Evidence for an α_3, β_3,γ, δ, I, II, ε, III_5 subunit stoichiometry of chloroplast ATP synthetase complex (CF_1–CF_0)

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1. INTRODUCTION

Phosphorylation of ADP by energy-transducing membranes is catalyzed by reversible H^+-translocating ATP synthetases (F_1–F_0) having a common organization in that they are composed of an extrinsic ATPase unit (F_1) linked with an intrinsic, protontranslocating membrane unit (F_0) [1–3]. Although the ATP synthetases of mitochondria, bacteria and chloroplasts appear to have comparable functional properties [2], their subunit composition and stoichiometry have not yet been firmly established. However, most of the work with mitochondria and bacteria showed, α_3, β_3, γ, δ, ε subunits to be associated with the F_1-ATPases [4–8], whereas an α_2, β_2, γ, δ_{1-2}, ε_2 subunit stoichiometry is strongly favoured for the chloroplast ATPase (CF_1) [3]. Consequently, significant differences with respect to subunit arrangement and mechanism of ADP phosphorylation could exist among ATP synthetases. These findings prompted us to investigate the subunit stoichiometry of chloroplast ATP synthetases from plants grown in an atmosphere enriched with $^{14}CO_2$. The ATP synthetases were immunoprecipitated from Triton X-100 thylakoid membrane extracts and the polypeptides separated by SDS/urea gel electrophoresis. Based upon the distribution of radioactivity, we estimated that the CF_1 subunits α, β, γ, δ and ε were present in a ratio of 3:3:1:1:1 while the CF_0 polypeptides I–III occurred in a stoichiometric ratio of 0.3:1:5, respectively. The results are consistent with the view that subunit composition and stoichiometry of pro- and eucaryotic F_1-ATPases as well as of procaryotic F_0 units are strictly preserved throughout evolution.

2. MATERIALS AND METHODS

Seedlings of field beans (*Vicia faba*) [2×] and oat (*Avena sativa*) [150×] were grown for 2 weeks at 20°C in an air atmosphere (4000 cm^3) enriched with 0.096 MBq $^{14}CO_2 \cdot cm^{-1}$. The light:dark ratio was 16:8 h. The freshly harvested leaves were homogeneized at 4°C in a buffer of 0.075 M Tricine–NaOH (pH 7.8), 0.5 M sucrose, 1 mM Na-diethyldithiocarbamate and 2% human serum albumin. The chloroplasts of the cheese cloth-filtered homogenates were sedimented by centrifugation at 4000 ×*g* for 15 min and osmotically shocked by resuspending them in 0.5 mM Tricine–NaOH (pH 8.0). Nuclei and cell debris were sedimented at 800 ×*g* for 5 min and discarded before the swollen thylakoid membranes were sedimented at 20 000 ×*g* for 15 min. Immunoprecipitation of ATP synthetases from Triton X-100 membrane extracts was performed as in [9] using affinity-purified immunoglobulins raised against the *Vicia faba* CF_1 [10]. The immunoprecipitates and thylakoid membranes were solubilized in 50 mM Na-borate (pH 9.0), 2% (w/v) SDS, 8 M urea and 10 mM dithiothreitol and subjected to SDS–urea acrylamide gradient gel electrophoresis [11]. Slab gels with lengths up to 60 cm have also been used allowing the successful one-dimensional gel electrophoretic separation of almost all thylakoid polypeptides [12]. The ^{14}C-labeled proteins of desiccated gels were visualized by autoradiography on HS-11 X-ray sensitive film (VEB Fotochemische Fabrik, Berlin) and the radioactivity of gel segments was measured by liquid scintillation counting. The 1 mm gel slices were dissolved in H_2O_2 [13] and the dpm determined

after the addition of 10 ml scintillation liquid (Präwozell:toluene:PPO:dimethyl POPOP, 33% (v/v): 66% (v/v):5% (w/v):0.3% (w/v) in a LKB liquid scintillation spectrometer 81 000.

3. RESULTS AND DISCUSSION

Consistently with [9,14], the immunoprecipitated ATP synthetases from Triton X-100 extracts of metabolically radiolabeled thylakoid membranes are composed of 8 non-identical polypeptides, 5 coinciding with the CF_1 subunits α, β, γ, δ and ε while the components I – III were ascribed to CF_0 unit (fig.1). Because a significant part of subunit protein was still associated with immunoglobulin chains (fig.1), it was essential to reduce the SDS extracts with dithiothreitol leading to complete dissociation of protein complexes as well as disappearance of intramolecular disulfide bonds. This may explain the different amounts of protein associated with the subunit II band of SDS-solubilized immunoprecipitates which had been treated with or without dithiothreitol prior to electrophoresis. Gel electrophoresis on very long gels (fig.2) allowed a better estimation of subunit M_r-values for stoichiometric calculations. They were determined to be 59 000, 56 000, 34 000, 21 000 and 16 000 for the CF_1 subunits α, β, γ, δ and ε and 20 000, 17 000 and 7500 for CF_0 components I – III, respectively, in the case of *Vicia faba*. Slightly different M_r-values were found for *Avena sativa* ATP synthetase subunits (table 1) and it should be noted that a pair of bands differing in M_r by ~500 were seen in the region of the γ subunit which remained after treating the samples with dithiothreitol. Assuming that the uniformity of photosynthetic radiolabeling of cellular components is not influenced by protein turnover processes and, consequently, the radioactivity incorporated into polypeptides is proportional to their quantities, the stoichiometric ratios of subunits of immunoprecipitated ATP synthetases have been determined (table 1). Analyses revealed a stoichiometry of α_3, β_3, γ, δ, ε for CF_1–ATPase subunits independently of the source of the enzyme complex. However, the ratio of α:β:γ subunits changed to 2:3:1 if *Vicia faba* thylakoid membranes were isolated in the absence of protecting proteins. An identical subunit stoichiometry of α_3, β_3, γ, δ, ε has been observed for mammalian [15] and yeast [5,16] as well as bacterial [6,8] F_1–ATPases using the method of biosynthetic radiolabeling with amino acids, whereas a different stoichiometry of α_2, β_2, γ, δ_{1-2}, ε_2 was reported for pea CF_1 [17]. The latter finding very probably resulted from subunit dissociation induced by EDTA extraction or another degradation of the membrane-bound enzyme because physical and chemical methods gave similar molecular parameters for different CF_1 preparations [18–20] which had been obtained by quite similar isolation methods. In agreement with [17], preliminary results with *Vicia faba* indeed revealed that the CF_1 extracted with EDTA from ^{14}C-labeled thylakoid membranes contained the subunits α, β and γ in a ratio of 1.9, 2.1 and 1, respectively (unpublished). This further indicates that an α and β subunit dissociates from the enzyme during EDTA extraction.

The stoichiometric ratios of CF_0 subunits I – III were determined to be 0.2–0.3:0.7–0.9:4.6–4.8 for *Vicia faba* and *Avena sativa*, respectively (table 1). Since the amounts of subunit I and II were found to be constant among several preparations, their non-stoichiometrical appearance in the immunoprecipitates is presently difficult to explain. It can be excluded, however, from gel plotting results in combination with monospecific antibodies raised against the CF_1 subunits that these components represent degradation products of them (unpublished). Similar stoichiometric ratios have been obtained for the subunits of the TF_0 of a thermophilic bacterium. The subunit stoichiometry of TF_0 has been estimated to be I_1:II_2:III_5 [6] and later one II_{2-3}:III_{5-6} [7]. An even more complicated subunit composition shows the mitochondrial F_0 units consisting of 5–14 non-identical polypeptides [2,16,21].

Due to the possibility of subunit dissociation during immunoprecipitation, the ratios of chloroplast ATP synthetase polypeptides bound to thylakoid membranes have been investigated. The very long acrylamide gradient gels used allowed the separation and exact identification of ATP synthetase subunits out of ~130 different membrane polypeptides in the case of *Vicia faba* and *Avena sativa* (fig.2).

The analysis of radioactivity associated with the subunits revealed a stoichiometry of α_3, β_3, γ, δ_3, I, II, ε, III_{10-14} (table 1) generally confirming the results obtained by immunoprecipitation. It cannot be excluded, however, that the bands containing the subunits δ, I and II are still overlapped by >1

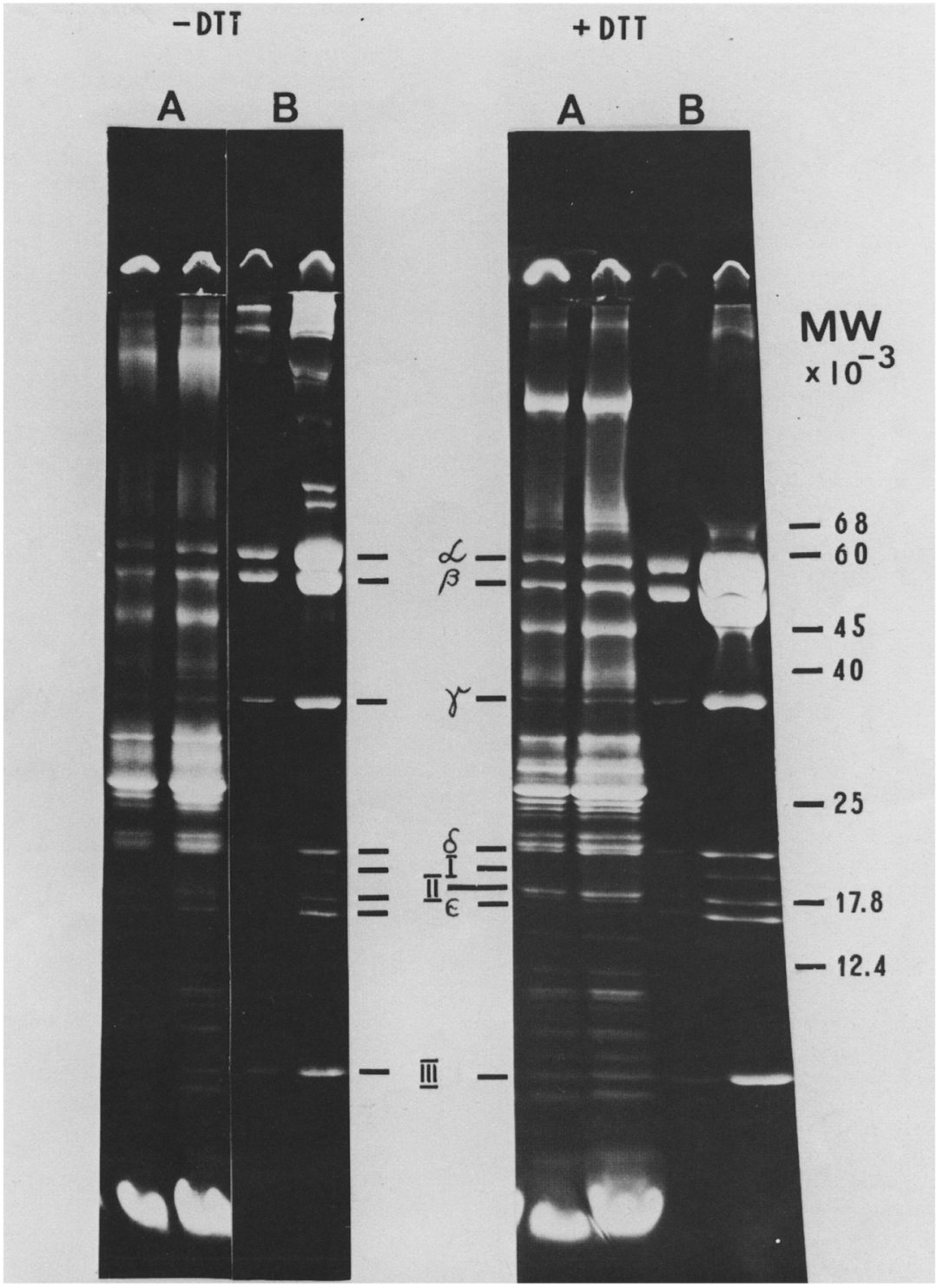

Fig. 1. Analysis by SDS–urea gel electrophoresis and autoradiography of metabolically ^{14}C-labeled *Vicia faba* chloroplast thylakoid membrane polypeptides (A) and immunoprecipitated ATP synthetase complex (B). The samples were solubilized in the presence or absence of dithiothreitol (DTT) as in section 2 and electrophoresed through an 8 – 18% acrylamide gradient gel containing 0.1% SDS and 5 M urea.

Table 1

Stoichiometry of chloroplast ATP synthetase subunits

Sub-unit	M_r × 10^{-3}	Observed stoichiometry of subunits[a]				Assumed stoichio-metry[g]
		ATP synthetase		Thylakoid membranes		
		^{14}C act. (dpm)[b]	stoichio-metry[c]	^{14}C act. (dpm)[b]	stoichio-metry[d]	
Vicia faba						
α	59	15 512	2.9	20 241	3.2	3
β	54	15 161	3.1	16 210	2.8	3
γ	33	2 990	1.0	3 539	1.0	1
δ	21	1 353	0.7	6 990	3.1	1
I	20	540	0.3	2 578	1.2	1
II	17	1 072	0.7	2 005	1.1	1
ε	16	1 450	1.0	1 725	1.0	1
III	7.5	3 125	4.6	8 448	10.5	5
Avena sativa						
α	57	12 815	3.1	9 551	3.3	3
β	56	10 934	2.7	8 249	2.9	3
γ {	36 / 35.5	2 613	1.0	1 830	1.0	1
δ	23	1 330	0.8	2 461	2.1	1
I	20	580	0.4	820	0.8	1
II	17	1 118	0.9	685	0.8	1
ε	16.5	1 310	1.1	825	1.0	1
III	7.5	2 600	4.8	5 235	13.7	5

[a] The ratios of subunits were estimated by dividing the relative amount of radioactivity for each subunit by its M_r and normalizing all values to subunit γ.

[b] The distribution of radioactivity among the subunits of a representative experiment

[c–f] The values represent mean values derived from a different number of preparations: [c] 4×; [d] 5×; [e] 2×; [f] 4×

[g] Values derived by rounding the observed stoichiometries to the nearest integer

polypeptide as was shown for the band of CF_0 subunit III (proteolipid) [22], although two-dimensional gel electrophoresis of thylakoid membrane polypeptides in the presence of 0.1% SDS in the first dimension and SDS and 5 M urea in the second dimension did not give any indication [26]. Minimally 10 polypeptides appeared to be associated with subunit III band of gel-electrophoretic membrane polypeptide profiles and ~1/2 of them seem to belong to the CF_0. The ATP synthetase proteolipid subunits were shown to bind dicyclohexylcarbodiimide [14] but are not phosphorylated in vivo [22], while components with the same M_r becomes phosphorylated on exposure of plants to light (fig.2C). Although the origin and the functions of CF_0 subunits I and II are still unknown and the number of copies of subunit I to III is subject to the greatest uncertainty, the subunit stoichiometry of the chloroplast ATP synthetase complex is proposed to be α_3, β_3, γ, δ, I, II, ε, III_5 yielding M_r ~490 000. Similar values have been obtained by hydrodynamic methods for bacterial [23] and mitochondrial [16] ATP synthetase preparations. In contrast to the results of physical and chemical investigations on soluble

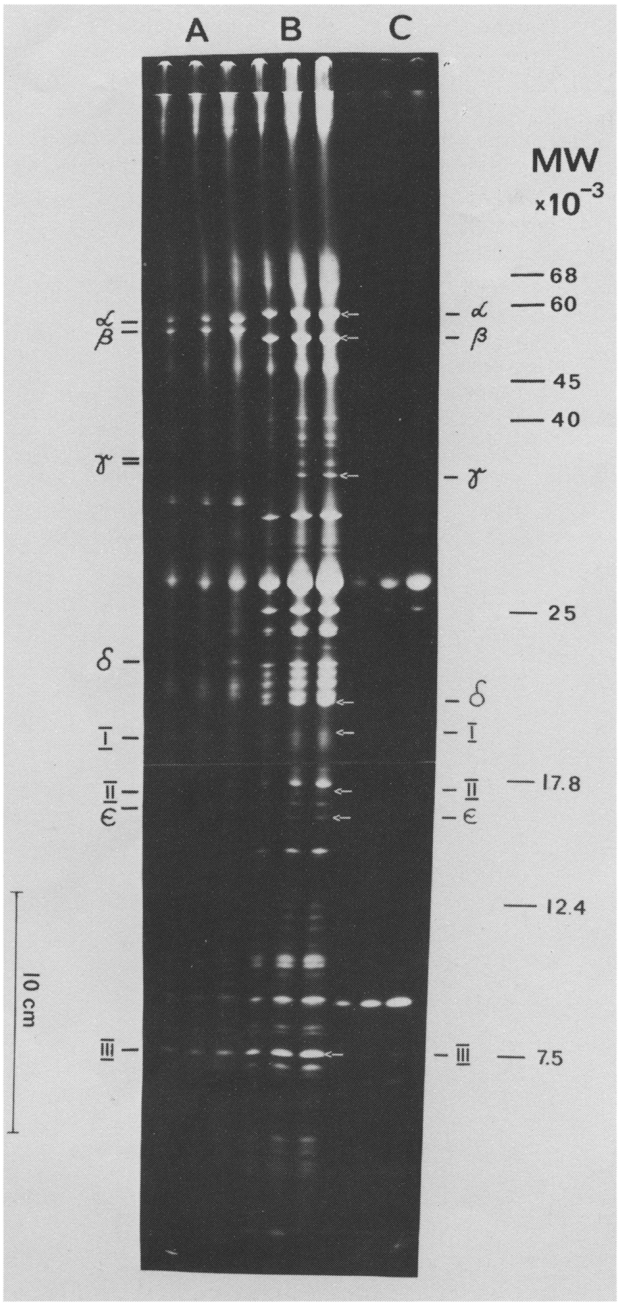

Fig.2. Gel electrophoretic separation of radiolabeled chloroplast thylakoid membrane polypeptides on very long slab gel and identified ATP synthetase subunits. ^{14}C-labeled thylakoid polypeptides of *Avena sativa* (A) and *Vicia faba* (B) as well as *Vicia faba* thylakoid polypeptides labeled in vivo with [^{32}P]orthophosphate (C) as in [22], except that 100 mM NaF_3 was added to all buffers, were electrophoresed with the corresponding, non-labeled ATP synthetases through a 4 cm 6% acrylamide stacking gel and a 60 cm 12–18% acrylamide gradient gel. The gel divided into 2 parts was autoradiographed.

CF_1-ATPases, suggesting a subunit stoichiometry of the α_2, β_2, γ-type [17,19,20,24], these data are fully consistent with a subunit model of α_3, β_3, γ, δ, ε proposed for F_1–ATPase in [25] and, furthermore, indicate a strict preservation of subunit composition and stoichiometry of F_1–ATPase units of pro- and eukaryotic H^+-translocating ATP synthetases throughout evolution. Work is in progress to identify the functions of the CF_0 subunits I and II by the use of immunological methods.

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REFERENCES

[1] Baird, B.A. and Hammes, G.G. (1979) Biochim. Biophys. Acta 549, 31–53.
[2] Filligame, R.H. (1980) Annu. Rev. Biochem. 49, 1079–1113.
[3] Nelson, N. (1981) Curr. Top. Bioenerg. 11, 1–33.
[4] Senior, A.E. (1975) Biochemistry 14, 660–664.
[5] Stutterheim, E., Henneke, M.A.C. and Berden, J.A. (1981) Biochim. Biophys. Acta 634, 271–278.
[6] Kagawa, Y., Sone, Y., Yoshida, M., Hirata, H. and Okamoto, H. (1976) J. Biochem. 80, 141–151.
[7] Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1978) Proc. Natl. Acad. Sci. USA 75, 4219–4223.
[8] Bragg, P.D. and Hou, C. (1979) Biochem. Biophys. Res. Commun. 72, 1042–1048.
[9] Süss, K.-H. (1980) FEBS Lett. 112, 255–259.
[10] Süss, K.-H. (1976) FEBS Lett. 70, 191–196.
[11] Süss, K.-H. and Brecht, E. (1980) Biochim. Biophys. Acta 592, 369–374.
[12] Schmidt, O. and Süss, K.-H. (1982) in preparation.

[13] Sierra, M.F. and Tzagoloff, A. (1973) Proc. Natl. Acad. Sci. USA 70, 3155–3159.
[14] Pick, U. and Racker, E. (1979) J. Biol. Chem. 254, 2793–2799.
[15] Catterall, W.A., Coty, W.A. and Petersen, P.L. (1973) J. Biol. Chem. 248, 7427–7431.
[16] Todd, R.D., Griesenbeck, T.A. and Douglas, M.G. (1980) J. Biol. Chem. 255, 5461–5467.
[17] Nelson, N. (1976) Biochim. Biophys. Acta 456, 314–338.
[18] Farron, F. (1970) Biochemistry 9, 3823–3828.
[19] Baird, B.A. and Hammes, G.G. (1976) J. Biol. Chem. 251, 6953–6962.
[20] Paradies, H.H., Schmidt, U.D. and Zimmermann, J. (1978) J. Biol. Chem. 253, 8972–8979.
[21] Galante, Y.M., Wong, S.-Y. and Hatefi, Y. (1981) Arch. Biochem. Biophys. 211, 643–651.
[22] Süss, K.-H. (1981) Biochem. Biophys. Res. Commun. 102, 724–729.
[23] Paradies, H.H., Mertens, G., Schmid, R., Schneider, E. and Altendorf, K. (1981) Biochem. Biophys. Res. Commun. 98, 595–606.
[24] Paradies, H.H. (1980) Biochem. Biophys. Res. Commun. 96, 1357–1363.
[25] Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. and Ui, N. (1979) J. Biol. Chem. 254, 9525–9533.
[26] Süss, K.-H (1978) Doctoral Thesis, Martin-Luther University Halle.