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Biochemistry

Biosynthesis and assembly of the proton-translocating adenosine triphosphatase complex from chloroplasts

(coupling factor/immunoprecipitation/*in vitro* synthesis/precursors/assembly)

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ABSTRACT **The H^+-translocating ATPase complex of chloroplasts consists of at least eight nonidentical subunits. Five of these (α, β, γ, δ, and ϵ subunits) collectively constitute the globular extramembranous CF_1 portion of the complex. The remaining three subunits (I–III) represent the membrane-embedded portion. Biosynthesis and assembly of these subunits were studied by pulse-labeling isolated spinach chloroplasts in the presence of cycloheximide or chloramphenicol and by translating total leaf RNA in a rabbit reticulocyte system. The labeled products were analyzed by immunoprecipitation with subunit-specific antisera or by isolating the entire H^+-translocating ATPase complex in a nearly pure state. We found that chloroplasts synthesize the α, β, γ, and ϵ subunits of CF_1, the membrane-embedded subunit I, and probably also the membrane-embedded subunit III. The δ subunit (and probably also subunit II) are imported from the cytoplasm via larger precursor forms. After isolated chloroplasts are labeled in the presence of cycloheximide, the chloroplast-made H^+-ATPase subunits are assembled into a complex that is indistinguishable from the authentic H^+-ATPase complex. This assembly indicates that isolated chloroplasts contain excess pools of the cytoplasmically made subunits.**

The proton-translocating ATPase complex of chloroplasts is an essential component of light-driven ATP synthesis. Like the analogous enzymes from mitochondria and bacteria, it consists of two major parts. One is a globular unit, termed CF_1, which is located on the outer surface of the thylakoid membrane and contains five different subunits (α, β, γ, δ, and ϵ; see ref. 1). The second unit, termed CF_0, is located in the membrane and contains three different subunits (I, II, and III). An isolation procedure for the chloroplast H^+-ATPase complex (i.e., the CF_1–CF_0 complex) has recently been reported (2, 3). If the isolated ATPase complex is incorporated into liposomes together with a purified preparation of photosystem I reaction center (4), it catalyzes the light-driven synthesis of ATP from ADP and P_i (5).

How is the H^+-ATPase complex formed *in vivo*? H^+-ATPases are among the most complicated enzymes known and their assembly *in vivo* thus poses a special challenge to the investigator. In addition, chloroplasts (like mitochondria) synthesize some of their own polypeptides (6, 7), and one would like to know whether some of these are subunits of the ATPase complex. Studies on the biosynthesis of CF_1 in three different laboratories have led to the conclusion that the α, β, and ϵ subunits of CF_1 (7–9) and subunit III (J. C. Gray, personal communication) are made by the chloroplast whereas γ and δ subunits are probably made outside and then imported (7–9).

Our own efforts to study the biosynthesis of a chloroplast H^+-ATPase were prompted by two considerations. First, we intended to study the site of synthesis of the various CF_1 subunits by recently developed specific immunoprecipitation methods by using antisera directed against each of the five CF_1 subunits. The earlier studies had relied on the chemical isolation of CF_1 from labeled cells or chloroplasts; this may cause complications by residual ribulosediphosphate carboxylase, whose large subunit (which is made in chloroplasts, ref. 7) has a molecular weight close to that of the CF_1 α and β subunits. Second, we hoped to follow the assembly of newly made CF_1 subunits into the entire H^+-ATPase complex by exploiting a procedure for isolating that complex on a small scale.

These technical improvements have indeed yielded new information. Here we show that isolated spinach chloroplasts also synthesize the CF_1 γ subunit and subunit I and that they can assemble these newly made H^+-ATPase subunits into an H^+-ATPase complex.

MATERIALS AND METHODS

Protein Synthesis by Isolated Chloroplasts. Spinach leaves were purchased from the local market, washed, placed on water, and illuminated overnight by a 150 W lamp from a distance of 1 m. Intact chloroplasts were prepared (10) and used within 30 min for protein-synthesis experiments. The reaction mixture contained in a volume of 0.8 ml: 80 μg of cycloheximide, 265 μmol of sorbitol, 5 μmol of Tris·HCl (pH 7.4), 40 μmol of KCl, 0.4 μmol of $MgCl_2$, 0.03 μmol of EDTA, 0.4 μmol of GTP, 4 μmol of ATP, 25 nmol of each of the 20 common amino acids except methionine, and 0.5 mCi of [^{35}S]methionine (100–300 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels). When indicated, 50 μg of chloramphenicol was also present. The reaction was started by addition of 0.2 ml of a chloroplast suspension containing 2 mg of chlorophyll per ml and was allowed to proceed for 45 min at 30°C with gentle shaking.

Immunoprecipitation. The reaction was stopped by the addition of 4 ml of cold acetone and immediate shaking on a Vortex mixer. After incubation for 10 min at 0°C, the suspension was centrifuged at $5000 \times g$ for 5 min and the supernatant was discarded. The pellet was suspended in 1 ml of a freshly prepared solution containing 1 mM each of *p*-aminobenzamidine, phenylmethylsulfonyl fluoride, *N*-tosyl-L-phenylalanyl chloromethane, and *N*-tosyl-L-lysyl chloromethane. Four milliliters of acetone chilled to −20°C was added and the suspension was incubated for 10 min at 0°C and centrifuged; the supernatant was discarded and the excess acetone was evaporated by a stream of nitrogen. (The two extractions by 80% acetone were necessary because residual chlorophyll prevented the interaction between the immune complexes and the *Staphylococcus* protein A in the subsequent immunoprecipi-

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Abbreviations: CF_1, chloroplast coupling factor 1; CF_0, membrane sector of the H^+-translocating ATPase from chloroplasts.

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tations.) The dry pellet was suspended in 0.7 ml of a solution containing 1 mM each of the different protease inhibitors and then solubilized by subsequent addition of 0.3 ml of a 20% (wt/vol) $NaDodSO_4$ solution. After incubation at room temperature for 15 min or at 100°C for 2 min, immunoprecipitation was carried out as described (11).

Assay for Assembly of Entire CF_1–CF_0 Complex. Chloroplasts were labeled with [^{35}S]methionine in a total volume of 5 ml as described above. The reaction was terminated by centrifugation at 10,000 × *g* for 10 min at 0°C. The pellet was homogenized in 2 ml of water and centrifuged at 10,000 × *g* for 10 min and the supernatant was used for measuring [^{35}S]-methionine incorporation into stroma proteins. The pellet was homogenized by a glass/Teflon homogenizer with 5 ml of 10 mM Tricine, pH 8/150 mM NaCl. After centrifugation at 10,000 × *g* for 10 min, the pellet was homogenized in 5 ml of 10 mM Tricine, pH 8/250 mM sucrose. Octyl-β-D-glucopyranoside and sodium cholate were added under gentle stirring to final concentrations of 1% and 0.5%, respectively (3). After 20 min at 0°C, the suspension was centrifuged at 200,000 × *g* for 1 hr. To the supernatant, saturated (at room temperature) ammonium sulfate was added to 37% saturation. After 20 min at 0°C, the suspension was centrifuged at 10,000 × *g* for 10 min. The pellet was discarded and the supernatant was brought to 48% saturation with saturated ammonium sulfate solution. After 20 min at 0°C, the suspension was centrifuged as before and all of the supernatant was carefully removed by suction. To the pellet was added 0.5 ml of a solution containing 30 mM Tris succinate (pH 6.5), 0.2% Triton X-100, 0.1 mM ATP, 0.5 mM EDTA, and 0.1% soybean asolectin (added as 4%, sonicated vesicles in 50 mM Tricine, pH 8). The sample was applied onto a linear 7–30% sucrose gradient containing all the components listed above. After centrifugation for 15–18 hr in a Spinco SW 41 rotor at 35,000 rpm, the bottom of the tubes was punctured and twelve 0.9-ml fractions were collected. Small aliquots of each fraction were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis on gels containing an exponential 12.5–17.5% gradient of polyacrylamide. Usually the pure H^+-ATPase complex appeared in gradient fractions 4–6.

Preparation of RNA from Spinach Leaves. About 500 g of spinach leaves were illuminated overnight as described above, washed, and frozen in liquid nitrogen. The frozen leaves were homogenized in a Waring blender for 1 min in 500 ml of 50 mM Tris·HCl, pH 7.6/150 mM NaCl/2 mM $MgCl_2$/2% Na-triisopropylnaphthalenesulfonate, filtered through eight layers of cheesecloth, and extracted for 20 min at room temperature with 250 ml of chloroform/phenol, 1:1 (vol/vol). After centrifugation at 10,000 × *g* for 10 min, the clear aqueous phase was removed and extracted twice more with an equal volume of chloroform/phenol. The NaCl concentration was adjusted to 0.2 M, and 2 vol of cold ethanol was added. After overnight incubation at −20°C, the suspension was centrifuged at 10,000 × *g* for 10 min and the pellet was resuspended in 50 ml of sterile 10 mM Tris·HCl (pH 7.4). The RNA was then precipitated with 3 M LiCl and further processed as described (12).

Other Methods. Leaf RNA obtained in this manner was translated in a nuclease-pretreated reticulocyte lysate as described (12) except that only 1 μg of nuclease per ml was used. Published methods were used for immunoprecipitation of labeled polypeptides from mixtures that had been dissociated with boiling $NaDodSO_4$ (11, 12), for preparing spinach CF_1 (a generous gift of D. W. Deters) (13), antibodies against each of the five CF_1 subunits (14), and [^{35}S]methionine (100–300 Ci/mmol) (15), for performing $NaDodSO_4$/polyacrylamide gel electrophoresis (16) followed by fluorography (17), and for measuring chlorophyll (18) and protein (19) concentrations.

RESULTS

Isolated Chloroplasts Synthesize α, β, γ, and ε Subunits of CF_1. Freshly isolated spinach chloroplasts were labeled with [^{35}S]methionine in the presence of 100 μg of cycloheximide per ml to block any protein synthesis on cytoplasmic ribosomes that might be present as contaminants. The chloroplasts were then extracted with 80% acetone, dissociated with $NaDodSO_4$, and subjected to immunoprecipitation with antisera directed against each of the five CF_1 subunits. The immunoprecipitated polypeptides were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by staining (Fig. 1 *Left*). As expected, the α, β, γ, and δ subunits and the ε subunit (not shown) had been specifically immunoprecipitated by the appropriate antiserum. Subsequent fluorography of the dried gel slabs showed that the α, β, γ, and ε subunits had been labeled by the isolated chloroplasts (Fig. 1 *Right*). No significant radioactivity was associated with the δ subunit even though the stained subunit was readily visualized in the immunoprecipitate. In contrast, the immunoprecipitated ε subunit was stained very weakly (see legend of Fig. 1), yet was clearly radioactive. No labeling of any of the subunits was observed in the presence of chloramphenicol, which blocks protein synthesis on chloroplast ribosomes.

Isolated Chloroplasts Not Only Synthesize CF_1 Subunits, but Also Assemble Them into a CF_1–CF_0 Complex. In the experiment depicted in Fig. 1, the labeled CF_1 subunits were precipitated from chloroplasts that had been dissociated in $NaDodSO_4$. As a consequence, no information was obtained about whether some of the CF_1 subunits made by the isolated chloroplasts had been assembled into an H^+-ATPase complex.

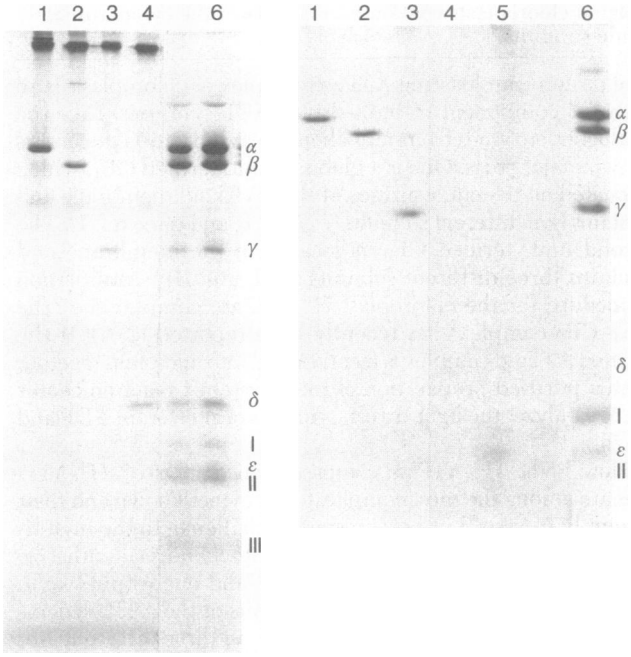

FIG. 1. Spinach chloroplasts synthesize the three largest CF_1 subunits. Isolated chloroplasts were labeled with [^{35}S]methionine. The reaction mixture was extracted with 80% acetone and dissociated with $NaDodSO_4$. Aliquots of the lysate were subjected to immunoprecipitation with antisera directed against each of the five CF_1 subunits (α, β, γ, δ, and ε). The immunoprecipitates were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis on 15% polyacrylamide gel slabs and the gels were either stained with Coomassie blue (*Left*) or fluorographed (*Right*). Track 1, α subunit; track 2, β subunit; track 3, γ subunit; track 4, δ subunit; track 5, ε subunit (because staining of this subunit was too weak for reproduction, only the fluorogram is shown); track 6, CF_1–CF_0 complex (isolated from chloroplasts labeled *in vitro*; see Fig. 2).

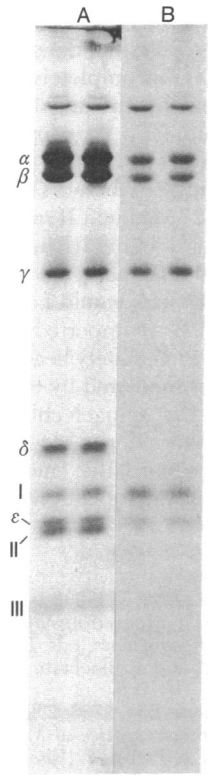

FIG. 2. Isolated chloroplasts can assemble newly synthesized CF_1 subunits into an H^+-ATPase complex. Isolated chloroplasts were labeled with [^{35}S]methionine and the H^+-ATPase complex was then isolated and analyzed by Na-$DodSO_4$/polyacrylamide gel electrophoresis followed by staining (*A*) and fluorography (*B*).

Indeed, recent data of Grebanier *et al.* (20) with isolated maize chloroplasts suggest that at least part of newly made α and β subunits of CF_1 are not assembled into a CF_1 molecule.

In order to test for assembly of newly made CF_1 subunits into an H^+-ATPase (i.e., CF_1-CF_0) complex, we performed the following four-step experiment: (*i*) isolated chloroplasts were labeled with [^{35}S]methionine in the presence of cycloheximide; (*ii*) the intact, functional H^+-ATPase complex was isolated from the labeled chloroplasts by a micromethod; (*iii*) the isolated complex was subjected to velocity sedimentation in a linear sucrose gradient; and (*iv*) each gradient fraction was analyzed for labeled H^+-ATPase polypeptides by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by fluorography. In addition, the fractions from the gradients were tested for ATP-P_i exchange activity upon incorporation into liposomes (5) and the staining intensity of each active fraction was compared with protein-bound radioactivity. Over 50% of the original H^+-ATPase was recovered in the peak fractions.

The result was clear-cut: CF_1 subunits made by isolated chloroplasts were recovered in the highly purified H^+-ATPase complex. By comparing the amount of labeled CF_1 subunits recovered in the H^+-ATPase complex with that isolated by immunoprecipitation of lysed chloroplasts with antisera against individual CF_1 subunits, we estimate that most of the newly labeled subunits had been incorporated into the H^+-ATPase. The relative amount of each subunit in the peak H^+-ATPase fractions agreed with that of highly active, reconstitutively active H^+-ATPase complex (3). Moreover, the peak of stainable H^+-ATPase complex coincided with a peak of labeled α, β, γ, and ϵ subunits. Again, no label was associated with the δ subunit (Fig. 2).

The experiment illustrated in Fig. 2 yielded an additional important result: the membrane-bound CF_0 subunit I had also been labeled by the isolated chloroplasts. Subsequent experiments (not shown) revealed that isolated chloroplasts also labeled subunit III—i.e., the chloroplast proteolipid (3, 21). However, labeling of this hydrophobic protein can be demonstrated only by conventional radioautography because it is leached out from the gels by the fluorographic procedure used here. Synthesis of subunit III by chloroplasts has also been found by J. C. Gray (personal communication). Isolated spinach chloroplasts can thus synthesize not only part of CF_1, but also part of the membrane sector of their H^+-ATPase complex.

CF_1 δ Subunit Is Made in Cytoplasm as a Larger Precursor. Because isolated chloroplasts did not label the CF_1 δ subunit, we suspected that it is made on cycloheximide-sensitive ribosomes outside the chloroplasts and then imported by "vectorial processing" (11). In order to test this, total spinach leaf RNA was translated in a reticulocyte lysate in the presence of [^{35}S]-methionine, the labeled translation products were subjected to immunoprecipitation with an antiserum against the CF_1 δ subunit, and the immunoprecipitate was analyzed by Na-$DodSO_4$/polyacrylamide gel electrophoresis followed by fluorography. The fluorogram revealed a labeled polypeptide that was about 8000 daltons larger than the mature δ subunit (Fig. 3). If taken in conjunction with the labeling experiments with isolated chloroplast, the result shown in Fig. 3 leaves little doubt that the CF_1 δ subunit is made outside the chloroplast. It is probably imported by a mechanism that is similar, or even identical, to that governing the import of the small ribulosediphosphate carboxylase subunit (22, 23).

Leaf RNA also directed the synthesis of very small amounts of radiolabeled polypeptides that were specifically immunoprecipitated by antisera against the α, β, and γ subunits of

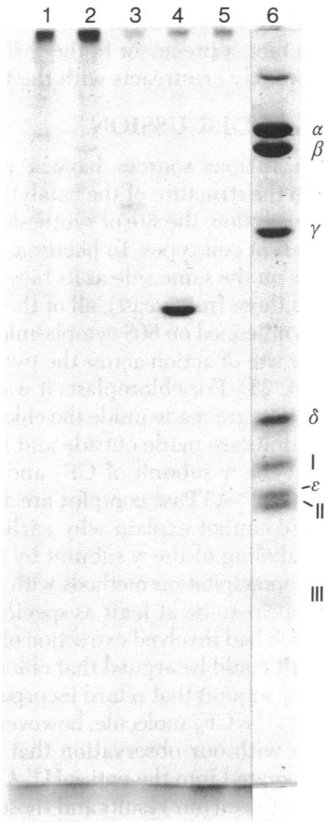

FIG. 3. Translation of total spinach leaf RNA yields a larger form of the δ subunit of CF_1. Spinach leaf RNA was translated in a reticulocyte lysate and subjected to immunoprecipitation by the antibodies against the five individual subunits of CF_1. The immunoprecipitates were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis on 15% polyacrylamide gel slabs and the gels were fluorographed. Track 1, α subunit; track 2, β subunit; track 3, γ subunit; track 4, δ subunit; track 5, ϵ subunit; track 6, stained CF_1-CF_0 complex. Track 6 is derived from the same gel. However, because this track was stained, its photograph had to be superimposed on the fluorogram (tracks 1–5).

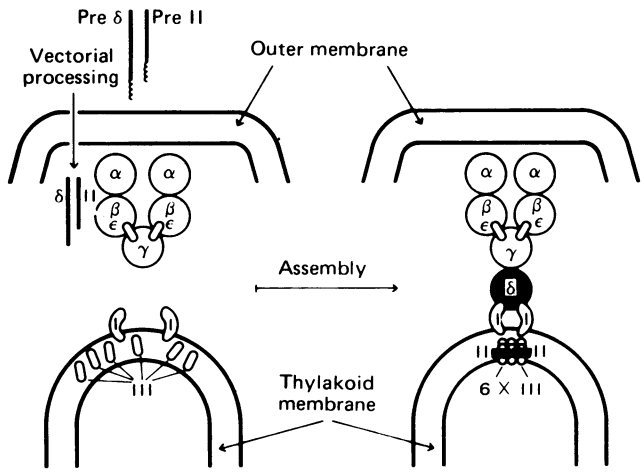

FIG. 4. Proposed model for the biosynthesis and assembly of the chloroplast H^+-ATPase complex.

CF_1. The polypeptides immunoprecipitated by the antisera against α and β subunits had the same electrophoretic mobility as the mature subunits. In contrast, the polypeptide immunoprecipitated by the antiserum against subunit γ was several thousand daltons larger than the mature CF_1 γ subunit. Because the amount of label associated with these three polypeptides was close to background, they were not characterized further. As a consequence, we cannot decide whether the CF_1 γ subunit is indeed made as a larger precursor or whether the polypeptide isolated by us is, in fact, a precursor to the γ subunit of mitochondrial F_1 that weakly crossreacts with the CF_1 γ subunit.

DISCUSSION

H^+-ATPases from various sources have a rather uniform structure. Although the structure of the catalytic part is highly conserved during evolution, the site of synthesis of the subunits varies among different cell types. In bacteria, the location of the catalytic part is on the same side as its biosynthesis. In mitochondria (such as those from yeast), all of the subunits of the catalytic part are synthesized on 80S cytoplasmic ribosomes and transported to their site of action across the two mitochondrial membranes (12, 24, 25). For chloroplasts it was reported that the α, β, and ϵ subunits are made inside the chloroplast whereas the remaining subunits are made outside and imported (7–9). We now show that the γ subunit of CF_1 and subunits I and (probably) III of the H^+-ATPase complex are also made inside the chloroplast. We cannot explain why earlier workers had failed to observe labeling of the γ subunit by isolated chloroplasts. The immunoprecipitation methods with subunit-specific sera used by us appear to be at least as specific as the earlier methods (7–9), which had involved extraction of assembled CF_1 from chloroplasts. It could be argued that chloroplasts contain large pools of free γ subunit that retard incorporation of newly made γ subunit into the CF_1 molecule; however, this possibility is not in harmony with our observation that newly made γ subunit was incorporated into the entire H^+-ATPase complex. The discrepancy between our results and those of others (7–9) thus requires further study.

In conclusion, no less than six out of the eight CF_1–CF_0 subunits are chloroplast products. The δ subunit of CF_1 and most probably also subunit II of CF_0 are cytoplasmic products and have to be transported into the chloroplast. Fig. 4 depicts a proposed model for the biosynthesis and assembly of the chloroplast H^+-ATPase complex.

What is the biological significance of these findings? It is tempting to speculate that the import of the CF_1 δ subunit and of subunit II could form the basis of a regulatory mechanism that maintains photophosphorylation while the H^+-ATPase is being assembled. This assembly poses two potential dangers: hydrolysis of ATP inside the chloroplast by incompletely assembled CF_1 and generation of an H^+ leak by an "ungated" CF_0 portion that had not yet combined with CF_1. We propose (Fig. 4) that the internally made α, β, γ, and ϵ subunits of CF_1 are initially assembled as an incomplete complex whose ATPase activity is suppressed by the ϵ subunit (26, 27). Subunit II might be required to assemble six molecules of subunit III into a functional H^+ pore. In the absence of subunit II, such a channel might not be stable and no significant H^+ leak would be induced. When the CF_1 subunit and subunit II are imported, the H^+ channel induced by subunit II might immediately become gated by the attachment of CF_1, which is mediated by the δ subunit (27, 28). In fact, our data suggest that spinach chloroplasts contain excess pools of the two externally made H^+-ATPase subunits; these pools might safeguard the energy-producing machinery of chloroplasts during chloroplast protein synthesis.

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