J Comp Physiol B (1987) 157:373–380

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Glycolysis and the regulation of cryoprotectant synthesis in liver of the freeze tolerant wood frog

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Accepted January 16, 1987

Summary. Wood frogs, *Rana sylvatica,* were sampled after freezing at −4 °C (a short time course from 2 to 70 min after the appearance of the freezing exotherm) and thawing (20 h at 3 °C after 70 min of freezing) and the regulation of liver glycolysis with respect to cryoprotectant glucose synthesis was examined. Within 5 min of the initiation of freezing, cryoprotectant concentrations in blood and liver had begun to increase. This was correlated with a rapid rise in the levels of hexose monophosphates in liver, including a 2.5 fold increase in glucose-6-P and 10 fold rise in fructose-6-P contents within the first 5 min post-exotherm. Contents of fructose-1,6-P_2, fructose-2,6-P_2, triose phosphates, P-enolpyruvate, and pyruvate did not significantly change over the course of freezing. Thawing sharply reduced the levels of hexose monophosphates in liver but raised P-enolpyruvate content by 2.3 fold. Changes in the contents of glycolytic intermediates over the freeze/thaw course are consistent with an inhibitory block of glycolysis at phosphofructokinase during freezing in order to facilitate a rapid glycogenolysis and production of cryoprotectant; during thawing, however, glycolysis appears to be inhibited at the level of pyruvate kinase.

Possible regulatory control of cryoprotectant synthesis by covalent modification of liver glycolytic enzymes was examined. Glycogenolysis during freezing was facilitated by an increase in the percentage of glycogen phosphorylase in the active *a* (phosphorylated) form and also by an increase in the total amount $(a+b)$ of enzyme expressed. For phosphofructokinase, kinetic changes as a result of freezing included a 40% reduction in K_m for fructose-6-P, a 60% decrease in K_a for fructose-2,6-P_2, and a 2 fold increase in I_{50} for ATP. These changes imply a freezing-induced covalent modification of the enzyme but are not, apparently, the factors responsible for inhibition of glycolytic flux at the phosphofructokinase locus during glucose synthesis. Kinetic parameters of pyruvate kinase were not altered over the freeze/thaw course.

Introduction

A number of species of terrestrially-hibernating frogs, in northern climates, readily tolerate freezing of extracellular water as part of their winter hardiness strategy (Schmid 1982; Storey 1985, 1986; Storey and Storey 1986a). We have initiated studies of vertebrate freeze tolerance using the wood frog, *Rana sylvatica,* as our model system (Storey 1984, 1985, 1986; Storey and Storey 1984, 1985a, b, 1986b). This species supercools to only −2 to −3 °C but can survive for days or weeks at lower temperatures (−6 to −8 °C) in the frozen state with 48% of total body water as ice (Storey 1984). Unlike freeze tolerant insects, but perhaps because of their larger body mass and usually well protected hibernation sites, freeze tolerant frogs show no anticipatory accumulation of cryoprotectants over the autumn months. Instead, cryoprotectant synthesis (glucose in *R. sylvatica,* glycerol in *Hyla versicolor*) is triggered only in direct response to the initiation of ice formation in the body (Storey and Storey 1985a, b). Ice nucleation occurs in the body extremities but within 5 min of the appearance of a freezing exotherm, liver glycogenolysis is stimulated and glucose export into blood and other tissues has begun. Glucose concentration in liver rises very quickly, from control levels of about 5 μmol/g wet weight, to approximately 80 μmol/g within the first 1.5 h (Storey and Storey 1985b). Glucose synthesis is facilitated by a rapid activa-

tion of glycogen phosphorylase in liver, the percentage of phosphorylase in the active *a* form rising to 100% within the first hour after nucleation (Storey and Storey 1985b).

The present study undertakes an examination of glycolysis and the state of its regulatory enzymes in liver over a course of freezing and thawing to gain a better picture of the regulation involved in glucose production.

Materials and methods

Animals. Immature adult *R. sylvatica* (average weight = 1.97 ± 0.38 g) were collected on damp evenings in mid-September 1984 (before any overnight frosts) from roadsides in the Ottawa area. Animals were held at 3 °C for 8 weeks without feeding in plastic containers filled with damp sphagnum moss. To freeze frogs, individuals were removed, weighed, and then placed on a pad of paper toweling with their abdomen in contact with a thermistor. Frog and thermistor were secured in place with a broad band of masking tape and were then placed in an incubator at −4 °C. Cooling was monitored using a YSI telethermometer attached to a linear recorder. About 15 min was required to cool a frog to its supercooling point. The initiation of freezing was visualized as an instantaneous jump from the supercooling point (average = −1.63 ± 0.70 °C) to the freezing point (−0.67 ± 0.14 °C). Length of freezing was timed from the appearance of this exotherm. Two types of control animals were sampled: a) animals taken directly from 3 °C, and b) animals attached to the thermistor as above and chilled in the incubator to a temperature of 0 °C.

A second experiment was designed to study the effects of thawing on liver enzyme activities. Frogs, acclimated as above to 3 °C, were moved in their plastic boxes (containing damp sphagnum moss) to an incubator at −4 °C. After freezing for 24 h at this temperature, animals were returned to 3 °C and were sampled over a time course of thawing.

Both control and freezing exposed frogs were killed by double pithing. When possible (except for 30 and 70 min frozen animals) blood samples were taken as described previously (Storey and Storey 1984). Tissues were then rapidly dissected out, frozen in liquid nitrogen, and transferred to −80 °C for storage.

Chemicals. Biochemicals and coupling enzymes were from Sigma Chemical Co., St. Louis or Boehringer Mannheim Corp., Montreal.

Metabolite measurements. Perchloric acid extracts of tissues were prepared as previously described (Storey and Storey 1984) with the following changes: a) dilution with 6% perchloric acid solution was 1:5 w/v, and b) extracts were neutralized with a mixture of 3 M KOH/0.3 M Tris base/0.4 M KCl. Glycogen, glucose, and fructose-2,6-P_2 were determined spectrophotometrically; glycogen by the method of Keppler and Decker (1974). All other metabolites were measured fluorometrically in coupled enzyme assays (Lowry and Passonneau 1972).

For measurement of fructose-2,6-P_2 content the method of van Schaftingen (1984) was used. This involves the preparation of a basic extract of tissues (using NaOH at 80 °C) followed by assaying fructose-2,6-P_2 content by its activating effect on pyrophosphate-linked phosphofructokinase. That enzyme activation was due to fructose-2,6-P_2 content only was confirmed when, after acid treatment, samples failed to activate the enzyme.

Enzyme modification during freezing. Possible covalent modification of three glycolytic enzymes as a result of freezing was tested by examining key kinetic parameters which are, in other systems, indicative of the phosphorylated or dephosphorylated enzyme forms.

Frozen liver samples were rapidly weighed and homogenized (1:5 w/v) in icecold 50 mM imidazole-HCl buffer, pH 7.5 containing 15 mM 2-mercaptoethanol, 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) using a Polytron PT10 homogenizer. The well-suspended homogenate was sampled directly for measurement of glycogen phosphorylase activity. For phosphofructokinase and pyruvate kinase, homogenates were centrifuged at 27000 *g* for 30 min at 4 °C. The resulting supernatant was dialyzed against homogenization buffer (minus PMSF) for 2 h at 3 °C, with one change of buffer, and was then used for the measurement of enzyme parameters.

Enzyme activities were determined in coupled enzyme assays using a Gilford recording spectrophotometer. All assays were performed at 21 °C.

Glycogen phosphorylase: Assay conditions were: 50 mM potassium phosphate buffer, pH 7.0, 2 mg/ml glycogen (previously dialyzed), 0.4 mM NADP, 4 μM glucose-1,6-P_2, 15 mM $MgCl_2$, and excess phosphoglucomutase and glucose-6-P dehydrogenase. Phosphorylase *a* and total phosphorylase (*a* + *b*) were determined in the absence vs presence of 1.6 mM AMP. *Phosphofructokinase:* V_{max} assay conditions were: 50 mM imidazole-HCl buffer, pH 7.0, 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM NADH, 0.2 mM ATP, 20 mM fructose-6-P, and excess dialyzed aldolase, triosephosphate isomerase, and glycerol-3-P dehydrogenase. Affinities for fructose-6-P ($S_{0.5}$) were determined from Hill plots. Inhibition constants (I_{50}) for Mg.ATP (1:1) were determined from plots of *V* vs [Mg.ATP] at subsaturating (0.8 mM) fructose-6-P. Activation constants (K_a) for fructose-2,6-P_2 were determined from plots of V vs [fructose-2,6-P_2] at 0.4 mM fructose-6-P. *Pyruvate kinase:* Maximal assay conditions were: 50 mM imidazole-HCl buffer, pH 7, 100 mM KCl, 10 mM Mg_2Cl, 0.2 mM NADH, 5 mM ADP, 5 mM P-enolpyruvate, and excess dialyzed lactate dehydrogenase. Affinities for P-enolpyruvate ($S_{0.5}$) were determined from Hill plots in the presence/absence of 0.2 mM fructose-1,6-P_2. Inhibition constants (I_{50}) for L-alanine were determined from plots of *V* vs [alanine] at subsaturating (0.05 mM) P-enolpyruvate concentration.

Results

In a previous study we monitored the cooling and freezing pattern for wood frogs of about 2 g in size held at a constant −3 °C. The freezing exotherm persisted for 2.5 to 3 h before body temperature equilibrated to air temperature (Storey and Storey 1985b). Based on this result, we chose a time course of up to 70 min beyond the initial appearance of the exotherm for the present study of metabolic regulation in liver. The effects of thawing for 20 h at 3 °C following freezing for 70 min were also determined.

Figure 1 shows the effect of a short time course of freezing on the concentrations of glucose in liver and blood and on liver glycogen content. Because of the extent of freezing, blood could be sampled from one frog only at the 30 min point and not

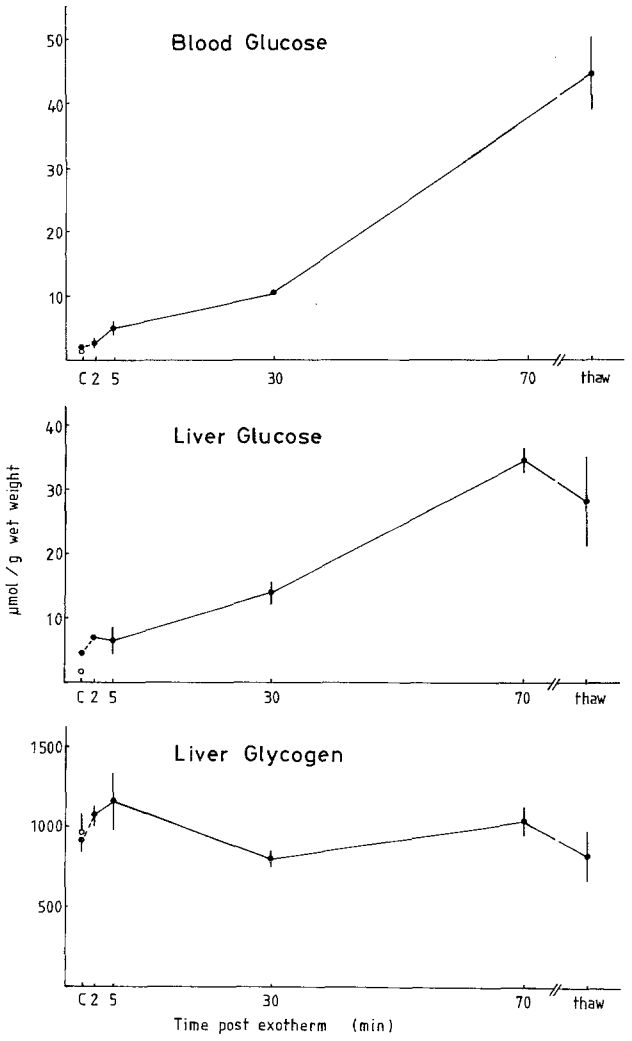

Fig. 1. Levels of glucose in blood and liver and liver glycogen content over a short course of freezing, followed by thawing, in *Rana sylvatica*. Individual animals were exposed to freezing for the times indicated, freezing timed from the appearance of the exotherm. Thawed animals were exposed to freezing for 70 min followed by 20 h thawing at 3 °C. Two types of control animals were sampled: animals sampled from a constant 3 °C (○), and animals attached to the thermistor and chilled in the incubator to 0 °C (●). Liver glycogen is expressed in glucose units. Results are means ± SEM, $n=3$ (except for blood glucose at 30 min, $n=1$); where error bars are missing on control and 2 min values, these were too small to indicate

at all at 70 min. However, as Fig. 1 shows, both liver and blood glucose contents were significantly elevated above those of controls within 5 min of the initiation of freezing. Glycogen content of livers showed no significant change over the short course of freezing but due to the high and variable amounts of glycogen in liver and the small amounts of glucose synthesized over this short time course, this result was not unexpected. Over a course of days and weeks of freezing, however, we have shown an inverse relationship between liver glycogen and tissue glucose contents (Storey and Storey 1986b).

Figure 2 shows the changes in concentrations of intermediates of glycolysis in liver over the course of freezing and thawing. The concentration of hexose monophosphates increased rapidly over the first 5 min post-exotherm and continued to climb over the full 70 min freezing exposure. For fructose-6-P, a 10 fold rise occurred within 5 min. Contents of fructose-2,6-P_2 and of the triosephosphates, however, showed no significant changes over the freezing exposure. The rise in fructose-6-P, with no change in fructose-1,6-P_2 levels, indicates a block in flux through glycolysis at the phosphofructokinase locus. Freezing had no significant effect on the contents of P-enolpyruvate or pyruvate in liver.

After thawing, liver hexose monophosphate concentrations dropped dramatically, consistent with the termination of glucose synthesis (Fig. 2). However, P-enolpyruvate concentration rose by 2.3 fold while pyruvate content remained stable; these results suggest an inhibition of glycolysis at the pyruvate kinase locus with the transition from frozen to thawed.

Levels of fructose-2,6-P_2, a potent activator of phosphofructokinase, were measured in liver and in other tissues (Table 1). Short term freezing did not affect the content of the bisphosphate in any tissue (values for 2, 5, 30, and 70 min samples were, therefore, combined).

Covalent modification is an effective mechanism for altering the activity of enzymes, often as the response to extracellular or environmental stimuli. Phosphorylation of regulatory enzymes typically alters one or more kinetic parameters; these are well documented for regulatory enzymes of glycolysis (glycogen phosphorylase, phosphofructokinase, and pyruvate kinase) and are widely used to infer changes in the phosphorylation state of these enzymes (Cohen 1980; Engstrom 1978; Sakakibara and Uyeda 1983). The effects of freezing exposure on selected kinetic parameters of the three enzymes in *R. sylvatica* liver are shown in Tables 2 to 4.

The initiation of freezing stimulated a rapid modification of glycogen phosphorylase, increasing the percentage of enzyme in the active *a* (phosphorylated) form from 41% in liver of control animals to 73% 5 min after the appearance of the freezing exotherm and to 94% after 70 min of freezing exposure (Table 2). Similar results have been found in our previous studies (Storey and

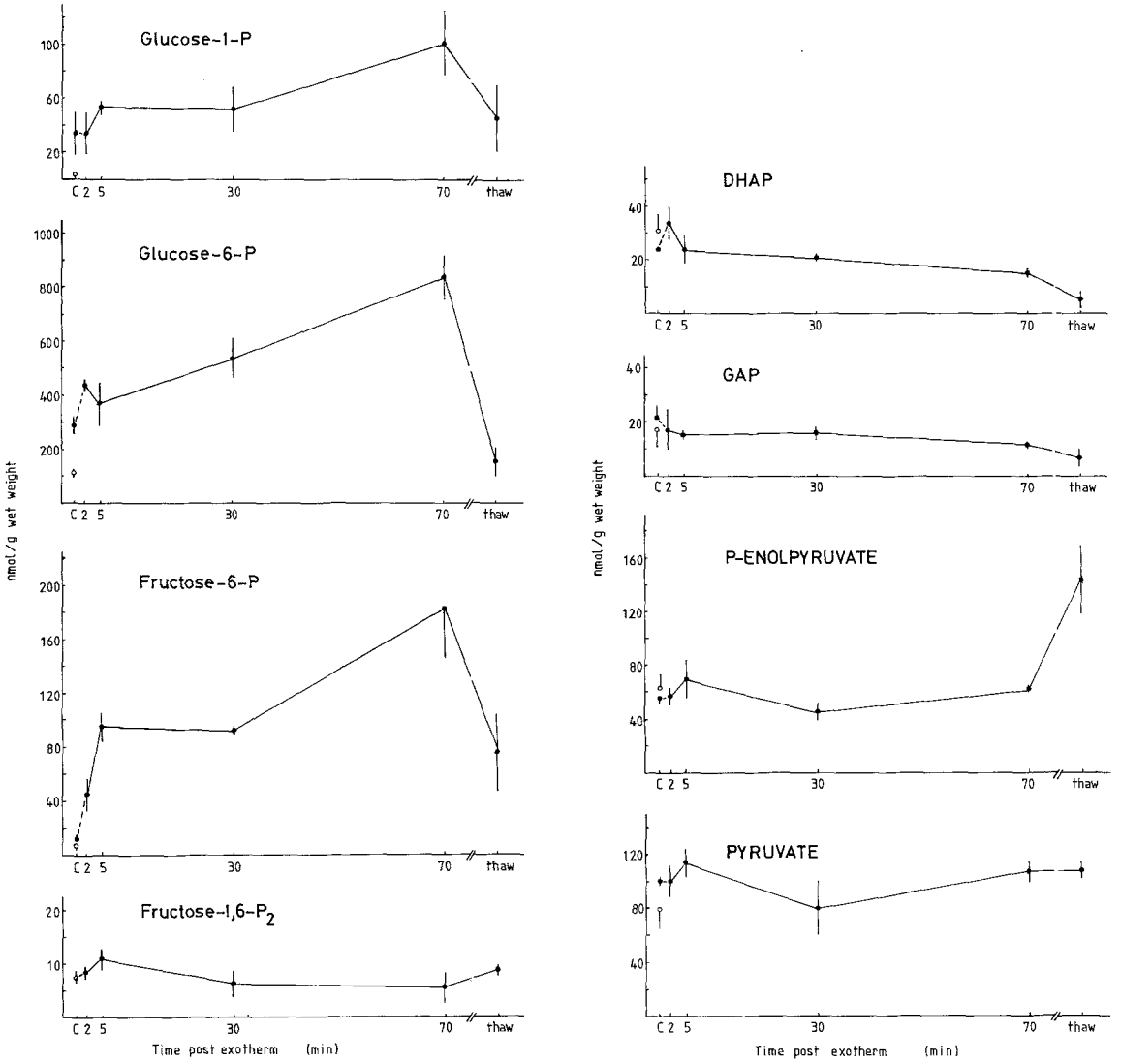

Fig. 2. Effect of a short course of freezing, followed by thawing, on the concentrations of some intermediates of glycolysis in liver of *Rana sylvatica*. Details are as in Fig. 1 legend

Storey 1984, 1985b). In addition to increasing the percentage of *a*, freezing also stimulated a rise in the total $(a+b)$ content of phosphorylase in liver. A 3 fold rise in total phosphorylase content was found at 70 min; again we have noted this previously (Storey and Storey 1984). After thawing for 20 h at 3 °C, both the percentage of phosphorylase in the *a* form and total phosphorylase content had returned to control levels. To ensure that changes in phosphorylase activity were not due to handling or temperature stresses but truly triggered by freezing, we compared phosphorylase in a second group of animals: 4 °C controls, −0.3 °C controls (animals handled and cooled to −0.3 °C before sampling), and 24 h at −2.1 °C; values for total phosphorylase and percent *a* were 4.00 ± 0.5 (14%), 2.63 ± 0.41 (19%), and 7.68 ± 0.41 (65%) U/g wet weight (all $n=4$), respectively.

The effects of short term freezing exposure on selected kinetic parameters of phosphofructokinase in liver are shown in Table 3. With the initiation of freezing, the K_m for fructose-6-P decreased by 40%; this was first seen at 5 min post-exotherm and persisted at the 70 min sampling time (n_H also increased significantly at 70 min). At the same times, the I_{50} for ATP doubled and the K_a for fructose-2,6-P_2 decreased to 40% of control values. All parameters showed a tendency to reverse towards control values after 24 h of thawing although values, in most cases, were intermediate between those of controls and 70 min freezing.

Table 4 shows the kinetic properties of pyru-

Table 1. Levels of fructose-2,6-bisphosphate in tissues of control, frozen, and thawed immature adult *R. sylvatica*

	Tissue			
Condition	Liver	Leg muscle	Kidney	Heart
Control	487±90	2200±238	2677±559	267±35
Freezing	545±108	3087±456	2374±247	262±51
Thawed	646±168	1982±540	3940±745	197±26

Results are given in pmol/g wet weight, means ± SEM, $n=6$ for control (values for 3 °C and 0 °C controls combined), $n=12$ for freezing exposed (values for 2, 5, 30, and 70 min post-exotherm combined since no significant changes in fructose-2,6-P_2 contents occurred at any of the sampling times), and $n=3$ for one day of thawing at 3 °C after 70 min freezing exposure

Table 2. Effect of short term freezing exposure on glycogen phosphorylase in *R. sylvatica* liver

		Phosphorylase		
		a	total	%*a*
Control	3 °C	1.45±0.49	3.44±0.53	41
Freezing	2 min	3.05±0.65	4.75±0.76	62
	5 min	3.30±0.40	4.90±0.43	73
	70 min	9.80±1.10	10.4 ±0.82	94
Thawed		1.90±0.35	4.30±0.83	43

Data are units/g wet weight, means ± SEM, $n=3$. Control values at 0 °C are not available for this experiment

vate kinase in *R. sylvatica* liver. The enzyme showed hyperbolic kinetics, unlike the mammalian liver enzyme (Engstrom 1978). Freezing exposure did not change enzyme maximal velocity, K_m for P-enolpyruvate, Hill coefficient, or I_{50} for alanine (values for these parameters for 2, 5, and 70 min freezing exposure were, therefore, combined). Fructose-1,6-P_2, however, had a slightly greater effect on K_m for P-enolpyruvate in liver from freezing exposed vs control animals; 0.2 mM fructose-1,6-P_2 reduced K_m 23% in control liver and 51% in liver of freezing-exposed animals.

To further investigate covalent modification as a regulatory factor in the response of liver metabolism to freezing, we performed a second experiment. Frogs were frozen for 1 day and were then sampled over a time course of thawing at 3 °C. Kinetic parameters for glycogen phosphorylase and phosphofructokinase are shown in Table 5 along with liver glucose concentrations. When frogs were thawed, cryoprotectant levels were rapidly reduced; we have previously shown that cryoprotectant from all tissues is returned to the liver and restored as glycogen (Storey and Storey 1986b). Correlated with the fall in liver glucose content was a strong depression of glycogen phosphorylase activity affecting both the percentage of enzyme in the *a* form and the total ($a+b$) activity of the enzyme. From a high of 10.4 U/g and 94% in the *a* form during the early minutes of glucose synthesis (Table 2), activities fell to 6.2 U/g and 44% *a* after 1 h of thawing and to a low point of 0.3 U/g and 7% *a* after 18 h of thawing (Table 5). After 4 days of thawing both total activity and percent *a* had returned to a level similar to that in liver of control frogs prior to freezing exposure (Table 2). Phosphofructokinase did not show a rapid change in kinetic parameters back to those which characterized the enzyme in control animals. K_m for fructose-6-P remained low over the course of thawing, similar to values in liver of animals frozen for 70 min (Table 3). With the exception of the 18 h value, the Hill coefficient remained high, as in frozen frogs. K_a for fructose-2,6-P_2 remained at an intermediate value and did not change over the time course of thawing. I_{50} for ATP was somewhat variable and showed no trend

Table 3. Effect of short term freezing exposure on the kinetic properties of phosphofructokinase in *Rana sylvatica* liver

		V_{max}	K_m (F6P)	n_H	I_{50} (ATP)	K_a (F2,6P_2)
Control	3 °C	0.94±0.13	2.04±0.11	1.12±0.11	0.52±0.06	45±9
	0 °C	0.93±0.08	1.85±0.28	1.06±0.10	0.83±0.02	58±7
Freezing	2 min	1.03±0.15	1.27±0.28	1.42±0.12	0.75±0.16	22±4
	5 min	1.33±0.05	1.22±0.08[a]	1.24±0.13	1.16±0.33[b]	18±1[b]
	70 min	1.14±0.15	1.16±0.05[a]	1.67±0.11[c]	1.13±0.17[b]	20±1[c]
Thawed		1.20±0.11	1.31±0.43	1.40±0.26	0.76±0.10	29±9

Results are means ± SEM of determinations on liver samples from 3 animals at each time point. Units are U/g wet weight for V_{max}, mM for K_m and I_{50}, and nM for K_a; n_H is the Hill coefficient. Results are significantly different from control (3 °C) by Student's *t*-test

[a] $P<0.01$

[b] $P<0.02$

[c] $P<0.05$

Table 4. Effect of short term freezing exposure on the kinetic properties of pyruvate kinase in *Rana sylvatica* liver

	V_{max}	K_m (PEP)		n_H	I_{50} L-alanine
		minus FDP	plus 0.2 mM FDP		
Control	21.4±1.1	0.082±0.002	0.063±0.002	1.09±0.09	0.122±0.021
Freezing	21.0±1.0	0.076±0.009	0.039±0.006[a]	1.00±0.05	0.181±0.037
Thawed	18.5±0.2	0.086±0.005	0.056±0.012	1.21±0.29	0.116±0.049

Results are means ± SEM, $n=6$ for control (values for 3 °C and 0 °C animals combined), $n=9$ for freezing exposed (values for 2, 5, and 70 min post-exotherm combined since no changes in enzyme parameters occurred over the freezing time course), and $n=3$ for thawed animals. Units are U/g wet weight for V_{max}, and mM for K_m and I_{50}; n_H is the Hill coefficient in the absence of FDP and was not significantly altered in the presence of FDP. FDP is fructose-1,6-P_2

[a] Significantly different from control, $P<0.05$

Table 5. Changes in glucose concentration and the kinetic properties of glycogen phosphorylase and phosphofructokinase in liver of *Rana sylvatica* over a time course of thawing at 3 °C following 1 day of freezing at −4 °C

	Time thawed			
	1 h	2.5 h	18 h	4 d
Glucose, μmol/g	169 ±7.5	136 ±1.0	95 ±6.5	25 ±10
Glycogen phosphorylase				
% *a*	44 ±5	23 ±3	7 ±2	41 ±3
total *a*+*b*	6.2±0.5	1.5±0.2	0.3±0.1	2.2±0.2
Phosphofructokinase				
V_{max}	1.0±0.01	1.1±0.06	1.2±0.01	1.1±0.09
K_m fructose-6-P	0.9±0.02	1.3±0.03	1.0±0.15	1.2±0.30
n_H	1.7±0.2	1.8±0.3	1.2±0.2	1.5±0.3
I_{50} ATP	0.9±0.10	0.7±0.04	1.3±0.1	1.1±0.01
K_a (fructose-2,6-P_2)	27 ±7	31 ±3	26 ±8	35 ±13

Frogs were held at −4 °C for 24 h and then removed to 3 °C and thawed for the times indicated. Results are means ± SEM for $n=3$ determinations. Units are U/g wet weight for V_{max}, mM for K_m and I_{50}, and nM for K_a; n_H is the Hill coefficient

over the course of thawing. Overall, results were very similar to the data found for a single time point of thawing, as seen in Table 3. Just as freezing had no effect on the kinetic parameters of pyruvate kinase in frog liver, thawing also produced no changes in the kinetics of the enzyme; mean values (data from all time points combined, $n=12$) were $V_{max}=22.4\pm1.45$ U/g, K_m (P-enolpyruvate) $=0.085\pm0.004$ mM (0.055 ± 0.003 mM in the presence of 0.2 mM fructose-1,6-P_2), $n_H=1.06\pm0.04$, and I_{50} L-alanine $=0.13\pm0.01$ mM, none of these significantly different from values in Table 4.

Discussion

The present study confirms our previous results on freeze tolerance in *R. sylvatica* (Storey and Storey 1985b) in showing that glucose synthesis by liver is initiated within the first 5 min after the appearance of the freezing exotherm. Synthesis proceeded linearly over the 70 min time course of freezing exposure.

Synthesis of glucose in liver was preceded by a rapid rise in the contents of hexose monophosphates, as occurs in other systems. Content of fructose-1,6-P_2 was not increased, however, despite a 10-fold rise in fructose-6-P content over the first 5 min post-exotherm and a 20-fold rise by 70 min. Contents of triose intermediates were similarly unaffected over the course of freezing. The different effects of freezing exposure on the liver contents of the substrate vs product of phosphofructokinase indicates an inhibitory block in glycolysis at this locus during glucose synthesis (Williamson 1970). This block allows glycogenolysis to be directed into the synthesis of glucose.

The initiation of cryoprotectant glucose synthesis in liver obviously requires fast-acting controls on the key enzymes involved. One such regulatory factor could be covalent modification via phos-

phorylation/dephosphorylation reactions. To search for probable covalent modification some kinetic properties of glycogen phosphorylase, phosphofructokinase, and pyruvate kinase in liver were assayed over the time course of freezing. All three of these enzymes are subject to protein phosphorylation, producing major changes in enzyme kinetic and regulatory properties, in mammalian liver (Cohen 1980; Engstrom 1978; Sakakibara and Uyeda 1983).

Covalent modification of glycogen phosphorylase was obvious and occurred extremely rapidly, the percentage of enzyme in the phosphorylated *a* form rising from 41% in control to 73% within 5 min of the appearance of the freezing exotherm. Glucose synthesis, then, is facilitated by a phosphorylation-mediated activation of glycogen phosphorylase to activate glycogenolysis. A second component to phosphorylase activation was also noted; this may or may not be the result of covalent modification of the enzyme. Freezing stimulated a 3-fold increase in the total activity of phosphorylase expressed. The sum total of the changes in total enzyme activity and percent activity in the *a* form resulted in a 6.75 fold increase in the total amount of active enzyme (1.45 vs 9.8 units/g of the *a* form) in liver 70 min after the appearance of the freezing exotherm. This supports the rapid output of cryoprotectant from liver during the early minutes/hours of freezing (Storey and Storey 1986b). Thawing had the opposite effect, radically depressing both total phosphorylase and the percentage of phosphorylase *a*, to a low point of only 0.02 units/g of *a* expressed after 18 h of thawing. The activation of phosphorylase during freezing appears to be a twofold, time-dependent process: the *b* to *a* conversion happening within 2 to 5 min but the increase in the total enzyme activity expressed requiring a longer time (apparent at 70 min in the present study). The molecular mechanisms underlying the control of total phosphorylase activity are currently under study in our laboratory. Of note is that fact that this is not a problem of recovery of enzyme activity since glycogen phosphorylase was routinely measured in the well-suspended homogenate.

Phosphofructokinase in *R. sylvatica* liver showed kinetic changes over the time course of freezing including a lowered K_m for fructose-6-P, an increased I_{50} for ATP, and a lowered K_a for fructose-2,6-P_2. These kinetic changes on their own could, presumably, activate phosphofructokinase during freezing in frog liver. Both the direction of change and the degree of change (generally about 2-fold) in kinetic parameters were similar to the effects of covalent modification on mammalian liver phosphofructokinase (Sakakibara and Uyeda 1983) and, based on the mammalian liver model, freezing in frog liver may result in the conversion of phosphofructokinase from a high-phosphate to a low-phosphate form. However, an apparent activation of phosphofructokinase, based on freezing-induced changes in kinetic properties, is at odds with the metabolite data which clearly show an inhibition of glycolytic flux at the phosphofructokinase locus during freezing-stimulated cryoprotectant synthesis. Obviously, then, additional regulatory factor(s) must be involved in phosphofructokinase control in this situation. These could include changes in the levels of allosteric modulators during freezing, or changes to the in vivo enzyme (e.g. subunit association/dissociation, enzyme binding to subcellular particles, enzyme susceptibility to proteolysis) during freezing with the additional possibility of changes to the phosphorylation state making the enzyme more or less susceptible to these regulatory processes. The present study examined one of these potential regulatory factors: control by fructose-2,6-P_2, a potent activator of phosphofructokinase. However, levels of this compound did not change significantly over the freezing exposure and, in addition, levels in vivo were, at all times, 10 to 20 fold higher than the K_a of the enzyme such that the enzyme in vivo may be always fully activated with respect to this compound.

Kinetic studies of pyruvate kinase in liver of *R. sylvatica* revealed an enzyme with hyperbolic kinetics; the mammalian liver enzyme typically shows sigmoidal kinetics (Engstrom 1978). Phosphorylation of the mammalian enzyme lowers enzyme V_{max}, increases K_m for P-enolpyruvate, decreases activation by fructose-1,6-P_2, and increases inhibition by alanine, all effects which result in a reduced enzyme activity. Freezing had no effect on any of these parameters for *R. sylvatica* liver pyruvate kinase, demonstrating that a covalent modification of the enzyme is not part of any regulatory control of the enzyme with respect to freezing and cryoprotectant synthesis. However, the metabolite data provide evidence of an inhibitory block in glycolysis at the level of pyruvate kinase during thawing; levels of P-enolpyruvate rose 2.3 fold with no change in pyruvate content in the thawed state. A metabolic block at the level of pyruvate kinase could be important in preventing the oxidation of the very high content of glucose cryoprotectant (levels in liver can be 200–400 μmol/g after freezing exposure (Storey and Storey 1984, 1986b)) and help to direct the

reconversion of glucose into glycogen in the thawed state. Again, however, factors other than covalent modification must be sought to explain glycolytic flux control at pyruvate kinase in liver of freeze tolerant frogs.

Acknowledgements. The excellent technical assistance of Mrs. S. Walters is greatly appreciated. Thanks to J.M. Storey for performing the frog freezing experiments. Thanks to Mr. I. McMurray for collecting wood frogs. Supported by a grant from the Canadian Liver Foundation.

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