FREEZE TOLERANCE

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Summary

Many organisms on Earth must endure prolonged exposures to subzero temperatures below the equilibrium freezing point of their body fluids. Uncontrolled ice formation in biological tissues has multiple damaging effects, both physical and metabolic. Hence, organisms have developed strategies to preserve life at temperatures below 0 °C. These include (1) anhydrobiosis (extreme desiccation removes all bulk water that can freeze), (2) vitrification (instead of crystallizing into ice, water solidifies into an amphorous glass), (3) freeze avoidance (multiple mechanisms, including the use of antifreeze agents, are used to greatly suppress the temperature at which biological water freezes), and (4) freeze tolerance (the growth of ice in extracellular fluid spaces is regulated

while the liquid state of the cytoplasm is preserved). Freeze tolerance is the winter hardiness strategy of many types of microorganisms, plants, and animals and the present article considers the phylogenetic diversity of freeze-tolerant organisms and summarizes our current understanding of the genetic, biochemical, and physiological adaptations that support freezing survival. These include the use of ice-nucleating proteins to initiate and regulate ice formation, the action of antifreeze proteins in molding crystal shape and inhibiting recrystallization, low molecular-weight carbohydrate cryoprotectants that provide colligative resistance to cell volume shrinkage during extracellular ice formation while protecting and stabilizing cellular macromolecules, the role of freeze-induced gene expression in producing proteins that aid survival, metabolic adaptations that provide ischemia resistance when freezing cuts off external oxygen supplies to cells, and mechanisms that allow heart beat, breathing, and other vital signs to be interrupted during freezing but restart spontaneously after thawing. Studies of the biochemical mechanisms of natural freezing survival also have multiple applications such as for the development of tissue/organ cryopreservation technology, the improvement of frost resistance in agricultural crops, and commercial snowmaking.

1. Introduction

Earth is a cold place. About 90% of the volume of the oceans is colder than 5 °C and the surface waters of polar seas drop to about –1.9 °C (the freezing point of seawater) for much of the year. Terrestrial environments are even colder. Polar regions cover about 14% of Earth's surface with winter temperatures that can range from –30 to –70 °C; winter in temperate zones can also reach lows of –30 °C or below. Yet despite the lethal effect of freezing for many organisms (think of the devastation of a late autumn garden after the first hard frost), life endures in all cold places. Indeed, there are many examples of organisms that can live only in cold environments. For example, psychrophilic bacteria typically cannot grow at temperatures much above 15 °C but can grow well at subzero temperatures. Some snow algae cannot grow above 10 °C whereas the growth of certain yeasts has been reported at temperatures as low as –18 °C. Viable bacteria have been isolated from subsurface permafrost in the Antarctic where they may have spend millennia in a continuous deep freeze at –25 to –30 °C and the Antarctic cold deserts are home to an amazing variety of yeasts and cyanobacteria that endure extreme desiccation and wide yearly temperature variation (–55 to +10 °C).

Organisms that live at subzero temperatures fall into two major groups—those that sustain all normal life functions at these low temperatures and those that interrupt normal life processes, often entering dormant life phases, in order to endure seasonal or intermittent subzero exposures. The vibrant community of organisms—microbes, plants, animals—living in polar seas are an excellent example of the former. Teleost fish in this environment spend all or most of their lives at –1.9 °C even though the osmolality of their body fluids suggests that they should freeze at about –0.5 °C. However, some unique adaptations, notably the presence of antifreeze proteins in their body fluids, keep them from freezing. For many terrestrial organisms, however, the often extreme subzero temperatures of winter are met with an interruption of feeding, growth, and activity and adaptive strategies including dormancy and cold hardiness are employed to preserve and protect the organism until warmer temperatures return in the spring.

One of the strategies of winter cold hardiness is freeze tolerance, the ability to endure the freezing of extracellular body fluids. Before discussing freeze tolerance as the main topic of this article, a summary of other options is important. As a general rule, organisms deal with any environmental stress at multiple levels including behavioral, physiological, and biochemical responses. Behavioral responses are typically the first line of defense and that is broadly illustrated by organismal responses to subzero temperatures. In advance of winter, most organisms seek full or partial shelter from the impending cold. For some, this is accomplished by migration, both long (birds or butterflies may fly thousands of miles) or short (garter snakes in Manitoba, Canada travel several kilometers to mass by the thousands in frost-free underground caves). Many animals go underground to hibernate below the frostline whereas perennial plants concentrate their reserves in underground roots and tubers while sacrificing aboveground foliage. Some animals hibernate underwater (e.g., many frogs and turtles) or winter as aquatic life stages (e.g., dragonfly nymphs) and are safe unless the water in their pond or stream freezes to the bottom. Other organisms gain partial protection from the full force of winter by hibernating at or near the ground surface. With a layer of leaf litter and a deep blanket of snow, temperatures at the soil surface can often remain close to 0 °C even when air temperatures above the snowpack drop below –20 °C. Furthermore, in this microhabitat, organisms are well buffered from rapid changes in temperature. Many organisms take refuge in this subnivean (under the snow) environment, some in well-developed forms (e.g., insects, spiders, frogs; many perennial plants grow new leaf rosettes before the old foliage dies back) and some as embryonic forms (seeds, spores, cysts). Although temperatures under the snow can still fall to subzero values, the insulation of the snowpack means that organisms have less severe thermal challenges to endure. In northern boreal forests, for example, temperatures under the snowpack may not drop below about –8 °C all winter. A final group of organisms experience virtually no thermal buffering and must endure deep and prolonged cold. The most obvious members in this group are trees and shrubs that require protection for their twigs and buds as well as many insects and other arthropods that winter in arboreal sites.

1.1. Low temperature and freezing injury

Why is protection from subzero temperatures necessary? Chilling injuries are one factor. Death due to chilling below 0 °C without freezing has been well investigated in insects and hypothermia can often be lethal for nonhibernating species of mammals. Indeed, humans cannot withstand the cooling of core organs much below 25 °C. Chilling injuries derive from a disruption of the normal integration of metabolic functions. All metabolic reactions are temperature dependent and most increase/decrease in rate by about twofold for every 10 °C increase/decrease in temperature (Q_{10} = 2). Some reactions have lower Q_{10} values and some substantially higher values of 3 or 4. Reactions with high Q_{10} values are substantially impaired during cooling, often causing disruption of the metabolic pathway or function in which they participate. The temperature sensitivity of metabolic reactions arises because temperature alters the strength of the hydrophobic and hydrophilic weak bonds that are key to the structure and function of macromolecules. For example, weak bonds are major determinants of protein conformation, the association of subunits within a protein

or of multiple proteins within a polymer, enzyme-substrate binding, and enzyme-membrane interactions.

Differential effects of low temperatures on opposing metabolic functions are often the most devastating. For example, the electrical potential difference across the plasma membrane, which is key to functions such as cell sensitivity to stimuli, nerve transmission, or muscle contraction, is maintained by the opposing actions of ATP-driven ion pumps that move ions (e.g., Na^+, K^+, Ca^{2+}, H^+) across membranes "uphill" against their chemical gradient versus ion channels that facilitate "downhill" movements along concentration gradients. Collectively, ion pumps use a huge proportion of total cellular energy; the Na^+/K^+ ATPase alone is responsible for 5–40% of total ATP turnover depending on cell type. For organisms that are not cold adapted, cold exposure can seriously impair the production of ATP, the cellular energy currency, and this, in turn, impairs the activity of ion pumps, with little effect on ion channels, and quickly leads to a dissipation of membrane potential difference. This causes further problems, particularly uncontrolled Ca^{2+} influx into cells which triggers the action of numerous Ca^{2+}-stimulated proteases that cause major destruction.
These problems caused by chilling can cause major injury or even death for nonadapted organisms if they are cooled below their normal low temperature limits. For example, summer-active ground squirrels are just as sensitive to hypothermia as are humans but during a period of autumn cold acclimation various adjustments are made to their macromolecules that allow them to maintain metabolic integration when their body temperature sinks to near 0 °C during winter hibernation. Similar processes of cold acclimation prepare organisms to maintain metabolic integration at subzero temperatures. The biochemistry of cold acclimation is an enormous topic on its own and will receive little attention in this article. Briefly, the process can include changes in the relative activities of various enzymes and metabolic pathways, synthesis of new protein/enzyme variants that function optimally at low temperatures, and adjustments to membrane composition and lipid fuel depots to raise their content of unsaturated lipids so that fluidity is maintained at very low temperatures. Thus, properly cold-acclimated organisms have little to fear from a decrease in temperature to subzero values. The major risk is from freezing.

For most organisms, internal ice formation has multiple damaging or lethal effects (Figure 1). (1) Ice crystals can cause direct physical damage to cells and tissues. Shearing and squeezing stresses can damage or break individual cells that are trapped among growing ice crystals. In multicellular organisms, ice expansion can burst delicate capillaries so that upon thawing vascular integrity is compromised and the organism suffers severe internal bleeding. (2) Ice growth inside of cells damages subcellular architecture and disrupts or destroys the compartmentation of metabolic pathways. The severity of this damage is why cytoplasmic freezing is avoided even by freeze tolerant organisms. Only a couple of exceptions to this have ever been found; the most conclusive demonstration of survival after intracellular freezing coming from studies of the Antarctic nematode, *Panagrolaimus davidi*. (3) Delivery of oxygen and fuel supplies from extracellular sources is cut off when cells are entrapped in ice or when the blood plasma of multicellular organisms freezes. (4) Freezing in extracellular spaces removes pure water into growing ice crystals and leaves behind a highly concentrated extracellular fluid. This sets up a steep osmotic gradient across cell membranes which

causes an outflow of water and net shrinkage of cells. Dehydration, elevated intracellular ionic strength, and cell volume reduction all have negative effects on cell morphology and metabolic function, and this is compounded by problems of too rapid swelling of cells during thawing. For organisms in nature, cell volume effects are typically the first and most important consequences of freeze/thaw. Shrinkage below a critical minimum cell volume can cause irreparable damage to cell membranes due to compression stress. Most freeze-tolerant organisms in nature do not experience dehydration or ionic strength changes extreme enough to directly affect macromolecules but these factors are of consequence in the field of cryopreservation and have been addressed during the development of protocols for cell freezing (e.g., sperm, blood) in liquid nitrogen (–196 °C).

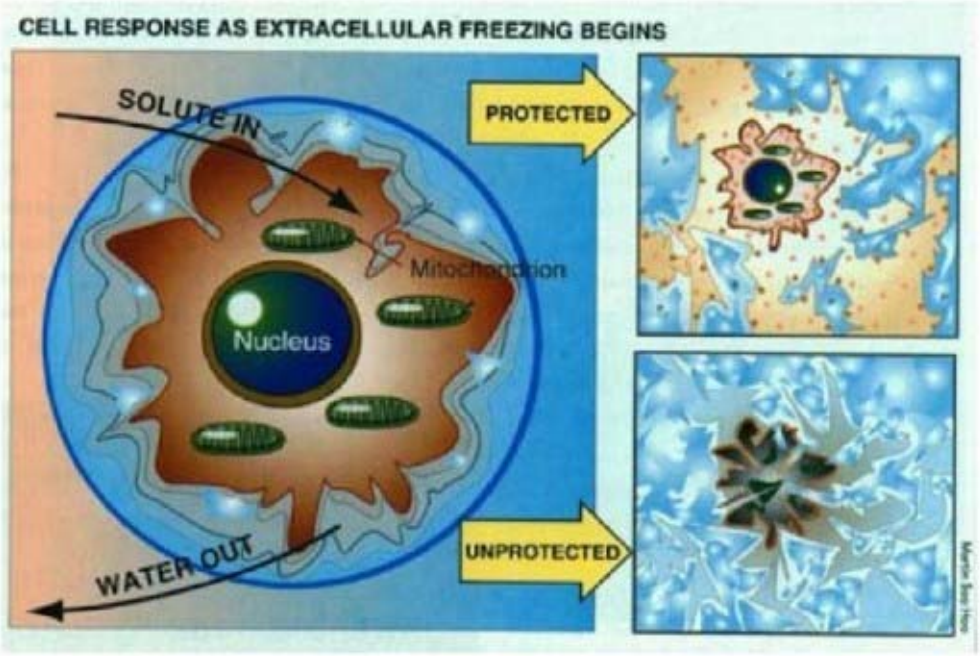

Figure 1. Cell responses to extracellular freezing

For an unprotected cell, extracellular ice nucleation results in a rapid growth of ice in large crystals. Solutes are excluded from the crystal and the osmolality of the remaining extracellular fluid rises quickly. Cells respond by loosing water. Cell shrinkage beyond critical minimum cell volume results in permanent damage to cell membranes so that upon thawing the integrity of the plasma membrane is lost. Freeze tolerant organisms use various protective strategies. Freezing is seeded by ice-nucleating proteins (P) at a temperature just under the equilibrium FP of body fluids so that ice growth is slow and controlled. Antifreeze proteins (brown circles) regulate the shape of crystal growth and inhibit recrystallization so that crystal size stays small. Low molecular weight carbohydrate cryoprotectants (orange circles) such as glycerol or glucose act in a colligative manner to minimize cell volume reduction and others such as trehalose (T) or proline stabilize the membrane bilayer structure. From Storey and Storey (1998) with permission.

2. Strategies for Survival at Subzero Temperatures

The potential problems caused by ice in cellular systems have led to the development of several strategies for subzero survival. Four general categories can be defined although they include some overlap. Each takes a different approach to preserving life at subzero temperatures and addressing the potentially serious injuries that can be done by ice. These are: (1) anhydrobiosis—literally meaning "life without water," this strategy involves extreme desiccation to eliminate all bulk water from the system and place organisms in a dormant, virtually ametabolic state. With no free water, no ice can form. (2) Vitrification—instead of freezing as crystals of ice, water solidifies into an amorphous glass. (3) Freeze avoidance—potent antifreezes are used to maintain the liquid state of body fluids, often to temperatures approaching –40 °C. (4) Freeze tolerance—controlled ice growth in extracellular body fluids is permitted although the liquid state of the cytoplasm is preserved. Freeze tolerance is the focus of the current article but before discussing the mechanisms of freezing survival in detail, a brief summary of the three alternatives is instructive.

2.1. Anhydrobiosis

One way to avoid damage due to internal freezing is to minimize or even eliminate all freezable, bulk water from an organism leaving behind only minuscule amounts of water that form bound or vicinal water shells around macromolecules. Hence, there is no free water left to form ice crystals. This is a drastic option that involves extreme dehydration and during which an organism cannot remain active or maintain any normal metabolic functions. It is unheard of among animals of greater than microscopic size and is rarely seen in the vegetative parts of plants except for a small group of "resurrection" plants. However, anhydrobiosis is a common way of ensuring the survival of the dormant stages of many organisms including eggs, cysts, spores, and seeds. In a dry state these can remain viable for many years, enduring both heat and cold. Many can be stored without harm in liquid nitrogen.

Anhydrobiosis (also called cryptobiosis) is best known as a means of survival in seasonally arid environments, the classic example being the encysted embryos of brine shrimp (*Artemia*). These can be dried to the point where water content is less than 0.1 g per g cysts (compared with about 4 g H_2O per g dry mass or 80% water for most active organisms). Anhydrobiotic organisms are virtually ametabolic since there is no bulk water remaining to support the diffusion of metabolic intermediates or the activity of enzymes. Desiccation places extreme stress on cellular macromolecules because hydration shells are normally very important for maintaining their functional conformation. To deal with this, high concentrations of polyhydric carbohydrates are synthesized during drying; for example, in brine shrimp cysts the three-carbon polyol, glycerol, and the disaccharide, trehalose, accumulate to about 4 and 14%, respectively, of the dry weight. Glycerol stabilizes protein structure whereas trehalose is particularly effective in stabilizing the lipid bilayers of membranes.

2.2. Vitrification

Vitrification is a process by which water is solidified, not into a crystal, but into an amorphous glass. The glass incorporates all of the dissolved solutes present in the water and hence vitrified cells are not under osmotic, ionic strength, or volume stresses as

frozen ones are. In the laboratory, the requirements for achieving a glass transition are daunting and include the need for extremely high concentrations of solutes (~40% solutions), rapid cooling to the glass transition temperature (Tg) that is often well below –30 °C, and warming rates in the order of 30–50 °C/min to prevent devitrification, the instantaneous crystallization of ice that can occur during warming at any temperature between the Tg and the melting point (MP) of the solution.

In nature, vitrification occurs under less rigorous conditions probably because of other contributing factors such as the substantial dehydration of systems that undergo natural vitrification and the accumulation of high concentrations of sugars that have particularly high glass transition temperatures (e.g., trehalose in animals, sucrose, raffinose, or stachyose in plants). Indeed, in both desiccation tolerant resurrection plants and *Artemia* there is strong evidence that vitrification occurs during drying at temperatures well above 0 °C and that the sugars have dual protective roles in both promoting vitrification of any remaining water and directly stabilizing proteins and membranes through hydrogen bonding to polar residues in the dry macromolecular assemblages. Sugar glasses also form in many plant seeds and, of direct relevance to cold hardiness, are key to subzero survival in the twigs of various subarctic woody plants such as poplar and birch. In poplar, for example, sugar glasses form below –20 °C and twigs that are cold hardened at –20 °C to optimize sugar production can subsequently endure exposure to liquid nitrogen.

Vitrification, unlike freezing, is not physically destructive of tissue organization or subcellular architecture and, hence, the technique has been pursued as method for the ultralow storage of medically important cells, tissues, and organs. Although vitrified storage of cell suspensions has been relatively easy to accomplish, the successful preservation of vitrified organs remains elusive. There are several reasons for this including the low tolerance of mammalian organs for substantial dehydration, the requirement for extremely high amounts of cryoprotective agents (such as dimethylsulfoxide, ethylene glycol, glycerol) which may have cytotoxic effects, and the need for very fast and even cooling and warming throughout the entire organ mass to both induce vitrification during cooling and avoid devitrification during warming.

2.3. Freeze Avoidance

Most animals and plants of greater than microscopic size (with the possible exception of their eggs or seeds) cannot endure dehydration to the extent needed to utilize either anhydrobiosis or vitrification as a means of subzero survival. A third option for winter survival is freeze avoidance, which involves suppression of the temperature at which biological water freezes. One or more mechanisms are employed to lower the temperature at which body fluids freeze to a value that is well below the anticipated environmental minima experienced by the organism. Many insects and other arthropods have optimized this strategy and some show the exceptional ability to remain liquid down to –40 °C or even lower. Cold-water marine fish also use this strategy to survive in seawater that can cool to –1.9 °C compared with the normal freezing point of the blood of teleost fishes which is about –0.5 °C. Deep supercooling also occurs within the primordium tissue of the buds and xylem tissues of many woody plants.

The freeze avoidance strategy is built upon two of the physical properties of water solutions. The first is a colligative property of solutions—the greater the concentration of dissolved solutes, the lower the freezing point (FP) of the solution. By accumulating high concentrations of solutes, freeze-avoiding organisms can substantially reduce the equilibrium FP of their body fluids. The equilibrium FP is the temperature at which an ice crystal added to a solution begins to grow. Typically, compatible solutes are used that are nonionic, soluble at very high concentrations, readily equilibrate across biological membranes, and do not perturb protein conformation. The solutes used are often the same ones used in anhydrobiosis or natural vitrification and, apart from their colligative effects, they are also highly effective at stabilizing macromolecules against low temperature and low water stresses. In animals, glycerol is by far the most common of the naturally occurring low molecular weight antifreezes but various other polyhydric alcohols and sugars are used by some species.

The second property of water solutions that is important to freeze avoidance is the phenomenon of supercooling (also called undercooling), the ability of water solutions to chill to temperatures below the equilibrium FP without freezing. All biological fluids supercool to some extent; for example, small volumes of human plasma can be chilled to near –15 °C before freezing. Indeed, under controlled laboratory conditions pure water can actually be cooled to –40 °C before the random assembly of water molecules into proto-ice nuclei occurs so frequently that homogeneous nucleation (spontaneous crystallization) cannot be avoided. However, very deep supercooling of most biological fluids does not normally occur because crystallization is typically stimulated by the action of heterogeneous nucleators including solutes, particles or surfaces that act to orient water molecules into the crystal lattice shape and trigger freezing. Thus, supercooled solutions are inherently unstable and successful long-term freeze avoidance requires the removal or masking of nucleators as well as strategies to block the growth of ice crystals beyond a microscopic size. Strategies that address both of these principles of freeze-avoidance—colligative suppression of the temperature at which body fluids freeze and stabilization of the supercooled state—have been developed by cold-hardy organisms.

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Biographical Sketches

Ken Storey has been a Professor of Biochemistry at Carleton University in Ottawa since 1979. He received his BSc from the University of Calgary and his PhD from the University of British Columbia. His research explores fascinating and unusual metabolic adaptations that allow animals to endure and exploit some of the world's harshest environments. In addition to the biochemistry of freezing survival, he studies the molecular mechanisms that control mammalian hibernation, that allow some animals to live without oxygen, and that support months of dormancy by other species. The author of over 400 research papers and the editor of four books on metabolic adaptations to environmental stress, Dr. Storey is also a popular speaker at universities and conferences worldwide. Research trips have taken him to study air-breathing fish up the Amazon river, deep ocean squid off Hawaii, and diving biochemistry of Weddell seals in Antarctica. He is an elected fellow of the Royal Society of Canada and the recipient of multiple research awards and fellowships including the 2001 Canada Research Chair in Molecular Physiology.

Jan Storey is a Research Associate in Biochemistry at Carleton University in Ottawa. She received a BSc from the University of Manitoba and a MSc from the University of British Columbia. Together with her husband, she has explored unique and fascinating examples of animal adaptation with particular interests in the biochemistry of frog and insect freeze tolerance. A major portion of her time is devoted to scientific writing including numerous popular articles.