

# Isolation and identification of a fourth subunit in the membrane part of the chloroplast ATP-synthase

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The subunit composition of highly active purified ATP-synthase from chloroplasts,  $CF_0F_1$ , was investigated by SDS gel electrophoresis. An additional subunit of  $CF_0$  was detected with an apparent molecular mass of 20 kDa. It is stained weakly with Coomassie blue but very strongly with silver. This subunit was isolated on a preparative SDS gel and the N-terminal amino acid sequence analyzed. It shows that the 20 kDa protein is identical with the protein encoded by the spinach chloroplast gene *atpI*, called subunit IV [(1986) Mol. Genet. 203, 117-128]. However, in comparison to the gene-derived sequence, the first 18 amino acids are missing, indicating N-terminal processing.

Chloroplast; ATP-synthase;  $CF_0F_1$ ;  $CF_0$  subunit; Amino acid sequence

## 1. INTRODUCTION

The membrane-bound ATP-synthase from chloroplasts,  $CF_0F_1$ , catalyzes ATP synthesis/hydrolysis coupled with a transmembrane proton transport. Like other ATP-synthases of the  $F_0F_1$  type, it has a hydrophilic part,  $CF_1$ , which contains the nucleotide-binding sites and a hydrophobic part,  $CF_0$ , which is inserted into the membrane and is supposed to act as a proton channel. General agreement has been reached as to the subunit composition of  $CF_1$  ( $\alpha, \beta, \gamma, \delta, \epsilon$ ) (reviews [1,2]). The subunit composition of  $CF_0$  has not been clarified as yet: either three [3-7] or four [7-10] subunits

have been reported with apparent molecular masses in the range of 15 kDa for I, 13 kDa for II, 8 kDa for III, and, in some reports, 19 kDa for a fourth subunit. Recently, the nucleotide sequence of a 4 kb piece of the spinach plastid chromosome was analyzed and an amino acid sequence homologous to subunit a of the *E. coli*  $F_0$  was found [11].

During the last years  $CF_0F_1$  has been isolated, purified, and reconstituted into asolectin liposomes [7,12]. These preparations show high rates of ATP synthesis ( $200\text{ s}^{-1}$  [13]) and ATP hydrolysis ( $20\text{ s}^{-1}$  [14]); i.e. this enzyme has practically the same activity as in the thylakoid membrane. Here, we have used this highly active enzyme to investigate the subunit composition of  $CF_0$ .

## 2. MATERIALS AND METHODS

$CF_0F_1$  was isolated as in [7] with the modification described in [15]. SDS gel electrophoresis of  $CF_0F_1$  (for analytical and preparative gels) was carried out basically as described [16,17]. The stack-

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**Abbreviations:**  $CF_0F_1$ , proton translocating ATP-synthase from chloroplasts; HPLC, high-pressure liquid chromatography

ing gel contained 3.75% polyacrylamide and the separation gel 15% polyacrylamide. SDS concentration was 0.1%. The staining solution contained 1 g/l Coomassie blue R250 in 25% (v/v) methanol, 7.5% (v/v) acetic acid. The gel was stained for 1 h at room temperature and then destained with 7.5% acetic acid for 24 h. Silver staining of the gel was carried out as described by Oakley et al. [18].

Preparative SDS gel electrophoresis was carried out as follows: 3.3 nmol  $CF_0F_1$  was layered on the top of the gel and electrophoresis run for 5 h at a constant voltage of 150 V. The protein bands were made visible by incubation of the gel for 10 min in 4 M sodium acetate. The protein band at 20 kDa was cut out and dialysed for 12 h against 62 mM Tris-Cl (pH 7.8), 10% glycerol and 0.1% mercaptoethanol as described by Irrgang et al. [23]. Electroelution was carried out for 18 h at 20°C and 7.5 mA, and thereafter the protein was dialysed against 1.0 l of 0.1% SDS for 48 h with 5 changes of buffer. The solution was then freeze-dried in aliquots.

For all dialysis steps and electroelution, benzylated cellulose tubing (cut-off 2.0 kDa, Sigma D 7884) was used. The membrane was heated to 100°C in 0.1 M EDTA for 1 h before use. N-terminal amino acid sequencing was performed by automated solid-phase Edman degradation. 1–2 nmol of the 20 kDa protein was coupled to aminopropyl glass via 1,4-phenylene diisocyanate using the on-column immobilisation technique [19]. Sequence determinations were performed by the double coupling method using 4-*N,N*-dimethylaminoazobenzene 4'-isothiocyanate and phenylisothiocyanate [20]. 4-*N,N*-Dimethylaminoazobenzene 4'-thiohydantoins were identified by two-dimensional thin-layer chromatography on polyamide [21] and, in addition, by HPLC on a reversed-phase column eluted with a methanol gradient [22]. For the first coupling step, phenylisothiocyanate was used exclusively to mask the support matrix and minimize background interference in the next cycles. The N-terminal residue was therefore not identified.

### 3. RESULTS

In fig.1, lanes 2 and 3 show the Coomassie-stained SDS gel of  $CF_0F_1$  and  $CF_1$ , respectively. In addition to the bands labeled I, II and III, a weak,

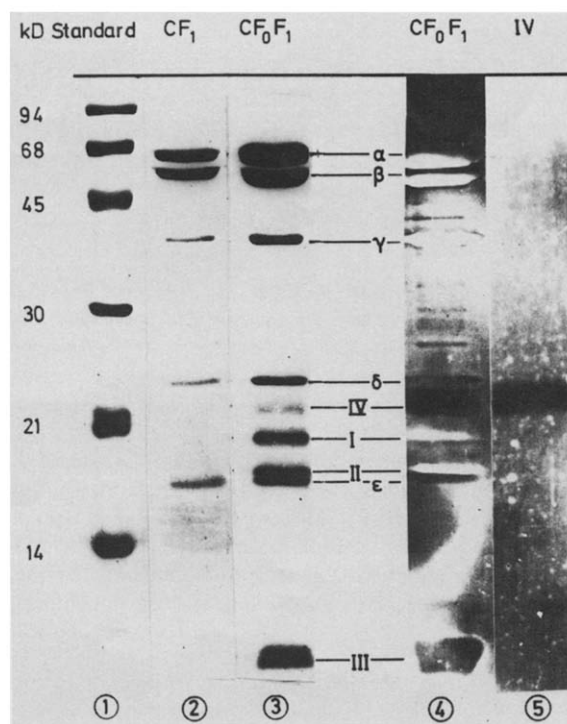


Fig.1. SDS gel electrophoresis of the purified chloroplast ATP-synthase,  $CF_0F_1$ . Lanes: 1, molecular mass standards stained with Coomassie blue; 2,  $CF_1$  stained with Coomassie blue (10  $\mu$ g protein); 3,  $CF_0F_1$  stained with Coomassie blue (30  $\mu$ g protein); 4,  $CF_0F_1$  stained with silver (30  $\mu$ g protein); 5, the isolated 20 kDa band (subunit IV) stained with silver (~1  $\mu$ g protein). For further details see section 2 and text.

diffuse band IV is observed with an apparent molecular mass of 20 kDa. Such a band has also been observed by others. However, because it appears to be so weak, it could not be excluded that this band represents an impurity in the preparation. In fig.1, lane 4 shows a gel of the same  $CF_0F_1$  preparation after silver staining. Obviously, the same bands are observed; however, some proteins ( $\alpha, \beta, \gamma, \delta, I, II$ ) are negatively stained (white) and some ( $\epsilon, III$ ) positively stained (black). The band at 20 kDa is also positively stained and has now become the most intensely stained one. This seems to indicate that it is not an impurity. Therefore, we have isolated this band for further characterization as described in section 2.

An aliquot from the freeze-dried preparation after isolation was taken for analytical SDS gel

GIQALIFATLAAAYIGDSL DGHK  
G1QALIFATLAAAYIGESMEGHK

It has been proposed that subunit a from *E. coli* plays an important role in the coupling of proton transport to ATP synthesis/hydrolysis [32,33]. Since the mechanism of chemiosmotic ATP synthesis/hydrolysis appears to be similar in all  $F_0F_1$  type ATP-synthases, the presence of subunit IV in  $CF_0F_1$  as a homologue of subunit a of *E. coli*  $F_0F_1$  is a very welcome result.

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