IDENTIFICATION OF CHLOROPLAST THYLAKOID MEMBRANE POLYPEPTIDES: COUPLING FACTOR OF PHOTOPHOSPHORYLATION (CF_1) AND CYTOCHROME *f*

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1. Introduction

Recently much information has become available concerning the polypeptide composition of chloroplast thylakoid membranes from many sources using different sodium dodecyl sulfate (SDS)-acrylamide-gel electrophoresis systems [1,2]. From all electrophoretical resolved membrane polypeptides only the pigment–protein complexes I and II [1,2] and the α and β subunits of CF_1 [3] have been clearly identified in the polypeptide profiles. The nature of the other polypeptides which are subunits of associated proteins [4] is questionable. If the gel electrophoresis is used as a tool in the study of structure–function relationships of photosynthetic membranes their identification becomes necessary.

This paper presents data on the characterization of purified cytochrome *f* and coupling factor (CF_1). For the identification of the polypeptides of these proteins in the membrane polypeptide spectra their electrophoretic mobilities were examined after separation on two different gel systems. It was found that four subunits of CF_1 ($\alpha,\beta,\gamma,\epsilon$) and the subunit of cytochrome *f* are equivalent to distinct peaks in the membrane polypeptide profiles.

2. Material and methods

2.1. *Extraction and purification of coupling factor (CF_1) and cytochrome f*

Envelope- and stroma-free chloroplast thylakoid membranes from Vicia faba were purified by the procedure as described before [5].

The CF_1 was extracted with 1 mM EDTA, pH 8.0, and purified in two steps. Step 1: to 1 litre of EDTA extract were added 10 ml of 1 M Tris–HCl, pH 8.0, and ATP to a final concentration of 0.1 mM. After short incubation the protein was precipitated at room temperature by slow addition of solid ammonium sulfate to a final concentration of 2 M. The precipitate was collected by centrifugation (20 000 × *g*, 20 min) redissolved in 5 mM Tris–HCl, pH 8.0, and dialyzed again. Step 2: the protein solution was applied to a DEAE (52)–cellulose column (2.5 × 35 cm) which had been equilibrated with 5 mM Tris–HCl, pH 8.0, containing additionally 0.1 M NaCl and 0.1 mM ATP. The CF_1 was eluted in two peaks between 0.2 and 0.3 M by a linear gradient from 0.1–0.3 M NaCl.

Cytochrome *f* was purified together with cytochrome b_{559} by the procedure as described by Garewal et al. [6]. The cytochromes were stabilized by addition of 5 mM dithioerythreitol to all solutions. The protein solutions were concentrated using a Amicon ultrafiltration chamber and an XM-100 Diaflo membrane instead dry Sephadex G-25.

2.2. *Analytical methods*

The polypeptide composition of the membranes and of the pure proteins was analyzed by acrylamide-gel electrophoresis in the presence of 0.1% SDS [7]. Gel slabs (7 × 0.3 × 10 cm) were prepared in cuvettes from Ortec (type 4214) and consisted of either 11% acrylamide (gel system I) or 11% acrylamide containing additionally 5 M urea (gel system II) as described previously [4].

Thylakoid membranes were disintegrated and membrane proteins dissociated to their subunits with 30 mM Na-borate–HCl, pH 8.9, containing 1% SDS.

For mol. wt analysis 1% β-mercaptoethanol was added to the samples and heated at 100°C for 2 min prior to electrophoresis. Reference proteins (bovine serum albumin, catalase, ovalbumin, aldolase, chymotrypsinogen A, myoglobin and cytochrome *c*) were carboxymethylated with iodacetamide [8].

Amino acid analysis [9], ordinary gel electrophoresis [10] and crossed immunoelectrophoresis [11] were performed as described.

Immunization of rabbits with pure CF_1 was carried out by subcutaneous injection of 1 mg of protein emulsified with Freunds complete adjuvant (Difco). The procedure was repeated 4 weeks later. Blood was taken from the ear vein.

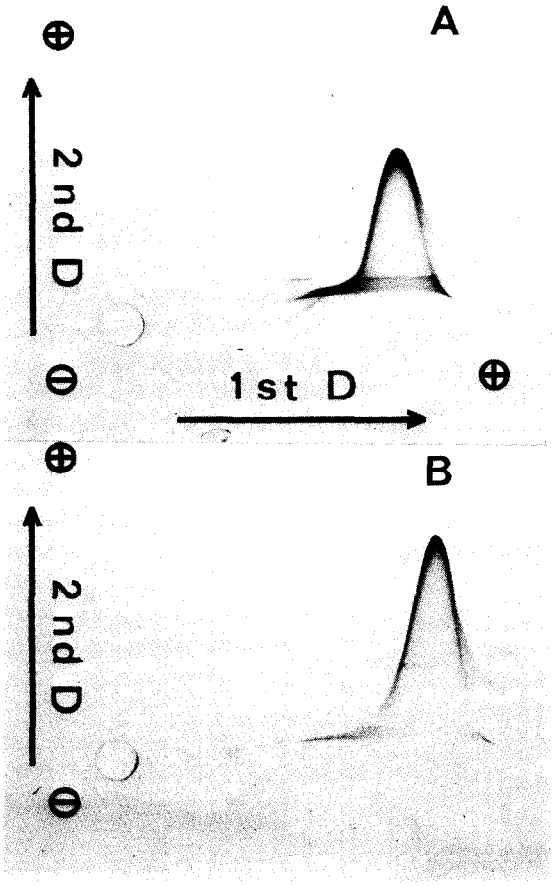

Fig.1. Crossed immunoelectrophoresis of pure CF_1 (A) and EDTA extract (B) using an anti-CF_1 serum. The samples are run in parallel in the electrophoresis.

3. Results and discussion

3.1. *Characterization of the purified proteins*

Both CF_1 containing fractions obtained by DEAE–cellulose chromatography were electrophoretically separated in a fast-moving main band and a slow-moving small band by the use of 6% acrylamide gels. These components show Ca^{2+}-ATPase activity after activation with 5 mM dithioerythreitol in 50 mM Tris–HCl, pH 8.0 [12]. Antibodies against the electrophorized protein preparation gave only one precipitation arc with pure CF_1 or EDTA extracts in the crossed immunoelectrophoresis (fig.1). X-ray-small-angle scattering measurements showed the enzyme to be pure and present as the monomer in solution at room temperature during more than 30 h (manuscript in preparation).

On the base of the amino acid composition (table 1) the mol. wt of CF_1 from Vicia faba was determined to be 279 000 (unpublished) by the numerical mathematical method of Katz [13]. The value is in agreement with data obtained by analytical ultracentrifugation [14,15] and by the X-ray measurements (unpublished).

Table 1
Amino acid composition of the coupling factor (CF_1) from Vicia faba

Amino acid	g Amino acid/100 g substance
Aspartic acid	8.29
Threonine	6.14
Serine	4.94
Glutamic acid	13.94
Proline	3.55
Glycine	4.65
Alanine	5.91
Valine	6.41
Isoleucine	6.89
Leucine	9.84
Tyrosine	3.86
Phenylalanine	3.74
Lysine	4.74
Histidine	0.60
Arginine	7.10
	90.60[a]

[a] Cysteine, methionine and tryptophan were not determined. The values were obtained by calculation from 24 and 48 h hydrolyses.

It was found by using the SDS–gel electrophoresis [3,16,17] that the CF_1 is composed of 5 subunits ($\alpha,\beta,\gamma,\delta,\epsilon$). In agreement with the spinach coupling factor [3,16,17] the mobilities of the three large subunits correspond to mol. wts of 59 000 (α), 55 000 (β) and 37 000 (γ). The two minor subunits were found to have mol. wts of 21 000 and 14 000 (fig.2c). The results correspond with an earlier observation [3]. When the samples of CF_1 were run without prior reduction of the interchain disulfide bonds with β-mercaptoethanol the 37 000 polypeptide splits into two bands. The fast-moving component (mol. wt 35 000) represents an oxidized form of the polypeptide.

Cytochrome *f* from Vicia faba extracted and purified by the Triton X-100–urea procedure [6] shows the same absorption spectrum as reported for the spinach protein [18] with maxima at 553.5, 531, 522, 420, 328, 284 and 278 nm (reduced form, absorbance ratio A_{420}:A_{278} between 0.9 and 1.0 for 3 preparations). For cytochrome *f* mol. wts between 31 000 and 34 000 have been determined by using SDS–gel electrophoresis [18–20]. Our preparations were found to consist of one polypeptide which has a mol. wt of 31 000 (fig.2d).

3.2. *Identification of the polypeptides in the spectra*

Nearly 25 membrane polypeptides could be separated on 11% acrylamide gels (gel system I) using a SDS–Na-borate–HCl buffer system (fig.2a, b). Machold and Aurich [21] have numerated the polypeptides in the electropherograms by consecutive letters beginning from the high-mol. wt region as shown in fig.2. The peaks B and B_1 had been identified

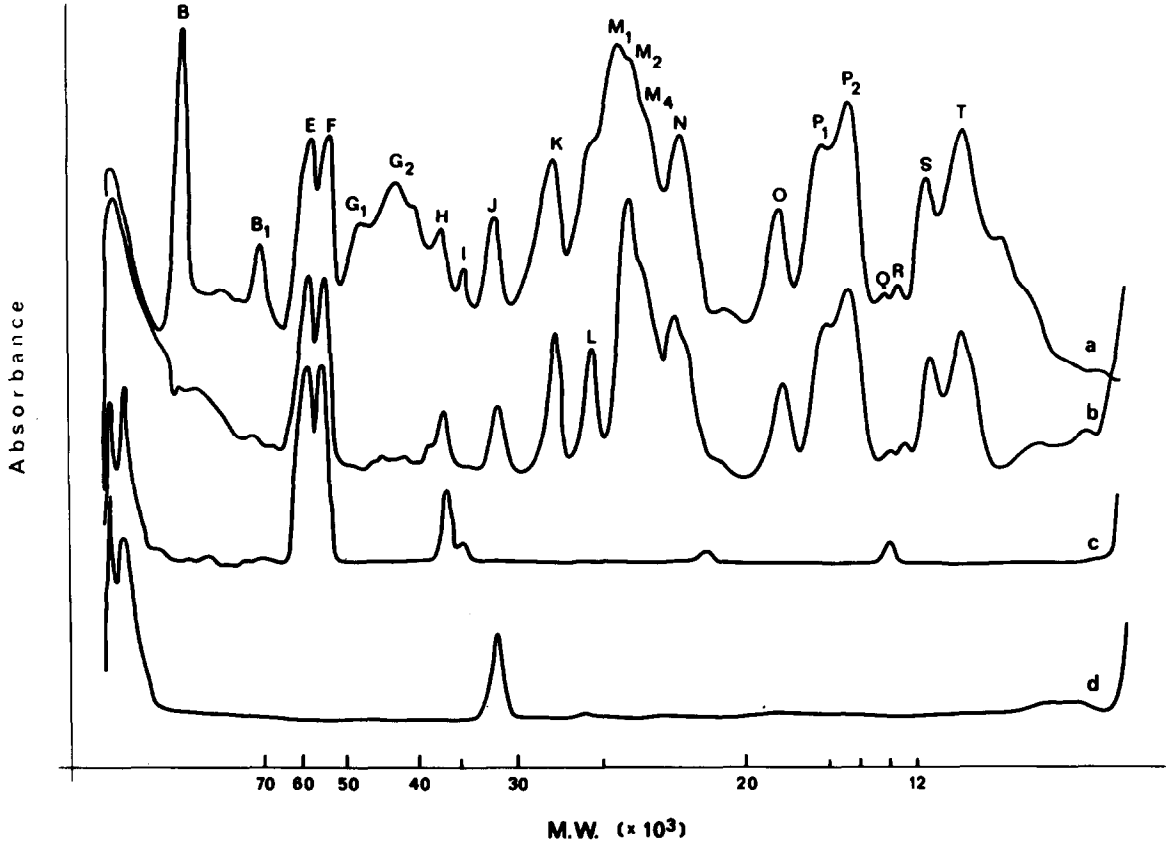

Fig.2. Densitograms of the thylakoid membrane polypeptides (a,b) of the CF_1 polypeptides (c) and of the polypeptide of cytochrome *f* (d) obtained on gel system I. Membranes and purified proteins were disintegrated with SDS as described. Without reduction with β-mercaptoethanol the polypeptides of the membranes is shown in curve a.

before [5,21] to be the pigment–protein complex I. Their protein moiety could not be resolved electrophoretically after heating the polypeptide mixture in presence of β-mercaptoethanol (fig.2b). The polypeptide area from M_1 to M_4 is consistent with pigment–protein complex II but contains other distinct polypeptides resolvable by two-dimensional gel electrophoresis (manuscript in preparation). To identify the other polypeptides by comparison of the electrophoretic mobilities both the coupling factor and cytochrome *f* were used as marker proteins and separated under the same conditions as the membrane polypeptide mixture.

In the membrane polypeptide spectra obtained on gel system I the three large subunits of coupling factor are equivalent to the peaks E (α), F (β) and H/I (γ) (see fig.2a, d). As reported previously [5] peak I could be quantitatively transformed into peak H by heat treatment in the presence of β-mercaptoethanol (fig.2a,b). Of the small CF_1 polypeptides only the ε subunit could be identified. It is equivalent to peak Q. By densitometric studies it was found that the ratio between this polypeptide and the subunit α and β are the same in the purified CF_1 as in the membrane polypeptide mixture. This shows that no loss of this subunit occurs during the purification of the protein.

In contrast to the polypeptides of the other two chloroplast cytochromes b_{559} and b_{563} which migrate near to the front during electrophoresis (unpublished)

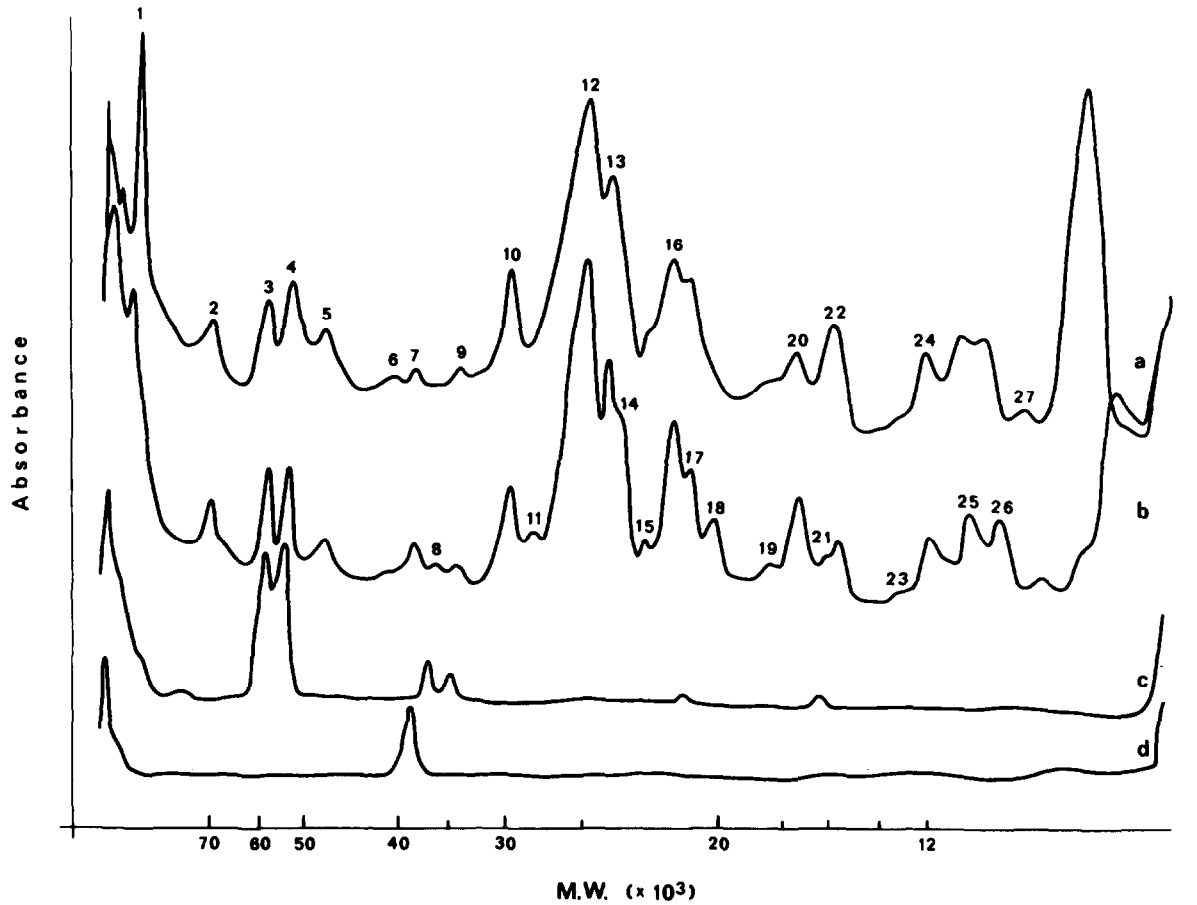

Fig.3. Densitograms of the thylakoid membrane polypeptides (a,b) of the CF_1 polypeptides (c) and of the polypeptide of cytochrome *f* (d) obtained on gel system II. The preparation of the samples was the same as in fig.2.

cytochrome *f* could be identified as peak J. A similar result was reported by Eaglesham and Ellis [22]. However, they could not identify the cytochrome as a distinct peak in their spectrum. The present finding is in contrast to the data of Nelson and Racker [20] who believe a fast-moving main polypeptide (probably K in our spectrum) to be cytochrome *f*.

An improvement of electrophoretic resolution especially for polypeptides with mol. wts < 25 000 was obtained on 11% acrylamide gels containing additionally 5 M urea [4]. Additional bands become visible compared with gel system I. For this reason we have enumerated the bands by consecutive numbers beginning from the high-mol. wt region (fig.3a,b). As described previously [4] the peaks 1 and 2 are consistent with pigment–protein complex I and the peaks 12 and 13 with the polypeptides II_a and II_b of pigment–protein complex II. Using gel system II for mol. wt calculation the α, β, γ and δ subunits of CF_1 were found to have the same values as determined on gel system I. For the ϵ polypeptide a mol. wt of 16 000 was found. In the membrane polypeptide profile the α and β subunits of CF_1 are equivalent to the peaks 3 and 4. Without reduction of the disulfide bonds the γ subunit shows the same mobility like peak 9. After reduction with β-mercaptoethanol the γ subunit splits into the two polypeptide components 8 and 9. The remaining part of polypeptide 9 could not be transformed in the slow-moving polypeptide by further reduction or by carboxymethylation. Using gel system II it was not possible to identify the two small CF_1 subunits in the spectrum.

Cytochrome *f* was found to have a slower mobility (mol. wt 38 000) than the γ subunit of CF_1 in contrast to the results obtained on gel system I. A possible explanation for this behaviour could be that urea and SDS compete under these conditions for the binding sites at the polypeptide chain leading to a loss of charge and a lower electrophoretic mobility. This behaviour may be caused by the high content of polar amino acids in cytochrome *f* [20].

Table 2 shows all peaks identified up to now in both gel systems used for the electrophoretical separation of thylakoid membrane polypeptides.

Finally it can be concluded that in the polypeptide spectra of purified thylakoid membranes all subunits of coupling factor of photophosphorylation and the polypeptide subunit of cytochrome *f* are present.

Table 2
Identified polypeptides in the spectra obtained on the gel systems I and II

Protein	Gelsystem I peak termed	Gelsystem II peak termed
Pigment–protein complex I	B, B_1	1, 2
Pigment–protein complex II	M-area	12, 13
Coupling factor:		
α-subunit	E	3
β-subunit	F	4
γ-subunit	I, H	8, 9
ϵ-subunit	Q	–
Cytochrome *f*	J	7

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