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Enzyme Dynamics of the Resurrection Plant *Selaginella lepidophylla* (Hook. & Grev.) Spring during Rehydration[1]

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ABSTRACT

The activities of 10 enzymes involved in carbohydrate metabolism were measured in both desiccated and rehydrated fronds of the desiccation-tolerant pteridophyte *Selaginella lepidophylla* (Hook. & Grev.) Spring. Enzyme conservation was defined as the ratio of desiccated to hydrated frond enzyme activity. The mean level of conservation was 74%, with nine of the 10 enzymes showing significant activity increases ($P < 0.05$) during hydration. The mean of photosynthetic enzyme conservation was significantly lower ($P = 0.05$) than the mean for glycolytic and respiratory enzymes combined. Chloramphenicol inhibited the normal activity increase in ribulose bisphosphate carboxylase and (NADPH)triose-P dehydrogenase but not pyruvate kinase upon rehydration. Cycloheximide did not affect the normal activity increase for these three enzymes. It is concluded that substantial enzyme conservation is beneficial for rapid resumption of metabolic activity in resurrection plants.

The ability to recover complete physiological activity following repeated protoplasmic dehydration of fully differentiated tissues is an adaptation unique to desiccation-tolerant plants. The physiological and molecular aspects of metabolic reactivation during rehydration of these plants are not fully understood. In a general sense however, the following interrelated features have been suggested to be essential (2): (a) desiccation-induced damage must be limited to a non-lethal level; (b) partial conservation of potential metabolic function, including structural and molecular components, must occur; and (c) the damage caused by desiccation must be repaired during rehydration.

A variety of cellular components appear to maintain potential function after desiccation. Ultrastructural conservation has been documented for thylakoid membranes and associated Chl complexes (29), mitochondria (1, 2), and other cell membrane systems (2, 15, 19). Elements of the protein synthesizing apparatus including mRNA, tRNA, and ribosomes are also conserved (8, 16, 20, 28). Finally, there have been a number of reports of conserved enzyme activity extractable from desiccated tissue (2, 5, 6, 11, 23, 27, 30).

The ability to conserve these cellular components is undoubtedly of great ecological significance. Most resurrection plants grow in environments where droughts are common; thus, rapid resumption of metabolic activity is essential for survival (10). While many enzymes may be conserved to some degree and play important roles in specific aspects of physiological recovery, only enzymes directly involved in carbohydrate metabolism related to the redevelopment of whole-plant respiratory and photosynthetic activity were chosen for assay. This choice was made because of the ecological importance of rapid carbon acquisition to the success of these plants in desert environments.

The purpose of this study was to examine the dynamics of a variety of carbohydrate metabolism enzymes during a hydration cycle in the resurrection plant *Selaginella lepidophylla*. Specifically, this study was undertaken: (a) to survey the probable extent of carbohydrate metabolism enzyme conservation in desiccated plant tissue, (b) to determine if levels of enzyme conservation were correlated with previously determined whole-plant CO_2 exchange patterns during rehydration, and (c) to examine the potential increase in enzyme activity during rehydration for selected enzymes using both organelle- and cytoplasm-directed protein synthesis inhibitors.

MATERIALS AND METHODS

Plant Collection and Storage. Desiccated plants of *Selaginella lepidophylla* (Hook. & Grev.) Spring were collected in August 1980 and May and December 1981 from a Chihuahuan desert site in west Texas described previously (10). Plants were stored air-dry in the laboratory at room temperature under subdued light until used.

Crude Enzyme Extraction. Crude enzyme was prepared from both desiccated fronds and from fronds rehydrated in distilled H_2O. Each sample consisted of 0.5 g air-dry weight of detached frond material. Both treatments were incubated at 25°C for 24 h with constant illumination (500 $\mu E\ m^{-2}\ s^{-1}$). Crude enzyme was extracted following Eickmeier (12) by homogenizing samples for 1 min at 4°C in 20 ml of an extraction buffer which contained: 100 mM Tris-HCl (pH 8.1), 0.1 mM EDTA, and 5 mM freshly dissolved reduced glutathione. The homogenate was filtered through Miracloth, centrifuged for 20 min at 30,000*g* and 4°C, and the supernatant used for enzyme assays within 2 h.

Enzyme Assays. *Malate Dehydrogenase (EC 1.1.1.37).* Malate dehydrogenase was assayed following Davies (7). The assay mixture contained (in 2 ml): 200 mM K-phosphate (pH 7.8), 150 μM NADH, and 75 μM oxaloacetic acid in 0.6% disodium EDTA (w/v). The assay used 30 μl of crude enzyme. The decrease in O.D. at 340 nm was followed for 3 min at 25°C. This assay and all subsequent spectrophotometric assays used a Varian 635 LC spectrophotometer and were corrected for control values.

(NADH)Triose-P Dehydrogenase (EC 1.2.1.12). (NADH)-Triose-P dehydrogenase was assayed following Bovarnick *et al.* (4). The assay mixture contained (in 2 ml): 100 mM Tris-HCl (pH 8.1), 5 mM $MgCl_2$, 2.2 mM reduced glutathione, 2 mM ATP, 70 μM NADH, 1 mM 3-phosphoglyceric acid, and 0.12 unit of phosphoglycerate kinase (EC 2.7.2.3; 1 unit converts 1.0 μmol 3-phosphoglyceric acid to 1,3-diphosphoglyceric acid/min at 25°C). The assay used 400 μl of crude enzyme. The decrease in O.D. at 340 nm was followed for 3 min at 25°C.

(NADPH)-Triose-P Dehydrogenase (EC 1.2.1.13). (NADPH)Triose-P dehydrogenase was assayed as described

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above for (NADH)triose-P dehydrogenase except that 70 μM NADPH was added to the reaction mixture instead of NADH.

Pyruvate Kinase (EC 2.7.1.40). Pyruvate kinase was assayed following Kachmar and Boyer (17). The assay mixture contained (in 1.5 ml): 90 mM K-phosphate (pH 7.8), 6.8 mM $MgSO_4$, 2.2 mM ADP, and 2.2 mM phosphoenolpyruvate. The reaction was initiated with the addition of 0.5 ml of diluted crude enzyme (1:3 v/v with distilled H_2O) and was incubated for 15 min at 25°C. Controls used 0.5 ml of distilled H_2O. Pyruvate standard solutions (2 ml of 500, 250, 125, 62.5, and 31.25 μM) were assayed as well. All assays were terminated with 1 ml of 0.1% (w/v) dinitrophenylhydrazine in 2 N HCl. After 10 min, 7 ml of 0.43 N NaOH was added to all assays and incubated for 10 min at room temperature. Assay mixtures were then centrifuged at 2000 rpm for 15 min and the O.D. at 510 nm was determined for the supernatants and pyruvate concentrations determined from a standard curve.

Phosphoglycerate Kinase (EC 2.7.2.3). Phosphoglycerate kinase was assayed following Bovarnick *et al.* (4). The assay conditions and reaction mixture were identical to those for (NADH)triose-P dehydrogenase except that (NADH)triose-P dehydrogenase was used instead of phosphoglycerate kinase in the assay mixture.

Ribulose Bisphosphate Carboxylase (EC 4.1.1.39). RuBP[2] carboxylase was assayed following Kennedy (18). The assay mixture contained (in 100 μl): 25 mM tricine (pH 8.1), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 5.5 mM RuBP, 5 μM 6-phosphogluconate, and 50 mM $NaH^{14}CO_3$ (0.5 μCi). Assay mixtures were preincubated for 5 min after the addition of 25 μl of crude enzyme but before the addition of substrate. Assays were initiated with RuBP and incubated at 25°C for 2 min. Assays were terminated with 50 μl of glacial acetic acid. Controls were terminated after preincubation. Aliquots of the reaction mixture were dried on Whatman GF/A filters and incorporated label measured via liquid scintillation.

Citrate Synthase (EC 4.1.3.7). Citrate synthase was assayed following Parvin (21). For this assay, crude enzyme was prepared in the absence of GSH to eliminate competition with the assay product CoASH as measured with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Instead, the extraction buffer contained 1% (w/v) BSA and 0.16% (w/v) polyvinylpyrrolidone (25). The reaction mixture contained (in 2 ml): 100 mM Tris-HCl (pH 8.0), 0.25 mM DTNB, 0.2 mM oxaloacetate, and 0.1 mM acetyl-CoA. The assay used 100 μl of crude enzyme. The increase in O.D. at 412 nm was followed for 3 min at 25°C and corrected for control values.

Aconitase (EC 4.2.1.3). Aconitase was assayed following Fansler and Lowenstein (14). The reaction mixture contained (in 1 ml): 20 mM Tris-HCl (pH 7.8), 100 mM NaCl, and 0.5 mM *cis*-aconitate (neutralized to pH 7.0 with 6 N NaOH). A volume of 10 μl of crude enzyme was used. The reaction was initiated with the addition of *cis*-aconitate and the O.D. at 240 nm was followed for 3 min at 25°C.

Enolase (EC 4.2.1.11). Enolase was assayed following Spring and Wold (26). The reaction mixture contained (in 1 ml): 50 mM Tris-HCl (pH 7.8), 1 mM $MgSO_4$, 1 mM D-glyceric-2-phosphate, 0.1 mM KCl, and 10 μM EDTA. The assay used 50 μl of crude enzyme. The O.D. at 240 nm was followed for 3 min at 25°C.

Ribose-5-P Isomerase (EC 5.3.1.6). Ribose-5-P isomerase was assayed following Domagk and Alexander (9). The initial reaction mixture contained (in 400 μl): 75 mM Tris-HCl (pH 8.4), 7.5 mM ribose-5-P, 0.75 mM EDTA, and 0.375 mM 2-mercaptoethanol. Assays were initiated with 100 μl of crude enzyme diluted with assay buffer (1:10 v/v) and incubated for 15 min at 25°C. After incubation the following were added to each assay mixture: 5 ml 75% (v/v) H_2SO_4 in distilled H_2O, 0.2 ml 0.1% (w/v) carbazole in absolute ethanol, and 0.2 ml 30 mM cysteine. The resulting mixture was incubated at 25°C and the O.D. at 546 was determined and corrected for control values.

Protein Synthesis Inhibitor Experiments. Fronds were hydrated as before except that either chloramphenicol (CAP) or cycloheximide (CHI) was added to the hydration medium at 250 mg ml^{-1} or 25 mg ml^{-1}, respectively. Desiccated fronds and fronds rehydrated in the absence of inhibitor were used as controls. Protein synthesis inhibitor concentrations were taken from Eickmeier (12). Crude enzyme preparation and enzyme assays followed methods described earlier.

Enzyme Conservation Calculation and Statistical Analyses. Enzyme activities were expressed in terms of standard enzyme units (EU) per g air-dry weight. An enzyme unit was defined as either 1 μmol of substrate consumed or product formed per min at 25°C. Enzyme conservation was calculated as (enzyme activity of desiccated fronds/enzyme activity of hydrated fronds) × 100. Each enzyme assayed was replicated four times for both desiccated and hydrated frond treatments. Enzyme activities of desiccated and hydrated fronds were compared using a one-tailed *t*-test. The effects of protein synthesis inhibitors were examined with one-way ANOVA. All statistical procedures follow Sokal and Rohlf (24).

RESULTS AND DISCUSSION

All enzymes examined in *S. lepidophylla* exhibited activity in desiccated fronds (Table I). Significant increases in enzyme content occurred after 24 h of hydration for nine of the 10 enzymes assayed. The average conservation for the 10 enzymes was 74%, and ranged from 27% for (NADPH)triose-P dehydrogenase to 97% for malate dehydrogenase.

Enzyme conservation in desiccated leaf tissue has been reported in a variety of other desiccation-tolerant taxa. Stewart and Lee (27) found that enzymes in the bryophyte *Acrocladium cuspidatum* were highly conserved. Plants desiccated for 8 d at 8% RH showed an average conservation of 94% for 10 enzymes. This average is greater than that for *S. lepidophylla*, and the conservation difference may reflect the shorter desiccation period used in the bryophyte study. In contrast, the *S. lepidophylla* material had been desiccated for several months in the laboratory and probably longer than that in the field prior to collection.

Enzmyme conservation has also been reported for the bryophyte *Tortula ruralis*. Sen Gupta (23) found a 70% conservation of RuBP carboxylase along with substantial conservation for glutamate oxaloacetate transaminase, malate dehydrogenase, and phosphoenolpyruvate carboxylase. In addition, both catalase and superoxide dismutase conservation has been found in this species by Dhindsa and Bewley (unpublished work cited in Ref. 2). The desiccation-tolerant angiosperm *Xerophyta viscosa* exhibited 40% conservation of RuBP carboxylase activity, even though the fraction 1 protein content does not appear to decline with leaf desiccation (6). Gundel (cited in Ref. 2) has also found conservation of a variety of enzymes in desiccated leaves of the angiosperm *Myrothamnus flabellifolia*.

A general comparison of enzyme conservation in *S. lepidophylla* by function indicates that conservation of photosynthetic enzymes was significantly less (60%) than that for respiratory enzymes (82%). After an arcsin square root transformation for percent data, a t-test of means gave a *t*-value of 1.95 ($df = 7$) which represents a significant difference precisely at the $P = 0.05$ probability level. Phosphoglycerate kinase was not included in this analysis because it has photosynthetic and glycolytic functions which were not differentiated. This result is consistent with that of Cowan *et al.* (5) which suggests a greater sensitivity of

[2] Abbreviations: RuBP, ribulose bisphosphate; CAP, chloramphenicol; CHI, cycloheximide.

Table I. *Enzyme Activities of Desiccated Fronds and Fronds Hydrated for 24 Hours in the Resurrection Plant S. lepidophylla*

Enzyme	Enzyme Activity		Conservation	*t* Value
	Desiccated fronds	Hydrated fronds		
	enzyme units g^{-1} dry wt		%	
Aconitase	1.35 ± 0.05[a]	1.88 ± 0.07	72	12.62***[b]
Citrate synthase	1.76 ± 0.03	2.05 ± 0.13	86	2.15*
Malate dehydrogenase	2.89 ± 1.39	2.97 ± 1.62	97	0.09 NS
Enolase	35.1 ± 2.9	40.0 ± 2.6	88	2.44*
Pyruvate kinase	1.26 ± 0.07	1.89 ± 0.19	67	6.26***
(NADH)Triose-P dehydrogenase	1.13 ± 0.05	1.40 ± 0.12	81	4.33***
Phosphoglycerate kinase	3.74 ± 0.52	4.61 ± 0.75	81	1.92*
Ribose-5-P isomerase	7.56 ± 0.87	9.24 ± 0.84	82	3.94***
RuBP carboxylase	0.60 ± 0.04	0.96 ± 0.10	62	6.82***
(NADPH)Triose-P dehydrogenase	0.48 ± 0.23	1.80 ± 1.23	27	2.09*

[a] Mean ($n = 4$) ± SE. [b] Unpaired, one-tailed *t* test, $df = 6$; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, NS = not significant.

photosynthetic than respiratory processes to desiccation for the lichens *Ramalina celastri* and *Peltigera polydactyla*.

The reduced conservation of Calvin cycle enzymes in *S. lepidophylla* may have important physiological consequences during plant rehydration. RuBP carboxylase, which is usually considered a major rate limiting step in photosynthetic carbon assimilation with respect to both chloroplast (22) and whole-leaf (3) levels, has the second lowest conservation level of the enzymes examined in addition to low absolute activity in desiccated fronds. (NADPH)Triose-P dehydrogenase shows even lower conservation and content in dry tissue (Table I). This latter result is consistent with the finding of Stewart and Lee (27) that (NADPH)triose-P dehydrogenase was least conserved in *A. cuspidatum*. This enzyme may be particularly sensitive to desiccation, and may be rate-limiting to photosynthetic carbon reduction early in rehydration. The delayed onset of photosynthetic activity relative to respiratory activity seen in *S. lepidophylla* upon rehydration (10) may be partly due to the loss of potential activity of RuBP carboxylase and (NADPH)triose-P dehydrogenase during the previous dehydration event.

Zeigler (30), on the other hand, concluded that the delay in the development of respiratory and photosynthetic activity of *M. flabellifolia* was due not to the lack of soluble enzyme activity but to the lack of intact membrane structures involved in electron transport. This conclusion does not seem warranted in the present case as respiratory activity develops very rapidly upon rehydration in *S. lepidophylla* (10). Furthermore, this species shows a considerable conservation of both chloroplast and mitochondrial ultrastructure (1). Also, both respiratory CO_2 evolution and subsequent net photosynthetic CO_2 uptake can occur in *S. lepidophylla* during rehydration in the presence of protein synthesis inhibitors at concentrations that cause significant reductions in [^{35}S]methionine incorporation (12). This suggests that conserved enzyme function, in the absence of *de novo* protein synthesis, is of great physiological significance and can influence patterns of cell metabolism early in the rehydration cycle.

Table II. *Effects of Protein Synthesis Inhibitors on Enzyme Activity of Selected Enzymes in the Resurrection Plant S. lepiodphylla*

Treatment	Enzyme Activity		
	(NADPH)Triose-P dehydrogenase	RuBP carboxylase	Pyruvate kinase
	enzyme units g^{-1} dry wt		
Desiccated	1.67 ± 0.22[a]	0.60 ± 0.02	0.92 ± 0.12
24 h Hydrated	4.43 ± 0.65	0.98 ± 0.05	1.35 ± 0.06
24 h Hydrated + 250 mg ml^{-1} CAP	2.25 ± 0.44	0.75 ± 0.04	1.51 ± 0.20
24 h Hydrated + 25 mg ml^{-1} CHI	4.09 ± 0.63	0.96 ± 0.03	1.54 ± 0.11
F value ($df = 3, 12$)	6.91**[b]	21.42***	4.69*
$LSD_{0.05}$[c]	1.59	0.12	0.41

[a] Mean ($n = 4$) ± SE. [b] Statistical significance notation follows that for Table I. [c] $LSD_{0.05}$ = least significant difference at $P = 0.05$, mean differences that exceed this value are significantly different from each other.

The effects of protein synthesis inhibitors on the increased enzyme activity normally observed during rehydration were examined with the three enzymes showing the lowest degree of conservation. Increases in enzyme content above desiccated levels were significantly inhibited upon rehydration for both (NADPH)triose-P dehydrogenase and RuBP carboxylase (Table II). The (NADPH)triose-P dehydrogenase content was not significantly different from desiccated controls and RuBP carboxylase content, while increasing relative to desiccated controls, was 58% below hydrated control values. Cycloheximide treatment did not significantly affect the normal increase in activity for either enzyme. The large subunit of RuBP carboxylase is synthesized in the chloroplast and its inhibition by chloramphenicol has been documented; on the other hand, inhibition of (NADPH)triose-P dehydrogenase activity by CAP was unexpected as its synthesis is reportedly cytoplasmic (13). We have no convincing explanation for this unusual result. Neither CAP nor CHI significantly affected the activity of pyruvate kinase upon rehydration. While conserved activity of the enzymes is important to early physiological reactivation as described above, these results indicate that *de novo* synthesis of some, but perhaps not all, enzymes may also contribute to enhanced physiological activity upon rehydration.

In summary, the conservation of potential enzymic function in desiccated fronds is both quantitatively high and widespread among enzymes involved with important aspects of carbohydrate metabolism. In general, however, photosynthetic enzymes showed a lower degree of conservation than those associated with respiration. This difference may explain the rapid resumption of respiratory activity and the delayed development of photosynthetic activity seen in this and other resurrection species upon

rehydration. Finally, the results indicate that while enzyme conservation may be important in the physiological reactivation process, *de novo* synthesis of some enzymes during rehydration also contributes to full physiological recovery in desiccation-tolerant plants.

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