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6-Phosphogluconate Dehydrogenase from a Freeze Tolerant Insect: Control of the Hexose Monophosphate Shunt and NADPH Production during Cryoprotectant Synthesis

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Kinetic properties of 6-phosphogluconate dehydrogenase (6PGDH) from the freeze-tolerant gall fly larvae, *Eurosta solidaginis*, are strongly affected by temperature and by the presence of the cryoprotectants glycerol and sorbitol. The enzyme was purified 563-fold with a final sp. act. of 13.5 U/mg protein and a 26% yield. K_m values for both substrates 6-phosphogluconate (6PG) and $NADP^+$ increased with a drop in assay temperature. The addition of either polyol served to lower these values even in the presence of high salt. 6PGDH appears to be the rate limiting step in the hexose monophosphate shunt (HMS) of this cold-hardy insect providing the reducing power in the form of NADPH needed for the production of sorbitol. The only inhibitor found for 6PGDH was KCl. Inhibition of the enzyme increased with a decrease in temperature. Polyols did not reduce KCl inhibition but they did serve to lower the substrate affinity values for 6PG and $NADP^+$ in the presence of high concentrations of salt. An increase in the activation energy determined by an Arrhenius plot showed that there was a conformational change in 6PGDH at temperatures below 5°C. This, along with the inhibition of the enzyme by high salt concentrations, could effectively shut down the biosynthesis of sorbitol at low temperature. Therefore, it is evident that through the regulation of this HMS enzyme *E. solidaginis* is able to (1) produce the reducing power needed for sorbitol synthesis, and (2) control the production and cessation of sorbitol synthesis.

Eurosta solidaginis Polyol biosynthesis Pentose phosphate cycle Hexose monophosphate shunt Cryoprotectants Insect cold hardiness

INTRODUCTION

Larvae of the gall fly *Eurosta solidaginis* have been extensively used as a model system for studies of the adaptations supporting insect freeze tolerance (Baust and Nishino, 1991; Storey and Storey, 1991, 1992). One of the striking features of cold hardiness in this species is the production of dual cryoprotectants, glycerol and sorbitol, and the distinctly independent regulation of the biosynthesis and catabolism of each (Morrissey and Baust, 1976; Storey and Storey, 1983, 1986). The two polyols are synthesized from the large reserves of glycogen that are accumulated by the larvae during summer feeding. Glycerol production occurs during early autumn facilitated by temperatures of about 10–15°C, whereas sorbitol synthesis commences in late autumn and only in response to a temperature trigger in the 0–5°C range (Storey and Storey, 1986). Glycerol and sorbitol reach final concentrations of 200–400 and 75–150 mM respectively (Storey *et al.*, 1981; Storey and Storey, 1986). Catabolism of the two polypols also differs. Sorbitol pools can vary up and down over the winter (interconverted with glycogen) in response to ambient temperature change and are finally restored as glycogen in early spring (Storey and Storey, 1986). However, glycerol pools, once synthesized, are maintained throughout the winter. Glycerol is catabolized in the spring but the carbon is not returned to glycogen and instead is probably incorporated into lipid pools or oxidized as an aerobic fuel (Storey and Storey, 1986).

The biosynthesis of polyols requires reducing power in the form of NADPH or NADH to support the activities of polyol dehydrogenase and glycerol-3-phosphate dehydrogenase. NADPH is generated by the two reactions of the hexose monophosphate shunt (HMS), glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.43) (transhydrogenase reactions can convert NADPH to NADH when required) that produce a net of 2 mol of

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NADPH per mol of glucose 6-phosphate (G6P) converted to ribulose 5-phosphate (Ri5P). Radiotracer studies with several cold-hardy species have shown that carbon flow through the HMS is increased relative to flux through glycolysis during active cryoprotectant synthesis (Kageyama, 1976; Wood and Nordin, 1980; Tsumuki *et al.*, 1987). Calculations show that sufficient reducing equivalents for glycerol and sorbitol synthesis result when 86 or 46%, respectively, of total G6P is processed via the HMS before entering the polyol pool (Storey and Storey, 1988).

The reaction catalyzed by 6PGDH is:

$$\text{6-Phosphogluconate} + \text{NADP}^+ + \text{H}_2\text{O} \rightarrow$$
$$\text{Ribulose 5-Phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2.$$

The reaction is considered to be essentially irreversible *in vivo* because of the loss of CO_2. The very large change in free energy that accompanies this reaction makes this the first committed step of the HMS (Baquer *et al.*, 1988). It is obvious, then, that controls on the activity of 6PGDH could be a key factor in the overall regulation of polyol synthesis in cold-hardy insects. The present paper analyzes the kinetic properties of purified 6PGDH from *E. solidaginis*, focusing in particular on the effects of temperature and polyols, and their interactions, on enzyme properties.

MATERIALS AND METHODS

Gall fly larvae were collected in September and October from fields around the Ottawa area. The galls were returned to the lab and larvae were immediately removed, frozen in liquid nitrogen and then transferred to −80°C until used. All chemicals and biochemicals were purchased from Boehringer–Mannheim Corp. Montreal, PQ or Sigma Chemical Company, St Louis, Mo.

Larvae (1 g, *c.* 20 animals) were homogenized 1:5 (w/v) in 20 mM imidazole–HCl, pH 7.0 + 15 mM 2-mercaptoethanol using an Ultra Turrax homogenizer; a few crystals of solid phenylmethylsulfonyl fluoride (PMSF) were added immediately prior to homogenization. The preparation was then centrifuged at 27,000 ***g*** for 25 min in a Sorvall RC-5B refrigerated centrifuge at 5°C. The supernatant was removed and subjected to polyethylene glycol (PEG) fractionation. Solid PEG (7000–9000 mol. wt) was added slowly to the homogenate whilst stirring until PEG concentration rose to 8% w/v. After stirring at 21°C for 20 min, the enzyme preparation was centrifuged as before for 10 min. The supernatant was removed and more solid PEG was added to adjust the solution to 20% PEG. After stirring and centrifuging again the supernatant was discarded and the pellet was saved. The 6PGDH-containing pellet was resuspended in 1 ml of homogenizing buffer.

The enzyme preparation was then loaded onto a phosphocellulose column (1 × 3 cm) equilibrated in homogenization buffer. The column was washed with two 10 ml aliquots of homogenization buffer and then the enzyme was eluted with a gradient of 0–400 mM KCl in homogenizing buffer. 1 ml fractions were collected and assayed for 6PGDH activity and peak tubes were pooled. The pooled enzyme was diluted 1:1 (v/v) with homogenization buffer to lower the ionic strength and loaded onto a column of Blue Dextran (1 × 3 cm) equilibrated in homogenizing buffer. The column was washed twice as above and the enzyme was eluted with a gradient of 0–400 mM KCl in homogenizing buffer. 1 ml fractions were collected and the tubes with the highest 6PGDH activity were combined and utilized as the 6PGDH source for all kinetic studies.

6PGDH activity was monitored by following $NADP^+$ reduction at 340 nm using a Pye Unicam SP 8–100 recording spectrophotometer with a circulating water bath attached for temperature control of the cuvettes. Optimal assay conditions were 20 mM imidazole–HCl buffer (pH 7.0 at 22°C), 0.5 mM 6-phosphogluconate (6PG), and 0.2 mM $NADP^+$. One unit of enzyme activity is defined as that which catalyzes the reduction of 1 μmol of $NADP^+$/min at 22°C. Substrate affinity constants (K_m) were determined using Lineweaver–Burk plots (1/v vs 1/[*S*]). I_{50} values (the concentration of inhibitor that reduces maximal enzyme activity by 50%) for salt (KCl) were calculated using Job plots (V_o/V vs [*I*]). Several metabolites were tested at concentrations of 0.1–20 mM to determine whether they activated or inhibited 6GPDH. These included ATP, ADP, AMP, proline, aspartate, alanine, glutamate, and PEP. Product inhibition by NADPH was tested over the range from 0.001 to 0.04 mM NADPH.

SDS–PAGE was performed on purified samples of 6PGDH as in Laemmli (1970) using a 10% w/v acrylamide gel. Protein was stained with 0.25% w/v Coomassie brilliant blue R. The subunit molecular weight was determined from a plot of R_f vs log mol. wt of protein standards.

The native molecular weight of 6PGDH was determined via Sephacryl S-300 gel filtration with a column buffer consisting of 50 mM KH_2PO_4, pH 7.2, 15 mM 2-mercaptoethanol, 0.1% w/v NaN_3, and 10% v/v glycerol. A 100 μl aliquot of purified 6PGDH was loaded onto the column (0.5 × 45 cm) and 1 ml fractions were collected and assayed for enzyme activity under optimal assay conditions. The standards were loaded in a similar manner and their elution peaks were detected via either enzyme activity assays monitored at 340 nm for rabbit muscle phosphofructokinase (360,000) and aldolase (160,000) or via absorbance at 280 nm for catalase (232,000), bovine serum albumin (68,500), and bovine heart cytochrome c (13,370). The native molecular weight of 6PGDH was determined from a plot of K_a vs log mol. wt of the protein standards.

Protein concentration was determined by the Coomassie blue dye-binding method with the Bio-Rad prepared reagent and bovine γ-globulin as the standard. Data are means ± SEM for analyses on $n = 3$ separate preparations of purified 6PGDH. Statistical significance

was determined using a Student's t-test or a two tailed Dunnett's test.

RESULTS

Enzyme purification

The activity of 6PGDH in *E. solidaginis* was calculated to be 2.89 ± 0.17 U/g wet wt. *E. solidaginis* 6PGDH was purified 563-fold with a 26% yield to a final specific activity of 13.5 U/mg protein (Table 1). The purification scheme was simple, quick and highly reproducible. The enzyme eluted in a single peak at a concentration of 200 mM KCl from both the phosphocellulose and Blue Dextran columns. Purified *E. solidaginis* 6PGDH was found to be homogenous as determined by the presence of a single band when the purified preparation was analyzed via SDS–PAGE (Fig. 1).

Molecular weight determination

The native molecular weight of *E. solidaginis* 6PGDH was determined using Sephacryl S-300 gel filtration. The mean value was determined to be $230{,}000 \pm 18{,}500$ Da ($n = 4$). Subunit molecular weight was determined from SDS–PAGE, giving a value of $47{,}000 \pm 2400$ Da (Fig. 1).

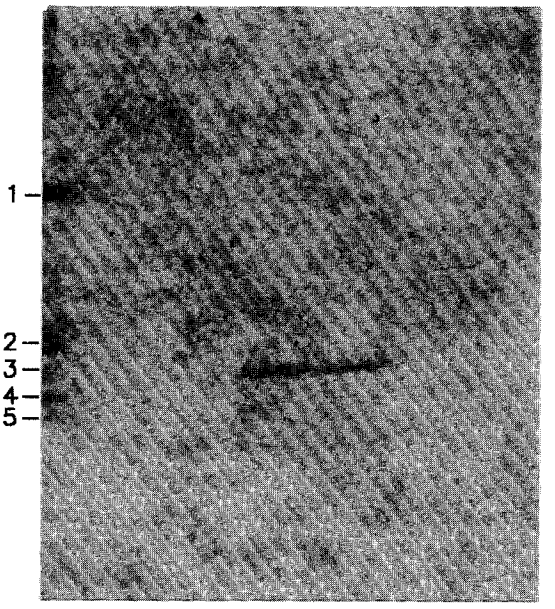

FIGURE 1. Subunit molecular weight determination for *E. solidaginis* 6PGDH. SDS–PAGE used the following standards; 1, glycogen phosphorylase *b* (97,400); 2, bovine serum albumin (66,000); 3, ovalbumin (45,000); 4, glyceraldehyde 3-phosphate dehydrogenase (36,000); and 5, bovine RBC carbonic anhydrase (29,000).

Substrate affinity

Table 2 presents affinity constants for 6-phosphogluconate (6PG) for purified 6PGDH. The enzyme was assayed at high (22°C) and low (5°C) temperatures and in the absence vs presence of added polyols (500 mM glycerol and/or 250 mM sorbitol, amounts similar to the natural levels of these cryoprotectants in *E. solidaginis*) or KCl. In the absence of added effectors, K_m 6PG was 63.7 μM when assayed at 22°C. This increased 2-fold when the assay temperature was lowered to 5°C. With the addition of either glycerol or sorbitol to the assay, K_m decreased significantly, the effect being more marked at 5°C. Addition of both polyols together caused a further 25% decrease in K_m at 22°C, but the combined effect was not marked at 5°C. However, the presence of 500 mM KCl in the assay had the opposite effect on K_m 6PG, greatly increasing K_m by 7-fold at 22°C and 4-fold at 5°C. The effect of high salt was partially counteracted by the further addition of both polyols to the assay mixture.

Substrate affinity coefficients for $NADP^+$ are shown in Table 3. Effects of temperature, polyols and KCl on K_m $NADP^+$ followed similar trends to those seen for K_m 6PG. The K_m for $NADP^+$ in the absence of additives at 22°C was 6.4 μM and this increased 2.5-fold when the enzyme was assayed at 5°C. In the presence of 500 mM glycerol K_m $NADP^+$ decreased by 50% at 22°C but in the presence of sorbitol or glycerol + sorbitol, K_m increased. However, when assayed at 5°C, the addition of glycerol had no significant effect on K_m $NADP^+$, but in the presence of sorbitol, K_m decreased 35%. As was seen for K_m 6PG, the addition of 500 mM KCl significantly raised K_m $NADP^+$ at both temperatures by 3.3-fold at 22°C and by 1.5-fold at 5°C. The effects of high KCl were again partially reversed by the further addition of glycerol and sorbitol when the enzyme was assayed at 22°C but not at 5°C.

Inhibitors of enzyme activity

A variety of compounds were assessed as potential effectors of 6PGDH. The following neither activated nor inhibited 6PGDH activity: ATP-Mg^{2+}, ADP, AMP, proline, glutamate, alanine, aspartate, and phosphoenolpyruvate up to concentrations of 10 mM added to the assay mixture. Overall, no enzyme activators were found. Product inhibition by NADPH was tested but high levels of NADPH had little effect on the enzyme. No inhibition was observed up to 10 μM and inhibition was $<30\%$ at levels up to 40 μM NADPH.

TABLE 1. Purification of 6-phosphogluconate dehydrogenase from *Eurosta solidaginis*

	Total protein (mg)	Total activity (U)	Percent yield	Fold purification	Specific activity (U/mg)
Crude homogenate	363	8.84	—	—	0.024
20% PEG	276	4.59	52	—	0.017
Phosphocellulose	3.84	4.24	48	45.8	1.1
Blue Dextran	0.17	2.29	26	562.5	13.5

TABLE 2. Effect of polyols and KCl on 6PGDH affinity for 6-phosphogluconate at two assay temperatures

Condition	K_m 6PG (μM) 22°C	5°C
Control	63.7 ± 1.7	119 ± 3.4[b]
+500 mM Glycerol	53.0 ± 3.0[a]	59.8 ± 0.49[a]
+250 mM Sorbitol	51.9 ± 1.9[a]	52.0 ± 1.7[a]
+500 mM Glycerol and 250 mM Sorbitol	38.3 ± 0.25[a]	63.9 ± 2.2[a,b]
+500 mM KCl	451 ± 6.1[a]	471 ± 22.4[a]
+500 mM Glycerol, 250 mM Sorbitol, 500 mM KCl	119 ± 2.1[a]	232 ± 4.0[a,b]

Mean ± SEM, $n = 3$–5 determinations on three separate enzyme preparations. Assays were run with 0.2 mM NADP+ in 20 mM imidazole, pH 7.0.

[a]Significantly different from the corresponding control value at the same temperature, two-tailed Dunnett test, $P < 0.01$.

[b]Significantly different from the corresponding value with the same additions at 22°C, Student's t-test, $P < 0.01$.

TABLE 4. Inhibition of 6PGDH by KCl in the presence or absence of added polyols at two assay temperatures

Condition	I_{50} KCl (mM) 22°C	5°C
Control	526 ± 8.0	316 ± 7.7[b]
500 mM Glycerol	435 ± 7.6[a]	329 ± 7.3[b]
250 mM Sorbitol	458 ± 10.0[a]	314 ± 9.8[b]
500 mM Glycerol and 250 mM Sorbitol	476 ± 4.4[a]	313 ± 7.3[b]

Mean ± SEM of $n = 3$–5 determinations on three separate enzyme preparations. Assays at both temperatures included 20 mM imidazole (pH 7.0), 0.5 mM 6PG, and 0.2 mM NADP+.

[a]Significantly different from the control value at the same temperature, two-tailed Dunnett test, $P < 0.01$.

[b]Significantly different from the corresponding value at 22°C, Student's t-test $P < 0.01$.

The only significant inhibitor of 6PGDH found was KCl. Table 4 shows I_{50} values for KCl at 22 and 5°C and the effects of added polyols on KCl inhibition. At 22°C I_{50} KCl was 526 mM and this decreased by 40% to 316 mM at 5°C. The addition of glycerol, sorbitol, or glycerol + sorbitol significantly lowered I_{50} KCl at 22°C but none of the polyols had a significant effect on the I_{50} at 5°C.

pH effects

Figure 2 shows the effect of pH on enzyme activity, assayed at both 22 and 5°C in imidazole buffer. At both temperatures, the enzyme showed a sharp optimum with maximal activity at pH 7.0 at 22°C and pH 7.31 at 5°C. The shift in optimum at 5°C is exactly the result predicted from the effect of temperature on the pH of imidazole buffers (pH increases by 0.018 unit per 1°C decrease in temperature). This optimum is lower than the optima of pH 7.5 or 8.3 reported for the enzyme from other sources (Pearse and Rosemeyer, 1974a; Rippa and Signorini, 1975; Del-mar Medina-Puerta, 1988).

Arrhenius plot

The effect of temperature on 6PGDH activity is depicted in Fig. 3 as an Arrhenius plot. The plot was linear over the range from 35 to 5°C with a calculated activation energy (E_a) for this interval of 55.6 ± 2.1 kJ/mol. Below 5°C there was a sharp decrease in enzyme activity that resulted in a 2-fold increase in E_a to 125.8 ± 3.4 kJ/mol for the 1–5°C interval.

TABLE 3. Effect of polyols and KCl on 6PGDH affinity for NADP+ at two assay temperatures

Condition	K_m NADP+ (μM) 22°C	5°C
Control	6.38 ± 0.24	16.6 ± 0.24[b,c]
+500 mM Glycerol	3.32 ± 0.13[a]	14.8 ± 0.49[c]
+250 mM Sorbitol	8.33 ± 0.29[a]	10.5 ± 0.22[c]
+500 mM Glycerol and 250 mM Sorbitol	10.8 ± 0.75[a]	12.1 ± 0.06
+500 mM KCl	21.3 ± 0.49[a]	24.5 ± 3.7[b]
+500 mM Glycerol, 250 mM Sorbitol, 500 mM KCl	15.8 ± 0.25[a]	23.3 ± 3.3[b]

Mean ± SEM, $n = 3$—5 determinations on three separate enzyme preparations. Assays were run with 0.2 mM 6PG in 20 mM imidazole, pH 7.0.

[a]Significantly different from the corresponding control value at the same temperature via two-tailed Dunnett's test, $P < 0.01$.

[b]Significantly different from the corresponding value at the same temperature via two-tailed Dunnett's test, $P < 0.05$.

[c]Significantly different from the corresponding value with the same additions at 22°C, Student's t-test, $P < 0.01$.

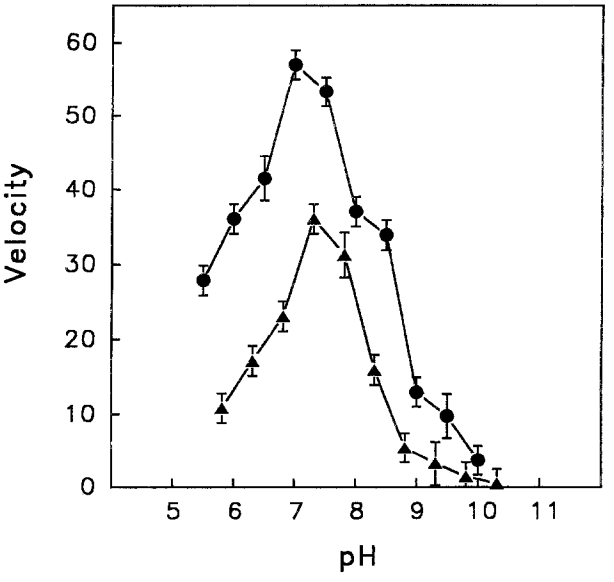

FIGURE 2. The effect of pH on 6PGDH activity at 22°C (●) and 5°C (▲). Reactions contained 0.55 U/mg protein/ml of purified 6PGDH and all activities are relative to the peak activity at 22°C. Data are means ± SEM of three separate enzyme preparations. Assays conditions were 20 mM imidazole, 0.5 mM 6PG, and 0.2 mM NADP+. All buffers were pH'd at 22°C; pH values at 5°C were calculated using +0.018 U/°C for imidazole (pH increases by 0.018 unit per 1°C decrease in temperature).

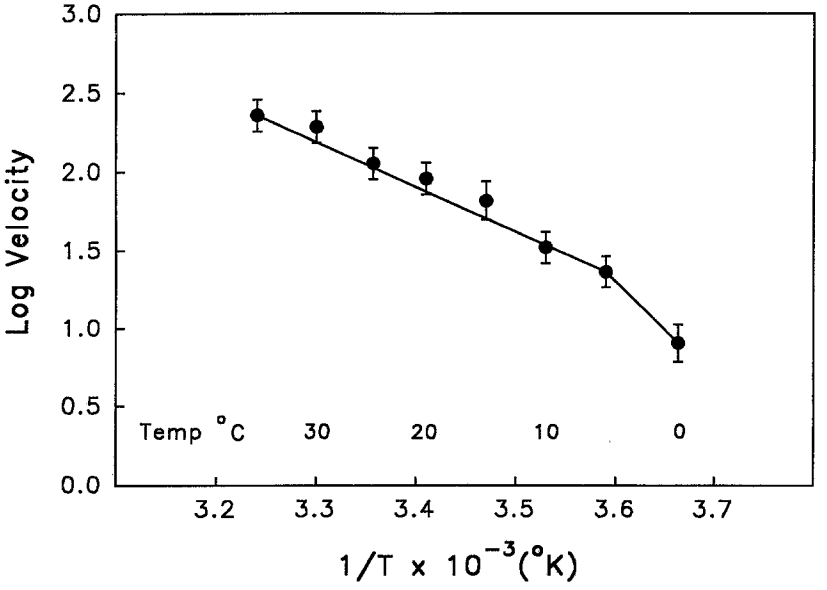

FIGURE 3. Arrhenius plot for *E. solidaginis* 6PGDH. Activity at each temperature was measured under conditions including 20 mM imidazole (adjusted to pH 7.0 at 22°C), 15 mM 2-mercaptoethanol, 0.5 mM 6PG and 0.2 mM $NADP^+$. Data are means ± SEM of three separate enzyme preparations.

DISCUSSION

In response to low temperature most cold hardy insects convert their glycogen reserves into large pools of polyol cryoprotectants; for example, many freeze-avoiding species accumulate glycerol in amounts over 2 M that may represent about 20% of the total body weight of the animal (Storey and Storey, 1991, 1992). Such a major undertaking requires efficient coordination and control over the pathways involved. A critical part of polyol synthesis is the provision of reducing power in the form of NADPH and NADH for use in the conversion of sugars to polyhydric alcohols. This reducing power is provided by two reactions of the HMS. The percentage of the total carbon passing through the HMS must be regulated for optimum conversion efficiency of glycogen to polyols (since 1 CO_2 is lost for every G6P that enters the pathway) and to maintain a balance between NADPH output and use. Since CO_2 is produced by the 6PGDH reaction, this enzyme is said to be the first committed step of the HMS. Thus, regulatory influences on its activity should have an impact on the overall biosynthesis of polyols in cold-hardy insects.

E. solidaginis 6PGDH was purified to homogeneity by an efficient purification scheme that gave a good yield (26%) and a final specific activity of 13.5 U/mg protein. This value compares favorably with the final specific activities calculated for purified 6PGDH from other sources. These include *Candida utilis*, 41.8 U/mg; human erythrocytes, 10–27 U/mg; and sheep liver, 21.2 U/mg (Rippa and Signorini, 1975; Silverberg and Dalziel, 1973; Pearse and Rosemeyer, 1974a; Dallocchio *et al.*, 1985). Values for the subunit (47,000) molecular weight are similar to the values reported for purified 6PGDH from other sources (47,000–52,000) (Silverberg and Dalziel, 1973); Pearse and Rosemeyer, 1974b; Rippa and Signorini, 1975). However, the native molecular weight of 6PGDH from yeast, sheep liver, and mammalian erythrocytes is about 100,000 indicating a dimeric structure for these 6PGDH enzyme forms (Silverberg and Dalziel, 1973; Pearse and Rosemeyer, 1974b; Rippa and Signorini, 1975). The native molecular weight of *E. solidaginis* 6PGDH (230,000) suggests that the enzyme from this insect is a tetramer.

The kinetic constants of *E. solidaginis* 6PGDH substrates differed somewhat from those documented in the literature for other 6PGDH sources. The K_m for 6PG, 63.7 ± 1.7 μM (at 22°C), was higher than the range of 18–54 μM reported by other authors. The K_m of $NADP^+$ (6.4 μM at 22°C) for *E. solidaginis* 6PGDH was below the values reported for the yeast and erythrocyte enzymes (20–30 μM) but greater than the values obtained for the bass liver enzyme (0.9 μM) (Pearse and Rosemeyer, 1974a; Rippa and Signorini, 1975; Dallocchio *et al.*, 1985; Del-mar Medina-Puerta *et al.*, 1988). However, the $NADP^+$ K_m value for *E. solidaginis* 6PGDH was similar to that found for crab hepatopancreas 6PGDH (4–5 μM) (Robert and Gray, 1972). Although levels of 6PG have not measured in *E. solidaginis*, the levels of glucose-6-phosphate (G6P), the substrate for the first enzyme of the HMS, rise sharply to about 0.4 mM during active sorbitol synthesis in the larvae (Storey and Storey, 1983). This value is 8-fold higher than the K_m for G6P (52 μM) of *E. solidaginis* glucose-6-phosphate dehydrogenase (G6PDH) (Storey *et al.*, 1991), and suggests, therefore, that G6PDH probably operates under saturating substrate conditions during periods of polyol synthesis at low ambient temperature (Storey *et al.*, 1991). The lower activity of 6PGDH, compared with G6PDH, in *E. solidaginis* plus the effect of low temperature in raising K_m 6PG to 119 μM indicate that 6PGDH could be rate-limited by substrate availability, particularly at low temperature.

The effect of polyols on *E. solidaginis* 6PGDH are probably important for increasing enzyme affinity for 6PG at low temperature and facilitating the continued accumulation of cryoprotectants. Glycerol biosynthesis by *E. solidaginis* larvae *in vivo* occurs at warmer temperatures (about 10–15°C) and, indeed, glycerol levels in an outdoor population rose to over 250 mM before sorbitol synthesis began (Storey and Storey, 1986). These high endogenous glycerol levels would help to increase enzyme affinity for 6PG over the low temperature range (5 to −5°C) in which sorbitol synthesis occurs (Storey and Storey, 1983, 1986). Thus, the K_m for 6PG at 5°C decreased by 2-fold to about 60 μM in the presence of added 500 mM glycerol. Polyols were also effective in reducing the negative effect that high salt concentrations had on 6PGDH activity. Inhibition of 6PGDH by salt is a common feature of this enzyme from other sources (Pearse and Rosemeyer, 1974). *E. solidaginis* larvae must deal with wide variations in intracellular ion concentrations as the result of extracellular freezing; cellular ionic strength rises several-fold when up to 65% of total body water is sequestered as extracellular ice (Storey, 1990). The counteracting effect of high polypols in relieving salt inhibition of 6PGDH could, therefore, be helpful in stabilizing enzyme function in the frozen state.

Data from the Arrhenius plot shows that 6PGDH activity drops sharply at temperatures below 5°C and indicates that the enzyme undergoes a conformational change at low temperatures. Furthermore, the addition of glycerol and sorbitol in combination was much less effective in relieving the inhibitory effects of high KCl on the enzyme at 5°C, compared with 22°C. These two factors may lead to a large drop in 6PGDH activity in the frozen animal, and in turn block the carbon flow through the HMS. The above inhibitory effects may also diminish the ability of the HMS to produce NADPH at temperatures below 0°C and/or in the frozen state, and may be one key reason that polyol synthesis occurs largely prior to freezing exposures in cold-hardy insects.

Although polyols had positive effects on the K_m for 6PG, glycerol and sorbitol did not significantly affect the K_m for $NADP^+$ which increased several-fold at low temperature, and in the presence of KCl at both temperatures. Furthermore, the two polyols did not modify the I_{50} value for KCl at 5°C, and at 22°C KCl inhibition was actually enhanced (I_{50} decreased about 20%) in the presence of both polyols. Thus, polyols gave no relief from the inhibitory effects of high salt on *E. solidaginis* 6PGDH.

NADPH is a strong product inhibitor and regulator of 6PGDH activity in many tissues (Ayala *et al.*, 1990, 1991; Kaloyianni and Kalomenopoulou, 1990; Dallocchio *et al.*, 1985). Contrary to these studies, however, *E. solidaginis* 6PGDH did not show significant product inhibition by NADPH. The reason(s) for product inhibition by NADPH *in vitro* are not clear. Perhaps the lack of product inhibition by NADPH helps promote polyol synthesis in this cold-hardy insect. This also may be an advantageous effect in cold-hardy insect systems where polyol synthesis is the major metabolic event proceeding during the autumn months, and several hundred millimolar amounts of these compounds must be produced.

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