Cell-free synthesis of putative precursors for envelope membrane polypeptides of spinach chloroplasts

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Protein synthesis in the presence of [^{35}S]methionine was carried out in a wheat germ cell-free system programmed with spinach leaf poly(A)$^+$ RNA. After translation, identification of the putative precursors of the envelope membrane polypeptides was performed by immunoprecipitation using antibodies against 3 envelope membrane polypeptides: M_r 22000, 29000 (phosphate translocator) and 36000. The identities of the immunoprecipitated precursors are established by performing competition studies. The results show that the precursor of the M_r 22000 envelope membrane polypeptide is of M_r ~10000, that of the phosphate translocator (M_r 29000) of M_r ~11000 and that of M_r 36000 polypeptide of M_r ~2000 larger than the authentic protein.

Precursor *In vitro synthesis* *Chloroplast envelope* *Polypeptide* *Phosphate translocator* *Membrane labeling* *(Spinach chloroplast)*

1. INTRODUCTION

The chloroplast envelope consists of two membranes of which the inner membrane is the functional barrier between the cytoplasm and the stroma space. A number of enzyme activities are associated with the chloroplast envelope (review [1]). One of the main functions of the inner envelope membrane is to regulate the metabolite flow between the cytosol and the chloroplast catalyzed by different transport systems. The most important transporter located in the inner envelope membrane is the phosphate translocator catalyzing the export of fixed carbon in exchange with phosphate from the chloroplast into the cytosol [2]. This membrane protein with apparent M_r 29000 as determined by SDS gel electrophoresis has been isolated and its exchange properties investigated by reconstitution into liposomes [3,4]. Although no further polypeptides of the chloroplast envelope have been functionally identified as yet, it could be shown that another major envelope polypeptide with apparent M_r 36000 is located in the inner envelope membrane and that a polypeptide with apparent M_r 22000 is the major protein of the outer envelope membrane [5]. Recently we have shown that most of the envelope membrane proteins including the M_r 36000, 29000 and 22000 polypeptides are coded by nuclear genes and therefore are supposed to be synthesized as precursors on soluble cytosolic ribosomes and inserted into the chloroplast envelope in a post-translational manner [6]. There is no general rule for the structure of precursors. In the case of mitochondria, some are synthesized as larger molecules and cleaved to smaller mature forms whereas others have the same apparent M_r as the functional protein (reviews [7–9]). So far in higher plants cell-free synthesis and transfer into chloroplasts could only be demonstrated for some stromal proteins and thylakoid membrane polypeptides [10–14]. Stroma and thylakoid membrane proteins account for about 50% each of the total chloroplast protein, whereas the envelope membrane represents less than 1%. This might explain why there is no information as to the nature

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of the precursor forms of envelope membrane proteins.

We report here for the first time the identification of in vivo synthesized precursors of 3 polypeptides from the chloroplast envelope, all of which are larger than their mature counterparts.

2. MATERIALS AND METHODS

Antisera were raised against the M_r 22000, 29000 and 36000 membrane polypeptides of the chloroplast envelope. The polypeptides were separated on an SDS-polyacrylamide gel and electroeluted from the gel at room temperature for 48 h at 25 mA. Subsequently the samples were dialyzed and dye was removed by extraction with a chloroform–methanol–water mixture [14]. Rabbits were injected with these antigens and antisera collected after the third boost. The activities of antisera were assayed by the immune replica technique [16] using peroxidase-coupled swine immunoglobulins (Boehringer Ingelheim, FRG) as the second antibody.

Purified envelope membrane polypeptides were ^3H labeled by using the modified reductive methylation procedure in [17] which does not alter the electrophoretic mobility of the labeled protein. Four μl of 0.2 M formaldehyde were added to 1 mg membrane protein in 0.2 M sodium borate buffer (pH 9.0) (final volume 0.3 ml). After 30 s, 10 μl NaB^3H_4 (2 mCi, spec. act. 10 Ci/mmol, Amersham Buchler) were added followed by 3 2-μl sequential additions of $NaBH_4$ (5.5 mg/ml) at 1-min intervals. To ensure complete reduction of the formaldehyde, an additional 15 μl $NaBH_4$ were added. Subsequently membranes were washed twice with 0.2 M sodium borate buffer, once with 10 mM Tricine buffer (pH 7.6) and stored at −85°C.

RNA from spinach leaves (*Spinacea oleracea*, US hybrid 424 from Ferry-Morse Seed Co., Mountain View, CA; 3–4 weeks old, grown in water culture as in [18]) was isolated as in [19]. An oligo(dT)–cellulose column was used to select for poly(A)$^+$ DNA which was precipitated overnight in 70% ethanol, 0.2 M sodium acetate, 10 mM Tris–HCl (pH 5.5), washed twice with 66% ethanol and then dissolved in sterile distilled H_2O and stored at −85°C.

The reaction mixture for the translation in a wheat-germ cell-free system contained 20 mM Hepes–KOH (pH 7.1), 100 mM KCl, 2 mM magnesium acetate, 0.6 mM spermine, 1 mM ATP, 8 mM creatine phosphate, 25 μg/ml creatine kinase, 25 μM CTP, 25 μM GTP, 1 mM dithiothreitol, 25 μM each of 19 amino acids minus methionine, 2.5 mCi/ml [^{35}S]methionine (spec. act. >600 Ci/mmol, New England Nuclear), about 100 μg/ml poly(A)$^+$ RNA and 0.25 vol. of wheat-germ extract. The translation reaction was carried out at 25°C for 90 min. Then the mixture was centrifuged for 45 min at 160000 × *g* and the supernatant stored at −85°C. After incubation of a standard 200 μl protein-synthesizing mixture, radioactivity recovered in total trichloroacetic acid-insoluble polypeptides was 3–5 × 10^7 cpm as compared to 2–3 × 10^6 cpm in the absence of added RNA. Thirty μl of this translation mixture were dissociated with 7.5 μl of 15% (w/v) SDS, heated to 90°C for 3 min and diluted to 640 μl with Triton buffer (1.5% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 10 mM methionine, 1 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3, 50 mM Tris–HCl (pH 8.0) and centrifuged for 20 min at 100000 × *g*. Pre-immune serum (10 μl) was added to the supernatants. After incubation for 1 h at 4°C, 50 μl of 10% (w/v) glutaraldehyde-fixed *Staphylococcus aureus* cells (Calbiochem) were added [20] and the mixtures incubated for 1 h at 4°C followed by centrifugation at 12000 × *g* for 4 min. Antiserum (10 μl) was added to the supernatants and the mixtures incubated overnight at 4°C. Then, 50 μl of 10% (w/v) fixed *S. aureus* cells were added and the sample rotated for 1 h at 4°C. Cells were recovered by centrifugation, 50 μl bovine serum albumin (150 mg/ml) added and the cells washed 3 times by resuspending in buffer containing 10 mM Tris–HCl (pH 7.5), 0.3 M NaCl, 10 mM methionine, 1% Triton X-100 and 0.1% SDS, twice with the same buffer without SDS and 3 times with 10 mM Tris–HCl (pH 7.5) and finally extracted for 3 min at 100°C with SDS-electrophoresis sample buffer containing 5% SDS. Cells were removed by centrifugation and the supernatants analyzed by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide slab gel electrophoresis and fluorography of the dried gels were as in [21,22]. The reference proteins were purchased from Amersham Buchler.

3. RESULTS AND DISCUSSION

Protein synthesis in the presence of [^{35}S]methionine was carried out in a wheat germ cell-free system programmed with spinach leaf poly(A)$^+$ RNA. Following translation, identification of the putative precursors of the envelope membrane polypeptides was performed by immunoprecipitation using antibodies directed against the different envelope polypeptides, SDS-gel electrophoresis and fluorography as outlined in section 2. These translation products were compared with the mature envelope membrane polypeptides immunoprecipitated from radioactively labeled envelope membranes.

Using uniformly ^{3}H-labeled envelope membranes it could be shown that under the experimental conditions the authentic M_r 22000, 29000 and 36000 polypeptides could be specifically immunoprecipitated and identified with antisera raised against the protein studied (fig.1). The same results were obtained when using the immunoblotting method [15] or in vivo ^{35}S-labeled intact chloroplasts prepared as in [6] instead of ^{3}H-labeled envelope membranes (not shown).

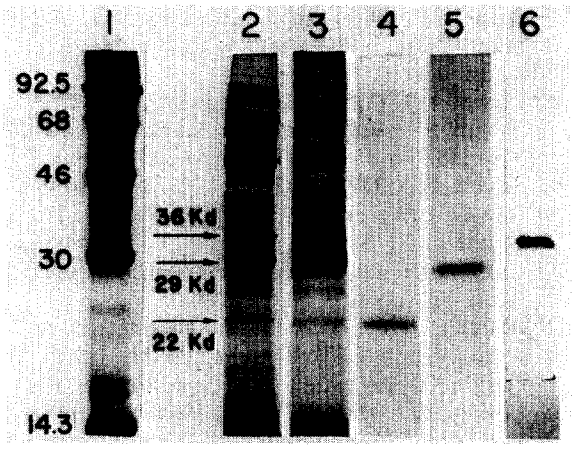

Fig.1. Immunoprecipitation of ^{3}H-labeled mature envelope membrane polypeptides. Lane 4–6, immunoprecipitate with antiserum directed against the M_r 22000 (lane 4), M_r 29000 (lane 5) and M_r 36000 polypeptides (lane 6); lane 1, relative M_r markers. The numbers on the left indicate $M_r \times 10^{-3}$; lane 2, total envelope membrane polypeptides stained with Coomassie brilliant blue; lane 3, fluorogram of ^{3}H-labeled total envelope membrane polypeptides.

In the following ^{35}S-labeled products of the messenger-dependent wheat germ system which are supposed to contain the putative precursors of the envelope polypeptides were immunoprecipitated with antibodies against the M_r 22000, 29000 and 36000 polypeptides. Fig.2 is a typical fluorogram showing immunoprecipitates of the translation mixture reacted with the different antisera. With antibodies against the M_r 22000, 29000 and 36000 polypeptides respectively, labeled polypeptides of M_r 32000 (lane 1), M_r 40000 (lane 3) and M_r 38000 (lane 5) were specifically immunoadsorbed. No radioactive polypeptides were immunoadsorbed using pre-immune sera (not shown). These results show that the 3 envelope membrane polypeptides studied are made as precursors of substantially greater M_r as compared to the mature proteins shown in fig.1: the precursor of the M_r 22000 outer envelope membrane polypeptide is of M_r ~10000, that of the phosphate translocator (M_r 29000 polypeptide) of M_r ~11000 and that of M_r 36000 of M_r ~2000 larger than the authentic protein (table 1).

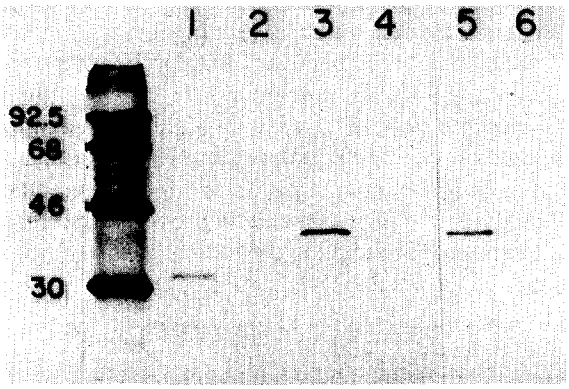

Fig.2. Fluorography of an SDS-polyacrylamide gel showing immunoprecipitation of putative envelope membrane polypeptide precursors from the total cell-free translation products synthesized in a wheat-germ system with spinach leaf poly(A)$^+$ RNA. The X-ray film was exposed for 4 days (lane 1–4) and 7 days (lane 5,6) at −85°C. Lane 1, precursor of the M_r 22000 polypeptide; lane 3, precursor of the M_r 29000 polypeptide; lane 5, precursor of the M_r 36000 polypeptide; lane 2,4,6, immunoprecipitation performed as for lane 1,3,5 except that 5 μg unlabeled mature M_r 22000 (lane 2), M_r 29000 (lane 4) and M_r 36000 polypeptides (lane 6) were present during incubation with antibody. Immunoprecipitation was performed as described in section 2.

Table 1

Molecular masses of in vitro synthesized putative precursors for envelope membrane polypeptides and their mature counterparts (kDa)

Mature protein	Precursor
22	32
29	40
36	38

To establish the identity of the immunoprecipitated precursor forms, competition studies were performed. For this purpose, during the incubation of the in vitro synthesized ^{35}S-labeled products with the specific antibodies, 5 μg of isolated non-radioactive M_r 22000, 29000 (phosphate translocator) and 36000 polypeptides were also present. As shown also in fig.2, the non-radioactive polypeptides can completely displace the precursor from the immunocomplex (lane 2,4,6). One might argue that this competition is an unspecific effect of an envelope membrane protein but it can be demonstrated that this displacement is indeed specific and that the precursors are not displaced by other proteins. Fig.3 shows that the labeled M_r 40000 precursor of the M_r 29000 polypeptide (lane 1) is only displaced by the non-radioactive M_r 29000 polypeptide (lane 2) but not by the M_r 36000 polypeptide (lane 3) and vice versa (lane 4–6). These results clearly prove the identities of the immunoprecipitated precursors and indicate

Fig.3. SDS-polyacrylamide slab gel electrophoresis and fluorography of the envelope membrane polypeptide precursors. Lane 1–3, immunoprecipitate from translation products with antiserum directed against the M_r 29000 and 36000 polypeptides (lane 4–6), respectively. In lane 2 and 6, 5 μg of non-radioactive M_r 29000 polypeptide and in lane 3 and 5, 5 μg of non-radioactive M_r 36000 polypeptide were added before immunoprecipitation.

that the studied envelope polypeptides are synthesized on cytoplasmic polysomes as higher M_r precursors containing an amino acid extension of about 80, 90 and 16 amino acid residues for the M_r 22000, 29000 and 36000 polypeptides, respectively. Studies to elucidate the mode of posttranslational transfer of these polypeptides into the envelope membrane are currently under investigation.

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