

Glutathione Status and Protein Synthesis during Drought and Subsequent Rehydration in *Tortula ruralis*¹

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ABSTRACT

Glutathione status and its relationship to protein synthesis during water deficit and subsequent rehydration have been examined in the drought-tolerant moss, *Tortula ruralis*. During slow drying there is a small decrease in total glutathione but the percentage of oxidized glutathione (GSSG) increases. During rapid drying there is little change in total glutathione but a small increase in GSSG. On rehydration of slowly dried moss, GSSG rapidly declines to normal level. But when rapidly dried moss is rehydrated, there is an immediate, sharp increase in GSSG as a percentage of total glutathione. After 2 hours of rehydration GSSG starts declining and reaches a normal level in about 6 hours. When an increasing degree of steady state water deficit is imposed on the moss tissue with polyethylene glycol 6000, there is a progressive decrease in protein synthesis but an increase in oxidized glutathione. When 5 millimolar GSSG is supplied exogenously during rehydration of rapidly dried or slowly dried moss, protein synthesis is strongly inhibited. *In vitro* protein synthesis supported by moss mRNA is also inhibited by more than 85% by 150 micromolar GSSG. The role of glutathione status in water deficit-induced inhibition of protein synthesis is discussed.

Drought-tolerant moss, *Tortula ruralis*, can be dried rapidly or slowly to less than 20% of its original fresh weight without loss of its ability to recover on subsequent rehydration (2). However, the rates of metabolic responses on rehydration vary with the speed of the previous drying. For example, the consumption of O₂ (17) level of lipid peroxidation and solute leakage (10) are considerably greater following rapid drying than following slow drying. On the other hand, the rate of protein synthesis on rehydration is much slower following rapid drying than following slow drying (3). This difference in rates of protein synthesis is particularly intriguing because while slowly dried moss loses its polyribosomes completely, the rapidly dried tissue retains them substantially (6). Furthermore, active mRNA has been shown to be present in both rapidly dried and slowly dried *T. ruralis* (8). The underlying causes of why the tissue with conserved polyribosomes has a lower rate of protein synthesis than the one which does not conserve any detectable level of polyribosomes are not understood at present.

This investigation was undertaken to determine the changes in glutathione status of the tissue during slow or rapid drying and during subsequent rehydration. GSH plays a major role in maintaining the redox state of cells (15, 18) and its oxidized

form, glutathione disulfide (GSSG), is known to inhibit protein synthesis *in vitro* (16). It was argued that the reported increase in the availability of oxidants during rehydration of rapidly dried moss (10) would cause changes in the glutathione status of the tissue. The results obtained demonstrate that during rehydration of the rapidly dried moss GSSG, as percent of total glutathione, increases sharply. GSSG also increase during steady state water deficit. Furthermore, GSSG inhibits both *in vivo* and *in vitro* protein synthesis. Mediation by GSSG of water deficit-induced damage to the protein synthesizing apparatus is suggested and discussed.

MATERIALS AND METHODS

Plant Material. *Tortula ruralis* (Hedw.) (Gaertn, Meyer, and Scherb) was collected, stored and prepared for experiments as described elsewhere (2). The apical 1 cm part of the gametophyte was used as experimental material.

Administration of Drought Stress. Slow or rapid dehydration was imposed on 500 mg fresh moss tissue using methods already described (4, 7). Briefly, slow dehydration was administered by placing tissue samples over a stirred, saturated solution of ammonium nitrate contained in a desiccator (65% RH). Rapid desiccation was imposed by placing the tissue over activated silica gel particles in a desiccator (RH of nearly 0%). A final weight of less than 20% of original fresh weight was obtained in about 8 h of slow drying and in less than 30 min of rapid drying.

Administration of Steady State Stress. Polyethylene glycol 6000 was used as osmoticum to impose steady state water stress as described previously (7). Five hundred mg fresh moss was placed in 5 ml of PEG solution with varying water potential. After 1.5 h, 20 μ Ci of [4,5-³H]leucine (60 Ci/mmol) was added in experiments when rate of protein synthesis was studied. In a parallel experiment after 1.5 h of preincubation of moss in PEG solution, the level of GSSG as percent of total glutathione was determined.

Determination of Total Glutathione and Oxidized Glutathione. Enzymic methods described recently (1) were used to determine total glutathione (GSH + GSSG) in terms of GSH equivalents, and oxidized glutathione (GSSG). Tissue samples consisted of 0.5 g fresh moss or equivalent weight of dried moss. Tissue was homogenized in 2.5 ml of 5% 5-sulfosalicylic acid and a 10,000g supernatant was obtained and used to determine total glutathione in GSH equivalents by the DTNB-GSSG reductase method. A standard curve was obtained by using authentic GSH at concentrations of 0 to 5 μ M. The assay was monitored by following the rate of formation of TNB² at 412 nm.

For the determination of GSSG, 2-vinylpyridine was used to trap the GSH present in the 5-sulfosalicylic acid supernatant

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² Abbreviations: TNB, 5-thio-2-nitrobenzoate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

solution (2 $\mu\text{l}/100 \mu\text{l}$). Then the GSSG present was assayed by the DTNB-GSSG reductase method. Authentic GSSG was used to prepare the standard curve.

Preparation of mRNA from Dried Moss. Five hundred mg of dried moss was used to prepare first total RNA and then, from the latter, fractionate poly (A)+ mRNA by oligo(dT)-cellulose chromatography as described previously (8). mRNA isolated from both rapidly dried and slowly dried moss was used to study the effect of GSSG on *in vitro* protein synthesis.

***In Vivo* Protein Synthesis.** Leucine incorporation into proteins was used as a measure of protein synthesis. Duplicate samples of 250 mg dried moss were placed in 5 ml distilled H_2O with or without 5 mM GSSG in 5 cm diameter Petri dishes. This moss regains its original fresh weight almost completely within 2 min of rehydration. After 5 min, 20 μCi of [4,5- ^3H]leucine (60 Ci/mmol) were added. Incorporation was allowed for different times as indicated in the respective experiments. At the end of incorporation, samples were washed with a cold (4°C) solution containing 0.2 mg/ml carrier leucine. Proteins were extracted and their amount and radioactivity determined as described previously (9).

***In Vitro* Protein Synthesis.** The effect of GSSG on *in vitro* protein synthesis supported by mRNA from dried moss was studied in a rabbit reticulocyte lysate system purchased from Amersham Corp. (Arlington Heights, IL). Total volume of the reaction mixture was 50 μl and contained varying concentration of GSSG, 2 μg of mRNA, and 50 μCi of [^{35}S]methionine (1400 Ci/mmol). A detailed protocol provided by the supplier was followed.

RESULTS

Changes in the level of GSSG as percent of total glutathione during slow or rapid drying are shown in Figure 1A. It can be seen that during slow drying there is a steady increase in GSSG in the first 4 h of drying. It increases from about 1.5% to nearly 15% of total glutathione. After the first 4 h of drying GSSG increases further, rather sharply, to more than 20%. During rapid drying GSSG increases from 1.5% to nearly 5%. Total glutathione content decreased from 4 μmol to 3.5 $\mu\text{mol}/\text{g}$ of original fresh weight during slow drying but remained unchanged during rapid drying (data not shown).

On rehydration, sharp changes in GSSG are observed following slow or rapid drying (Fig. 1B). During rehydration of the slowly dried moss GSSG declines rapidly and reaches the control level of the fresh moss in about 2 h. In the case of rapidly dried moss, on rehydration there is an immediate large increase in GSSG during the first 2 h. It increases from about 3% to 45% of the total glutathione. Thereafter it shows a sharp decline and reaches a level of 10% of the total glutathione in the next 4 h. At the end of 10 h of rehydration GSSG is almost at the control level. Thus, it may be concluded that on rehydration GSSG rapidly declines in the case of slowly dried moss, but shows a large, transitory increase in the case of rapidly dried moss. Total glutathione content during rehydration increased from 3.5 to 4.2 $\mu\text{mol}/\text{g}$ of original fresh weight in the case of slowly dried moss but did not change much in the case of rapidly dried moss (data not presented).

To explore the relationship, if any, between glutathione status and protein synthesis, it was considered worthwhile to compare the kinetics of protein synthesis to those of GSSG changes during rehydration. The results on protein synthesis are shown in Figure 1C. Rates of protein synthesis in fresh and slowly dried moss are similar, particularly after the initial 1 or 2 h. In the case of rapidly dried moss, however, the rate of protein synthesis on rehydration is very low during the first 2 h but progressively increases thereafter. After 4 or 5 h of rehydration, it is similar to the rate in fresh or slowly dried moss.

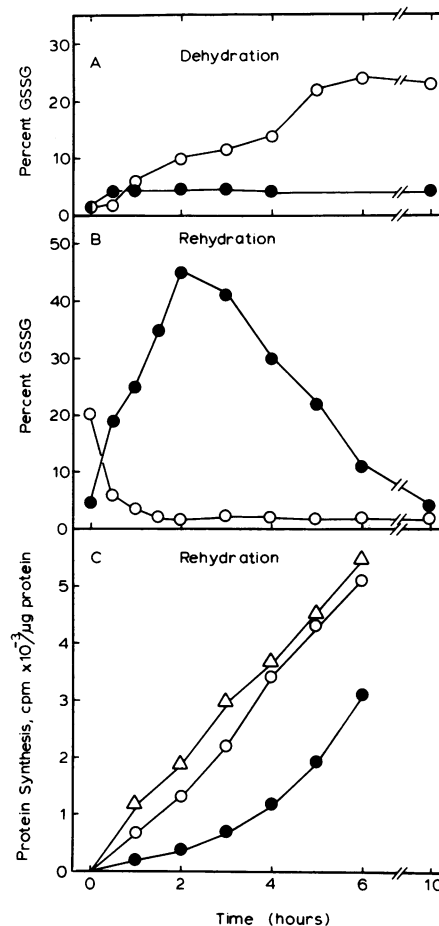


Fig. 1. Oxidized glutathione content, as percent of total glutathione, during dehydration (A) and subsequent rehydration (B), and protein synthesis during rehydration (C) in moss subjected to slow drying (O) rapid drying (●), or no drying-fresh moss (Δ). Each value is a mean of two replicates which deviated from the mean by less than 10%.

To determine whether a steady state water deficit would also be accompanied by an increase in GSSG, as is observed during air drying (Fig. 1A), moss was subjected to different degrees of steady state water stress by using PEG 6000 as osmoticum. After 90 min of preincubation in the osmoticum, radioactive leucine was added to determine protein synthesis and parallel samples were removed, washed, and used to determine total glutathione and percent GSSG (Fig. 2). Protein synthesis steadily declines with the decline in water potential and reaches a minimum at -50 bars. Percent GSSG shows an increase with the decrease in water potential, reaches a maximum at about -30 bars, and changes little thereafter. Thus, it can be concluded that during steady state water deficit protein synthesis declines and percent GSSG increases.

The effect of 5 mM GSSG on *in vivo* protein synthesis during 2 h of rehydration of slowly dried or rapidly dried moss is shown in Figure 3. GSSG inhibits protein synthesis strongly in both slowly dried and rapidly dried moss. While the inhibition is persistent in the case of rapidly dried moss, it appears to be partially reversed after the first hour in the case of slowly dried moss. Therefore, it can be concluded that exogenously supplied GSSG causes a strong inhibition of *in vivo* protein synthesis.

To determine whether GSSG would inhibit *in vitro* protein synthesis, moss mRNA was incubated with [^{35}S]methionine in a rabbit reticulocyte lysate in the presence or absence of GSSG. Results are shown in Figures 4 and 5. Figure 4 shows that increasing concentrations of GSSG cause an increasing inhibition

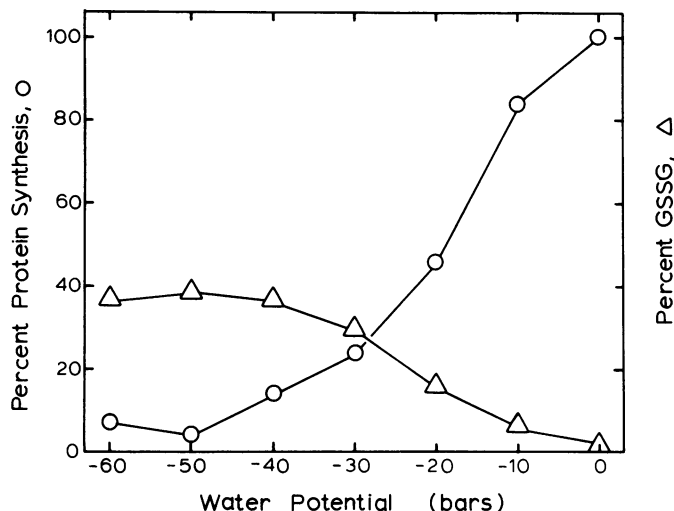


FIG. 2. Decrease in protein synthesis (O) and increase in oxidized glutathione content, as percent of total glutathione (Δ) with decreasing water potential of the bathing solution. Value for 100% control in protein synthesis was 970 cpm per μg protein during 1 h of incorporation. Each value is a mean of two replicates which deviated from the mean by less than 10%.

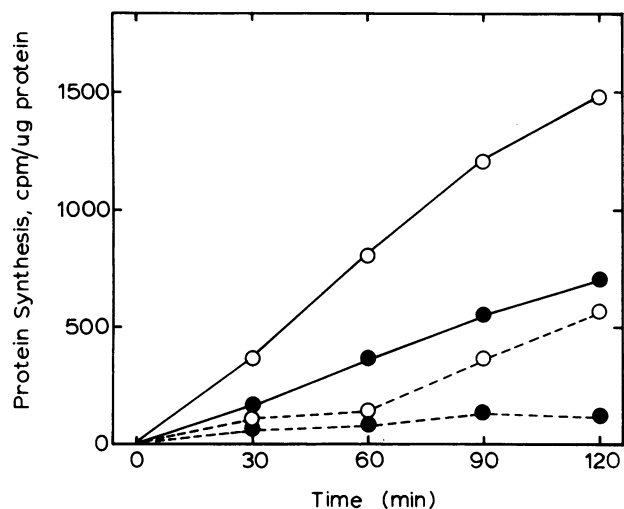


FIG. 3. *In vivo* protein synthesis during rehydration of slowly dried moss (O) and rapidly dried moss (●) in the absence (—) or presence (---) of 5 mM oxidized glutathione. Each value is a mean of two replicates which deviated from the mean by less than 10%.

of amino acid incorporation. Nearly 85% inhibition is obtained with 150 μM GSSG. Higher concentrations of GSSG produce a maximum of slightly more than 90% inhibition. The time course of *in vitro* protein synthesis in the presence or absence of 150 μM GSSG is shown in Figure 5. Incorporation is linear with time during the first 20 min in both the presence and absence of GSSG. The rate of incorporation steadily declines thereafter. It should be noted that GSSG causes more than 80% inhibition of *in vitro* protein synthesis.

DISCUSSION

The present study shows that (a) GSSG increases during slow drying and during rehydration following rapid drying of *T. ruralis*, and (b) GSSG inhibits *in vivo* as well as *in vitro* protein synthesis.

This study was aimed at understanding why the rapidly dried

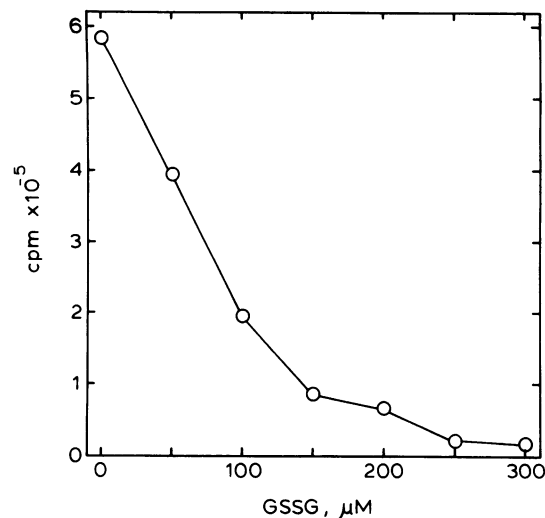


FIG. 4. Inhibition of *in vitro* protein synthesis, supported by moss mRNA, by oxidized glutathione. See "Materials and Methods" for technical details. Incorporation was allowed for 60 min. Each value is a mean of two replicates which deviated from the mean by less than 10%.

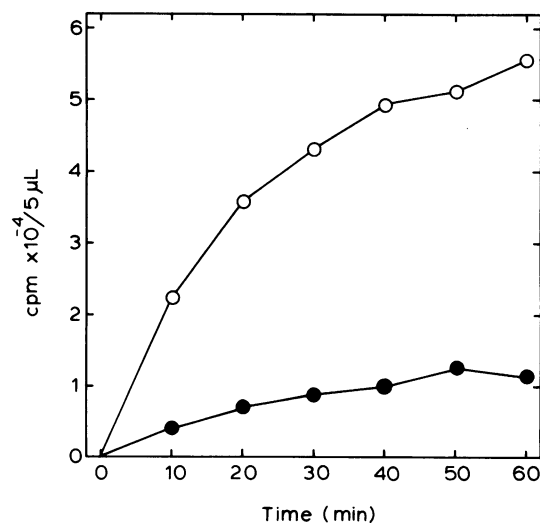


FIG. 5. Time course of *in vitro* protein synthesis supported by moss mRNA, in the absence (O) or presence (●) of 150 μM oxidized glutathione. Each value is a mean of two replicates which deviated from the mean by less than 10%.

moss with conserved polyribosomes synthesizes proteins on rehydration at a much slower rate than the slowly dried moss which does not conserve any detectable polyribosomes (6). Since the rapidly dried moss, on rehydration, is known to consume elevated levels of O_2 (17) and to undergo increased oxidative damage (10) it was argued that the slower rate of protein synthesis may reflect some oxidative damage to the protein synthesizing apparatus. The results of the present study strongly support such a possibility and point to the importance of glutathione status to protein synthesis. Small decrease in total glutathione content was observed during slow drying. The reason for this decrease is not clear. It is possible that some glutathione may have been used in the formation of mixed disulfides (15).

Reduced glutathione, GSH, plays many protective functions in cellular metabolism. It can directly react with free radicals and become itself oxidized to GSSG (15). It is also a substrate for glutathione peroxidase which catalyzes the removal of lipid hydroperoxides, products of the routinely occurring process of lipid peroxidation (15, 18). As a result of the glutathione peroxidase

reaction, GSH is oxidized to GSSG. The -SH groups of proteins are also protected by GSH (15, 18). The increase in GSSG observed in the present study may be due to either one or both of the above mentioned possibilities, direct GSH reaction with free radicals and GSH oxidation by glutathione peroxidase. The sharp increase in GSSG observed on rehydration of rapidly dried moss coincides in time with the reported burst in O₂ consumption (17) and increase in lipid peroxidation (10). It also coincides in time with the decreased rate of protein synthesis: as GSSG starts declining, the rate of protein synthesis increases. The possibility of a causal relationship between increased GSSG and decreased protein synthesis is strongly supported by the observation that GSSG inhibits *in vivo* and *in vitro* protein synthesis. Partial reversal of GSSG-induced inhibition of *in vivo* protein synthesis after 1 h of rehydration in slowly dried moss (Fig. 3) may be due to reduction of GSSG to GSH. Inhibition of *in vitro* protein synthesis by GSSG has been demonstrated in rabbit reticulocyte lysates (11, 12, 16). However, in these studies endogenous mRNA served as a template. In the present study such inhibition has been demonstrated using exogenous mRNA. The kinetics of inhibition *in vivo* and *in vitro* are different (Figs. 3 and 5). The reasons for this difference are unclear at present.

The progressive increase in GSSG with increasing steady state water deficit suggests that GSSG may mediate the water deficit-induced inhibition of protein synthesis reported by several workers (5, 9, 19). The mechanism of GSSG inhibition of *in vitro* protein synthesis in reticulocyte lysates has been the subject of several studies. It has been shown (11, 12) that GSSG activates a translational inhibitor, a protein kinase, which phosphorylates and thereby inactivates the initiation factor eIF-2. It has been suggested that sugar phosphates, NADPH, and thiol-reducing systems are required to maintain a high rate of protein synthesis probably by keeping the inhibitor in the inactive state (13, 14). This probably means that such a level of reductants is needed to keep the GSSG concentration from increasing above a certain level. It should, perhaps, be mentioned that NADPH is a coenzyme of GSSG reductase. This suggested importance of NADPH to protein synthesis is noteworthy in light of the observation (4) that resumption of nonautotrophic CO₂ fixation, a source of NADPH generation (20), on rehydration occurs at near normal levels in slowly dried moss but only at about 60% of normal levels in the case of rapidly dried moss. The latter takes about 2 h to restore nonautotrophic CO₂ fixation to normal levels.

In summary, the present study provides evidence that glutathione status may be an important factor in the regulation of protein synthesis under water stress conditions. A mechanism for drought stress action on protein synthesis and several other metabolic processes, based on oxidation injury, is being proposed separately (RS Dhindsa, unpublished data).

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