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Purification and characterization of E37, a major chloroplast envelope protein

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We have purified to homogeneity E37, the second major polypeptide of the inner membrane of the chloroplast envelope. The protein was retained on a Mono S column at pH 7, indicating it is a basic protein. After cyanogen cleavage, the protein was partially sequenced at 2 different sites. The sequence is compared with the deduced amino acid sequence of a cDNA coding for a 37 kDa envelope polypeptide recently published by Dreses-Werringloer et al. (Eur. J. Biochem. (1991) 195, 361–368.)

Chloroplast envelope; Inner envelope membrane, E37 polypeptide; Membrane protein purification; Amino acid sequence; Spinach chloroplast

1. INTRODUCTION

Chloroplasts are limited by a pair of distinct membranes known as the envelope which separates the organelle from the surrounding cytosol. This double membrane system is involved in the regulation of photosynthesis and in chloroplast development, i.e. the transport of plastid proteins synthesized in the cytoplasm [1–4] and the synthesis of plastid components such as glycerolipids (phosphatidic acid, diacylglycerol, galactolipids, phosphatidylglycerol and sulfolipid), terpenoid compounds (carotenoids, prenylquinones) and protochlorophyll [2–4]. The identification of protein markers of the outer or inner envelope membrane allowed the development of methods for the separation and the characterization of each envelope membrane [5–7]. The analysis of the fractions pointed out that the 2 major envelope polypeptides, named E30 and E37, are inner membrane associated [6]. E30 has been identified as the phosphate translocator and mediates the export of trioses phosphate against phosphate during photosynthesis [8–10]. It has been shown that E37 is probably a peripheral protein as it is extracted from the membrane by NaOH [11]. Unfortunately the function of this polypeptide is still unknown. In addition, several other minor polypeptides of 37 kDa are also present in envelope membranes [12]. For instance a NADPH:protochlorophyllide reductase has been localized in the chloroplast envelope [12,13] and identified as a 37 kDa polypeptide [12]. However NADPH:protochlorophyllide reductase has been shown to be distinct from E37 [12]. Recently the molecular cloning and sequencing of a cDNA coding for a 37 kDa envelope polypeptide was reported [14]. Herein we describe the purification to homogeneity of E37 and the amino acid sequencing of fragments of the purified protein obtained after cleavage by cyanogen bromide. These data indicate that this major envelope protein corresponds to the cDNA sequence previously reported.

Abbreviation: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid

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2. MATERIALS AND METHODS

2.1. *Chloroplast envelope isolation*

Intact chloroplasts purified from spinach (*Spinacia oleracea* L.) leaves were lysed in a hypotonic medium and the total envelope membrane was then purified on a sucrose gradient as described in [15]. Envelope fractions were kept in liquid nitrogen.

2.2. *Solubilization of chloroplast envelope membranes*

Envelope membranes (final concentration: 0.6–0.7 mg protein·ml^{-1}) were incubated for 15 min on ice in medium A containing 50 mM MOPS, pH 7.8, 1 mM DTT, 6 mM CHAPS. The supernatant was collected after centrifugation at 245 000 × *g* for 15 min.

2.3. *Purification of E37*

Solubilized envelope proteins (approximately 10 mg of protein) were loaded on a DEAE-Trisacryl M (LKB) column (5–6 ml) equilibrated with medium A and allowed to flow through the column at 18 ml/h. The column was subsequently washed with the same medium. The non-adsorbed proteins were collected and pooled. The pooled fraction was pH-adjusted to 7 with a few drops of 1 N HCl and layered on a Mono S HPLC HR 5/5 column (Pharmacia) equilibrated with medium B (Medium A at pH 7 instead of pH 7.8) with a flow rate of 30 ml/h. The column was washed with medium B until the 280 nm absorbance returned to baseline. Proteins were then eluted from the column by a gradient of KCl (up to 1 M) in medium B. Fractions of 1.5 ml were collected and analysed by SDS-PAGE. All purification steps were performed at 4°C.

2.4. *Analysis of fractions and protein determination*

Proteins were precipitated on ice in 10% (w/v) trichloracetic acid for 1 h and analysed by SDS-PAGE at room temperature in a 7.5–15% acrylamide gradient as in [6]. Immunoblotting experiments were performed as in [16]. Antibodies against E37 were prepared as described in [11]. Anti-E37 reacts with a single envelope polypeptide after two-dimensional polyacrylamide gel electrophoresis [12]. The amount of proteins in each fraction was measured with BCA reagent (Bio-Rad) using BSA as a standard.

2.5. *Cyanogen bromide cleavage*

The Mono S-purified E37 was precipitated with 10% (w/v) trichloroacetic acid on ice. The resulting precipitate (~40 μg) was recovered by centrifugation and redissolved in 200 μl of 80% (v/v) formic acid at 25°C. After solubilization 600 μl of ethanol were added with 3 μl of 5 M NaCl followed by the addition of 2.5 ml of diethylether and the temperature was brought down to −20°C to precipitate the protein. The dried pellet was resuspended in 400 μl of 70% (v/v) formic acid. Argon was blown over the solution for 2 min and a crystal of BrCN was added. After 42 h at 25°C in the dark the sample was freeze-dried. The cleavage products were further purified by SDS-PAGE on a 15–20% acrylamide gradient gel. They were then electrotransferred from the gel onto a Problott membrane (Applied System) at 50 V for 35 min in 10 mM CAPS, 10% methanol according to the Applied System manufacturer. The membrane was briefly rinsed with water before staining with Ponceau S solution (5 mg/ml in 5% trichloracetic acid). A thin polypeptide band of ~13 kDa and a broader one of ~10 kDa were excised and subjected to NH_2-terminal sequencing on a model 477A gas-liquid-phase protein sequenator (Applied Biosystems) equipped with a model 120A on-line phenylthiohydantoin amino acid analyzer.

3. RESULTS AND DISCUSSION

The 37 kDa envelope protein was purified from envelope membranes after solubilization with 6 mM CHAPS and ion exchange chromatography combining 2 columns with opposite charges: DEAE-Trisacryl and Mono S (see section 2). This protein did not bind to DEAE-Trisacryl unlike most of the other envelope proteins (Fig. 1A). In contrast it was well retained on Mono S column and recovered with 0.2 M KCl (Fig. 1A and B). Analysis of the fractions by SDS-PAGE and Coomassie blue staining shows that after Mono S chromatography the protein was pure (Fig. 1A). Minor protein contaminants of 34 kDa and 40 kDa that were present after DEAE-Trisacryl chromatography were eliminated by Mono S chromatography (Fig. 1A). Carotenoids were also eliminated in the void volume of Mono S (result not shown). The purified polypeptide was identified as E37 by immunoblotting (Fig. 2). Some traces of immunoreactivity were also observed in the purified fraction near 70 kDa (Fig. 2). However this reactivity was not observed in the envelope fraction and furthermore no 70 kDa polypeptide could be detected with Coomassie blue in the purified fraction. It is not clear whether these bands are aggregates of E37 or different proteins. Table I shows that the yield of purified E37 was close to 2% of the total envelope proteins that we started from. Considering the loss of protein at each step of the purification this means that E37 represents more than 2% of the envelope proteins. In fact, by scanning envelope proteins on a SDS-PAGE gel, we

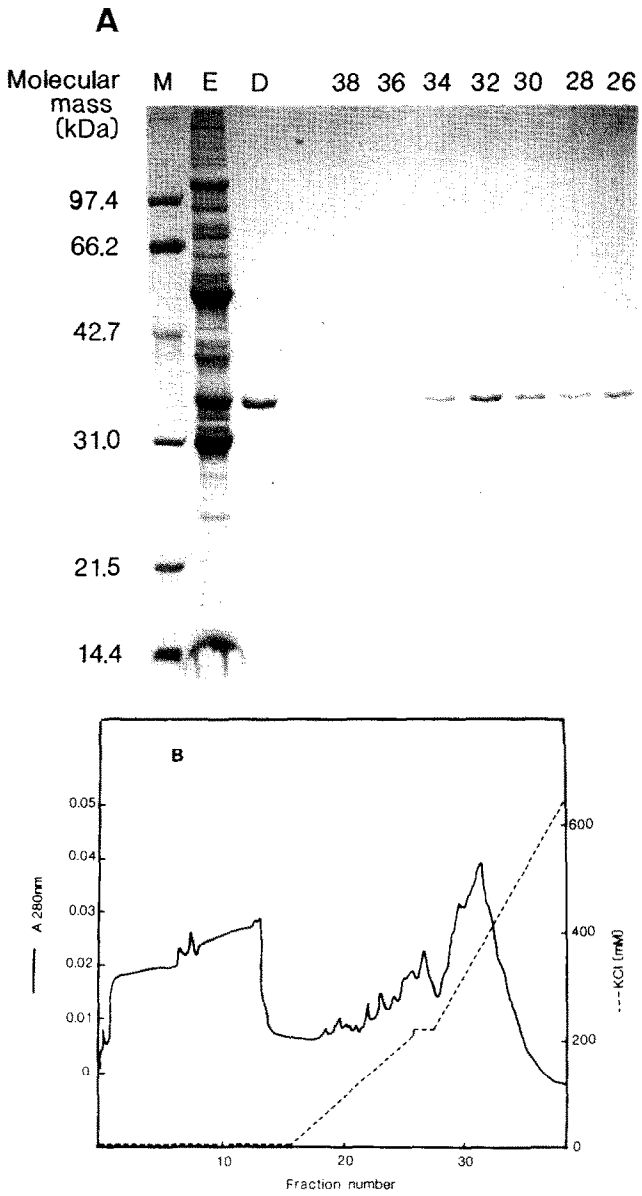

Fig. 1. Purification of the 37 kDa envelope protein. A. SDS-PAGE (7.5–15% acrylamide) of polypeptides from different stages in the purification of the protein. Proteins were stained with Coomassie blue. Lane M: molecular mass markers (Bio-Rad); lane E: envelope proteins (40 μg of protein); lane D: DEAE flow-through fraction (3 μg of protein); following lanes: proteins eluted in fractions 26 to 38 of the Mono S column (see part B) (proteins corresponding to 50 μl of each fraction were loaded on each lane). B. Purification of E37 by Mono S chromatography. Solid line: absorbance at 280 nm; dashed line: KCl concentration.

calculated that E37 represents 5–10% of total envelope proteins.

The NH_2-terminal sequencing of the protein did not give positive results, therefore the purified protein was subjected to CNBr cleavage for amino acid sequencing of the fragments (see section 2). After SDS-PAGE and electrotransfer on nylon membrane, several cleavage products were visualized by Ponceau S (results not

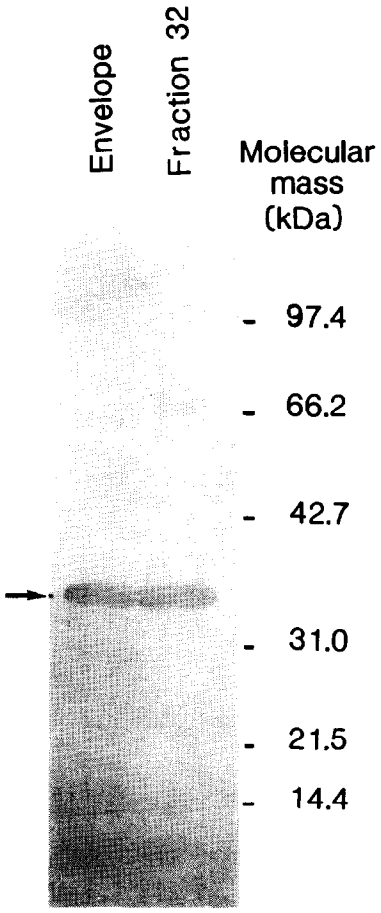

Fig. 2. Western blot analysis of envelope protein and of the purified 37 kDa envelope protein using antibodies raised against E37. 30 μg of envelope protein and 150 μl of fraction 32 (see Fig. 1) were resolved by SDS-PAGE, transferred to nitrocellulose and screened with antibodies (see section 2). The arrow indicates the position of the 37 kDa envelope protein stained with Ponceau S.

shown). Two well-resolved fragments with a good staining intensity were recovered and NH_2-terminal-sequenced. A 21 amino acid long sequence was obtained from the 13 kDa fragment while a shorter 4 amino acid sequence could be read on the 10 kDa fragment (Fig. 3). The comparison of these 2 partial sequences of the protein with the deduced amino acid sequence of a cDNA coding for a 37kDa envelope polypeptide [14] shows complete sequence homology (Fig. 3). Thus the full cDNA sequence recently published by Dreses-Werringloer et al. [14] can be conclusively attributed to

Table I

Purification protocol of E37

	Amount of protein	Yield
Envelope	14 mg	100%
Solubilized envelope	10 mg	74%
DEAE flow-through fraction	600 μg	4.2%
Mono S pooled fraction (fr 28 to 34)	270 μg	1.9%

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1         10        20        30        40        50
MACSMLNGVDKLALISGKTPNRLRFSGSDFTGSYKLPRLNLPPNSRNLRA
                    ↑
51        60        70        80        90        100
KTLTTVTKCTLSASERPASQPRFIQNKQEAFWFYRFLSIVYDNIINPGHW

101       110       120       130       140       150
TEDMRDVALEPADLNNRNMLVVDVGGGTGFTTLGIIKHVDPKNVTILDQS

                  MLVVDVGGGTGFTTLGIIKXVDP

151       160       170       180       190       200
PHQLAKAKAKKPLKECRIIEGDAEDLPFPTDYADRYVSAGSIEYWPDPQR

201       210       220       230       240       250
GIREAYRVLKLGGKACLIGPVYPTFWLSRFFADVWMLFPKEEEYIEWFQK

                                    MLFPK

251       260       270       280       290       300
AGFKDVQLKRIGPKWYRGVRRHGLIMGCSVTGVKPASGDSPLQLGPKVED

301       310       320       330       340       350
VQKPVHPLVFLYRFLLGALASTYYVLVPIYMWIKDKIFPKGMPL
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Fig. 3. Comparison of the partial amino acid sequence of two CNBr fragments of E37 **(in bold letters)** with the deduced amino acid sequence of a cDNA coding for a 37 kDa envelope protein published by Dreses-Werringloer et al. [14].

the major inner envelope polypeptide E37. No similarity with any known protein sequence could be found by these authors. However the hydrophobicity distribution analysis revealed a moderate hydrophobic character [14]: indeed E37, unlike phosphate translocator, is not chloroform–methanol extractible [11]. On the other hand, only one hydrophobic stretch was found. This segment is located near the C terminus of the protein and is long enough to span the membrane [14]. However this seems unlikely since the protein was shown to be NaOH-extractible [11], suggesting that it is probably a peripheral protein.

The lack of binding of E37 to DEAE-Trisacryl and the binding to Mono S at pH 7 indicates that the protein is basic, which is uncommon for envelope proteins and in general for membrane proteins. This result is in complete agreement with the theoretical isolectric point (pK_i = 9.49) we have calculated from the amino acid composition of E37 deduced from the cDNA sequence.

The function of E37, a major protein of the inner envelope membrane, is still not known. Taking into account its abundance and localisation, and by comparison with the phosphate translocator, we thought that it could be an anion translocator involved in the photorespiratory carbon and nitrogen cycles or in the transport of reducing equivalents across the chloroplast inner membrane [3]. However, the fact that E37 has only one hydrophobic stretch and that it is NaOH extractible, indicate that it is probably not a translocator.

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