Biochimica et Biophysica Acta 851 (1986) 217–222
Elsevier

BBA 42104

Cyanobacterial and chloroplast F_1-ATPases: cross-reconstitution of photophosphorylation and subunit immunological relationships

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(Received April 24th, 1986)

Key words: Coupling factor; Photophosphorylation; ATPase; Reconstitution; (Spirulina, Spinach, Chloroplast)

The photosynthetic F_1-ATPase from the cyanobacterium *Spirulina platensis* was recently purified in a five-subunit, reconstitutively active form (Hicks, D.B. and Yocum, C.F. (1986) Arch. Biochem. Biophys. 245, 220–229). Here we report on the similarities of the cyanobacterial F_1 to the higher plant chloroplast F_1 (CF_1), as judged by two distinct methods. The ability of each coupling factor to reconstitute photophosphorylation in photosynthetic membranes depleted of F_1 content by 2 M NaBr treatment was tested. Addition of either the homologous enzyme (e.g., *Spirulina* F_1, *Spirulina* membranes) or the heterologous enzyme (e.g., spinach CF_1 *Spirulina* membranes) to depleted membranes increased the rate of phenazine methosulfate-dependent cyclic photophosphorylation from nearly zero to up to 70 μmol ATP/h per mg Chl. Antibodies against four subunits of CF_1 (α, γ, δ and ε) and against β of *Escherichia coli* F_1 were reacted with the *Spirulina* enzyme by protein blotting. The α, β and γ subunits of *Spirulina* F_1 cross-reacted with antibodies against the corresponding subunits from spinach. The cross-reactivity of the γ subunit correlated with previous observations that *Spirulina* membrane ATPase activity can be modulated by light and dithiothreitol, in a similar fashion to their effect on the enzyme from spinach chloroplasts. The ability of cyanobacterial and chloroplast enzymes to restore activity to heterologous membranes in the absence of immunological similarities between their respective δ and ε subunits may suggest that structural features other than particular amino acid sequences of these subunits are paramount in their roles in binding F_1 to the membrane and in sealing proton leaks.

Introduction

F_1-ATPases are a distinctive class of ATPases which contain the catalytic sites for ATP synthesis in energy-transducing membranes of bacteria, mitochondria and chloroplasts. Water soluble, this group of proteins is bound specifically to a hydrophobic membrane polypeptide assembly, F_0, which functions to selectively allow protons to flow down their electrochemical gradient to F_1, thereby coupling the transmembrane protonmotive force with ATP synthesis [1–3]. As might be expected for an enzyme carrying out a complicated function, the structure of an F_1-ATPase is complex. On SDS polyacrylamide gels, F_1-ATPases can be resolved into five sizes of subunits, named α, β, γ, δ and ε in order of decreasing molecular weight, with a likely stoichiometry of 3 : 3 : 1 : 1 : 1 [4]. While all five subunits are required for the expression of

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Abbreviations: F_1, Factor 1 or ATP synthase; CF_1, chloroplast F_1; Chl, chlorophyll; F_0, hydrophobic membrane polypeptide assembly.

ATP synthesis in oxidative and photophosphorylation, purified F_1-ATPases lacking some of their subunits will hydrolyze ATP. Thus, it is clear that particular subunits probably play specific roles in the proper functioning of F_1-ATPases in vivo. At the same time, however, similar names for subunits of ATPases from different organisms may not play the same role. For example, the ε subunit from chloroplasts is an inhibitor of ATPase activity and may function as such in vivo [5], while a completely distinct protein serves this function mitochondria, the ATPase inhibitor polypeptide [6]. Subunit structure and function have been investigated by a variety of means, including immunological techniques, isolation and sequence of the genes encoding specific subunits and biochemical analyses. These studies indicate the β subunit is highly conserved and probably contains the catalytic site, while the α subunit, the only subunit other than β that binds nucleotides, is somewhat less conserved. The γ subunit may act as a gate for protons conducted through F_0. In addition, the γ subunit has a special role in the chloroplast F_1 (CF_1) in the cycling of the enzyme from an inactive to an active form [7,8]. A disulfide group in γ is reduced when CF_1 is activated either by light when membrane-bound or by dithiothreitol in the isolated state [7,8]. Interestingly, the thiol groups participating in this reaction are absent in the *Escherichia coli* enzyme. No cross-reactivity was observed between anti γ of CF_1 and *E. coli* F_1 or in the reciprocal experiment by protein blotting, while anti γ of yeast mitochondrial F_1 recognized *E. coli* γ, but not the corresponding subunits of spinach CF_1 or rat liver mitochondrial F_1 [9]. Much less is known about the two smallest subunits, δ and ε. Although they play a role in binding F_1 to F_0, recent work indicates that the absence of one or the other does not preclude this binding; however, the membranes are leaky to protons when these subunits are missing [10]. The δ subunit of *E. coli* F_1 is not related to a mitochondrial F_1 subunit, but instead is related to oligomycin-sensitive conferral protein (oscp), while the mitochondrial ε subunit has no counterpart in the *E. coli* enzyme [11].

In this report we have investigated the similarities of the photosynthetic F_1 ATPases from a cyanobacterium, *Spirulina platensis*, and from spinach chloroplasts. The endosymbiotic hypothesis of the origin of chloroplasts [12] argues that chloroplasts developed from an endocytotic event involving either close relatives of cyanobacteria or cyanobacteria themselves, which are the only extant free-living prokaryotes that carry out oxygenic photosynthesis. The sequence data from proteins such as cytochrome *f* and ferredoxin support this hypothesis; more recently, even stronger evidence has been found in which analyses of transfer and ribosomal RNAs indicate that cyanobacterial and chloroplast RNAs are more closely related to each other than are the chloroplast RNAs to the cytoplasmic RNAs of the same cells [13,14].

Materials and Methods

Spirulina F1 was purified following procedures detailed in Refs. 15 and 16, after extraction from pyrophosphate-washed membrane vesicles of *Spirulina platensis*. All solutions at every step in the procedure contained the protease inhibitors phenylmethylsulfonyl fluoride, *p*-amino-benzamidine, and L-1-tosylamide-2-phenylethylchloromethyl ketone. Spinach CF_1 was purified by a similar procedure. Purified *Spirulina* F_1 and CF_1 were electrophoresed on SDS polyacrylamide gels (12.5%), transferred, and decorated as described in Ref. 9. 10 μl serum were used for each of the two lanes on the nitrocellulose, in 20 ml solution containing 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA and 0.5% bovine serum albumin. 1 μCi of ^{125}I-protein A, low-specific activity was used per two lanes. Exposure was for 1 day when 10 μg samples were used and for two days in the case of 2.5 μg samples.

F_1-depleted *Spirulina platensis* membrane vesicles and chloroplast thylakoids were prepared by 2 M NaBr treatment according to Ref. 17. Reconstitution of phenazine methosulfate-dependent cyclic photophosphorylation was assayed by incubation of F_1-deficient membranes (33 μg Chl) with the specified amount of F_1 for 15 min on ice in a medium consisting of 50 mM Tricine (pH 8.0)/25 mM $MgCl_2$/0.2 mg/ml bovine serum albumin; aliquots (13 μg Chl) of the reconstituted membranes were assayed for ATP synthesis by a 1 min illumination (white light, approximately $3 \cdot 10^3$

$J \cdot m^{-2} \cdot s^{-1}$) of the membranes in a reaction mixture containing 50 mM Tricine (pH 8.0)/50 mM NaCl/5 mM sodium phosphate (containing about $(8-10) \cdot 10^5$ cpm/μmol)/1 mM ADP/6.7 mM $MgCl_2$/1 mg per ml bovine serum albumin, and either 0.4 mM PMS in the case of *Spirulina* membranes or 0.05 mM PMS in the case of spinach chloroplast thylakoids. Extraction of unreacted ^{32}Pi was by the method of Avron [18] and $AT^{32}P$ was detected by Cerenkov counting [19].

Results

We have recently purified and characterized the F_1-ATPase from the cyanobacterium *Spirulina platensis* [15]. *Spirulina* F_1 is a latent ATPase whose activity is greatly stimulated by trypsin digestion or exposure to alcohols and stimulated to a lesser extent by dithiothreitol [20]. These enzymatic properties are similar to those of the isolated spinach chloroplast F_1 (CF_1), except that dithiothreitol stimulates CF_1-ATPase activity to much higher levels than are observed with *Spirulina* F_1.

On SDS polyacrylamide gradient gels, *Spirulina* F_1 is resolved into five subunits with estimated molecular weights of 53400, 51600, 36000, 21100, 14700. A densitometer scan of *Spirulina* F_1 and spinach CF_1 (Fig. 1) shows that the subunits of *Spirulina* F_1 are close in size to CF_1 subunits (estimated sizes of 56.9, 51.4, 36.9, 21.3 and 16.0 kDa on gradient gels). Since a correspondence in size of two subunits does not necessarily imply that the subunits are equivalent, we have examined this issue by protein blotting experiments that have been used to identify relationships of subunits of F_1 ATPases from *E. coli*, yeast mitochondria, rat liver mitochondria, and spinach chloroplasts [9].

The subunits of *Spirulina* F_1 were separated on SDS polyacrylamide gels and reacted with antibodies against the α, γ, δ and ε subunits of spinach CF_1 and against the β subunit of *E. coli* F_1. Anti-β of *E. coli* F_1 was chosen because it exhibits less cross-reactivity with the α subunit than does anti-β of spinach CF_1. The results of these experiments are shown in Fig. 2. The three largest subunits of *Spirulina* F_1 cross-react with antibodies against α, β and γ. The recognition of

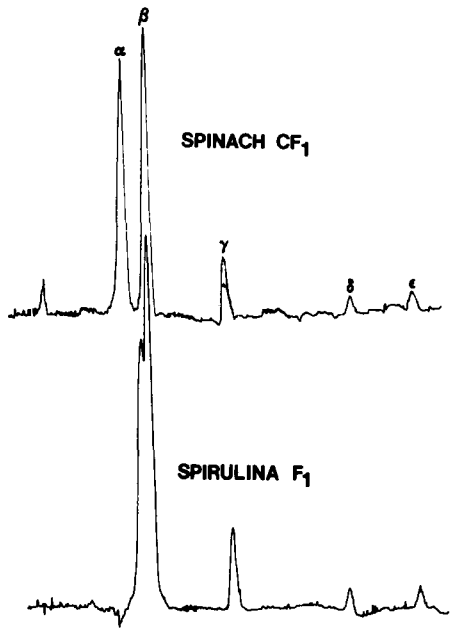

Fig. 1. Densitometer scan of *Spirulina* F_1 and spinach CF_1 on SDS 9–15% polyacrylamide gradient gels.

a large subunit of *Spirulina* F_1 by anti-β is consistent with amino acid sequence data of the β subunit from prokaryotic and eukaryotic F_1-ATPases that show that this subunit is highly conserved. Anti-α cross-reacts with a large subunit of *Spirulina* F_1 as well, but since the two large *Spirulina* subunits are poorly resolved, it is not possible to determine with the present data the relative migration of these subunits with respect to α and β subunits of spinach CF_1. Significantly, a subunit of similar M_r to the γ subunit of CF_1 cross-reacts with anti-γ of CF_1 as shown in Fig. 2A, when 2.5 μg of ATPase are applied to the gel and, more prominently, in Fig. 2B where 10 μg of ATPase are loaded on the gel. This is the first observation of cross-reaction between anti-γ of CF_1 and another F_1-ATPase with the exception of other chloroplast coupling factors from other eukaryotic sources. Antibodies against the δ and ε subunits did not recognize *Spirulina* F_1 subunits. The sensitivity of protein blotting is indicated in Fig. 2B where it can be seen that some proteolysis has occurred to the δ subunit of spinach CF_1 which is not detectable on Coomassie-stained polyacrylamide gels.

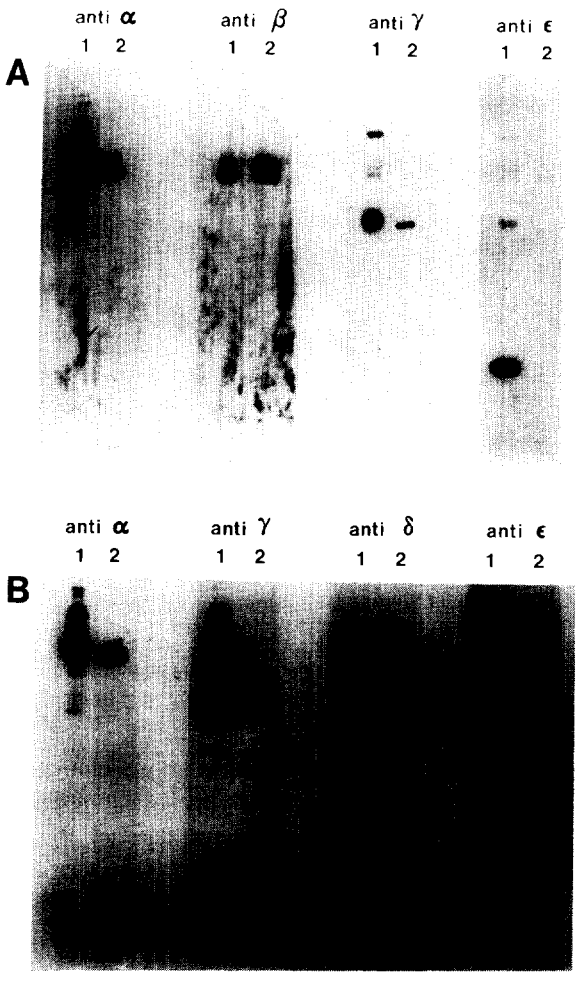

Fig. 2. Immunological cross-reactivity of subunits of *Spirulina* F_1 with antibodies against individual subunits of spinach CF_1 and *E. coli* F_1. Spinach CF1 (lane 1) and *Spirulina* F_1 (lane 2) were electrophoresed, transferred and decorated as described [7]. Antibodies against α, γ, δ and ε subunits were prepared from spinach CF_1; anti-β was from *E. coli* F_1. (A) 2.5 γ coupling factor; (B) 10 μg coupling factor.

As a functional test of the relatedness of cyanobacterial and chloroplast ATPases, we assayed each enzyme for its ability to reconstitute photophosphorylation in photosynthetic membranes of *Spirulina* that were depleted of coupling factor content by 2 M NaBr treatment. Nelson and Eytan have shown that this treatment extracts greater than 90% of the CF_1 population from spinach chloroplast thylakoids, while lowering the rate of phenazine methosulfate-dependent cyclic photophosphorylation too close to zero [21]. Pro-

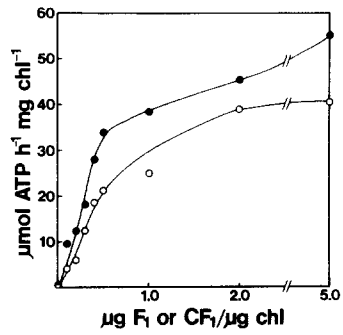

Fig. 3. Reconstitution of photophosphorylation in NaBr-treated *Spirulina* membrane vesicles by spinach CF_1 and *Spirulina* F_1. *Spirulina* F1 (○———○) or spinach CF_1 (●———●) was incubated with *Spirulina* membranes (33 μg Chl) for 15 min on ice and aliquots (13 μg Chl) were assayed for $AT^{32}P$ synthesis as described in Materials and Methods.

tein blotting of NaBr spinach thylakoids indicates that greater than 95% of the β and δ subunits are extracted by this procedure [10]. When CF_1 is added back to the depleted membranes, substantial rates of photophosphorylation can be restored. Similarly, the ATPase activity of *Spirulina* membranes declines over 90% by NaBr treatment and photophosphorylation is almost completely abolished [15]. Purified *Spirulina* F_1 reconstitutes light-driven ATP synthesis in the F_1-depleted *Spirulina* membranes yielding rates of up to about 50 μmol ATP synthesized/h per mg Chl under saturating conditions, or about 1/4 or 1/3 of the rates observed in pyrophosphate-washed membranes (150–200 μmol ATP/h per mg Chl). Spinach CF_1 was tested for its ability to recon-

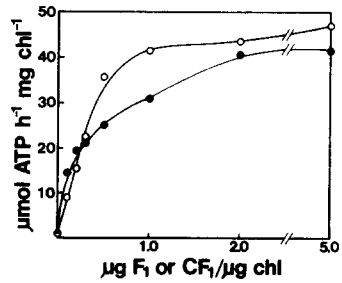

Fig. 4. Reconstitution of photophosphorylation in NaBr-treated spinach chloroplast thylakoids by spinach CF_1 and *Spirulina* F_1. *Spirulina* F_1 (○———○) or spinach CF_1 (●———●) was incubated with spinach chloroplast thylakoids (33 μg Chl) for 15 min on ice and aliquots (13 μg Chl) were assayed for $AT^{32}P$ synthesis as described in Materials and Methods.

TABLE I

MAXIMUM RATES OF PHOTOPHOSPHORYLATION OBSERVED WITH F_1-DEFICIENT MEMBRANES OF *SPIRULINA PLATENSIS* AND SPINACH CHLOROPLASTS RECONSTITUTED WITH HOMOLOGOUS OR HETEROLOGOUS COUPLING FACTORS

Source of F_1	F_1-deficient membrane	
	Spirulina platensis	spinach chloroplasts
Spirulina F_1	52.3 [a]	50.8
Spinach CF_1	74.2	65.7

[a] μmol ATP synthesized/h per mg Chl.

stitute activity in depleted *Spirulina* membrane vesicles. As shown in Fig. 3, spinach CF_1 successfully restored photophosphorylation activity which was dependent on the amount of protein added to the membranes. Little difference in the reconstitutive activity of the eukaryotic and prokaryotic enzymes was observed in different experiments, although different variables of the reconstitution procedure were not optimized for each enzyme.

The ability of spinach CF_1, a eukaryotic enzyme, to reconstitute photophosphorylation in photosynthetic membranes of *Spirulina platensis*, a prokaryote, encouraged us to carry out the reciprocal experiment. We found that, under our assay conditions, *Spirulina* F_1 was as active a coupling factor as spinach CF_1 in restoring ATP synthesis to NaBr-treated spinach chloroplast thylakoids. The relationship of activity to the amount of coupling factor added back to the spinach thylakoids is plotted in Fig. 4. Table I lists the highest activities observed in the different reconstituted systems.

Discussion

The cardinal feature of the chloroplast ATPase is the latency of its hydrolytic activity. Membrane-bound CF_1 is activated by illumination in the presence of a sulfhydryl reagent, and under these conditions, a disulfide group in tryptic fragments S1 and S2 of the γ subunit are reduced to free thiols [22]. Photosynthetic membranes of cyanobacteria also exhibit latent hydrolytic activity (e.g., *Anabaena variabilis* [23], *Synechococcus lividus* [24], *Spirulina maxima* [25], and *Spirulina platensis* [20]) and we have shown that the purified *Spirulina platensis* ATPase is a latent ATPase which can be activated by procedures that elicit CF1 activity (i.e., dithiothreitol, trypsin, alcohols) [20]. When membrane-bound, *Spirulina* F_1 is also activated by light in the presence of dithiothreitol.

These observations suggest that chloroplast and cyanobacterial ATPases may be closely related and the observations presented in Results are consistent with this prediction. The three largest subunits of *Spirulina* F_1 are antigenically related to the α, β and γ subunits of CF_1; the cross-reactivity of the *Spirulina* and CF_1 subunits indicate that cyanobacterial ATPase activity may be regulated by the same mechanism that appears to control activation/deactivation of CF_1, namely a reduction/oxidation of a disulfide group in the γ subunit, presumably by a thioredoxin-like system [26].

The ability of each coupling factor to reconstitute ATP synthesis in a heterologous membrane indicates that not only is the binding of the ATPase specific for the heterologous F_0, but that formation of a proton gradient and presumably a catalytically active hybrid species (i.e., *Spirulina* F_1–chloroplast F_0, spinach CF_1–*Spirulina* F_0) takes place. Recently, it was shown that the F_1-ATPase from the thermophilic bacterium PS3 reconstituted photophosphorylation in lettuce chloroplasts partially depleted of CF_1 by EDTA treatment but not in thylakoids treated with NaBr [27]. However, the bacterial ATPase was capable of restoring light driven H^+ uptake in NaBr-treated thylakoids to about the same extent that was observed with native CF_1. These authors in Ref. 27 argued that the bacterial F_1 bound specifically to chloroplast F_0 but did not form a catalytically active species, concluding that exogenously added coupling factor, whether the native or the bacterial enzyme, acted solely to plug proton leaks, while catalysis in these reconstituted systems was proposed to be carried out by residual CF_1 remaining bound to the thylakoids after treatment. While the results reported here do not speak directly to this issue, the observation that ATP synthesis can be reconstituted by both heterologous and homologous ATPases to approximately the same extent in NaBr-treated membranes may be indicative of a phylogenetically closer relationship of cyanobac-

teria and chloroplasts than between PS3 and chloroplasts.

Binding of F_1 to F_0 is a complex, incompletely understood phenomenon. In bacteria, a role for the two smallest subunits, δ and ε in binding, has been demonstrated, but, in chloroplasts, a CF_1 complex devoid of both of these subunits binds to membranal CF_0 in a specific manner as shown by competition experiments with native, 5-subunit CF_1 [10]. However, the subunit-deficient CF_1 did not reconstitute formation of the proton gradient or ATP synthesis [10]. These authors also showed that CF1-ε exhibited these same characteristics while CF-δ only partially restored proton gradient formation and ATP synthesis. The capacity of the cyanobacterial and chloroplast enzymes for heterologous reconstitution of photophosphorylation, while their smallest subunits show no immunological cross-reactivity may indicate that structural features other than particular amino acid sequences are involved in the correct binding of these subunits to the membranal F_0, or may indicate that the antibodies prepared against the chloroplast δ and ε subunits react with domains not showing amino acid sequence homology.

In conclusion, these data show the coupling factor F_1 ATPases from spinach chloroplasts and the cyanobacterium *Spirulina platensis* to be closely related on the basis of two distinct criteria, subunit immunological recognition and cross-reconstitutive ability. We infer from these data support for the close evolutionary relationship of cyanobacteria and chloroplasts that has emerged from sequence data of proteins and nucleic acids of cyanobacteria and chloroplasts [13,14].

Acknowledgement

This research was supported by the U.S. Department of Agriculture, Competitive Research Grants Office (5901-0410-8-0103-0 and 82-CRCR-1-1127).

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