



Reversible Phosphorylation of Fructose 1,6-Bisphosphatase Mediates Enzyme Role in Glycerol Metabolism in the Freeze-Avoiding Gall Moth *Epiblema scudderiana*

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Fructose-1,6-bisphosphatase (FBPase) from larvae of the freeze-avoiding gall moth *Epiblema scudderiana* occurs in two forms, which are interconverted by reversible phosphorylation and separable by CM-cellulose column chromatography. The phosphoenzyme has properties that would make it the more active form *in vivo*. Compared with the dephosphorylated form, the phosphoenzyme had three-fold lower values for K_m fructose-1,6-bisphosphate and K_a Mg^{2+} and lower sensitivities to allosteric inhibitors (I_{50} values for fructose-2,6-bisphosphate and AMP were 50% and 10-fold higher, respectively). The proportions of the two enzyme forms in the larvae changed with the seasons and with acclimation to warm (15°C) vs cold (4°C) temperatures. The phosphorylated enzyme predominated (70% of total activity) in early autumn and during the spring, as well as in warm acclimated larvae, all situations where gluconeogenesis via FBPase would be favoured. During the autumn cold-hardening period when the larvae are actively synthesizing the antifreeze, glycerol, the ratio of the two enzyme forms changed to about 50:50. This, plus allosteric inhibition and low temperature effects on enzyme kinetics, would effectively suppress FBPase activity and prevent futile recycling of glycerol carbon back into glycogen during the winter months when the 2 M pool of polyol must be sustained for antifreeze protection. Acclimation studies suggested that low temperature itself might be the signal that triggers enzyme dephosphorylation, and this could integrate control over FBPase with the well-known phosphorylation-mediated activation of glycogen phosphorylase by low temperature in cold-hardy insects. © 1997 Elsevier Science Ltd

Insect cold hardiness Polyol metabolism Gluconeogenesis Protein kinase Antifreeze

INTRODUCTION

Larvae of the goldenrod gall moth, *Epiblema scudderiana* (Clemens), use a freeze avoidance strategy to endure prolonged exposures to subzero temperatures during the winter. During autumn cold-hardening, supercooling point is pushed to -38°C and the larvae synthesize huge amounts of glycerol, levels of the polyol rising to over 2000 $\mu\text{mol/g}$ fresh mass or approx. 19% of total larval mass (Rickards *et al.*, 1987). Glycerol is the most common of several polyhydric alcohols that occur in cold-

hardy insects (Zachariassen, 1985), many of its physical features suiting it for antifreeze or cryoprotectant actions (Storey and Storey, 1988). Various metabolic considerations also favour the use of glycerol, including the near quantitative conversion of glycogen to glycerol that preserves the net carbohydrate pool of the organism and the close links between glycerol metabolism and the central glycolytic pathway (Storey and Storey, 1988).

The synthesis of glycerol as a winter protectant in insects is fuelled from reserves of glycogen that are accumulated in the fat body during late summer feeding. During the autumn cold-hardening period, the activities of various enzymes needed for glycerol synthesis are elevated and production is stimulated as ambient temperatures drop. The trigger temperature for most species, including *E. scudderiana*, is within the range of 0–5°C, with maximal rates of glycogen to glycerol conversion

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between 0 and -10°C (Kelleher *et al.*, 1987). Once initiated by autumn cold temperatures, the build-up of glycerol pools is one way, and subsequent warm exposure does not impede the continuing accumulation of the polyol (Churchill and Storey 1989a). This situation changes later in the winter when the larvae begin to respond to warming with a rapid catabolism of glycerol and restorage of the carbon into glycogen reserves. However, they still respond to recooling with renewed glycerol synthesis. By early spring the situation shifts again: the catabolic response to warming is even stronger and the glycerol synthesis response to cold is even weaker. The larvae thus clearly show seasonal changes in the metabolic "poise" of their glycerol-synthesizing and glycerol-catabolizing pathways (Churchill and Storey, 1989a), and this must come from programmed changes in the key regulatory enzymes that control the pathways involved. For example, an analysis of glycogen phosphorylase showed that not only did total enzyme activity decline from autumn through to late winter, but the stimulatory effect of low temperature on the enzyme (percentage converted to the active *a* form) also faded (Churchill and Storey, 1989a).

A metabolic locus that is very important in glycerol metabolism is the interconversion of fructose-6-phosphate (F6P) and fructose 1,6-bisphosphate (F1,6P₂). The enzymes interconverting these two metabolites effectively control the partitioning of carbohydrate between the hexose phosphate and triose phosphate pools (because F1,6P₂ is readily equilibrated with the triose phosphates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, via the aldolase reaction). The ATP dependence of the 6-phosphofructo-1-kinase reaction (PFK), which converts F6P to F1,6P₂, means that the biosynthesis of glycerol is a major energetic cost to the larvae and, therefore, one which needs to be closely regulated. However, PFK is functionally irreversible *in vivo* and so the reconversion of glycerol to glycogen involves fructose 1,6-bisphosphatase (FBPase) (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) which plays a key role in gluconeogenesis in all animals (Tejwani, 1983). FBPase can be regulated in at least three ways: (a) by changing the amount of enzyme present; (b) by reversible protein phosphorylation which alters enzyme substrate and effector affinities; and (c) by powerful allosteric regulators including fructose 2,6-bisphosphate (F2,6P₂) and AMP as inhibitors and bivalent cations as activators (Tejwani, 1983; Pilkis *et al.*, 1987). Furthermore, for poikilothermic animals, temperature change can be an added factor that regulates enzyme function; for example, the low temperature triggering of glycerol accumulation in many insects is due to a low temperature activation of glycogen phosphorylase (Storey and Storey, 1991).

In recent studies we have shown that three of these mechanisms clearly contribute to the regulatory control of FBPase in *E. scudderiana*. Seasonal changes in enzyme maximal activities alter the activity ratio,

FBPase/PFK, from 0.2 in mid-September to 6.8 in early March (Joanisse and Storey, 1994), levels of allosteric inhibitors (F2,6P₂ and AMP) are greatly increased during active glycerol synthesis (Churchill and Storey, 1989b), and low assay temperature alters enzyme substrate affinity and greatly increases sensitivity to allosteric inhibitors (Holden and Storey, 1995). In the present study we examine the fourth potential regulatory influence on FBPase — reversible protein phosphorylation via the action of protein kinases and protein phosphatases. The results demonstrate that both seasonal and temperature-dependent changes in the distribution of the enzyme between phosphorylated and dephosphorylated forms occurs, and that this mode of regulation is important for regulating glycolytic vs gluconeogenic flux to achieve glycerol synthesis vs catabolism.

MATERIALS AND METHODS

Animals and chemicals

Galls containing the larvae of the moth *E. scudderiana* were gathered in the Ottawa area in early September 1989. Galls were stored in sacks outdoors where they experienced ambient winter temperatures. On various dates over the 1989–90 winter, groups of galls were brought into the lab and placed in holding incubators set at the current outdoor temperature. Within the next few hours, galls were opened and larvae removed, quickly frozen in liquid nitrogen, and then transferred to -80°C for storage. In October 1989, other galls were placed in constant temperature incubators set at either 15°C or -4°C , and held there for three weeks. The -4°C temperature was chosen because it stimulates rapid glycerol synthesis (Churchill and Storey, 1989b), whereas at 15°C levels of the cryoprotectant would remain low. All biochemicals were purchased from Boehringer–Mannheim Corp. (Montreal, PQ, Canada) or Sigma Chemical Co. (St Louis, MO, U.S.A.), unless otherwise stated.

Enzyme preparation and purification

Samples of frozen larvae were homogenized 1:10 w/v in buffer A (15 mM imidazole–HCl, 75 mM NaF, 3.5 mM EDTA, 3.5 mM EGTA, 15 mM 2-mercaptoethanol, 20% v/v glycerol, pH 7.5) with a few crystals of the protease inhibitor, phenylmethylsulfonyl fluoride, added. After centrifugation for 20 min at 24,000g in a Sorvall RC-5B refrigerated centrifuge at 5°C , the supernatant was removed and applied to a phosphocellulose column equilibrated in buffer A. The column was washed with 20 ml of buffer A and then FBPase was eluted with a gradient of 0–1 M KCl in buffer A; 1 ml fractions were collected and assayed. The five peak fractions were pooled and desalted by centrifugation (1 min at top speed on a desk-top centrifuge) through a column of G-25 Sephadex equilibrated in buffer B (10 mM imidazole–HCl, 50 mM NaF, 2.5 mM EGTA, 2.5 mM EDTA, 15 mM 2-mercaptoethanol, 20% v/v glycerol, pH 7.9).

The eluant from the G-25 column was then layered onto a CM-cellulose column equilibrated in buffer B. The column was washed with 20 ml of buffer B and eluted with a linear gradient of 0–300 mM KCl in buffer B; 1 ml fractions were collected and assayed. Peak fractions were then collected, pooled, and desalted by centrifugation through a G-25 column equilibrated in buffer B.

In vitro phosphorylation and dephosphorylation incubations

The CM-cellulose column showed that FBPase eluted as two peaks. To determine if these peaks corresponded to phosphorylated and dephosphorylated forms of FBPase, enzyme samples were incubated *in vitro* under conditions that would promote either phosphorylation or dephosphorylation of the enzyme. Samples of the frozen larvae were homogenized in buffer B and centrifuged as previously described. The supernatant was removed, divided into two equal portions, and then stored on ice. The first portion was desalted by centrifugation through a G-25 column equilibrated in buffer C (buffer B minus EGTA and EDTA). A 0.5 ml aliquot of the eluant was incubated in 0.5 ml of buffer C containing 1 mM cyclic 3'5' adenosine monophosphate (cAMP), 10 mM MgCl₂, and 10 mM ATP, adjusted to pH 7 in order to promote phosphorylation of the enzyme. The second portion was desalted by centrifugation through a G-25 column in buffer D (buffer B minus NaF, EGTA, and EDTA) and then a 0.5 ml aliquot was incubated with 0.5 ml of buffer D containing 10 mM MgCl₂, adjusted to pH 7 in order to promote dephosphorylation of the enzyme. Enzymes were incubated at 4°C for 24 h and then desalted by centrifugation through a G-25 column equilibrated in buffer B. The preparations were then chromatographed on phosphocellulose and CM-cellulose columns as described earlier.

Enzyme assay and kinetics

FBPase activity was measured by following F1,6P₂ consumption in a coupled enzyme assay that converted NADP⁺ to NADPH and was monitored at 340 nm on a Gilford 260 spectrophotometer with a water-jacketed cell holder that maintained the assay temperature at 22°C. Optimal assay conditions were determined to be 0.1 mM F1,6P₂, 5 mM MgSO₄, 0.2 mM NADP⁺ and 1 unit each of phosphoglucosomerase and glucose-6-phosphate dehydrogenase. Kinetic studies were performed on enzyme purified from larvae that were acclimated in the laboratory at –4°C. K_m values were determined from Michaelis–Menten plots. The K_a for MgSO₄ was determined from plots of V_{max} vs [activator] at suboptimal F1,6P₂ concentration, and I₅₀ values for AMP and F2,6P₂ were determined from plots of V/V_{max} vs [inhibitor]. All values were reported as means ± SEM for at least three determinations. Statistical differences were determined using the Student's *t*-test (two-tailed).

RESULTS

Enzyme preparation and in vitro incubations

Partial purification of FBPase from –4°C acclimated *E. scudderiana* larvae involved initial chromatography on phosphocellulose from which the enzyme eluted in a single peak at approx. 200 mM KCl (data not shown). The enzyme was subsequently applied to a CM-cellulose ion exchange column and on this column eluted in two distinct peaks at approx. 60 and 120 mM KCl, respectively (Fig. 1). To determine whether these forms represented isozymes or were phosphorylated vs dephosphorylated forms of a single enzyme, crude enzyme extracts were incubated *in vitro* under conditions that promoted the actions of either endogenous protein phosphatases or protein kinases. After *in vitro* incubation, extracts were then subjected to phosphocellulose and CM-cellulose chromatography as described above. Figure 2 shows that when enzyme extracts were incubated under conditions that promoted phosphorylation by cAMP-dependent protein kinase, virtually all of the enzyme activity shifted into a single peak that eluted at 63 ± 2 mM KCl. In contrast, when enzyme extracts were incubated under conditions that promoted dephosphorylation, activity shifted into a peak that eluted at 110 ± 7 mM KCl. These two peaks corresponded to those seen in Fig. 1, and demonstrate that the two peaks of FBPase activity occurring naturally in the larvae can be identified as phosphorylated (eluting first) and dephosphorylated (eluting second) forms of the enzyme.

Seasonal and temperature-dependent changes in the phosphorylation state of FBPase

To determine whether the proportion of phosphorylated vs dephosphorylated FBPase in the larvae changed with acclimation state or in a seasonal pattern that might be related to cryoprotectant synthesis, FBPase was ana-

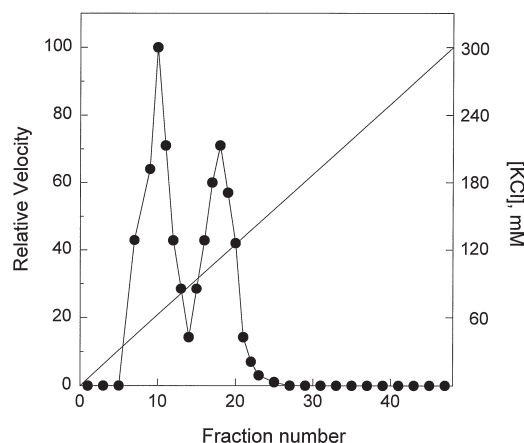


FIGURE 1. Elution profile of *E. scudderiana* FBPase on CM-cellulose. Peak fractions from phosphocellulose chromatography of the enzyme from –4°C acclimated larvae were pooled, applied to CM-cellulose and eluted with a linear gradient of 0–300 mM KCl. Activities were assayed at 22°C and are shown relative to the velocity in the peak tube.

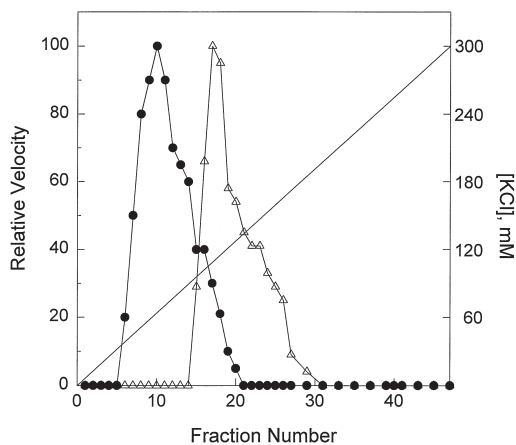


FIGURE 2. Effect of *in vitro* incubations under conditions promoting enzyme phosphorylation or dephosphorylation on the subsequent elution profile of *E. scudderiana* FBPase on CM-cellulose. The representative plot shows results from two separate incubations. The filled circles represent incubation under conditions promoting endogenous cAMP-dependent protein kinase activity. Open triangles show enzyme incubated under conditions that promote endogenous phosphatase activity. Enzyme was eluted with a linear gradient of 0–300 mM KCl. Other information as in Fig. 1.

lysed in extracts from larvae sampled from various conditions. After chromatography on CM-cellulose, the peak areas for each form were calculated and Fig. 3 shows the percentage of FBPase activity present in the phosphorylated vs dephosphorylated forms at each sampling time. Autumn-collected larvae acclimated to 15 vs -4°C showed distinctly different distributions of the two enzyme forms. At the warmer temperature 68% of enzyme activity was in the phosphorylated form and 32% in the dephosphorylated form. At -4°C , however, the

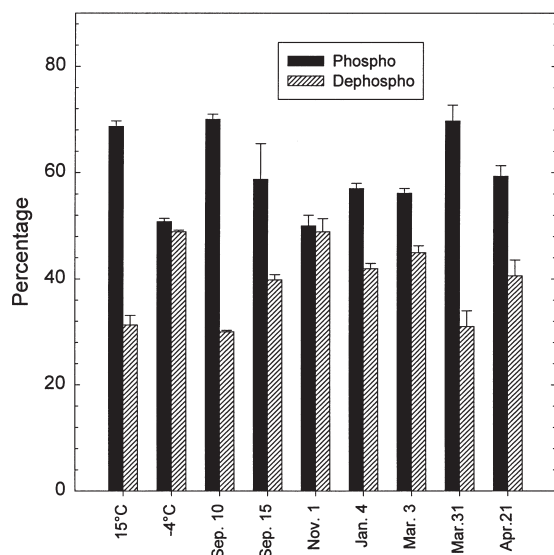


FIGURE 3. Seasonal changes in the percentage of *E. scudderiana* FBPase present in the phosphorylated (solid bars) vs dephosphorylated (shaded bars) forms in the larvae. The two enzyme forms were separated on CM-cellulose as described in Fig. 1, and peak areas were calculated. For temperature acclimation, October larvae were acclimated to 15 or -4°C for 3 weeks. Data are means \pm SEM, $n = 3$.

proportions of the two enzyme forms shifted until they were nearly equal. Proportions of the two enzyme forms also varied seasonally. In early September, the phosphorylated enzyme form predominated over the dephosphorylated (70 vs 30%) but the percentage of the phosphoenzyme decreased as the autumn progressed, and by November 1 the percentages of the two enzyme forms were equal. Over mid- to late winter the percentage of phosphorylated FBPase rose again to 55–58% and by late March the phosphoenzyme again comprised 70% of total activity. By late April the distribution had changed again to 59% phosphorylated, 41% dephosphorylated.

Kinetic analysis

Kinetic properties of the phosphorylated and dephosphorylated forms of FBPase were assessed using the enzymes that were partially purified from -4°C acclimated larvae (Fig. 1). Table 1 shows that the two enzyme forms had very different kinetic properties. The phosphoenzyme had a significantly greater affinity for its substrate, F1,6P₂, with a K_m value only one-third of the value for the dephosphorylated form. Similarly, the affinity of the phosphoenzyme for Mg^{2+} ion cofactor, was also greater with a K_a approx. one-third of the value for the dephosphorylated form. In contrast, the phosphorylated enzyme was much less susceptible to the effects of inhibitors, AMP and fructose-2,6-bisphosphate, than was the dephosphorylated enzyme. The I_{50} for F2,6P₂ of the phosphorylated enzyme was 50% higher than that of the dephosphorylated form, and the I_{50} for AMP was 10-fold higher. The combined effects of the kinetic differences between the two enzyme forms indicate that the phosphorylated enzyme form would be much more active *in vivo*.

DISCUSSION

The present study shows that FBPase in *E. scudderiana* exists in two forms, and that these are interconvertible by reversible protein phosphorylation. Conditions of *in vitro* incubation that promoted the action of endogenous cAMP-dependent protein kinase thus shifted enzyme activity into the form eluting off phosphocellulose at low ionic strength (63 mM), whereas incubation under conditions promoting protein phosphatase activity shifted the enzyme to elute at higher ionic strength (110 mM). Characterization of the kinetic properties of the two enzyme forms showed that phosphorylation strongly altered these. The phosphoenzyme is clearly the more active form with a greater affinity for substrate (K_m three-fold lower) and Mg^{2+} (K_a three-fold lower) and lower sensitivity to inhibition by F2,6P₂ (I_{50} 50% higher) or AMP (I_{50} 10-fold higher) (Table 1). These characteristics agree with the properties of the phosphorylated and dephosphorylated forms of FBPase from mammalian liver (Vidal *et al.*, 1986) but are opposite to the effects of phosphorylation on yeast FBPase (Gancedo *et al.*, 1983; Pohlig and Holzer, 1985). Some authors have reported

TABLE 1. Properties of the phosphorylated and dephosphorylated forms of partially purified FBPase from -4°C acclimated *E. scudderiana*

	Phosphorylated	Dephosphorylated
K_m F1,6P ₂ (μM)	25 ± 6	$76 \pm 16^*$
K_a Mg ²⁺ (mM)	5.6 ± 0.4	$17.1 \pm 1.28^\dagger$
I_{50} F2,6P ₂ (μM)	0.95 ± 0.10	$0.60 \pm 0.10^*$
I_{50} AMP (μM)	63.0 ± 0.75	$6.0 \pm 0.15^\dagger$

Data are means \pm SEM, $n = 3-5$ preparations of the purified enzymes. Assays were at 22°C .

*Significantly different from the corresponding value for the high phosphate form using a two-tailed Student's t -test, $P < 0.05$; $^\dagger P < 0.005$.

that the phosphoenzyme from rat liver showed a higher affinity for F1,6P₂ and less inhibition by F2,6P₂ and AMP than the dephosphorylated form (Ekdahl and Ekman, 1984; Vidal *et al.*, 1986), although Pilkis *et al.* (1987), in summarizing the field, showed that there was still considerable uncertainty about the effects of phosphorylation on the properties of the mammalian liver enzyme and its role in regulating enzyme function.

FBPase is an important regulatory enzyme in carbohydrate metabolism, and along with PFK-1 sits at a critical locus that controls carbon interconversion between the hexose phosphate and triose phosphate portions of glycolysis. When active, FBPase facilitates gluconeogenic flux whereas the enzyme is inactivated via the actions of allosteric inhibitors, AMP and F2,6P₂ (Vidal *et al.*, 1986; Hue and Rider, 1987; Pilkis *et al.*, 1987), and covalent modification (Meek and Nimmo, 1984; Vidal *et al.*, 1986; Ekdahl, 1992) when glycolysis needs to be promoted. Based on the known patterns of carbohydrate metabolism in *E. scudderiana*, it is proposed that gluconeogenic flux, mediated by an active FBPase, should be important at two different seasons: (a) during late summer and early autumn when glycogen reserves are being laid down; and (b) during the spring clearance of glycerol pools when a significant portion of total carbon is reconverted to glycogen. Note that glycerol also appears to have other fates in the spring such as oxidation as an aerobic fuel or use as a substrate for biosynthesis (Storey and Storey, 1991), fates that would make use of the NADPH or NADH generated by polyol dehydrogenase or glycerol-3-phosphate dehydrogenase, respectively. In contrast, inhibition of FBPase is needed during the autumn period of active glycerol accumulation in order to achieve an energy-efficient, near-stoichiometric conversion of glycogen into glycerol. During polyol synthesis in cold-hardy insects, glycogen-derived carbon can reach the glycerol pool in one of two ways. Some of the carbon flows through glycolysis and the ATP-dependent PFK-1 reaction to reach the triose phosphates that lead into the reactions of glycerol synthesis. A high proportion of total carbon, however, is channelled through the hexose monophosphate shunt (HMS) in order to generate the NADPH-reducing equivalents that are needed for the conversion of sugar to polyhydric alcohol. The outputs of the HMS are F6P and glyceraldehyde-3-phosphate (GAP). The former is processed via PFK-1 whereas the latter can be directly channelled into glycerol via the

GAP phosphatase and polyol dehydrogenase reactions. What is critical for overall efficiency, however, is that GAP is not allowed to return to the hexose phosphate pool. That depends on inhibition of the FBPase and/or aldolase reactions. An analysis of purified aldolase from *E. scudderiana* showed the presence of a single enzyme form whose K_m increased two-fold at 5°C vs 22°C , and that was somewhat more susceptible to inhibition by inorganic phosphate and glycerol-3-phosphate at low temperatures (Holden and Storey, 1994). However, with substrate concentrations *in vivo* that are at least 100-fold higher than K_m values, it is obvious that substrate is always saturating for aldolase and that little regulation of this equilibrium enzyme is possible (Churchill and Storey, 1989b; Holden and Storey, 1994). Regulatory control over gluconeogenic flux from the triosephosphates is thus undoubtedly centered on FBPase.

This study, when combined with our previous data, shows that multi-faceted controls are applied to regulate FBPase in *E. scudderiana*, and thereby to help regulate the glycogen and glycerol pools that together represent approx. 20% of the total body mass of the larvae. The present study shows that one of the mechanisms of control is reversible protein phosphorylation, the kinetic analysis indicating that the phosphoenzyme would be the more active form *in vivo* with its greater affinity for substrate and lower susceptibility to allosteric inhibitors. The changing proportions of the phosphorylated and dephosphorylated over the seasons or with temperature acclimation show that the phosphoenzyme predominated under conditions where gluconeogenesis should be active, whereas the proportion of the dephosphorylated enzyme rose under conditions where gluconeogenesis needs to be inhibited. The phosphorylated form thus predominated (approx. 70% of total activity) during early autumn when larvae were still feeding and accumulating glycogen reserves. The same situation was also found when October larvae were acclimated to a warm temperature (15°C) at which glycerol is not synthesized. However, the proportion of the phosphoenzyme dropped to approx. 50% during later autumn when glycerol would be rapidly accumulating (Rickards *et al.*, 1987), and was also low when larvae were placed at -4°C , a temperature that quickly stimulates glycerol production (Churchill and Storey, 1989a). The percentage of phosphorylated enzyme rose again during late winter and spring when

glycerol pools are being catabolized and carbon is being channelled back into glycogen reserves.

Changes in the phosphorylation state of FBPase can also be integrated with other controls on the enzyme to provide effective control over gluconeogenesis in the larvae. In addition to the seasonal changes in enzyme distribution between phosphorylated and dephosphorylated states, the maximal activity of FBPase in the larvae rose several-fold from autumn to spring, undoubtedly due to increased protein synthesis. Furthermore, the ratio of FBPase to PFK activities changed dramatically over the seasons to shift the metabolic poise of carbohydrate metabolism from predominantly glycolytic in the autumn to predominantly gluconeogenic in the spring. In the early autumn, PFK activity is high (approx. 2.5 U/g wet mass) whereas FBPase is very low (approx. 0.5 U/g ww), a situation that would facilitate glycerol synthesis (Joanisse and Storey, 1994). By January, however, FBPase activity had risen by 14-fold, and by March PFK activity had fallen by more than half so that the ratio of maximal activities, FBPase/PFK, changed from 0.2 in the autumn to 6.8 in late winter. Coupled with a strong rise during the spring in the percentage of larval FBPase present in the more active, phosphorylated form, this would clearly favour gluconeogenic flux in the larvae during the spring.

Changes in the levels of allosteric modifiers in the larvae add another level of control to FBPase, one which when integrated with the seasonal changes in the amount of FBPase protein and its phosphorylation state, would provide even tighter control over glycolytic vs gluconeogenic flux in the larvae. When polyol synthesis was stimulated by abruptly moving *E. scudderiana* larvae to -4°C , levels of the allosteric inhibitor of FBPase, F_{2,6}P₂, rose from 0.1 nmol/g wet weight in controls to 2 nmol/g ww within 6 h and remained at approx. 4–8 nmol/g ww over several days, while glycerol accumulated (Churchill and Storey, 1989b). AMP levels also rose by three-fold within the first 1.5 days at the lower temperature to approx. 200 nmol/g ww. Compared with the I₅₀ values shown in Table 1, *in vivo* levels of either of these metabolites would strongly inhibit FBPase activity and would be particularly effective on the dephosphorylated enzyme form whose content increases under seasonal or low temperature conditions where glycerol synthesis is favoured. In contrast, both F_{2,6}P₂ and AMP are powerful activators of PFK, and would stimulate the activity of this enzyme to facilitate the flow of carbon arising from activated glycolysis into glycerol synthesis.

The present results also provide more evidence of the role of temperature change in the regulation of enzyme activity in cold-hardy insects. The study by Holden and Storey (1995) found temperature-dependent changes in the kinetic properties of purified FBPase. Although the K_m for FBPase dropped by three-fold at 5°C compared with 22°C, the effects of allosteric inhibitors were potentiated at low temperature; I₅₀ values for AMP and F_{2,6}P₂ dropped at 5°C to just 42 and 12%, respectively,

of their values at 22°C. Furthermore, high glycerol (2 M, approx. natural levels at mid-winter) overrode the inhibitory effects of F_{2,6}P₂ at high assay temperature but not at low, an effect that would potentiate FBPase activity when temperatures rise in the spring and glycerol pools need to be cleared. The effect of temperature acclimation on the FBPase phosphorylation state suggests that temperature change may also stimulate a change in the phosphorylation state of the enzyme, and may mediate the seasonal change in the ratio of the phosphorylated vs dephosphorylated enzyme forms. A low-temperature trigger is clearly responsible for the phosphorylation-mediated activation of glycogen phosphorylase that initiates polyol synthesis in many insect species (for review see Storey and Storey, 1991), including *E. scudderiana* (Churchill and Storey, 1989a). For phosphorylase, this arises because of differential temperature effects on the activities of phosphorylase kinase and phosphorylase phosphatase (Hayakawa and Chino, 1983; Hayakawa, 1985). Not surprisingly, temperature-dependent reversible phosphorylation controls regulate glycogen synthetase activity in the opposite way (Hayakawa and Chino, 1982). The present study provides the first firm indication that temperature change may also coordinate changes in the phosphorylation state of other enzymes of carbohydrate metabolism in cold-hardy insects, apart from those that directly control glycogen breakdown or synthesis. PFK and pyruvate kinase might also be controlled in this way as isoelectrofocusing revealed the presence of two forms of each enzyme in the larvae, the proportions of which changed dramatically between fall, winter and spring (Joanisse and Storey, 1995); whether these represent phosphorylated and dephosphorylated enzyme forms remains to be determined.

REFERENCES

- Churchill T. A. and Storey K. B. (1989a) Seasonal variation in the temperature-simulated interconversion of glycogen and glycerol pools in a freeze avoiding moth larva. *Cryo-Lett.* **10**, 127–136.
- Churchill T. A. and Storey K. B. (1989b) Metabolic correlates to glycerol biosynthesis in a freeze-avoiding insect, *Epiblema scudderiana*. *J. Comp. Physiol. B* **159**, 461–472.
- Ekdahl K. N. (1992) Further studies on the phosphorylation and kinetics of rat liver fructose-1,6-bisphosphatase: importance of three-dimensional structure of a substrate to protein kinase A. *J. Biochem.* **112**, 719–723.
- Ekdahl K. N. and Ekman P. (1984) The effect of fructose-2,6-bisphosphate and AMP on the activity of phosphorylated and unphosphorylated fructose 1,6-bisphosphatase from rat liver. *FEBS Lett.* **167**, 203–207.
- Gancedo J. M., Mazon M. J. and Gancedo C. (1983) Fructose-2,6-bisphosphate activates the cAMP-dependent phosphorylation of yeast fructose 1,6-bisphosphatase *in vitro*. *J. Biol. Chem.* **258**, 5998–5999.
- Hayakawa Y. (1985) Activation mechanism of insect fat body phosphorylase by cold. *Insect Biochem.* **15**, 123–128.
- Hayakawa Y. and Chino H. (1982) Temperature-dependent activation or inactivation of glycogen phosphorylase and synthase of fat body of the silkworm *Philosamia cynthia*: the possible mechanism of the temperature-dependent interconversion between glycogen and trehalose. *Insect Biochem.* **12**, 361–366.

- Hayakawa Y. and Chino H. (1983) Insect fat body phosphorylase kinase is Ca^{2+} -independent and acts even at 0°C . *Biochim. Biophys. Acta* **746**, 14–17.
- Holden C. and Storey K. B. (1994) Purification and characterization of aldolase from the cold hardy insect *Epiblema scudderiana*: enzyme role in glycerol biosynthesis. *Insect Biochem. Mol. Biol.* **24**, 265–270.
- Holden C. and Storey K. B. (1995) Fructose-1,6-bisphosphatase from a cold-hardy insect: control of cryoprotectant glycerol catabolism. *Arch. Insect Biochem. Physiol.* **28**, 225–235.
- Hue L. and Rider M. H. (1987) Role of fructose 2,6-bisphosphate in the control of glycolysis in mammalian tissues. *Biochem. J.* **245**, 313–324.
- Joanisse D. R. and Storey K. B. (1994) Enzyme activity profiles in an overwintering population of freeze avoiding gall moth larvae, *Epiblema scudderiana*. *Can. J. Zool.* **72**, 1079–1086.
- Joanisse D. R. and Storey K. B. (1995) Temperature acclimation and seasonal responses by enzymes in cold-hardy gall insects. *Arch. Insect Biochem. Physiol.* **28**, 339–349.
- Kelleher M. J., Rickards J. and Storey K. B. (1987) Strategies of freeze avoidance in larvae of the goldenrod gall moth *Epiblema scudderiana*: laboratory investigations of temperature cues in the regulation of cold hardiness. *J. Insect Physiol.* **33**, 581–586.
- Meek D. W. and Nimmo H. G. (1984) Effects of phosphorylation on the rat liver fructose-1,6-bisphosphatase. *Biochem. J.* **222**, 125–130.
- Pilkis, S. J., Claus, T. H., Kountz, P. D. and El-Maghrabi, M. R., Enzymes of the fructose 6-phosphate — fructose-1,6-bisphosphate substrate cycle. In *The Enzymes*, Vol. 18, ed. P. D. Boyer and E. G. Krebs. Academic Press, New York, 1987, pp. 3–46.
- Pohlig G. and Holzer H. (1985) Phosphorylation and inactivation of yeast fructose-1,6-bisphosphatase by cyclic AMP-dependent protein kinase from yeast. *J. Biol. Chem.* **260**, 13818–13823.
- Rickards J., Kelleher M. J. and Storey K. B. (1987) Strategies of freeze avoidance in larvae of the goldenrod gall moth, *Epiblema scudderiana*: winter profiles of a natural population. *J. Insect Physiol.* **33**, 443–450.
- Storey K. B. and Storey J. M. (1988) Freeze tolerance in animals. *Physiol. Rev.* **68**, 27–84.
- Storey, K. B. and Storey, J. M., *Biochemistry of cryoprotectants. In Insects at Low Temperature*, ed. D. Denlinger and R. E. Lee. Chapman and Hall, New York, 1991, pp. 64–93.
- Tejwani G. A. (1983) Regulation of fructose-bisphosphatase activity. *Adv. Enzymol.* **54**, 121–194.
- Vidal H., Roux B. and Riou J. P. (1986) Phosphorylation- and ligand-induced conformational changes of fat liver fructose-1,6-bisphosphatase. *Arch. Biochem. Biophys.* **248**, 604–611.
- Zachariassen K. E. (1985) Physiology of cold tolerance in insects. *Physiol. Rev.* **65**, 799–832.

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