ATP-dependent proteolysis in pea chloroplasts

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Proteins newly formed from labeled amino acids by isolated intact pea chloroplasts are not entirely stable. Between 20 and 35% of the labeled protein is degraded over a 20–30 min incubation period in pulse-chase experiments. Protein degradation is prevented when the chloroplast ATP level drops, as in the dark without added ATP. Degradation is stimulated by adding ATP directly or by generating it in photophosphorylation. Susceptible new proteins are not stabilized against further additions of ATP, during incubation under ATP-deficient conditions.

ATP-dependent protease *Chloroplast* *Protein regulation* *Chloroplast biogenesis* *Protein synthesis*

1. INTRODUCTION

Intracellular protein hydrolysis has been studied extensively in animal cells [1]. Two major proteolytic mechanisms have been characterized. One is the lysosome system, studied most intensively for liver cells. The analogous system in plant cells is probably represented by proteases in vacuoles [2]. The second is ATP-dependent proteases, examined primarily in the cell-free activity found in lysed reticulocytes. A protease dependent on ATP has also been studied in great detail in *Escherichia coli* [1]. The ubiquity of this system was suggested by its recent discovery in liver mitochondria [3].

The proteases of chloroplasts have not been investigated in any detail, and no ATP-dependent proteolysis was reported before this. These organelles synthesize many of their own proteins [4] and rapid rates were recently measured in both light-driven [5] and in Mg-ATP driven [6] translation. Peptide hydrolysis in chloroplasts must occur, however, in several functions. Processing proteases that remove the transit peptide from newly imported precursor proteins have been detected [7] but not yet isolated or studied in detail. Instability of the apoprotein of the light harvesting chlorophyll-protein complex in the absence of chlorophylls *a* and *b* was ascribed to a chloroplast protease [8] but the specific enzyme has not yet been detected directly, or studied. In *Chlamydomonas reinhardtii* the imported small subunit of ribulosebisphosphate carboxylase is also reported to be destroyed by proteolysis when not enough large subunit is present to permit assembly [9]. More recently, the rapid turnover of the 32 kDa atrazine binding protein [10] has been ascribed to proteolysis of oxidatively damaged molecules (Kyle and Arntzen, personal communication). Proteolysis of phosphorylase from spinach leaves, dependent on added ATP, was discovered recently [11], but its subcellular localization has not yet been investigated.

The current work started with the observation of apparent instability of some of the newly synthesized proteins made by isolated intact pea chloroplasts [6]. We have extended these observations, and find that destruction of some of the newly made proteins in isolated pea chloroplasts depends on the ATP status of the organelle. The same finding was made simultaneously and independently, as detailed in [12].

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2. MATERIALS AND METHODS

Pea plants (Progress No. 9, Agway) were grown for 8 days in a growth chamber as in [5]. The plants were kept for 16 h in darkness, then illuminated for 30 min prior to harvest. Homogenization, and collection of intact chloroplasts were described earlier. Chloroplasts were incubated at 0.1 mg chlorophyll/ml at 27°C in the dark with Mg-ATP, or in the light, in a medium containing 32 mM Hepes-KOH (pH 8.3), 350 mM sorbitol, 0.8 mM dithiothreitol, 0.2 mM $MgCl_2$ and a radioactive amino acid. Measurement of chlorophyll, and of incorporation of the amino acid into protein, were as in [5,6]. When radioactive proteins of the thylakoids and of the soluble stroma components were measured separately, the intact chloroplasts at the end of the incubation were lysed in a hypotonic buffer (10 mM Na pyrophosphate, pH 7.5), and the membranes washed 4 times with the same hypotonic buffer.

The thylakoids and soluble proteins (supernatant from the initial breakage) were applied to separate wells of a 10–16% acrylamide gradient gel with SDS. Electrophoresis was performed at room temperature with a constant 100 V. The gels were dried, and fluorographed with the aid of 'Enhance' (New England Nuclear).

Radioactive chemicals used included [^{3}H]leucine (50 Ci/mmol), [^{3}H]lysine (45 Ci/mmol), [^{3}H]glycine (7 Ci/mmol) and [^{35}S]methionine (about 1450 Ci/mmol). ^{3}H-Labeled chemicals were from ICN, and [^{35}S]methionine from Amersham.

3 .RESULTS AND DISCUSSION

Pulse-chase studies were performed with isolated intact chloroplasts, to observe the extent of degradation of newly made proteins. Translation was allowed to occur using [^{3}H]leucine at 0.2 mM and 410 μCi/μmol, for an initial period of 5–10 min. The reaction was then diluted 100-fold with unlabeled leucine, by adding 20 μmol/ml; thus continuing translation would not show up as significant additional cpm in the protein fraction. Control experiments showed that adding this much leucine did not inhibit continuing incorporation of [^{3}H]lysine (not shown).

Both in the dark with added Mg-ATP at 10 mM (fig.1) and in light-driven protein synthesis (fig.2) the newly synthesized, radioactive protein, was seen to be degraded once the cold leucine was added. To test the possibility that degradation was due to the addition of excessive amounts of non-radioactive leucine, protein synthesis was also stopped by adding either chloramphenicol or EDTA. In both cases a similar extent of degradation was found (not shown). The loss of protein radioactivity is slow, continuing for 20–120 min after addition of unlabeled leucine. However the total amount lost seemed to come to an end-point, with 20–35% of the original total protein radioactivity degraded. The extent of proteolysis seemed to be similar at every point along the time-course for leucine incorporation, in the light or in the dark (fig.1, 2). In other experiments, using 6 mM ATP, 15, 17 and 31% degradation of newly labeled proteins was found using [^{3}H]lysine, [^{3}H]glycine and [^{35}S]methionine, respectively, during 80-min chase periods.

It was considered possible that the protein loss might represent to a large extent oxidatively damaged 32-kDa atrazine-binding protein [10], which turns over very rapidly in the light. If so, the degradation might be prevented by the electron

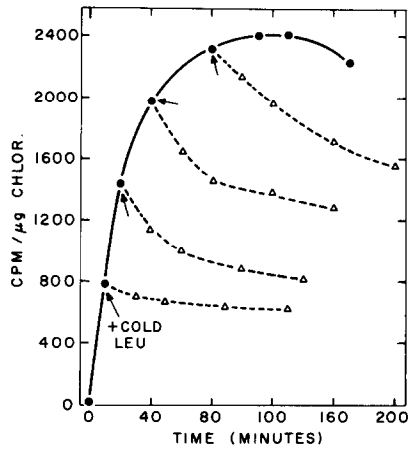

Fig. 1. Loss of newly synthesized protein during Mg-ATP-driven protein synthesis by pea chloroplasts in the dark. The arrows point to the time at which [^{3}H]leucine present in the medium was diluted by addition of a 100-fold excess of unlabelled leucine. Duplicate aliquots were taken at later time points as shown on the graph. ATP was present at 10 mM; $MgCl_2$ at 11.5 mM.

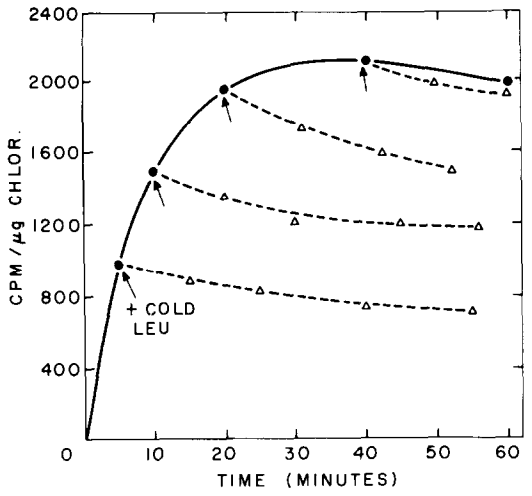

Fig. 2. Loss of newly synthesized protein during light-driven translation by chloroplasts. Conditions as in fig. 1, except that the $MgCl_2$ concentration was 0.2 mM and no ATP was added.

transport inhibitor, dichlorophenyldimethylurea (DCMU), which would prevent half-reduced quinones, capable of interacting with O_2, from forming. The addition of DCMU did indeed prevent degradation of newly formed protein (table 1). However, DCMU may inhibit cyclic as well as non-cyclic electron flow under oxidative conditions [13], and thus could be preventing any continuing light-driven ATP synthesis. Cyclic electron flow can be restored by adding phenazine methosulfate and using white light [13]. This reagent did indeed reverse a large part of the protection by DCMU (table 1), and the protection was completely reversed by adding ATP, which readily penetrates young pea chloroplasts [14,15]. Thus the protection given by DCMU appears to be a matter of inhibiting ATP formation dependent on light-driven electron transport; and the ATP level seems to be a more direct factor than the electron transport process.

This is further confirmed by the results shown in table 2. Intact chloroplasts were incubated with [^{3}H]leucine in the light for 15 min, cold leucine was added and degradation was followed at different levels of energy supply. It can be seen that very little degradation occurred in darkness; twice as much under low light intensity (170 μEinstein/m^2 per s) and 4 times as much at high light intensity (900 μeinstein/m^2 per s). In the dark, even more extensive proteolysis occurred by 60 min if Mg-ATP (10 mM) was added; and the greatest protein loss occurred with 5 mM Mg-ATP added on top of strong light.

Table 1

DCMU protection of newly made protein, and reversal by ATP or cyclic electron flow

Treatment	Degradation of newly synthesized protein (%)
Control	27.5
5 μM DCMU	18.9
10 μM DCMU	10.6
20 μM DCMU	8.5
20 μM DCMU + 10 mM Mg-ATP	34.4
20 μM DCMU + 50 μM PMS	20.2
20 μM DCMU + 100 μM PMS	22.1
20 μM DCMU + 200 μM PMS	19.7
20 μM DCMU + 500 μM PMS	14.4

Intact chloroplasts were incubated in the light with [^{3}H]-leucine for 15 min. A 100-fold excess of unlabeled leucine was added, and the system was divided into separate aliquots with additions of DCMU, Mg-ATP or phenazine methosulfate (PMS) as shown. The chloroplasts were incubated for 60 min more in the light, then assayed for radioactive protein. The labeling at 15 min was 1520 cpm/μg chlorophyll

The time-course for loss of radioactive protein

Table 2

Dependence of proteolysis on ATP levels

Treatment	Degradation of newly synthesized protein (%)
Dark	5.7
Low light	11.0
Bright light	23.0
Dark + 5 mM Mg-ATP	29.0
Bright light + 5 mM Mg-ATP	34.0

Conditions and procedure as in table 1. The [^{3}H]leucine incorporation came to 1700 cpm/mg chlorophyll prior to the addition of unlabeled leucine

in darkness, without added ATP is, shown in fig. 3 (solid line). A small extent of degradation occurs in the first 10–15 min; subsequently, in darkness the new protein appears to be quite stable. However, on adding ATP at any point, proteolysis starts immediately, coming to an end-point after about 30 min, with 25% of the initial protein degraded. This experiment shows that the same fraction of the newly formed protein remains in an unstable form during incubation in darkness without ATP.

The newly formed protein sensitive to ATP-dependent proteolysis may represent an intermediate pool which has not gone through a stabilization step. If so, the stabilization process(es) may also require ATP, and so cannot proceed under the conditions shown in fig. 3. Alternatively the sensitive proteins may be ones that are intrinsically imperfect and so always susceptible to proteolysis. They might also be components of large complexes (in which they would be stable) which cannot be formed under these conditions due to, for example, the absence of cytoplasmically synthesized components.

Both stroma and thylakoid proteins were lost during incubation with ATP. For instance, in one experiment in which these classes of proteins were separated prior to counting, 35% was lost from the stroma components, and 26% from the thylakoids, during an 80-min incubation period in light and with the presence of 5 mM ATP.

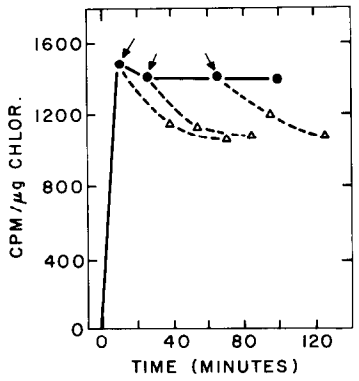

Fig. 3. Stability of newly formed protein in the dark until ATP is added. Intact chloroplasts were incubated in the light with [^{3}H]leucine for 10 min, then the light was turned off and a 200-fold excess of unlabeled leucine added. At different times, as shown, an aliquot was removed, ATP (5 mM) and $MgCl_2$ (5.7 mM) were added, and counts in total protein were followed in duplicate aliquots at the times shown.

Possible selectivity of degraded proteins was examined by fluorography of labeled proteins, spread out on SDS-polyacrylamide gradient gels. Decrease of radioactivity was seen with both stroma and thylakoid proteins. There were no specific protein bands that seemed to be completely degraded during the proteolytic period, and also none that seemed completely immune. There appeared to be differences in the extent of degradation of some of the polypeptides compared to others, but it has not yet been possible to put these differences on a quantitative basis. Some more specific differences were noted in [12].

A question of primary interest is whether the ATP-dependent protease in chloroplasts will resemble the prokaryotic or eukaryotic enzyme, since their mechanisms of action are probably quite different [1]. As with whole cells, the true physiological function of the enzyme remains to be determined. Since it has been detected in terms of destroying newly formed proteins, its function might be to remove polypeptides that are improperly formed, or incompletely assembled. Further work is needed to assess these or other possibilities.

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REFERENCES

[1] Hershko, A. and Ciechanover, A. (1982) Annu. Rev. Biochem. 51, 335–364.
[2] Matile, P. (1978) Annu. Rev. Plant Physiol. 29, 193–213.
[3] DeSautels, M. and Goldberg, A.L. (1982) Proc. Natl. Acad. Sci. USA 79, 1869–1873.
[4] Ellis, R.J. (1983) in: Subcellular Biochemistry (Roodyn, D.B. ed.) Vol. 9, pp. 237–261,Plenum, New York.
[5] Fish, L.E. and Jagendorf, A.T. (1982) Plant Physiol. 70, 1107–1114.
[6] Fish, L.E., Deshaies, R. and Jagendorf, A.T. (1984) Plant Sci. Lett., in press.

[7] Chua, N.-H. and Schmidt, G.W. (1979) J. Cell Biol. 81, 461–483.
[8] Bennett, J. (1981) Eur. J. Biochem. 118, 61–70.
[9] Schmidt, G.W. and Mishland, M.L. (1983) Proc. Natl. Acad. Sci. USA 80, 2632–2636.
[10] Edelman, M. and Reisfeld, A. (1978) in: Chloroplast Development (Akoyunoglou, G. and Argyroudi-Akoyunoglou, J.H. eds) pp. 641–652, Elsevier, Amsterdam, New York.
[11] Hammond, J.B.W. and Preiss, J. (1984) Plant Physiol., in press.
[12] Malek, K., Bogorad, L., Ayers, A. and Goldberg, A.L. (1984) FEBS Lett. 166, 253–257.
[13] Jagendorf, A.T. and Margulies, N.M. (1960) Arch. Biochem. Biophys. 90, 176–183.
[14] Robinson, S.P. and Wiskich, J.T. (1977) Plant Physiol. 59, 422–427.
[15] Stankovic, Z.S. and Walker, D.A. (1977) Plant Physiol. 59, 428–432.