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Regulatory properties of phosphofructokinase in the eggs of the silkworm, *Bombyx mori*

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Phosphofructokinase (PFK) was purified from non-diapause eggs (incubated at 25°C) of the silkworm, *Bombyx mori*, by using hydroxylapatite, Sephacryl S-300, and ATP-agarose column chromatography. PFK activity separated into two distinct peaks on Sephacryl S-300, the molecular masses being 310 and 185 kDa; SDS-PAGE showed that both were composed of a single 48 kDa subunit. When eggs were cold-acclimated, different profiles occurred; eggs acclimated at 0°C exhibited two broad peaks of PFK activity centered at 310 and 90 kDa, whereas eggs held at 5°C showed activity primarily in a broad peak at 90 kDa. Partially purified PFK showed a sigmoidal saturation curve for its substrate, fructose-6-phosphate (F6P) with an $S_{0.5}$ of 1.02 ± 0.27 mM at pH 7.0. PFK was activated by fructose-2, 6-bisphosphate, AMP and inorganic phosphate, and inhibited by a high concentrations of its second substrate, ATP. Diapause eggs showed low and constant PFK activity (about 0.2 U/g eggs) over 9-days after oviposition whereas when diapause initiation was prevented by HCl treatment one day after oviposition, PFK activity had doubled by day 6 and rose to 5-fold higher than initial values by day 9. To determine if the mechanism of this activity increase could be a change in the phosphorylation state of the enzyme, homogenates of day 6 and day 9 eggs were incubated under conditions that promoted the action of cAMP-dependent protein kinase or protein phosphatase and the effect of incubation on the $S_{0.5}$ for F6P was monitored. Diapause egg PFK showed a high $S_{0.5}$ of about 4 mM which was unchanged by incubation under phosphorylating conditions and but was strongly reduced under dephosphorylating conditions. PFK from HCl-treated eggs showed a decrease in $S_{0.5}$ over time to 3.4 mM at day 6 and 0.9 mM at day 10 that was virtually the same as the effect of incubation under dephosphorylating conditions. Hence, the diapause form of the enzyme appears to be the phosphorylated form and the increase in activity when diapause was prevented by HCl-treatment can be linked to dephosphorylation of PFK. Thus, PFK activity in *Bombyx* eggs appears to be regulated by a variety of factors including temperature effects on the native state of PFK subunit, reversible protein phosphorylation, and the effects of allosteric modulators.

Key words: 6-Phosphofructo-1-kinase, diapause, egg development, allosteric regulation, reversible protein phosphorylation

Introduction

Diapausing eggs of the silkworm, *Bombyx mori*, have the ability to endure adverse environ-

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mental circumstances, particularly low temperature. Chief among the biochemical adjustments to support diapause survival is the conversion of egg glycogen reserves into large pools of sorbitol and glycerol at the initiation of diapause (CHINO, 1958; YAGINUMA and YAMASHITA, 1977; FURUSAWA and SHIKATA, 1982; FURUSAWA *et al*, 1982). Polyol accumulation may provide protection against freezing or desiccation or both. At the initiation of diapause phase, sorbitol synthesis from stored glycogen is stimulated by the conversion of glycogen phosphorylase from its inactive *b* form to the active *a* form under anaerobic conditions (YAMASHITA *et al*., 1975). The pathway leading from glycogen to sorbitol is coupled to the pentose phosphate pathway, which supplies the reducing power (NADPH) for sorbitol synthesis from glucose (CHINO, 1960; KAGEYAMA and OHNISHI, 1971; TAKAHASHI *et al*., 1974; SUZUKI and MIYA, 1975; KAGEYAMA, 1976). In these eggs, activity of glucose-6-phosphate (G6P) dehydrogenase is about twice as high as that of phosphofructokinase (PFK) (SUZUKI and MIYA, 1975) and amounts of G6P equivalent to about 10% of the accumulated sorbitol are oxidized through the pentose phosphate pathway. Another fraction of G6P is eventually metabolized via the pentose phosphate pathway to glycerol (YAGINUMA *et al*., 1990). A similar carbon flux occurs in non-diapause eggs which accumulate sorbitol and glycerol when temperatures fall below 10°C (FURUSAWA *et al*., 1987, 1992). To operate these pathways, the PFK locus of glycolysis must be regulated. Although some glycolytic flux may continue, inhibitory control over PFK is necessary to divert most carbon flow into the pentose phosphate pathway or the reactions that convert G6P to sorbitol.

PFK has been cited as a key enzyme regulating the production of cryoprotectants in a number of cold-hardy insects including glycerol synthesis in *Papilio machaon* and *Monema flavescens* (HAYAKAWA and CHINO, 1982) and trehalose production in *Philosamia cynthia* and *Trichiocampus populi* (HAYAKAWA and CHINO, 1982). PFK activity is modulated by low temperatures. In *Eurosta solidaginis* larvae, the sequential synthesis of glycerol and then sorbitol is induced by lowering temperatures (STOREY *et al*., 1981). This is attributed to control at the PFK locus, the enzyme being active at warmer temperatures (10-15°C) to allow carbon flow into the pathway of glycerol synthesis but inhibited by a combination of factors at colder temperatures (5 to -5°C) which diverts carbon into sorbitol synthesis (STOREY, 1982). In silkworm eggs, changes in PFK activity and their influence on carbohydrate metabolism have been investigated during both diapause and embryonic development (KAGEYAMA and OHNISHI, 1971). At the initiation of diapause, PFK activity is at a trace level, but activity was considerably higher in non-diapause eggs (SUZUKI and MIYA, 1975, 1977) and in diapause eggs treated with HCl to break dormancy (KAGEYAMA and OHNISHI, 1971). However, the regulatory mechanism(s) responsible for the change in PFK activity between diapause and developing states have not been clear. In the present study, we purified PFK from non-diapause eggs, and characterized the kinetics of the enzyme, the influence of acclimation temperature on PFK properties, and enzyme regulation by reversible protein phosphorylation.

Materials and Methods

Animals: The polyvoltine race (N_4) was programmed to produce non-diapause eggs by exposure to high temperature (25°C) in the dark (24D: 0L) during the embryonic life of the maternal generation. Eggs laid within a 3 h period (i.e. at an identical developmental stage), were pooled and used for each experiment. The eggs were maintained under the following regimes: (1) continuous exposure to 25°C to proceed embryonic development, or (2) continuous chilling at 5°C or 0°C starting 24 hours after oviposition. When these eggs were transferred to 25°C and incubated for 12 days, the hatching ability of more than 90% and about 80% were observed in 5°C- and 0°C-acclimated eggs, respectively (FURUSAWA *et al*., 1989).

The bivoltine race (Shunrei x Shogetsu),

reared on fresh mulberry leaves, was programmed to produce diapause eggs by high temperature (25°C) and a long day photoperiod (16L: 8D) during the embryonic life of the maternal generation. Eggs laid within a 3 hour period (i.e. at an identical developmental stage) were pooled. Some of the diapause eggs were treated with HCl (Sp. gr., 1.075, 46°C for 5 min) after keeping them at 25°C for 20 h after oviposition to obtain embryonic developmental eggs (artificial non-diapause eggs). Eggs were then allowed to develop at 25°C and the eggs hatched on the 9th or 10th day after the HCl-treatment.

Enzyme assay: Phosphofructokinase (PFK) activity was monitored by following NADH oxidation at 340 nm using a Gilford 240 recording spectrophotometer or a Shimadzu spectrometer UV-1600 at 22-25°C. Standard assay conditions were 20 mM imidazole-HCl buffer (pH 7.0), 5 mM fructose-6-phosphate (F6P), 2 mM ATP, 50 mM KCl, 5 mM $MgSO_4$, 0.12 mM NADH, 0.5 unit (U) aldolase, 0.5 U triosephosphate isomerase and 2 U glycerol-3-phosphate dehydrogenase in a final volume of 1 ml. 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 concentrations as indicated. Activator constants (Ka) were determined using double reciprocal plots of $(1/V\text{-}V_0)$ versus 1/[activator].

Enzyme preparation and purification: The homogenizing buffer (HB) contained 20 mM imidazole-HCl, pH 7.0, 15 mM 2-mercaptoethanol and 30% v/v glycerol. A few crystals of solid phenylmethylsulfonyl fluoride (protease inhibitor) were added immediately prior to homogenization. Samples of frozen eggs (usually 3.5 g) were ground with 4 volumes of HB using a mortar and pestle, and subsequently homogenized with an Ultra Turrex homogenizer. The homogenate was centrifuged at 27,000 x *g* for 20 min. These subsequent procedures were carried out on ice or 4°C. The pellet was discarded and the supernatant was passed through a small column of Sephadex G-25 (equilibrated with HB) by centrifugation at 3,000 rpm for 1 min. The filtrate was loaded onto a hydroxylapatite column (1.2 cm x 2 cm height or 1.8 cm x 3.5 cm) equilibrated in HB. PFK was eluted with a linear gradient of 0-500 mM potassium phosphate (KPi) in HB buffer. One or 2 ml fractions were collected, and 50 to 100 μl of the eluent was used to assay PFK activity. Peak fractions were pooled and then applied to a Sephacryl S-300 column (1.5 cm x 40 cm) which was equilibrated in 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 30% v/v glycerol. PFK was eluted with the same buffer and 100 to 200 μl of each 1 ml fraction was used to measure activity. Pooled peak fractions were desalted by centrifugation through Sephadex G25 as above and then applied to an ATP-agarose column (1 cm x 2 cm) equilibrated in HB. The column was sequentially washed with 10 ml of HB, 2 ml of 500 mM KCl in HB, 5 ml of HB, 5 ml of 2.5 mM F6P in HB, 5 ml of HB, and 5 ml of 2.5 mM ADP in HB. Then PFK was eluted with 5 ml of 5 mM F6P plus 5 mM ADP in HB and collected in 1 ml fractions. These fractionations were carried out at a room temperature (approximately 22°C). Each fraction was concentrated in a Centricon-30 by centrifugation at 5,000 rpm at 4°C overnight. Purified enzyme was used to determine subunit molecular mass of PFK. A 40 μl aliquot of each fraction was boiled for 3 min in a equal volume of sample loading buffer and then applied to a 10% (w/v) acrylamide gel and SDS-polyacrylamide electrophoresis was carried out by the method of LAEMMLI (1970).

Molecular mass determination: The native molecular mass of PFK was determined by Sephacryl S-300 gel filtration chromatography. Standards were run in the same manner as indicated above for enzyme purification and were detected by activity assays at 340 nm for rabbit muscle PFK (360 kDa), pyruvate kinase (240 kDa), aldolase (160 kDa), hexokinase (99 kDa), and malate dehydrogenase (90 kDa), or by absorbance at 280 nm for bovine heart cytochrome c (14.5 kDa). The molecular weight of PFK was deter-

mined from a plot of Ka versus log molecular mass for the protein standards.

To determine subunit molecular mass of PFK, a 40 μl aliquot of each fraction on the ATP-agarose column chromatography was boiled for 3 min in a equal volume of sample loading buffer and then applied to a 10% (w/v) acrylamide gel and SDS-polyacrylamide electrophoresis was carried out by the method of LAEMMLI (1970).

Reversible phosphorylation of PFK: One gram of eggs was ground in a mortar with 5 ml of HB and then one volume of homogenate was mixed with an equal volume of one of three buffers, all containing 20 mM imidazole-HCl (pH 7.0), 15 mM 2-mercaptoethanol and 10% (v/v) glycerol (Buffer D). Buffer A was designed to inhibit both protein kinases and protein phosphatases and consisted of Buffer D plus 100 mM NaF, 5 mM EDTA and 5 mM EGTA. Buffer B promoted enzyme phosphorylation and contained Buffer D plus 100 mM NaF, 10 mM $MgCl_2$, 10 mM ATP and 1 mM cAMP. Buffer C promoted enzyme dephosphorylation and contained Buffer D plus 10 mM $MgCl_2$. Each mixture was incubated at 4-5°C for 18 hours, and then homogenized with an Ultra Turrex homogenizer. The homogenate was centrifuged at 27,000 x g for 20 min at 5°C. The pellet was discarded, and the supernatant was passed through a column of Sephadex G-25 by centrifugation at 3,000 rpm for 1 min, followed by assay of PFK activity.

Protein content determination: Protein contents of the HCl-treated eggs and non-diapause eggs were estimated by the method of LOWRY *et al.* (1951).

Results

Enzyme purification and molecular weight determination of PFK.

PFK was purified from embryonic developmental eggs (6-day old) of the non-diapause type (kept at 25°C). After desalting, highspeed supernatant (1.3 ml; total activity, 14.9 x 10^{-2} U) was applied to a hydroxylapatite column; PFK activity eluted between 110-200 mM KPi and had a maximum activity (3.38 x 10^{-2} U/ml) at 140 mM KPi with a single peak. Active fractions (between 110 and 200 mM KPi) were pooled and total activity in these 4 ml was 8.11 x 10^{-2} U (54.4% recovery). The enzyme was then applied to a Sephacryl S-300 column and eluted from the gel filtration column in two main peaks (Fig. 1). The estimated molecular weights of Peaks I and II of PFK were about 310 kDa and 185 kDa, respectively. For further purification, the PFK fractions in Peak II were pooled, desalted by centrifugation through Sephadex G25 and then applied to an ATP-agarose column. After extensive washing with the sequence of buffers described in the Materials and Methods, PFK was eluted with 5 ml of 5 mM F6P plus 5 mM ADP in HB and 1 ml fractions were collected. Each fraction was concentrated, and analyzed on SDS-PAGE to check its purity and the subunit molecular weight (Fig. 2). A single band was detected on the gels stained with Coomassie brilliant blue (Fig. 2a), whereas several minor contaminants of the PFK preparation were noted with silver stain (Fig. 2b). In both cases, the major band representing the PFK subunit had a molecular weight of about 48 kDa. From these results, it appears that the native PFK from *B. mori* eggs exists in both a tetramer (peak II) or hexamer (peak I) form during embryonic development of non-diapause eggs. Due to low final yields of purified enzyme with this method, kinetic studies of PFK could not be carried out with the pure enzyme so for these studies a partially purified enyzme was used after the hydroxylapatite column chromatography.

To assess the characteristics of PFK of cold-acclimated non-diapause eggs, samples of eggs were kept at 25°C for 24 hours after oviposition, and then continuously stored at 5°C or 0°C for 30 days. After fractionation on a hydroxylapatite column, PFK in these samples was analyzed by gel filtration on Sephacryl S-300 (Fig. 1). In the non-diapause eggs at 5°C and 0°C, PFK activity was present in two broad peaks centered on 310 kDa and 90 kDa. This contrasts with the enzyme distribution in embryonic developmental

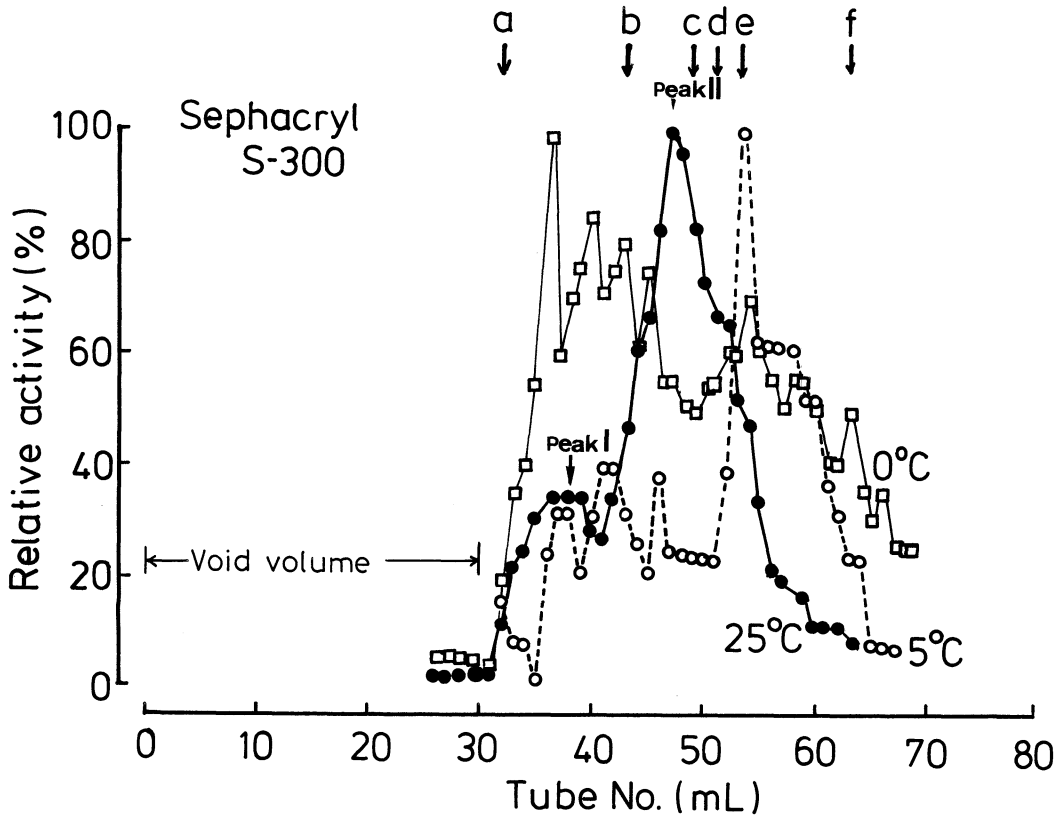

Fig. 1. Elution profiles of Sephacryl S-300 column chromatography for PFK activity from diapause eggs of *B. mori*. The eggs were kept at 25°C for 6-days after oviposition for embryonic development (solid circles), or were kept at 25°C for the first 24 hours after oviposition and then chilled at either 5°C (open circles) or 0°C (open squares). The enzyme was partially purified by chromatography on hydroxylapatite before applying to the Sephacryl S-300 column. Activity in each enzyme preparation is plotted relative to the fraction containing the highest activity; 4.1 x, 1.15 x, and 1.77 x 10^{-2} U/ml in the eggs kept at 25, 5 and 0°C, respectively. Relative activity is expressed as per cent to the maximum activity of 4.1 x 10^{-2} U/ml (25°C, Tube No. 47), 1.15 x 10^{-2} U/ml (5°C, Tube No. 54) and 1.77 x 10^{-2} U/ml (0°C, Tube No. 36). Arrows indicate the elution volumes of standards: rabbit muscle PFK (a; 360 kDa), pyruvate kinase (b; 240 kDa), aldolase (c; 160 kDa), hexokinase (d; 99 kDa) and malate dehydrogenase (e; 90 kDa), and cytochrome c (f; 14 kDa).

eggs at 25°C. The relative distribution of PFK activity also differed between the three groups. The 185 kDa PFK was the majority in non-diapause eggs, whereas the 90 kDa enzyme in the eggs kept at 5°C. The PFK became more divergent in the eggs kept at 0°C. Thus, it appears that low temperature exposure of non-diapause eggs induces changes in the native state of PFK subunits.

Substrate affinity.

Extracts of 6-day old eggs of non-diapause (N_4) and of HCl-treated diapause eggs (Shunrei x Shogetsu) were fractionated by hydroxylapatite column chromatography, and then the partially purified enzymes was used for analysis of enzyme substrate affinities at pH 6.5, pH 7.0 and 8.0. The affinity for F6P of PFK from non-diapause eggs was higher than that of the HCl-treated diapause eggs at all pH values (Fig. 3). $S_{0.5}$ values (Hill coefficients, n_H, are in brackets) were 2.78 ± 0.15 mM (3.05 ± 0.06), 1.02 ± 0.27 mM (1.61 ± 0.08), and 0.97 ± 0.27 mM (2.20 ± 0.48) at pH 6.5, 7.0 and 8.0, respectively, for PFK from

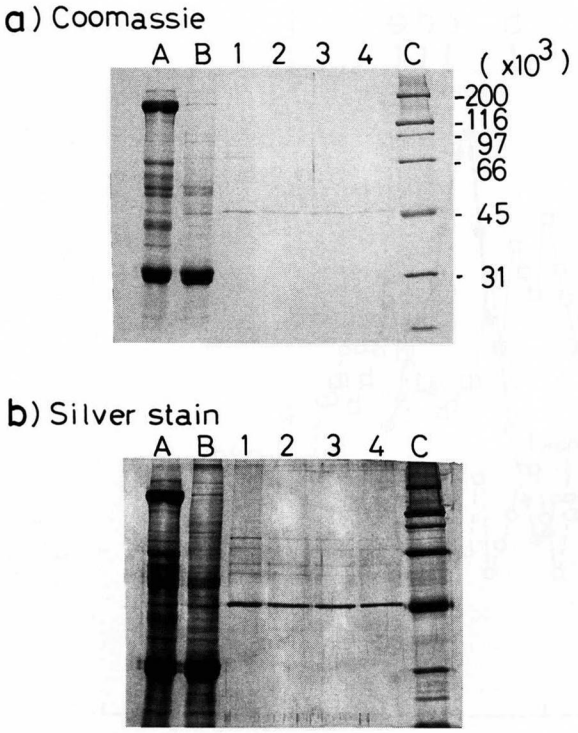

Fig. 2. SDS-PAGE analysis of PFK at different steps of purification. Lanes are: A, crude supernatant; B, pooled peak after hydroxylapatite chromatography; C, molecular weight standards; 1-4: fractions from ATP agarose-affinity column. Standard proteins used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa).

non-diapause eggs (Fig. 3a), compared with 7.69 mM (1.50), 4.85 mM (1.36) and 3.29 mM (1.20) at pH 6.5, 7.2 and 8.0, respectively, in the HCl-treated diapause eggs (Fig. 3b). The substrate saturation curves for F6P for PFK from non-diapause eggs were sigmoidal at all three pH values, but those for PFK from the HCl-treated diapause eggs rather hyperbolic.

Allosteric activators and inhibitors.

The effects of the potent allosteric activator, fructose-2,6-bisphosphate (F2,6P_2), on PFK from diapause (HCl-treatment) and non-diapause eggs are shown in Fig. 4. PFK from HCl-treated eggs and non-diapause eggs was strongly activated by F2,6P_2 with Ka values of 0.1 μM and 0.33 μM, respectively. However, PFK from diapause eggs showed no activation by F2, 6P_2, even at concentrations as high as 2.2 μM which fully activated the enzyme from the other two sources.

AMP is also an allosteric activator of PFK and Ka values were determined to be 0.13 μM and 10.3 μM for the HCl-treated diapause eggs and non-diapause egg (N_4), respectively (data not shown). AMP had no effect on diapause egg PFK. Pi also activated with Ka values of 9.98 mM for PFK of non-diapause eggs (6-day old), but Pi has no effect on PFK of diapause eggs. For Pi activation, a hyperbolic curve was obtained when PFK from 6-day old non-diapause eggs was assessed, but the curve was sigmoidal for the enzyme from 30-day old non-diapause eggs at 5°C and 0°C.

PFK was subject to substrate inhibition by ATP (Fig. 5). For PFK from HCl-treated diapause eggs, inhibition occurred when levels rose above about 2 mM ATP at either pH 6.5, 7.2 and 8.0. PFK from non-diapause eggs (N_4) was somewhat less inhibited by ATP, particularly at pH 8.0, where inhibition began to occur over 10 mM ATP. The I_{50} values for ATP of HCl-treated diapause eggs were 3.2, 2.2 and 5.0 mM ATP at pH 6.5, 7.2 and 8.0, respectively, whereas for PFK from non-diapause eggs values were all at least 3-fold higher at 9.4, 7.8, and 16.3 mM, respectively. The I_{50} values for ATP of diapause eggs were 3.8 mM ATP at pH 6.5, and 5.0 mM at 7.2 and 8.0, respectively. Figure 5 also shows ATP substrate affinity. For HCl-treated eggs, Km values for ATP were 0.1 mM at pH 6.5 and pH 7.0 and 0.2 mM at pH 8.0 whereas in non-diapause eggs Km values were 0.08, 0.42 and 4.29 mM at pH 6.5, 7.2 and 8.0, respectively. In diapause eggs, Km values for ATP were 0.1 mM at pH 6.5 and pH 7.0 and 0.5 mM at pH 8.0.

Phosphorylation and dephosphorylation.

In diapause eggs, PFK activity fluctuated between 0.1-0.22 U/g eggs up to 9-days after oviposition (Fig. 6). In embryonic developmental eggs which were prevented from diapause initia-

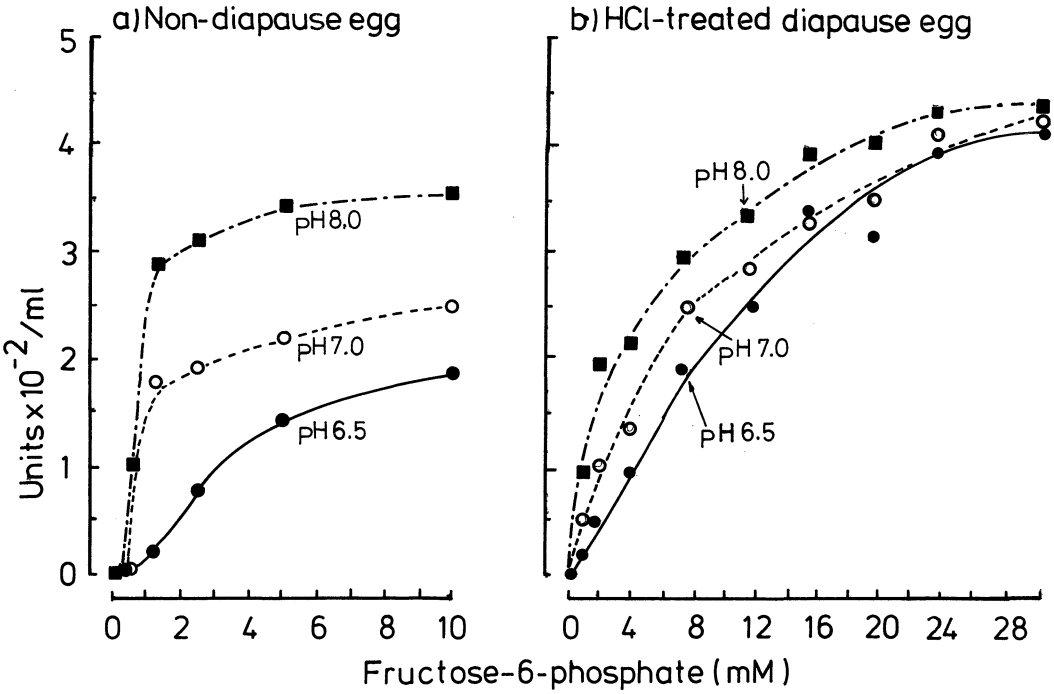

Fig. 3. Effect of pH on the initial rate of the PFK-catalyzed reaction at 25°C. PFK from 4 g aliquots of polyvoltine, non-diapause eggs (a), or HCl-treated diapause eggs, 6-days old (b), was partially purified by hydroxylapatite chromatography (as described in Material and Methods) followed by desalting through Sephadex G-25. A 50 μl aliquot of enzyme extract was mixed with 950 μl of 20 mM imidazole-HCl buffer (pH 6.5, 7.0 or 8.0) containing 2 mM ATP, 50 mM $MgSO_4$, 50 mM KCl, 0.12 mM NADH, 0.5 U aldolase, 0.5 U triosephosphate isomerase, 2 U glycerol-3-phosphate dehydrogenase, and different concentrations of F6P as shown in the figure. Data show means for n=3 separate preparations; SEM bars are contained within the symbols.

tion by the HCl treatment, PFK activity was about 0.25 U/g eggs from day 0 through day 5 after oviposition. Thereafter, activity increased, rising by about 2-fold on day 6 and then increasing again on day 9 to a maximum of 0.98 U/g just before hatching. In natural non-diapause eggs (N_4) which were kept continuously at 5°C, PFK activity was maintained at a low level of 0.1 U/g eggs for at least 40 days (data not shown). In the embryonic developmental eggs (N_4), PFK activity was less than 0.1 U/g eggs 1 day after oviposition, and then increased gradually up to about 0.4 U/g on day 6, and to a maximum level of 0.85 U/g just before hatching. When these activities were expressed as U/mg protein, parallel patterns of activity change were seen, showing that the rise in PFK activity was due to a specific induction of this enzyme and was not the result of a general rise in overall protein content of the developing larvae.

The increase of PFK activity in the HCl-treated embryonic developmental versus diapause eggs could be due either to increased enzyme synthesis or to the conversion of the existing enzyme from a less active to a more active state. This latter could be the result of reversible protein phosphorylation which is known to regulate PFK from other sources. In order to determine whether *B. mori* egg PFK could be modified by reversible phosphorylation, crude enzyme extracts from 6 or 9-day old diapause and HCl-treated eggs kept at 25°C were incubated with one of three buffer types. Buffer A was designed to inhibit both protein kinases and protein phosphatases, Buffer B promoted enzyme phosphorylation, and Buffer C promoted enzyme dephos-

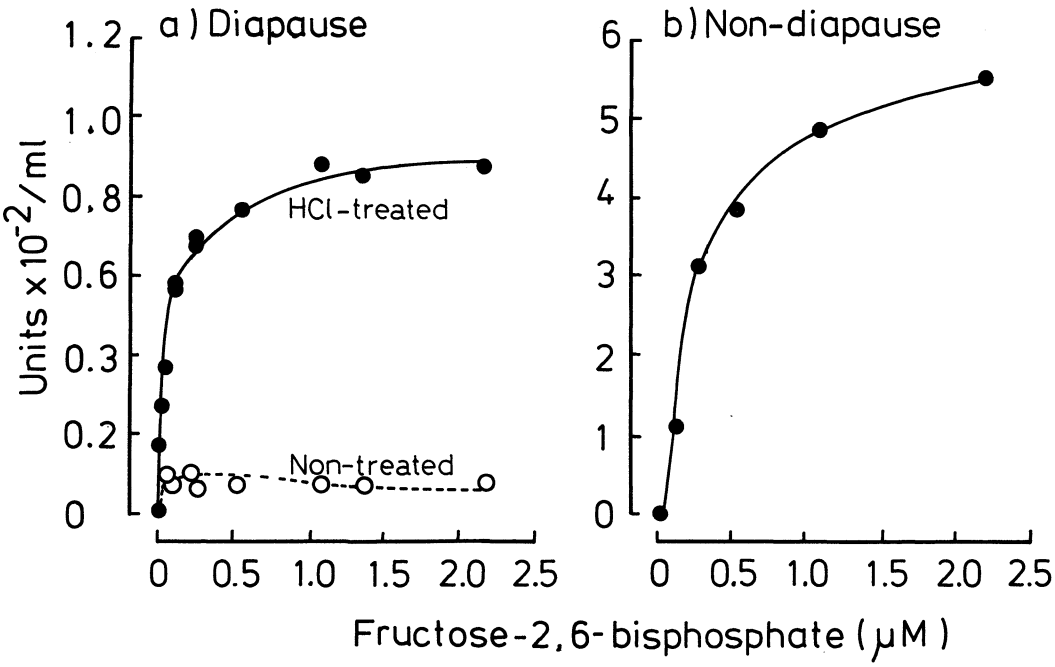

Fig. 4. Effect of $F2,6P_2$ on the initial rate of the PFK-catalyzed reaction. PFK was partially purified from diapause eggs, both HCl-treated and untreated (a), or non-diapause eggs (b) as described in Figure 3. Standard assay conditions at 25°C were used except that the final concentration of F6P was 3 mM for assays of diapause egg PFK and 0.8 mM for assays with non-diapause egg PFK. Results are means of duplicate determinations performed on two separate preparations.

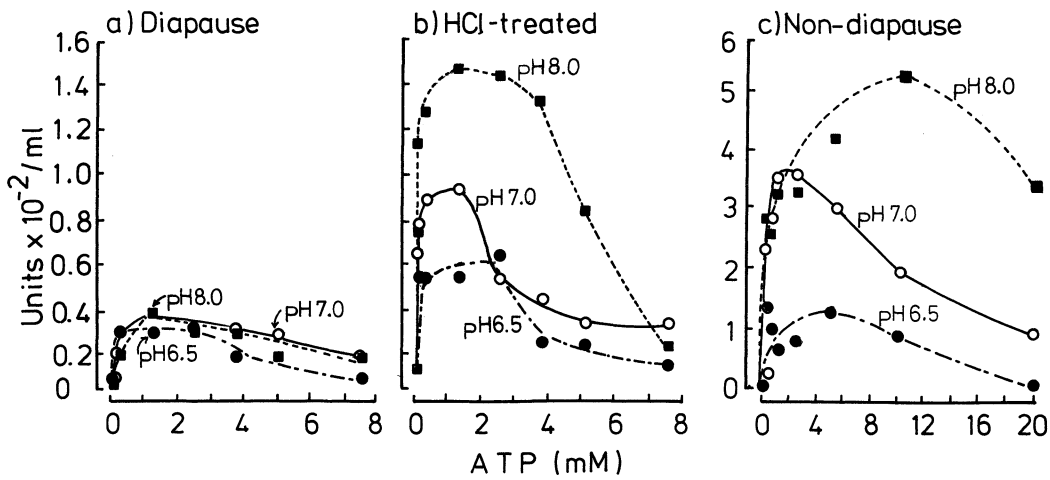

Fig. 5. Effect of ATP on the initial rate of the PFK reaction. PFK was prepared from 4 g samples of diapause eggs (a), HCl-treated diapause eggs (b), or non-diapause eggs (c), all sampled 6 days after oviposition as described in Fig. 3. Aliquots of extract (100 μl), were assayed under standard assay conditions at 25°C except for different pH values of the imidazole buffer (pH 6.5, 7.0 or 8.0) and varying ATP concentration. Results are mean values of duplicate determinations performed on two separate preparations.

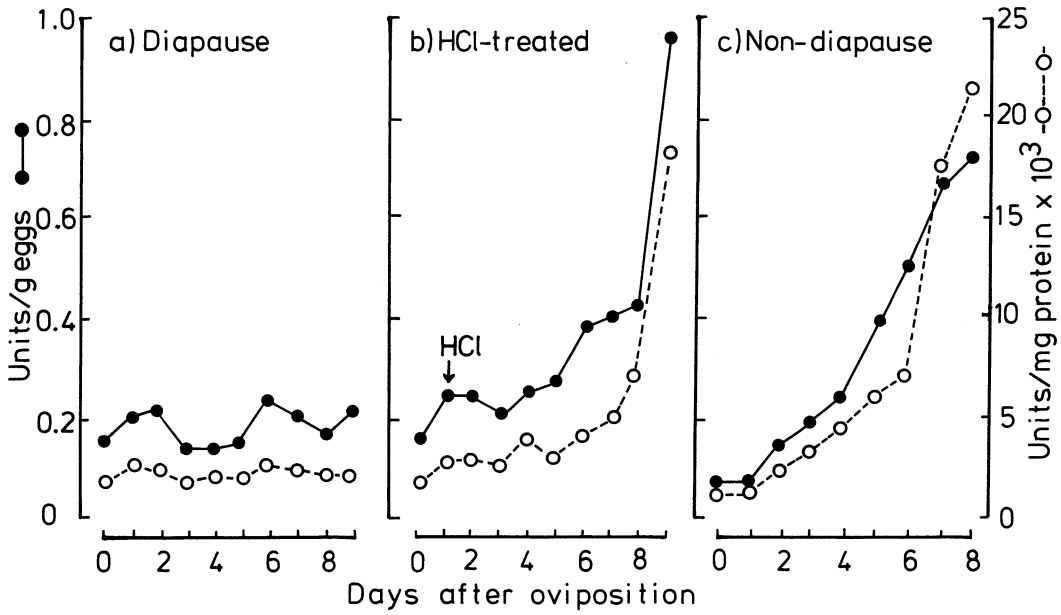

Fig. 6. Changes in PFK activity in diapause eggs (a), HCl-treated diapause eggs (embryonic developmental eggs) (b), and non-diapause eggs (c). PFK was measured under standard assay conditions at 25°C and data are expressed both as U/g eggs (filled circle, left scale) and as U/mg protein (open circle, right scale). Results are means of duplicate determinations performed on two separate preparations.

phorylation. Effects of incubation on enzyme substrate affinity for F6P were then assessed (Table 1), F6P affinity being a key parameter that is typically modified by the phosphorylation state of the enzyme. By inhibiting both protein kinases and protein phosphatases, homogenization in Buffer A preserves the phosphorylation state of PFK as it was at the time of sampling. Table 1 shows that PFK in both 6-and 9-day old diapause eggs had similar F6P affinity constants with values of about 4-4.5 mM for enzyme prepared in buffer A. Incubation of extracts from diapause eggs under conditions promoting enzyme phosphorylation (Buffer B) had no effect

Table 1. Effect of Phosphorylation and/or dephosphorylation bufferes on enzyme affinities ($S_{0.5}$) for F6P and Hill coefficient (n_H) of PFK of diapause and HCl-treated eggs

Parametr	Buffer	6-day old eggs		9-day old eggs	
		Diapause	HCI-treated	Diapause	HCI-treated
$S_{0.5}$ (mM)	Buffer A	4.46±0.75	3.39±0.86	3.88±0.59	0.93±0.22[b)]
	Buffer B	3.76±0.41	3.24±0.46	5.33±0.98[a)b)]	1.92±0.41[a)b)]
	Buffer C	2.61±0.39[a)]	1.23±0.25[a)]	3.40±0.93	1.33±0.30
n_H	Buffer A	1.05±0.13	1.41±0.23	1.24±0.07	0.86±0.09[b)]
	Buffer B	1.49±0.11[a)]	2.39±0.38[a)]	2.61±0.75[a)b)]	1.31±0.08[a)b)]
	Buffer C	0.87±0.12[a)]	0.90±0.23[a)]	0.83±0.15[a)]	0.94±0.14

The diapause eggs and the HCl-treated eggs 6-and 9-day old,which prevented from diapause initiation for embryonic development, were homogenized with HB and then one volume of homogenate was mixed an equal volume of Buffer A, B or C as shown in Materials and Methods. Each mixture was incubated at 4-5°C for 18 hours, and $S_{0.5}$ and n_H were estimated from the Hill plot. Results are means (±SEM) of triple determinations performed on two separate preparations. The values attached with symbols a and b are significantly different from the corresponding Buffer A and 6-day old eggs in each diapause or HCl-treated eggs, respectively, by the Student's *t*-test. $P<0.05$

on the $S_{0.5}$ F6P of PFK from 6-day old eggs but significantly elevated n_H whereas the enzyme from 9-day old eggs showed significant increases in both $S_{0.5}$ (by 40%) and n_H (by 2-fold) after incubation under *in vitro* phosphorylation conditions. By contrast, incubation under dephosphorylating conditions (buffer C) reduced both the $S_{0.5}$ F6P (by 40%) and n_H of PFK from 6-day old diapause eggs. Dephosphorylation did not affect the $S_{0.5}$ of PFK from 9-day old diapause eggs but n_H was significantly reduced by this treatment. PFK from HCl-treated eggs showed a similar pattern of responses to phosphorylation and dephosphorylation when the enzyme in 6-day old eggs was examined. Hence, phosphorylation incubations did not affect $S_{0.5}$ for F6P but, again, increased n_H whereas dephosphorylation incubations again strongly reduced both $S_{0.5}$ (by 60%) and n_H. PFK in 9-day old, HCl-treated eggs incubated in Buffer A showed a much lower $S_{0.5}$ for F6P than in any of the other cases, in particular, being only 25-30% of the corresponding value in 6-day old HCl-treated eggs or 9-day old diapause eggs. Incubation under phosphorylating conditions doubled the $S_{0.5}$ and significantly raised n_H. By contrast, incubation under dephosphorylating conditions had no effect on enzyme parameters.

Discussion

The profile of PFK activity from 6-day old eggs exhibited two peaks with molecular masses of 310 kDa and 185 kDa on gel filtration (Sephacryl S-300) (Fig. 1). After affinity chromatography of the 185 kDa protein, SDS-PAGE showed a single major subunit band with a molecular mass of 48 kDa. Thus, it appears that the enzyme in non-diapause eggs can exist in either tetramer (185 kDa) or hexamer (310 kDa) forms. PFK from Bumblebee (*Bombus atratus*) flight muscle has a molecular weight of 360 kDa which was a tetramer with subunit molecular weight of 80 kDa (LEITE *et al.*, 1988). The native molecular mass of PFK from the whole body of the Lepidopteran larva *Epiblema scudderiana* was 420 ± 20 kDa, the enzyme being a tetramer with subunit molecular mass of 70-95 kDa (HOLDEN and STOREY, 1993). Thus, there seems to be considerable variation among insects species in both the native and subunit sizes of PFK. Furthermore, the non-diapause egg PFK from *B. mori* was uniquely interesting in that tertiary and/or quaternary structure of the enzyme clearly varied with the physiological state of the eggs. Thus, developing eggs at 25°C contained primarily the tetrameric enzyme with some hexamer (Fig. 1) but eggs incubated at 5 or 0°C largely lacked the tetrameric form and showed PFK distributed divergently between 310 kDa and 90 kDa forms. This suggests that the tetrameric enzyme may be dissociated into dimers by low temperature. The tetramer of PFK from *Thermus thermophilus* (XU *et al.*, 1990) and from rabbit muscle (BOCH and FRIEDEN, 1976a, 1976b; FRIEDEN *et al.*, 1976) is the active form and its dimer is inactive. Tetramer-dimer conversion is dependent on pH, temperature and allosteric effectors. This could be an effective regulatory mechanism for enzyme and glycolytic control in the developing eggs in response to environmental temperatures. Lowering the amount of active tetrameric PFK in the eggs at low temperature may be one way of reducing/inhibiting enzyme activity (and hence, glycolytic flux) at low temperature in a manner that is readily reversible when temperature rises again. Indeed, although the dimer (90 kDa) form of *B. mori* egg PFK (Fig. 1) may be an inactive form *in vivo*, activity was detectable *in vitro*, probably due to a conversion of dimer to tetramer under the 25°C assay conditions. Similar low temperature stimulated dissociation of polymers into monomers has been reported for glycogen phosphorylase, pyruvate carboxylase and glyceraldehyde 3-phosphate dehydrogenase in mammalian tissues (BEYER, 1972).

Analysis of partially purified PFK from 6-day old non-diapause eggs showed that the enzyme exhibited sigmoidal F6P saturation curves (Fig. 3), as is common for PFK from other sources. The enzyme also was activated by F2, $6P_2$ (Fig. 4), AMP and inorganic phosphate, and exhibited substrate inhibition by high levels of ATP (Fig. 5). These properties are typical of

PFK from numerous sources which is typically activated by ammonium ion, inorganic phosphate, $F2,6P_2$ and AMP, and inhibited by citrate and high levels of substrate ATP (RAMAIAH, 1974; UEDA, 1979; HERS *et al.*, 1982; HUE and RIDER, 1987). Thus, *B. mori* egg PFK *in vivo* would be controlled by numerous factors including changes in intracellular pH, ion distribution, and the levels of positive and negative allosteric modulators (HORTWITS and HETTINGEN, 1979) as well as factors such as subunit association/dissociation and reversible protein phosphorylation.

The properties of PFK from diapause eggs that were treated with HCl to prevent them entering diapause showed various properties quite similar to those of the enzyme from non-diapause eggs. Like the non-diapause egg PFK, the enzyme from HCl-treated eggs show strong activation by $F2,6P_2$ (Fig. 4), high affinity for ATP and inhibition by ATP at higher substrate levels (Fig. 5) although PFK from HCl-treated eggs had lower I_{50} values for ATP. F6P substrate kinetics were sigmoidal but with a lower affinity for F6P ($S_{0.5}$ 7.7, 4.9 and 3.3 mM at pH 6.5, 7.0 and 8.0, respectively) and a higher Hill coefficient than the corresponding values for PFK in non-diapause eggs ($S_{0.5}$ 2.8, 1 and 1 mM) (Fig. 3). These data implies the PFK is one of the key enzyme to proceed the embryonic development of *B. mori* egg.

Except for the F6P affinities and ATP I_{50} values which show that the two enzyme types are not exactly alike, these are characteristics of an active PFK and this correlates well with the rising activity of PFK found in 6-day old HCl-treated eggs, which follows a similar pattern to that in the non-diapause eggs of the same age (Fig. 6). PFK in diapause eggs, by contrast, showed markedly different properties that would be consistent with a largely inactive enzyme and the low enzyme activity *in vivo* (Fig. 6). Most strikingly, PFK from diapause eggs (kept at 25°C) was not affected by $F2,6P_2$ (Fig. 4), which is the primary activator that regulates PFK activity with respect to the need for carbohydrate catabolism in support of biosynthesis. Sensitivity to $F2,6P_2$ in developing eggs (both non-diapause and HCl-treated) integrates PFK activity with the demand for glycolytic carbon for biosynthesis in the developing embryo. PFK from diapause eggs also showed quite strong inhibition by ATP (I_{50} 3.8-5 mM at the three assay pH values) which was similar to the inhibitory effect of ATP on HCl-treated diapause eggs (I_{50} 2.2-5.0 mM) but lower than the values for non-diapause eggs (I_{50} 7.8-16.3 mM).

Another key mechanism of PFK control in animal system is post-translational modification of the enzyme via the incorporation of covalently bound phosphate. Changes to the phosphorylation state of PFK alter the measured kinetic constants of the enzyme (STOREY, 1990). Many animals have the ability to retreat into a hypometabolic or dormant state as a defense against harsh environmental conditions, sharply reducing their metabolic rate during these periods. In many instances, reversible protein phosphorylation of rate-limiting enzymes including PFK has been shown to be one of the key mechanisms mediating metabolic depression (STOREY and STOREY, 1990). In *B. mori* diapause eggs, anaerobic conditions occur in the eggs with the initiation of diapause, and trigger the activation of glycogen phosphorylase (YAMASHITA *et al.*, 1975). The glucose-6-P derived from stored glycogen is converted into sorbitol which accumulates in high levels and helps to impart cold tolerance and desiccation resistance to the eggs (CHINO, 1960; KAGEYAMA and OHNISHI, 1971; TAKAHASHI *et al.*, 1974; SUZUKI and MIYA, 1975; KAGEYAMA, 1976). Sorbitol accumulation is facilitated by a block on carbon flux through glycolysis at the PFK locus which causes a diversion of hexose phosphates into the reactions of sorbitol synthesis. Indeed, as Fig. 6 shows, diapause eggs exhibit a very low activity of PFK. Treatment with HCl, however, which prevents diapause initiation and allows the embryo to develop, leads to a strong increase in PFK activity in the eggs between 6-9 days after treatment, similar to the activity of PFK in non-diapause eggs at the same time (Fig. 6). During this period

in HCl-treated artificial non-diapause eggs and non-diapause eggs the storage glycogen is gradually consumed (YAMASHITA *et al.*, 1975), and the activity of NAD-sorbitol dehydrogenase rises (YAGINUMA and YAMASHITA, 1979); this suggests that both glycogen and sorbitol pools are being catabolized to fuel aerobic metabolism. Elevated PFK activity when diapause is prevented by HCl-treatment would support carbohydrate catabolism from both of these sources as a means of enhancing glycolytic rate in support of the energy needs during embryogenesis.

The mechanism of PFK activation in HCl-treated eggs, as seen in Fig. 6, is suggested by the incubation studies summarized in Table 1. PFK in many animals is regulated by reversible phosphorylation which can interconvert the enzyme between high and low activity states characterized by changes in enzyme properties including F6P affinity, sensitivity to ATP inhibition, and Ka values for allosteric effectors. Indeed, stable changes in these properties, as the result of a change in physiological state, can frequently be used as a diagnostic tool to infer changes in the phosphorylation state of the enzyme. The present data in Table 1 do just that. Incubation of the enzyme in buffer A containing inhibitors of both protein kinases and protein phosphatases shows the state of the native enzyme in both diapausing and HCl-treated eggs. In the diapause state (in both 6- and 9-day eggs), PFK shows a high $S_{0.5}$ for F6P (low substrate affinity) and almost hyperbolic relationship between F6P concentration and velocity. PFK from the 6-day old HCl-treated eggs also shows the same properties but by 9-days old, PFK kinetic properties had changed substantially with $S_{0.5}$ for F6P and n_H reduced by 73 and 40%, respectively. Incubation under conditions that promote phosphorylation of the enzyme by cAMP-dependent protein kinase had either no effect (6-day old) or raised (9-day old) $S_{0.5}$ F6P value and in all cases, significantly increased the n_H, compared with untreated enzyme from the same source. By contrast, incubation under conditions that promoted phosphatase action significantly reduced both the $S_{0.5}$ and n_H of PFK from 6-day old eggs, both diapause and HCl-treated. Phosphatase treatment of PFK from 9-day old diapause eggs did not change $S_{0.5}$ but again significantly reduced n_H. With 9-day old HCl-treated eggs, however, phosphatase treatment had no significant effect on the enzyme, compared with the untreated control (buffer A). Taken together, the lack of effect of protein kinase treatments on 6-day old eggs and the lack of effect of phosphatase treatments on 9-day old HCl-treated eggs strongly support the conclusion that the native enzyme in 6-day old eggs is the high phosphate form whereas the enzyme in HCl-treated 9-day old eggs is the low phosphate form. Thus, entry into the diapause state results in the phosphorylation of PFK, producing an enzyme form that would be less active *in vivo* (Fig. 6) due to a high $S_{0.5}$ F6P, reduced cooperativity (Table 1), and lack of sensitivity to activators such as $F2,6P_2$ (Fig. 4). When diapause is prevented by HCl treatment, PFK is dephosphorylated and converted to a more active form with a high affinity for F6P (Table 1) and high sensitivity to allosteric activation by $F2,6P_2$ (Fig. 4), properties that closely resemble those of PFK in non-diapause eggs. Thus, one of the results of HCl treatment in disrupting diapause initiation is a gradual dephosphorylation of PFK over several days that converts the enzyme to a high substrate affinity form (Table 1) (as in non-diapause eggs; Fig. 3) and increases enzyme activity (Fig. 6).

In summary, the present study documents two biochemical mechanisms that are used to regulate PFK *in vivo* in *B. mori* eggs: reversible dissociation of subunits and reversible protein phosphorylation. Further studies are needed to explore both of these mechanisms in diapause and non-diapause eggs including their relationship with each other, the influence of temperature and allosteric effectors on both mechanisms, and a determination of the full complement of regulatory controls that act on the enzyme during the transitions to and from the diapause state.

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古澤壽治・小西　綾・坂野大介・小谷英治・杉村順夫・JANET M. STOREY・KENNETH B. STOREY：家蚕卵のホスホフルクトキナーゼの活性調節

家蚕卵のホスホフルクトキナーゼ(PFK)の性状を知るため，多化性品種(N_4)の卵を25°Cで保護し，産卵後6日目の卵から，Hydroxylapatite，Sephacryl S－300，及びATP-agaroseカラムによってPFKを精製した。その結果，PFKは48 kDaをサブユニットとし，主として4量体から成り，この他に6量体がみられた。しかし，5°Cや0°Cに保護した卵(N_4)のPFKは6量体，および2量体として存在した。また胚発育卵のPFKは，frukutose-2,6-bisphosphate(F 2,6 P_2)，AMPや無機リン酸によって活性化されたが，休眠卵PFKはほとんど活性化されなかった。さらに，休眠卵(二化性)と胚発育卵の卵齢経過に伴うPFK活性について検討したところ，休眠卵では低活性であったのに反し，胚発育卵では産卵6日目から孵化まで，急激に上昇した。そして，休眠卵や胚発育6日目の卵のPFKはリン酸化状態にあり，その後胚発育後期(9日目)までにPFKは脱リン酸化されることによって，活性が上昇すると推察した。以上の結果は，家蚕卵のPFK活性は，PFKサブユニット会合に対する温度の影響，F 2,6 P_2やAMPのエフェクター，さらにリン酸化／脱リン酸化によって調節されていることを示唆している。