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

Petra Fromme, Peter Gräber and Johann Salnikow*

Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Straße des 17 Juni 135, 1000 Berlin 12 and **Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, Franklinstr. 29, 1000 Berlin 10, Germany*

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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 *atp*I, 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^{-1} [13]) and ATP hydrolysis (20 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 compositon of CF_0.

Correspondence address: P. Fromme, Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Straße des 17 Juni 135, 1000 Berlin 12, Germany

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

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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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, 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 (α,β,γ,δ,I,II) are negatively stained (white) and some (ϵ,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.

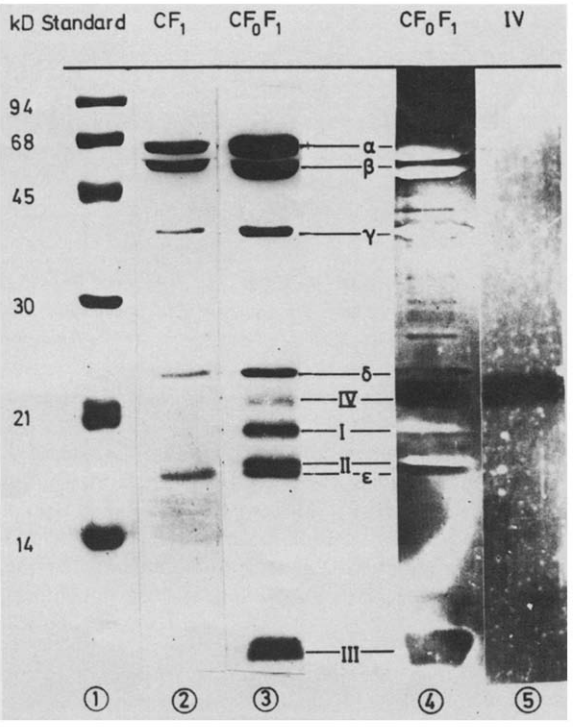

Fig.1. SDS gel electrophoresis of the purified chloroplast ATP-synthase, CF_0F_1. Lanes: 1, molecular mass standards stained with Coomassie blue; 2, CF_1 stained with Coomassie blue (10 μg protein); 3, CF_0F_1 stained with Coomassie blue (30 μg protein); 4, CF_0F_1 stained with silver (30 μg protein);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

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

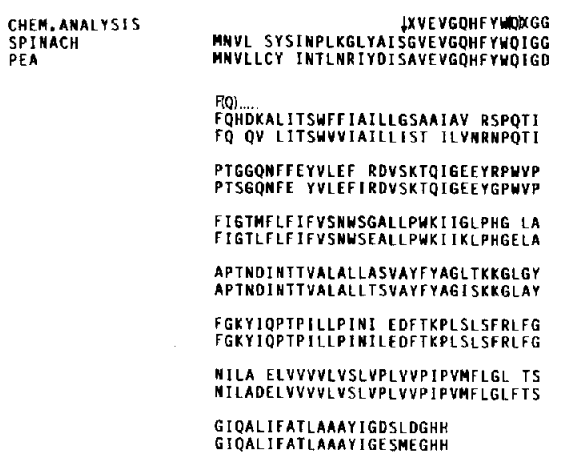

Fig.2. Comparison of amino acid sequence of subunit IV deduced from the DNA sequence of spinach chloroplasts [11] and pea chloroplasts [24]. At the top the amino acid sequence resulting from protein-chemical analysis is shown.

electrophoresis in order to check the purity of the isolated subunit. Fig.1 (lane 5) shows the silver-stained gel. The isolated protein was pure. By comparing the integrated peak of a densitometer scan of the isolated subunit IV with the corresponding peak from the total CF_0F_1 (30 μg), the yield of the isolated subunit could be calculated. About 50% of the theoretical value was found. The main losses are presumably due to incomplete electroelution.

The isolated protein was subjected to N-terminal amino acid sequence analysis. The following sequence was determined:

1 5 10
X-Val-Glu-Val-Gly-Gln-His-Phe-Tyr-Trp-(Gln)-
15
X-Gly-Gly-Phe-(Gln)...

In position 6 and 10 peaks corresponding to the amino acid Asn were observed in about equivalent concentrations; without further studies, however, it is premature to decide at present if true amino acid dimorphism or only artifacts of unknown origin are encountered. Comparison of the determined amino acid sequence with that of subunit IV deduced from spinach and pea chloroplast DNA sequences [11,24] shows complete identity (fig.2). This means that the 20 kDa protein is a product of the chloroplast gene *atp*I called 'subunit IV' [11]. The protein-chemical data imply, however, proteolytic removal of the first.

4. DISCUSSION

It is shown here that, in highly active CF_0F_1 preparations, in addition to the well-established subunits ($\alpha,\beta,\gamma,\delta,\epsilon$,I,II,III) a ninth subunit is also present which shows on SDS gel an apparent molecular mass of 20 kDa. We have isolated this protein and subjected it to partial amino acid sequence analysis from the N-terminus. The determination of a 15 amino acid sequence allows the unequivocal identification of this protein as the gene product of the plastid gene *atp*I, which is homologous to subunit a from *E. coli* F_0; however, the first 18 amino acids are missing. This implies posttranslational processing by cleavage of a Ser–Gly bond.

N-terminal processing of the plastid proteins like the one observed in this study has been described recently for other polypeptides of the photosynthetic apparatus such as the 18 and 23 kDa proteins in the photosynthetic oxygen-evolving complex [25,26]. Furthermore, it should be mentioned that when the N-terminal amino acid sequence of subunit I from CF_0 is compared to the sequence deduced from the plastid DNA, the first 17 amino acids are missing. This implies that also in this case a processing occurs (with cleavage between alanine and glycine [10,11,27]).

The physiological role of these proteolytic phenomena is not yet known. It can be argued, however, that processing might be required for the assembly or functional integrity of the whole complex, for instance for the correct insertion of subunits I and IV into the thylakoid membrane during the formation of CF_0F_1.

The isolated subunit IV runs on SDS gels at an apparent size of 20 kDa, whereas the molecular mass of the processed gene product should be 25 kDa. Presumably, this is due to the fact that on SDS gels molecular masses of hydrophobic proteins are frequently underestimated [28–30]. Alternatively, the lower molecular mass could also be explained by additional C-terminal processing, which has been demonstrated for the 32 kDa protein (herbicide binding) of photosystem II [31]. This would be a problem for further investigations.

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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