Plant Physiol. (1980) 66, 1119–1122
0032-0889/80/66/1119/04/$00.50/0

Model for Stress-induced Protein Degradation in *Lemna minor*[1]

Received for publication December 17, 1979 and in revised form May 12, 1980

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ABSTRACT

Transfer of *Lemna minor* fronds to adverse or stress conditions produces a large increase in the rate of protein degradation. Cycloheximide partially inhibits stress-induced protein degradation and also partially inhibits the protein degradation which occurs in the absence of stress. The increased protein degradation does not appear to be due to an increase in activity of soluble proteolytic enzymes. Biochemical evidence indicates that stress, perhaps acting via hormones, affects the permeability of certain membranes, particularly the tonoplast. A general model for stress-induced protein degradation is presented in which changes in membrane properties allow vacuolar proteolytic enzymes increased access to cytoplasmic proteins.

Previous work (5) has shown that the transfer of fronds of *Lemna minor* to adverse or stress conditions leads to a reduction in protein synthesis, a large increase in protein degradation, and a corresponding loss of soluble protein. The adverse conditions used included starvation (growth on distilled H_2O), nitrogen deficiency (NO_3^- omitted from the growth medium), osmotic stress (growth medium made 0.5 M with respect to mannitol), and, in addition, the special case of isotopic stress (growth medium containing 50% v/v deuterium oxide) (6). The increased protein degradation induced by stress appears to be relatively unspecific, affecting many enzymes, although to varying degrees. Fronds maintained in adverse conditions adapt in such a way that protein synthesis is increased and the initially rapid rate of proteolysis is decreased. The stress-induced protein degradation can be envisaged as a strategy to degrade proteins and provide amino acids for the synthesis of an enzyme complement more suitable for growth in adverse conditions (8).

The work described here was undertaken to elucidate the mechanism of the stress-induced degradation.

MATERIALS AND METHODS

Plant Material and Growth Media. *Lemna minor* was maintained in sterile culture as described by Trewavas (23). The composition of the various stress media has been given previously (5, 6).

Estimation of Protein Degradation. First-order rate constants of degradation kd[3] and half-lives ($t_{1/2}$) of soluble proteins were determined by the tritiated water (^3H_2O) technique of Humphrey and Davies (18). In experiments concerning the effect of cycloheximide on protein degradation, kd and $t_{1/2}$ were also measured by labeling fronds with [^{14}C]leucine (3.8×10^4 Bq ml^{-1}) in complete growth medium for 2 days and observing the loss of ^{14}C from proteins following transfer of fronds to unlabeled medium containing 10 μM cycloheximide. Although this underestimates protein degradation due to recycling of the leucine (10), it is a useful comparative method.

Assay of Proteinase and Peptidase Activity. Proteolytic activity was measured by autodigestion of extracted *Lemna* protein. Fronds (1.0–2.0 g fresh weight) were frozen and ground in 50 mM Tris buffer (pH 8.0) containing 3.5% (w/v) NaCl and 8 mM mercaptoethanol. The extracts were clarified by centrifuging at 20,000*g* for 15 min, then desalted by passage through Sephadex G-25 (12.0 × 3.5 cm columns). Reaction mixtures consisted of 1.0 ml desalted extract and 6.5 ml either 25 mM Tris buffer (pH 7.1) or 25 mM citrate buffer (pH 5.2), both containing 8 mM mercaptoethanol. Mixtures were incubated at 37 C for 2 h, after which 1.5-ml aliquots were removed, protein was precipitated by the addition of 0.25 ml 40% (w/v) trichloroacetic acid, and the free amino acid content of the supernatants was measured by a ninhydrin reaction. Proteinase activity is expressed as μmol amino acid released/g fresh weight·h. These assays measure proteolytic activity of the soluble fraction. There may well be proteolytic activity associated with the insoluble fraction.

Peptidase activity was measured as the *p*-nitroaniline released from leucine-*p*-nitroanilide or benzylarginine-*p*-nitroanilide (2). The assay mixture contained 0.5 ml desalted *Lemna* extract and 0.9 ml 25 mM Tris buffer (pH 7.1) to which was added 0.1 ml 3 mM leucine-*p*-nitroanilide or benzylarginine-*p*-nitroanilide (dissolved in dimethyl formamide). The *A* at 405 nm was measured initially and after 2 h incubation at 37 C. Incubation was terminated by the addition of 0.5 ml 30% (w/v) acetic acid. Peptidase activity is expressed as ΔA_{405}/g fresh weight·h.

In all experiments, total protein (as trichloroacetic acid-precipitable material) was determined by the method of Lowry *et al.* (20).

RESULTS AND DISCUSSION

Effect of Cycloheximide on Protein Degradation. The effect of 10 μM cycloheximide on protein degradation in complete growth medium and in medium lacking NO_3^- ($-NO_3^-$ medium) was investigated. Degradation was measured by two methods, as described under "Materials and Methods" and the results are given in Table I. The increase in kd induced by transfer of *Lemna* fronds from complete to $-NO_3^-$ medium is partially inhibited by cycloheximide, indicating that *de novo* protein synthesis may be necessary for enhanced degradation. Also, cycloheximide partially inhibits protein degradation under conditions of no stress (in complete medium), indicating either that some *de novo* protein synthesis is needed for normal proteolysis or that cycloheximide directly or indirectly inhibits proteolysis (15).

The effect of 10 μM cycloheximide on the acidic and neutral proteinase activity isolated from *Lemna* fronds growing in complete medium was examined. Proteolytic activity was measured as

[1] This research was supported by Grant AG 83/14 from the Agricultural Research Council, United Kingdom.

[3] Abbreviations: kd, rate constant of degradation; Bq, Becquerel.

Table I. *Effect of 10 μM Cycloheximide on Protein Degradation in L. minor Growing in Complete Medium (no Stress) or Subjected to Nitrate Deprivation ($-NO_3^-$ Medium)*

Degradation was estimated by two different techniques (10, 18).

Growth Medium	Method	kd	$T_{1/2}$
		day^{-1}	*days*
Complete	^3H_2O	−0.10	7.0
Complete + CHX[a]	^3H_2O	−0.06	11.6
Complete	[^{14}C]Leu	−0.07	10.5
Complete + CHX	[^{14}C]Leu	−0.05	13.6
$-NO_3^-$	^3H_2O	−0.25	2.8
$-NO_3^-$ + CHX	^3H_2O	−0.12	5.8
$-NO_3^-$	[^{14}C]Leu	−0.18	3.8
$-NO_3^-$ + CHX	[^{14}C]Leu	−0.13	5.3

[a] Cycloheximide.

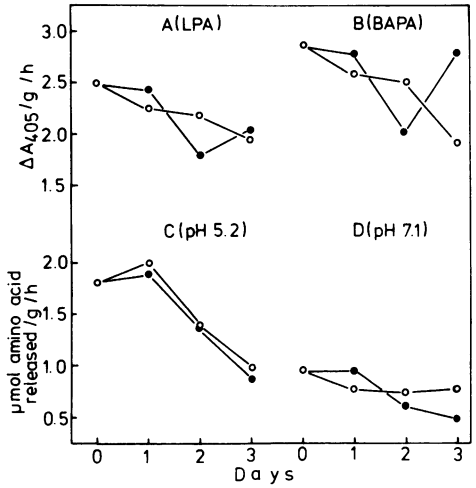

FIG. 1. The effect of stress on the peptidase activity against leucine-*p*-nitroanilide (LPA) (A) and benzylarginine-*p*-nitroanilide (BAPA) (B) and on the acidic (C) and neutral (D) proteinase activity of *Lemna minor*. Fronds were transferred to $-NO_3^-$ medium (○——○) or 0.5 M mannitol medium (●——●) and harvested daily, and the enzyme activities were measured.

described under "Materials and Methods" and the *in vitro* addition of cycloheximide was found to have no significant effect on the activity at pH 5.2 or 7.1 (data not presented). The inhibition of proteolysis by cycloheximide is thus not due to a direct effect on the soluble proteolytic enzymes.

Effect of Stress on Proteinase and Peptidase Activity. Perhaps the most obvious way in which stress could increase protein degradation is by stimulating the synthesis of proteolytic enzymes. Such a mechanism would be consistent with the observed inhibition by cycloheximide (Table I). Thus, proteinase and peptidase activity were measured in fronds transferred to $-NO_3^-$ medium or to a medium made 0.5 M with respect to mannitol. Samples were assayed for enzyme activity at daily intervals. The results (Fig. 1) show a slight (10–15%) increase in acidic proteinase activity, but this rise is transient. In general, the activities of the proteinases and peptidases decline following the transfer to adverse growth conditions, making it unlikely that the proteolytic enzymes are directly responsible for the increased protein degradation. Another mechanism must thus be sought.

Effect of Stress on Vacuolar System. A possible alternative explanation for stress-enhanced protein breakdown would be that stress increases the availability of proteins to the degradative apparatus. This would be analogous to the situation in animals where the increased intracellular protein catabolism induced by, for example, nutritional deprivation is due to an increase in lysosomal fragility and in the number of autophagic vacuoles (1, 12). Despite some evidence to the contrary (4), the vacuoles of plants can be equated with lysosomes (21, 27) and several proteolytic enzymes appear to be located in the vacuole (3). Furthermore, direct evidence for protein hydrolysis in plant vacuoles has been obtained (22). The possibility of stress-induced changes in the properties of the tonoplast in *Lemna* was investigated using a technique (Davies, D. D., Oliver, J. and Cooke, R. J., unpublished data) developed to measure the partitioning of amino acids between the vacuole and the cytosol.

When *Lemna* fronds are placed on a medium containing ^3H_2O, the soluble amino acids become rapidly labeled at the C-2 position, due to exchange reactions catalyzed by transaminases (9, 16). If labeled fronds are transferred back to unlabeled medium, the reverse reactions occur and 2-^{3}H is rapidly lost. The kinetics of the 2-^{3}H loss from soluble amino acids is shown in Fig. 2. Although other explanations are possible, such kinetics is consistent with a

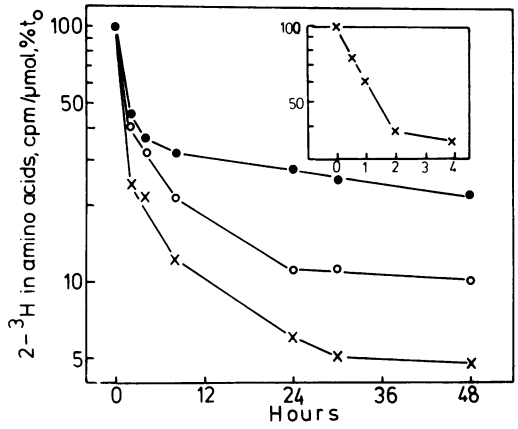

FIG. 2. The loss of 2-^{3}H from the soluble amino acids of *Lemna minor* exposed to ^3H_2O for different lengths of time. Fronds were grown on complete medium containing ^3H_2O (3.8×10^7 Bq ml^{-1}) for 6 h (×——×), 24 h (○——○), or 48 h (●——●), washed, and transferred to unlabeled medium (t_0). Samples were removed at intervals, the soluble amino acids were isolated (17), and the 2-^{3}H content was measured (18). Inset shows in greater detail the loss of 2-^{3}H occurring over the first 4 h.

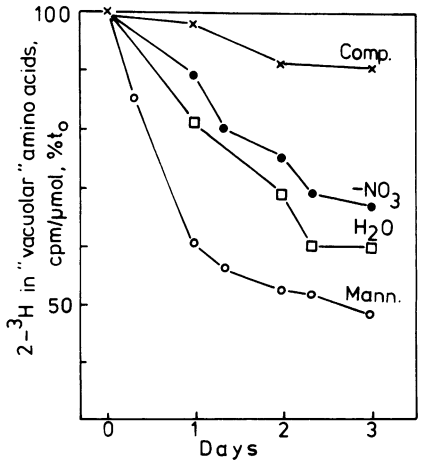

FIG. 3. The effect of stress on the 2-^{3}H content of vacuolar amino acids in *Lemna minor*. Fronds were grown on complete medium containing ^3H_2O (3.8×10^7 Bq ml^{-1}) for 48 h, washed, and placed on unlabeled complete medium for a further 48 h. At time t_0, fronds were transferred to unlabeled complete medium (×——×), $-NO_3^-$ medium (●——●), distilled H_2O (□——□), or 0.5 M mannitol medium (○——○). At intervals, samples were collected, the soluble amino acids were isolated (17), and the 2-^{3}H content was determined (18).

Table II. *Comparison of in Vivo Losses of Protein from Lemna Fronds under Conditions of Stress with in Vitro Rates of Proteolysis*

The *in Vivo* loss of protein was obtained from Cooke *et al.* (5) and *in Vitro* proteolysis was measured as described under "Materials and Methods."

Growth Conditions	Net Protein Loss	Proteolysis: Amino Acids Released	
		pH 5.2	pH 7.1
	μg/g·h	μg/g·h	
Complete medium		216	108
− NO_3^- medium			
1 day	4	259	89
2 days	12	168	82
3 days	21	98	86
0.5 M mannitol medium			
1 day	31	257	108
2 days	21	168	65
3 days	25	86	55

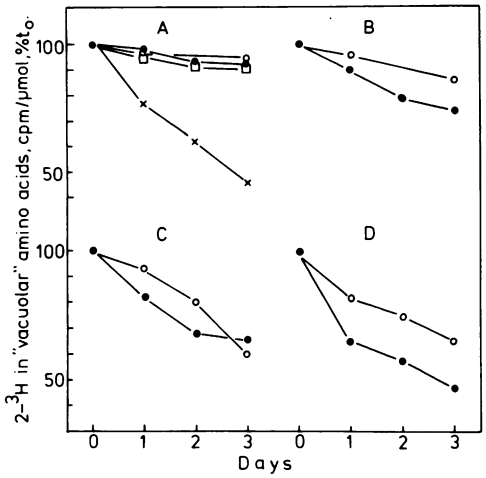

FIG. 4. The effect of 5 μM ABA, 2 μM N^6-BA and 10 μM cycloheximide on the loss of 2-^{3}H from vacuolar amino acids of *Lemna minor* grown in complete medium or under conditions of stress. A, complete: (●——●), control; (○——○), +BA; (×——×), +ABA; (□——□), + cycloheximide. B, $-NO_3^-$: (●——●), control; (○——○), +BA. C, distilled H_2O: (●——●), control; (○——○), +BA. D, 0.5 M mannitol: (●——●), control; (○——○), +BA. Fronds were grown on complete medium containing ^3H_2O (3.8×10^7 Bq ml^{-1}) for 48 h, washed, and transferred to unlabeled complete medium for a further 48 h. At time t_0, fronds were transferred to the appropriate unlabeled medium. Samples were collected at intervals, the soluble amino acids were isolated (17) and the 2-^{3}H content was determined (18). The values for the $t_{1/2}$ (in days) of total soluble protein under the different conditions (5) were as follows: complete, 7.0; complete + ABA, 4.1; complete + BA, 7.7; complete + cycloheximide, 11.6; $-NO_3^-$, 2.8; $-NO_3^-$ + BA, 5.0; H_2O, 2.1; H_2O + BA, 3.8; mannitol, 2.5; mannitol + BA, 5.8.

two-compartment model. There is a rapid exponential loss of 2-^{3}H from amino acids undergoing exchange reactions catalyzed by transaminases present in the cytosol. The very slow subsequent loss of 2-^{3}H from amino acids is due to the leakage of amino acids from a compartment lacking transaminases into the cytosol. When fronds are maintained on ^3H_2O for long periods, the relative amount of slowly exchanging 2-^{3}H increases with time, indicating that these amino acids have accumulated in a compartment lacking transaminases. It seems reasonable to assume that this compartment is the vacuole.

The effect of stress on the presumptive "vacuolar" pool of amino acids was investigated by maintaining *Lemna* fronds on complete growth medium containing ^3H_2O (3.8×10^7 Bq ml^{-1}) for 2 days and then transferring the fronds to unlabeled complete medium for a further 2 days to ensure that all 2-^{3}H-labeled amino acids were removed from the cytosol and that the 2-^{3}H-labeled amino acids remaining were in the vacuole (Fig. 2). The fronds then were transferred to a stress medium (total starvation, $-NO_3^-$, or 0.5 M mannitol). At intervals samples were removed, the soluble amino acids were isolated (17), and the 2-^{3}H content was measured (18). The results (Fig. 3) show that, although there was a slight loss of 2-^{3}H from vacuolar amino acids in fronds maintained on complete medium, there was a much greater loss when the plants were stressed. This is interpreted as indicating that the various stress conditions have altered the permeability of the tonoplast and allowed an increased efflux of vacuolar amino acids into the cytoplasm, where the transaminases ensure a rapid loss of 2-^{3}H.

We concluded that stress may alter the properties of the tonoplast. This allows a possible explanation for stress-induced increases in proteolysis, if it is assumed that leakage of amino acids from the vacuole can be equated with passage of larger molecules. Stress is envisaged as altering the properties of the tonoplast in such a way as to allow increased interaction between cytoplasmic proteins and degradative enzymes. A somewhat similar mechanism to account for the rapid, large increase in proteolysis brought about by transfer of *Lemna* fronds to medium made 50% (v/v) with respect to deuterium oxide has been proposed (6, 7). In such isotopic stress, extensive damage of the tonoplast is revealed by electron microscopy, correlating with the increase in protein breakdown. *Lemna* fronds can adapt to the deuterium oxide medium and, in such fronds, the rapid rate of proteolysis decreases and the tonoplast is apparently "repaired." In the study presented here, electron micrographs of stressed fronds did show some evidence of damage to membranes. We feel that the micrographs do not merit publication because of the difficulties of establishing unequivocally that the observed changes are solely due to the applied stress (7).

Rate of Proteolysis during Stress. To sustain the argument that stress-induced protein degradation is due to an effect on the permeability of the tonoplast, it is necessary to establish that there is always sufficient proteolytic activity to account for the net loss of protein. By assuming the average mol wt of an amino acid to be 120, *in vivo* rates of net protein loss under stress (5) have been compared with *in vitro* proteolytic activity. These results (Table II) show that there is always sufficient soluble proteolytic activity to account for the observed *in vivo* rates of protein loss.

Effect of Hormones on Permeability of Tonoplast. The possibility that hormones may mediate the interaction between stress and the tonoplast was investigated since there is evidence (5, 24) that ABA and N^6-BA affect kd. The effect of these compounds on the loss of 2-^{3}H from amino acids, which is interpreted as an alteration of tonoplast permeability, was examined. The results (Fig. 4) show that ABA causes an increase in the loss of 2-^{3}H from vacuolar amino acids, whereas N^6-BA reduces the loss caused by stress conditions. The effects (Figs. 3 and 4) of different stresses and hormones on tonoplast permeability correlate well with their effects on kd (5; see legend of Fig. 4).

A Model for Stress-induced Protein Degradation. In animal cells, it is believed that there are dual protein catabolic pathways, which are envisaged as (*a*) the normal degradative machinery, a poorly understood but specific process which may or may not involve the lysosomes; and (*b*) lysosomal autophagy, an indiscriminate process responsible for the enhanced proteolysis brought about by nutritional deprivation and certain other conditions (1, 11–14, 19). This is based partly on the observation that, although inhibitors, such as puromycin and cycloheximide, inhibit enhanced protein breakdown, they have little or no effect on basal degradation (14). Newly synthesized proteins are thought to be

needed for the formation of autophagic vacuoles, which fuse with the lysosome system. Protein synthesis inhibitors block this system, but leave another pathway of degradation unaffected (19).

We concluded that, in *Lemna*, the control of stress-induced proteolysis may be located in the tonoplast, mediated by the action of hormones. In yeast, the protein composition of the tonoplast is altered by the growth rate and physiological status of the cells (25). Something similar may occur in *Lemna*, with stress changing the properties of the tonoplast and causing the increased interaction of cytoplasmic proteins and degradative enzymes. Whether or not there is a separate system for basal protein degradation is unclear. Cycloheximide inhibits both normal and stress-enhanced protein breakdown (Table I) and it thus seems unnecessary to postulate dual pathways. Such an effect of cycloheximide on basal proteolysis suggests that some form of autophagy may be involved. The rate and specificity of degradation then will depend partly on the properties of this transport system and partly on the activities of the proteolytic enzymes in the vacuole. Recently, van der Wilden *et al.* (26) demonstrated the presence of autophagic organelles in mung bean cotyledons.

Any mechanism for stress-induced protein degradation has to account for the fact that *Lemna* can adapt to unfavorable conditions (5). Changes in vacuolar membrane properties provide a way in which such adaptation could occur. This simple model is unlikely to explain fully the complexities of protein degradation; nevertheless, it is consistent with present knowledge and provides a basis for further experiments.

Acknowledgment—We gratefully acknowledge the technical assistance of Jane Oliver.

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