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GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN COLD HARDY INSECTS: KINETIC PROPERTIES, FREEZING STABILIZATION, AND CONTROL OF HEXOSE MONOPHOSPHATE SHUNT ACTIVITY

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Abstract—Properties of glucose-6-phosphate dehydrogenase were assessed from the larvae of three insect species, the freeze tolerant *Eurosta solidaginis*, the freeze avoiding *Epiblema scudderiana*, and warm-acclimated *Tenebrio molitor*. Maximal enzyme activities were 16–17 fold higher in the cold hardy larvae than in *T. molitor* in line with the key role of G6PDH in providing NADPH for the synthesis of cryoprotectant polyols in these species. K_m values for glucose-6-P and NADP were determined at both high (24°C) and low (4°C) temperatures for all three enzymes. Temperature decrease had the greatest effect on *T. molitor* G6PDH increasing K_m glucose-6-P by 3-fold and K_m NADP by 2-fold; K_m values for G6PDH from the cold hardy species were less temperature-sensitive. The addition of polyols (glycerol, sorbitol) or KCl caused selected changes in the K_m values for both substrates in all species. Cryoprotectant action in the freezing protection of G6PDH was also examined, comparing G6PDH from *E. solidaginis*, *E. scudderiana* and yeast. A range of polyols (glycerol, sorbitol), other carbohydrates (trehalose, glucose, lactate) and amino acids (alanine, glutamate, proline) were effective in protecting activity during freezing. Without cryoprotectant, enzyme activity after 1 h freezing at −77°C was <10% compared to controls. Low concentrations of protectants (typically <50 mM) gave complete protection during freezing. Values for cryoprotectant concentrations giving half-maximal recovery of activity ranged from as low as 7–10 mM for trehalose to 20–25 mM for most other compounds.

Key Word Index: *Eurosta solidaginis*; *Epiblema scudderiana*; *Tenebrio molitor*; hexose monophosphate shunt; polyol synthesis; cryoprotectants; freeze denaturation of enzymes

INTRODUCTION

Glucose-6-P dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49) (G6PDH) catalyzes the first reaction of the hexose monophosphate shunt and is one of the two NADPH-generating steps in the shunt. The enzyme is effectively rate-limiting in controlling flux into the shunt under most physiological conditions (Levy, 1979). G6PDH is frequently subject to coarse control by changes in enzyme maximal activity in response to dietary or other physiological stimuli. Fine control is generally the result of potent product inhibition by NADPH and the effective thermodynamic irreversibility of the reaction because of the rapid hydrolysis of the D-glucono-δ-lactone-6-P product (Levy, 1979).

In cold-hardy insects flux through the hexose monophosphate shunt is critically important for generating the reducing equivalents needed for the synthesis of polyol cryoprotectants. Indeed, it can be calculated that for glycerol synthesis from glycogen 86% of the total carbon flow must be routed through the shunt (before exiting as fructose-6-P or glyceraldehyde-3-P) to generate the required reducing power (Storey and Storey, 1988). For synthesis of hexitols, such as sorbitol, the value is lower but still nearly half (46%) of the total carbon flow. With glycerol levels that may be as high as 2 M or 20% of fresh weight in fully cold-hardened insects, the importance of efficient regulation of hexose monophosphate shunt activity is obvious.

The present study examines G6PDH from two cold-hardy insect larvae, the freeze tolerant goldenrod gall fly, *Eurosta solidaginis* (Fitch) (Diptera, Tephritidae), and the freeze avoiding goldenrod gall moth, *Epiblema scudderiana* (Clemens) [Lepidoptera, Olethreutidae]. *E. solidaginis* larvae produce both glycerol and sorbitol, often in a nearly 2:1 ratio, with midwinter levels of 250–400 μmol/g wet wt glycerol and 100–150 μmol/g sorbitol (Morrissey and Baust, 1976; Storey *et al.*, 1981; Storey and Storey, 1986). *E. scudderiana* accumulates glycerol alone in amounts of 2000–2300 μmol/g (Rickards *et al.*, 1987; Churchill and Storey, 1989a). During the synthesis of polyols from glycogen, hexose monophosphate shunt activity is enhanced (Kageyama, 1976; Wood and Nordin, 1980; Tsumuki *et al.*, 1987) and, as the gateway to this pathway, G6PDH clearly becomes an important enzyme locus in the metabolism of cold-hardy insects. In the present study, we analyze the kinetic properties of G6PDH from these two species, and the influence of both low temperature and polyols on the enzyme. We also compare the enzyme from cold-hardy insect

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Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase; G6P, glucose-6-phosphate.

larvae with G6PDH from larvae of a warm-acclimated insect species.

For freeze tolerant animals the preservation of enzyme/protein structure and function during the freezing process is an additional requirement. Because freezing is restricted to extracellular spaces only, intracellular enzymes are not in direct contact with ice but the influence of ice is felt as the dehydration of the cell and the greatly increased concentration of solutes in the remaining liquid fraction. Many authors have studied the denaturing effects of freezing on proteins and enzymes and the actions of polyhydric alcohols and other compounds in preserving structure and function after freezing (Fink, 1986; Gekko, 1983; Carpenter and Crowe, 1988). In most of these studies the focus has been on protein chemistry and purified enzymes have been used as model systems in order to examine the physical processes involved in freeze denaturation and molecular actions of solutes in stabilizing protein structure. Enzymes from naturally freeze tolerant animals have never been studied in this manner, however. Two questions can be asked: (1) Does an enzyme from a freeze tolerant animal have a greater inherent stability to freezing than the homologous enzyme from another source? and (2) Do the naturally-occurring cryoprotectants offer specifically enhanced cryopreservation of enzymes compared to other, chemically-similar compounds? The present study examines these questions by comparing the effects of freezing on G6PDH from freeze tolerant versus intolerant species.

MATERIALS AND METHODS

Chemicals and animals

Biochemicals were purchased from Sigma Chemical Co., St Louis, Mo. or Boehringer Mannheim, Montreal, PQ; yeast G6PDH was from Boehringer. Goldenrod galls containing larvae of *E. solidaginis* and *E. scudderiana* were collected in the autumn of 1988 from fields around Ottawa and were acclimated in the lab to a constant 3°C for several weeks. Galls were then opened and the larvae removed, frozen in liquid nitrogen, and transferred to −77°C for storage. *T. molitor* larvae were obtained from a Carleton University colony that is maintained at room temperature; larvae were frozen and stored as above.

Enzyme kinetic studies

Frozen larvae were rapidly weighed and homogenized (1:2 w/v for *T. molitor*; 1:5 w/v for the others) in buffer containing 20 mM imidazole–HCl, pH 7.2 (adjusted at 24°C) and 15 mM 2-mercaptoethanol. *E. solidaginis* and *E. scudderiana* preparations used a glass/glass hand-held homogenizer and centrifugation in an Eppendorf microcentrifuge at 12,000 *g* for 10 min at 5°C. *T. molitor* preparations used a Polytron PT10 homogenizer and centrifugation in a Sorvall RC5B centrifuge at 21,000 *g* for 10 min at 5°C; the low activity of G6PDH in *T. molitor* necessitated the higher centrifugation speed to produce sufficient amounts of clear supernatant. Low molecular weight metabolities were then removed by centrifuging the supernatant fractions through 5 mL columns (a maximum of 0.5 mL enzyme extract loaded per column) of Sephadex G-25 (equilibrated in homogenization buffer) in an IEC benchtop centrifuge at top speed for 1 min (Helmerhorst and Stokes, 1980). The resulting filtrate was used as the enzyme source for kinetic studies.

Standard conditions for maximal G6PDH activity were: 20 mM imidazole–HCl buffer pH 7.2 (at 24°C), 4 mM $MgSO_4$, 1 mM glucose-6-P (G6P), and 0.5 mM NADP (1 mM for *T. molitor*). Enzyme kinetic constants were assessed at both 24 and 4°C using a Pye Unicam SP 8–100 recording spectrophotometer attached to a Haake KT33 circulating water bath for temperature control of the cuvettes. Arrhenius plots were constructed using measurements of the maximal activities of G6PDH at seven temperatures between 2 and 40°C.

Enzyme freezing studies

All enzyme preparations and cryoprotectant and KCl stocks were made in freezing buffer: 20 mM imidazole–HCl, pH 7.2 (at 24°C) with 15 mM 2-mercaptoethanol. Purified yeast G6PDH was diluted 1:200 v/v with this buffer to produce a stock solution of 3.5 U/ml; bovine serum albumin was added to this in a concentration of 1 mg/ml to provide a soluble protein concentration that was similar to that in the enzyme extracts of whole larvae. Extracts of *E. solidaginis* and *E. scudderiana* were prepared as above but using a 1:10 w/v homogenate. Low molecular weight compounds (including endogenous cryoprotectants) were again removed by passage through Sephadex G-25 spun columns; for *E. scudderiana* extracts had to be passed through two such columns in series in order to remove all of the endogenous glycerol. For freezing tests, 50 μl aliquots of enzyme preparation were combined in Eppendorf polypropylene microcentrifuge tubes with appropriate aliquots of cryoprotectants and KCl and then made up to a final volume of 200 μl with freezing buffer. Tubes were capped, mixed, and then frozen for 1 h in a freezer at −77°C. Equivalent control tubes were maintained throughout on ice. After freezing, tubes were allowed to thaw for a few minutes at room temperature, and then were mixed and stored on ice. Enzyme activity was assayed in all samples using the standard assay conditions, outlined above. The recovery of enzyme activity after freezing was expressed as a percentage of activity in the equivalent control sample.

For each cryoprotectant, plots relating recovery of G6PDH activity vs cryoprotectant concentration were constructed using 8–10 different concentrations of cryoprotectant, with $n = 3$ replicates of each experiment using different preparations of enzyme. P_{50} values, the amount of protectant providing 50% recovery of enzyme activity after freezing, were calculated by using a computer program to fit the data to a modified Hill equation.

RESULTS

G6PDH in three insect species

Table 1 shows the maximal activities of G6PDH in the three insect larvae along with substrate affinity constants at 24°C. The two cold hardy species had much higher maximal activities of the enzyme, 16–17 fold greater than the activity in *T. molitor*. All three enzymes showed high affinity for both substrates; *E. scudderiana* had the lowest K_m G6P (21.3 μM) and *E. solidaginis* the highest, 3-fold greater. K_m NADP was lowest (3.1 μM) in *E. solidaginis* and 6-fold higher in *T. molitor*.

Table 1. Maximal activities (μmol G6P utilized min^{-1} g wet $wt^{-1} \pm$ SEM, $n = 3$) and kinetic constants for glucose-6-P dehydrogenase from different sources, assessed at 24°C

	E. solidaginis	*E. scudderiana*	*T. molitor*
Units/g	4.38 ± 0.03	4.02 ± 0.09	0.25 ± 0.02
K_m G6P, μM	62.9 ± 1.2	21.3 ± 0.13	31.4 ± 3.5
K_m NADP, μM	3.13 ± 0.03	9.50 ± 0.6	17.9 ± 2.0

Table 2. Effects of temperature and of cryoprotectants and salt on the kinetic constants for *E. solidaginis* glucose-6-P dehydrogenase

Addition	K_m G6P, μM		K_m NADP, μM		Activation energy	Q_{10}
	24°C	4°C	24°C	4°C	cal/deg mol	0–10°C
No addition	63 ± 1.2	52 ± 1.9†	3.1 ± 0.03	5.3 ± 0.1†	13,025 ± 215	2.34 ± 0.03
Sorbitol, 250 mM	71 ± 3.3*	66 ± 0.8*†	4.3 ± 0.1*	7.1 ± 0.2*†	ND	
Glycerol, 500 mM	59 ± 1.6	42 ± 4.6†	3.6 ± 0.23*	10.3 ± 0.2*†	ND	
Sorbitol and glycerol, 250/500 mM	58 ± 2.5	48 ± 0.9†	4.0 ± 0.23*	9.7 ± 0.2*†	14,889 ± 287*	2.65 ± 0.04*
KCl, 600 mM	123 ± 1.5*	53 ± 1.2†	23 ± 0.7*	31 ± 1.4*†	12,525 ± 191	2.27 ± 0.02
Sorbitol, glycerol and KCl, 250/500/600 mM	125 ± 0.9*	101 ± 4.1*†	22 ± 1.9*	20 ± 1.2*	13,073 ± 119	2.34 ± 0.02

Values are means ± SEM, $n = 3$ determinations on separate preparations of enzyme.
*Significantly different from the corresponding control (no additions) value by the Student's t-test, $P < 0.05$.
†Significantly different from the corresponding value at 24°C, $P < 0.05$.
ND—not determined.

Temperature and cryoprotectant effects on the properties of E. solidaginis G6PDH

Table 2 shows the K_m for G6P and NADP of *E. solidaginis* G6PDH assayed at 24 and 4°C and in the presence or absence of added cryoprotectants and/or KCl. In the absence of additions, the enzyme assayed at 4°C showed a significant decrease in K_m G6P and a significant increase in K_m NADP, as compared to the enzyme at 24°C. These same qualitative effects of low temperature on K_m G6P and K_m NADP were also seen in the presence of added cryoprotectants or salt, with one exception (K_m NADP in the presence of sorbitol, glycerol and KCl was unchanged at low temperature). The addition of natural cryoprotectants to the assay had some effects on enzyme kinetic properties. In the presence of 250 mM sorbitol, K_m G6P increased significantly but was unaffected by the addition of glycerol (500 mM) or the addition of both polyols together. K_m NADP was increased significantly in the presence of polyols in all cases, with the effect being much more pronounced at 4°C. The addition of KCl raised the K_m NADP by 6–7 fold and increased K_m G6P at 24°C. Sorbitol and glycerol, when added together with KCl, did not change the effects of KCl on enzyme properties except to cause an increase in K_m G6P at 4°C.

Arrhenius plots for *E. solidaginis* G6PDH were in all cases linear over the wide temperature range tested (2–40°C) with no indication of breaks in the plot. The activation energy was increased slightly in the presence of glycerol + sorbitol but was not affected by the other conditions tested (Table 2). Q_{10} values for the interval 0–10°C were calculated; the value was 2.34 for the control condition and was significantly higher (by 13%) only in the presence of glycerol + sorbitol.

Temperature and cryoprotectant effects on the properties of E. scudderiana G6PDH

Table 3 shows the effects of temperature and polyols on the K_m values for *E. scudderiana* G6PDH. In the absence of effectors, the enzyme assayed at 4°C showed an increase in K_m G6P and no change in K_m NADP as compared to the assay at 24°C. The addition of the natural cryoprotectant glycerol in increasing amounts led to a progressive decrease in the K_m G6P and increased the K_m NADP, the effects being somewhat more pronounced at 24°C. KCl had little effect on the K_m G6P but doubled the K_m NADP at both temperatures. Glycerol + KCl added together appeared to negate the effects of each compound individually when assayed at 24°C but at 4°C glycerol + KCl had strong effects resulting in a 3-fold decrease in K_m G6P and a 2-fold increase in K_m NADP.

Arrhenius plots for *E. scudderiana* G6PDH were linear over the 2–40°C range with no breaks. The activation energy decreased progressively with the addition of increasing amounts of glycerol and glycerol + KCl together had the greatest effect on activation energy. Q_{10} values for the 0–10°C interval showed the same pattern, decreasing from 2.47 for the enzyme without additions to 1.71 for the enzyme in the presence of glycerol + KCl.

Temperature and cryoprotectant effects on the properties of T. molitor G6PDH

In the absence of effectors, low temperature strongly reduced substrate affinities of *T. molitor* G6PDH, K_m values increasing by 3-fold for G6P and by 2-fold for NADP (Table 4). A similar qualitative effect of low temperature also occurred in most instances when cryoprotectant or KCl were present. The addition of polyols, glycerol in an amount

Table 3. Effects of temperature and of cryoprotectants and salt on the kinetic constants for *E. scudderiana* glucose-6-P dehydrogenase

Addition	K_m G6P, μM		K_m NADP, μM		Activation energy	Q_{10}
	24°C	4°C	24°C	4°C	cal/deg mol	0–10°C
No addition	21 ± 0.1	31 ± 3.0†	9.5 ± 0.6	7.5 ± 0.8	13,838 ± 191	2.47 ± 0.03
Glycerol, 500 mM	13 ± 0.7*	29 ± 0.7†	16 ± 1.1*	16 ± 1.9*	11,137 ± 382*	2.03 ± 0.05*
Glycerol, 2 M	6.2 ± 0.9*	14 ± 1.1*†	32 ± 2.0*	14 ± 1.2*†	9,058 ± 502*	1.81 ± 0.05*
KCl, 100 mM	28 ± 0.9*	25 ± 0.9†	19 ± 1.5*	14 ± 0.7*†	12,810 ± 262*	2.31 ± 0.04
Glycerol and KCl, 2 M/100 mM	18 ± 0.6*	10 ± 0.9*†	10 ± 1.3	17 ± 1.4*†	8,580 ± 95*	1.71 ± 0.04*

Values are means ± SEM, $n = 3$ determinations on separate preparations of enzyme.
*Significantly different from the corresponding control (no additions) value by the Student's t-test, $P < 0.05$.
†Significantly different from the corresponding value at 24°C, $P < 0.05$.
ND—not determined.

Table 4. Effects of temperature and of cryoprotectants and salt on the kinetic constants for *T. molitor* glucose-6-P dehydrogenase

Addition	K_m G6P, μM		K_m NADP, μM		Activation energy	Q_{10}
	24°C	4°C	24°C	4°C	cal/deg mol	0–10°C
No addition	31 ± 3.5	94 ± 1.2†	18 ± 2.0	38 ± 8.0†	15,654 ± 1100	2.80 ± 0.20
Glycerol, 2 M	10 ± 1.0*	36 ± 0.9*†	12 ± 2.5	21 ± 4.0	14,100 ± 430	2.45 ± 0.07
Sorbitol and glycerol, 250/500 mM	20 ± 1.6*	53 ± 0.9*†	28 ± 3.0*	43 ± 1.0†	14,603 ± 884	2.60 ± 0.20
KCl, 100 mM	58 ± 0.4*	51 ± 1.0*†	37 ± 5.0*	52 ± 6.0	20,482 ± 2247*	4.00 ± 0.30*
Glycerol and KCl 2 M/100 mM	22 ± 1.1*	93 ± 4.0†	27 ± 1.3*	51 ± 7.0†	13,240 ± 454*	2.39 ± 0.09*
Sorbitol, glycerol and KCl 250/500/100 mM	30 ± 2.0	49 ± 9.0*†	79 ± 13*	79 ± 9.0*	12,249 ± 382*	2.19 ± 0.06*

Values are means ± SEM, $n = 3$ determinations on separate preparations of enzyme.
*Significantly different from the corresponding control (no additions) value by the Student's t-test, $P < 0.05$.
†Significantly different from the corresponding value at 24°C, $P < 0.05$.
ND—not determined.

similar to the natural levels in *E. scudderiana* or glycerol + sorbitol in amounts similar to the natural levels in *E. solidaginis*, reduced the K_m G6P in a manner that correlated with total polyol concentration. KCl had the opposite effect on K_m G6P at 24°C and KCl plus polyols had opposing effects that resulted in intermediate values for K_m. K_m NADP of *T. molitor* G6PDH was largely unaffected by polyols or salt when assayed at 4°C but at 24°C K_m NADP increased significantly under all conditions except in the presence of glycerol alone.

Arrhenius plots for *T. molitor* G6PDH were again linear over the 2–40°C range but with activation energy somewhat higher than for the other two species. The addition of KCl increased the activation energy by 30% but the addition of polyols with KCl reversed this. Q_{10} values for the 0–10°C range followed the same pattern with Q_{10} rising from 2.8 in the absence of additions to 4.0 in the presence of KCl.

Freezing protection of G6PDH

To determine whether G6PDH from freeze tolerant vs freeze intolerant organisms was differently affected by freezing or responded differently to the addition of cryoprotectants during freezing, G6PDH from three sources (*E. solidaginis*, *E. scudderiana* and yeast) was frozen in the presence of varying amounts of putative cryoprotectant substances. In the absence of cryoprotectants, Fig. 1 shows that virtually all enzyme activity was lost with 1 h freezing at −77°C (in buffer containing 20 mM imidazole pH 7.2, 15 mM 2-mercaptoethanol and with 100 mM KCl). Parenthetically, a preliminary time course showed that this level of activity loss was attained after 10 min of freezing with a half-time of only about 3 min. Enzyme activity was stabilized during freezing by the addition of cryoprotectants and Fig. 1 shows the effects of selected protectants on the three enzymes. In all cases, we found that 100% recovery of activity (compared to identical control preparations maintained on ice throughout) was achieved when sufficiently high amounts of cryoprotectant were added; in most cases this required as little as 50 mM protectant.

The relationship between cryoprotectant concentration and activity recovered after freezing was a sigmoidal one, and in most cases, cryoprotection rose from 0 to 100% over a very narrow concentration range (Fig. 1). To quantify the protection provided by different compounds, we calculated a P_{50} value, the amount of protectant providing half maximal freezing protection. To do this we used an adaptation of

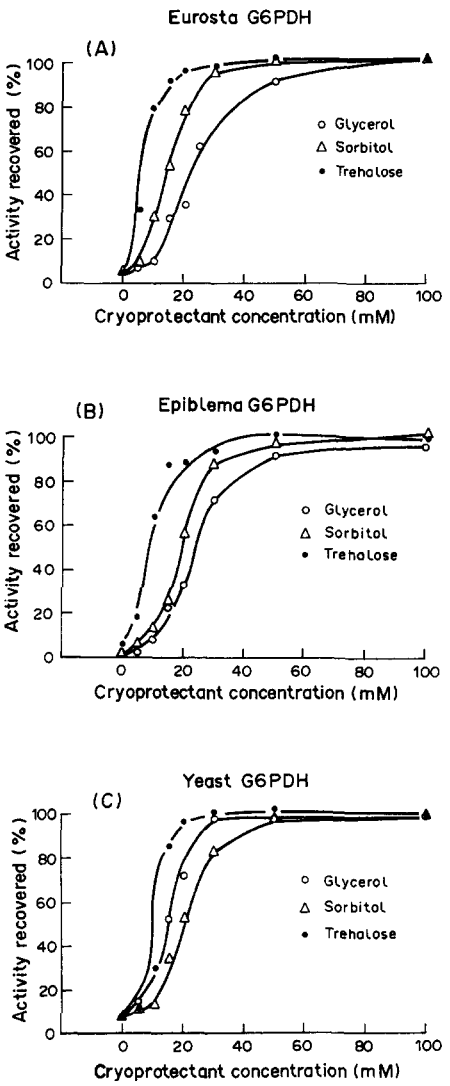

Fig. 1. Effect of cryoprotectant concentration on the recovery of G6PDH activity after freezing for 1 h at −77°C. (A) *E. solidaginis* G6PDH; (B) *E. scudderiana* G6PDH; (C) purified yeast G6PDH. Effects of three protectants are shown: (○) glycerol, (△) sorbitol, and (●) trehalose. Percent recovery is calculated relative to paired samples for each cryoprotectant concentration that were kept on ice throughout. Data show representative curves ($n = 1$) for protection by each compound; equivalent curves ($n = 3$) were used to determine the P_{50} values reported in Table 4. All freezing tests were done in the presence of 100 mM KCl added.

the Hill equation (for sigmoidal relationships) that is normally used to describe activator effects on enzymes and takes into account the fact that enzyme velocity in the absence of activator (or protectant, in this case) is greater than zero. Table 5 shows the calculated P_{50} values for freezing protection of the three enzymes by 11 different compounds. Most of the compounds tested provided good cryoprotection of G6PDH with P_{50} values ranging from 6–30 mM. Glycerol + sorbitol together (tested as a 2:1 molar mix) showed P_{50} values not significantly different from those for glycerol alone but significantly higher ($P < 0.005$) than the P_{50} values for sorbitol alone for both *E. solidaginis* and *E. scudderiana* G6PDH. Only L-alanine and taurine stood out as requiring distinctly higher levels to provide half-maximal freezing protection. Trehalose, by contrast, provided cryoprotection at significantly lower concentrations than any other compound, the P_{50} values being < 10 mM in all cases. There was also a species-specific pattern to the cryopreservation. In almost all cases, the P_{50} values were highest for *E. scudderiana* and lowest for *E. solidaginis* G6PDH.

Table 6 shows the effects of KCl on the recovery of yeast G6PDH after freezing and the interaction of salt concentration with the protective actions of three cryoprotectants. The recovery of enzyme activity after freezing increased progressively with increasing salt concentration in the absence of added cryoprotectants. P_{50} values for glycerol and L-proline showed no dependence on KCl concentration whereas P_{50} for glucose increased slightly with increasing salt.

Table 5. Freezing protection of glucose-6-P dehydrogenase by various low molecular weight compounds. P_{50} is defined as the amount of cryoprotectant that provides half-maximal recovery of enzyme activity after 1 h freezing at −77°C

	P_{50} values (mM)		
	E. solidaginis	*E. scudderiana*	Yeast
Glycerol	20.7 ± 1.6	24.6 ± 0.9	16.9 ± 2.1‡
Sorbitol	14.5 ± 0.8	18.4 ± 0.6†	18.3 ± 0.9†
Glycerol/sorbitol*	23.6 ± 0.6	26.7 ± 1.5	19.7 ± 1.1†‡
Glucose	17.2 ± 0.6	19.4 ± 0.5†	15.9 ± 0.6‡
Trehalose	6.7 ± 0.02	9.0 ± 1.0†	9.8 ± 1.0†
L-Lactate	18.6 ± 1.0	24.5 ± 0.3†	19.5 ± 1.3
L-Proline	12.9 ± 0.8	20.4 ± 2.6†	11.9 ± 0.5
L-Glutamate	14.1 ± 1.0	20.3 ± 1.0†	16.8 ± 1.0
L-Alanine	20.5 ± 0.9	19.2 ± 2.0	48.6 ± 7.0†
Taurine	23.6 ± 3.2	49.1 ± 10.1†	74.7 ± 12.8†
Glucose-6-P	17.5 ± 1.1	28.9 ± 2.3†	18.3 ± 0.6†

Data are means ± SEM of P_{50} determinations on $n = 3$ separate samples from each species with 8–10 different concentrations of cryoprotectant used for each P_{50} determination (see Fig. 1). All freezing tests were done in the presence of 100 mM KCl.

*Glycerol/sorbitol was tested in a 2:1 molar mix and the P_{50} values reported refer to the total polyol concentration present.

†Significantly different from the corresponding value for *E. solidaginis* G6PDH by the Student's *t*-test, $P < 0.05$.

‡Values for *E. scudderiana* and yeast G6PDH are significantly different, $P < 0.05$.

Table 6. Effect of KCl concentration on the recovery of yeast glucose-6-P dehydrogenase activity after freezing

	KCl concentration		
	0 mM	100 mM	600 mM
% Recovery without cryoprotectants	5.9 ± 0.8%	8.7 ± 1.5%	25.1 ± 1.8*%
P_{50} values for cryoprotectants			
Glycerol	17.6 ± 1.1	16.9 ± 2.1	17.4 ± 1.4
Glucose	10.2 ± 0.7	15.9 ± 0.6*	17.8 ± 1.3*
L-Proline	13.2 ± 1.4	11.9 ± 0.5	12.6 ± 0.7

*Significantly different from the corresponding value with 0 mM KCl by the Student's *t*-test, $P < 0.005$.

DISCUSSION

Flux through the hexose monophosphate shunt and the associated polyol production is undoubtedly aided in the two cold hardy insect species by the high relative activities of G6PDH in these animals, maximal activity in the whole larvae being 16–17 fold greater than in the *T. molitor* larvae maintained at constant warm temperature. It should be noted that *T. molitor* larvae in nature also undergo cold hardening for the winter months but the primary adaptation involved is the synthesis of antifreeze proteins (Duman *et al.*, 1982); the species does not produce polyols. G6PDH activity in *E. solidaginis* (4.4 U/g) was also high relative to the activities of other enzymes that would compete for hexose phosphates, glucose-6-phosphatase (1 U/g) and phosphofructokinase (0.9 U/g) (Storey and Storey, 1981), and this would help to promote the high relative flux through the hexose monophosphate shunt (compared to glycolysis) that is seen during active polyol synthesis in this species (Tsumuki *et al.*, 1987).

The substrate affinity constants of G6PDH from the cold-hardy species, measured at 24°C, were not distinctly different from the range of values reported for G6PDH from other insect species (Cherfurka, 1958; Horie, 1967; Takahashi *et al.*, 1974) or from various bacterial and mammalian sources (Levy, 1979). However, several other factors may aid enzyme performance during active polyol synthesis. These include: (1) substrate availability, (2) temperature effects on enzyme properties, and (3) the interactions of temperature and polyols on the enzyme. Substrate availability is likely to play an important role in G6PDH function and hexose monophosphate shunt activity. Cold activation of glycogen phosphorylase, the initial step in polyol synthesis (Storey and Storey, 1988), leads to a rapid rise in G6P levels. For example, triggering of sorbitol synthesis at 3°C in *E. solidaginis* produced a 2.5 fold increase in G6P within 2 h to a level of 400 μM that was maintained over several days while rates of polyol synthesis were high (Storey and Storey, 1983). Cold activation of glycerol biosynthesis in *E. scudderiana* had a comparable effect, a 4.5 fold rise in G6P to 450 μM which was again maintained over several days (Churchill and Storey, 1989b). Compared to K_m G6P values at 4°C for *E. solidaginis* (42–101 μM) (Table 1) and *E. scudderiana* (10–31 μM) (Table 2), it is apparent that during active polyol synthesis, G6PDH probably functions with no limitation by G6P availability.

Temperature effects on G6PDH are the second factor in enzyme control. Neither *E. solidaginis* nor *E. scudderiana* G6PDH showed unfavourable effects of low temperature on enzyme maximal activity. Arrhenius plots were linear between 2 and 40°C and calculated Q_{10} values for the interval 0–10°C were 2.34 for *E. solidaginis* and 2.47 for *E. scudderiana* G6PDH (Tables 2 and 3), values that are typical of the effects of temperature change on the activity of most enzymes and biological processes (Hochachka

and Somero, 1984). However, the activity of G6PDH from the warm-adapted *T. molitor* was more strongly reduced by decreasing temperature; activation energy and the Q_{10} of 2.8 were both significantly higher than for the other species. In *E. solidaginis* a comparison can also be made with the Q_{10} values for other enzymes from this species. Q_{10} for *E. solidaginis* glycerol-3-P dehydrogenase was 2.16 and for glutamate dehydrogenase was 2.0 indicating that the relative activities of many enzymes in the cell may decrease in concert with decreasing temperature (Storey and Storey, 1982; Male and Storey, 1982). However, phosphofructokinase from *E. solidaginis* was distinctly different with a Q_{10} of 3.6 (Storey, 1982). The strong reduction of phosphofructokinase activity at low temperature that this represents is one of the contributing factors that blocks glycolytic flux and directs carbon flow into the two reactions, G6PDH and glucose-6-phosphatase, that lead to sorbitol synthesis at temperatures $<5°C$.

Low temperature effects on G6PDH K_m values are also important to enzyme control. In the absence of cryoprotectants or KCl, *T. molitor* G6PDH showed much larger changes in K_m values for both substrates with the change in assay temperature from 24 to 4°C and K_m values for both substrates increased (K_m G6P by 3-fold, K_m NADP by 2-fold). By contrast, K_m values for *E. solidaginis* or *E. scudderiana* G6PDH were more strongly conserved at low temperature and in each species, K_m for one substrate increased and for the other substrate decreased at low temperature. It should be noted that K_m values at low temperature were all determined under conditions where the pH of the imidazole assay buffer (set to pH 7.2 at 24°C) was allowed to rise with decreasing temperature, following alphastat predictions (i.e. pH would be about 7.5 at 4°C) (Reeves, 1972). Such limited effects of temperature change on K_m values are common for enzymes from ectotherms that must function over a wide range of body temperatures (Hochachka and Somero, 1984). They are also certainly adaptive for a cold hardy animal and for pathways of polyol metabolism that must specifically function at low environmental temperatures.

The third factor that may influence enzyme function during low temperature polyol synthesis is the interaction between enzyme kinetic properties, temperature, and added polyols. The addition of polyols had some significant effects on enzyme properties. For *E. scudderiana*, which synthesizes glycerol *in vivo* at ambient temperature of $+5$ to $-5°C$, the addition of glycerol reduced the Q_{10} of the enzyme and lowered the K_m G6P in the presence or absence of salt. Both of these effects would enhance enzyme function at low temperature while glycerol accumulates *in vivo*. By contrast, the kinetic properties of *E. solidaginis* G6PDH were relatively unaffected by polyols, a feature that has also been noted for *E. solidaginis* glycerol-3-P dehydrogenase (Storey and Storey, 1982).

Most enzymes are denatured by freezing in dilute solution and many studies have examined the actions of low molecular weight metabolites (particularly sugars and sugar alcohols) in protecting the structure and/or function of enzymes during freezing (Gekko, 1983; Fink, 1986; Carpenter and Crowe, 1988). These studies have typically utilized highly purified enzymes from commercial sources in order to study the physical principles involved in the freezing preservation of protein structure. Surprisingly, however, no studies have ever analyzed the freezing sensitivity and freezing stabilization by low molecular weight protectants of enzymes from cold hardy animals. Although the intracellular environment of freeze tolerant animals never contains ice naturally, proteins in these animals experience the same physical stresses in their immediate environment (a decrease in water content, increases in the concentrations of salts and other solutes) whether ice forms inside or outside the cell. We reasoned, therefore, that an analysis of the freezing behaviour and relative protective effects of cryoprotectants on G6PDH from a freeze tolerant insect vs a freeze avoiding insect vs the enzyme from a non-cold hardy source (yeast) should show whether there were adaptive changes to proteins that could improve their stability at low temperature or in a frozen cell. A comparison of the effect of freezing on the two insect enzymes shows that G6PDH from the freeze tolerant insect was, indeed, somewhat more stable to freezing. Thus, a greater percentage of enzyme activity was recovered after freezing in the absence of added cryoprotectant ($6.5 \pm 0.81\%$ for *E. solidaginis* vs $2.23 \pm 0.33\%$ for *E. scudderiana*) and the P_{50} values for protection of *E. solidaginis* G6PDH were in almost all cases significantly lower than the corresponding values for *E. scudderiana* G6PDH (Fig. 1, Table 5). These differences in P_{50} values indicate that less cryoprotectant was required to stabilize the *E. solidaginis* enzyme against freeze denaturation. However, the yeast enzyme showed freezing stability and protection by polyol and sugar cryoprotectants that differed little from the results for *E. solidaginis* G6PDH. Thus, the proposal that proteins from freeze tolerant species may be more stable to freezing stresses remains in doubt.

The results also indicate that the natural cryoprotectants found in cold hardy insects (glycerol, sorbitol) have no particular advantages as enzyme stabilizers; a range of other carbohydrates and amino acids as well as the substrate of G6PDH, glucose-6-P, were equally effective cryoprotectants at similar concentrations. The only uniformly poor protectant tested was taurine, a compound that frequently occurs as a natural osmolyte in salt-tolerant invertebrates. The most effective protectant was the disaccharide, trehalose, the blood sugar of insects. A number of cold hardy insects do, in fact, accumulate high levels of trehalose as their cryoprotectant (reviewed by Storey and Storey, 1988). A somewhat surprising result was the very low levels of cryoprotectants that were required to provide freezing protection of G6PDH. P_{50} values for most compounds were about 20 mM and in most cases 100% protection during freezing was achieved with about 50 mM protectant. The mode of action of low molecular cryoprotectants in the freezing stabilization of proteins is by the preferential exclusion of these compounds from the surface of the protein; as a result of this the native state of the protein, as opposed to the denatured state, is the more thermodynamically favourable one (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1989). If

about 50 mM protectant is all that is needed to stabilize G6PDH during freezing in a dilute protein solution, then it is obvious that the 500–2000 mM levels of cryoprotectants *in vivo* (in addition to normal cellular pools of other metabolites such as sugars and amino acids) are far in excess of the requirement for the protection of proteins such as G6PDH during natural freezing exposures. However, the high levels of cryoprotectants may be useful in protecting more labile enzymes; for example, P_{50} values for *E. solidaginis* and *E. scudderiana* phosphofructokinase are several-fold higher than those reported for G6PDH (A. Lindholm and K. Storey, unpublished data). More likely, however, it is the other important function of cryoprotectants that determines their concentration *in vivo* and this is their colligative actions in freezing point and supercooling point depression in freeze avoiding insects and in cell volume regulation in freeze tolerant insects.

For glycerol, sorbitol/glucose, and trehalose, compounds with 3, 6, and 12 carbons, respectively, the P_{50} values for the insect G6PDH's decreased as both molecular weight and total hydroxyl groups per molecule (3, 6 and 8, respectively) increased. Therefore, cryoprotective effect on G6PDH seems to be more closely related to the total hydroxyl equivalents present, as previously postulated by Baust and Morrissey (1977), than to the number of molecules in solution. For example, at the P_{50} values, the total hydroxyl groups available for cryoprotection from glycerol vs trehalose are 62.1 (20.7×3) vs 53.6 (6.7×8) for *E. solidaginis* G6PDH and 73.8 vs 72 for *E. scudderiana* G6PDH. Thus, equivalent protection of proteins is available from much lower concentrations of trehalose compared to glycerol. But, whereas the protection of proteins from freeze denaturation is related to total hydroxyl groups, the colligative function of cryoprotectants in freezing point and supercooling point depression or cell volume regulation are strictly dependent upon the number of molecules in solution. For such colligative actions glycerol is the best compound to use for its production creates the largest number of molecules from a fixed reserve of fat body glycogen. This is probably the major reason why glycerol is the most common cryoprotectant among cold hardy insects (Storey and Storey, 1988).

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