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Communication

Drought Stress, Enzymes of Glutathione Metabolism, Oxidation Injury, and Protein Synthesis in *Tortula ruralis*[1]

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ABSTRACT

The activities of glutathione reductase (EC 1.6.4.2), glutathione peroxidase (EC 1.11.1.9), and glutathione S-transferase (EC 2.5.1.18) were found to increase during slow drying or during rehydration following rapid drying of the drought-tolerant moss *Tortula ruralis*. Little change was observed in the activity of malate deydrogenase (NAD^+ oxidoreductase, EC 1.1.1.37) during dehydration or subsequent rehydration. When the tissue was treated with cycloheximide, actinomycin D, or cordycepin, the increase in the activities of glutathione reductase and glutathione S-transferase was largely prevented while effect on glutathione peroxidase was much smaller. Concomitantly, oxidized glutathione (GSSG) as percentage of total glutathione increased. GSSG level was correlated positively with the levels of lipid peroxidation and solute leakage and negatively with the rate of protein synthesis. The results show that GSSG level is a good indicator of oxidation stress and provide support to the suggestion that GSSG mediates, at least in part, the drought stress-induced inhibition of protein synthesis.

The drought-tolerant moss *Tortula ruralis* may be dried rapidly in less than 30 min, or slowly in 8 to 10 h, to less than 20% of the original fresh weight (7). On rehydration, the moss regains nearly 99% of its original fresh weight in less than 2 min, and many chemicals that are normally not taken up by the tissue can be taken up along with mass flow of water (4). Polyribosomes are conserved during rapid drying but not during slow drying (7). But the rate of protein synthesis on rehydration is considerably lower in RD[2] than in SD moss (5). It has been suggested that GSSG, which inhibits *in vivo* and *in vitro* protein synthesis in this moss and accumulates during rehydration following rapid drying (5), mediates the drought stress-induced inhibition of protein synthesis. However, the relationship of the endogenous GSSG to the rate of *in vivo* protein synthesis is unclear. One way to explore this relationship is to modulate the endogenous GSSG content and then determine if the correlation between GSSG content and the cellular processes of interest is still maintained.

The present investigation was undertaken with three main objectives: (a) to study the changes in activities of enzymes of GSH utilization and regeneration, GR (EC 1.6.4.2), GP (EC 1.11.1.9), and GST (EC 2.5.1.18); (b) to determine if modulation of the activities of these enzymes with inhibitors of transcription and translation is possible and, if so, then determine its effects on endogenous GSSG content; and (c) to determine if the altered GSSG content shows any correlation with the levels of LP, solute leakage, and protein synthesis. The results obtained show that the activity of all these three enzymes increases during slow drying and during rehydration following rapid drying, and that this increase can be largely prevented, particularly in the case of GR, by applying inhibitors of translation and transcription. Furthermore, the low level of GR activity is correlated with increased levels of GSSG, LP, and solute leakage, and a decreased rate of protein synthesis.

MATERIALS AND METHODS

Plant Material

Gametophytes of *Tortula ruralis* (Hedw.) (Gaertn, Meyer, and Scherb) were collected, stored, and prepared for experiments as described by Bewley (2). The apical 1 cm part of the plant was used as experimental material. Fresh moss was blotted dry with paper towels to remove any surface water. This procedure facilitated the uptake of test chemicals.

Administration of Drought Stress

Samples (250 mg) of fresh moss were subjected to either slow drying in an atmosphere of 65% RH or rapid drying over activated silica gel particles (nearly 0% RH). A similar final weight of less than 20% of the original fresh weight was achieved in 8 to 9 h of slow drying and in less than 30 min of rapid drying. For details see Dhindsa and Bewley (6).

Inhibitor Treatment

To load the moss tissue with inhibitors before drying, 250 mg fresh moss was incubated in 2 mL distilled water with or without 20 μg/mL CH, 50 μg/mL actinomycin D, or 50 μg/mL cordycepin for 2 h. The moss was then washed, blotted dry, and either used immediately as fresh moss for experiments or subjected to drying.

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[2] Abbreviations: RD, rapidly dried; SD, slowly dried; CDNB, 1-chloro-2,4-dinitrobenzene; CH, cycloheximide; DNB, 2,4-dinitro benzene; GSH, reduced glutathione; GSSG, oxidized glutathione; GP, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; LP, lipid peroxidation; MDA, malondialdehyde; MDH, malate dehydrogenase (NAD^+ oxidoreductase).

Determination of Total Glutathione, GSSG, and Level of LP

The determination of total glutathione in GSH equivalents and GSSG was according to enzymic methods (1) as previously used with moss tissue (5). GSSG content was calculated as percentage of the total glutathione. The level of LP was measured as MDA as described (8).

Determination of Solute Leakage

Radiolabeled β-alanine was used to determine solute leakage since it is not incorporated into proteins. Fresh moss was incubated in 2 mL distilled water containing 5 μCi of β-[1-^{14}C]alanine (55 mCi/mmol) for 2 h. The moss was then washed with a cold (4°C) solution of 100 μg/mL carrier β-alanine followed by a quick rinse with distilled water and blotted to dry the tissue surface. It was then either used as fresh moss to study leakage, or subjected to slow or rapid drying. To determine leakage, 250 mg fresh moss or its equivalent in variously dried moss was placed in 5 mL distilled water on a shaker. Leakage was allowed for 2 h and the radioactivity leaked into the bathing solution was determined by liquid scintillation spectrometry. At the end of the leakage period, and after sampling the aliquot of the bathing medium for determination of the leaked radioactivity, the tissue was killed in a water bath held at 100°C for 15 min. The solution was brought to the original volume and an aliquot was used to determine the total radioactivity. The leaked radioactivity was expressed as percentage of the total radioactivity.

In Vivo Protein Synthesis

Incorporation of radiolabeled leucine into TCA-precipitable fraction was used as an index of protein synthesis. Fresh moss (250 mg) or its equivalent in dried moss was placed in 5 mL distilled water containing 20 μCi of [4,5-^{3}H] leucine (60 Ci/mmol) for 2 h. When a longer treatment with another chemical or period of rehydration was required, radiolabeled leucine was added for the last 2 h. At the end of incorporation, tissue was washed with cold (4°C) solution of 100 μg/mL carrier leucine. Procedures for extraction of proteins and for determination of protein content and radioactivity were as described (5).

Enzyme Assays

Enzyme extract of the moss tissue was prepared by grinding 250 mg fresh moss or its equivalent of dried moss in 2 mL 50 mM k-phosphate buffer (pH 7.0), in a ground glass homogenizer. The homogenate was centrifuged at 15,000*g* for 10 min and the supernatant obtained was used as enzyme source. The protein content of the enzyme extract was determined as above (5).

GR was assayed by the procedure of Carlberg and Mannervik (3). A 3-mL reaction mixture contained 100 mM phosphate buffer (pH 7), 1 mM GSSG, 1 mM EDTA, 0.1 mM NADPH, and 25 to 50 μL enzyme extract. The reaction was started by adding the enzyme extract. The rate of NADPH oxidation was followed by monitoring the decrease in *A* at 340 nm with a recording spectrophotometer. Two blanks, one without the enzyme extract and the other without GSSG, were used as controls.

GP was assayed with the procedure of Hochstein and Utley (11) by coupling its reaction with that catalyzed by GR. The 3-mL reaction mixture contained 20 mM phosphate buffer (pH 6.8), 1.86 mg GSH, 22.5 units of glutathione reductase, 0.39 mg KCN, 0.6 mg NADPH, 0.0015% H_2O_2, and 50 to 100 μL enzyme extract. The reaction was started by adding the enzyme extract and the rate of oxidation of NADPH was followed as above.

GST was assayed by monitoring the formation of the conjugate between GSH and CDNB which absorbs maximally at 340 nm with an extinction coefficient of 9.6 mM^{-1} cm^{-1} (10). The 3-mL reaction mixture contained 100 mM phosphate buffer (pH 6.5), 1 mM GSH, 1 mM CDNB, and 50 to 100 μL enzyme extract. The reaction was started by adding the enzyme and the rate of formation of the conjugate, GS-DNB, was followed by monitoring the increase in absorbance at 340 nm.

MDH has been included since its activity did not change during dehydration and subsequent rehydration. Its activity was assayed by the procedure of Ting (15) by following the rate of NADH oxidation.

RESULTS

The changes in the activities of GR, GP, and GST during slow or rapid drying of *Tortula ruralis* are shown in Figure 1. None of the enzyme activities changes during rapid drying. During slow drying, there is little change in any of the enzyme

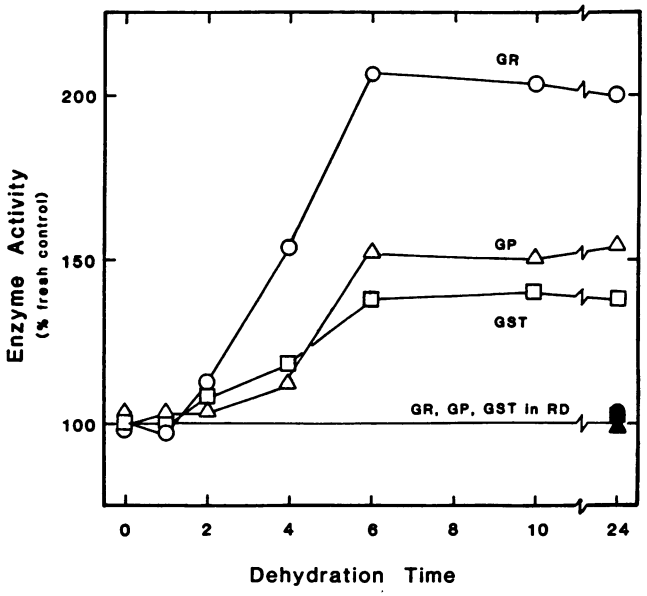

Figure 1. Changes in activities of GR, GP, and GST during slow (○, △, □) or rapid drying (●, ▲, ■). Each value is a mean of two replicates none of which deviated from the mean by more than 10%. The 100% values for fresh control tissue were: GR, 68 nmol NADPH oxidized per min per mg protein; GP, 185 nmol NADPH oxidized per min per mg protein; GST, 58 nmol of GS-DNB formed per min per mg protein. Activity of malate dehydrogenase changed little and remained close to 5.5 μmol of NADH oxidized per min per mg protein.

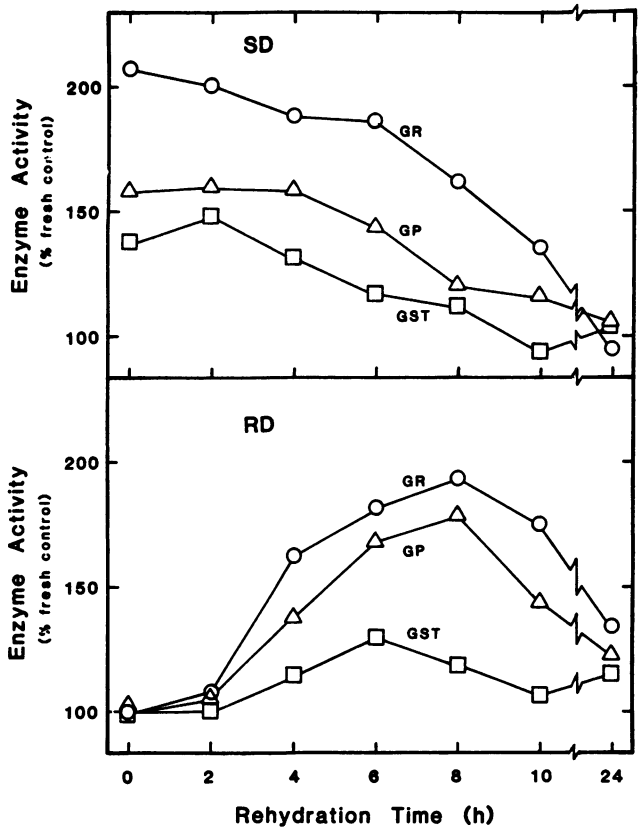

Figure 2. Changes in activities of GR, GP, and GST during rehydration following slow drying (SD, upper panel) or rapid drying (RD, lower panel). Each value is a mean of two replicates, none of which deviated from the mean by more than 10%. For 100% values in fresh control tissue, see legend to Figure 1. The activity of malate dehydrogenase changed little during rehydration (data not presented).

activities during the first 2 h. Thereafter, GR activity increases sharply, reaches a maximum by about 6 h, and remains at this level throughout drying. The activities of GP and GST also increase but increase is smaller than that in GR activity. Thus, the activity increases to about 200%, 150%, and about 135% of that in the fresh control tissue, for GR, GP, and GST, respectively. Furthermore, each enzyme activity reaches a maximum at 6 h of drying. The activity of MDH remained unchanged at 5.5 μmol NADH oxidized per min per mg protein.

The activities of GR, GP, and GST during rehydration of the SD and RD moss are shown in Figure 2. In the case of SD moss (upper panel), the enzyme activities decline gradually during rehydration and reach control levels by 24 h of rehydration. In the case of RD moss, changes in the enzyme activities are rather interesting. While there is little change in any enzyme activity during the first 2 h of rehydration, the activities start increasing thereafter and reach a maximum between 6 to 8 h.

Again, the maximum percentage increase occurs in GR, followed by GP and GST. After reaching the maxima, the activities start decreasing. But at 24 h, the activities are still higher than in the fresh control. Again, the activity of MDH remained unchanged at control levels (data not shown).

When moss was pretreated with CH (20 μg/mL) and then subjected to slow drying, the activities of GR and GST decreased to about 80% of those in the fresh control tissue—in sharp contrast to their increases shown in Figure 1. Pretreatment with actinomycin D (50 μg/mL) or cordycepin (50 μg/mL), followed by slow drying, allowed only 20 to 25% increases in GR and GST activities compared to the larger increases shown in Figure 1. Since the desiccation-induced damage to protein synthesis is manifested on rehydration, the effects of CH, actinomycin D, and cordycepin on the activities

Table I. *Effects of CH, Actinomycin D, and Cordycepin on Activities of GR, GP, and GST, Levels of GSSG Content, Lipid Peroxidation (MDA), and Solute Leakage and on Rate of Protein Synthesis at 4 h of Rehydration of T. ruralis*

The tissue was predried slowly or rapidly, in the absence or presence of inhibitors of protein and RNA synthesis. Samples treated with the inhibitors during rehydration were predried in their absence. Each value for enzyme activity is a mean of two replicates none of which deviated from the mean by more than 10%. For 100% values of enzyme activities in fresh control tissue, see legend to Figure 1. Each value for GSSG, MDA, solute leakage, and protein synthesis is a mean of three replicates ± SE. The 100% fresh control value for the rate of protein synthesis was 560 cpm/μg protein·h.

Rehydration Treatment	GR	GP	GST	GSSG	MDA	Solute Leakage	Protein Synthesis
	% fresh control			*% total*	*nmol/g dry wt*	*% total*	*% fresh control*
Fresh control	100	100	100	5 ± 0.95	315 ± 24	18 ± 1.5	100
SD	170	145	120	4 ± 0.75	398 ± 42	17 ± 1.8	98 ± 9.5
SD + CH pretreatment	75	105	69	31 ± 2.5	1655 ± 210	38 ± 3.2	19 ± 2.2
SD + actinomycin D pretreatment	95	110	106	20 ± 1.8	1118 ± 145	34 ± 3.9	25 ± 3.2
SD + cordycepin pretreatment	108	102	98	22 ± 1.8	1090 ± 126	37 ± 3.3	22 ± 2.4
SD + CH	108	125	97	15 ± 1.3	828 ± 92	29 ± 3.5	18 ± 1.7
SD + actinomycin D	110	115	105	6 ± 0.8	388 ± 45	18 ± 1.6	88 ± 9.0
SD + cordycepin	115	120	99	6 ± 0.5	410 ± 36	18 ± 1.8	92 ± 8.4
RD	180	175	118	10 ± 1.2	320 ± 26	21 ± 2.2	85 ± 9.3
RD + CH	75	91	87	41 ± 3.7	2180 ± 260	58 ± 6.2	12 ± 1.8
RD + actinomycin D	82	95	83	39 ± 4.1	2265 ± 315	55 ± 7.0	14 ± 1.6
RD + cordycepin	78	98	89	42 ± 3.6	2150 ± 265	56 ± 6.2	15 ± 1.8

of GR, GP, and GST, and on the levels of GSSG, LP, solute leakage, and protein synthesis during rehydration of the three glutathione enzymes under study have declined during 4 h of rehydration following slow drying, in agreement with the data in Figure 2. In the samples pretreated with cycloheximide during slow drying, the activities decline further during rehydration. While the activity of GP decline to about the control level, those of GR and GST decline to levels considerably below that in the control. In the moss pretreated with actinomycin D or cordycepin, the activities have declined during rehydration and are more or less equal to those of fresh control moss. In the moss which was SD in the absence of inhibitors but is rehydrated in the presence of inhibitors, enzyme activities do not differ much from those in the control.

In the case of RD moss, the activities of GR and GP are about 80% higher than in the fresh control while the activity of GST is only about 20% higher. When RD moss is rehydrated in the presence of inhibitors, the enzyme activities are lower than those in the control tissue. However, the decline in GP is the smallest.

The effects of the inhibitors of protein and RNA synthesis on LP, solute leakage, and protein synthesis during rehydration are also shown in Table I. The SD moss has similar GSSG content, MDA level, solute leakage, and rate of protein synthesis as the fresh control moss. However, when moss is SD in the presence of CH, actinomycin D, or cordycepin, and the levels of GSSG, MDA, and solute leakage on rehydration are high while the rate of protein synthesis is low. The results are similar when the moss, dried without inhibitors, is rehydrated for 4 h in the presence of CH, although the levels of GSSG, MDA, and solute leakage are not as high. The addition of actinomycin D and cordycepin during rehydration has little effect on the levels of GSSG, MDA, solute leakage, and protein synthesis. The RD moss after 4 h of rehydration shows nearly similar levels of GSSG, MDA, solute leakage, and protein synthesis as the fresh or SD moss. However, when RD moss is rehydrated for 4 h in the presence of CH, actinomycin D, or cordycepin, the levels of GSSG, MDA, and solute leakage are the highest while the rate of protein synthesis is the lowest.

DISCUSSION

The timing of the increase in GR and GP activities observed in the present study may explain the reported changes in levels of GSSG, LP, and solute leakage during dehydration and subsequent rehydration of the moss tissue (4, 5, 8). GSSG accumulates during slow drying but is rapidly metabolized on rehydration (5). The increase in GR activity observed in the present study probably explains the rapid decline in GSSG through its conversion to GSH. Because GSSG is a potent inhibitor of protein synthesis (5, 9, 12), its rapid decline on rehydration probably underlies the ability of the SD moss to resume protein synthesis on rehydration at a rate similar to that in the fresh moss (5). This is supported by the observation that in the tissue treated with actinomycin D and cordycepin the GSSG level does not decline and protein synthesis rate is decreased. In the RD moss, the levels of LP, solute leakage, and GSSG are known to increase during rehydration and decline within about 4 h (5, 8). The rate of protein synthesis is very low immediately on rehydration but rapidly increases as GSSG level declines (5). The increase in GR and GP activities during rehydration of RD moss are expected to mediate the decline in GSSG and LP levels. While GP may metabolize lipid hydroperoxides and generate GSSG, GR may reduce GSSG to GSH (13, 14). When these enzyme activities are prevented from increasing by actinomycin D and cordycepin, the levels of GSSG and LP remain high and the rate of protein synthesis low. The role of GST is not clear in the present study but it may be involved in protection of protein sulfhydryl groups (13) during desiccation. Collectively, these results show that endogenous GSSG level is negatively correlated with the rate of protein synthesis and thus support the suggestion (4, 5) that the drought stress-induced inhibition of protein synthesis is mediated by GSSG generated due to oxidation stress.

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