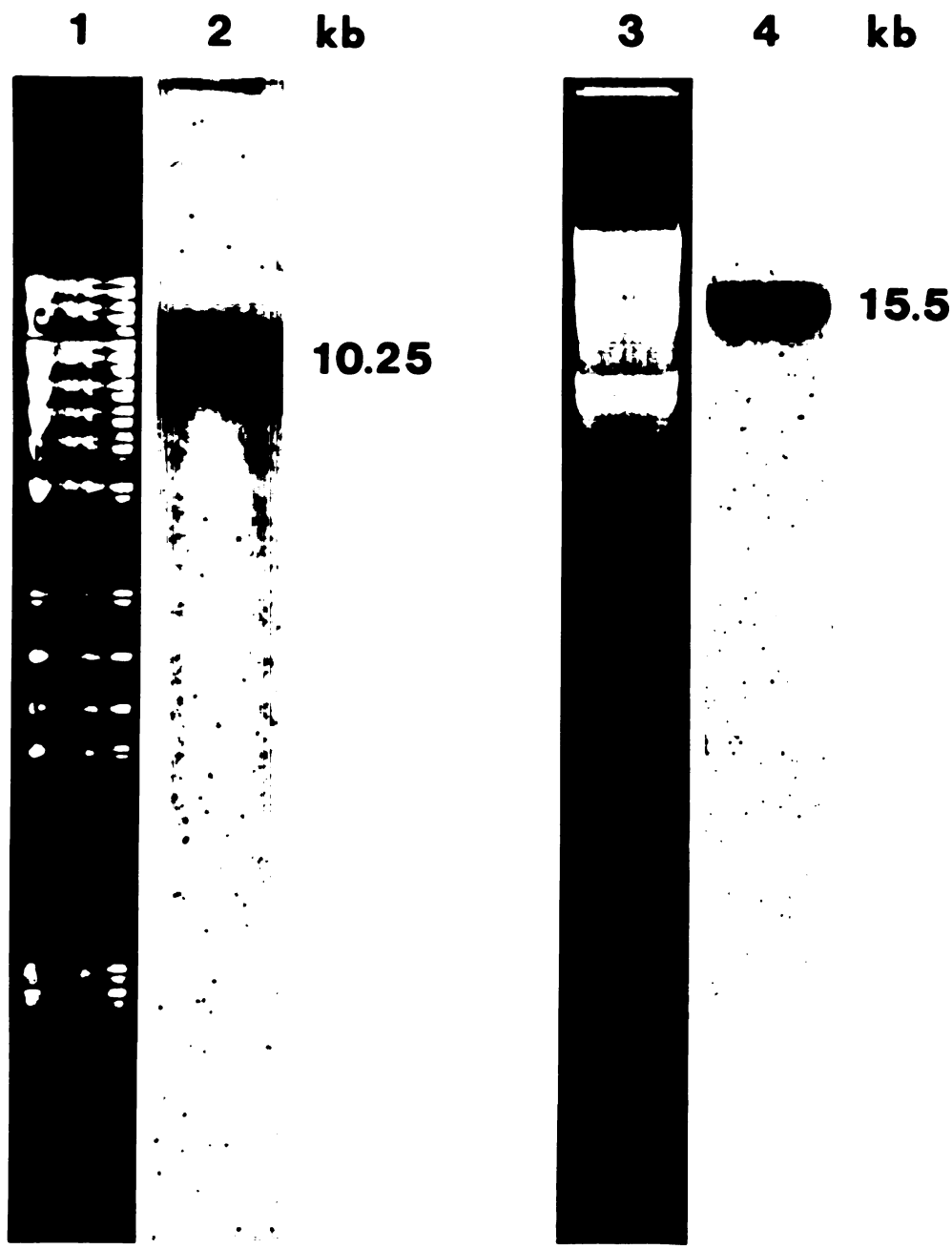
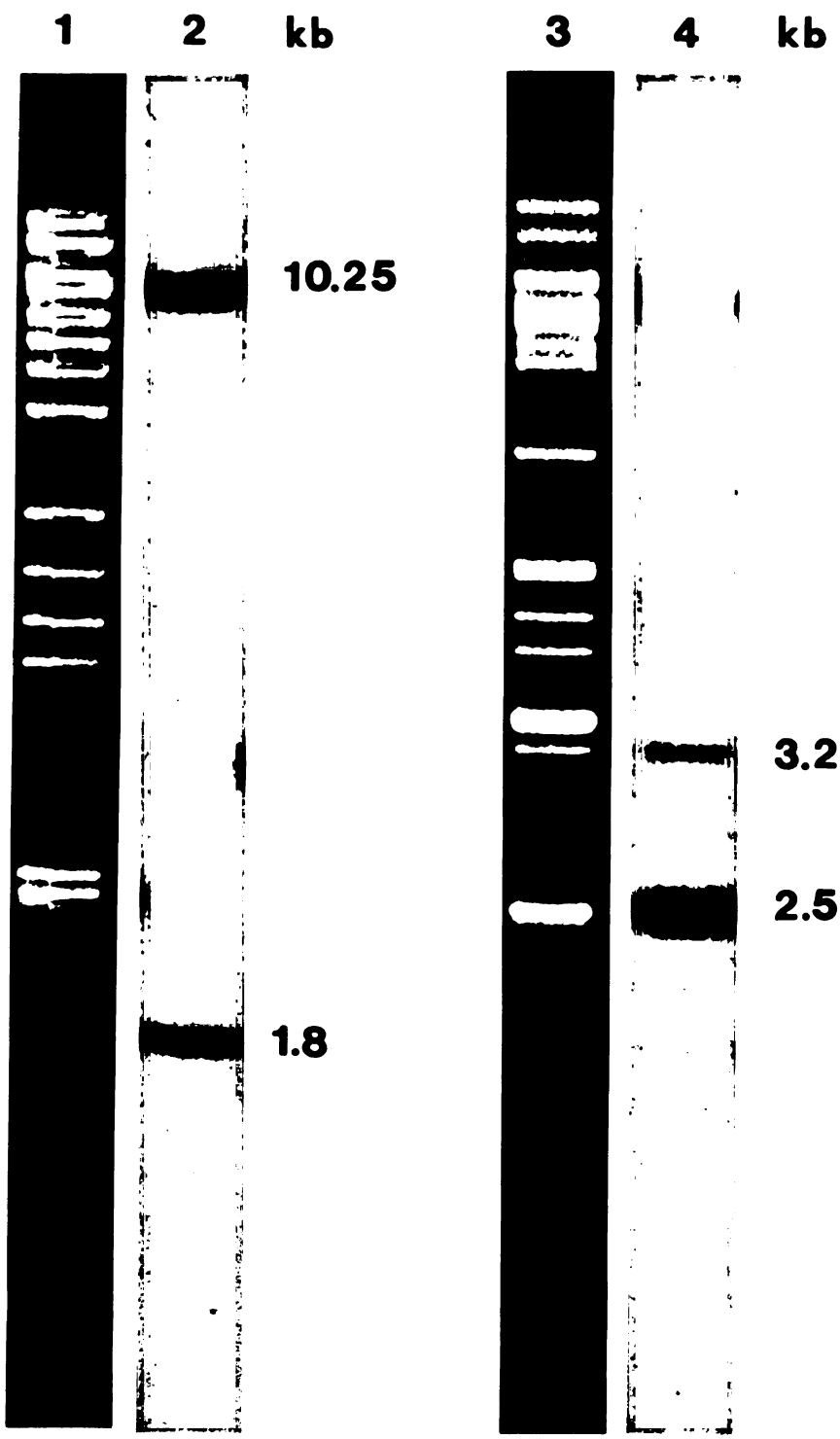

1
2
kb
10.25
3
4
kb
15.5

Figure 20. Hybridization of cyanelle DNA with pBS40. Cyanelle DNA was digested with PstI or EcoRI and the DNA fragments were separated by electrophoresis in an agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized with pBS40. 1: Cyanelle DNA digested with PstI. 2: Autoradiograph of cyanelle DNA digested with PstI and hybridized with pBS40. 3: Cyanelle DNA digested with EcoRI. 4: Autoradiograph of cyanelle DNA digested with EcoRI and hybridized with pBS40.


1
2
kb
10.25
1.8
3
4
kb
3.2
2.5

was hypothesized that the 5' ends of these genes are located near each other in the cyanelle DNA. However, the hybridization experiments were not conclusive because there appeared to be several poorly resolved EcoRI fragments of ca. 2.5 kb. It was therefore possible that the probes for the large subunit and the β subunit hybridized with different EcoRI fragments. The smallest fragment which clearly, on the basis of the hybridization results, contains the large subunit and part of the β subunit is Ps4 (10.25 kb). If the two genes were located at opposite ends of this fragment they would be separated by 8-9 kb.

ii. Cloning the gene for the large subunit of RUBISCO

Numerous unsuccessful attempts were made to clone Ps4 (10.25 kb), the smallest DNA fragment that contained the copmplete sequence of the large subunit. Attempts to clone Ba5 (15.5 kb) were also unsuccessful. Though plasmids of 15-20 kb transform less efficiently than smaller plasmids (71), the size does not appear to be the major impediment to cloning Ps4 and Ba5. Larger PstI fragments [Ps1 (17.1 kb), Ps2 (12.75 kb), and Ps3 (11.25 kb)] have been successfully cloned in pBR322 (described in chapter 4). To test the hypothesis that a factor other than size was interferring with cloning, Ps3 and Ps4 were isolated together and ligated into the PstI site of pBR322. Of 49 colonies, that were tetracycline-resistant and ampicillin-sensitive, 24 had

recombinant plasmids that carried Ps3. The other 25 colonies either had plasmids that contained smaller PstI fragments or did not have plasmids. No plasmids carrying Ps4 were recovered. These results suggest that the failure to clone Ps4, and perhaps Ba5, is more likely related to the DNA sequence than to the size of the fragment.

The Cyanophora sequences identified as the rbcL and atpB genes on the basis of their hybridization with the rbcL and atpB sequences, hybridized under relatively stringent hybridization conditions. That hybridization occurred under stringent conditions suggests that the Cyanophora sequences share considerable homology with the maize sequences. Because the rbcL had been cloned from maize (41), spinach (190), tobacco (55), Euglena (170), and Chlamydomonas (108,47) and the atpB gene had been cloned from spinach (190,189) and maize (96), it seemed unlikely that the Cyanophora rbcL and atpB gene sequences were interferring with the cloning of of Ps4 and Ba5. Because no DNA fragment that contained the entire rbcL gene was successfully cloned (attempts to clone BaSa8 also failed) and because it was desirable to avoid fragments that contained excess flanking sequence that might inhibit cloning, the Cyanophora rbcL gene was cloned in pieces.

In order to clone the EcoRI fragments which hybridized with pZR48 and with pY440, cyanelle DNA was restricted with EcoRI and the DNA fragments separated by electrophoresis in

an 0.8 % agarose gel. Ec14, Ec15, Ec16, and Ec17, were isolated, purified, and ligated to dephosphorylated pBR328. Ec14, Ec15, and Ec16, which did not resolve on the preparative agarose gel, were treated as a single fragment. Three clones each containing a different EcoRI fragment (Ec15, Ec16 or Ec17) of ca. 2.5 kb and one clone carrying Ec14 (3.2 kb) were obtained. Plasmids were isolated from these clones, purified by equilibrium density centrifugation on CsCl-ethidium bromide gradients, restricted with EcoRI, blotted to nitrocellulose filter paper, and hybridized with a portion of the large subunit of RUBISCO (pY440) and with the β and ε subunits of the ATP synthase (pZR48) of maize. Ec14, Ec15, and Ec17 did not hybridize with pY440 or with pZR48 (data not shown). Ec16 hybridized with pY440 and pZR48. The recombinant plasmid that carries Ec16 was designated pCp001.

Because pZR48 and pBS40 hybridize to the same PstI, BamHI, and EcoRI fragments, and because pZR48 and pY440 hybridize to Ec16, I conclude that the 5' ends of the large subunit of RUBISCO and the β subunit of the ATP synthase are located in Ec16. pY440 and the 840 bp fragment of maize chloroplast DNA hybridized with a single PstI (Ps4) and a single BamHI (Ba5) fragment, whereas pZR48 and pBS40 hybridized with two PstI (Ps4 and Ps17) and two BamHI (Ba5 and Ba8) fragments. Digestion of pCp001 with BamHI, PstI, and HindIII showed that Ec16 contains a single site for each

enzyme. Restriction endonuclease mapping of Ec16 (pCp001) together with the results of hybridization experiments permitted Ec16 to be aligned relative to Ps4 and Ps17 and to Ba8 and Ba5. Figure 21B shows the position of Ec16 and other cloned fragments of cyanelle DNA that contain the large and small subunits of RUBISCO.

Figure 21A shows a restriction endonuclease map of Ba8 and Ba5, which was constructed in order to identify cyanelle DNA fragments that contain the 3' half of the rbcL gene and fragments that contain the rbcS gene. Because Ba5, Ps4, Bg11, and BaSa8 were not cloned, these fragments were isolated from agarose gels and redigested with restriction endonucleases to construct the map. Ba8 is carried by a recombinant plasmid designated pCp020.

Digestion of Ba5 with EcoRI generates fragments of ca. 9.6 kb, 2.36 kb, 2.3 kb, and 0.63 kb. When BaSa8, a DNA fragment that extends from the left end of Ba5 to the SalI site in Ba5 (Figure 21A), is digested with EcoRI, fragments of 5.5 kb, 2.3 kb, 0.63 kb and 0.27 kb are produced. The 0.27 kb EcoRI fragment was not observed when Ba5 was digested with EcoRI because it was electrophoresed off of the agarose gel. Digestion of pCp001 with BamHI generates fragments of 2.3 kb and 0.20 kb. From these results, I conclude that either the 0.63 kb or the 0.27 kb EcoRI fragment is adjacent to Ec16. DNA sequencing of Ec16 (results presented in chapter 3) localized the initiation

Figure 21. A. A restriction endonuclease map of the restriction fragments Ba8 and Ba5 of cyanelle DNA. The solid bars represent the locations of the rbcL, rbcS, and atpB genes with the 5' ends indicated. B. Restriction endonuclease fragments of cyanelle DNA carried by the recombinant plasmids pCp001, pCp024, pCp025, and pCp029.

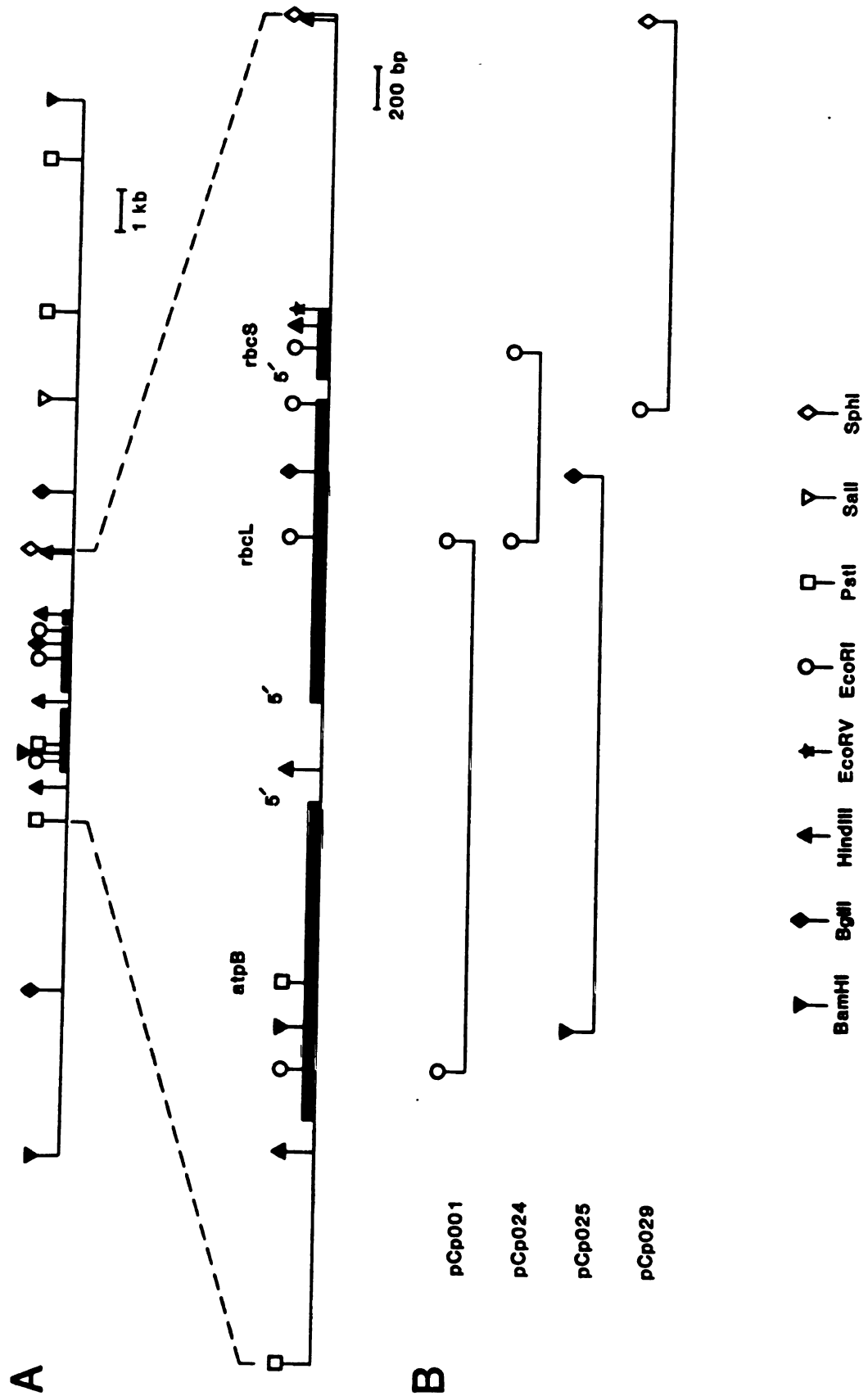

A
1 kb
atpB
rbcL
rbcS
5′
5′
5′
B
200 bp
pCp001
pCp024
pCp025
pCp029
BamHI
BglII
HindIII
EcoRV
EcoRI
PstI
SalI
SphI

Figure 21

codon (based on the homology of the derived amino acid sequence to that of maize) of the large subunit to a position 330 bp to the right of the HindIII site in Ec16. The coding sequences of the rbcL genes of plants, eukaryotic green algae, and cyanobacteria range from 1416-1431 bp and are highly conserved (45,47,116,137,161,163,190). The large subunit of Cyanophora hybridized with the maize large subunit under stringent hybridization conditions suggesting that it is homologous to the large subunit of maize and to large subunits of other species. It was expected, therefore, that the coding sequence of the cyanelle large subunit gene would be approximately the same size as that of other species. A coding sequence of 1431 bp would place the termination codon of the Cyanophora rbc approximately 0.67 kb to the right of the Ec16 fragment. It therefore seemed likely that remaining portion of the large subunit coding sequence was present on the 0.63 kb and 0.27 kb EcoRI fragments. In order to clone these fragments, the BaSa8 fragment of cyanelle DNA was partially digested with EcoRI and ligated to dephosphorylated pBR322. Colonies were screened by isolating plasmid from 5-ml cultures and digesting it with EcoRI. In this manner a recombinant plasmid (designated pCp024) carrying both the 0.63 kb and the 0.27 kb EcoRI fragments was selected.

Localization of the BglII sites in Ba5 and BaSa8 (Figure 21A) indicated that a BglII site is located ca.

2.6 kb from the left end of Ba5, i.e., ca. 0.3 kb to the right of Ec16. Restriction of pCp024 with EcoRI generates fragments of 0.63 kb and 0.27 kb, whereas restriction with BglII+EcoRI generates fragments of 0.31 kb, 0.32 kb, and 0.27 kb. From this result it was hypothesized that Ec16 and the 0.63 kb fragment are adjacent in the cyanelle DNA. In order to verify that these fragments are adjacent, the 2.6 kb DNA fragment that extends from the BamHI site in Ec16 to the BglII site in the 0.63 kb EcoRI fragment was cloned in pKC7. Transformants were screened by isolating plasmid from 5-ml cultures and digesting it with BamHI+BglII. The recombinant plasmid that carries the 2.6 kb BamHI-BglII fragment was designated pCp025.

In order to verify that the DNA fragment carried by pCp025 overlaps Ec16 and the 0.63 kb EcoRI fragment of pCp024, pCp025 was restricted with EcoRI+BglII, EcoRI+HindIII, and BamHI+HindIII, pCp001 was restricted with BamHI+HindIII and EcoRI+HindIII, and pCp024 was restricted with EcoRI+BglII. Table 11 shows that digestion of pCp025 with BamHI+HindIII or with EcoRI+HindIII generates fragments of the same size as the fragments generated by restriction of pCp001 with BamHI+HindIII or with EcoRI+HindIII. Digestion of pCp024 with EcoRI+BglII produces fragments of 0.32 kb, 0.31 kb, and 0.27 kb, whereas digestion of pCp025 with EcoRI+BglII produces a fragment of 0.31 kb. From this result I conclude that the 0.63 kb EcoRI is adjacent to

Table 11. DNA fragments generated by digestion of recombinant plasmids pCp001, pCp024, and pCp025 with restriction endonucleases. Recombinant plasmids were restricted with EcoRI, EcoRI+BglII, BamHI+HindIII, and EcoRI+HindIII. The DNA fragments were separated by electrophoresis in a 1.5 % agarose gel. Phage λ DNA digested with HindIII and pBR322 digested with HinfI served as molecular weight standards. DNA fragments that consist of vector DNA or of vector plus insert DNA are not included in the table. Molecular weights are in kilobasepairs.

	restriction endonuclease			
plasmid	EcoRI	EcoRI+BglII	BamHI+HindIII	EcoRI+HindIII
pCp001	2.5	2.5	1.26	1.35, 1.12
pCp024	0.63, 0.27	0.31, 0.32, 0.27		0.63, 0.27
pCp025		0.31	1.26	1.12

Ec16, and that the 2.6 kb BamHI-BglII fragment overlaps Ec16 and the 0.63 kb EcoRI fragment. It could not be determined from these results if the 0.27 kb EcoRI fragment carried by pCp024 was cloned in the same orientation, relative to the 0.63 kb fragment, as in the cyanelle DNA.

iii. Cloning of the gene for the small subunit of RUBISCO

DNA sequencing of the large subunit of RUBISCO (results presented in chapter 3) showed that the 0.27 kb EcoRI fragment carried by pCp024 contains 21 bp of the 3' end of the large subunit, a spacer of 108 bp, and an open reading frame of 120 bp that is terminated by the EcoRI site at the right end of the fragment. The open reading frame was tentatively identified as part of the sequence of the small subunit of RUBISCO because its deduced amino acid sequence is 60 % homologous to the amino acid sequence of the small subunit from Anacystis (162). In order to clone a DNA fragment containing the complete coding sequence of the cyanelle rbcS, Bg11, the 3.6 kb BglII fragment located within Ba5, was isolated. Bg11 was digested with EcoRI+SphI and ligated to pBR322. Ampicillin-resistant colonies were screened by isolating plasmid from 5-ml cultures and digesting it with EcoRI+SphI. Three types of recombinant plasmids were found: plasmids that contained both the 0.27 kb EcoRI and the 1.5 kb EcoRI-SphI fragments; plasmids that contained only the 1.5 kb EcoRI-SphI fragment; and

plasmids that contained only the 0.27 kb EcoRI fragment. A recombinant plasmid, designated pCp029, containing both the 0.27 kb EcoRI fragment and the 1.5 kb EcoRI-SphI fragment was selected for use in the DNA sequencing of the small subunit of RUBISCO.

Discussion

The large subunit of RUBISCO and the β subunit of the ATP synthase have been localized on specific restriction fragments of cyanelle DNA by heterologous hybridization with portions of the genes encoding the large subunit and the β subunit from the chloroplast DNA of maize. Two recombinant plasmids, pCp001 and pCp024, which together contain the complete sequence of the large subunit of the cyanelle RUBISCO were constructed. The EcoRI fragment (Ec16) of cyanelle DNA carried by pCp001 codes for the 5' ends of the large subunit of RUBISCO and of the subunit of the ATP synthase. Pcp024 carries two EcoRI fragments, one of 0.63 kb and one of 0.27 kb. A third recombinant plasmid, pCp025, contains a 2.6 kb fragment of cyanelle DNA that spans the junction between the EcoRI fragment carried by pCp001 and the 0.63 kb fragment carried by pCp024. A DNA sequence identified as the sequence of the small subunit of RUBISCO has been cloned in a recombinant plasmid designated pCp029. pCp024 and pCp029 both contain the 0.27 kb EcoRI fragment of the cyanelle DNA that contains the 3' end of the

coding sequence of the large subunit, a spacer of 108 bp and the 5' end of the coding sequence of the small subunit.

The fragments of cyanelle DNA carried by the recombinant plasmids pCp001, pCp024, pCp025, and pCp029 were used in the DNA sequencing of the large and small subunits of RUBISCO and 210 nucleotides of the 5' end of the β subunit of the ATP synthase (results presented in chapter 3). Sequencing of Ec16 showed that it contains the 5' ends of both the β subunit and the large subunit corroborating the results of heterologous hybridizations between portions of the genes encoding the maize chloroplast large subunit and β subunit and the cyanelle DNA. The results from the heterologous hybridizations and from DNA sequencing are combined in Figure 21A which shows the locations of these genes on a restriction map of Ba5 and Ba8. The positions of the large and small subunits of RUBISCO and the β subunit of the ATP synthase are represented by solid bars with the 5' ends indicated.

The 3' end of the β subunit has not been localized. pZR48, which contains the complete sequence of the β and ε subunits of the maize chloroplast ATP synthase, hybridized to Ps17 and Ps4. Ps4 contains the 5' end of the β subunit which, based on the DNA sequence, is 160 bp to the left of the HindIII site in Ec16. The coding sequence of the gene for the β subunit of the maize chloroplast is ca. 1.5 kb. If the coding sequence of the cyanelle β subunit is of

comparable size to that of maize, then its 3' end would lie in Ps17 ca. 240 bp to the left of the Ec16. The position of the 3' end of the subunit as presented in Figure 21A was "localized" by assuming that its coding sequence is 1500 bp.

The identity of the 3.2 kb EcoRI fragment that hybridized with pZR48, pBS40, and pY440 is uncertain. When the 3.2 kb EcoRI fragment, Ec14, was cloned it did not hybridize with either pY440 or pZR48. An examination of Figures 17 and 20 shows that the hybridization of pY440, pZR48 and pBS40 to the 3.2 kb EcoRI fragment is weaker than their hybridization to Ec16. It is possible that in digests of cyanelle DNA Ec14 was contaminated with a small amount of Ec16 which was responsible for the hybridization. It is also possible that Ec14 was contaminated with a partial restriction product composed of Ec16 (2.5 kb) and the 0.63 kb EcoRI fragment or of Ec16 and the 0.63 kb and 0.27 kb EcoRI fragments.

APPENDIX B

APPENDIX B

GROWTH MEDIA FOR *CYANOPHORA PARADOXA*

Medium CYB

for 1000 mls

NH_4 acetate	200 mg
Na_{2++} βglycerophosphate	50 mg
Fe^{++} (as Cl)	0.4 mg
Ca (as Cl)	10 mg
KCl	30 mg
$NaNO_3$	255 mg
KNO_3	303 mg
$MgSO_4 \cdot 7H_2O$	100 mg
vitamin B_{12}	1 µg
CYB metal mix+	10 ml
vitamin mix S3++	5 ml
Bicine buffer	420 mg
pH 7.8	

+ CYB metal mix. 1 ml contains: H_3BO_3, 1.14 mg; $FeCl_3 \cdot 6H_2O$, 48 mg; $MnSO_4 \cdot 1H_2O$, 112 µg; $ZnSO_4 \cdot 7H_2O$, 22 µg; $CoSO_4 \cdot 7H_2O$, 4.8 µg; Na_2EDTA, 1 mg.

++ Vitamin mix S3. 1 ml contains: thiamine, 0.05 mg; biotin, 0.1 µg; *p*-aminobenzoic acid, 1 µg; folic acid, 0.2 µg; nicotinic acid, 0.01 mg; thymine, 0.3 mg; inositol, 0.5 mg; Ca pantothenate 0.01 mg.

Bold's 3N Bristols (169)

for 1000 mls

$NaNO_3$	75 mg
$CaCl_2$	25 mg
$MgSO_4 \cdot 7H_2O$	75 mg
K_2HPO_4	75 mg
KH_2PO_4	175 mg
NaCl	25 mg
P_{II} metals+	6 ml
vitamin B_{12} (150 µg/ml)	1 ml
soil extract (169)	40 ml

+ P_{II} metals. 1 ml contains: Na_2EDTA, 0.75 mg; $FeCl_3 \cdot 6H_2O$, 0.097 mg; $MnCl_2 \cdot 4H_2O$, 0.041 mg; $ZnCl_2$, 0.005 mg; $CoCl_2 \cdot 6H_2O$, 0.002 mg; Na_2MoO_4, 0.004 mg.

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