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PHOSPHOFRUCTOKINASE FROM THE OVERWINTERING GALL FLY LARVA, *EUROSTA SOLIDAGINIS*: CONTROL OF CRYOPROTECTANT POLYOL SYNTHESIS

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Abstract—Kinetic and regulatory properties of phosphofructokinase (PFK) from the larvae of the gall fly, *Eurosta solidaginis*, are strongly affected by temperature. Arrhenius plots show an activation energy of 19,804 ± 1330 cal/mole and a very high Q_{10} of 3.64 (between 10 and 0°C). Enzyme affinity for ATP and Mg^{2+} showed a slight positive modulation with decreasing assay temperature but enzyme affinity for fructose-6-P was strongly negatively modulated, $S_{0.5}$ for fructose-6-P rising from 4.4 ± 0.5 mM at 25°C to 7.7 ± 0.7 mM at 5°C. Metabolite modulators of PFK showed differential effects on enzyme affinity for fructose-6-P at high vs low temperature. AMP (0.05 mM) activation produced an 8.2 fold decrease in $S_{0.5}$ for fructose-6-P at 25°C but only a 3.3 fold decrease in $S_{0.5}$ at 5°C. Two enzyme inhibitors, glycerol-3-P and sorbitol, however, showed stronger effects at 5°C compared to 25°C. At physiological levels of both inhibitors, 4 mM glycerol-3-P and 250 mM sorbitol, $S_{0.5}$ for fructose-6-P was increased to 14.1 mM at 25°C and to 22.3 mM at 5°C; AMP only partially reversed these inhibitory effects.

Control at the PFK locus appears to be responsible for the differential, temperature dependent synthesis of glycerol and sorbitol as cryoprotectants in *E. solidaginis*. Low temperature inactivation of PFK by direct temperature effects on enzyme fructose-6-P affinity and regulation by modulators as well as increased levels of inhibitors, glycerol-3-P and sorbitol, *in vivo* at low temperatures could result in a severe restriction of flux through the PFK locus at low temperature blocking low temperature glycerol synthesis and potentiating sorbitol production.

Key Word Index: Phosphofructokinase, polyol synthesis, overwintering, *Eurosta solidaginis*, low temperature enzyme regulation

INTRODUCTION

THE THIRD instar larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch), which overwinter inside stem galls on goldenrod plants, display both physiological and biochemical adaptations for cold hardiness. Included are a tolerance to extracellular freezing and the accumulation of high levels of two polyhydric alcohols, glycerol and sorbitol, for intracellular cryoprotection (BAUST, 1981). The two polyols accumulate sequentially, in a temperature dependent manner, during low temperature acclimation of the larvae (MORRISSEY and BAUST, 1976; STOREY *et al.*, 1981a). At higher temperatures glycogenolysis leads to the synthesis of glycerol; levels of 0.5 to 0.6 M have been measured in haemolymph (MORRISSEY and BAUST, 1976). As temperature falls below about 10°C, however, glycerol concentrations reach a plateau with no further synthesis occurring below 5°C (STOREY *et al.*, 1981a). At 5°C, a second activation of glycogenolysis is initiated, this time with carbon flux directed into sorbitol synthesis (STOREY *et al.*, 1981a) resulting in haemolymph sorbitol levels of up to 0.2 M (MORRISSEY and BAUST, 1976). Polyol synthesis in *E. solidaginis* during low temperature acclimation requires regulation at two levels: (a) a control of glycogenolytic flux rate probably via regulation of phosphorylase activity and (b) a differential control of carbon flow through glycolysis producing triose phosphates for glycerol synthesis at high temperatures vs hexose phosphates for sorbitol synthesis at low temperatures. Phosphofructokinase (E.C. 2.7.1.11), catalyzing the reaction

$$\text{fructose-6-phosphate} + \text{ATP} \xrightarrow{Mg^{2+}} \text{fructose-1,6-diphosphate} + \text{ADP},$$

is ideally situated for controlling the temperature dependent partitioning of carbon flow into glycerol vs sorbitol production.

Phosphofructokinase (PFK) occurs in a single isozymic form in *E. solidaginis* with no alteration in isozyme form during low temperature acclimation of the larvae (STOREY *et al.*, 1981b). In the present study, the kinetic and regulatory properties of the enzyme have been examined with particular emphasis on temperature effects on enzyme properties. The results showed that *E. solidaginis* PFK is strongly inactivated at low temperatures. Q_{10} for the enzyme is 3.64 (between 10 and 0°C) while enzyme affinity for substrate, fructose-6-P, is reduced, activator effects by AMP are decreased and inhibitor effects are enhanced as temperature is lowered. All of these effects would result in a strong decrease in carbon flow through the PFK locus at low temperatures blocking low temperature synthesis of glycerol and potentiating the diversion of carbon flow into sorbitol synthesis.

MATERIALS AND METHODS

Chemicals and animals

All biochemicals and enzymes, including beef heart PFK, were purchased from Sigma Chemical Co.

Gall fly larvae were collected and acclimated as previously described (STOREY *et al.*, 1981a) and stored frozen at −80°C until use. Larvae acclimated to 0°C for one week were routinely used as the source of PFK for kinetic studies.

Enzyme preparation

Whole larvae were homogenized (1:5 w/v) in 20 mM imidazole buffer, pH 7.5 using a Polytron homogenizer. The homogenate was centrifuged at 27,000 *g* for 30 min at 4°C. The superatant was removed and heated at 55°C for 10 min, then chilled in ice and recentrifuged. This heat treatment destroys adenylate kinase (AK) activity (>95% of AK activity, originally 4 units/g wet wt., was removed) while leaving PFK activity unaltered. Removal of AK activity (AMP + ATP $\rightleftharpoons$ 2 ADP) is required in order to study AMP effects on PFK. The supernatant from the second centrifugation was then run through a Sephadex G-25 (2 cm i.d. × 10 cm) to remove small molecular weight contaminants. The resulting preparation was used for kinetic studies.

Enzyme assay

Enzyme activity was monitored at 340 nm using a Pye Unicam SP1800 recording spectrophotometer with water jacketed cell holder for temperature regulation, For low temperature studies, cuvettes were pre-equilibrated in a water bath to the desired temperature. Standard assay conditions for kinetic studies were: 20 mM imidazole buffer, pH 7.0, 10 mM fructose-6-P, 2 mM ATP, 0.1 mM NADH, 5 mM $MgSO_4$, 100 mM KCl and excess dialyzed aldolase, triose-P isomerase and glycerol-3-P dehydrogenase. In assays testing fructose-1,6-P_2 effects on PFK activity the above coupling enzymes were replaced by dialyzed pyruvate kinase and lactate dehydrogenase with 1 mM P-enolpyruvate added to the assay mixture.

In all studies, buffer pH was adjusted to pH 7.0 at 25°C and then pH was allowed to change with changing assay temperature; for imidazole this results in a 0.017 unit increase in pH per 1°C decrease in temperature (WILSON, 1977). Assay pH was allowed to vary with temperature in order to best mimic the *in vivo* conditions under which PFK would operate; it is now well known that intracellular pH of ectotherms varies with environmental temperature in order to preserve the fractional dissociation state of histidine imidazole groups at a constant value (YANCEY and SOMERO, 1978; SOMERO, 1981).

Kinetic constants (K_m) for ATP and Mg^{2+} were determined from direct linear (CORNISH-BOWDEN, 1979) or Hanes plots while $S_{0.5}$ for fructose-6-P was determined from Hill plots. In studies of AMP activation of PFK, AMP was added to cuvettes after initial rates in the absence of AMP had been recorded. Determinations of I_{50} for ATP (inhibitor concentration reducing enzyme velocity by 50%) was made at constant co-substrate levels, 10 mM fructose-6-P and 5 mM $MgSO_4$ by the method of JOB *et al.* (1978).

Conversion of fructose-6-P to glucose-6-P by endogenous phosphoglucoisomerase in the partially purified PFK preparation during the course of enzyme assays was monitored and appropriate corrections made to fructose-6-P concentrations in the calculation of fructose-6-P affinity constants. AMP used by residual adenylate kinase was also measured; during the course of an assay less than 2% of added AMP was used up, even at lowest (0.05 mM) AMP levels. Inhibitor effects were tested for their specificity for the PFK reaction by using the product sampling method of NEWSHOLME *et al.* (1970).

Results are reported as means ± S.E.M. with determinations made on at least three separate preparations of enzyme.

RESULTS

Enzyme activity and Arrhenius plots

The maximal activity of PFK in the *E. solidaginis* larvae was 1.9 μ-mole fructose-6-P utilized/min g^{-1} wet wt. at 25°C. PFK along with phosphorylase and hexokinase are the glycolytic enzymes with lowest activities in *E. solidaginis* suggesting that PFK, as it is in other systems, may be a key regulatory site in glycolysis in these larvae.

The activity of PFK from *E. solidaginis* decreases dramatically with temperature. At 5°C, enzyme activity was reduced to 0.19 units/g wet wt., a ten fold decrease in activity over a 20°C temperature range. Fig. 1 shows an Arrhenius plot for *E. solidaginis* PFK. The plot is straight over the range 20–0°C but deviates from this straight line at higher temperatures. Calculated activation energy was 19,804 ± 1330 cal/mole (n = 3) with a Q_{10} of 3.35 between 20 and 10°C rising to 3.64 over the range 10–0°C. In contrast, Fig. 1 shows that beef heart PFK is much less strongly inactivated by low temperature. The enzyme showed an activation energy of 10,438 ± 815 cal/mole and a Q_{10} of 2.0. Glycerol (0.5 M) and sorbitol (0.25 M), the two cryoprotectants produced by *E. solidaginis* and whose functions may include the stabilization of protein structure and function at low temperature, had no effect upon the slope of the Arrhenius plot for *E. solidaginis* PFK.

pH optima

E. solidaginis PFK has a pH optimum of 7.8. This shifted to 8.1 with addition of the activator, AMP (at

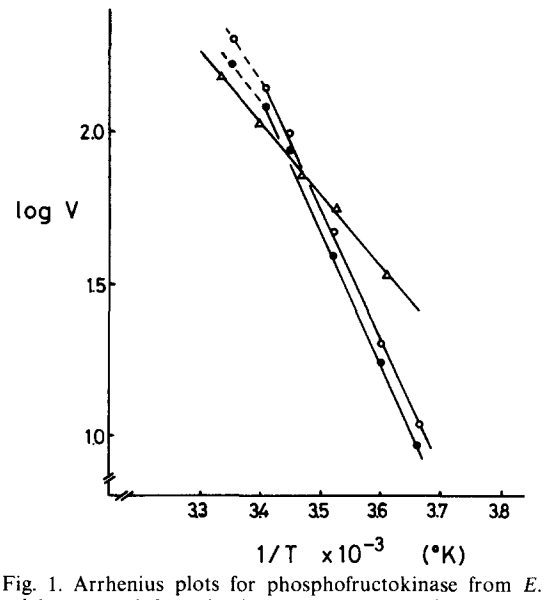

Fig. 1. Arrhenius plots for phosphofructokinase from *E. solidaginis* and from beef heart. Enzyme activities were measured at constant conditions: 30 mM fructose-6-P, 2 mM ATP, 5 mM $MgSO_4$ and 20 mM imidazole buffer, pH 7.0 (pH at 25°C). Symbols are: ○, *E. solidaginis* PFK; ●, *E. solidaginis* PFK in the presence of 500 mM glycerol and 250 mM sorbitol; △, beef heart PFK.

0.5 mM). Enzyme activity and pH optimum was similar in either Tris–HCl or imidazole–HCl buffers but phosphate buffer (all buffers 20 mM) strongly reduced enzyme activity. Imidazole buffer was used in all enzyme studies because the pH vs temperature characteristics of this buffer are the same as those of the major intracellular buffering agent, the imidazolium group of histidine (WILSON, 1977).

Substrate affinity constants

Like PFK from other sources, the *E. solidaginis* enzyme shows hyperbolic saturation kinetics for the substrates ATP and Mg^{2+}. K_m for Mg^{2+} was 0.54 ± 0.06 mM at 25°C but decreased at lower temperatures to 0.20 ± 0.03 mM at 5°C. K_m for ATP decreased similarly at lower temperatures from 0.035 ± 0.003 mM at 25°C to 0.015 ± 0.002 mM at 5°C. ATP inhibited PFK activity at high concentrations; optimal ATP levels were 2 mM while I_{50} for ATP was 10 mM at 25°C and 6 mM at 5°C.

Fructose-6-P kinetics of *E. solidaginis* PFK were hyperbolic at higher pH values (pH 8) but became increasingly sigmoidal as pH decreased, a situation which is also found for PFK from other sources. The Hill coefficient, *n*, increased from 1.0 at pH 8 to 1.2 at pH 6.67. The affinity constant for fructose-6-P, $S_{0.5}$, was also affected by pH, decreasing with increasing pH: $S_{0.5}$ = 10.6, 4.4, 2.4 and 0.85 mM at pH 6.67, 7.0, 7.4 and 8.0, respectively (all at 25°C). Addition of the activator, AMP (0.5 mM), produced hyperbolic saturation kinetics and reduced $S_{0.5}$ for fructose-6-P by about ten fold to 1.0, 0.3, 0.2 and 0.11 mM at pH 6.67, 7.0, 7.4 and 8.0, respectively.

Modifier effects on fructose-6-P kinetics

Table 1 shows the actions of positive and negative effectors of PFK activity on the $S_{0.5}$ for fructose-6-P at two temperatures, 25 and 5°C. In the absence of effectors, decreasing temperature resulted in an increase in $S_{0.5}$ from 4.4 ± 0.5 mM at 25°C to 7.0 ± 0.7 mM at 5°C, an 82% increase. This temperature effect alone would function to reduce enzyme activity *in vivo* at low temperature.

AMP is a potent activator of PFK activity. For *E. solidaginis*, the activating effects of AMP decreased with decreased temperature. At subsaturating fructose-6-P (2 mM) and low, physiological levels of AMP (0.05 mM) this resulted in enzyme activity ratios, $V_{+\,AMP}/V_{control}$, of 3.72, 2.12 and 1.86 at 25, 15 and 5°C, respectively. This same effect is illustrated by AMP effects on $S_{0.5}$ for fructose-6-P as shown in Table 1. Low AMP (0.05 mM) reduced $S_{0.5}$ by 8.2 fold to 0.54 mM at 25°C but a lesser effect was found at 5°C where $S_{0.5}$ was reduced by only 3.3 fold to 2.8 mM. High AMP (0.5 mM) had a similar effect at both temperatures, however, maximally activating the enzyme and decreasing $S_{0.5}$ for fructose-6-P by fourteen fold. AMP also reduced *n* to 1.0, producing hyperbolic kinetics, at both temperatures.

Effects of other metabolites on PFK activity were tested. *E. solidaginis* PFK did not show product activation by ADP or fructose-1,6-P_2 nor was the enzyme inhibited by citrate (5 mM). *E. solidaginis* PFK was, however, inhibited by sorbitol, one of the cryoprotectant polyols, but not by glycerol (500 mM). Glycerol-3-P was also an effective inhibitor of enzyme activity. Glycerol-3-P and sorbitol, at concentrations similar to physiological levels (4 mM and 250 mM, respectively), raised $S_{0.5}$ for fructose-6-P and increased *n* (Table 1). The effects of both of these inhibitors were greater at lower temperature. Glycerol-3-P raised $S_{0.5}$ for fructose-6-P by 1.8 fold at 25°C and by 2.2 fold at 5°C while sorbitol inhibition produced a 1.5 and 1.7 fold increase in $S_{0.5}$ at 25 vs 5°C. In both cases, low (0.05 mM), physiological levels of AMP reversed the inhibition. Inhibition by the two compounds was additive; in the presence of both glycerol-3-P and sorbitol, $S_{0.5}$ for fructose-6-P was raised to 14.1 mM at 25°C and 22.3 mM at 5°C. Addition of AMP under these conditions produced only a partial reversal of the inhibition, a greater reversal occurring at 25 than at 5°C.

Temperature alone and temperature/modifier interactions combine to affect *E. solidaginis* PFK such that enzyme activity is strongly inhibited at low temperature. These effects were observed using PFK isolated

Table 1. Effect of modifiers on fructose-6-P kinetics of *E. solidaginis* phosphofructokinase at high and low temperature

Condition	25°C		5°C	
	$S_{0.5}$	*n*	$S_{0.5}$	*n*
Control	4.4	1.1	7.0	1.2
+0.05 mM AMP	0.54	1.0	2.1	1.0
+0.50 mM AMP	0.30	1.0	0.51	1.0
+Mg·G3P	7.8	1.1	15.3	1.3
+Mg·G3P and 0.05 mM AMP	0.54	1.0	2.9	1.0
+Sorbitol	6.62	1.2	11.7	1.2
+Sorbitol and 0.05 mM AMP	0.54	1.0	2.6	1.0
+Mg·G3P and Sorbitol	14.1	1.2	22.3	1.2
+Mg·G3P, Sorbitol and 0.05 mM AMP	2.3	1.0	10.2	1.0

Affinity constants, $S_{0.5}$, are given in mM; *n* is the Hill coefficient.

Assays were performed at cosubstrate concentrations of 2 mM ATP and 5 mM $MgSO_4$ with 20 mM imidazole buffer, pH 7.0 (pH at 25°C) and effectors at the following concentrations: 4 mM glycerol-3-P (+4 mM $MgSO_4$) and 250 mM sorbitol. Results are the average of determinations on at least three separate preparations of enzyme with reproducibility ± 15%.

from larvae acclimated to 0°C. PFK from larvae acclimated to other temperatures could, however, display different kinetic properties. To examine this possibility PFK was prepared from larvae acclimated to 25 and 0°C with all steps in the preparation (homogenization, centrifugation, dialysis for 2 hr (heat treatment was omitted)) done at 25 or 0°C, respectively in order to preserve any temperature-dependent conformational differences in the enzyme from the two sources. Each preparation was then studied at both 25 and 0°C. At 25°C both preparations showed identical K_m values for ATP and Mg^{2+} and identical $S_{0.5}$ and n for fructose-6-P. Kinetics for both enzymes were also identical at 0°C indicating that the kinetic properties of *E. solidaginis* PFK are not altered when larvae are acclimated to different temperatures.

DISCUSSION

E. solidaginis phosphofructokinase is similar to PFK from other animal sources in a number of ways including a pH optimum of near 8, fructose-6-P saturation kinetics which are hyperbolic at high pH but sigmoidal at lower pH, substrate inhibition by ATP at high concentrations and an activation by AMP (MANSOUR, 1972; UYEDA, 1979). Like PFK from other invertebrate sources, but unlike the mammalian enzyme, the larval enzyme does not show product activation by either ADP or fructose-1,6-P_2 (STOREY and HOCHACHKA, 1975; STOREY, 1976) and like PFK from other insects, the enzyme is not inhibited by citrate (WALKER and BAILEY, 1969; NEWSHOLME *et al.*, 1977). *E. solidaginis* PFK differs from the enzyme from many other sources, however, in the strong effect of temperature on enzyme activity (a Q_{10} of 3.64 compared to Q_{10} of 2 for beef heart PFK or other invertebrate PFK's (FREED, 1971; STOREY and HOCHACHKA, 1975)) and in showing major regulatory effects by glycerol-3-P and sorbitol.

Temperature and temperature-modulator interactions on *E. solidaginis* PFK combine to effectively limit enzyme activity at low temperatures. Compensatory strategies to potentiate enzyme activity and preserve metabolic flux rates at low temperatures are often present in poikilotherms but may not be applicable to *E. solidaginis* PFK. Thus the same, single enzyme form of PFK was present in all larvae whether acclimated to 24 or −30°C; no evidence for "warm" vs "cold" isozymes of the enzyme was found (STOREY *et al.*, 1981b). In addition, no kinetic differences in the enzyme isolated from larvae acclimated to different temperatures were found suggesting the absence of kinetically distinct temperature-dependent conformational "isozymes" of the enzyme as has been noted in other systems (SOMERO, 1969). Although a positive thermal modulation was found for ATP and Mg^{2+} kinetics (K_m values for these substrates decrease with decreasing temperature), fructose-6-P kinetics were strongly negatively affected by low temperature. Indeed, enzyme affinity for fructose-6-P decreased nearly two fold at 5°C compared to 25°C, an effect which apparently overrides an opposing effect by the increase in buffer pH at low temperature. At constant temperature, increasing pH decreased $S_{0.5}$ for fructose-6-P; when buffer pH increased with decreasing temperature (from 7.0 at 25°C to 7.35 at 5°C (WILSON, 1977)), however, $S_{0.5}$ increased. Enzyme kinetic studies in which assay pH is allowed to change with temperature (compared to maintaining constant pH at different temperatures) are now thought to more closely mimic the situation *in vivo* (SOMERO, 1981) as it is now well known that blood and intracellular pH of heterotherms varies with changes in ambient temperature (YANCEY and SOMERO, 1978; WILSON, 1977; SOMERO, 1981).

In addition to the direct effect of temperature on fructose-6-P affinity, the interacting effects of temperature and modulators further reduce PFK affinity for fructose-6-P at low temperatures. AMP, at physiological levels (0.05 μmol/g wet wt (STOREY *et al.*, 1981a)) is a less effective activator of PFK at 5 than at 25°C while inhibitor effects by glycerol-3-P and sorbitol are enhanced at low temperature. Glycerol-3-P and sorbitol effects would be potentiated *in vivo* by the much higher levels of these compounds in larvae acclimated to low temperatures. Cessation of glycerol synthesis in the larvae is accompanied by an increase in glycerol-3-P levels from 0.2 μmol/g wet wt in larvae acclimated to 15°C to 1.6 μmol/g in larvae at −30°C (STOREY *et al.*, 1981a). Sorbitol synthesis, when initiated by exposure to temperatures below 5°C, results in a rapid increase in the level of this polyol from 0.8 to 97.4 μmol/g wet wt (STOREY *et al.*, 1981a). The net effect of temperature and these three modulators on fructose-6-P kinetics could produce as much as a twenty fold increase in $S_{0.5}$ at 5 compared to 25°C ($S_{0.5}$ is 0.54 mM at 25°C with 0.05 mM AMP, low glycerol-3-P and no sorbitol but rises to 10.2 mM at 5°C with 0.05 mM AMP, high glycerol-3-P and high sorbitol (Table 1)), an effect which could severely limit PFK activity *in vivo* at low temperatures.

The negative effects of low temperature at the PFK locus may be the major factor behind the observed, differential, temperature-dependent synthesis of glycerol and sorbitol as cryoprotectants in *E. solidaginis*. Glycerol production, which requires glycolytic flux to

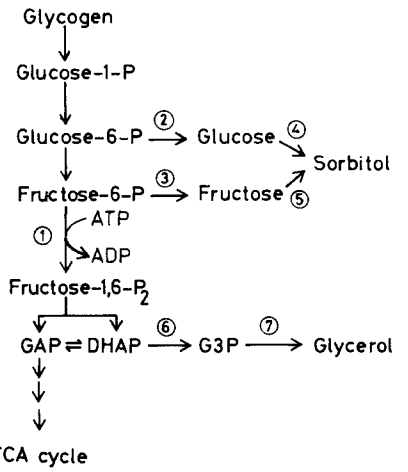

Fig. 2. Proposed metabolic pathways of glycerol and sorbitol production in *E. solidaginis* larvae showing the central role of phosphofructokinase as the site directing carbon flow into glycerol versus sorbitol synthesis. Enzymes are: 1. Phosphofructokinase, 2. Glucose-6-phosphatase, 3. Fructose-6-phosphatase, 4. Polyol dehydrogenase, 5. Sorbitol dehydrogenase, 6. Glycerol-3-P dehydrogenase, 7. Glycerol-3-phosphatase.

the level of the triose phosphates, proceeds at higher temperatures (Fig. 2). Accumulation is in part anticipatory, beginning before the larvae are first exposed to low temperatures (BAUST, 1981) and continues with low temperature acclimation but ceases when temperature falls below about 10°C (STOREY *et al.*, 1981a). A second activation of glycogenolysis occurring at about 5°C does not result in further glycerol synthesis but leads to sorbitol production instead (STOREY *et al.*, 1981a). Glycerol production is probably prevented by a low temperature blockage of glycolysis at the PFK locus but sorbitol, which is derived from the hexose phosphates, does not require an active PFK for its synthesis. The low temperature inactivation of PFK is likely to be the major factor responsible for the diversion of carbon into sorbitol synthesis.

The functional significance of the dual cryoprotectant system of *E. solidaginis* is not known. It may well be that glycerol and sorbitol are separately and specifically synthesized and that the regulatory properties of PFK, seen in the present study, are specific adaptations for controlling the separate synthesis of each polyol. The two separate activations of glycogenolysis seen during low temperature acclimation of the larvae, one leading to glycerol synthesis and one to sorbitol synthesis (STOREY *et al.*, 1981a), suggest separate and specific demands for each polyol. The regulation of *E. solidaginis* PFK by glycerol-3-P and sorbitol, controls which appear to be unique to the *E. solidaginis* enzyme, may be the mechanism to specifically accomplish the separate synthesis of the two polyols.

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