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Short Sequence-Paper

Sequence of the γ-subunit of *Spirulina platensis*: A new principle of thiol modulation of F_0F_1 ATP synthase?

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Abstract

The gene encoding the γ subunit of *Spirulina platensis* F_0F_1, the relative of the chloroplast F_1 subunit responsible for thiol activation, has been cloned and sequenced. As in other cyanobacteria, a specific couple of cysteines like those involved in thiol modulation of the chloroplast enzyme was not found. Instead, two cysteine residues were identified in the *Spirulina* subunit at positions unique amongst all so far sequenced γ subunits. Involvement of these cysteines in the thiol-modulation of the *Spirulina* enzyme reported before (Hicks and Yocum (1986) Arch. Biochem. Biophys. 245, 230–237, and Lerma and Gomez-Lojero (1987) Photosynth. Res. 11, 265–277) would manifest a re-invention of a regulatory mechanism.

Keywords: ATPase, F_0F_1-; Regulation; γ Subunit; Cyanobacterium

F-type ATPases are the key enzymes in the utilization of transmembrane electrochemical potential differences for ATP synthesis. They consist of two main entities, a membrane integral F_0-part and an F_1-part, which binds to F_0 and protrudes from the membrane surface. Bacterial and chloroplast F_1 typically has five different subunits, named α, β, γ, δ, and ε in order of decreasing masses and present in 3:3:1:1:1 stoichiometry. F_0 shows more variation, having always one *a* subunit (named IV in chloroplasts), two *b* subunits in most bacteria, and perhaps one *b* and one *b'* subunit in photosynthetic bacteria and chloroplasts (here: subunits I and II). Finally, there are 6 to 12 proteolipid subunits (*c* in bacteria, subunit III in chloroplasts) present per F_0. Transfer of the coupling ions through F_0 is probably mechanically coupled to the catalytic events, occuring in three catalytic sites in F_1 [1–3].

From a thermodynamical view, either ATP synthesis or hydrolysis should occur in dependence on the phosphate potential encountered by any F_0F_1 enzyme. Such reversibility, however, would be unfavourable, for instance, in chloroplasts, where ATP synthesis, but not ATP hydrolysis, should take place. Several regulatory mechanisms have been demonstrated to act on F_0F_1 enzymes in different species. Inhibitory subunits in F_1 may suppress or attenuate ATP hydrolysis. Under conditions favourable for ATP hydrolysis, ADP binds tightly into a catalytic site and inhibits ATPase activity [4]. After imposition of a protonmotive force sufficient to drive ATP synthesis, this inhibitory ADP is released from the enzyme. The inhibitory subunit can also dissociate (IF_1 in mitochondria [5]) or change its binding location (e.g., ε in chloroplasts [6]). In chloroplast F_0F_1, a special pair of two closely spaced cysteines in the γ subunit builds up a disulfide bridge under oxidizing conditions [7]. Upon reduction of this disulfide, the enzyme's threshold for activation by the electrochemical potential of protons across the thylakoid membrane is substantially lowered [8].

In accordance with the endosymbiontic theory, proposing that cyanobacteria and chloroplasts share a common ancestor [9], their respective ATP synthases are closely related [10]. One distinct difference however manifests in the absence of a short stretch of 9 amino acids in the γ-subunit of cyanobacterial F_0F_1 as compared to the chloroplast enzymes. Since this stretch contains the two

Abbreviations: ATCC, American Type Culture Collection; bp, base-pairs; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PCC, Pasteur Culture Collection; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tricine, *N*-tris(hydroxymethyl)methyl glycine; Tris, *N*-tris(hydroxymethyl)aminomethane.

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cysteines responsible for the thiol modulation of F_0F_1 in chloroplasts [11], such a regulatory process, which was accordingly not found in *Synechococcus* sp. PCC 6716 [12], was so far supposed to be absent in cyanobacteria. In 1986, a 4- to 10-fold acceleration of ATP hydrolysis was reported for membranes prepared from *Spirulina platensis* after incubation with DTT in the light [13]. Subsequently, Hicks et al. demonstrated immunological cross-reactivity of *S. platensis* F_0F_1 α, β, and γ subunits with antisera raised against respective spinach subunits [14]. Later, F_0F_1 from *Spirulina maxima* membranes was also shown to be activatable by DTT treatment [15]. Bakels et al. recently examined the activation of *S. platensis* F_0F_1 and found an unexpected low threshold for ATP synthesis after illumination of the membranes in the presence of DTT as compared to untreated membranes [16].

In the light of these evidences, *Spirulina* F_0F_1 appears to be thiol-modulated, a regulatory mechanism so far uncommon to cyanobacterial F-ATPases. Therefore we determined the primary structure of this enzyme's γ subunit as the paramount candidate for such a feature. Degenerated primers were designed according to the consensus of conserved regions near the 3′ ends of cyanobacterial *atpA* genes, coding for F_1 α, near the 5′ end of *atpC*, and more inside of *atpC*, encoding F_1 γ subunits. Sequencing of pieces obtained from PCR runs with *S. platensis* ge-

Fig. 1. Preparation of *Spirulina platensis* F_1. The figure shows a silver-stained SDS-PAGE gel (PhastSystem, Pharmacia) of a preparation of *Spirulina platensis* F_1. Lane (a) shows spinach CF_1 as standard, lane (b) F_1 prepared from *S. platensis*. The arrow marks the band subjected to protein-sequencing. *S. platensis* was grown under continuous light in medium 1679 SOT (recipe obtained from the ATCC online catalogue on Internet). Cells from 2 liters of culture at an A_{730} of 1.0 were pelleted and resuspended to 0.1 mg/ml chlorophyll in a buffer, containing 20 mM Hepes at pH 7.5, 0.4 M sucrose, 15 mM NaCl, 3 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM PMSF, 1 mM Benzamidine, and 0.1 mM TPCK. The cells were broken by 5 min sonication in a Branson B15 Cell Disruptor at 80% output on ice. All further procedures were carried out on ice or in refrigerated centrifuges (4° C). After 10 min centrifugation at $1000 \times g$, supernatants were collected and centrifuged again 1 h at $100\,000 \times g$. The pellets were resuspended to 1 mg/ml chlorophyll in 20 mM Hepes (pH 7.5), 0.4 M sucrose, and 15 mM NaCl. To remove phycobilisomes, this suspension was diluted to 0.2 mg/ml chlorophyll in 10 mM $Na_2P_4O_7$ and centrifuged another 30 min at $100\,000 \times g$. F_1 was extracted by resuspension of the pellet to 0.1 mg/ml chlorophyll and 30 min incubation in 50 mM sucrose, 2 mM Tris, adjusted to pH 7.5 by addition of solid Tricine. After another 30 min centrifugation at $100\,000 \times g$, the supernatant was loaded on a Resource Q column (Pharmacia). The column was equilibrated with 25 mM Tris-HCl and developed with 1 M NaCl in the same buffer. F_1 eluted around 260 mM NaCl as judged by ATPase measurements and SDS PAGE.

nomic DNA as a template revealed the anticipated similarities to respective cyanobacterial genes. Non-degenerated primers were synthesized and used to generate new and independent clones covering different regions of *atpC*. To unravel the C-terminus of the protein, a primer was synthesized representing a region near the 3′ end of the sequence known so far, and pointing downstream. Another highly degenerated 54mer was constructed according to a consensus of the 3′ ends of the known cyanobacterial sequences, pointing upstream. From the first stop codon present in the known sequences on to the end of the primer, the degenerated sequence comprised stop codons as well as those coding for appropriate amino acids as found in the consensus. Since the sequenced pieces were all obtained by means of PCR, care was taken to sequence every single base from several independent clones generated by different runs. By this procedure, we attempted to avoid the propagation of sequence mistakes introduced by PCR faults.

In order to confirm the correct localization of the start codon we subjected the *Spirulina* F_1 γ subunit to protein sequencing. F_1 was prepared according to the procedure outlined in the legend to Fig. 1. Protein sequencing revealed the sequence SNLKAIRDRIQSVK for the N-terminus of the γ subunit, confirming the DNA sequence and the putative identification of the start codon. The complete sequence of the *Spirulina platensis* γ subunit is shown in Fig. 2 aligned to five different consensus sequences, derived from sets of genes encoding F_1 γ subunits from cyanobacteria, chloroplasts, mitochondria, Gram-negative and Gram-positive bacteria, respectively. The *S. platensis* γ subunit shows high similarity to other known γ subunits, especially those from cyanobacteria. Most strikingly, the regulative loop of 9 amino acids containing the two cysteines in chloroplasts (marked by a bar in Fig. 2), is completely absent from *S. platensis* γ. Only one conserved cysteine was found at position 90, and additionally two cysteines occurred at locations unique to the *Spirulina* protein. In order to examine the evolutionary position of the *S. platensis* γ subunit in relation to those from other species, we calculated the phylogenetic tree shown in Fig. 3. This analysis placed *S. platensis* γ into one monophyletic group together with all the other cyanobacterial sequences. Since it was generated by means of a degenerated primer, the sequence at the very 3′ end of the gene might still be erroneous. This does not, however, influence the phylogenetic analysis, which was by omission of the 5′ and 3′ ends restricted to a slightly smaller part of the subunit (indicated by squared brackets in Fig. 2).

Fairly high identity amongst all γ subunits sequenced so far exists in two stretches close to both ends of the molecules. This is not surprising upon inspection of the recently published 2.8 Å crystal structure of mitochondrial F_1 [17]. The conserved regions apparently make up two long helices, extending through the whole F_1 part and

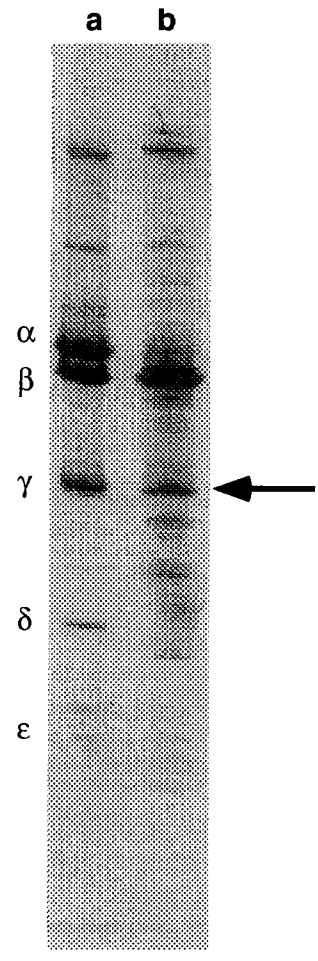

Fig. 2. Alignment of γ subunits from different species. Alignments in order to minimize gaps were carried out by the GCG PILEUP program [20]. Most sequences used in this work were obtained from the NCBI WWW GenBank server via Mosaic 2.0 for Apple Macintosh (for accession codes, see legend to Figure 3). The sequences of the following species were compiled into consensus sequences: chloroplasts: *S. oleracea, A. thaliana, N. tabacum, P. sativum*; cyanobacteria: *A.* 7120, *S.* 6301, *S.* 6716, *S.* 6803; mitochondria: *B. taurus, H. sapiens, S. cerevisiae*; Gram-positives: *B. caldotenax, B. stearothermophilus, B* PS3, *B. megaterium, B. firmus*; Gram-negatives: *E. coli, V. alginolyticus, T. ferrooxidans*. The nucleotide sequence of *S. platensis* γ has been submitted to EMBL and assigned the accession number Z46799.

making numerous contacts to other subunits. The less well conserved middle part of the subunit seems to protrude out of the bulk of F_1 and was not resolved in the crystal structure. It is in this part of the protein where the chloroplast subunits contain an extra stretch of 9 amino acids, including the two cysteines involved in thiol modulation. It appears to us that cyanobacteria lost this regulative stretch during evolution, since in the sequences of all other bacteria as well as of mitochondria, the respective gap is much larger than 9 amino acids, e.g., 37 residues in Gram-positives. If the regulative sequence was present in the common ancestor of chloroplasts and cyanobacteria, any other putative structural prerequisites perhaps needed for thiol modulation might still be present in cyanobacterial F_0F_1.

Consistently, the introduction of the regulative stretch into the γ subunit of *Synechocystis* sp. PCC 6803 yielded a mutant F_1 with highly DTT-sensitive ATPase activity [18]. Bakels et al. stressed a physiological argument in order to explain the need of *S. platensis* for a thiol-modulated ATP synthase [16]. The reduced threshold for F_0F_1 activity in this alkalophilic bacterium should according to them enable synthesis of ATP under conditions of a low electrochemical potential of the proton across the cytoplasmic membrane, i.e., at a high H/ATP ratio. One could argue that there are other alkalophilic bacteria like *B. firmus* [19] which certainly do not show thiol-modulation of their F-ATPases. On the other hand, Gram positive *Bacillae*are grouped far away from cyanobacteria and chloroplasts in the evolutionary tree shown in Fig. 3. Their F_0F_1 may simply lack those above-mentioned putative prerequisites needed for thiol-modulation in excess of a cysteine-pair in γ, which would then prevent any re-invention of such regulation as might have occurred in *Spirulina*. Thiol-modulation in *Spirulina* then might play the same role as in chloroplasts, i.e., raising the enzyme's activation threshold upon oxidation of the cysteines and thereby preventing wasteful ATP-hydrolysis.

Amongst the 29 sequences examined during this work, two cysteines are uniquely found in *S. platensis*. They are located in weakly (Cys-67) or not at all (Cys-138) conserved regions of the subunit and appear now to be the most likely candidates for the observed activation, since

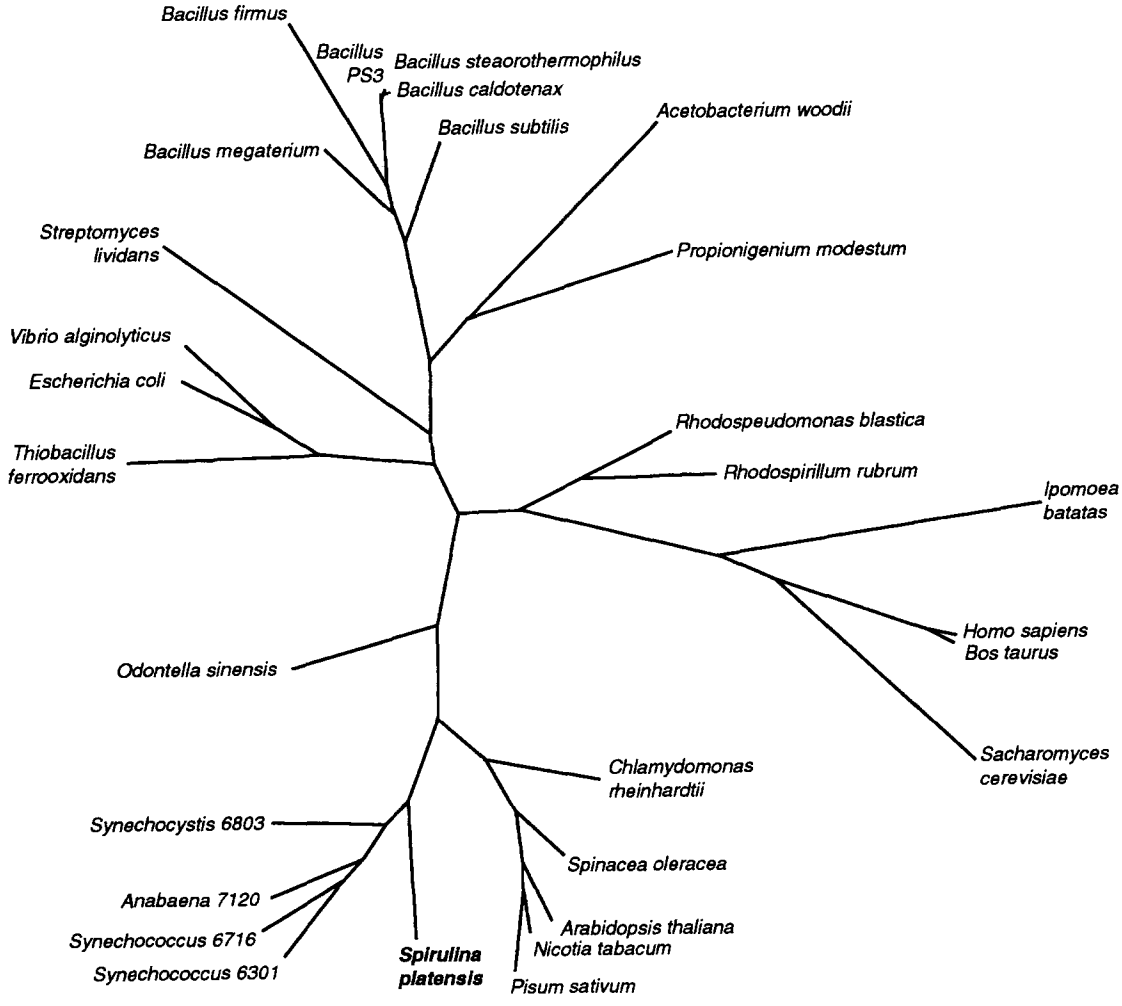

Fig. 3. Phylogenetic relations of F_1 γ subunits. The tree was constructed from 28 different sequences found in GenBank release 84.0, plus the *S. platensis* sequence reported here. Nucleotide sequences starting at position 10 according to the *S. platensis* numbering and ending at position 932 were aligned by means of the GCG PILEUP program [20]. Calculations of phylogenetic trees were carried out by the program DNAML of J. Felsenstein's PHYLIP program package [21] running on a DEC AXP 3400. The branch lengths of the resulting tree indicate phylogenetic distances between the γ subunits of any two species. GenBank accession codes for references are: *S. oleracea* (SOATPC), *A. thaliana* (ATHATPC1), *N. tabacum* (NTATPC), *P. sativum* (PSATPC), *C. rheinhardtii* (CREATPS), *O. sinensis* (OSATPASE), *A.* 7120 (ANAATP1), *S.* 6301 (SYATPS), *S.* 6716 (S6716ATPG), *S.* 6803 (SSATP1), *B. taurus* (BOVATPS), *H. sapiens* (HUMMTATP3), *S. cerevisiae* (SCU09305), *I. batatas* (IPBF1ATPG), *R. blastica* (RBATP), *R. rubrum* (RPATP), *B. caldotenax* (BACASG), *B. stearothermophilus* (BACASO), *B.* PS3 (PS3TF0F1), *B. megaterium* (BACATPA), *B. firmus* (BACATP), *B. subtilis* (BSATPASE), *E. coli* (ECOUNC), *V. alginolyticus* (VAUNC), *T. ferrooxidans* (TFEUNCAH), *P. modestum* (PMUNC2), *A. woodii* (AWU10505), *S. lividans* (SLATPSYNA).

we did not find in *S. platensis* γ the two typical cysteines responsible for thiol-modulation in chloroplasts. We are currently constructing *Synechocystis* sp. PCC 6803 mutants which have cysteines introduced at the respective positions in order to check for any reinstatement of thiol-modification.

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References

[1] Fillingame, R.H. (1990) Molecular mechanics of ATP synthesis by F_1F_0-type hydrogen ion-transporting ATP synthases. In: Bacteria, Vol. 12, 345–391 (Krulwich, T.A., ed.), pp. 345–391, Academic Press, San Diego.
[2] Senior, A.E. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 7–41. .
[3] Nelson, N. (1991) Trends Pharmacol. Sci. 12(2), 71–75. .
[4] Bickel-Sandkötter, S. and Wessels, T. (1994) Biol. Chem. Hoppe. Seyler. 375, 3–9. .
[5] Lebowitz, M.S. and Pedersen, P.L. (1993) Arch. Biochem. Biophys. 301, 64–70. .
[6] Richter, M.L. and McCarty, R.E. (1987) J. Biol. Chem. 262, 15037–15040. .
[7] Nalin, C.M. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7275–7280. .
[8] Junesch, U. and Gräber, P. (1985) Biochim. Biophys. Acta 809, 429–434. .
[9] Gray, M.W. (1989) Trends Genet. 5, 294–299. .
[10] Lill, H. and Nelson, N. (1991) Plant. Mol. Biol. 17, 641–652. .
[11] Moroney, J.V., Fullmer, C.S. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7281–7285. .
[12] Bakels, R.H.A., Van Walraven, H.S., Scholts, M.J.C., Krab, K. and Kraayenhof, R. (1991) Biochim. Biophys. Acta 1058(2), 225–234. .
[13] Hicks, D.B. and Yocum, C.F. (1986) Arch. Biochem. Biophys. 245, 230–237. .
[14] Hicks, D.B., Nelson, N. and Yocum, C.F. (1986) Biochim. Biophys. Acta 851, 217–222. .
[15] Lerma, C. and Gomez-Lojero, C. (1987) Photosynth. Res. 11, 265–277. .
[16] Bakels, R.H.A., Van Walraven, H.S., Krab, K., Scholts, M.J.C. and Kraayenhof, R. (1993) Eur. J. Biochem. 213, 957–964. .
[17] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628. .
[18] Werner-Grüne, S., Gunkel, D., Schumann, J. and Strotmann, H. (1994) Mol. Gen. Genet. 244, 144–150. .
[19] Sturr, M.G., Guffanti, A.A. and Krulwich, T.A. (1994) J. Bacteriol. 176, 3111–3116.
[20] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Research 12, 387–392.
[21] Felsenstein, J. (1989) PHYLIP 3.2 Manual, Berkeley, University of California Herbarium.