Immunological detection of proteins similar to bacterial proteases in higher plant chloroplasts

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Despite numerous demonstrations of protein degradation in chloroplasts of higher plants, little is known about the identity of the proteases involved in these reactions. To identify chloroplast proteases by immunological means, we investigated two proteins: ClpP, a protein similar to the proteolytic subunit of the bacterial ATP-dependent Clp protease, for which a gene is found in the chloroplast genome [Maurizi, M. R., Clark, W. P., Kim, S. H. & Gottesman, S. (1990) J. Biol. Chem. 265, 12546-12552] and PrcA, a cyanobacterial Ca2+-stimulated protease [Maldener, I., Lockau, W., Cai, Y. & Wolk, P. (1991) Mol. & Gen. Genet. 225, 113-120]. We expressed the clpP gene from rice in Escherichia coli, purified its product, and generated antibodies against the product. Western blot analysis revealed the ClpP protein in different leaf extracts. Analysis of fractionated barley chloroplasts revealed that the protein was associated with the stromal fraction. The expression of ClpP is light independent and tissue specific, as it was found in green and etiolated barley leaves, but not in roots. A second protein, similar to the cyanobacterial protease PrcA, was also detected in chloroplasts. Antibody against this protease recognized proteins in various leaf extracts. When pea chloroplasts were fractionated, the antibody only recognized a stromal protein. The expression of this protein is regulated by light, as it was found in green leaves but not in etiolated leaves. The tissue specificity of PrcA was similar to that of ClpP in that it could not be detected in root extracts.

Keywords: chloroplast; Clp protease; protein degradation; proteolysis.

The degradation of chloroplast proteins has been demonstrated in numerous cases. The best-known example is the D1 protein of the photosystem II reaction center, which exhibits one of the highest rates of turnover in the chloroplast. Under high light intensity, the D1 protein is damaged and its rapid degradation is an essential step in the repair mechanism which allows its substitution by a newly synthesized protein (Mattoo et al., 1989). Rapid degradation also occurs when a multi-subunit complex lacks one of its protein components. In Chlamydomonas, when chloroplast protein synthesis, including that of the large subunit of rubisco, is inhibited, the imported nuclear-encoded small subunit is rapidly degraded (Schmidt and Mishkind, 1983). Rapid degradation of rubisco subunits has also been observed in tobacco mutant XV1 where a point mutation in the large subunit prevents its assembly to a holoenzyme (Avni et al., 1989). In a Lema mutant in which the translationally active levels of mRNA of the Rieske Fe-S protein were greatly reduced, the other subunits of the cytochrome b-f complex were expressed, but their products failed to accumulate due to increased degradation rates (Bruce and Malkin, 1991). The availability of prosthetic groups can also affect protein stability. When plants are grown in the dark, chlorophyll synthesis is arrested. In the absence of chlorophyll, newly synthesized light-harvesting chlorophyll-a/b-binding protein is unstable (Apel and Kloppstech, 1980; Bennett, 1981). Chlorophyll is also necessary for the stabilization of the newly synthesized chlorophyll apo-proteins D1 and CP43 of photosystem II (Mullet et al., 1990). Even ions can affect the degradation rate of a protein. In Chlamydomonas grown in a copper-deficient medium, the synthesis, transport and processing of pre-apoplastocyanin is unaffected. However, the mature plastocyanin is rapidly degraded (Merchant and Bogorad, 1986). In addition, it is widely accepted that leaf senescence is accompanied by a sharp increase in protein degradation within the chloroplast (reviewed by Dalling and Nettleton, 1986; Gepstein, 1988), which leads to loss of photosynthetic capacity.

Despite the multitude of protein degradation reactions in the chloroplast, very little is known about the mechanisms involved. With the exception of the D1 polypeptide of photosystem II, which has been reported to possess autoproteolytic activity (Misra et al., 1991; Shipton and Barber, 1991), the enzymes involved in the aforementioned reactions are not known. Furthermore, it is not clear what degree of overlap exists between the possible different proteolytic systems. ATP-dependent proteolysis has been demonstrated in isolated chloroplasts (Liu and Jagendorf, 1984; Malek et al., 1984) but a corresponding protease has not been isolated. However, there is good genetic evidence for the existence of at least one such protease. An ORF in the chloroplast genome (Shinozaki et al., 1986; Ohyama et al., 1986; Hiratsuka et al., 1989) shows high similarity to ClpP, the proteolytic subunit of the Escherichia coli Clp protease (Gray et al., 1990; Maurizi et al., 1990; Clarke et al., 1994). A protein similar to a regulatory subunit of Clp protease has also been found in plants. Two nuclear genes in tomato have been identified (Gottesman et al., 1990) that have a high degree of similarity to the bacterial gene and whose predicted amino acid sequences contain N-terminal extensions which have been sug-
gested to be involved in targeting these proteins to the chloroplast. A full-length cDNA clone of the regulatory subunit was targeted to the chloroplast stroma (Moore and Keegstra, 1993).

Because of the limited information on the identity of chloroplast proteases, we attempted to identify such proteins. The prokaryotic characteristics of chloroplasts and the identification of genes similar to an E. coli protease, suggested that components of the proteolytic machinery in chloroplasts may resemble those of prokaryotic organisms. We therefore immunoblotted plant leaf extracts with antibodies to identify potential chloroplast proteases. We describe here the immunological detection of ClpP, an ATP-dependent protease, and PrcA, an ATP-independent protease previously described in cyanobacteria (Lockau et al., 1988), in the chloroplasts of higher plants.

MATERIALS AND METHODS

Preparation of protein extracts, intact chloroplasts, stromal fractions and thylakoid fractions. Pea seedlings (Pisum sativum var. Alaska), barley seedlings (Hordeum vulgare), tomato plants (Lycopersicon esculentum) and Arabidopsis plants (Arabidopsis thaliana) were all grown under standard conditions, unless otherwise stated. Leaves or roots were frozen in liquid nitrogen, then finely ground with a mortar and pestle. The ground tissue was resuspended and homogenized in 50 mM Tris/HCl, pH 8.0, 5% (mass/vol.) glycerol, 0.1 M KCl, 10 mM EGTA, 0.5 mM dithiothreitol and 1.5% (mass/vol.) polyvinylpyrrolidone. Protein concentration was determined by means of the Bradford method (Bradford, 1976) and samples were prepared for SDS/PAGE by addition of 1 vol. solubilization buffer (Adam and Hoffmann, 1993). Intact chloroplasts were isolated on Percoll gradients as described (Cline, 1986). Stromal and thylakoid fractions were obtained by freezing and thawing intact chloroplasts, followed by a 10-min centrifugation at 10,000 g at 4°C. The resulting supernatant contained the stromal and envelope proteins and the pellet, which contained the thylakoid fraction, was resuspended to the supernatant volume in 50 m\(\text{M} \) Hepes/KOH, pH 8.0, 0.33\(\text{M} \) sorbitol. Both fractions were prepared for SDS/PAGE by addition of 1 vol. solubilization buffer (Adam and Hoffmann, 1993).

Expression of rice clpP in E. coli. The clpP gene from rice (Hiratsuka et al., 1989), generously donated by Prof. M. Sugiyara of Nagoya University, was subcloned into the XbaI and EcoRI restriction sites of pBluescript SK- (Stratagene). By means of a site-directed mutagenesis technique (Kunkel, 1985) an NdeI restriction site was introduced at the codon for the initiating Met. The gene was then digested with NdeI and EcoRI and ligated into the corresponding sites of the pBT7-7 expression vector. Competent E. coli cells, strain BL21(DE3), were transformed with this construct and expression of the gene was induced by addition of 0.5\(\text{mM} \) isopropyl-thio-\(\beta\)-d-galactoside. After 4 h, cells were collected by centrifugation, resuspended in 10\(\text{mM} \) Tris/HCl, pH 8.0, 120\(\text{mM} \) NaCl, 1\(\text{mM} \) EDTA, and frozen. The cells were thawed, lysed by means of a sonicator and centrifuged at 8000 g for 15 min. The resulting supernatant that contained the expressed protein was resolved by stained SDS/PAGE (Schagger et al., 1988) and ClpP was electrophoretically eluted from the gel. To verify the identity of the purified protein, it was subjected to automated Edman degradation on an Applied Biosystems 476 sequencer and the resulting sequence was found to match that predicted from the nucleic acid sequence.

Antibody preparation and Western blot analysis. Purified ClpP protein was used to prepare polyclonal antibodies in rabbits by means of standard techniques (Harlow and Lane, 1988). The antibody preparation was further purified by a modification of a previously described method (Johnson et al., 1985). Purified ClpP (100\(\mu\)g) was resolved by SDS/PAGE and blotted onto a poly(vinylidene difluoride) membrane (Millipore). Antibodies (100\(\mu\)l, 150 mg/ml, in 20\(\text{mM} \) Tris/HCl, pH 7.5, 50\(\text{mM} \) NaCl, 20% (mass/vol.) fetal calf serum) were incubated with the membrane for 90 min at room temperature. The membrane was washed three times for 5 min in 20\(\text{mM} \) Tris/HCl, 50\(\text{mM} \) NaCl and the bound antibodies were released from the membrane by washing with the membrane with 3.5 ml 0.2\(\text{M} \) glycine, pH 2.5. The antibody solution was neutralized with 1.75\(\text{mL} \) 1\(\text{M} \) sodium phosphate, pH 9.0, 5% fetal calf serum. Prior to Western blot analysis, the neutralized antibody solution was diluted with 6 ml H\(\text{O}, 1\) ml fetal calf serum and 3 ml 20\(\text{mM} \) Tris/HCl, pH 7.5, 50\(\text{mM} \) NaCl. Antibody against the cyanobacterial PrcA protein was generously donated by Prof. W. Lockau of Humboldt University, Berlin. Western blot analysis was carried out by means of conventional methods (Harlow and Lane, 1988). Proteins were resolved on 12% acrylamide gels, blotted onto nitrocellulose paper, incubated with the primary antibody (300\(\mu\)g/ml) overnight at 4°C, then incubated with a goat anti-rabbit IgG conjugated to alkaline phosphatase (20\(\mu\)g/ml). The immune complex was visualized by development of the blots with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Harlow and Lane, 1988).

RESULTS

Detection of ClpP in chloroplasts, Maurizi et al. (1990) detected peptides that reacted with an antibody raised against E. coli ClpP in spinach leaf extract. To investigate ClpP expression in chloroplasts, we generated an antibody against the plant clpP gene product. The clpP gene from rice (Hiratsuka et al., 1989) was expressed in E. coli cells and the resulting protein was purified by means of SDS/PAGE (see Materials and Methods). The identity of the purified protein was verified by N-terminal amino acid sequencing. We found that the initiating Met was processed in the bacterial cells, but the amino acid residues identified in the subsequent nine cycles of Edman degradation perfectly matched the sequence predicted from the gene (data not shown). The antibody raised in rabbits was further purified by adsorption to purified ClpP (see Materials and Methods) and used for Western blot analysis. Immunoblotting of higher-plant leaf extracts with these antibodies revealed a strong interaction with tomato and barley proteins of 27-29 kDa (Fig. 1). It is not clear whether the different intensities observed represent different levels of the protein or different cross-reactivities of the antibody with the proteins from different species. A
duplicate blot, incubated with pre-immune serum, showed no cross-reacting bands (data not shown). The strong interaction between the antibody and the barley protein prompted us to carry out further related experiments in barley. In all experiments described, results similar to those with barley extracts were observed with pea extracts.

The localization of the *clpP* gene to the chloroplast genome implies that, if expressed, its product will be localized in the chloroplast. When isolated barley chloroplasts were fractionated and the proteins resolved on gels, blotted and probed with the anti-ClpP IgG, the cross-reacting protein was found in the stroma (Fig. 2), consistent with the hydrophilic nature of ClpP in *E. coli*. Occasionally, variable amounts of ClpP associated with thylakoids were also detected (data not shown), but these could be removed by treatment of the thylakoids with 0.1 M NaOH which releases stromal proteins that adsorb to thylakoid membranes non-specifically during fractionation (Huang et al., 1992).

The expression of ClpP protein appeared to be independent of either the developmental stage of the plastid or of light. Western blot analysis of protein extracts from barley leaves, germinated either in the light or in the dark, revealed ClpP in both extracts (Fig. 3). Thus, ClpP protein is present in plastids of green and etiolated leaves, and its accumulation in leaves does not require light. Accumulation of ClpP in leaves in the dark suggested that it may also be expressed in other tissues grown in the dark, such as roots. However, when roots of light-germinated barley seedlings were analyzed, we could not detect any cross-reacting polypeptides (Fig. 3). Similar results were observed in pea seedlings, while shoots showed an intermediate level of expression (data not shown), which suggests that ClpP protein is expressed in a tissue-specific manner. This pattern of expression was also observed at the mRNA level. Northern blot analysis revealed similar levels of ClpP transcripts in both green and etiolated pea leaves, but not in roots (Ostersetzer, O. and Adam, Z., unpublished results).

We attempted to further characterize the cross-reacting protein in barley chloroplasts by immunoprecipitation of the protein. However, our antibodies failed to precipitate any protein from barley stroma and were unable to interact with any protein in ELISA. Thus, it seems that while this antibody preparation can efficiently interact with denatured ClpP, it is incapable of recognizing the protein in its native form. This behavior can probably be attributed to the finding that over-expressed ClpP is found in an aggregated, inactive form that cannot be renatured (Shanklin, J. personal communication).

**Detection of PrcA in chloroplasts.** The observation that chloroplasts contain ClpP, a protein similar to a bacterial protease, increased the possibility that protein similar to other bacterial proteases exist in chloroplasts. To investigate the presence of other proteases, we probed plant leaf extracts with an antibody raised against a cyanobacterial Ca$^{2+}$-stimulated protease, PrcA (Lockau et al., 1988; Maldener et al., 1991). Proteins extracted from either pea, tomato, barley or *Arabidopsis* leaves were all recognized by the anti-PrcA IgG (Fig. 4). The interacting proteins had molecular masses in the range of 43-49 kDa, which is close to the reported size of the cyanobacterial protein (52 kDa). With pre-immune serum, we could not detect any cross-reacting proteins (data not shown). When isolated pea chloroplasts were fractionated and analyzed, all the protein was found associated with the stromal fraction (Fig. 5). The stromal location of PrcA is consistent with the hydrophilic characteristics of the cyanobacterial protein (Lockau et al., 1988; Maldener et al., 1991). In addition to a sharp band at 45 kDa, we occasionally observed a diffuse band at 63 kDa, which was not observed in total leaf extracts. The nature of this band is not known, but a high-molecular-mass band of the pre-protein was also observed occasionally in cyanobacteria (Lockau, W., personal communication). Further analysis of PrcA revealed that, unlike ClpP, its
accumulation is regulated by light. PrcA was found in leaves of pea seedlings germinated in the light, but it could not be detected in leaves of etiolated seedlings (Fig. 6). PrcA was also absent from roots of light-germinated seedlings (Fig. 6). The light-dependency of PrcA expression was not limited to pea: accumulation of the protein was also observed in leaves of barley seedlings germinated in the light, but not in etiolated leaves (data not shown).

**DISCUSSION**

Although a number of chloroplast proteases have been purified to a considerable degree (Liu and Jagendorf, 1986; Kuwabara and Hashimoto, 1990; Kuwabara, 1992; Boshnell et al., 1993; Casano et al., 1994), their genes have not been cloned. Moreover, links between most of these reported activities and chloroplast-specific protein-degradation events have not been established. To identify additional chloroplast proteases, we reasoned that since chloroplasts have prokaryotic characteristics, chloroplast proteases may resemble bacterial proteases. We therefore analyzed chloroplast proteins for the potential existence of proteins similar to known prokaryotic proteases. Results presented in this work indicate that chloroplasts contain a protein similar to a cyanobacterial Ca2+-stimulated protease (Lockau et al., 1988) and a protein similar to the bacterial ClpP gene product. The clpP gene is found in the chloroplast genome (Ohyama et al., 1986; Shimozaki et al., 1986; Hiratsuka et al., 1989; Gray et al., 1990; Maurizi et al., 1990; Clarke et al., 1994) and is expressed, and the corresponding protein accumulates. Both proteases are expressed in leaves and not in roots, but their expression is differentially affected by light. While ClpP is expressed in both green and etiolated leaves, the PrcA protein is found only in green leaves.

The roles that these two proteases play in chloroplast biology is unclear. Although the cyanobacterial Ca2+-stimulated protease has been studied previously (Lockau et al., 1988; Maldener et al., 1991), no specific function could be assigned to it. Disruption of the prcA gene in cyanobacteria did not result in a distinguishable phenotype. Thus, Prca may function in cyanobacteria as a general-purpose protease, the loss of which can be compensated for by other proteases. ATP-dependent degradation of newly synthesized proteins in chloroplasts has been previously demonstrated (Liu and Jagendorf, 1984; Malek et al., 1984) but the proteases involved have not been identified. Thus, chloroplast Clp protease could be involved in this process. We were recently able to detect ATP-stimulated degradation of a mis-targeted protein in the chloroplast stroma (Halperin and Adam, 1996). We are currently investigating the possible involvement of Clp protease in this process.

In bacteria, protein degradation by ClpP is dependent on its cooperative interaction with a regulatory subunit that is capable of binding and hydrolyzing ATP (Katayama-Fujimura et al., 1987; Hwang et al., 1988; Maurizi, 1991). A protein similar to a regulatory subunit of Clp protease has been identified in the nuclear genome of plants (Gottesman et al., 1990; Moore and Keegstra, 1993). Recently, this gene product was shown to enter the chloroplast in a manner typical of nuclear-encoded chloroplast proteins (Moore and Keegstra, 1993). Additional support for a chloroplastic location of this gene product comes from the finding that cDNA clones from pea (Moore and Keegstra, 1993) and Arabidopsis (Franklin, A. and Hoffman, N., personal communication) were identified with different preparations of antibodies raised against chloroplast proteins. Together with our demonstration that ClpP protein accumulates in chloroplasts, these results provide evidence for the existence of the two protein components of Clp protease in chloroplasts of higher plants.

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