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Triggering of cryoprotectant synthesis by the initiation of ice nucleation in the freeze tolerant frog, *Rana sylvatica*

Janet M. Storey and Kenneth B. Storey*
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

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Summary. The triggering of cryoprotectant synthesis was examined in the freeze tolerant wood frog, *Rana sylvatica*. A slow decrease in ambient temperature (1 °C every 2 days) from 3° to −2.1 °C was used to search for a specific trigger temperature. None was found. Instead it was found that, despite subzero temperature, animals which remained in a supercooled unfrozen state had low blood glucose (1.66±0.44 μmol/ml) while those which had frozen had high blood glucose (181±16 μmol/ml). These results indicate that it is the initiation of ice nucleation, rather than a specific subzero temperature, which triggers cryoprotectant glucose synthesis. This was confirmed by monitoring the freezing curves for individual frogs with sampling of blood and tissues at various times relative to the initiation of nucleation (detected as an instantaneous temperature jump from −3 to −1 °C). Animals sampled before nucleation had low blood and liver glucose contents and a low percentage of liver phosphorylase in the *a* form. Within 4 min of the initiation of freezing, however, blood glucose had jumped to 16 μmol/ml and liver glucose to 39.5 μmol/g wet weight. Glucose in both compartments continued to increase as the time of freezing increased correlated with an increase in liver phosphorylase *a* content from 47% before nucleation to 100% after 50 min of freezing. The results clearly demonstrate that freeze tolerant frogs have no anticipatory synthesis of cryoprotectant as a preparation for winter but rather can translate the initiation of extracellular ice formation into a signal which rapidly activates cryoprotectant production by liver.

* To whom all correspondence should be addressed

Introduction

In 1982 Schmid reported a natural tolerance for freezing during the winter hibernation of terrestrial frogs. Four species inhabiting northern North America have proven to be freeze tolerant: the wood frog, *Rana sylvatica*, the gray tree frog, *Hyla versicolor*, the spring peeper, *H. crucifer*, and the chorus frog, *Pseudacris triseriata* (Schmid 1982; MacArthur and Dandy 1982; Storey 1985). Although a variety of invertebrates, especially terrestrial insects (Ring 1980), show a tolerance for extracellular freezing these are the only vertebrate animals known to have this capability. As such these frogs offer unique opportunities for study particularly with respect to organ specific adaptations for freezing survival.

We have begun studies of the biochemical adaptations for freeze tolerance in two species, *R. sylvatica* and *H. versicolor* (Storey 1984; Storey and Storey 1984, 1985). Both species supercool only to −2 or −3 °C but can survive extended periods of time frozen at moderate (−6 to −10 °C) subzero temperatures. As is typical of freeze tolerant invertebrates (Ring 1980) low molecular weight carbohydrates are accumulated as cryoprotectants, glucose in *R. sylvatica* and glycerol in *H. versicolor*. Glucose is a very uncommon cryoprotectant amongst cold tolerant animals (Ring 1980; Danks 1978) perhaps because the normally strict control over blood sugar levels by hormones is at odds with the accumulation of molar levels of cryoprotectant in blood and tissues. None-the-less, levels of glucose as high as 0.26 M in immature adults and 0.55 M in mature females have been measured in blood of *R. sylvatica* after

freezing exposure (Storey and Storey 1984; Storey 1985). Cryoprotectant glucose appears to be largely manufactured by the liver from stores of glycogen (Storey and Storey 1984). Although freezing exposed frogs have high levels of glucose when thawed, attempts to stimulate cryoprotectant synthesis with various low temperature acclimation regimens, as has been done with freeze tolerant insects, have failed. Neither a temperature decrease of 1 °C per day from 23 °C to 0 °C nor weeks of exposure to a constant 3 °C raised cryoprotectant levels in *R. sylvatica* (Storey and Storey 1984). However, subsequent exposure to subzero temperatures (−2 to −4 °C), which resulted in freezing, elevated glucose levels in blood and tissues.

The present studies were designed to pinpoint the trigger for cryoprotectant synthesis in *R. sylvatica*. Two potential triggers were possible: 1) a direct temperature trigger at a set subzero temperature, or 2) triggering by the initiation of ice nucleation itself. The results show that temperature is not a trigger for cryoprotectant synthesis. Instead, unique amongst freeze tolerant species, synthesis is directly triggered by the initiation of extracellular ice formation.

Materials and methods

Animals and chemicals

Sexually immature adult wood frogs were collected along roadsides in wooded rural areas around Ottawa on rainy nights from late September through mid-October. Animals were held in the lab, without feeding, in plastic boxes containing damp sphagnum moss at 3 °C for at least 1 month before use.

Biochemicals and coupling enzymes were from Sigma Chemical or Boehringer Mannheim.

Trigger temperature experiment

Boxes containing frogs (average weight = 1.74 ± 0.55 g) and moss were transferred to an adjustable temperature incubator at 3 °C and held for 3 days after which control animals were sampled. The set temperature of the incubator was then lowered in increments of 1 °C every 2 days and animals were sampled after the 2 day exposure to each temperature. Exact temperature at each sampling time was recorded as the average temperature from 4 thermistors placed at various positions in the boxes.

Individual freezing experiment

Individual frogs (average weight = 1.62 ± 0.25 g) were placed on a pad of paper toweling. A thermocouple was positioned on the back of the animal and then thermocouple and animal were secured with a broad band of masking tape. Each animal was then placed in an incubator set at −3 °C and the course of cooling and freezing was monitored using a YSI telethermometer.

Animal sampling

Frogs which were frozen were thawed at room temperature for 20–30 min before sampling in order that blood could be sampled. All animals were killed by double pithing. Blood was sampled from the severed aorta using a heparinized capillary tube and was immediately mixed with perchloric acid. Selected tissues were then rapidly dissected out, frozen in liquid nitrogen and stored at −80 °C until use.

Biochemical analyses

Perchloric acid extracts of tissues and blood were prepared as described by Storey and Storey (1984). Metabolite concentrations and phosphorylase activities were determined by coupled enzyme assays as described by Storey and Storey (1984).

Results

Triggering temperature experiment

Figure 1 shows the effect of decreasing temperature at the rate of about 1 °C every 2 days on the concentration of glucose in frog blood. Temperatures above −1 °C had no effect on blood glucose concentration, the average blood sugar content being 1.66 ± 0.44 µmol/ml. After 2 days at −1.2 °C remaining frogs were about equally divided between those which were still in the thawed state and those which had frozen. As can be seen from Fig. 1 the thawed frog sampled still had a low blood glucose level while blood glucose in the

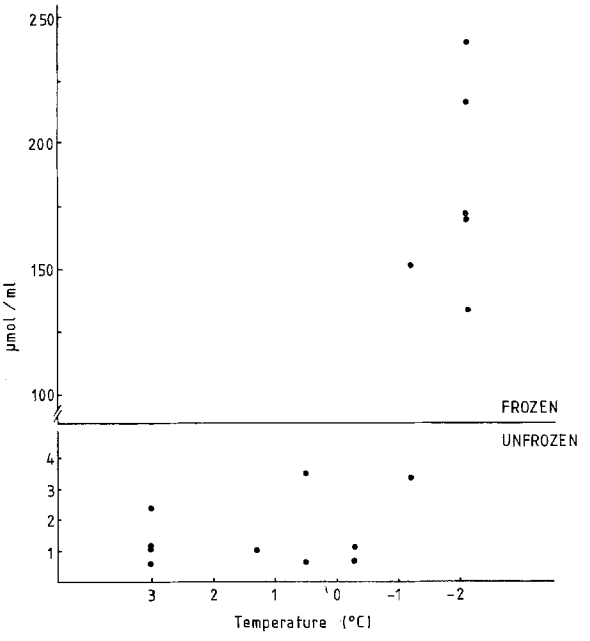

Fig. 1. Levels of blood glucose in *R. sylvatica* as a function of temperature decrease. Animals were sampled after 2 days exposure to each temperature. Data points are for individual animals. The status of the animals, thawed *versus* frozen, upon removal from the incubator is also shown

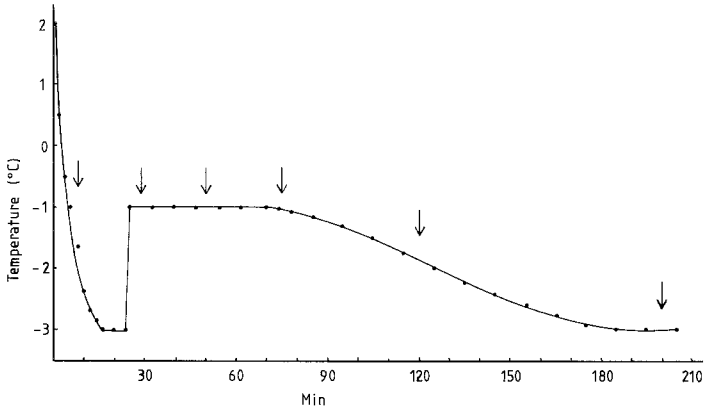

Fig. 2. Composite freezing curve for immature adult *R. sylvatica* showing the relative time points at which individual animals were sampled. Individual frogs varied greatly in the length of time (2–45 min) spent in the initial supercooled state at −3 °C; other characteristics of the freezing curves were very similar between individuals. Individuals were therefore sampled at times relative to the initiation of nucleation (temperature jump to −1 °C)

frozen animal was 152 μmol/ml. After 2 days at −2.1 °C all remaining frogs were frozen and all animals had elevated blood glucose, the average glucose content in frozen animals being 181 ± 16 μmol/ml. Thus the data suggest that temperature is not the trigger for glucose synthesis but that freezing is.

Liver glycogen and glucose contents were also quantitated in these animals. Glycogen content averaged 567 ± 72 μmol/g wet weight (expressed as glucose units) in thawed animals (those at temperatures of −0.3 °C or above) but decreased to 263 ± 33 μmol/g in animals frozen at −2.1 °C. Liver glucose content behaved oppositely being 2.16 ± 0.44 μmol/g in the thawed animals and 223 ± 29 μmol/g in the frozen animals.

To further confirm that it was the initiation of freezing rather than temperature itself which stimulated cryoprotectant synthesis, several frogs were placed in a moss-filled box and put in the incubator at 0 °C. After 2 days at this temperature, temperature was lowered to −2 °C and allowed to equilibrate (as determined from 4 thermistors placed at different points in the box). After 2 h at −2 °C, animals were examined. Two completely unfrozen animals were sampled as well as 2 animals which had begun to freeze (legs and arms stiff, skin icy but abdomen still soft). These were analyzed for blood and liver glucose contents. Unfrozen animals had blood glucose levels 0.55 and 1.0 μmol/ml and liver glucose of 2.43 and 4.38 μmol/g wet weight. Partially frozen animals, however, had blood glucose levels of 58.8 and 29.1 μmol/ml and liver glucose of 64.3 and 53.1 μmol/g.

Individual freezing

The triggering of cryoprotectant synthesis was also examined by monitoring freezing curves for individual frogs. Animals were chilled in an incubator at −3 °C in contact with a thermocouple. Figure 2 shows a composite freezing curve based on the average freezing behaviour of individuals. Nine animals monitored supercooled to an average of −3.0 ± 0.04 °C and froze at −1.07 ± 0.11 °C. Animals varied most strongly in the time spent in the supercooled state at −3 °C (from 2 to 90 min). Other characteristics of freezing were very similar including 15–30 min to chill to −3 °C, 40–50 min spent freezing on the plateau region of the curve at −1 °C and about 180 min in total for the complete freezing of the animal and return of temperature to −3 °C. These similarities are undoubtedly the result of the similar body weight of all specimens.

Figure 2 also shows the time at which selected individual animals were sampled. Metabolite and enzyme data for these animals are given in Table 1. The animal sampled prior to ice nucleation at a body temperature of about −1 °C had low levels of blood and liver glucose and a 45% phosphorylase *a* content. This was typical of control animals in previous experiments (Fig. 1 and Storey and Storey 1984). However within 4 min of the initiation of nucleation (indicated in Fig. 2 by the temperature jump to −1 °C) both blood and liver glucose were elevated to levels 3.3 and 6.6 times the control values, respectively. Over the subsequent minutes of freezing phosphorylase *a* content in liver increased dramatically and glucose continued to climb in blood and liver. After approximately 1.5 h of the initiation of freezing phosphorylase *a* content began to decline although remaining higher than that in control animals. Liver glucose content remained high, however, and blood glucose continued to rise suggesting a steady output of glucose from liver had been established. Liver glycogen content was high in all animals except for the animal frozen longest. This reflects the fact

Table 1. Levels of selected metabolites in liver and leg muscle of *R. sylvatica* sampled at times relative to the initiation of extracellular freezing as illustrated in Fig. 2

Time	Blood	Liver				Leg muscle		
	Glucose	Glucose	Glycogen	Lactate	Phosphorylase a	Glucose	Glycogen	Lactate
Prior to nucleation	5.0	6.0	665	0.9	47%	1.5	68	4.5
Post nucleation: 4 min	16.3	39.5	627	1.5	52	3.6	47.5	11.6
25 min	29.0	44.2	812	1.0	92	2.1	51.8	4.3
50 min	–	47.6	722	3.2	100	2.9	50.5	5.3
95 min	40.0	77.7	692	0.8	61	2.1	57.2	3.3
175 min	65.7	78.0	448	8.9	64	5.8	84.4	2.6

Metabolite data are given as μmol/ml for blood and μmol/g wet weight for tissues, glycogen is expressed in glucose units. Glycogen phosphorylase activity is given as the percentage of total activity in the active *a* form. Results are for individual frogs, with all 8 parameters quantitated in the same individual

that blood and liver glucose concentrations in these animals after about 3 h of freezing, although elevated, do not yet approach the 200–300 μmol/g wet weight found when cryoprotectant levels are equilibrated throughout the animals (Storey and Storey 1984; Storey 1985); a major decrease in liver glycogen content in these early hours is not therefore expected. Lactate in liver was only elevated in the animal frozen longest (about 3 h from freezing initiation). Elevation of lactate at this time may suggest that this is the point at which anoxic/ischaemic conditions begin to prevail in the animal as freezing starts to restrict blood flow. Metabolite levels in leg muscle are also shown in Table 1. Muscle showed only a small accumulation of glucose by the end of the period of extracellular freezing suggesting that longer times or a slower rate of freezing may be required for muscle to accumulate significant amounts of cryoprotectant. Lactate did not accumulate in muscle over the freezing period.

Although data in Table 1 are for individual animals at each time point only, we have subsequently confirmed the activation of glucose synthesis in liver by freezing initiation with additional experiments. Glucose levels in liver were 1.7 ± 0.4, 6.9 ± 0.3, 14.2 ± 1.4 and 34.4 ± 1.8 ($n = 3$) for animals chilled to 0 °C in the apparatus (control), and 2, 30 and 70 min post exotherm, respectively.

Discussion

The data in the present study show that temperature itself is not the trigger for cryoprotectant synthesis in *R. sylvatica*. This confirms and extends data from a previous study in which two different temperature acclimation schemes failed to induce anticipatory synthesis of cryoprotectant in unfrozen frogs (Storey and Storey 1984). Instead cryoprotectant synthesis in these frogs begins coincident with the initiation of ice nucleation. Thus, when frogs were held at subzero temperature, those in a supercooled unfrozen state retained low blood and tissue glucose while frogs which had frozen at the same temperature had high glucose in tissues and blood. Data from the freezing of individual frogs showed that within 4 min of the initiation of ice nucleation liver glucose production has been activated and glucose output into the blood had begun.

To our knowledge this stimulation of cryoprotectant synthesis using as a trigger the initiation of extracellular ice formation is unique amongst freeze tolerant animals. Freeze tolerant invertebrates typically utilize temperature (cool but not subzero) and/or photoperiod cues to achieve a gradual production of cryoprotectants and other cold hardiness features (ex. thermal hysteresis proteins) in an anticipatory manner over the autumn months (Storey and Storey 1983; Duman and Horwath 1983). The lack of an anticipatory response with respect to cryoprotectant production in frogs may reflect one of two things: 1) hibernation sites may protect frogs from freezing for most, or sometimes even all, of the winter so the metabolic energy involved in cryoprotectant synthesis/catabolism may be wasted in some instances, or 2) the very rapid rate with which glucose can be accumulated in frog blood and tissues when freezing is initiated may preclude a need for anticipatory synthesis. In this regard the amounts of cryoprotectant which can be produced within 3 h in frogs at subzero temperatures (Table 1) accumulate in freeze tolerant gall fly larvae only after several days at considerably higher trigger temperatures (13 °C for glyc-

erol, 3 °C for sorbitol) (Storey and Storey 1983). Thus very rapid rates of cryoprotectant production in the frog coupled with what is probably a very slow rate of freezing in the protected natural hibernation sites could probably ensure an adequate synthesis and distribution of cryoprotectant during the course of extracellular freezing.

The primary event in the activation of cryoprotectant synthesis in the frog is the activation of glycogen phosphorylase in liver (but not in other organs) (Table 1; Storey and Storey 1984). Both total phosphorylase activity and the percentage of the enzyme in the active *a* form rise dramatically with freezing exposure (Storey and Storey 1984). The phosphorylase *b* to *a* conversion is achieved by protein phosphorylation and this is typically stimulated by the actions of Ca^{2+} or cAMP mediating the effects of nervous or hormonal stimulation. How is ice nucleation in an extracellular fluid compartment detected and translated into a stimulation of liver glycogen phosphorylase activity? As yet we have no data on this although some speculations might be made. Our observations are that the first sites at which ice formation can be detected are in or under the skin, particularily on the legs or arms. It is not likely then that liver is responding directly to ice formation in its immediate environment. Rather, detection may occur at the level of receptors in the skin (perhaps due to a rapid outflow of cell water when extracellular freezing begins). Alternately, in instances where animals have supercooled to a significant extent before freezing (see Fig. 2) (although extensive supercooling is not required as illustrated by the frog frozen at −1.2 °C in Fig. 1) the instantaneous jump in body temperature which occurs upon nucleation may be the signal detected. The very rapid activation of liver glucose synthesis (Table 1) suggests that transmission of the signal to liver is probably by nervous stimulation or through the action of catecholamine hormones. Further studies will monitor catecholamines in blood and levels of second messengers in liver during freezing in an attempt to pinpoint the molecular events of cryoprotectant triggering.

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