FEBS Letters 379 (1996) 302–304 FEBS 16605

Molecular chaperones are present in the thylakoid lumen of pea chloroplasts

Thomas Schlicher, Jürgen Soll*

Botanisches Institut, Universität Kiel, Am Botanischen Garten 1–9, D-24118 Kiel, Germany

Received 15 December 1995

Abstract A soluble protein fraction was obtained from pea chloroplast thylakoids, which represents highly enriched lumenal components. Using antisera against chaperonin 60 (cpn60), chaperonin 10 (cpn10) and the heat shock cognate protein of 70 kDa (hsc70) we are able to demonstrate, that the thylakoid lumen contains a separate set of molecular chaperones, which is distinct from the stromal one. In contrast to the α and β subunits of cpn60 present in the stroma the lumen contains only one cpn60 isoform of distinct isoelectric point. Furthermore the lumenal cpn10 is of 'normal' size and not like its stromal counterpart of a double-domain tandem architecture. The immunoreactive hsc70 isoforms in the lumen seem also to be different from the stromal ones. Thus, chloroplasts seem to contain the largest number of molecular chaperone isoforms present in one organelle.

Key words: Pisum sativum L.; Chaperone; Thylakoid lumen; Protein assembly

1. Introduction

Chloroplasts are highly structured organelles responsible for photosynthesis in green parts of plants. Most of the chloroplast polypeptides constituents are nuclear encoded, synthesized in the cytosol and posttranslationally translocated into the organelles [1]. Chloroplasts contain three distinct membrane systems, termed the outer and the inner envelope and the thylakoid membrane and also three soluble compartments, the interenvelope space, the stroma and the thylakoid lumen. Nuclear coded proteins are routed to all of these chloroplast subcompartments by distinct pathways [2,3]. This process requires as a final event folding and assembly of these proteins into biologically active units. Molecular chaperones such as hsc70 homologues and the cpn60/cpn10 family are known to be essential for translocation [4,5], folding and assembly steps involved in these reactions [6–8].

The cpn60/cpn10 system operating in the chloroplast stroma [9–12] is unique in comparison to other organelles or prokaryotes [13–15]. Stromal cpn60 exists in two isoforms α and β in the chloroplasts [11,12] and its co-chaperonin 10 is comprised of two distinct cpn10 like domains, fused in tandem to give a binary co-chaperonin structure [9,16]. The reasons for the presence of the special composition of the stromal cpn60/cpn10 is unknown in the moment, because it has been demonstrated that stromal proteins can be folded by heterologous non chloroplast cpn60/cpn10 systems [16,17].

The thylakoid lumen houses a number of proteins, of which most prominent are the nuclear encoded components of the oxygen evolving enzyme complex (OEE) associated with photosystem II and also plastocyanin, a mobil electroncarrier between the cytochrome *b/f* complex and photosystem I [2]. Following thylakoid transfer the OEE subunits must be assembled into the functional complex. We thus wanted to know, if this chloroplast subcompartment contained the molecular chaperones necessary for folding and assembly. Our data demonstrate that the thylakoid lumen contains a 'normal' cpn60/cpn10 system distinct from the unusual stromal system and novel hsc70 homologues. The coexistence of two chaperonin systems in one organelle could have provoked the differential development (evolution) of its components.

2. Materials and methods

2.1. Antisera

Antisera against OEE33 from pea [18] and SSU from tobacco [19] were raised in chicken. OEE33 and SSU were synthesized by overexpression in *E. coli* using the pET-vector system (Novagen, Madison/USA) and purified as insoluble inclusion bodies [20]. An antiserum was raised in a rabbit against a 21 amino acids long peptide of stromal cpn10 (DEEGNRIPLPVCSGNTVLYSC) coupled to rabbit serum albumin [9]. The antiserum to hsp70 from tomato is described in [21] (gift from L. Nover, Frankfurt). The antiserum to cpn60 from *Neurospora* mitochondria is described in [22] (gift from U. Hartl, New York).

2.2. Isolation and fractionation of chloroplasts

Purified chloroplasts were isolated from 12-day-old pea plants by standard procedures [23]. The chloroplasts equiv. to 5 mg chlorophyll were lysed in 75 ml 50 mM NaP_i-buffer pH 7.4, 10 mM NaCl. After 30 min on ice intact chloroplasts were separated from lysed organelles by centrifugation through a 40% Percoll cushion [23]. Thylakoids were removed from the top of the Percoll cushion, diluted with NaP_i-buffer and recovered by centrifugation (5 min, 4000 × *g*) and washed twice (5 min, 2000 × *g*). The thylakoid pellet was resuspended in 10 mM NaP_i-buffer pH 7.4, 5 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose and treated with the protease thermolysin at 1 mg protease per mg chlorophyll for 10 min on ice [23]. The protease treatment was stopped by the addition of excess EDTA pH 7.4 (50 mM). Thylakoids were recovered by centrifugation as above washed four times in the presence of EDTA, followed by four washes without EDTA. The final membrane pellet was resuspended to give a chlorophyll concentration of 0.2 mg × ml^{-1}. Thylakoid membranes were fragmented by three cycles in a French press at 107 pa [24]. The last cycle contained in addition 5 mM EDTA. A membrane free supernatant containing the lumenal proteins was obtained by centrifugaton (30 min, 200,000 × *g*).

A stromal extract was obtained by lysis of intact chloroplasts in 10 mM HEPES-KOH pH 7.4 and centrifugation (30 min, 200,000 × *g*). This procedure does not result in the lysis of the thylakoid network and subsequent release of lumenal proteins [24].

2.3. Electrophoresis and Western blotting

Two-dimensional gel elctrophoresis was essentially done as described

*Corresponding author. Fax: (49) (431) 880 2517.

Abbreviations: cpn, chaperonin; hsc, heat shock cognate protein; Rubisco, ribulose-1,5-bis-phosphate carboxylase oxygenase; SSU, small subunit of Rubisco; OEE, oxygen evolving enzyme complex; OEE33, OEE of 33 kD; Str, stroma fraction; Lu, thylakoid lumen fraction.

SSDI 0014-5793(95)01534-5

[25]. The second dimension SDS-PAGE was done using a 10% polyacrylamide separating gel. Polypeptides were transferred to nitrocellulose filters using a semidry blotting procedure (Pharmacia, according to the manufacturers recommendations). The filters were treated and immundecorated with antisera as described [23]. An alkaline phosphatase stain in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium was used for detection.

3. Results and discussion

Methods, which were originally developed to obtain inside-out thylakoid vesicles [24], were used in this study to isolate soluble proteins from the thylakoid lumen and to probe them for the presence of molecular chaperones, i.e. cpn60/cpn10 and hsc70 by immunological methods. The protein composition of the chloroplast stroma is distinct from that of the thylakoid lumen (Fig. 1a). Antisera to marker proteins of each compartment were used to assess a possible crosscontamination. OEE33, a component of the lumenal oxygen evolving enzyme complex associated with photosystem II was only detectable in that fraction but not in the stroma fraction, while the small subunit of the CO_2 fixing enzyme Rubisco was only detectable in the stroma fraction (Fig. 1b). We conclude from these data, that crosscontaminations were beyond detectability and were therefore unlikely to influence our analysis.

The identical preparations of lumenal and stromal extracts were then tested by immundecoration of nitrocellulose filters after one-dimensional SDS-PAGE for the presence of hsc70, cpn60 and cpn10 homologues. As shown in Fig. 2 the chloroplast stroma contains the clearly distinguishable cpn60 α and β isoforms (Fig. 2a, lane 1) while only one polypeptide crossreacts in the thylakoid lumen fraction with the cpn60 antiserum (Fig. 2a, lane 2). The lumenal cpn60 is of similar size as the larger stromal cpn60 α isoform. These results can be interpreted in several ways. (i) The presence of a cpn60 isoform in the lumen is due to crosscontamination. We feel that this is unlikely, due to our control experiments presented in Fig. 1.

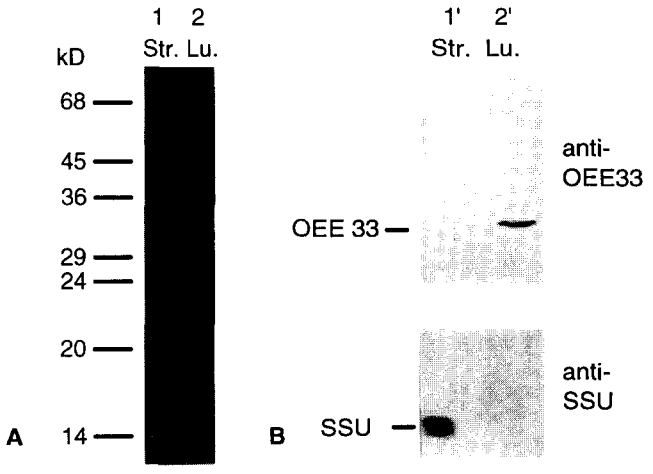

Fig. 1. The stroma and the thylakoid lumen from pea chloroplasts contain distinct sets of soluble proteins. (A) A coomassie brilliant blue obtained SDS-PAGE of the stromal (Str., lane 1) and lumenal (Lu., lane 2) polypeptide composition is shown. Numbers on the left indicate molecular weight markers in kDa. (B) Equal amounts of protein (20 μg) from the stromal (lane 1') or lumenal (lane 2') fraction was separated by SDS-PAGE and blotted. The lower part of the blot was immundecorated using an anti-SSU antiserum and the upper part with anti-OEE33 antiserum.

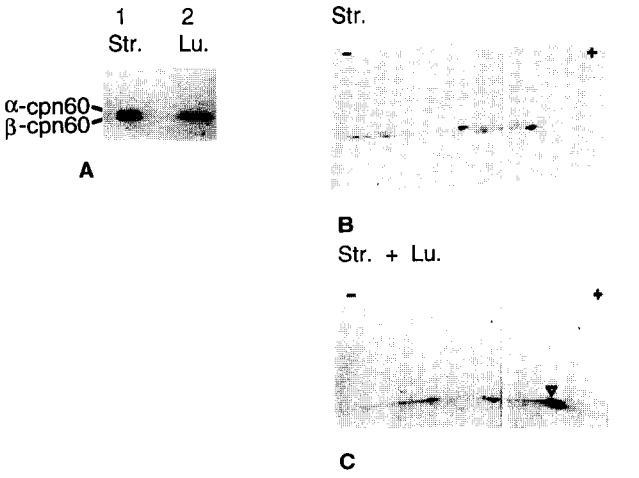

Fig. 2. The thylakoid lumen contains a distinct cpn60 isoform. (A) Stromal (lane 1) or lumenal proteins (35 μg each) were separated by SDS-PAGE, blotted and immundecorated using an anti-cpn60 antiserum. (B) Stromal proteins (32 μg) were separated by two-dimensional electrophoresis, blotted and immundecorated with anti-cpn60 antiserum. The position of the anode (+) and kathode (−) is indicated. (C) Stromal (32 μg) and lumenal (37 μg) proteins were mixed prior to two-dimensional electrophoresis and immundecorated with anti-cpn60 antiserum as in B. The position of the lumenal cpn60 isoform is indicated by an arrow.

(ii) The lumenal cpn60 is identical to the stromal cpn60 α subunit, but it has been transported across the thylakoid membrane into the lumen by an as yet unknown mechanism. (iii) The lumenal cpn60 represents indeed an independent, sofar undescribed gene product. To distinguish between these possibilities polypeptides were separated by two-dimensional gel-electrophoresis, transfered to nitrocellulose filters and immundecorated. The stromal cpn60 α and β isoforms are clearly separated (Fig. 2b). This is in accordance with the calculated isoelectric points of the cpn60 α and β isoforms from pea chloroplasts of 4.6 and 5.1, respectively [11,12]. In order to clearly demonstrate a possible difference between the cpn60 isoforms, lumenal and stromal proteins were mixed prior to electrophoresis. As shown in (Fig. 2c) we can now clearly detect three immunoreactive polypeptides (the position of the additional lumenal cpn60 isoform is indicated by an arrow). From these results we conclude, that the chloroplast thylakoid lumen contains a new cpn60 homologue. This protein is most likely a nuclear gene product since the plastid genome does not contain an open reading frame, which could code for this new cpn60. The isolectric point of the lumenal cpn60 is similar to that of the stromal α isoform, i.e. around 4.6.

Cpn60 function is assisted through interaction with its co-chaperonin cpn10 [6]. We thus wanted to know, if the thylakoid lumen contained its own cpn10 protein and if this was of the tandem architecture like the stromal isoform. As shown (Fig. 3a, lane 2) the thylakoid lumen contains a cpn10 like protein, which is recognized by a peptide specific antiserum raised against a peptide of the stromal cpn10. The size of the lumenal cpn10 like protein on SDS-PAGE is about 10–12 kDa similar to most other cpn10 proteins. In contrast the stroma contains two proteins, which are recognized by the cpn10 antiserum. The higher molecular weight form at around 24 kDa (Fig. 3a, lane 1) represents the mature double-domain stromal cpn10

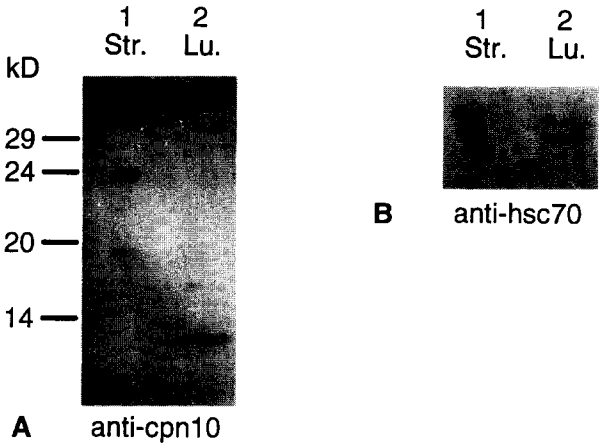

Fig. 3. The thylakoid lumen contains proteins homologue to cpn10 and hsc70. (A) The lower part of the blot shown in Fig. 2A was immundecorated with an anti-cpn10 antiserum. (B) Stromal (30 μg) and lumenal (20 μg) proteins were separated and subjected to immundecoration by an anti-hsc70 antiserum as outlined in Fig. 1.

[9]. Much less abundant was a lower molecular weight protein (Fig. 3a, lane 1). This form is also detected after import and processing of the precursor form of stromal cpn10 into chloroplasts [9]. This indicates that the lower molecular weight form of stromal cpn10 could represent a degradation product. The exact nature was, however, not investigated further. From these data we conclude, that the thylakoid lumen contains a new cpn10 like protein, which is distinct from the stromal double-domain cpn10.

The family of hsc70 proteins also plays an important role in folding of proteins but also in translocation of proteins across membranes, e.g. mitochondria or endoplasmic reticulum [26]. Translocation of proteins across the thylakoid membrane into the lumen occurs by different mechanisms, however, at least one requires the hydrolysis of ATP [2]. This raised the possibility, that hsc70 homologues could be involved in one of these translocation pathways. We therefore tested the lumenal protein fraction for hsc70 like proteins by immunoblotting. As shown in Fig. 3b the thylakoid lumen contains at least two hsc70 like proteins (Fig. 3b, lane 2). These can be distinguished from their soluble stromal counterparts (Fig. 3b, lane 1), which have been described as 75 kDa hsc and 78 kDa hsc [27]. We propose, that one of the possible functions of the lumenal hsc70 is to pull proteins across the thylakoid membrane as proposed for other systems [4,5,26].

4. Conclusion

We have demonstrated in this report that the thylakoid lumen contains its own set of cpn60/cpn10 proteins. The composition of the lumenal system resembles that of the 'normal' systems known from other cellular compartments [6,28] and is unlike the unique stromal cpn60/cpn10 system. It is tempting to speculate, that the unique stromal cpn60/cpn10 proteins arose in evolution in order to avoid a crossing or mixture of the two plastidal systems in vivo. The lumenal cpn60/cpn10 are most likely coded on nuclear genes and have to be transported through the stroma into the lumen. It will be interesting to see, what kind of translocation mechanism is involved in this to hinder premature interception of these lumenal chaperonins in the stroma by their stromal 'relatives'.

The presence of hsc70 like proteins in the lumen predicts also the occurence of those polypeptides, which cooperate with hsc70 in function, i.e. DnaJ and GrpE [6,7]. We have no antisera at the moment to test this hypothesis. However, a stromal DnaJ protein was recently detected (Schlicher and Soll, unpublished), corroborating our idea.

Acknowledgements: We thank S. Ihle for excellent technical assistance. We are indepted to Drs. Cline, Hartl, Klein, Nover for providing antisera or cDNA-clones. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

References

[1] Soll, J. (1995) Bot. Acta 108, 277–282.
[2] Robinson, C. and Klösgen, R.B. (1994) Plant Mol. Biol. 26, 15–25.
[3] Keegstra, K., Olsen, L.J. and Theg, S.M. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 471–501.
[4] Stuart, R.A., Cyr, D.M., Craig, E.A. and Neupert, W. (1994) Trendes Biochem. Sci. 19, 87–92.
[5] Pfanner, N., Craig, E.A. and Meijer, M. (1994) Trends Biochem. Sci. 19, 368–372.
[6] Gething, M.-J. and Sambrook, J. (1992) Nature 355, 33–45.
[7] Schröder, H., Langer, Th., Hartl, F.-U. and Bukau, B. (1993) EMBO J. 12, 4137–4144.
[8] Blumberg, H. and Silver, P.A. (1991) Nature 349, 627–629.
[9] Bertsch, U., Soll, J., Seetharam, R. and Viitanen, P.V. (1992) Proc. Natl. Acad. Sci. USA 89, 8696–8700.
[10] Viitanen, P.V., Schmidt, M., Buchner, J., Suzuki, T., Vierling, E., Dickson, R., Lorimer, G.H., Gatenby, A. and Soll, J. (1995) J. Biol. Chem. 270, 18158–18164.
[11] Martel, R., Cloney, L.P., Pelcher, L.E. and Hemmingsen, S.M. (1990) Gene 94, 181–187
[12] Hemmingsen, S.M., Woolford, C., van der Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W. and Ellis, R.J. (1988) Nature 333, 330–334.
[13] Azem, A., Kessel, M. and Goloubinoff, P. (1994) Science 265, 653–666.
[14] Schmidt, M., Rutkat, K., Rachel, R., Pfeifer, G., Jaenicke, R., Viitanen, P., Lorimer, G. and Buchner, J. (1994) Science 265, 656–659.
[15] Matthew, J.T., Viitanen, P.V. and Lorimer, G.H. (1994) Science 265, 659–666.
[16] Bertsch, U. and Soll, J. (1996) Plant Mol. Biol., in press.
[17] Baneyx, F., Bertsch, U., Kalbach, C.E., van der Vies, S.M., Soll, J. and Gatenby, A.A. (1995) J. Biol. Chem. 270, 10695–10702.
[18] Cline, K., Henry, R., Li, C. and Yuan, J. (1993) EMBO J. 12, 4105–4114.
[19] Klein, R.R. and Salvucci, M.E. (1992) Plant Physiol. 98, 546–553.
[20] Paulsen, H., Rümmler, U. and Rüdiger, W. (1990) Planta 181, 201–211.
[21] Neumann, D., zur Nieden, U., Manteuffel, R., Walter, G., Scharf, K. and Nover, L. (1987) Eur. J. Cell Biol. 43, 71–81.
[22] Ostermann, J., Horwich, A.L., Neupert, W. and Hartl, F.-U. (1989) Nature 341, 125–130.
[23] Waegemann, K. and Soll, J. (1991) Plant J. 1, 149–159.
[24] Akerlund, H.-E. and Andersson, B. (1983) Biochim. Biophys. Acta 725, 34–40.
[25] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[26] High, St. and Stirling, C.J. (1993) Trends Cell Biol. 3, 335–339.
[27] Marshall, J.S., DeRocher, A.E., Keegstra, K. and Vierling, E. (1990) Cell Biol. 87, 374–378.
[28] Lubben, Th.H., Gatenby, A.A., Donaldson, G.K., Lorimer, G.H. and Viitanen P.V. (1990) Proc. Natl. Acad. Sci. USA 87, 7683–7687.