

The nuclear-encoded polypeptide Cfo-II from spinach is a real, ninth subunit of chloroplast ATP synthase

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Proton-translocating F-ATP synthases from chloroplasts contain a *nuclear-coded* subunit, Cfo-II, that lacks an equivalent in the corresponding *E. coli* complex. Three recombinant phages that code for the entire precursor of this subunit have been isolated from λ gt11 cDNA expression libraries made from polyadenylated spinach RNA using a two-step strategy. The reading frame of 222 amino acid residues includes 147 residues for the mature protein (*M*, 16.5 kDa) and a transit sequence of 75 residues (*M*, 8.0 kDa). Secondary structure predictions indicate a bitopic protein, anchored by a single N-terminal transmembrane segment and a C-terminal hydrophilic region that probably reaches into CF₁. Cfo-II precursor made in vitro can be imported into isolated, intact chloroplasts and assembled into ATP synthase. This protein is a real subunit of the plastid enzyme and a distinctive characteristic of ATP synthases involved in photosynthetic processes. Unique features are (i) that the gene for Cfo-II (*atpG*) appears to be a duplication of *atpF* encoding Cfo-I, the homologues of the genes for subunits *b'* and *b* in photosynthetic bacteria, (ii) that it represents the first instance that *one* copy of the various duplicated loci found in plastid chromosomes has been phylogenetically translocated to the nucleus, and (iii) that it operates with a bipartite (import/thylakoid-targeting) transit peptide but *without* an intermediate cleavage site for the stroma protease, suggestive of a way of membrane integration different from that of its plastome-encoded counterpart Cfo-I. With these data, the first complete sequence for a chloroplast ATP synthase of a higher plant (spinach) is available.

Photosynthesis; F-ATP synthase; Subunit Cfo-II gene (*atpG*); Phylogeny; Spinach

1. INTRODUCTION

Proton gradients across membranes play a crucial role in cellular energy transduction. The enzymes directly utilizing this electrochemical potential are F-type ATP synthases which are found in photosynthetic as well as in respiratory electron transport chains and located in exposed regions of thylakoid membranes as well as in inner mitochondrial or eubacterial membranes. All these enzymes possess a characteristic bipartite structure including a membrane-embedded part, Fo or Cfo, which operates in the vectorial transfer of protons across the membrane, and a peripheral catalytic sector, F₁ or CF₁, that protrudes into the organelle (or bacterial) matrix. The catalytic part of all of these enzymes is invariant and composed of five subunits (α – ϵ); the membrane sector is less well defined. The eight subunits of the F-ATP synthase from *E. coli* include three Fo components (designated a–c). Prokaryotic photosynthetic and eukaryotic F-ATP synthases may contain a greater number of subunit species than that of *E. coli*, those of chloroplasts, purple bacteria and cyanobacteria nine, mitochondrial enzymes even more (e.g. summarized in [1,2]).

The molecular biology of ATPases/synthases as well as structural and functional data have recently provided penetrating insight into proton pump families and their evolution (cf. [2]). Apparently, all F-ATP synthases originated in the eubacterial kingdom and evolved from a common ancestor [2,3]. All genes for subunits of these enzymes (*atp*) sequenced from bacteria and plastids so far are organized in operons. Their arrangement is highly conserved in prokaryotes and even plastid chromosomes maintained the basic eubacterial gene arrangement, although operon structure has occasionally been altered. The genes for Fo and F₁ are always clustered in the order *atpI*–H–F and *atpD*–A–C–B–E. They exist in separate operons in two purple bacteria suggestive of a modular evolution of both sectors [3,4], but in a single, compact operon in *E. coli* [5]. Cyanobacteria also house two operons, the *atp* operons 1 and 2, that, however, differ in two details. With purple bacteria, they share two different (duplicated and diverged) genes (*atpF* and *atpG*) for subunits *b* and *b'* that are related with the *b* subunit of *E. coli*, and *atpB/atpE* encoding the subunits β and ϵ respectively, form an own transcription unit [6–8]. Basically the same arrangement in these two gene clusters is found in plastid chromosomes [9,10] except that loci are missing which now originate in nuclear genes due to intracellular gene translocations during evolution [6,11,12]. This implies that eukaryotic (chloroplast and mitochondrial) F-ATP synthases are

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genetically bipartite. Chlorophytes lack genes for three subunits, *atpC* (γ), *atpD* (δ) and for Cfo-II, in their plastid chromosomes, chromophytic algae (chlorophyll *a/c*-lineage) only *atpC* [13].

The identity, phylogeny and functional role of the nuclear-coded subunit Cfo-II remains to be settled. Although copurification of this component with isolated ATP synthases that has been repeatedly demonstrated in 1D- or 2D-polyacrylamide gels (cf. Fig. 1 in [14]; [11,12] and references therein), and a serum directed against this subunit that abolishes ATP synthesis [15] have suggested that Cfo-II is a constituent of chloroplast ATP synthases, its association with the enzyme has remained controversial, since it appeared to lack an equivalent in *E. coli*. In principle, Cfo-II could therefore represent a phylogenetic gain, and because of this a different category of nuclear-encoded chloroplast proteins [8], or one of the subunits not found in *E. coli* [16], or simply by an artifact. The work described here resolves this enigma. For preliminary accounts see [16–18].

2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea* var. Monatol) was grown in controlled environment at 20°C and in 12 h light/dark cycles. Biochemicals were purchased from Boehringer (Mannheim) or Biolabs Inc. (Bad Schwalbach, Taunus), α -labelled [32 P]dATP (spec.act. 3,000 Ci/mmol) and [35 S]methionine (spec.act. 800 Ci/mmol) from Amersham (Braunschweig). The cDNA libraries, the techniques used to isolate and sequence [19] plasmid DNA as well as to perform agarose and polyacrylamide gel electrophoresis have previously been described [11,12,16]. Sequence data were analysed with computer programs (PC Gene) delivered by Genofit (Geneva, Switzerland). Intact chloroplasts were isolated from developing spinach leaves, purified in Percoll (Pharmacia, Freiburg i. Br.) gradients, washed and used for in organello import experiments according to [20]. Two polyclonal antisera were used at dilutions 1/500 to 1/1000, a trispecific serum raised against spinach Cfo-II (its IgGs reacting with the plastome-encoded subunits ϵ and Cfo-I [12] did not interfere with the screening of the polyA RNA-based libraries) and a monospecific Cfo-II antiserum which was kindly provided by Dr. R. Berzborn (Bochum).

3. RESULTS AND DISCUSSION

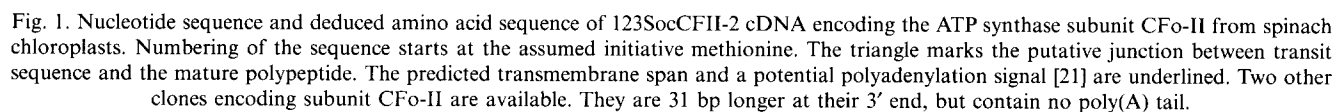
Clone selection. The recombinant phage 123SocCFII-1 which bears a cDNA clone for the ATP synthase subunit Cfo-II from spinach was initially selected serologically from a λ gt11 expression library [16] using a trispecific ([12] and see above) antiserum elicited against subunit Cfo-II. It was checked (i) on the basis of hybrid release translation experiments and immunoprecipitation of polypeptides that were synthesized in RNA-programmed cell-free rabbit reticulocyte or wheat germ translation assays, and (ii) after transport of the precursor protein made from hybrid-selected RNA in vitro into isolated, unbroken spinach chloroplasts [16]. The phage had a cDNA insert of 430 nucleotides which hybridized to a mRNA species of approximately 950 bp [16].

Attempts to isolate a 'full-length' cDNA corresponding to these messages using an RNA copy of a 181 nucleotide 5' terminal *EcoRI*–*PvuII* fragment of 123SocCFII-1 insert as a hybridization probe against 10^5 recombinant phage failed initially because of a gene-internal *EcoRI* restriction site. Sixteen positive clones were obtained which ended at the same position within the available N-terminal sequence of 32 amino acid residues [15], consistent with the absence of an *EcoRI* linker at this terminus. Fill-in synthesis of the 3' recessed ends of phage DNA by the Klenow-fragment of *E. coli* DNA polymerase I in the presence of α - 32 P]dATP consistently yielded two signals (sizes ca. 430 and > 350 bp; data not shown).

The sequence of the combined inserts (822 bp) of the phage 123SocCFII-2 is presented in Fig. 1. The favorable location of the *EcoRI* cleavage site within that part of the cDNA that encodes the determined N-terminal amino acid sequence, and the position of the *EcoRI* linkers allowed only one orientation of the two fragments. Computer-assisted translation shows that the only open reading frame of the sequence spans 666 nucleotides which translate into a protein of 222 amino acid residues. The canonical sequence environment of the first codon, 5'-CXX/ATG/G-3', fits the eukaryotic translation initiation consensus [21] and the deduced sequence starts with residues Met-Ala which is the preferred N-terminal dipeptide sequence of chloroplast transit sequences [22]. The determined N-terminal Cfo-II sequence is identical with amino acids 76–107 deduced from the cDNA nucleotide sequence [15], except for leucine at position 4 which is replaced by a glutamic acid residue. Therefore, the transit peptide is predicted to contain 75 amino acid residues (M_r 8.0 kDa), the mature protein 147 residues corresponding to a molecular weight of 16.5 kDa. This value agrees well with the estimate of 16 kDa obtained by gel electrophoresis [11,12]. Copy number determinations suggest that Cfo-II originates in a single-copy gene per haploid spinach genome ([18] and data not shown), consistent with the isolation of only a single class of cDNA clones (cf. Fig. 1).

As an additional step towards identifying the isolated cDNAs we have monitored whether 35 S-labelled translation product from RNA synthesized on linearized (ClaI) p6SocCFII-2 template in a rabbit reticulocyte-based cell-free assay can be both imported and assembled by intact spinach chloroplasts. During incubation of a postribosomal supernatant containing the primary translation product for subunit Cfo-II with illuminated, unbroken chloroplasts, the polypeptide is taken up, correctly processed and assembled into ATP synthase indistinguishable from authentic spinach Cfo-II by its size in several gel systems (Fig. 2).

A search of the complete EMBL DNA data base identified homology only to the ATP synthase subunit Fo-*b'* from *Rhodospirillum*, *Anabaena*, *Synechococcus*,



F-ATP synthases represent one of few examples known in which evolution left appropriate footprints in a single structure so that they can serve as milestones

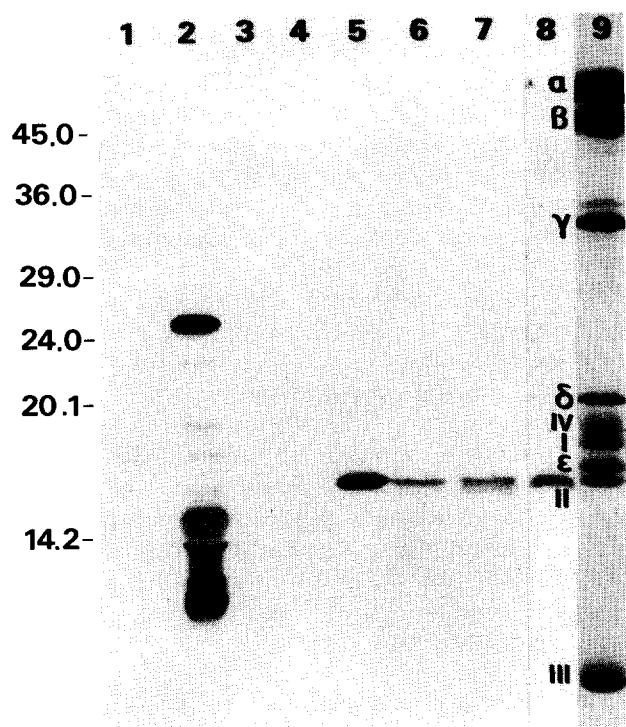


Fig. 2. In vitro transcription-translation of p6SocCFII-2 RNA and import of the in vitro CFo-II precursor product into isolated spinach chloroplasts. Fluorograph (lanes 1–8) of a 15% denaturing SDS-containing polyacrylamide gel with 2.5 M urea. (Lane 1) Control translation products without added RNA, (2) the translation products of p6SocCFII-2 RNA in a rabbit reticulocyte lysate, (3) products in the supernatant from intact chloroplasts after import of the CFo-II precursor and thermolysin treatment of the organelles, (4) stroma, (5) thylakoid fraction and (6, 7) thylakoid fractions after treatment with different concentrations of thermolysin (a detectable degradation product of 3.0 kDa, representing the N-terminal plus transmembrane residues, has migrated off the gel), (8) spinach chloroplast ATP synthase reisolated after import of the CFo-II precursor into chloroplasts. Lane 9: Spinach chloroplast ATP synthase purified on a sucrose gradient, analyzed for polypeptide composition on SDS/urea polyacrylamide gels and visualized by Coomassie Blue staining. Left: Molecular weight standards.

for phylogenetic dating. Fig. 5 compares the arrangement of ATP synthase genes in various bacterial and plastid chromosomes. The fact that the overall gene order is the same in all these organisms (cf. [2,3]) provides one of the best arguments that the organelles evolved by endosymbiosis [2,4], notwithstanding differences in organizational detail. The available data suggest that both sectors of the enzyme underwent separate evolutionary pathways [2-4] and that *atp* operons have been structurally modified only by infrequent events at discrete sites. They indicate four principal steps in their evolution: (i) the fusion of ancestral modules, probably coding separately for Fo or F₁ [4], (ii) gene duplication (*atpA/atpB*, *atpF/atpG*), (iii) operon fragmentation, and (iv) in eukaryotes, the intracellular translocation of genes. Unfortunately, there is not yet sufficient information to fix precisely all relevant points of divergence.

For instance, the *E. coli* operon is frequently considered to be ancient [8,13], but it may be a derived, streamlined version of *atp* operons. If so, it is not clear whether purple bacteria [4] or cyanobacteria [2] represent the more ancient case. We favor the former alternative, since it is in line with the idea that both subcomplexes were generated separately. It is also supported by the findings that *atpC* may be separated from or attached to the *atp1* cluster in cyanobacteria, depending on the organism [4,6], and an additional gene can be located between *atpC* and *atpB* in purple bacteria (cf. [4]). Apparently, both gene interfaces flanking *atpC* are prone to fragmentation rather than to fusion, consistent with the *atp* operon anatomy (Fig. 5). In spite of ambiguities, some basic temporal order of divergence can be inferred from occasional events that persisted in evolution. All organisms studied share *atpA/atpB* which indicates that they originated early in evolution [2]. *AtpG/atpF* are exclusively found in ATP synthases operating in photosynthesis and exist at the prokaryotic level. This implies that 'photosynthetic' and 'respiratory' F-ATP synthases must have been separated relatively early. The existence of both genes in purple bacteria suggests that this duplication has not only preceeded the appearance of eukaryotes, but also the proposed fusion of the Fo- and F₁ encoding modules. Within eukaryotes, intracellular gene translocation has occurred in at least two, possibly three or four independent events since both *atpG* and *atpD*, which are nuclear genes in chlorophyta, still reside in plastid chromosomes in the chlorophyll *alc*-line (Fig. 5). In the diatom *Odontella sinensis* and the brown alga *Dictyota dichotoma*, only subunit γ originates in a nuclear gene [13]. There is compelling evidence that chromophytic algae are the result of secondary endosymbioses (that is, their plastids are descendants of eukaryotic endosymbionts engulfed by various heterotrophic eukaryotes [26,27]). It remains, however, to be

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Fig. 3. Binary comparison of CFo-I and -II from spinach and of subunits *b* and *b'* of the ATP synthase from various species. Numbers in columns refer to the percentage of identical amino acids between subunits using the method of [23] with a window size of 20 residues; a dash marks alignments where no homology between two proteins was found. The similarity is approximately twofold greater, if conservative replacements are taken into account. Where the comparison was made between sequences of unequal length, the percentage was calculated from the longer one. Sequences are taken from [4-9,13,24] and this paper.

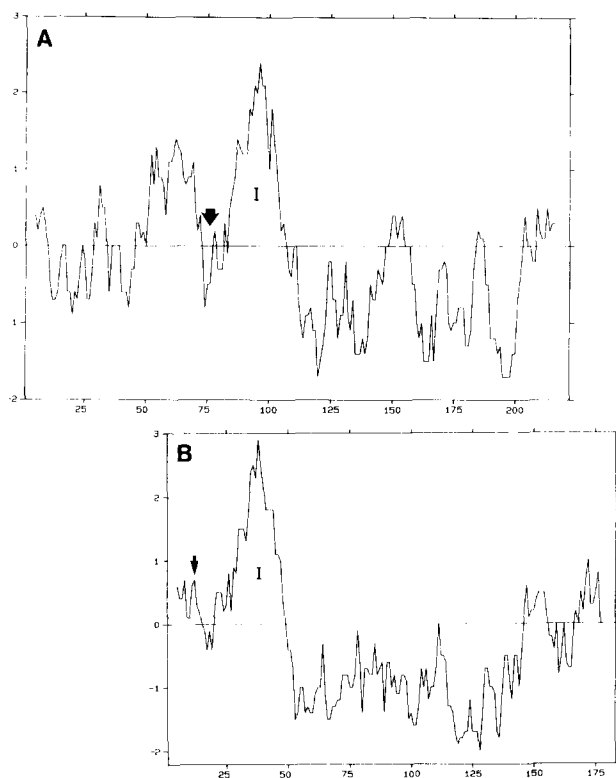


Fig. 4. Hydropathy plots [25] of (A) subunit CFo-II and (B) CFo-I from spinach ATP synthase. The blots were produced with a window average across 19 residues. The arrows indicate the processing sites between transit peptide (CFo-II) or N-terminal residues (CFo-I) and mature protein. The potential transmembrane segment is marked by (I).

settled whether the *alc*-lineage represents a phylogenetic intermediate to the development of the chlorophyll *alb*-line, split off before *atpG* and *atpD* had been translo-

cated, or whether the two lines evolved independently. This important point could be elucidated with appropriate plant material, possibly including studies on the origin and relationship of the various antenna systems that have been attached to the phylogenetically basic and highly conserved photosynthetic reaction centers [28,29]. Fig. 5 summarizes some novel aspects of late F-ATP synthase evolution; for the early evolution see [2].

We have previously noted that the genes for various thylakoid membrane proteins must have evolved by a series of duplications of certain primordial DNA segments [30]. Besides *atpA/atpB* and *atpF/atpG*, these include *psaA/psaB*, *psbB/psbC*, *psbA/psbD* and *psbE/psbF* encoding components of photosystems I and II, respectively. All pairs still reside in plastid chromosomes, except *atpF* and *atpG* which are deposited in different subcellular compartments. This offers the unique opportunity to probe into possible changes of regulatory mechanisms involved in intracellular differentiation due to gene transfer.

3.4. The transit sequence, aspects of assembly

Chloroplast transit sequences fall into two major classes [22], mere import sequences (or stroma-targeting transit peptides) which are removed by a stroma-located metallo-protease during or after import, and relatively long (ca. 70–85 amino acid residues), bipartite stroma-targeting/thylakoid-translocating transit peptides which are processed in two steps involving an additional protease located on the inner side of stroma thylakoids [31]. The thylakoid-targeting domains of the latter presequences are characterized by a hydrophobic core of variable length (13–17 residues) and flanking polar or hydrophilic residues on either side [22] which are critical for correct interaction with, partition into and translo-

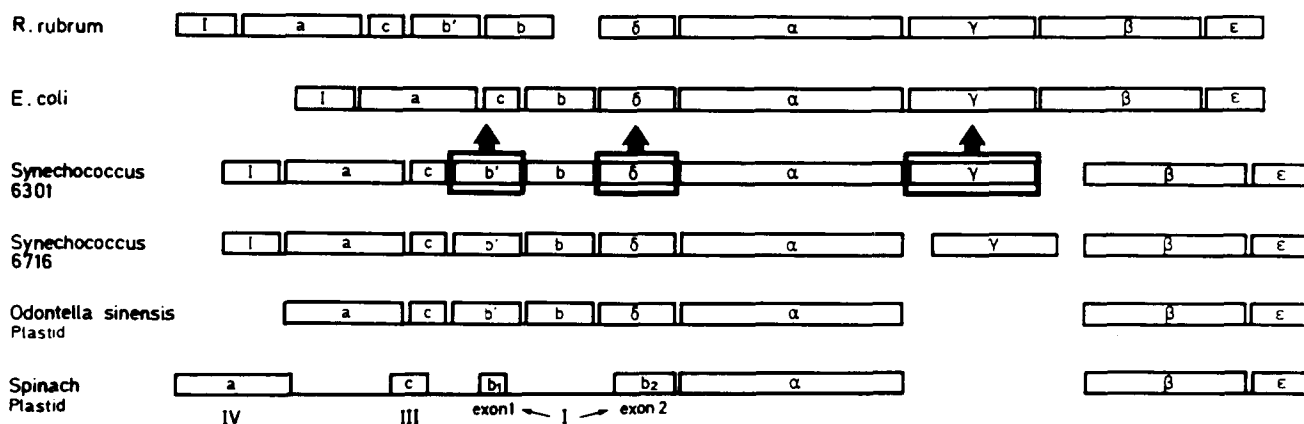


Fig. 5. Physical maps of the *atp* operons from *E. coli* [5], *Rhodospirillum rubrum* [4] cyanobacteria (cf. [4,6–8], *Odontella sinensis* [13] and the spinach plastid chromosome [9]. Segmented operons are individually represented. Transcription runs from left to right in each instance. The maps are aligned with the approximate intergenic distances so that all loci are put at equivalent positions. The coding regions are designated α to ϵ for CF₁ (F₁) subunits *a* (IV), *b* (I), *b'* and *c* (III) for those constituting CFo (Fo). The arrowed boxes in the *Synechococcus* 6301 scheme designate those genes that are nuclear chlorophyta.

cation across the thylakoid membrane [32]. Composite transit peptides are restricted to hydrophilic, luminal proteins including plastocyanin, the 16, 23 and 33 kDa polypeptides of the oxygen-evolving complex [22]. Integral proteins, such as the chlorophyll *alb* and CP24 apoproteins [33,34], or the Rieske Fe/S protein of the cytochrome complex [35], generally carry stroma-targeting transit sequences and integrate *via* internal, uncleaved hydrophobic epitopes (e.g. [33,34]).

Although CFo-II is an integral protein and does not traverse the thylakoid membrane, an intriguing feature of its transit peptide is that it resembles those of the extrinsic luminal proteins (Fig. 4) suggesting that its transitory hydrophobic domain and not (only?) its transmembrane segment ensures or contributes to its correct insertion and integration into the thylakoid membrane. This differs from CFo-I which is of plastid origin, and not made with a typical transitory thylakoid-targeting segment [9] as cytochrome *f*, which is also bitopic and, although synthesized within the organelle as well, is made as a precursor with an N-terminal extension reminiscent of thylakoid-targeting domains of nuclear-coded luminal proteins [35]. In wheat, CFo-I loses posttranslationally 17 (hydrophilic) N-terminal residues ([36]; cf. also Fig. 4). These do not exhibit any similarity with transit or targeting sequences. Although this finds an analogue in CFo-IV [37], their role, if any, is unclear. CFo-I has therefore both to integrate and assemble *via* its internal (uncleaved) hydrophobic epitope which in this instance also serves to anchor the protein in the membrane. Analysis of the CFo-II presequence shows that it operates indeed as a composite transit peptide but that the synthesis of the mature component occurs in only one processing step and not in two or even three as might be anticipated from the comparison with CFo-I (Michl D., personal communication). CFo-II represents therefore a first case that an intrinsic polypeptide exists with a bipartite transit peptide and that such a presequence operates without an intermediate cleavage site.

The biological significance for the outlined differences between the various bitopic thylakoid proteins is not clear and probably finds a phylogenetic explanation [28,34]. It is unlikely that they reside in different integration modes (co- or posttranslational) as a consequence of the different intracellular location of genes, or in positional differences of the hydrophobic domain which is N-terminal in the CFo subunits but C-terminal in cytochrome *f*. Whatever the cause, it is obvious that bitopic proteins, because of their relatively simple transmembrane arrangement and the fact that even such proteins can use different routing and integration modes, provide appealing models for studying subcellular differentiation, for delineating epitopes functional in intraorganelle sorting and topogenesis of structure as well as their evolution due to intracellular gene translocation in eukaryotes.

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