Regulation of phosphofructokinase and the control of cryoprotectant synthesis in a freeze-avoiding insect

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Phosphofructokinase (PFK) from larvae of the freeze-avoiding gall moth *Epiblema scudderiana* was purified 711-fold using ATP-agarose affinity chromatography to a final specific activity of 23 U/mg protein. The native molecular mass of the enzyme was 420 000 ± 20 000 Da. The enzyme showed an optimum pH of 8.13 ± 0.21 at 22°C and 8.19 ± 0.11 at 5°C. Arrhenius plots of PFK activity showed a sharp break at 9°C. $S_{0.5}$ values for fructose 6-phosphate showed positive thermal modification, decreasing with decreasing assay temperature; the opposite was true for ATP-Mg^{2+}. PFK was activated by fructose 2,6-bisphosphate, AMP, and inorganic phosphate; activator effects were temperature-dependent. The enzyme was inhibited by ATP-Mg^{2+}, citrate-Mg^{2+}, and phosphoenolpyruvate. The positive effects of low temperature on enzyme kinetic properties would promote PFK activity to channel glycolytic carbon flow into the production of glycerol during cold-stimulated cryoprotectant synthesis.

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De la phosphofructokinase (PFK) prélevée chez des larves d'*Epiblema scudderiana*, un papillon gallicole réfractaire au gel, a été rendue 711 fois plus concentrée par chromatographie d'affinité à l'ATP-agarose lui conférant une activité spécifique finale de 23 U/mg protéine. La masse moléculaire originale de l'enzyme était de 420 000 ± 20 000 Da. L'enzyme avait un pH optimal de 8,13 ± 0,21 à 22°C et de 8,19 ± 0,11 à 5°C. La courbe d'Arrhenius de l'activité de la phosphofructokinase a démontré qu'il y avait une coupure brusque à 9°C. La valeur $S_{0,5}$ pour le fructose-6 phosphate a mis en lumière une modification thermique positive, décroissant parallèlement au décroissement de la température de l'expérience; l'inverse se produisait dans le cas de l'ATP-Mg^{2+}. La PFK est activée par le fructose 2,6-biphosphate, l'AMP et le phosphate inorganique; l'effet des activateurs dépend de la température. L'enzyme est inhibée par l'ATP-Mg^{2+}, le citrate-Mg^{2+} et le phosphoénolpyruvate. Les effets positifs d'une température faible sur les propriétés cinétiques de l'enzyme peuvent favoriser l'activité de la PFK de façon à orienter l'utilisation du carbone glycolytique vers la production de glycérol au cours de la synthèse de la substance cryoprotectrice stimulée par le froid.

[Traduit par la rédaction]

Introduction

Many insects endure winter exposure to deep subzero temperatures without freezing. To do so they use a variety of biochemical adaptations including the addition of antifreeze proteins to body fluids and a buildup of extremely high concentrations of low molecular weight carbohydrates (sugars or polyols) that act as cryoprotectants (Storey and Storey 1992). Larvae of the gall moth *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae) accumulate glycerol as their cryoprotectant in concentrations of 2 M or more, representing about 19% of the total body mass of the insect (Rickards et al. 1987). As a result, the supercooling point of the larvae is lowered to −38°C in midwinter compared with −12°C in the absence of cryoprotectant, allowing the species to overwinter without freezing in exposed galls on the woody stems of goldenrod plants (Rickards et al. 1987).

The synthesis of glycerol as a cryoprotectant by this and other insect species is fueled from massive stores of glycogen that are accumulated by the fat body during summer feeding (Miller 1976; Storey and Storey 1991). During the autumn the activities and ratios of glycogenolytic and hexose monophosphate cycle enzymes are optimized for the efficient and quantitative conversion of stored glycogen into glycerol as the weather cools. Most cold-hardy insects respond to a temperature trigger in the range 0–5°C, with maximal rates of glycogen to cryoprotectant conversion occurring between 0 and −5°C (Storey and Storey 1991, 1992). Cold activation of glycogen phosphorylase initiates glycogen breakdown, and via regulatory controls applied at key loci of glycolysis carbon is channeled into the accumulation of glycerol (in most species) or other polyhydric alcohols (Storey and Storey 1991).

6-Phosphofructo-1-kinase (PFK) is an important regulatory, and often rate-limiting, enzyme of the glycolytic pathway and gates the commitment of sugar phosphates (derived from glycogen or glucose) into the triose phosphate portion of the pathway. The enzyme is subject to a wide variety of regulatory mechanisms including allosteric controls by powerful activators and inhibitors, post-translational modification by protein phosphorylation and dephosphorylation, enzyme aggregation, and enzyme binding subcellular macromolecules (e.g., F-actin) (Hue and Rider 1987; Pilkis et al. 1987; Storey 1988). Insects that produce glycerol as their cryoprotectant require a cold-active PFK that facilitates triose phosphate formation. The importance of control at the PFK locus has been illustrated by the much greater activities of the enzyme in insects that produce glycerol than in trehalose-producing species (Hayakawa and Chino 1982) and by a sharp increase in the product of PFK, fructose 1,6-bisphosphate, upon cold exposure, indicating an activation of flux through the enzyme (Churchill and Storey 1989). The importance of PFK control is further illustrated in dual-polyol-producing species by the strong temperature-dependent controls on PFK that facilitate glycerol synthesis at moderate temperatures (10–15°C) but block enzyme activity at lower temperatures to divert carbon flow into the synthesis of sorbitol instead (Storey 1982; Storey and Storey 1991).

The present study examines the kinetic and regulatory prop-

TABLE 1. Purification of PFK from *Epiblema scudderiana*

	Total protein (mg)	Total activity (U)	% yield	Fold purification	Specific activity (U/mg)
Crude homogenate	192	6.62	—	—	0.033
10% PEG	75.0	3.85	58.0	1.60	0.051
ATP-agarose	0.044	1.01	15.0	711	23.2

erties of partially purified PFK from *E. scudderiana* and analyzes the interactions of enzyme properties with temperature and high glycerol concentrations to determine the factors that are important in enzyme control during cryoprotectant synthesis.

Materials and methods

Animals and chemicals

Galls containing *E. scudderiana* caterpillars were collected in the autumn of 1989. In the laboratory the galls were placed at either 15 or −4°C; these acclimation temperatures were chosen because at 15°C the insects do not synthesize glycerol, whereas at −4°C glycerol production occurs at a high rate (Rickards et al. 1987). After acclimation for 3 weeks, galls were opened and the larvae were removed, frozen in liquid nitrogen, and then transferred to −80°C until use. Biochemicals were purchased from Boehringer-Mannheim Corp., Montreal, Quebec, or Sigma Chemical Co., St. Louis, Missouri. Sephacryl S-300 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Protein was determined by the method of Bradford (1976) using the Bio-Rad Laboratories prepared reagent and a standard of bovine gamma globulin.

Enzyme assay and kinetic studies

PFK activity was monitored by following NADH oxidation at 340 nm using a Gilford 240 recording spectrophotometer with a Lauda K-2/R water bath attached for temperature control of the cuvettes; kinetic properties were assessed at both 22 and 5°C. Optimal assay conditions were 50 mM imidazole-HCl buffer (pH 7.2 at 22°C), 10 mM fructose 6-phosphate (F6P), 0.5 mM ATP:Mg^{2+} (1:1 mixture of ATP and $MgCl_2$), 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM NADH, and 1 U aldolase, 1 U triosephosphate isomerase, and 2 U glycerol-3-phosphate dehydrogenase in a final volume of 1 mL (Storey 1984). Substrate affinity constants were determined from Hill or Hanes plots. I_{50} values (the concentration of inhibitor that reduces enzyme velocity by 50%) were determined from plots of velocity versus [inhibitor] at [F6P] as indicated. Activator constants (K_a) were determined using double reciprocal plots of $(1/V - V_0)$ versus 1/[activator]. All kinetic parameters are given as the mean ± SEM for $N = 3-6$ separate preparations of enzyme.

Enzyme preparation and purification

Samples of frozen larvae (1 g, or approximately 20 animals) were homogenized 1:5 (w/v) in ice-cold buffer using an Ultra Turrax homogenizer. The homogenizing buffer (HB) contained 50 mM imidazole-HCl, pH 7.2, 15 mM 2-mercaptoethanol, and 40% v/v glycerol. A few crystals of solid phenylmethylsulfonyl fluoride (protease inhibitor) were added immediately prior to homogenization. The homogenate was centrifuged at 27 000 × *g* for 25 min at 5°C in a Sorvall RC-5B refrigerated centrifuge. The pellet was discarded and solid polyethylene glycol 8000 (PEG) was added slowly to the supernatant to a final concentration of 4% w/v. The solution was stirred gently at 22°C for 20 min and then centrifuged for 10 min at 27 000 × *g* at 5°C. The pellet was discarded and more solid PEG was added to the supernatant to adjust the PEG concentration to 10% w/v. After stirring and centrifugation as above the PFK-containing pellet was then resuspended in a small volume of HB, usually 0.5 mL.

The enzyme was then loaded onto an ATP-agarose affinity column (1 × 2 cm) equilibrated with HB. The column was washed in sequence with 1 mL of HB, 1 mL of 1 M KCl in HB, and 5 mL of HB. PFK was then eluted in a single peak with a linear gradient of 0−5 mM each of F6P + ADP in HB containing 500 mM KCl. One-millilitre fractions were collected and assayed for PFK activity. Peak tubes were pooled, stored in 40% glycerol, and used as the source of PFK for all kinetic studies.

Molecular mass determination

The native molecular mass of PFK was determined by Sephacryl S-300 gel filtration chromatography. The column buffer consisted of 50 mM KH_2PO_4, 15 mM 2-mercaptoethanol, 0.1% w/v NaN_3 and 10% v/v glycerol, pH 7.2. A 100-μL aliquot of crude supernatant was loaded onto the column and fractions of 0.5 mL were collected. Standards were run in the same manner and detected by activity assays at 340 nm for rabbit muscle PFK (360 000), rabbit muscle aldolase (160 000), and rabbit liver fructose 1,6-bisphosphatase (140 000) or by absorbance at 280 nm for bovine blood hemoglobin (64 500) and bovine heart cytochrome *c* (13 370). The molecular weight of PFK was determined from a plot of K_a versus log molecular mass for the protein standards.

Isoelectrofocusing

Column isoelectrofocusing was performed by the method of Vesterberg (1971), using an LKB Products 8101 (110 mL) column and pH 3.5 to 10 LKB ampholines in a sucrose density gradient with column development at 500 V for 14 h. Samples of crude supernatant of larvae acclimated to both −4 and 15°C were analyzed. One-millilitre fractions were collected and assayed for PFK activity as described above.

Results

Enzyme purification

PFK was purified 711-fold in two steps: polyethylene glycol fractionation and ATP-agarose affinity chromatography (Table 1). The affinity chromatography alone produced 450-fold purification, with the enzyme eluting in a single sharp peak at about 1.2 mM F6P + ADP. This highly purified preparation showed a final specific activity of 23.2 U/mg protein with an overall yield of 16%. Polyacrylamide gel electrophoresis of the preparation showed a major band of PFK protein with a single minor contaminating band (data not shown).

Substrate affinities

Affinity constants for F6P and ATP-Mg^{2+} are shown in Table 2. F6P kinetics were sigmoidal with calculated Hill coefficients greater than 1. $S_{0.5}$ F6P was high at 22°C but decreased 4-fold when the assay temperature was lowered to 5°C, showing that the enzyme has a much higher affinity for F6P at low temperature. The Hill coefficient was also reduced at 5°C. $S_{0.5}$ F6P was also determined in the presence of 2 M glycerol but the polyol did not affect enzyme affinity for F6P at either assay temperature. PFK affinity for ATP-Mg^{2+} showed a hyperbolic relationship. K_m ATP-Mg^{2+} was also affected by temperature change, increasing 6.5-fold at 5°C compared with 22°C (Table 2).

TABLE 2. Substrate affinity constants for *E. scudderiana* PFK

	22°C	5°C
$S_{0.5}$ F6P (mM)	6.13±0.18	1.49±0.07*
n_H	1.95	1.33
K_m ATP-Mg^{2+} (mM)	0.015±0.001	0.097±0.003*
n_H	1.00	1.04

NOTE: Data are given as the mean ± SEM for 3–6 separate enzyme preparations; n_H is the Hill coefficient.
*Significantly different from the corresponding value at 22°C by Student's *t* test, $P < 0.005$.

TABLE 3. Allosteric inhibitors of *E. scudderiana* PFK

	I_{50} (mM)	
Inhibitor	22°C	5°C
PEP	1.42±0.07	6.88±0.19*
Mg^{2+}-citrate	6.58±0.41	8.63±0.45
Mg^{2+}-ATP	10.4±0.52	11.4±0.56

NOTE: Data are given as the mean ± SEM for 3–6 separate enzyme preparations. Substrate concentrations were 0.5 mM ATP-Mg^{2+} and F6P at 10 mM at 22°C and 5 mM at 5°C.
*Significantly different from the corresponding value at 20°C by Student's *t* test, $P < 0.025$.

TABLE 4. Allosteric activators of *E. scudderiana* PFK

	22°C		5°C	
	K_a (μM)	n_H	K_a (μM)	n_H
$F2,6P_2$	0.170±0.003	1.80	0.590±0.070*	1.20
AMP	16.6±0.350	1.00	35.3±0.640*	1.00
P_i	1230±66.5	1.00	na	

NOTE: Data are given as the mean ± SEM for determinations on 3–6 separate enzyme preparations; n_H is the Hill coefficient; na, no activation by P_i at 5°C.
*Significantly different from the corresponding value at 5°C by Student's *t* test, $P < 0.005$.

Allosteric inhibitors

Many compounds were tested as potential inhibitors of *E. scudderiana* PFK (each one at 5, 10, and 20 mM), including phosphoenolpyruvate (PEP), citrate, citrate-Mg^{2+}, ATP, ATP-Mg^{2+}, pyruvate, lactate, glycerol, ADP, succinate, proline, alanine, malate, inorganic phosphate (P_i), and glutamate. The only effective inhibitors identified were PEP, citrate-Mg^{2+}, and ATP-Mg^{2+} (Table 3), all exerting their inhibitory effects with respect to F6P. Inhibition by citrate-Mg^{2+} and ATP-Mg^{2+} exhibited high I_{50} values that were unchanged by assay temperature, but inhibition by PEP was much reduced at 5°C, the I_{50} value dropping 4.8-fold compared with the value at 22°C.

Allosteric activators

PFK was activated by AMP, fructose 2,6-bisphosphate ($F2,6P_2$), and P_i (Table 4). As also occurs for PFK from other sources, $F2,6P_2$ was an extremely powerful activator, with K_a values of only 0.17 μM at 22°C and 0.59 (4.5-fold higher) at 5°C. K_a values for AMP were similarly affected by temperature, increasing 2-fold at low versus high temperatures. PFK was sensitive to activation by P_i (K_a 1.23 mM) at 22°C, but P_i did not activate the enzyme at low temperature.

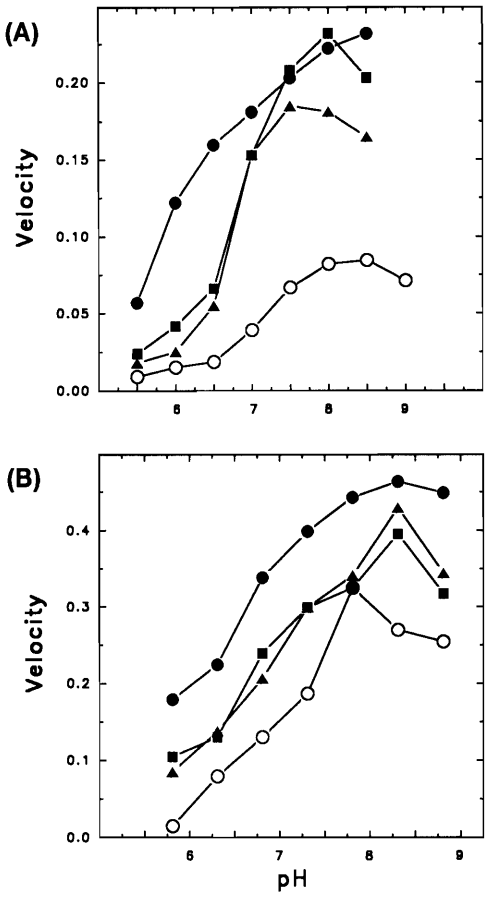

FIG. 1. Effect of pH on PFK activity at 22°C (A) and 5°C (B) under four assay conditions: ●, optimal substrates 10 mM F6P + 2 mM ATP-Mg^{2+}; ○, suboptimal F6P (0.5 mM) + 2 mM ATP-Mg^{2+}; ▲, suboptimal F6P (0.5 mM) + 2 mM ATP-Mg^{2+} + $F2,6P_2$ (0.5 μM at 22°C; 0.2 μM at 5°C); ■, suboptimal F6P (0.5 mM) + 2 mM ATP-Mg^{2+} + AMP (0.3 mM at 22°C; 0.2 mM at 5°C). Aliquots of imidazole buffer were adjusted to the desired pH values at 22°C; corresponding pH values of these buffers when chilled to 5°C were calculated assuming an increase of 0.018 pH units per 1°C decrease in temperature. Data are means for 3–5 different enzyme preparations; SEM error bars are contained within the symbols used.

pH curves

Figure 1 shows pH curves for PFK at 22°C and 5°C under four assay conditions: optimal [F6P], suboptimal [F6P], and suboptimal [F6P] in the presence of either $F2,6P_2$ or AMP as activator. At 22°C the pH optimum was 8.5 at both optimal and suboptimal F6P concentrations. The addition of activators shifted the optima to lower values, pH 8.0 in the presence of AMP and pH 7.5 in the presence of $F2,6P_2$. At 5°C the effect of pH change was different. At optimal [F6P], the pH optimum was 8.31, whereas suboptimal [F6P] gave a lower value, 7.81. In the presence of either activator, the optimum pH was 7.81.

Arrhenius plot

The effect of temperature on the activity of PFK is shown in Fig. 2 as an Arrhenius plot. The plot was discontinuous, with a distinct break at 9°C. The calculated activation energy for the 9–35°C interval was 40.4 ± 2.70 kJ/mol, whereas between 1 and 9°C the E_a value was 3-fold higher at 121 ± 13.3 kJ/mol.

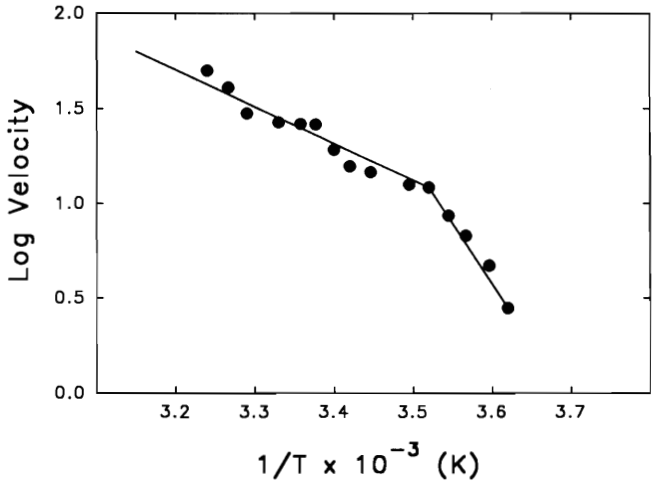

FIG. 2. Effect of temperature on *E. scudderiana* PFK: an Arrhenius plot of activity at optimal substrate concentrations in 2°C increments over the range 1–35°C. Data are means of $N = 4$ different enzyme preparations; SEM error bars are contained within the symbols used.

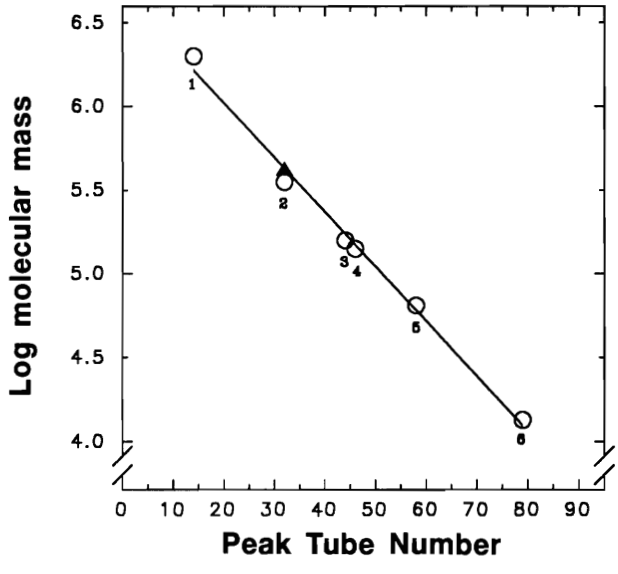

FIG. 3. Native molecular mass of *E. scudderiana* PFK determined by gel filtration of crude supernatant on Sephacryl S-300 with the following standards: 1, blue dextran; 2, rabbit muscle phosphofructokinase; 3, aldolase; 4, fructose 1,6-bisphosphatase; 5, bovine hemoglobin; 6, bovine heart cytochrome *c*.

Isoelectric focusing

Column isoelectrofocusing showed mean isoelectric points (pI) for *E. scudderiana* PFK of pH 5.81 for larvae acclimated to 15°C and 5.63 for larvae acclimated to −4°C ($N = 2$ trials for both).

Molecular mass determination

The native molecular mass for *E. scudderiana* PFK was determined to be 420 000 ± 20 000 Da ($N = 3$) by Sephacryl S-300 gel filtration (Fig. 3).

Discussion

The purification of *E. scudderiana* PFK is a fast and simple procedure that resulted in manyfold purification (711-fold) and a good yield (15%) of enzyme. Although the final preparation was not homogeneous, the final specific activity of the enzyme (23.2 U/mg protein at 22°C) compared favorably with the specific activity reported for flounder liver (24.0 U/mg) PFK but was lower than the specific activity of mammalian PFK (192 U/mg at 37°C) (Sand 1981; Pandian et al. 1983; Hachimori et al. 1986). Isoelectric focusing showed only one peak of PFK activity, indicating a single isozymic form in the larvae that did not change as a result of their acclimation to high (15°C) or low (−4°C) temperatures. The native molecular mass of *E. scudderiana* PFK was determined to be 420 000 ± 20 000 Da, similar to the value for the enzyme from various other sources (Paetkau and Lardy 1968; Pilkis et al. 1987; Starling et al. 1982; Storey 1982; Sale and Denton 1985). Although the subunit molecular mass of *E. scudderiana* PFK was not determined, the enzyme from other sources is a tetramer with subunit molecular masses of 70–95 kDa. Like PFK from other sources, *E. scudderiana* PFK showed an alkaline optimum pH of 8.5 at 22°C and 8.3 at 5°C (Sand 1981; Storey 1984). In the presence of enzyme activators the optimum shifted to a lower pH value at 22°C but not at 5°C.

Low-temperature-stimulated activation of glycogen phosphorylase initiates the glycogenolysis that provides the substrate for cryoprotectant synthesis in cold-hardy insects, but control over phosphorylase cannot determine which of several different polyols is the final product. Regulation at the PFK locus determines whether carbon is channeled into triose phosphates and hence to glycerol, the most common cryoprotectant, or is shunted (due to PFK inhibition) into pathways producing disaccharides (trehalose, sucrose), hexitols (sorbitol, mannitol), or the 4- or 5-carbon polyols that are derived from intermediates of the hexose monophosphate shunt (e.g., ribitol, erythritol, threitol) (Storey and Storey 1991).

The present study shows that temperature and allosteric regulation are important factors in the control of *E. scudderiana* PFK with respect to its role in cryoprotectant synthesis. Low temperature (5°C) had a positive effect on enzyme affinity for F6P, lowering $S_{0.5}$ 4-fold compared with 22°C. This, combined with an increase in the levels of hexose phosphates in vivo as a result of low-temperature-induced glycogenolysis (e.g., the glucose-6-phosphate level rose 5-fold within 2 h when larvae were cold exposed; Churchill and Storey 1989), would promote increased flux through PFK. Furthermore $S_{0.5}$ F6P was not changed by the addition of 2 M glycerol to the assay, indicating that enzyme function would not be altered by the accumulation of glycerol to high levels as cryoprotectant biosynthesis proceeded. Although K_m ATP-Mg^{2+} responded oppositely to low assay temperatures, K_m values (0.015–0.097 mM) are much lower than the 2.5–3 μmol/g wet weight levels of ATP in the larvae (Churchill and Storey 1989), so that ATP would be saturating at both temperatures. In addition, substrate inhibition of *E. scudderiana* PFK by high ATP-Mg^{2+} levels was fairly weak, with I_{50} values of 10.4–11.4 mM which did not change significantly with temperature; these values were considerably lower than those for PFK from other sources (Sale and Denton 1985; Buckwitz et al. 1990; Whitwam and Storey 1991). This low inhibition by ATP would facilitate high flux through the PFK locus under conditions where the energy charge remained high, as is needed for a role in biosynthesis in glycerol-producing species.

Like PFK from other sources, *E. scudderiana* PFK was activated by $F2,6P_2$ and AMP (Hue and Rider 1987; Pilkis et al. 1987). Another common activator of PFK, P_i, affected

E. scudderiana PFK only at 22°C. Although K_a values for both activators rose at 5°C, they were still well within the concentration range of these compounds in vivo. Thus, K_a AMP was 16–35 μM compared with in vivo levels of 60–80 nmol/g wet weight in control larvae, and K_a F2,6P_2 was 0.17–0.59 μM compared with 0.2–0.4 nmol/g in control larvae (Churchill and Storey 1989). Furthermore, levels of both of these activators rise sharply during the initiation of glycerol biosynthesis as a result of cold exposure of *E. scudderiana* larvae. AMP rose to a sharp but transient peak of 200 μM after 1.5 d at low temperature, whereas F2,6P_2 increased to 2 nmol/g in just a few hours and continued to rise to 8 nmol/g over subsequent days, while the rate of glycerol biosynthesis was high (Churchill and Storey 1989). This 20- to 40-fold increase in F2,6P_2 is probably one of the most critical factors in activating and maintaining PFK activity during polyol biosynthesis. Indeed, this role of the F2,6P_2 signal in facilitating the use of carbohydrates for biosynthesis is also its primary role in other systems. F2,6P_2 rises in response to anabolic signals and, conversely, levels of the activator are sharply reduced when carbohydrate reserves must be spared, as in starvation or anoxia (Hue and Rider 1987). High F2,6P_2 levels during active glycerol synthesis probably also serve a second role, the inhibition of fructose-1,6-bisphosphatase (FBPase), the enzyme that reverses the PFK reaction (Hue and Rider 1987; Pilkis et al. 1987). Thus, oppositely directed allosteric controls by F2,6P_2 on PFK and FBPase can ensure a unidirectional flow of carbon into the glycerol pool at a time when concentrations of F1,6P_2, glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate are high and would otherwise facilitate gluconeogenesis (Churchill and Storey 1989).

In addition to inhibition by ATP-Mg^{2+}, *E. scudderiana* PFK was also inhibited by citrate-Mg^{2+} and PEP. As for ATP-Mg^{2+}, however, inhibition by both compounds was weak. For example, I_{50} values for PEP were 1.4 mM at 22°C and 6.9 mM at 5°C compared with levels of PEP in vivo of only 0.3–0.4 μmol/g wet weight (Churchill and Storey 1989).

The Arrhenius plot for *E. scudderiana* PFK showed two linear portions with a clear break at 9°C (Fig. 2) and a 3-fold increase in activation energy over the lower temperature range. A break in the Arrhenius plot for PFK has also been seen for the enzyme from other sources, although the temperatures at which the transition occurs are higher: 15°C for chicken liver, 28°C for flounder liver, and 20°C for PFK from *Eurosta solidaginis*, another cold-hardy insect (Kono and Uyeda 1974; Sand 1981; Storey 1982). Kono and Uyeda (1974) suggested that the transition may be due to a shift in the equilibrium between two different conformational states whose equilibrium constant is dependent upon temperature. The sharper decrease in enzyme activity with decreasing temperature below 9°C might seem to be unfavorable for the high rates of glycerol synthesis that occur naturally between about 5 and −5°C. However, the effect of low temperature in lowering $S_{0.5}$ for F6P, as well as the large increases in the levels of enzyme activators (AMP, F2,6P_2) in vivo at low temperature, are probably effective in maintaining high PFK activity during the period of active glycerol synthesis.

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