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The γ-subunit of ATP synthase from spinach chloroplasts

Primary structure deduced from the cloned cDNA sequence

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cDNA clones encoding the γ-subunit of chloroplast ATP synthase were isolated from a spinach library using synthetic oligonucleotide probes. The predicted amino acid sequence indicated that the mature chloroplast γ-subunit consists of 323 amino acid residues and is highly homologous (55% identical residues) with the sequence of the cyanobacterial subunit. The positions of the four cysteine residues were identified. The carboxyl-terminal region of the choloroplast γ-subunit is highly homologous with those of the γ-subunits from six other sources (bacteria and mitochondria) sequenced thus far.

H^+-ATPase; F_1; γ-Subunit; Cyanobacteria; cDNA; (Spinach chloroplast)

1. INTRODUCTION

ATP synthase (CF_0F_1) of chloroplast thylakoids catalyzes the light-driven synthesis of ATP from ADP and P_i [1,2]. The catalytic portion, CF_1, consists of five different subunits α, β, γ, δ and ε [3]. Isolated CF_1 is a latent ATPase that can be activated by a variety of methods [4], whereas the F_1 of mitochondria or bacteria already has ATPase activity without such treatments. The γ-subunit of CF_1 is believed to be important in the regulation of ATPase activity and the flow of protons through the proton pathway (CF_0) of the enzyme [1,4]. Conformational changes of the γ-subunit, which have been studied by examining alterations in reactivity to sulfhydryl reagents and sensitivity to trypsin [5–8], may be closely related to the regulatory roles of this subunit.

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The amino acid sequence of the *E. coli* γ-subunit [9,10] is known from the DNA sequence, and studies with mutants suggested that both the amino- and carboxyl-terminal regions of this subunit are required for assembly of F_1 and its catalytic function [11,12]. The primary sequences of the γ-subunits from *Rhodospirillum rubrum* [13], *Rhodopseudomonas blastica* [14], thermophilic bacterium PS3 [15], *Synechococcus* sp.6301 [16] and bovine mitochondria [17] have also been determined. Here, we cloned and sequenced cDNA coding for the γ-subunit of spinach chloroplasts, since determination of its primary sequence is essential for understanding the regulation of CF_1 and since it was of interest to compare its sequence with those of γ-subunits from other sources. From the predicted amino acid sequence, we identified conserved residues and discuss the significance of the regions containing cysteine residues, which have been studied extensively in relation to conformational change of CF_1 [8].

2. MATERIALS AND METHODS

Purified CF_1 from spinach was subjected to polyacrylamide

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gel electrophoresis in the presence of SDS [18], and the γ-subunit was recovered electrophoretically from the gel matrix. The amino-terminal sequence of the γ-subunit from the amino terminus to residue 20 (ANLRELRDRIGSVKNTQKIT) was determined with a gas-phase sequencer (Applied Biosystems). A 20-base oligonucleotide probe [5'-GTIAA(A/G)AA(T/C)ACI-CA(A/G)AA(A/G)AT-3'] (probe N) corresponding to the amino-terminal region between the Val-13 and Ile-19 residues and the 21-base oligonucleotide [5'-(T/C)TC(A/G)TC(T/C)T-CIGCIGC(A/G)TCIAC-3'] (probe S2) corresponding to tryptic peptide S2 [16] were synthesized on an Applied Biosystems DNA synthesizer.

Spinach (*Spinacia oleracea* var. Nobel) seeds were obtained from a local store and grown under conditions of high humidity. After 5–6 days in absolute darkness, the plants were placed under daylight and cotyledons were collected 10 h later. Total RNA was isolated from the tissue homogenate by phenol extraction, poly(A)$^+$ RNA being isolated by oligo(dT)-cellulose column chromatography [19].

Single-stranded cDNA was synthesized by oligo(dT) priming from mixtures with poly(A)$^+$ RNA as templates, and double-stranded cDNA by using *E. coli* DNA polymerase I, RNase H and DNA ligase [20]. Flush ends were generated with T_4 DNA polymerase, and the double-stranded DNA was treated with *Eco*RI methylase. After ligation with *Eco*RI linker, the resulting DNA was digested with *Eco*RI and passed through a Sephadex G-25 column. Flush ended DNA was also directly ligated with *Eco*RI adapter (Pharmacia). Both DNA preparations were ligated with pUC18 and recombinant plasmids were introduced into *E. coli* C600 [21]. About 10^4 colonies of both DNA preparations were screened by colony hybridization with probes N and S2 labeled at the 5'-end with ^{32}P. Nucleotide sequences were determined by the dideoxynucleotide chain-termination method [22]. Other reagents used were as described in [12] or of the highest grade commercially available.

3. RESULTS

We have identified two clones carrying parts of the cDNA for the CF_1 γ-subunit: pSG302 and pSG402 were identified in the cDNA library constructed using *Eco*RI linker and adapter, respectively (fig.1A). The recombinant plasmid pSG302 (carrying 750 bp cDNA) hybridized with both probe N and S2, while pSG402 (carrying 700 bp cDNA) hybridized with only probe S2. Both strands of the inserts of the two plasmids were sequenced and the cDNA coding for the γ-subunit was obtained (fig.1B). pSG402 had an internal *Eco*RI site, while pSG302 carried a sequence upstream of this *Eco*RI site, suggesting that methylation with *Eco*RI methylase was not sufficient for constructing the latter plasmid. The open reading frame was 987 bp long and encoded 329 amino acid residues. This sequence contained the amino-terminal sequence of the mature protein determined chemically (residues Ala-1 to Thr-20), and part of the transit sequence (residues -1 to -6). Thus, the mature protein consisted of 323 amino acid residues of molecular mass 35.7 kDa, which is in good agreement with the value of 35 kDa determined from the mobility of the protein on polyacrylamide gel electrophoresis in the presence of SDS [26]. We identified sequences having a similar amino acid composition to those of the four tryptic peptides (S1–S4) [23] (fig.1B). The amino acid sequence of tryptic peptide S2 [16] was identical with that of the residues between 205 and 215. The overall amino acid composition of the mature γ-subunit was essentially the same as that determined chemically [23].

4. DISCUSSION

It is of interest to compare the amino acid sequence of the γ-subunit of spinach chloroplasts with those of six other sources that have been determined so far [9,10,13–17]. The chloroplast sequence had the highest homology (55%) with that of the γ-subunit from the cyanobacterium *Synechococcus* sp., and lower homology with those of the subunits from *E. coli* (33%) and bovine heart (27%), supporting the close evolutionary relationship between chloroplasts and cyanobacteria. When all the γ-subunits thus far sequenced are aligned to obtain maximal homology, only 33 residues are identical. The carboxyl-

Fig.1. Nucleotide and deduced amino acid sequence of the γ-subunit of spinach chloroplast ATP synthase (CF_0F_1). (A) Partial restriction endonuclease map of the two cDNA clones pSG302 and pSG402. The reading frame for the γ-subunit is shown by an open box. Both strands of the cDNA clones were sequenced. Sites of restriction endonucleases: Ha, *Hae*III; H, *Hin*dIII; S, *Sal*I; E, *Eco*RI; B, *Bgl*II. (B) Nucleotide sequence and deduced amino acid residues of the cDNA coding for the γ-subunit. The amino-terminal sequence of the γ-subunit obtained chemically (residues Ala-1 to Thr-20) is underlined. Regions corresponding to the four tryptic fragments (S1–S4) [23] are also shown. Numbers in parentheses indicate amino acid residues from the amino terminus of the mature γ-subunit.

A

B

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          10         20         30         40         50         60         70         80         90
ACAAACCCAATCCAATGCGCAAACCTCCGTGAGCTACGAGACCGGATCGGATCAGTCAAAAACACGCAGAAGATCACCGAAGCAATGAAG
T  N  P  I  Q  C  A  N  L  R  E  L  R  D  R  I  G  S  V  K  N  T  Q  K  I  T  E  A  M  K
-6            -1          Amino terminal sequence ( 1 - 20 )

         100        110        120        130        140        150        160        170        180
CTCGTCGCCGCCGCTAAAGTCCGCCGTGCGCAAGAAGCCGTCGTAAACGGCCGCCCCTTCTCGGAGACTCTAGTCGAAGTTCTTTACAAC
L  V  A  A  A  K  V  R  R  A  Q  E  A  V  V  N  G  R  P  F  S  E  T  L  V  E  V  L  Y  N

         190        200        210        220        230        240        250        260        270
ATGAATGAACAGCTACAGACTGAGGATGTTGATGTTCCTCTGACGAAGATTCGGACGGTGAAGAAGGTGGCGTTGATGGTGGTTACCGGC
M  N  E  Q  L  Q  T  E  D  V  D  V  P  L  T  K  I  R  T  V  K  K  V  A  L  M  V  V  T  G

         280        290        300        310        320        330        340        350        360
GACCGTGGTCTTTGCGGCGGGTTTAATAATATGTTGCTGAAGAAGGCTGAGTCTAGGATTGCTGAGCTTAAGAAGCTTGGTGTTGATTAT
D  R  G  L  C  G  G  F  N  N  M  L  L  K  K  A  E  S  R  I  A  E  L  K  K  L  G  V  D  Y
                S3 (87-98)

         370        380        390        400        410        420        430        440        450
ACTATTATTAGTATTGGAAAGAAAGGAAACACTTATTTTATCCGGCGTCCTGAGATTCCCGTCGACAGGTACTTCGACGGAACAAACCTA
T  I  I  S  I  G  K  K  G  N  T  Y  F  I  R  R  P  E  I  P  V  D  R  Y  F  D  G  T  N  L

         460        470        480        490        500        510        520        530        540
CCAACCGCCAAAGAAGCACAAGCCATAGCAGACGACGTCTTCTCCCTATTCGTAAGCGAAGAAGTCGACAAAGTCGAAATGCTCTACACA
P  T  A  K  E  A  Q  A  I  A  D  D  V  F  S  L  F  V  S  E  E  V  D  K  V  E  M  L  Y  T

         550        560        570        580        590        600        610        620        630
AAATTCGTCTCTTTAGTAAAATCAGACCCAGTAATCCACACCCTACTCCCCCTCTCACCCAAAGGAGAAATTTGCGACATCAATGGAAAA
K  F  V  S  L  V  K  S  D  P  V  I  H  T  L  L  P  L  S  P  K  G  E  I  C  D  I  N  G  K
                                                                  S1 (196-204)

         640        650        660        670        680        690        700        710        720
TGTGTCGACGCAGCAGAAGACGAACTCTTCCGTCTCACAACAAAAGAAGGTAAGCTAACGGTAGAAAGAGACATGATCAAAACCGAAACA
C  V  D  A  A  E  D  E  L  F  R  L  T  T  K  E  G  K  L  T  V  E  R  D  M  I  K  T  E  T
          S2 (205-215)

         730        740        750        760        770        780        790        800        810
CCAGCATTTTCCCCAATTCTGGAATTCGAACAAGATCCTGCTCAAATTCTCGACGCTTTGCTTCCATTATACTTAAACAGTCAGATTTTG
P  A  F  S  P  I  L  E  F  E  Q  D  P  A  Q  I  L  D  A  L  L  P  L  Y  L  N  S  Q  I  L

         820        830        840        850        860        870        880        890        900
AGGGCTTTACAAGAATCACTTGCTAGTGAACTTGCTGCGAGGATGACTGCTATGAGTAATGCTACTGATAATGCGAATGAGTTGAAGAAG
R  A  L  Q  E  S  L  A  S  E  L  A  A  R  M  T  A  M  S  N  A  T  D  N  A  N  E  L  K  K

         910        920        930        940        950        960        970        980        990
ACGTTGTCTATTAATTATAATAGAGCGCGTCAGGCTAAGATTACTGGTGAGATCTTGGAGATTGTTGCTGGTGCTAATGCATGTGTTTGA
T  L  S  I  N  Y  N  R  A  R  Q  A  K  I  T  G  E  I  L  E  I  V  A  G  A  N  A  C  V  *
                                                S4 (308-323)

        1000       1010       1020       1030       1040       1050       1060       1070       1080
TTAATCAATTTGTTTTTACAATATGGTTATTTGTACTTACTTATTTTTGGTTAATAATTGTTGGTATAATTATAAATATGGATATATTAT

        1090       1100       1110       1120       1130       1140
GTACGTTTTTGTGATGGTTTGAAATGGACAGGGGATATATCAAGTGCAGAGAGATTAATT
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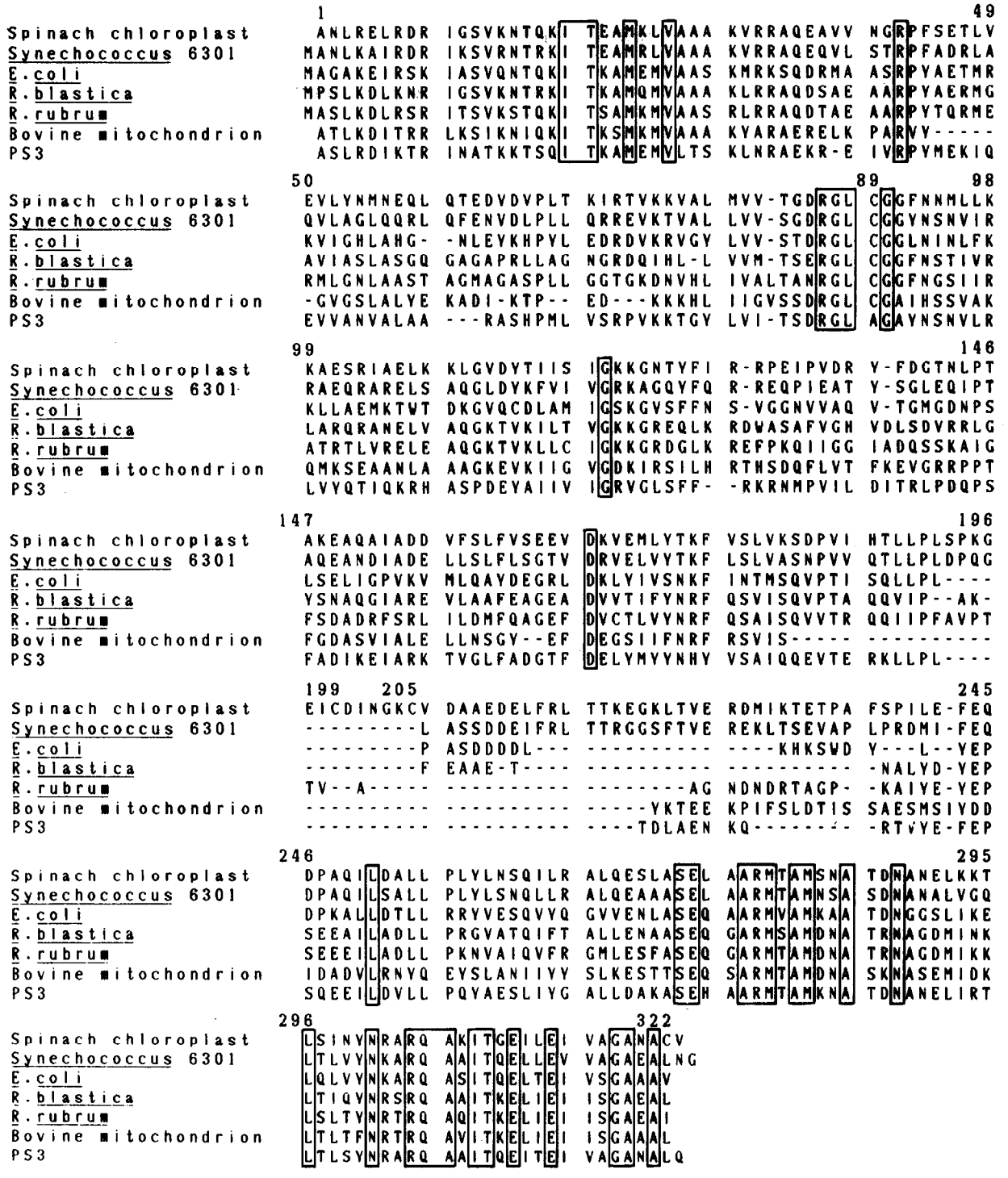

Fig.2. Alignment of amino acid sequences of γ-subunits from various sources. The sequences of the γ-subunits of *E. coli* [9,10], *R. rubrum* [13], *Rps. blastica* [14], thermophilic bacterium PS3 [15], *Synechococcus* sp. [16], beef heart mitochondria [17], and spinach chloroplasts were aligned to obtain maximal homology. Identical residues are boxed and gaps (– – –) have been inserted. Amino acid residues of the spinach subunit are numbered from the amino terminus.

terminal regions of the subunits are highly conserved in the seven species and seem to be essential for assembly and catalysis, consistent with the conclusion drawn from results with *E. coli* mutants. A nonsense mutant (Gln-269 ⟶ end) had an F_1 complex without ATPase activity on its membranes, although this complex was unstable after solubilization, whereas another mutant (Gln-261 ⟶ end) showed essentially no F_1 assembly [12] (*E. coli* sequence numbered from the second codon [12]). Residues Met-23 and Val-26 of the *E. coli* γ-subunit are conserved in other γ-subunits and this region also contains residues conservatively substituted. Consistent with these findings, a deletion of 7 amino acid residues (Lys-21 to Ala-27) was found to result in loss of F_1 assembly [11]. It will be of interest to replace the conserved residues using site-directed mutagenesis and study the altered function of the mutant subunits.

Consistent with the findings of McCarty and coworkers [23], the chloroplast γ-subunit was shown to have four Cys residues. Cys-89 corresponds to the cysteine residue in peptide S3 [23] that is modified with sulfhydryl reagents such as *N*-ethylmaleimide in the light. The modification resulted in inhibition of ATP synthesis and hydrolysis by CF_1 [24], suggesting that Cys-89 is essential for the CF_1 activity. As shown in fig.2, this Cys residue is conserved in all γ-subunits so far sequenced except that of the thermophilic bacterium PS3 [15], which has an Ala residue in the corresponding position. Four residues around Cys or Ala are conserved in all species, supporting the importance of this region. Cys-322 corresponds to the cysteine residue in peptide S4 [23] and is modified in the dark. Although conserved residues are clustered in the carboxyl-terminal region of the γ-subunits (fig.2), no cysteine was present at the homologous position of the γ-subunits from other sources, suggesting that Cys-322 is not essential for the catalysis. The two Cys residues forming the disulfide bond found in tryptic fragments S1 and S2 [23] correspond to Cys-199 and Cys-205, respectively, of the deduced sequence. The two Cys residues were in the extra-domain (around Ser-193 to Phe-237) which was not found in any other subunits except that of *Synechococcus* sp. Since reduction of the disulfide bond was associated with activation of ATP hydrolysis and synthesis by CF_1 [7,25], this extra-domain may be essential for the regulation of catalytic activity of CF_1. In the cyanobacterium, the extra-domain is 9 residues smaller and does not contain the two Cys residues. Further work on the role of the extra-domain in *Synechococcus* sp. appears of interest.

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