Volume 166, number 2 FEBS 1147 January 1984

Newly synthesized proteins are degraded by an ATP-stimulated proteolytic process in isolated pea chloroplasts

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Received 22 November 1983

Up to 20% of the [^{3}H]leucine-labeled proteins synthesized by isolated chloroplasts in the light was degraded during subsequent incubation for 20–40 min. The degradation of these radioactive proteins was more rapid in the light than in the dark and was at least 2-fold greater in the presence of 5 mM ATP in light or darkness. Exogenous amino acids did not influence degradation rates, although they promoted protein synthesis. Overall, proteins from thylakoid and stromal fractions were degraded at comparable rates. Analysis by electrophoresis in denaturing polyacrylamide gels revealed that many proteins decreased in both fractions. Certain low molecular mass stromal proteins were lost almost completely during a 90 min incubation in the presence of ATP, while others were unaffected or decreased only slightly. Thus chloroplasts, like eukaryotic and prokaryotic cells and mitochondria, contain an ATP-stimulated proteolytic system.

Protein degradation *ATP-stimulation* *Proteolytic system* *Pea chloroplast*

1. INTRODUCTION

Plant proteins are degraded continuously and at distinct rates [11]. The stability of proteins in chloroplasts has not been extensively studied, although there is evidence that different proteins within this organelle can turn over at different rates. For example, it has been shown [2] that the apoprotein of the light harvesting chlorophyll–protein complex can be rapidly degraded. In *Chlamydomonas reinhardtii*, nuclear gene-encoded subunits of ribulosebiphosphate carboxylase/oxygenase are rapidly degraded, if the chloroplast-derived subunits are not produced [3]. In addition, the 32 kDa thylakoid protein product of chloroplast Photogene 32 is produced from a 34.5 kDa precursor [4], by an as yet unidentified endoprotease. There are no published studies concerning proteases in the chloroplast [1], although proteolytic activity has been reported in etioplasts [5].

Protein degradation in animal cells occurs both in the lysosome (which appears analogous to the plant or yeast vacuole) and in the cytosol by an ATP-dependent degradative system [6]. One important function of this latter process is the selective degradation of abnormal proteins, which may arise by biosynthetic errors, mutation, or post-synthetic damage. A similar ATP-dependent degradative pathway exists in bacteria [7] and has been demonstrated recently in mammalian mitochondria [8]. The matrix of liver mitochondria contains an ATP-requiring, vanadate-sensitive proteolytic enzyme very similar to that in *E. coli* [9]. One function of the mitochondrial protease is to hydrolyze newly synthesized mitochondrial proteins that may be in some way defective or produced in the absence of subunits encoded in the nucleus [8].

The present studies have tested whether chloroplasts from higher plants also contain an ATP-requiring system for the rapid degradation of newly synthesized polypeptides. Such a pathway may be related to the degradatve systems found in bacteria and mitochondria and may serve similar functions. Evidence is presented here, and in [10],

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001457593/84/$3.00

that chloroplasts contain an ATP-stimulated proteolytic system that can hydrolyze newly synthesized chloroplast proteins. Preliminary reports of this [11] and a similar activity in *Chlamydomonas* [12] have appeared.

2. MATERIALS AND METHODS

2.1. *Isolation of intact chloroplasts*

Chloroplasts were isolated from 7-day-old pea shoots (*Pisum sativum*, L., cv. Progress no. 9, Burpee, Warminster, PA) grown at 26 ± 1°C in 12 h photoperiod (500 lx, cool white fluorescent tubes). Plastids were purified by centrifugation on Percoll gradients as in [13] with the following modifications: glutathione was not included in the gradients and the dithiothreitol concentration in the resuspension medium was 1 mM.

2.2. *Incubation for protein synthesis degradation*

Isolated chloroplasts were resuspended in 50 mM Hepes-KOH (pH 8.3) buffer containing 375 nM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 1 mM dithiothreitol to a chlorophyll concentration of 3 mg/ml. Chloroplast proteins were labeled in the light (2000 lx, from two BBA photoflood tungsten bulbs) at 26°C as in [13]. Chloroplasts (15 μg chlorophyll equivalent) were incubated with continuous shaking in the above buffer containing 5 μCi [^{3}H]leucine (NEN, 141 μCi/nmol). Incorporation of [^{3}H]leucine was interrupted by the addition of 5 μl of 30 mM leucine (to give a final concentration of 1 mM). ATP (5 mM, pH 7) and a protein amino acid mixture (0.1 mM) lacking leucine were included in some experiments, as indicated in the figure legends. Reactions were stopped by the addition of 150 μl of 10 mM sodium pyrophosphate (pH 7.4) at 4°C. Aliquots of 50 μl were spotted onto Whatman 3MM paper disks and washed as in [14]. Tritium counting efficiency of 10% was determined by two independent methods and was used in the calculation of dpm.

2.3. *Electrophoresis and fluorography*

Thylakoids samples for fluorography were obtained by washing the labeled chloroplasts (60 μg chlorophyll equivalent) 3 times with cold 10 mM sodium pyrophosphate (pH 7.4). The washed thylakoids were resuspended in 400 μl of sodium dodecyl sulfate buffer [62.5 mM Tris-HCl (pH 6.8), 2.3 (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol]. The stroma proteins were precipitated from the combined pyrophosphate washes with acetone at −20°C. Dry precipitates were dissolved in 400 μl of the above SDS buffer. Polyacrylamide gel electrophoresis was performed at 1 mA constant current for 4 h. The buffer system in [14] was used, with 4.5% stacking and 12.5% resolving gels. The gel was washed in 50% methanol–12% acetic acid for 1–2 h and stained overnight in 0.2% Coomassie brilliant blue R 250 dissolved in the above solvents. Destaining was for at least 12 h in 3 washes of 10% ethanol, 5% acetic acid. Prior to drying on 3MM paper, the gels were treated for 30 min with Enlightning (NEN) according to instructions. Dried gels were exposed to Kodak XAR-5 film for at least 7 days at −80°C.

3. RESULTS AND DISCUSSION

High rates of light-driven incorporation of [^{3}H]leucine into chloroplast protein by isolated organelles were observed (fig.1). This process was nearly linear for about 15 min without added amino acids (fig.1b) and for about 40 min when amino acids were included in the incubation medium (fig.1a). The addition of 5 mM ATP greatly decreased the light-driven incorporation (fig.1c). Similar effects of exogenous amino acids and ATP on light-driven incorporation of labeled amino acids have been reported in [16].

The following 'pulse-chase' experiments were designed to determine whether there is an energy-requiring degradative process in plastids, that may limit net incorporation in the light. Light-driven [^{3}H]leucine incorporation was arrested after 15 min by the addition of 5 μl of 30 mM non-radioactive leucine (1 mM final concentration). The addition of D-chloramphenicol (0.4 mM final concentration) was equally effective in stopping light-driven incorporation, although this treatment was not used routinely due to a slight inhibition of protein degradation (not shown). Protein degradation was measured by determining the amount of radioactivity remaining in the chloroplast proteins during subsequent 15–90-min incubations.

Up to 25% of the radioactive protein was degraded in the subsequent 40 min chase in the light and 19% was degraded in the dark (fig.2).

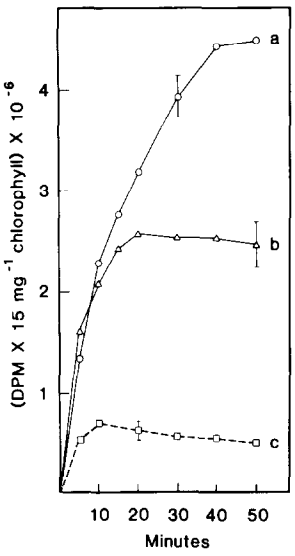

Fig. 1. The time course of light-driven incorporation of [^{3}H]leucine into protein by isolated chloroplasts. Incubation for curve (b) ((▲,△) was as described in section 2. The following additions were made in experiments (a and c): a mixture of 0.1 mM amino acids lacking leucine was included in experiments (a) (●,○) and (c) (■,□). ATP at 5 mM was included in (c) (dashed line). Each point is an average of at least two experiments with duplicates. Only the maximum SE is shown for each line.

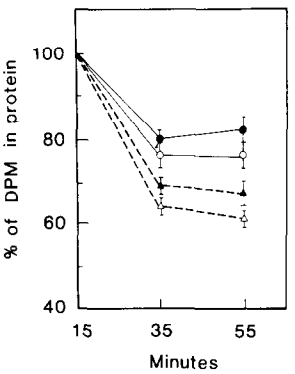

Fig. 2. Effect of light and exogenous ATP on the degradation of radioactive chloroplast protein. Chloroplast proteins were radioactively labeled for 15 min in the light with [^{3}H]leucine (without additional exogenous amino acids to an average of 1 860 000 dpm/15 μg chlorophyll = 100%). Non-radioactive leucine (1 mM final concentration) was added and the mixtures incubated further in the light (○,△) or in the dark (●,▲). ATP (5 mM final concentration) was added with leucine (▲,△) or was not included (——,○,●). Each point is an average of duplicates from 3 experiments ± SE.

The addition of 5 mM ATP resulted in at least a doubling of the degradation rates (fig. 2,3). This stimulation of degradation by exogenous ATP was similar in both the light and darkness (fig.2). The stimulation of proteolysis by ATP was measured only in the presence of Mg^{2+}. In most experiments 1 mM $MgCl_2$ was employed (5 mM having no additional effect); 5 mM ATP was ineffective in stimulating proteolysis with 0 or 0.1 mM $MgCl_2$ (not shown). Comparable ATP-stimulated protein degradation has been observed independently by authors in [10], although they detected less degradation in the dark than we did (fig.2). This difference may be due to our use of a lower $MgCl_2$ concentration (1 mM), or to slight differences in the development of the leaves from which chloroplasts were isolated. We could not detect an ATP-stimulated proteolytic activity in young seedlings which had not undergone expansion of the first leaf.

Animal and bacterial cells, as well as isolated mitochondria, selectively degrade abnormal proteins and this process appears to be ATP-dependent [6-9]. Provinding the chloroplasts with an exogenous source of amino acids might be expected to improve the metabolic state of the

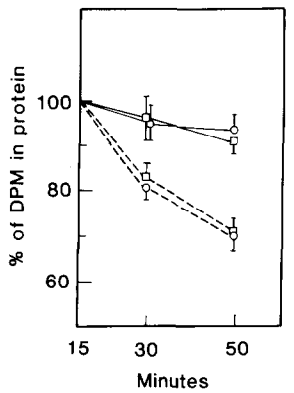

Fig. 3. Loss of radioactivity from proteins labeled in the presence or absence of an exogenously supplied mixture of amino acids. Chloroplast proteins were radioactively labeled for 15 min in the light without (○, 100% = 2 100 000 dpm/15 μg chlorophyll) or with (□, 100% = 2 440 000 dpm/15 μg chlorophyll) 0.1 mM mixture of protein amino acids lacking leucine. Non-radioactive leucine (1 mM final concentration) was added after 15 min without (——) or with ATP (– – –, 5 mM final concentration). Each point is an average of duplicates from 2 experiments ± SE.

isolated organelle (fig.1) and possibly minimize mistranslation or premature termination due to amino acid deficiency. Even though exogenous amino acids extended the period of incorporation of [^{3}H]leucine (fig.1a), no differences were measured in the rates of degradation of proteins labeled in the absence or presence of 0.1 mM amino acid mixture (fig.3). In other experiments, we attempted to induce production of 'defective' proteins by radioactively labeling chloroplast proteins in the presence of a valine analog, aminochlorobutyric acid (0.1 mM) (fig.4) or the arginine analog canavanine (25 μg/ml). However, proteins synthesized in the presence of these analogs were degraded in 5 mM ATP at the same rate as control proteins (fig.4, data for canavanine not shown). Similarly, attempts to induce production of incomplete polypeptides by labeling proteins for 15 min in 2 μg/ml puromycin, which inhibited incorporation by 30%, did not cause a significant increase in degradation in the presence of ATP (not shown). It should be stressed, however, that we have not demonstrated that defective proteins were in fact formed under these conditions. Thus, these experiments do not exclude the possibility that defective proteins are preferential substrates of the chloroplast ATP-stimulated system. For example, plant cells contain high levels of non-protein amino acids, and may have evolved efficient editing mechanisms to prevent incorporation of such analogs into proteins. Alternatively, the majority of proteins synthesized in organello may be in an abnormal conformation already, or may be present in a precursor form (e.g., [4]).

The nature of radioactive proteins degraded in the presence of ATP was examined by separating the stromal and thylakoid proteins by denaturing polyacrylamide gel electrophoresis and fluorography (fig.5). In both the stromal and thylakoid fractions an equal decrease in radioactive protein was detected (35% decrease in each fraction,

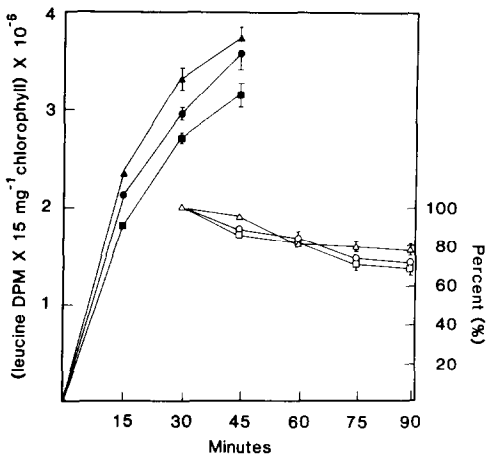

Fig. 4. Loss of radioactivity from proteins labeled in the presence of aminochlorobutyric acid, a valine analog. Chloroplast proteins were radioactively labeled (▲,■,●) in the light with added 0.1 mM mixture of protein amino acids minus leucine (△,▲), the same mixture lacking valine (□,■) and a mixture in which valine was replaced by 0.1 mM aminochlorobutyric acid (○,●). After 30 min incorporation, unlabeled leucine (1 mM final concentration) and ATP (5 mM final concentration) were added and radioactivity remaining was determined (△,□,○). Each point is an average of duplicates from 3 experiments ± SE.

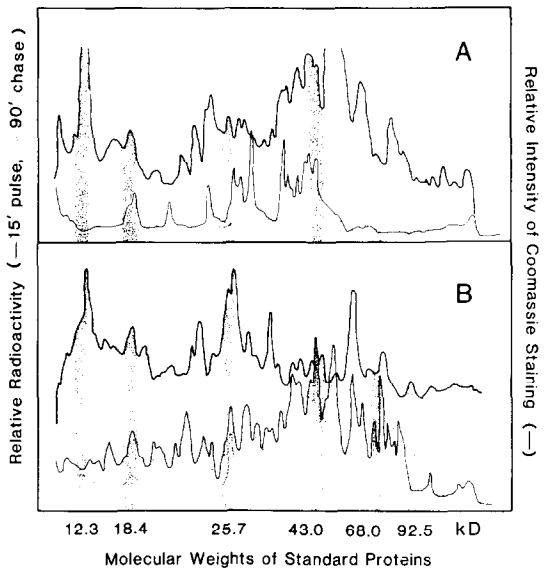

Fig. 5. Polyacrylamide gel analysis of the stability of labeled stromal (A) and thylakoid (B) proteins. Shown are densitometric scans of photographs of the stained gel (top solid line) and of the fluorographs of proteins labeled for 15 min (bottom solid line) and incubated in the light for an additional 90 min in the presence of 5 mM ATP and 1 mM leucine (dotted line). The traces of fluorographs of proteins incubated only in 1 mM leucine without ATP were comparable to the 15 min labeled control and are not presented. Shaded areas indicate the migration of stained and radioactive proteins of known molecular mass (in kDa). Similar observations were made on samples from 3 independent experiments.

following incubation in 5 mM ATP). Some unidentified low molecular mass proteins were extensively degraded in the stromal fraction in the presence of ATP (fig.5a) and, in contrast, some thylakoid proteins of various sizes were relatively unaffected (fig.5b). However, most stromal and thylakoid proteins were degraded to some extent. Thus a large number of chloroplast proteins, in addition to the nuclear-coded light-harvesting chlorophyll–protein complex [2] and the small subunit of ribulosebisphosphate carboxylase/oxygenase [3], may be susceptible to proteolysis in the chloroplast. Organizational and functional changes can be induced in the thylakoid membranes by exogenous ATP [17]. It remains to be investigated whether proteolytic steps are involved in these events.

Related studies with disrupted chloroplasts indicate that the high-energy bond of ATP is utilized by the plastid proteolytic system. For example, a non-hydrolysable ATP analog does not stimulate proteolysis, while nucleotide triphosphates other than ATP have only a slight effect. Experiments are underway, to characterize this proteolytic system and to elucidate its possible role in chloroplast development and function.

ACKNOWLEDGEMENTS

This research was supported in part by grants to L.B. from the National Science Foundation and from the Competitive Grants Office of the US Department of Agriculture. It was also supported in part by the Maria Moors Cabot Foundation of Harvard University. L.M. was a Fellow of the Natural Sciences and Engineering Research Council of Canada.

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