SITE OF SYNTHESIS OF SUBUNITS TO PHOTOSYSTEM I REACTION CENTER AND THE PROTON–ATPase IN *SPIRODELA*

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1. Introduction

Unlike their counterparts in mitochondria, many of the chloroplast inner-membrane proteins appear to be synthesized on organelle ribosomes. Some of these are, however, synthesized on cytoplasmic ribosomes and imported into the chloroplast by an energy-dependent process called 'vectorial processing [1,2]. Thus, biogenesis of the chloroplast inner membrane involves a close interaction between the nuclear and chloroplast genetic systems. The sites of synthesis of the chloroplast proton–ATPase complex have been studied in [3–6] but those of the photosystem I reaction center in higher plants have never been elucidated.

In this study we employed short-term in vivo pulse-labeling of *Spirodela oligorrhiza*. This aquatic higher plant rapidly incorporates [^{35}S]methionine into chloroplast proteins and can be grown in sterile conditions [7]. Our experiments, in the presence and absence of protein synthesis inhibitors followed by subsequent isolation of the protein complexes, revealed the following: Synthesis of subunits α, β and ϵ of CF_1 as well as subunit III (the chloroplast proteolipid, see [8]) of CF_0 was inhibited in the presence of D-*threo*-chloramphenicol but not cycloheximide, suggesting that these proteins are products of the chloroplast translation system. Subunits γ and δ of CF_1 and subunit II of CF_0 behaved in an opposite fashion suggesting that these components are products of the cytoplasmic translation system. The site of synthesis of subunit I of CF_0 was not resolved. In photosystem I reaction center, only subunit II was indicated as being synthesized on cytoplasmic ribosomes while subunits I, V and VIb were characterized as being chloroplast translation products. The site of synthesis of subunits III, IV and VIa remains to be clarified.

2. Materials and methods

2.1. *Growth, radiolabeling and chloroplast membrane isolation*

Axenic *Spirodela oligorrhiza* (Kurtz) Hegelm was cultured phototrophically in 250 ml flasks under steady-state light conditions (200 foot candles, cool-white fluorescent lights, 25°C) in half-strength Hutner's medium [9]. After transfer to fresh cultivation medium in Petri plates, plants were placed overnight at 25°C under 300 foot candles of white light. Fifty μCi/ml [^{35}S]methionine was added to the medium and growth allowed to continue for 3–6 h in the absence or presence of cycloheximide (50 μg/ml, final conc.) or D-*threo*-chloramphenicol (100 μg/ml, final conc.). The labeled fronds were washed with 2 changes of 5 ml ice-cold medium, combined with twice their wet weight of unlabeled fronds, and suspended in 3 vol. ice-cold STN solution (0.3 M sucrose, 0.01 M tricine–NaOH (pH 8) and 0.01 M NaCl). Thylakoid membranes were prepared by blending followed by differential centrifugation and resuspension in 0.1 ml STN solution containing 5 mM $MgCl_2$ (STN–Mg)/g fresh wt of original tissue [10].

2.2. *Purification of the proton–ATPase complex*

Chloroplast membranes, containing ~1 mg chl, were washed with ~10 ml solution containing 10 mM tricine–NaOH (pH 8). After centrifugation at 15 000 × *g* for 15 min the pellet was homogenized in 10 ml solution containing 10 mM tricine–NaOH (pH 8) and 0.15 M NaCl, and again centrifuged as above. Following an additional wash with 10 mM tricine–NaOH (pH 8), octyl-β-D-glucopyranoside and sodium cholate were added to final conc. 1% and 0.5%, respectively. After 20 min at 0°C the suspen-

sion was centrifuged at 200 000 × *g* for 1 h and the proton–ATPase complex purified as in [6].

2.3. *Purification of photosystem I reaction center*

Following the octyl-glucopyranoside–sodium cholate treatment and 200 000 × *g* centrifugation, the pellet was homogenized in a solution containing 25 mM tricine–NaOH (pH 8) and 2% Triton X-100 to give 0.5 mg chl/ml. After centrifugation at 20 000 × *g* for 10 min the supernatant was applied on a DEAE-cellulose column (0.6 × 10 cm) [11,12]. The column was washed with ~10 ml of a solution containing 20 mM Tris–HCl (pH 8) and 0.2% Triton X-100, and the reaction center protein complex eluted with the same solution containing 150 mM NaCl. A dark green fraction of ~1 ml was collected and applied on a linear sucrose gradient (5–30%) in a solution containing 20 mM Tris–HCl (pH 8) and 0.2% Triton X-100. After centrifugation for 15 h at 150 000 × *g* in a SW 41 rotor in a Spinco ultracentrifuge, the lower green band was collected as in [11,12]. Further steps were as in [6].

3. Results

The purified proton–ATPase complex from *Spirodela*, like that of spinach [6,13], was resolved into 8 subunits upon SDS–polyacrylamide gel electrophoresis (fig.1, tracks 1,2). The purified complex from plants untreated, or treated with chloramphenicol or cycloheximide is shown in tracks 5–7: the 3 stained patterns were similar. Autoradiograms of these are shown in tracks 2–4. In the untreated sample (track 2) all 8 subunits of the proton–ATPase complex were labeled. Two additional radioactive bands were present: one was the rapidly metabolized, 32 000 M_r photosystem II-regulatory polypeptide [10]; the second, the 26 000 M_r apoprotein of the light-harvesting chl *a*/*b* complex. In short term experiments with [^{35}S]methionine, these appear as the main, labeled membrane components in *Spirodela* [7]. Neither of these polypeptides have been observed in stained gels of the purified proton–ATPase complex; nor do they co-purify with the complex upon fractionation in sucrose gradients (not shown). The proton–ATPase complex purified from plants labeled in the presence of chloramphenicol (track 3) showed radioactivity mainly in the γ and δ subunits of CF_1 and in subunit II of CF_0. These subunits were not significantly labeled in the presence of cycloheximide while α, β, ϵ and III were (track 4). We note that synthesis of α and β subunits was strongly inhibited by chloramphenicol (cf. tracks 3,4) although total elimination of the bands did not occur. Subunit I, which was weakly labeled with [^{35}S]methionine in the untreated control (track 2), was not resolved in the inhibitor-treated samples.

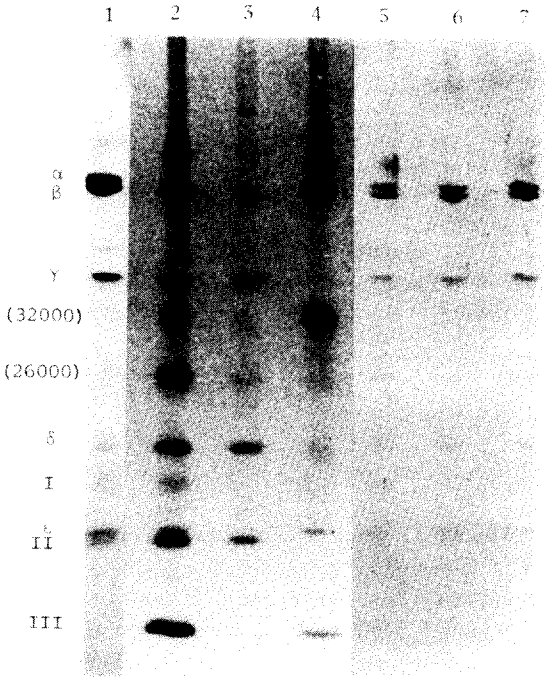

Fig.1. Effect of chloramphenicol and cycloheximide on the synthesis and assembly of the subunits of the proton–ATPase complex. Fronds of *Spirodela* were labeled with [^{35}S]methionine, in the presence and absence of protein synthesis inhibitors, and the proton–ATPase complex was isolated as in section 2. Samples of ~10 μg protein were applied on SDS–polyacrylamide 10–15% gradient gels. Tracks 2–4 are autoradiograms of the Coomassie blue-stained gels shown in tracks 5–7. Track 1 is from another stained gel of purified proton–ATPase complex (~30 μg protein). Tracks 2 and 5 contain samples from the control experiments (lacking protein synthesis inhibitors). Tracks 3 and 6 are samples from fronds labeled in the presence of 100 μg chloramphenicol/ml. Tracks 4 and 7 are samples from fronds labeled in the presence of 50 μg cycloheximide/ml.

The subunit structure of photosystem I reaction center from *Spirodela* is shown in fig.2. It is very similar to that from other higher plants [11,12,20–23]. As in the case of Swiss chard and spinach, subunit III, identifiable by its fluorescence upon excitation by red light [16], changed positions with subunit IV when

electrophoresed on slabs rather than tube gels (N. N., unpublished). In addition, subunit VI [11,12] was resolved into two stained bands on the slabs, and is referred to as VIa and VIb. To obtain more highly purified reaction center from small amounts of labeled chloroplasts, preparations of reaction center were reapplied on a second sucrose gradient. This resulted in a loss of subunit III from the complex as shown for other plants [11,12]. The SDS–polyacrylamide gel patterns of the purified reaction center complex, extracted from plants labeled in the absence and presence of protein synthesis inhibitors are shown in fig.3. The stained patterns are shown in tracks 1–3 and their autoradiograms in tracks 4–6. In the autoradiogram of the untreated control (track 6), subunits I, II and VIb were clearly labeled, subunit V weakly labeled, and subunits IV and VIa not detected by the [^{35}S]methionine probe. The radiolabeled 32 000 M_r and 26 000 M_r bands described in relation with fig.1 also appeared as minor contaminants in the photo

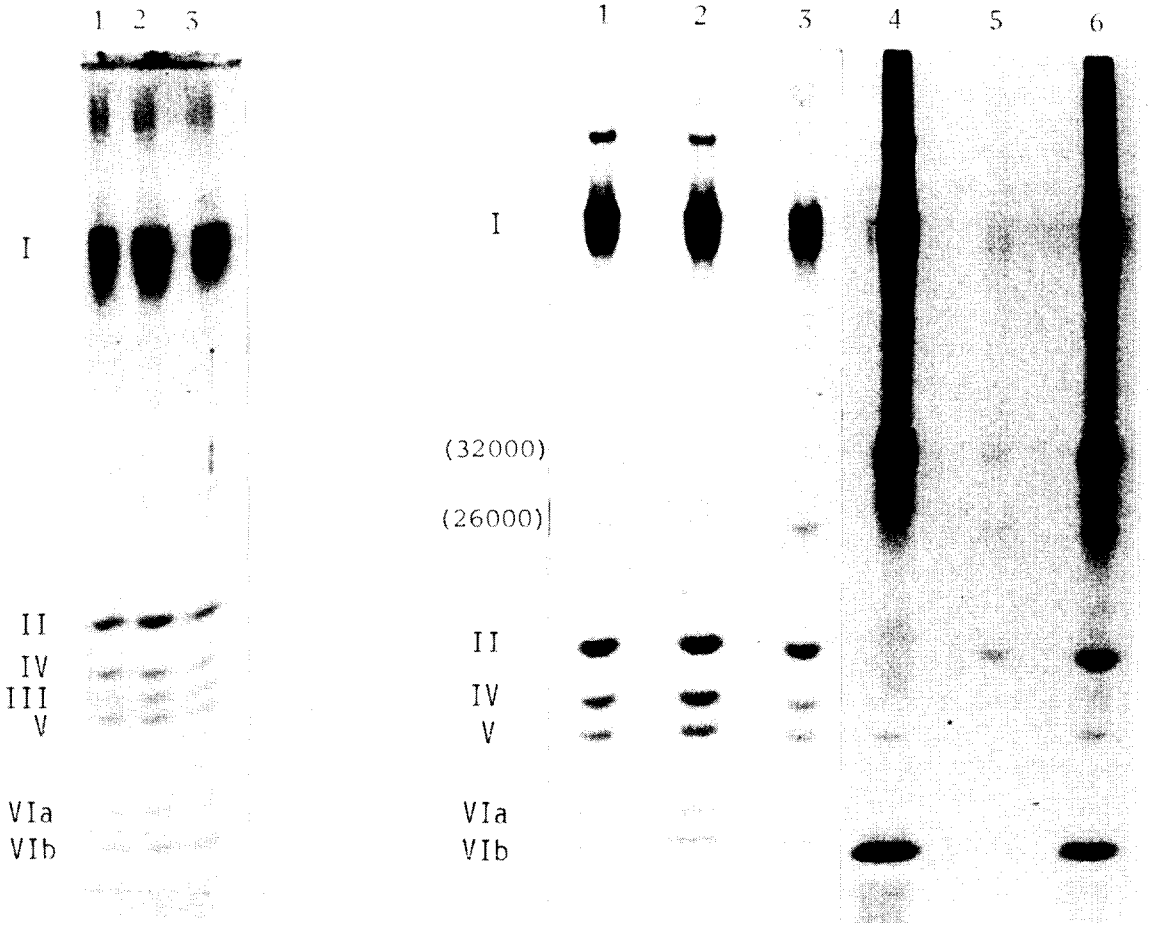

Fig.2. SDS–polyacrylamide gel patterns of photosystem I reaction center from *Spirodela* chloroplasts purified through one sucrose gradient. Purified photosystem I reaction center from 3 different preparations (tracks 1–3) containing ~30 μg protein each, was electrophoresed on a 10–15% polyacrylamide gel. The gel was stained with Coomassie blue and destained as in [6]. Roman numerals represent the subunits of the reaction center.

Fig.3. SDS–polyacrylamide gel patterns of photosystem I reaction center purified through two gradients of sucrose from fronds treated with and without protein synthesis inhibitors. Gel conditions as in fig.2. Tracks 4–6 are autoradiograms of the stained gels in tracks 1–3. Tracks 3 and 6 contain samples from the control experiment (lacking protein synthesis inhibitors). Tracks 2 and 5 are samples from fronds labeled in the presence of 100 μg chloramphenicol/ml. Tracks 1 and 4 are samples from fronds labeled in the presence of 50 μg cycloheximide/ml.

system I-reaction center fraction. These proteins did not copurify with the reaction center on sucrose gradients (not shown). Among those subunits revealed by the [^{35}S]methionine-label only subunit II was labeled in the presence of chloramphenicol (track 5) while subunits I, V and VIb were labeled in the presence of cycloheximide (track 4).

4. Discussion

Protein synthesis inhibitors, specific for the cytoplasmic or plastid translation systems, as well as synthesis of proteins by isolated chloroplasts have been the two main methods used to probe the subcellular site of synthesis of chloroplast proteins [4,24]. For chloroplasts from several cell types it was reported that the α, β and ϵ subunits of CF_1 are translated within the organelle whereas the γ and δ subunits are translated in the cytoplasm and imported into the chloroplast [3–5]. In [6] the γ subunit of CF_1 was synthesized by isolated spinach chloroplasts. Here, in vivo labeling of *Spirodela* plants in the presence of specific inhibitors, shows the γ subunit of CF_1 to be a product of cytoplasmic ribosomes and has to be transported into the chloroplasts. The apparent discrepancy among the various observations might be due to a silent gene for the γ subunit in the chloroplast that was activated in the isolated spinach chloroplasts. It also might be that the site of synthesis of the γ subunit of plastid proton–ATPase is different in various plant species, as has been suggested for the proteolipid of the mitochondrial ATPase from *Neurospora* and yeast [16]. This work also confirms findings that subunit III (proteolipid) of CF_0 is a chloroplast product [6,14], and that subunit II is synthesized outside the organelle on cytoplasmic ribosomes [6]. A possible biological significance for synchronizing the synthesis of the CF_1 δ subunit and CF_0 subunit II has been proposed [6].

The site of synthesis of individual subunits of purified, higher-plant photosystem I reaction center was studied here for the first time. The presence of polypeptides of cytoplasmic and chloroplastic origin in partially purified preparations of photosystems I and II in *Chlamydomonas* had already been indicated [25]. It was also known that subunit I of photosystem I reaction center in *Chlamydomonas* is a product of the chloroplast translation system [15] although its presence in the thylakoid membranes is under the control of both chloroplast and nuclear [18,19] genes in this alga. From our data with in vivo labeled *Spirodela*, sites of synthesis can be provisionally assigned to 4 of the 7 subunits. Subunits I, V and VIb appear to be translated within the organelle while subunit II is synthesized outside the plastid on cytoplasmic ribosomes. These findings require confirmation by other, complementary approaches, such as synthesis by isolated chloroplasts or cell-free translation of chloroplast mRNAs. It is noteworthy that no biochemical function in electron transfer was assigned to subunit II in the published model for photosystem I reaction center [12,20]. It is tempting to suggest that this cytoplasmically made subunit plays a role in the regulation of synthesis or assembly of the other chloroplast translated subunits in the reaction center. The biogenesis and assembly of the various subunits of photosystem I reaction center is under investigation in our laboratories.

Acknowledgements

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References

[1] Nelson, N. and Schatz, G. (1979) Proc. Natl. Acad. Sci. USA 76, 4365–4369.
[2] Grossman, A. R., Bartlett, S. G. and Chua, N.-H. (1980) Nature 285, 625–628.
[3] Mendiola-Morgenthaler, L. R., Morgenthaler, J. J. and Price, C. A. (1976) FEBS Lett. 62, 96–100.
[4] Ellis, R. J. (1977) Biochim. Biophys. Acta 463, 185–215.
[5] Bouthyette, P.-Y. and Jagendorf, A. T. (1978) Plant Cell Physiol. 19, 1169–1174.
[6] Nelson, N., Nelson, H. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 1361–1364.
[7] Edelman, M. and Reisfeld, A. (1980) in: Genome Organization and Expression in Plants (Leaver, C. J. ed) pp. 353–361, Plenum, New York.
[8] Nelson, N., Eytan, E., Natsani, B. E., Sigrist, H., Sigrist-Nelson, K. and Gitler, C. (1977) Proc. Natl. Acad. Sci. USA 74, 2375–2378.
[9] Posner, H. B. (1967) in: Methods in Developmental Biology (Witt, F. A. and Wessels, N. K. eds) pp. 301–317, Thomas Y. Crowell, New York.
[10] Mattoo, A. K., Pick, U., Hoffman-Falk, H. and Edelman, M. (1981) Proc. Natl. Acad. Sci. USA 78, in press.
[11] Bengis, C. and Nelson, N. (1975) J. Biol. Chem. 250, 2783–2788.

[12] Nelson, N. and Notsani, B. (1977) in: Bioenergetics of Membranes (Packer, L. et al. eds) pp. 233–244, Elsevier/North-Holland, Amsterdam, New York.
[13] Pick, U. and Racker, E. (1979) J. Biol. Chem. 254, 2793–2799.
[14] Doherty, A. and Gray, J. C. (1980) Eur. J. Biochem. 108, 131–136.
[15] Chua, N.-H. and Gillham, N. W. (1977) J. Cell Biol. 74, 441–452.
[16] Sebald, W. (1977) Biochim. Biophys. Acta 463, 1–27.
[17] Clayton, R. K. and Hazelkorn, R. (1972) J. Mol. Biol. 68, 97–105.
[18] Chua, N.-H. and Bennoun, P. (1975) Proc. Natl. Acad. Sci. USA 72, 2175–2179.
[19] Chua, N.-H., Matlin, K. and Bennoun, P. (1975) J. Cell Biol. 67, 361–377.
[20] Bengis, C. and Nelson, N. (1977) J. Biol. Chem. 252, 4564–4569.
[21] Satoh, K. (1979) Plant Cell Physiol. 20, 499–512.
[22] Mullet, J. E., Burke, J. J. and Arntzen, C. J. (1980) Plant Physiol. 65, 814–822.
[23] Mullet, J. E., Burke, J. J. and Arntzen, C. J. (1980) Plant Physiol. 65, 823–827.
[24] Gilham, N. W., Boynton, J. E. and Chua, N.-H. (1978) Curr. Top. Bioenerget. 8, 211–260.
[25] Bar-Nun, S. and Ohad, I. (1977) Plant Physiol. 59, 161–166.