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Purification and characterization of glycogen phosphorylase *A* and *B* from the freeze-avoiding gall moth larvae *Epiblema scudderiana*

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Abstract. The active *a* and inactive *b* forms of glycogen phosphorylase from cold-hardy larvae of the gall moth, *Epiblema scudderiana*, were purified using $DEAE^+$ ion exchange and 3′-5′-AMP-agarose affinity chromatography. Maximum activities for glycogen phosphorylases *a* and *b* were 6.3 ± 0.74 and 2.7 ± 0.87 μmol glucose-1-P·min^{-1}·g wet weight^{-1}, respectively, in −4 °C-acclimated larvae. Final specific activities of the purified enzymes were 396 and 82 units·mg protein^{-1}, respectively. Both enzymes were dimers with native molecular weights of 215000 ± 18000 for glycogen phosphorylase *a* and 209000 ± 15000 for glycogen phosphorylase *b*; the subunit molecular weight of both forms was 87000 ± 2000. Both enzymes showed pH optima of 7.5 at 22 °C and a break in the Arrhenius relationship with a two- to fourfold increase in activation energy below 10 °C. Michaelis constant values for glycogen at 22 °C were 0.12 ± 0.004 mg·ml^{-1} for glycogen phosphorylase *a* and 0.87 ± 0.034 mg·ml^{-1} for glycogen phosphorylase *b*; the Michaelis constant for inorganic phosphate was 6.5 ± 0.07 mmol·l^{-1} for glycogen phosphorylase *a* and 23.6 mmol·l^{-1} for glycogen phosphorylase *b*. Glycogen phosphorylase *b* was activated by adenosine monophosphate with a K_a of 0.176 ± 0.004 mmol·l^{-1}. Michaelis constant and K_a values decreased by two- to fivefold at 5 °C compared with 22 °C. Glycerol had a positive effect on the Michaelis constant for glycogen for glycogen phosphorylase *a* at intermediate concentrations (0.5 mol·l^{-1}) but was inhibitory to both enzyme forms at high concentrations (2 mol·l^{-1}). Glycerol production as a cryoprotectant in *E. scudderiana* larvae is facilitated by the low temperature-simulated glycogen phosphorylase *b* to glycogen phosphorylase *a* conversion and by positive effects of low temperature on the kinetic properties of glycogen phosphorylase *a*. Enzyme shut-down when polyol synthesis is complete appears to be aided by strong inhibitory effects of glycerol and KCl on glycogen phosphorylase *b*.

Key words: Cryoprotectant synthesis – Insect cold hardiness – Glycerol metabolism – Regulation of glycogenolysis – Gall moth, *Epiblema*

Introduction

Larvae of the gall moth *Epiblema scudderiana* Clemens (Lepidoptera: Olethreutidae) use the freeze-avoidance strategy of cold hardiness to overwinter inside galls on goldenrod stems. During autumn cold-hardening the larvae accumulate glycerol as a cryoprotectant in quantities as high as 2 mol·l^{-1} or more in body fluids, about 19% of total body mass, and this helps to push the supercooling point of the larvae to −38 °C (Rickards et al. 1987). The biosynthesis of glycerol utilizes as its substrate the large reserves of glycogen accumulated by the larvae during summer feeding (Rickards et al. 1987). Cryoprotectant synthesis takes place in the fat body of cold-hardy insects (Hayakawa and Chino 1981; Shimada 1982; Yi et al. 1987; Storey and Storey 1991) and is primarily regulated via control over the activity of the enzyme glycogen phosphorylase (Ziegler et al. 1979; Hayakawa 1985).

Glycogen phosphorylase exists in two forms, an active phosphorylated *a* form (GP*a*) and an inactive (or subactive) dephosphorylated *b* form (GP*b*). Interconversion of GP*a* and GP*b* via phosphorylase kinase and phosphorylase phosphatase is the major mechanism for changing the activity state of the enzyme, and these enzymes are themselves part of a phosphorylation cascade that is regulated by the actions of protein kinases and

Abbreviations: E_a, activation energy; GP*a*, glycogen phosphorylase *a*; GP*b*, glycogen phosphorylase *b*; *h*, Hill coefficient; I_{50}, concentration of inhibitor that reduces enzymes velocity by 50%; K_a, concentration of activator that produces half-maximal activation of enzyme activity; K_m, Michaelis-Menten substrate affinity constant; MW, molecular weight; PEG, polyethylene glycol; P_i, inorganic phosphate; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; V_{max}, enzyme maximal velocity

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second messengers (Newgard et al. 1989; Meinke and Edstrom 1991). In cold-hardy insects the activities of GP kinase and GP phosphatase are differentially sensitive to temperature change with the result that low temperature exposure (0–5 °C) causes a rapid net increase in GP*a* activity and thereby initiates glycogenolysis to fuel cryoprotectant synthesis (Hayakawa 1985). In *E. scudderiana* the percentage of total phosphorylase in the *a* form remains high throughout the period of active glycerol biosynthesis and then falls to low levels once maximal cryoprotectant levels have been reached (Churchill and Storey 1989a).

In addition to the increase in enzyme maximal activity brought about by the GP*b* to GP*a* conversion at low temperature, the kinetic or regulatory properties of *E. scudderiana* glycogen phosphorylase may be specifically adapted to promote high rates of glycogenolysis at low temperatures and enzyme function may be sensitive to the extremely high concentrations of glycerol that build up as the end product of glycogenolysis. In the present study GP*a* and GP*b* from *E. scudderiana* larvae are purified, then characterized according to physical and kinetic properties. Temperature, pH, glycerol, and salt effects on the enzymes are analyzed to produce a full picture of phosphorylase control during polyol biosynthesis.

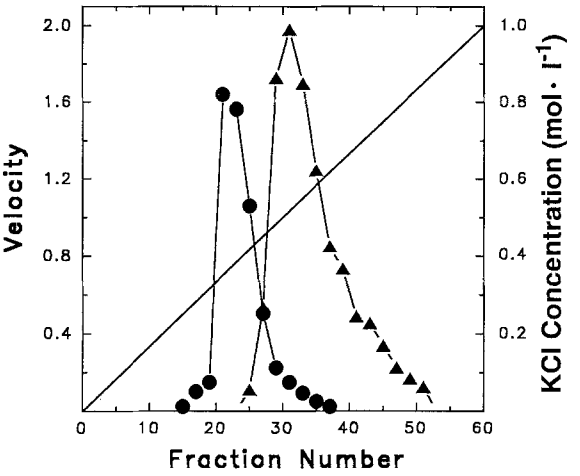

Fig. 1. DEAE-Sephadex column chromatography of *E. scudderiana* GP*a* and GP*b*. ▲ GP*a*; ● GP*b*. Velocity is in units·ml^{-1}·mg protein^{-1} with V_{max} values of 1.98 and 1.64 U·ml^{-1}·mg protein^{-1} for GP*a* and GP*b*, respectively. Data are means of $n=3$ determinations on separate preparations of enzyme; SEM bars are within the size of symbols used

Materials and methods

Animals and chemicals. Spindle-shaped goldenrod galls containing larvae of *E. scudderiana* were collected in the autumn of 1989. Galls were acclimated for 3 weeks in a laboratory incubator at either 15 or −4 °C. These temperatures were chosen because cryoprotectant biosynthesis by the larvae does not occur at 15 °C but glycerol builds up rapidly at −4 °C (Rickards et al. 1987). After acclimation, galls were quickly opened and the larvae were removed, frozen in liquid N_2 and then stored at −80 °C until use. Biochemicals were purchased from Boehringer-Mannheim Corp., Montreal, P.Q., Canada, or Sigma Chemical Company St. Louis Mo., USA. Sephacryl S-300 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad Laboratories prepared reagent and a standard of bovine gamma globulin.

Assay of phosphorylase a *and* b. Phosphorylase was assayed essentially as described by Morishima and Ueno (1990) using a Gilford 260 spectrophotometer with a water-jacketed cell holder for control of cuvette temperature and monitoring NADPH production at 340 nm. Optimal assay conditions for GP*a* were 50 mmol·l^{-1} KH_2PO_4 buffer (pH adjusted to 7.2 at 22 °C), 4 mg·ml^{-1} oyster glycogen, 15 mmol·l^{-1} $MgSO_4$, 5 µmol·l^{-1} glucose 1,6-bisphosphate, and 0.2 mmol·l^{-1} $NADP^+$ with 1 U glucose-6-phosphate dehydrogenase and 1 U phosphoglucomutase in a total volume of 1 ml. For total phosphorylase activity or the assay of purified GP*b*, the assay further contained 1 mmol·l^{-1} AMP; GP*b* activity in a mixture of the two forms was determined as total activity minus GP*a*. One unit of phosphorylase activity is defined as the amount of enzyme that utilizes 1 µmol $NADP^+$·min^{-1} at 22 °C.

Optimal yield of phosphorylase a *or* b *forms.* Prior to enzyme purification, tests were carried out to determine the animal source (15 or −4 °C-acclimated larvae) and the homogenization buffer that would produce the highest yield of either GP*a* or GP*b*. Samples of frozen larvae (approx. 0.2 g, generally five larvae) were homogenized 1:5 w/v using a Polytron homogenizer. One of four ice-cold buffers was used; all contained 15 mmol·l^{-1} imidazole-HCl and 15 mmol·l^{-1} 2-mercaptoethanol and were adjusted to pH 7.2 at 22 °C (Buffer D). Buffer A was designed to inhibit both protein kinases and protein phosphatases and contained Buffer D plus 50 mmol·l^{-1} NaF, 5 mmol·l^{-1} EDTA and 5 mmol·l^{-1} EGTA. Buffer B promoted enzyme phosphorylation and contained Buffer D plus 50 mmol·l^{-1} NaF, 1 mmol·l^{-1} Mg^{2+} and 2 mmol·l^{-1} ATP. Buffer C promoted enzyme dephosphorylation and contained Buffer D plus 2 mmol·l^{-1} Mg^{2+}. Homogenates were not centrifuged but were allowed to settle for 1 h on ice followed by assay of enzyme activity in the supernatant.

Purification of glycogen phosphorylase a *and* b. Optimization tests (above) showed that highest GP*a* activity could be obtained from −4 °C-acclimated larvae homogenized in buffer B. For purification frozen larvae (approx. 1 g or 20 larvae) were homogenized 1:5 w/v in buffer B and the homogenate was allowed to settle on ice for 1 h. The homogenate was then centrifuged at 12000 *g* in an Eppendorf microcentrifuge for 5 min. The pellet was discarded and the supernatant was fractionated using polyethylene glycol 8000 (PEG). Solid PEG was added to the supernatant to a final concentration of 2% w/v, mixed gently with a Lab Quake test tube inverter for 20 min at 22 °C, and then centrifuged at 27000 *g* for 10 min in a Sorvall RC-5B refrigerated centrifuge at 5 °C. The pellet was discarded and further PEG was added to the supernatant to adjust the concentration to 6% w/v. After mixing and centrifuging as above, the pellet containing GP*a* was resuspended in 0.5 ml buffer D and then dialyzed for 2 h against 5 mmol·l^{-1} imidazole buffer, pH 7.2 (buffer E).

The enzyme was then applied to a column (7 cm × 1.5 cm) of DEAE-Sephadex equilibrated in buffer E. The column was washed with 30 ml of buffer E and then the enzyme was eluted with a linear gradient of 0–1 mol·l^{-1} KCl in 40 ml of buffer E; 1 ml fractions were collected and assayed for enzyme activity. This column readily separates GP*a* and GP*b* activity (Fig. 1); fractions with high GP*a* activity and minimal or no GB*b* activity were pooled, placed in dialysis tubing, and concentrated against solid sucrose to a final volume of approximately 2 ml. The preparation was then dialyzed against 1 l buffer E for 2 h.

The enzyme was then applied to a column (2 cm × 1 cm) of 3′,5′-cyclic AMP agarose (Sigma) equilibrated in buffer E. The column was washed with 30 ml of buffer E and then enzyme was eluted with a linear gradient of 0–25 mmol·l^{-1} AMP in 40 ml

buffer E; 1 ml fractions were collected and assayed. The enzyme eluted in a single peak (with a maxima at 8.5 mmol·l^{-1} AMP) and peak fractions were pooled, concentrated against solid sucrose to a final volume of 4 ml, and then dialyzed for 2 h against buffer E. After addition of glycerol (10% v/v) the purified enzyme was stable for at least 2 weeks when stored at 4 °C.

The purification scheme for GB*b* followed the same steps except that the starting material was 15 °C-acclimated larvae, homogenization of larvae was in buffer A, and peak fractions of GB*b* were saved from the DEAE column.

Enzyme kinetics. Substrate affinity constants (K_m) and Hill coefficients (h) were determined using Hill plots. K_a values for AMP for GB*b* were calculated from plots of enzyme activity (at suboptimal glycogen and P_i concentrations) versus increasing AMP concentrations. I_{50} values (the concentration of inhibitor that reduced V_{max} by 50%) for KCl and glycerol were determined from plots of activity versus [inhibitor]. Kinetics were assessed at both 5 and 22 °C. For pH curves the buffer used was a mixture of 15 mmol·l^{-1} imidazole + 20 mmol·l^{-1} KH_2PO_4; pH was adjusted to 7.2 at 22 °C and pH at 5 °C was calculated based on a 0.018 pH unit increase per 1 °C decrease in temperature for imidazole. Data are means ± SEM for $n = 3$–5 separate preparations of purified enzyme. Statistical testing used the Student's t-test.

SDS-PAGE. SDS-PAGE was performed on purified GP*a* and GB*b* samples as in Laemmli (1970) using a 10% w/v acrylamide gel. Subunit MW standards were rabbit muscle glycogen phosphorylase *b* (MW 97400), bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde 3-phosphate dehydrogenase (36000), bovine erythrocyte carbonic anhydrase (29000), and bovine α-lactalbumin (14200). Protein was stained with 0.25% w/v Coomassie brilliant blue R. The subunit MW was determined from a plot of Rf versus log MW of the protein standards.

MW determination. The native MWs of GP*a* and GP*b* were determined by gel filtration on Sephacryl S-300 (40 × 0.5 cm). The column was equilibrated and developed in 50 mmol·l^{-1} KH_2PO_4 buffer containing 15 mmol·l^{-1} 2-mercaptoethanol, 0.1% w/v NaN_3, 10% v/v glycerol, pH 7.2. The column was calibrated with rabbit muscle phosphofructokinase (MW 360000), rabbit muscle aldolase (160000), rabbit liver fructose 1,6-bisphosphatase (140000), bovine blood hemoglobin (64500), and bovine heart cytochrome c oxidase (13370). MWs of GP*a* and GP*b* were determined from a plot of K_a versus log MW of the standards.

Results

Optimization of GPa and GPb yield

Crude homogenates of the larvae contain both phosphorylase kinase and phosphorylase phosphatase and by manipulating the contents of the homogenization buffer (±kinase inhibitors, ±phosphatase inhibitors) we aimed to optimize the available starting material before beginning purification of either GP*a* or GP*b*. Acclimation state of the larvae also strongly influenced GP*a* and GP*b* contents. Thus, when homogenized in buffer A (containing both kinase and phosphatase inhibitors) the ratio GP*a*:GP*b* was 9:91 for 15 °C-acclimated larvae and 72:28 for −4 °C-acclimated larvae, a result in line with the known effect of low temperature in stimulating phosphorylase activation and polyol synthesis in cold-hardy insects (Ziegler et al. 1979; Hayakawa 1985; Churchill and Storey 1989a). Maximal activity of GP*a* in −4 °C-acclimated larvae was 6.3 ± 0.74 μmol glucose-1-P·min^{-1}·g^{-1} wet weight compared with 2.7 ± 0.87 U·g^{-1} for GP*b*. Homogenization and incubation in buffer B (promoting enzyme phosphorylation) increased the percentage of GP*a* to 68 and 81% for 15 and −4 °C larvae, respectively, whereas incubation in buffer C (promoting dephosphorylation) had the opposite effect and %GP*a* fell to 7 and 47%, respectively. Final choices for optimizing the content of either the *a* or the *b* form for purification were −4 °C larvae homogenized in buffer B for GP*a* and 15 °C larvae in buffer A for GP*b* (%GP*b* was slightly higher in buffer C but total units of activity was reduced compared to buffer A). By manipulating enzyme activity in the crude homogenates prior to purification through the use of endogenous kinases/phosphatases we avoided the need to treat with commercial kinases/phosphatases to achieve a high purity of the *a* or *b* form, as has been done for mammalian phosphorylase (Fischer and Krebs 1959).

Purification of glycogen phosphorylase a *and* b. Table 1 shows the purification of the *a* and *b* forms of glycogen phosphorylase from *E. scudderiana*. The purification scheme for each enzyme was essentially the same, differ-

Table 1. Purification scheme for *Epiblema scudderiana* glycogen phosphorylase

Step	Total activity (units)	Total protein (mg)	Yield (%)	Fold Purification	Specific activity (U·mg^{-1})
Glycogen phosphorylase a					
Crude	13.9	44.0	–	–	0.321
PEG, 2–6%	11.9	13.6	85.0	2.82	0.884
DEAE-Sephadex	9.80	9.31	70.0	3.43	1.11
AMP-Agarose	4.61	0.012	33.0	1250	396
Glycogen phosphorylase b					
Crude	19.0	77.0	–	–	0.250
PEG, 2–6%	15.4	20.0	81.0	3.00	0.770
DEAE-Sephadex	6.93	2.81	36.0	9.91	2.50
AMP-Agarose	4.32	0.053	23.0	328	82.0

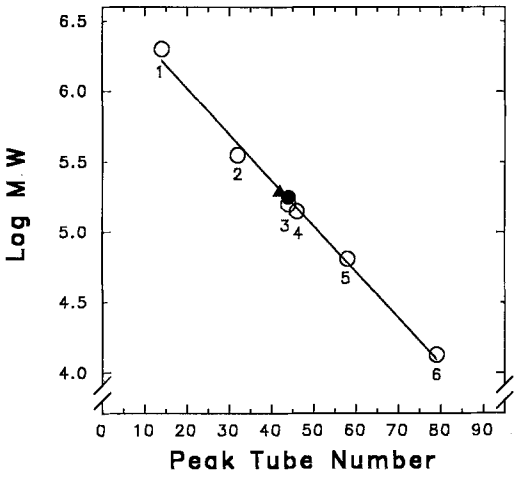

Fig. 2. Native molecular weight of *E. scudderiana* GP*a* and GP*b* determined by Sephacryl S-300 gel filtration. ▲ GP*a*; ● GP*b*; Standards (○): 1 blue dextran, 2 phosphofructokinase, 3 aldolase, 4 fructose 1,6-bisphosphatase, 5 hemoglobin, 6 cytochrome c oxidase

ing in the initial homogenization buffer used to maximize *a* or *b* content and in which activity peak was saved from the DEAE-Sephadex chromatography (Fig. 1). GP*a* was purified 1250-fold to a final specific activity of 396 $U \cdot mg^{-1}$ protein, whereas GP*b* was purified 328-fold to a final specific activity of 82 $U \cdot mg^{-1}$. The specific activity of GP*b* was high compared with values for the enzyme purified to homogeneity from other sources (Morishima and Sakurai 1985); however, SDS-PAGE indicated that the *E. scudderiana* GP*b* preparation was not completely pure. SDS-PAGE also showed a very minor additional protein band in the purified GP*a* preparation.

Molecular weights

The native MWs of GP*a* and GP*b* were 215000 ± 18000 and 209000 ± 15000 (both $n=3$), respectively (Fig. 2), values not significantly different from each other (Student's *t*-test). SDS-PAGE showed the same subunit MW of 87000 ± 2000 ($n=3$) for both enzymes.

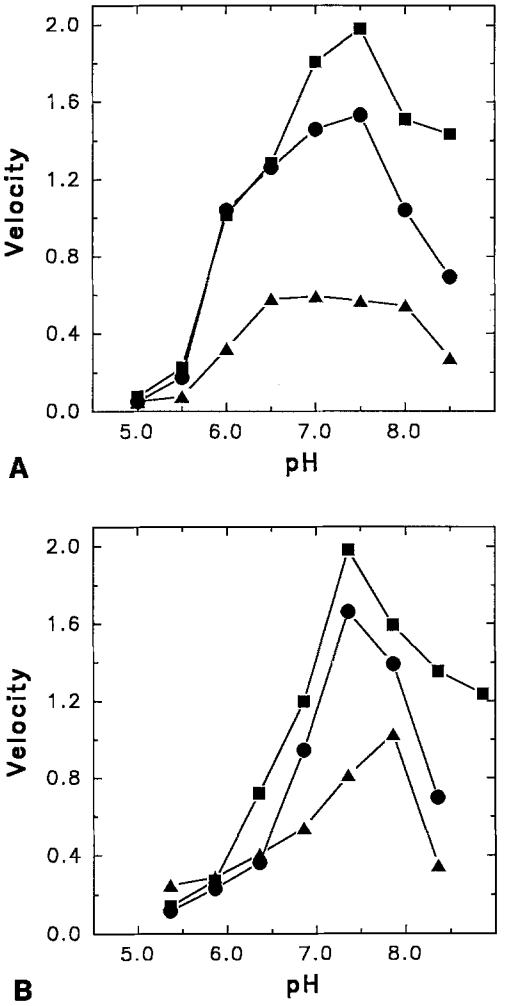

Fig. 3A, B. Plot of activity versus pH for *E. scudderiana* GP*a* at 22 °C (**A**) and 5 °C (**B**). ■ control, no additions; ● plus 0.5 $mol \cdot l^{-1}$ glycerol; ▲ plus 2 $mol \cdot l^{-1}$ glycerol. Reaction conditions for GP*a* assay are as in Materials and methods. Velocity is in units $\cdot ml^{-1} \cdot$ mg $protein^{-1}$ with V_{max} equal to 1.98 $U \cdot ml^{-1} \cdot mg\ protein^{-1}$. Data are means ±SEM, $n=3$ separate enzyme preparations; SEM bars are contained within the dimensions of the symbols used. All buffers were adjusted to specific pH values at 22 °C; the corresponding pH values of these buffers when cooled to 5 °C were calculated assuming a 0.018 pH unit increase per 1 °C decrease in temperature for imidazole buffer

Effect of pH on enzyme activity

Profiles of GP*a* activity versus pH are shown in Fig. 3a for assays at 22 °C and Fig. 3b for assays at 5 °C. Optimal activity at 22 °C was at pH 7.5, similar to the optima of 7.0–7.5 reported for phosphorylase from other animal sources (Fischer and Krebs 1959; Childress and Sacktor 1970; Morishima and Sakurai 1985). Maximal activity for all animal phosphorylases, including *E. scudderiana*, seems to be conserved at pH values between 7.0 and 7.5. The addition of 0.5 $mol \cdot l^{-1}$ glycerol to the assay did not change the optimum but enzyme velocity decreased as glycerol concentration increased. At 2 $mol \cdot l^{-1}$ glycerol the enzyme showed a broad optimum between pH 6.5 and 8.0. At 5 °C the pH optimum of GP*a* dropped to 7.31 under both control conditions (no glycerol) and in the presence of 0.5 $mol \cdot l^{-1}$ glycerol in the assay (Fig. 3b). In the presence of 2 $mol \cdot l^{-1}$ glycerol, however, the optimum was higher at pH 7.81.

Figure 4 shows pH versus activity profiles for GP*b*. At 22 °C the optimum was again pH 7.5; the addition of polyols had no effect on the optimum, but sharply reduced enzyme maximal activity (Fig. 4A). Unlike the situation for GP*a*, however, the pH optimum at 5 °C rose to 7.81 in the presence or absence of glycerol (Fig. 4B).

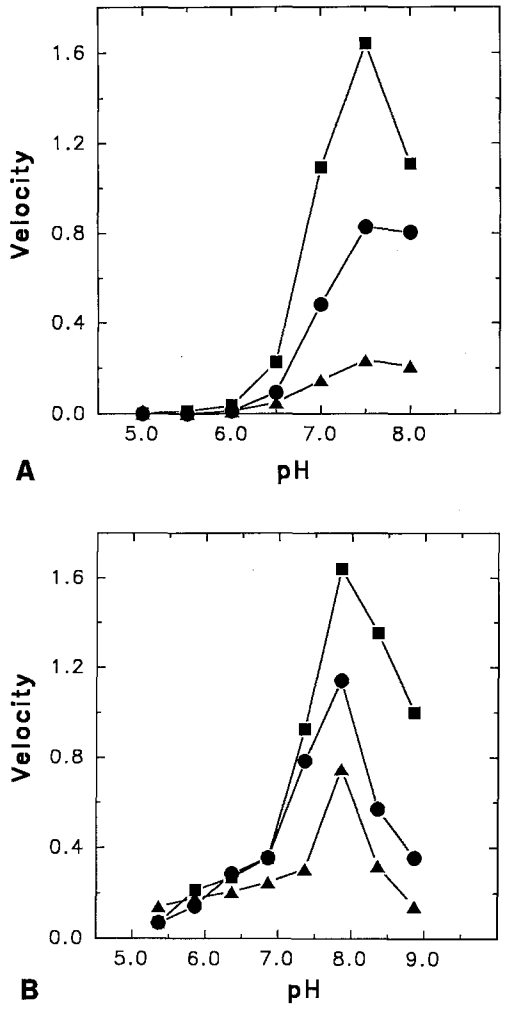

Fig. 4A, B. Plot of activity versus pH for *E. scudderiana* GP*b* at 22 °C (**A**) and 5 °C (**B**). ■ control, no additions; ● plus 0.5 mol·l^{-1} glycerol; ▲ plus 2 mol·l^{-1} glycerol. Reaction conditions for GP*b* assay are as in Materials and methods. Velocity is in units·ml^{-1}·mg protein^{-1} with V_{max} equal to 1.64 U·ml^{-1}·mg protein^{-1}. Data are means ± SEM, $n=3$ separate enzyme preparations; SEM bars are contained within the dimensions of the symbols used

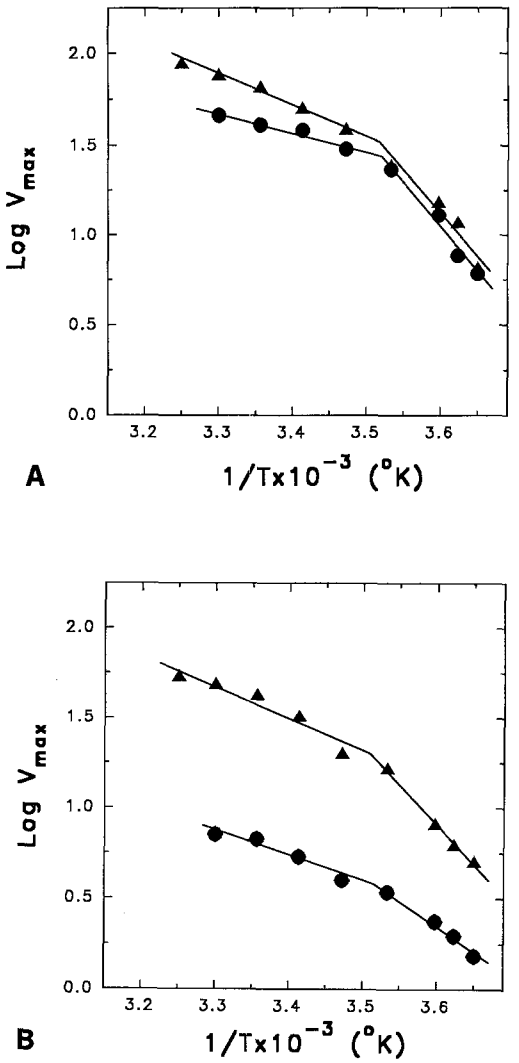

Fig. 5A, B. Arrhenius plots for *E. scudderiana* glycogen phosphorylase *a* (**A**) and *b* (**B**). Assays were run under V_{max} substrate conditions using phosphate buffer (in the presence of 1 mmol·l^{-1} AMP for GP*b*) and in the absence (▲) or presence (●) of 2 mol·l^{-1} glycerol. Velocity is in units·ml^{-1}·mg protein^{-1} with V_{max} equal to 1.98 and 1.64 U·ml^{-1}·mg protein^{-1} for GP*a* and GP*b*, respectively. Data are means for $n=4$ separate enzyme preparations; SEM bars are contained within the dimensions of the symbols used

Arrhenius plots

Figure 5 shows Arrhenius plots for *E. scudderiana* glycogen phosphorylase in the presence and absence of 2 mol·l^{-1} glycerol. For both GP*a* and GP*b* (Fig. 5A, B) the plots were discontinuous with a sharp change in slope between 15 and 10 °C in all cases. In the absence of glycerol E_a was 31 ± 1.5 kJ·mol^{-1} over the 15–35 °C range and increased significantly twofold to 77 ± 3.4 kJ·mol^{-1} between 1 and 10 °C ($P<0.005$). In the presence of 2 mol·l^{-1} glycerol E_a was 23 ± 1.3 kJ·mol^{-1} between 15 and 35 °C but increased fourfold to 97 ± 5.1 kJ·mol^{-1} over the range 1–10 °C ($P<0.005$). E_a values in the presence and absence of glycerol were also significantly different for each of the two temperature ranges ($P<0.01$).

The addition of glycerol strongly reduced the activity of GP*b* but, as seen in the GP*a* plots, the Arrhenius plots for GP*b* were again discontinuous with breaks between 15 and 10 °C (Fig. 5b). In the absence of glycerol E_a was 37 ± 2.1 kJ·mol^{-1} over the range 15–35 °C and increased significantly twofold to 86 ± 4.3 kJ·mol^{-1} when temperature decreased to the range 1–10 °C ($P<0.005$). When glycerol was present E_a decreased within both temperature ranges, being 28 ± 1.1 kJ·mol^{-1} between 15 and 35 °C and 1.5-fold higher at 56 ± 3.9 kJ·mol^{-1} between 1 and 10 °C ($P<0.005$).

Table 2. K_m values for glycogen of *E. scudderiana* GP*a* and GP*b*: effects of variable cosubstrate (phosphate) concentration, addition of glycerol, and temperature

Condition	K_m Glycogen (mg·ml^{-1})	
	GP*a*	GP*b*
22 °C		
50 mmol·l^{-1} P_i (optimal)	0.120±0.004	0.874±0.034[a]
20 mmol·l^{-1} P_i	0.132±0.008	2.74 ±0.046[a]
10 mmol·l^{-1} P_i	0.172±0.007	2.82 ±0.108[a]
50 mmol·l^{-1} P_i+		
0.5 mol·l^{-1} glycerol	0.073±0.002[c]	1.53 ±0.014[a,c]
2 mol·l^{-1} glycerol	0.302±0.006[c]	1.81 ±0.035[a,c]
5 °C		
50 mmol·l^{-1} P_i (optimal)	0.024±0.001[b]	0.193±0.009[a,b]
50 mmol·l^{-1} P_i+		
0.5 mol·l^{-1} glycerol	0.015±0.001[b,c]	0.117±0.011[a,b,c]
2 mol·l^{-1} glycerol	0.079±0.003[b,c]	0.086±0.009[b,c]

Data are means±SEM for $n=3$–5 separate enzyme preparations. [a] Significantly different from the corresponding value for GP*a* by the Student's *t*-test, $P<0.005$; [b] Significantly different from the corresponding value at 22 °C, $P<0.001$; [c] Significantly different from the corresponding value without glycerol, $P<0.005$

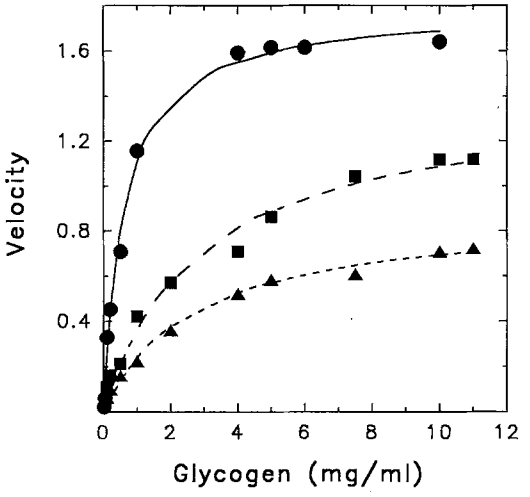

Fig. 6. Reaction velocity versus glycogen concentration for GP*b* at 22 °C at three different concentrations of inorganic phosphate. ● 50 mmol·l^{-1} P_i (optimal); ■ 20 mmol·l^{-1} P_i; ▲ 10 mmol·l^{-1} P_i. All assays included 1 mmol·l^{-1} AMP. Velocity is in units·ml^{-1}·mg protein^{-1} with V_{max} equal to 1.64 U·ml^{-1}·mg protein^{-1}. Data are means±SEM for $n=3$–5 determinations on separate enzyme preparations; error bars are enclosed within the symbols used

Substrate affinities

Table 2 shows K_m values for glycogen for both GP*a* and GP*b*. The relationship between glycogen concentration and enzyme velocity was hyperbolic (see Fig. 6 for GP*b*) with *h* not significantly different from 1 in all cases. GP*a* showed a substantially higher substrate affinity for glycogen than did GP*b*; K_m glycogen for GP*a* was 7.3- and 8-fold lower at 22 °C and 5 °C, respectively, than the corresponding value for GP*b* (at optimal phosphate concentration). The concentration of the cosubstrate, P_i, affected the glycogen K_m for both enzyme forms with K_m increasing as P_i concentration decreased. The effect was greater on GP*b*; at 10 mmol·l^{-1} P_i, K_m glycogen increased 220% for GP*b* compared with a 43% increase in K_m for GP*a*. Assay temperature also had a strong effect on K_m glycogen: low temperature (5 °C) sharply increased substrate affinity. GP*a* K_m glycogen was 5-fold lower at 5 °C compared with 22 °C (at optimal P_i), and similarly 4.5-lower for GP*b*.

Table 3. K_m values for inorganic phosphate for GP*a*: effects of variable glycogen concentration and addition of glycerol

[Glycogen]	K_m P_i (mmol·l^{-1})	Hill coefficient
22 °C		
0.5 mg·ml^{-1} (optimal)	6.49±0.07	2.30
0.2 mg·ml^{-1}	20.5 ±0.70	1.40
0.5 mg·ml^{-1}+0.5 mol·l^{-1} glycerol	8.65±0.22[b]	1.90
0.5 mg·ml^{-1}+2 mol·l^{-1} glycerol	10.4 ±0.19[b]	1.80
5 °C		
1 mg·ml^{-1} (optimal)	3.74±0.14[a]	1.31
1 mg·ml^{-1}+0.5 mol·l^{-1} glycerol	5.25±0.13[a,b]	1.60
1 mg·ml^{-1}+2 mol·l^{-1} glycerol	7.08±0.17[a,b]	1.00

Data are means±SEM for $n=3$–5 separate enzyme preparations. [a] K_m P_i at optimal [glycogen] significantly different from the corresponding value at 22 °C, $P<0.05$; [b] Significantly different from the corresponding value without glycerol, $P<0.001$

Table 2 also shows the effects of glycerol on K_m glycogen. During cryoprotectant synthesis by the larvae, glycogenolysis must go forward under ever-increasing concentrations of glycerol that reach at least 2000 µmol·g wet weight^{-1} in fully cold-hardened larvae (Rickards et al. 1987; Churchill and Storey 1989a). Glycerol effects on glycogen phosphorylase were, therefore, analysed at two concentrations: a concentration that is submaximal in vivo (0.5 mol·l^{-1}) and is characteristic of the phase of active glycerol biosynthesis, and a concentration that is maximal (2 mol·l^{-1}) in vivo and characteristic of the completion of cryoprotectant synthesis. Glycerol had novel effects on GP*a* K_m for glycogen; at 0.5 mol·l^{-1} glycerol K_m decreased by about 40% at both temperatures, whereas at 2 mol·l^{-1} glycerol the opposite effect was seen with K_m increasing 2.5–3 times compared with control values. For GP*b* K_m glycogen increased about twofold in the presence of glycerol at 22 °C, but the opposite effect occurred at 5 °C with K_m decreasing as glycerol increased.

Table 3 shows GP*a* substrate affinity for P_i. The relationship between velocity and P_i concentration was sigmoidal and more strongly so at 22 °C compared with 5 °C; for example, *h* was 2.3 and 1.3, respectively, at the two temperatures under optimal glycogen concentrations. Enzyme affinity for P_i (at 22 °C) was dependent on glycogen concentration, K_m increasing as [glycogen] decreased. K_m was also affected by temperature, decreasing by 40% at 5 °C compared with 22 °C under optimal glycogen concentrations. As for K_m glycogen, the K_m

Table 4. K_m values for inorganic phosphate for GP*b*: effects of variable glycogen concentration and addition of glycerol

[Glycogen]	K_m P_i ($mmol \cdot l^{-1}$)	Hill coefficient
22 °C		
4 $mg \cdot ml^{-1}$ (optimal)	23.6 ± 0.34	1.98
1 $mg \cdot ml^{-1}$	47.4 ± 0.19	1.87
4 $mg \cdot ml^{-1}$ + 0.5 $mol \cdot l^{-1}$ glycerol	61.3 ± 0.42^b	1.83
4 $mg \cdot ml^{-1}$ + 2 $mol \cdot l^{-1}$ glycerol	67.4 ± 1.6^b	1.80
5 °C		
4 $mg \cdot ml^{-1}$ (optimal)	14.5 ± 0.34^a	1.46

Data are means ± SEM for at least $n = 3$–5 separate enzyme preparations. [a] Significantly different from the corresponding value at 22 °C, $P < 0.05$; [b] Significantly different from the corresponding value without glycerol, $P < 0.001$
A combination of low enzyme activity plus high glycerol inhibition prevented analysis of glycerol effects on K_m at 5 °C

Table 5. Inhibition of *E. scudderiana* GP*a* and GP*b* by KCl

Condition	I_{50} KCl ($mmol \cdot l^{-1}$)	
	GP*a*	GP*b*
22 °C		
No glycerol	399 ± 4	178 ± 2^a
+ 0.5 $mol \cdot l^{-1}$ glycerol	208 ± 11^c	–
+ 2 $mol \cdot l^{-1}$ glycerol	161 ± 6^c	–
5 °C		
No glycerol	146 ± 8^b	$38 \pm 1^{a,b}$
0.5 $mol \cdot l^{-1}$ glycerol	$89 \pm 2^{b,c}$	–
2 $mol \cdot l^{-1}$ glycerol	$102 \pm 2^{b,c}$	–

Data are means ± SEM for at least $n = 3$–5 separate enzyme preparations. Assays were run with glycogen concentrations of 0.5 $mg \cdot ml^{-1}$ for GP*a* and 1 $mg \cdot ml^{-1}$ for GP*b*. [a] Significantly different from the corresponding value for GP*a* by the Student's *t*-test, $P < 0.05$; [b] Significantly different from the corresponding value at 22 °C, $P < 0.005$; [c] Significantly different from the corresponding value without glycerol, $P < 0.005$
A combination of low enzyme activity plus high glycerol inhibition prevented analysis of glycerol effects on I_{50} at 5 °C

for P_i was also affected by the addition of glycerol to the assay; K_m increased with increasing [glycerol] at both assay temperatures with K_m values at 2 $mol \cdot l^{-1}$ glycerol 1.6–1.9 times greater than in the absence of added glycerol.

Table 4 shows GP*b* affinity constants for P_i. The *b* form of the enzyme showed a lower affinity for P_i than did the *a* form, with GP*b* K_m values about fourfold higher than the corresponding values for GP*a* at both temperatures. Again, K_m P_i was increased when [glycogen] was decreased. K_m P_i for GP*b* was also dependent on temperature with affinity increasing at low temperature; K_m decreased by 40% at 5 °C compared with 22 °C. The Hill coefficient was also lower at 5 °C than at 22 °C. The addition of glycerol increased K_m for P_i at 22 °C threefold at 2 $mol \cdot l^{-1}$ glycerol.

Activation by AMP

AMP is a potent activator of GP*b* and increased the maximal activity of *E. scudderiana* GP*b* by about 20-fold. K_a for AMP at 22 °C was 176 ± 4 $\mu mol \cdot l^{-1}$ (at 4 $mg \cdot ml$ $glycogen^{-1}$, 50 $mmol \cdot l^{-1}$ P_i). K_a increased in the presence of glycerol to 338 ± 5 $\mu mol \cdot l^{-1}$ at 0.5 $mol \cdot l^{-1}$ glycerol and to 263 ± 10 $\mu mol \cdot l^{-1}$ at 2 $mol \cdot l^{-1}$ glycerol. Low temperature caused a significant increase in enzyme sensitivity to AMP, K_a dropping to 53 ± 1 $\mu mol \cdot l^{-1}$ ($P < 0.005$) at 5 °C (at 1 $mg \cdot ml$ $glycogen^{-1}$, 50 $mmol \cdot l^{-1}$ P_i).

Inhibition by KCl and glycerol

Table 5 shows that both GP*a* and GP*b* were inhibited by KCl, GP*b* being more strongly affected by the salt. Both enzymes showed an increase in inhibition by KCl (I_{50} decreased significantly) at low temperature; I_{50} decreased by a factor of 2 for GP*a* and 4.7 for GP*b* at 5 °C compared with 22 °C. KCl inhibition of GP*a* increased in the presence of glycerol, the I_{50} decreasing by 60% in the presence of 2 $mol \cdot l^{-1}$ glycerol at 22 °C and by 25% at 5 °C.

Strong inhibition by glycerol prevented analysis of glycerol effects on several properties of GP*b*. Measurements of I_{50} values for glycerol confirmed that the polyol was a much stronger inhibitor of GP*b* than of GP*a*. I_{50} values for glycerol were 383 ± 18 $mmol \cdot l^{-1}$ at 22 °C but fell significantly to 61 ± 2 $mmol \cdot l^{-1}$ at 5 °C ($n = 3$–5 for each; $P < 0.005$; at 1 $mg \cdot ml$ $glycogen^{-1}$, 50 $mmol \cdot l^{-1}$ P_i).

Discussion

The purification procedure used for *E. scudderiana* glycogen phosphorylase is a fast, two-step method that gives a high yield and a high purification factor and readily separates the *a* and *b* forms of the enzyme. Final specific activities for *E. scudderiana* GP*a* and GP*b* were 396 and 82 $U \cdot mg^{-1}$, respectively, much higher than specific activities previously reported for the insect enzyme from other sources: blowfly flight muscle, 12 $U \cdot mg^{-1}$ and silkmoth fat body, 47.3 $U \cdot mg^{-1}$ (Childress and Sacktor 1970; Morishima and Sakurai 1985).

The native (209–215 kDa) and subunit (87 kDa) MWs determined for GP*a* and GP*b* indicated that both forms of *E. scudderiana* glycogen phosphorylase are dimeric proteins; the slightly higher mean MW for GP*a* (215 kDa) can be attributed, at least in part, to the presence of covalently-bound phosphate on the enzyme. The subnit MW of *E. scudderiana* phosphorylase is a little lower than the 90–100 kDa reported for the enzyme from mammalian and other insect sources (Childress and Sacktor 1970; Titani et al. 1977; Dombradi et al. 1986; Morishima and Sakurai 1985; Van Marrewijk et al.

1985). Early studies on glycogen phosphorylase proposed that the conversion of GP*b* to GP*a* involved a dimer to tetramer transition (Keller and Cori 1953), but more recent studies have shown that the most active form of GP*a* in the presence of glycogen is a dimer (Huang and Graves 1970). Similarly, active GP*a* from *E. scudderiana* is a dimer, apparently with identical subunits, as has also been reported for the locust fat body and the fruit fly enzymes (Dombradi et al. 1986; Van Marrewijk et al. 1985). However, the active form of phosphorylase in some other insects (blowfly flight muscle, silkworm fat body) appears to be a monomer (Childress and Sacktor 1970; Morishima and Sakurai 1985).

The primary mode of phosphorylase regulation in animals is the interconversion between the active *a* and inactive *b* forms. A rapid increase in the amount of the *a* form has been well documented as a response to cold exposure in many species, including *E. scudderiana,* and initiates the glycogenolysis needed for cryoprotectant biosynthesis (Yamashita et al. 1975; Ziegler et al. 1979; Hayakawa and Chino 1982; Churchill and Storey 1989a; Storey and Storey 1991). Indeed, in the present study we found 72% GP*a* in −4 °C-acclimated larvae compared with only 9% GP*a* in 15 °C-acclimated animals. Our study of the kinetic properties of *E. scudderiana* GP*a* and GP*b*, however, show that a number of other factors, particularly temperature and glycerol effects on the kinetic properties of the enzymes, can also have important roles in regulating glycogenolysis.

Both GP*a* and GP*b* showed a dependence of the K_m for one substrate on the concentration of cosubstrate; thus, K_m glycogen increases as $[P_i]$ decreases (and vice versa). Such heterotropic interactions have also been reported for other insect phosphorylases (Childress and Sacktor 1970; Vaandrager et al. 1987). K_m values for both substrates for GP*a* are similar to values for other insect phosphorylases (Childress and Sacktor 1970; Morishima and Sakurai 1985; Vaandrager et al. 1987; Morishima and Ueno 1990) and well within the physiological concentration range of these compounds in vivo. K_m values for GP*a* for glycogen and P_i were much lower than the corresponding values for GP*b* at both temperatures, as is typical of phosphorylase from all sources, and further indicates that GP*a* is the active form. The $[P_i]$ in the larvae, as measured by the method of Atkinson et al. (1973), was found to be 45 ± 1.3 μmol·g wet weight^{-1} (C. Holden, unpublished results) and this compares favorable with GP*a* K_m values for P_i of 3.7–6.5 mmol·l^{-1} at optimal glycogen. Glycogen content of *E. scudderiana* larvae increases as high as 230 mg·g wet weight^{-1} in early autumn before cryoprotectant synthesis begins (Rickards et al. 1987), an amount far in excess of the GP*a* K_m values for glycogen of 0.02–0.12 mg·ml^{-1} (at optimal P_i) or the optimal glycogen concentrations of 0.5–1 mg·ml^{-1}. Thus, GP*a* activity appears unlikely to be limited by substrate availability in vivo.

With respect to GP*b* activity, larval glycogen content was also many-fold higher than the K_m glycogen values but the high K_m P_i values may mean that the enzyme is limited by P_i availability. Indeed GP*b* is often considered to be functionally inactive in vivo because of its low activity in the absence of allosteric activators and the often high, non-physiological concentrations of activators needed to maximize activity (Childress and Sacktor 1970; Stalmans and Hers 1975; Newgard et al. 1989). AMP levels in unstressed *E. scudderiana* are about 0.03–0.1 μmol·g wet weight^{-1}, compared with GP*b* K_a values for AMP of 0.05–0.18 mmol·l^{-1} (Churchill and Storey 1989b, c), and this suggests that GP*b* is probably subactive in vivo. However, AMP levels rise sharply (from 0.08 to 0.20 μmol·g^{-1}) in *E. scudderiana* in response to a low temperature shock that stimulated glycerol synthesis (Churchill and Storey 1987b). Thus, it is possible that in addition to the GP*b* to GP*a* conversion that is stimulated by low temperature, high AMP levels may also increase the activity of remaining GP*b*.

The kinetic properties of both GP*a* and GP*b* from *E. scudderiana* demonstrate that the enzyme is well designed for low-temperature function and this would potentiate the high rates of polyol synthesis that occur within the range 5 to −5 °C in this and other cold-hardy insects (Storey and Storey 1991). Both temperature and temperature-modulator interactions on kinetic properties facilitate low temperature function of the enzyme. Thus, GP*a* affinity for both substrates increased at 5 °C compared with 22 °C: glycogen affinity fivefold and P_i affinity by nearly twofold. GP*b* showed the same pattern and, furthermore, the K_a AMP dropped threefold at low temperature.

The K_m for glycogen of GP*a* also showed a novel response to the presence of glycerol. At intermediate glycerol levels (0.5 mol·l^{-1}) the presence of the polyol increased enzyme affinity for glycogen (K_m dropped by about 40%) whereas when high glycerol (2 mol·l^{-1}) was present the effect was reversed and K_m rose about threefold compared with K_m in the absence of glycerol (Table 2). During the period of active glycerol accumulation during the autumn cold-hardening it would be advantageous for the accumulating glycerol to have a positive feed-back effect on the rate of glycogenolysis via modulation of the K_m for glycogen. However, at very high glycerol concentrations the reverse effect, an inhibition by high glycerol, could be one factor that slows phosphorylase activity and/or promotes the reconversion of GP*a* to GP*b* when cryoprotectant levels reach their maximum in vivo, about 2000 μmol·g^{-1} (Churchill and Storey 1987a). Intermediate levels of glycerol (0.5 mol·l^{-1}) also has no effect on the pH optimum of the enzyme and relatively little effect on the maximum activity of GP*a* at either 22 or 5 °C, whereas high glycerol (2 mol·l^{-1}) reduced V_{max}, broadened the pH optimum at 22 °C and shifted the optimum to a higher value at 5 °C (Fig. 3). High glycerol also strongly depressed the maximum velocity of GP*b* at both temperatures as shown in the pH curves (Fig. 4) and the Arrhenius plot (Fig. 5) but did lower the K_m glycogen at 5 °C. Indeed, with a GP*b* I_{50} for glycerol of 61 mmol·l^{-1} at 5 °C, the very strong inhibitory effects of glycerol made it impossible to accurately assess various kinetic parameters of GP*b* in the presence of glycerol at low temperature.

High salt (KCl) strongly inhibited *E. scudderiana*, gly-

cogen phosphorylase although the *a* form appeared to be less sensitive to KCl than the *b* form. The larvae lose about 25% of their total body water during cold hardening (Rickards et al. 1987) so a reduced sensitivity of the *a* form to high ionic strength could be advantageous in the intracellular milieu of the overwintering larvae.

Analysis of Arrhenius plots of phosphorylase activity showed a distinct break in the relationship for both GP*a* and GP*b* and in the presence or absence of glycerol. The break occurred between 10 and 15 °C in all cases creating two linear parts of the plot with two- to fourfold higher K_as at temperatures below 10 °C than at temperatures of 15–35 °C. Such discontinuities in the Arrhenius relationship are not unusual for multimeric enzymes and indicate a temperature-induced change in conformation or subunit aggregation state of the enzyme. Phosphofructokinase from *E. scudderiana* and from the cold-hardy larvae of the gall fly *Eurosta solidaginis* shows a break in the Arrhenius plot in the range 10–15 °C, whereas *E. scudderiana* aldolase and fructose-1,6-bisphosphatase do not (Storey 1982; C. Holden, unpublished data). Calculated Q_{10} values for the interval 0–10 °C were 3.3 for GP*a* and 3.7 for GP*b* in the absence of glycerol indicating a fairly rapid decrease in enzyme maximal velocity with decreasing temperature over this range. This may seem to be at odds with the high rates of glycogenolysis and glycerol synthesis that occur in the larvae in vivo at low temperatures. However, effects of temperature on V_{max} may be outweighed by the low-temperature-stimulated conversion of GP*b* to GP*a* to produce at least 70% GP*a* in vivo during periods of active polyol synthesis (Churchill and Storey 1989a; this study) and by the increased enzyme affinity for both substrates seen at low temperatures.

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