Isolation of CF_0CF_1 from *Chlamydomonas reinhardtii* cw15 and the N-terminal amino acid sequences of the CF_0CF_1 subunits

Heike R. Fiedler[a], Roland Schmid[b], Stefan Leu[c], Noun Shavit[c], Heinrich Strotmann[a,*]

[a]*Institut für Biochemie der Pflanzen, Heinrich Heine Universität Düsseldorf, Universitätsstr. 1, D-40225 Düsseldorf, Germany*
[b]*Fachbereich Biologie/Chemie-Mikrobiologie-, Universität Osnabrück, Osnabrück, Germany*
[c]*Center of Bioenergetics, Life Science Department, Ben Gurion University, Beer Sheva, Israel*

Received 2 November 1995

Abstract **CF_0CF_1 was isolated from chloroplasts of the cell wall-deficient *Chlamydomonas reinhardtii* strain cw15. The subunit pattern was analyzed by SDS-gel electrophoresis and the N-terminal amino acid sequences of all nine subunits were determined by microsequencing. The amino acid sequences of subunits α, β, γ and ϵ match with those derived from the corresponding *Chlamydomonas* DNA sequences. In variance with the previously assumed N-terminus of β; however, it was found that the first 11 amino acids are lacking. The subunits δ, I, II, III and IV were identified by comparison with known sequences of homologous polypeptides of higher plant chloroplasts and cyanobacteria, respectively.**

Key words: *Chlamydomonas*; CF_0CF_1; Amino acid sequence

1. Introduction

F_0F_1-ATPases are involved as catalysts of ATP formation driven by an electrochemical proton gradient across thylakoid membranes, inner mitochondrial membranes and bacterial plasma membranes. The membrane-integral F_0 sector is responsible for proton translocation, the extrinsic F_1 is the catalytic part of the complex. Photosynthetic membranes contain four different F_0 subunits I (b), II (b′), III ($c_{\approx 10}$), IV (a) and five different F_1 subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ are present in F_0F_1-ATPases [1].

In higher plants six CF_0F_1 subunits namely α, β, ε, I, III and IV are encoded in the plastid genome and three of the subunits, namely γ, δ and II are nuclear encoded [2]. The same gene distribution is assumed for green algae. In *C. reinhardtii* six chloroplast encoded CF_0CF_1 genes (atpA, atpB, atpE, atpF, atpI, atpH) have been mapped by heterologous hybridization [3] In contrast to higher plants, where these genes are arranged in two conserved operons, in *C. reinhardtii* these genes are spread individually across the whole chloroplast genome. So far the DNA sequences of the genes atpA, atpB, atpC, atpE and atpH (EMBL accession number X90559) have been reported, but except for the γ subunit [4] no N-terminal protein sequences of CF_0CF_1 subunits from *Chlamydomonas* are available.

The unicellular green alga *Chlamydomonas reinhardtii* can be transformed in the plastid genome by biolistic techniques [5] and thus has become a model organism for molecular genetic studies of eukaryotic photosynthesis. The cell wall-deficient mutant CW15 [6] can be transformed as well and furthermore is a higly suitable source of photosynthetically active chloroplasts [7]. Therefore this *Chlamydomonas* strain appears an ideal organism for mutagenetic studies on CF_0CF_1 genes with the aim to learn more about the detailed mechanism of energy transfer, catalysis and regulation of F_0F_1 of photosynthetic membranes. To this end it is necessary to establish the preparative and analytical biochemistry of *Chlamydomonas* CF_0CF_1. Here we report on the purification of CF_0CF_1 from *Chlamydomonas reinhardtii* CW15 and the N-terminal sequences of all nine CF_0CF_1 subunits.

2. Materials and methods

Chlamydomonas reinhardtii CW15 was grown at 20°C in high salt medium [8] containing 0.2% acetate. The light intensity was 500 lux in a 14 h light/10 h dark cycle. The 6 litre cultures were bubbled with sterile air. The cultures were grown to a cell density of 6×10^6 cells/ml and then harvested by centrifugation, washed with medium A (250 mM sorbitol, 35 mM HEPES, pH 7.8, 1 mM $MnCl_2$, 5 mM $MgCl_2$ and 2 mM EDTA) and resuspended in 100 ml of the same medium at 4°C. All further steps were carried out at this temperature. The cells were passed two times through a Yeda Press at 5×10^5 N/m^2 and 5.5×10^5 N/m^2, respectively. The pellet gained by 1 min centrifugation at $3000 \times g$ was resuspended in 5 mM $MgCl_2$ to break intact chloroplasts and the thylakoids were sedimented by centrifugation.

2.1. Preparation of CF_0CF_1

For solubilization of the thylakoid membranes [9] the pelleted thylakoid membranes were resuspended in a medium containing 200 mM sucrose, 10 mM Tricine buffer, pH 8.0, 10 mM $MgCl_2$, 0.5% sodium cholate and 30 mM octylglucoside at a chlorophyll concentration of 2 mg/ml. After stirring for 30 min in the dark at 4°C, undissolved particles were removed by centrifugation at $120\,000 \times g$ for 1 h. The protein fraction which precipitated between 37% and 48% ammonium sulfate (pH 8.0) was re-dissolved in 1.2 ml of a solution containing 0.2% Triton X-100, 30 mM Tris-succinate, pH 6.5, 0.5 mM EDTA and 0.1 mM ATP. The solubilized protein was separated by centrifugation in a 10% to 50% sucrose gradient (total volume 12 ml) for 18 h at $170{,}000 \times g$ and fractionated in 0.6 ml samples. The polypeptide patterns of the fractions were analyzed by SDS-gel electrophoresis [10] on a 15% acrylamide gel. For amino acid sequencing the purest CF_0CF_1 fraction was separated by SDS-PAGE on a 15% acrylamide gel. The protein bands were transferred to PVDF membranes by electroblotting and the excised bands placed into the blot cartridge of a sequencer model 473A from Applied Biosystems. Microsequencing was performed using a faster modification of the standard cycle

2.2. ATPase activity

The activity of CF_0CF_1-containing protein fractions was measured as methanol-stimulated ATPase [11] at 37°C. The assay medium contained 25 mM Tris buffer pH 8.3, 25 mM NaCl, 2.5 mM $MgCl_2$, 35% methanol, 5 mM [γ-^{32}P]ATP and 5 μg protein/ml. Samples were taken after 30, 60 and 120 s, deproteinized by 0.5 M $HClO_4$ and analyzed for $^{32}P_i$ [12].

*Corresponding author. Fax: (49) (211) 311-3706.

SSDI 0014-5793(95)01332-6

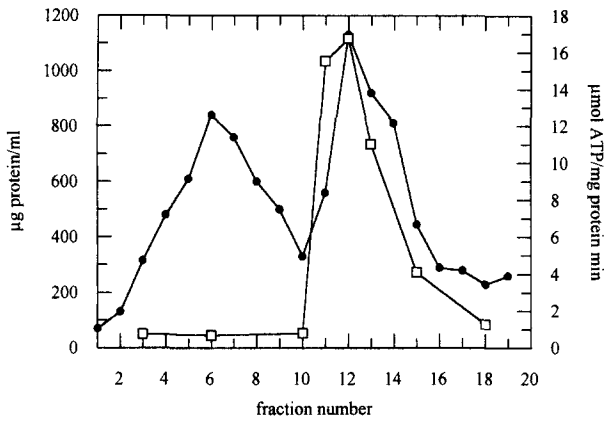

Fig. 1. Protein distribution (●) and ATPase activity (□) of solubilized thylakoid membranes from *Chlamydomonas reinhardtii CW15* after sucrose gradient centrifugation.

3. Results and discussion

Thylakoid membranes were solubilized and CF_0CF_1 was isolated by sucrose gradient centrifugation as described in section 2. The protein contents and the ATPase activity profile of the fractions are shown in Fig. 1. The highest activities are found in the fractions 11 and 12 (counting from top to bottom). The polypeptide pattern of fraction 11 obtained by SDS gel electrophoresis is shown in Fig. 2.

The CF_0CF_1 preparation shows eight predominant polypeptides and a few less stained bands. The bands marked with numbers 1 to 9 were subjected to N-terminal microsequencing. The obtained sequences are listed in Fig. 3 and compared with amino acid sequences derived from DNA sequences of *Chlamydomonas* or sequences from spinach and the cyanobacterium *Anabaena*.

Band 1. The apparent molecular weight of this polypeptide is 55 kDa. The sequence corresponds to the one derived from DNA sequence of atpA reported by Leu et al. [13], except that the N-terminal methionine is missing. Hence this protein is clearly the α subunit of *Chlamydomonas* CF_1. In variance to a previous report [14] and in agreement with another report [15] the *Chlamydomonas* CF_1 band with the highest apparent molecular weight obtained in a conventional SDS-gel system is subunit α rather than subunit β.

Band 2. The apparent molecular weight is 53 kDa. The N-terminal sequence identifies this polypeptide as β subunit. However, in variance to the amino acid sequence suggested from the DNA sequence [16], the first 11 amino acids are missing. Since at position 11 follows another methionine, this one rather than the previously assigned one seems to be the actual starting methionine. As in α subunit the N-terminal methionine is processed. Concerning the N-terminal end, *Chlamydomonas* β subunit seems to be more like β of cyanobacteria than β of higher plant chloroplasts.

Band 3. The apparent molecular weight of 38.5 kDa points to γ subunit and actually the N-terminal sequence agrees with the one expected for the mature γ protein [4]. The precursor protein of the nuclear encoded γ subunit from *Chlamydomonas* possesses a 35 amino acids import sequence [4].

Band 4. This polypeptide of an apparent molecular weight of 21 kDa is assigned subunit δ. This is confirmed by the N-terminal sequence which resembles δ from spinach chloroplasts [17] and *Anabaena* [18]. The atpD gene of *Chlamydomonas* resides in the nucleus and thus possesses a transit sequence (Berzborn et al., personal communication).

Band 5. The apparent molecular weight of this band which is not well stained by Coomassie Blue, is 18 kDa. The obtained N-terminal amino acid sequence resembles the amino acid sequence of subunit IV of CF_0 from spinach chloroplasts. The gene product of spinach atpI lacks the first 18 amino acids, suggesting posttranslational processing [19]. Since the start of the *Chlamydomonas* protein corresponds with the beginning of the mature spinach protein, it is possible that the *Chlamydomonas* protein is a processed product as well. However, the DNA sequence of atpI from *Chlamydomonas* has not been reported so far.

Band 6. The apparent molecular mass of this polypeptide is 17 kDa. The alignment of the N-terminal sequence with subunit

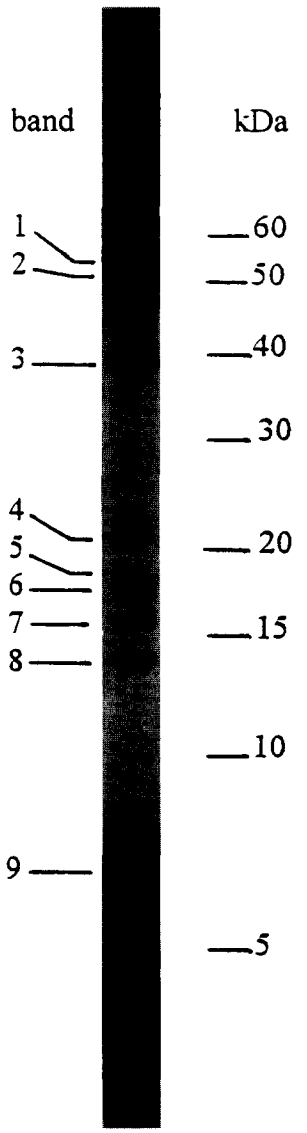

Fig. 2. SDS-PAGE of a CF_0CF_1-containing fraction from sucrose gradient centrifugation of solubilized thylakoid membranes from *Chlamydomonas reinhardtii* cw15. Fraction 11 of Fig. 1 (corresponding to about 30% sucrose) was analysed on a 15% acrylamide gel and stained with Coomassie Brillant Blue.

```
Band 1:                                                    AMRTPEELXXLI...
From Chlamydomonas-atpA [13]:                              MAMRTPEELSNLI...

Band 2:                                                 SDSIETKNMGRIVQIIGPV...
From Chlamydomonas-atpB [16]:           MPWGILIPLTMSDSIETKNMGRIVQIIGPV...

Band 3:                                    GLKEVRDRIASVKNTQKITDAMKLVAA...
Chlamydomonas-gamma [4]:                   GLKEVDNDIASVKNTQKIHTA.........
From Chlamydomonas-atpC [4]:            ...GLKEVRDRIASVKNTQKITDAMKLVAA...

Band 4:                                      RKNEVSESYAKALVELADEKGKLE...
From Spinach-atpD:                         ...VDSTASRYASALADVADVTGTLE...
From Anabaena-atpD:                        SKVANTEVAQPYAQALLSIAKSKSLTE...

Band 5:                                                    EVXVGQHYYXE...
Spinach-IV [19]:                                           XVEVGQHFYWQ...
From spinach-atpI [17]:                 MNVLSYSINPLKGLYAISGVEVGQHFYWQ...
From Anabaena-atpI [18]:                     MLNFLNFYSVPLAELEVGKHLYWQ...

Band 6:                                                 MGGFGFNTNVFETNIINLY...
From Spinach-atpF:                  KNVTDSFVFLGHWPSAGSFGFNTDILATNLINLS...
From Anabaena-atpF:    MGTFLLLLMAEASAVGGELAEGGAEGGFGLNTNILDTNLINLA...

Band 7:                                                    EAGKIFDFNTLPVMAGEEL...
From Spinach-atpG:                                      ...EIEKASLFDFNLTLPIIMAEF...
From Anabaena-atpG:                        THWITLLAVEKVAKEGGLFDLDATLPLMAIQF...

Band 8:                                                 SLQISILTPERPFWNGQADEIILPT...
From Chlamydomonas-atpE[3]:                             MSLQISILTPERPFWNGQADEIILPT...

Band 9:                                 f-MNPIVAAASVVSAGLAVGLAAIGPGMGQGTAA...
From Spinach-atpH:                        MNPLIAAASVIAAGLAVGLASIGPGVGQGTAA...
From Anabaena-atpH:                       MDPLVSAASVLAAALAVGLAAIGPGIGQGNAA...
```

Fig. 3. N-Terminal sequences of polypeptide bands of a CF_0CF_1 preparation of *Chlamydomonas reinhardtii CW15*. The numbers correspond to the one used in Fig. 2. The obtained sequences were aligned to amino acid sequences derived from known DNA sequences of ATP synthase subunits of *C. reinhardtii*, spinach [17] and *Anabaena* [18], respectively.

I of spinach and subunit b of *Anabaena* yields an obvious homology of band 6 with this F_0 subunit. The amino acid sequence derived from the DNA sequence of the spinach gene (atpF) is 17 amino acids longer in the N-terminal end than the actual subunit I of the assembled complex. The N-terminal extension was proposed to function as a leader sequence to guide the polypeptide into the thylakoid membrane and be split off after integration [20]. As the N-terminal end of the *Chlamydomonas* protein is similar to that of the spinach protein, it is possible that the precursor protein of *Chlamydomonas* possesses a leader sequence as well.

Band 7. The N-terminal amino acid sequence of this polypeptide of an apparent molecular weight of 15 kDa can be aligned to subunit II from spinach and subunit b′ of *Anabaena*. In spite of low overall sequence homology the similarity of the sequence of band 7 with subunit II/b′ is striking. In higher plants subunit II is nuclear encoded (atpG), but in the diatom *Odontella* [21] and in the red alga *Antithamnion* [22] subunit II as well as subunit δ are plastid-encoded proteins. Investigations are in progress to determine the location of the atpG gene in *Chlamydomonas*.

Band 8. The N-terminal amino acid sequence of the band 8 protein which has an apparent molecular weight of 13.5 kDa, completely agrees with the translated DNA sequence of ε subunit from *Chlamydomonas* [3].

Band 9. The apparent molecular mass of 7 kDa points to subunit III ('proteolipid'). Actually the obtained amino acid sequence is highly homologous to the sequences known for spinach [17] and *Anabaena* [18]. As the proteolipid from other sources [23] the N-terminal methionine of subunits III from *Clamydomonas* is formylated.

The N-terminal ends of the CF_0CF_1 subunits of *C. reinhardtii* are very similar to those of higher plant chloroplast and cyanobacterial CF_0CF_1. The present study will allow to localize and to identify the missing genes. To our knowledge this is the first series of N-terminal amino acid sequences of a chloroplast protein complex determined in *C. reinhardtii*.

Comparison of the N-terminal amino acid sequences with the

atpA: MetAlaMet
CTCGAGAAAG ATTTTAAAAA TAAACTTTTT TAATCTTTTA TTTATTTTTT ATGGCAATG

atpB: MetSerAsp
TAAAATAAGT TAAAATATGA ATGCCTTGGG GCATATTAAT TCCACTTACT ATGAGTGAT

atpE (X53977): MetSerLeu
TAAAGAATGT TCTAGCTTTA TCGCTTTTAG AAGTTTTATT TTTTTTTATT ATGAACCCT

atpH (X90559): MetAsnPro
AATATTCTTT GGTTGTTATC GATTTTATTG ATTCATTAGG AGGAAATACA ATGAACCCT

Fig. 4. Comparison of the DNA sequences preceeding the genes atpA, atpB, atpE and atpH.

four known plastid gene sequences hints at three different mechanisms of gene expression: (1) the atpA gene and the atpE gene are translated from the first methionine, but this methionine is removed; (2) the atpB gene is translated from the second methionine of the reading frame and the methionine is removed as well; (3) the atpH gene is translated from the first methionine and formylated. We have aligned the 50 bases preceeding the translation start of the four plastid genes to look for potential similarities and ribosome binding sites (Fig. 4). No similarities can be found, and no conventional ribosome binding site can be detected in these *Chlamydomonas* ATPase genes.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungs-bereiche 171 and 189) and by the German–Israeli Foundation (No. I-251-129.03/92).

References

[1] Strotmann, H. and Bickel-Sandkötter, S. (1984) Annu. Rev. Plant Physiol. 35, 97–120.
[2] Hermans, J., Rother, C., Bichler, J., Steppuhn, J. and Herrmann, R.G. (1988) Plant Mol. Biol. 10, 323–330.
[3] Woessner, J.P., Gillham, N.W. and Boynton, J.E. (1987) Plant Mol. Biol. 8, 151–158.
[4] Yu, L.M. and Selman, B.R. (1988) J. Biol. Chem. 263, 19342–19345.
[5] Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein T.M., Shark, K.B. and Sanford, J.C. (1988) Science 240, 1534–1538.
[6] Hyams, J. and Davies, D.R. (1972) Mut. Res. 14, 381–389.
[7] Mendiola-Morgenthaler, L., Leu, S., Boschetti, A. (1985) Plant Sci. 38, 33–39.
[8] Sueoka, N. (1960) Proc. Natl. Acad. Sci. USA 46, 83–87.
[9] Pick, U. and Racker, E. (1979) J. Biol. Chem. 254, 2793–2799.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Anthon, G.E. and Jagendorf, A.T. (1983) Biochim. Biophys. Acta 723, 358–365.
[12] Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272.
[13] Leu, S., Schlesinger, J., Michaels, A. and Shavit, N. (1992) Plant Mol. Biol. 18, 613–616.
[14] Lemaire, C. and Wollman, F.-A. (1989) J. Biol. Chem. 264, 10228–10234.
[15] Selman-Reimer, S., Merchant, S. and Selman, B.R. (1981) Biochemistry 20, 5476–5482.
[16] Woessner, J.P., Gillham, N.W. and Boynton, J.E. (1987) Gene 44, 17–28.
[17] Hennig, J. and Herrmann, R.G. (1986) Mol. Gen. Genet. 203, 117–128.
[18] McCarn, D.F., Whitaker, R.A., Alam, J., Vrba, J.M., Curtis, S.E. (1988) J. Bacteriol. 170, 3448–3458.
[19] Fromme, P., Gräber, P. and Salnikow, J. (1987) FEBS Lett. 218, 27–30.
[20] Bird, C.R., Koller, B., Auffret, A.D., Huttley, A.K., Howe, C.J., Dyer, T.A. and Gray, J.C. (1985) EMBO J. 4, 1381–1388.
[21] Pancic, P.G., Strotmann, H. and Kowallik, K.V. (1992) J. Mol. Biol. 224, 529–536.
[22] Kostrzewa, M. and Zetsche, K. (1992) J. Mol. Biol. 227, 961–970.
[23] Sebald, W. and Wachter, E. (1980) FEBS Lett. 122, 307–311.