Anhydrobiosis in invertebrates

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Abstract

Recent work on anhydrobiosis in invertebrates is reviewed. I introduce definition and classification of cryptobiosis, and review the distinctive features and extremely high stress tolerance of anhydrobiotic invertebrates. Most anhydrobiotic invertebrates have evolved various kinds of behavioral, morphological, physiological and physical adaptations to reduce water loss during induction of anhydrobiosis. Trehalose is known as a common compatible solute in anhydrobiotic organisms from unicellular organisms to invertebrates and higher plants. Trehalose may provide effective protection against desiccation because it has superior biochemical and physicochemical properties for stabilizing membranes and biomolecules including proteins and lipids. Recent work also indicates several possible kinds of molecules involved in induction of anhydrobiosis. The adaptations necessary for successful induction of and recovery from anhydrobiosis vary greatly among taxa of invertebrates. Understanding the diversity of anhydrobiosis in invertebrates would be a key to elucidate evolutionary scenarios in anhydrobiosis.

Key words: Anhydrobiosis; cryptobiosis; invertebrates; dehydration; trehalose

INTRODUCTION

Organisms have evolved various types of survival strategies against adverse conditions. Some species move away from sites with harsh conditions to conditions more favorable for growth and reproduction, as in migratory butterflies and locusts (Dingle, 1996). On the other hand, most organisms must cope in the same place with adverse conditions such as low and high temperatures, limited food availability, high salt concentration, anoxia and dehydration (Danks, 1987).

Dehydration is one of the most serious stresses in both aquatic and terrestrial organisms. Water is the major component of living organisms. The average amount of body water in invertebrates is around 70% (from 17% to 90%) and water makes up 95–99% of the total number of molecules (Edney, 1977; Hadley, 1994). Most organisms have only limited ability to survive water loss (Danks, 2000; Wharton, 2002b). Humans do not survive 14% loss of their body water and most organisms die when they lose 50% of the body water at the individual, organ or cellular level. Many desiccation-

tolerant organisms have evolved mechanisms to inhibit water loss, but most of them die soon after the water content declines below a critical level, which varies greatly among species. On the other hand, some organisms are able to survive for an extended period even after they are almost completely dehydrated. This particular biological state is termed anhydrobiosis, a kind of cryptobiosis.

In this review, I provide an overview of studies on invertebrate anhydrobiosis from ecological, physiological, biochemical and molecular perspectives. This paper also considers recent researches concerning the biophysical property of cell membranes and biomaterials for protection against various stresses.

DEFINITION AND CLASSIFICATION OF CRYPTOBIOSIS

Leeuwenhoek (1702) observed inactive animalcules (tardigrades and rotifers) of an oval shape in dry sediments from the gutters of roofs of houses (Wright, 2001). The animalcules started moving shortly after coming into contact with water. A

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Fig. 1. Classification of hypobiosis based on the metabolic level. The definition in Keilin (1959) was modified based on the current concept of cryptobiosis.

similar ability to tolerate dehydration was reported in Needham (1743), Spallanzani (1776) and Doyère (1842). Such a phenomenon is termed cryptobiosis (latent or hidden life), and is defined as the state of an organism when it shows no visible signs of life and when its metabolic activity is hardly measurable, or comes reversibly to a standstill (Keilin, 1959). The state of cryptobiosis appears to be a distinctive biological phase of life history.

Cryptobiosis is one particular adaptation against environmental stresses, but should not be confused with diapause. The latter at the larval, pupal and adult stages is arrested development resulting from reduced hormonal activities, which may persist even when favorable environmental conditions come (Tauber et al., 1986; Danks, 1987). The most common environmental cues inducing diapause are unstressful factors such as photoperiod, whereas cryptobiosis responds to the stressful factors themselves. Keilin (1959) classified these adaptations based on the level of metabolic depression (Fig. 1). Diapause is a kind of hypometabolic state (dormancy) as well as quiescence [Keilin (1959) categorized hibernation and aestivation in dormancy, however these two terms reflect the season without specifying the type of developmental suppression]. Cryptobiosis is a generic term for ametabolism, and can be further divided into five categories based on the factors that induce it: cryobiosis (induced by freezing), thermobiosis (low and high temperatures), osmobiosis (extremely high osmolarity), anoxybiosis (lack of oxygen) and anhydrobiosis (lack of water).

ANHYDROBIOTIC ORGANISMS AND THEIR DEVELOPMENTAL STAGES

Anhydrobiosis is the most common form of

cryptobiosis, and anhydrobiotic organisms are able to survive almost complete dehydration; 95 to 99% of the water content is lost in many cases (Crowe et al., 1992; Danks, 2000). Such organisms are found in many taxa ranging from unicellular organisms to higher invertebrates and plants; plant seeds, vegetative tissues of higher plants (resurrection plants), yeasts, bacteria, fungal spores, protozoans, eggs of turbellaria, nematodes, rotifers, tardigrades, spring-tails, cysts of primitive crustaceans including the brine shrimp (*Artemia salina*) and larvae of a chironomid midge, *Polypedilum vanderplanki* (Schmidt, 1948; Vegis, 1964; Van Gundy, 1965; Clegg, 1967 1978, 1979, 2001; Crowe and Clegg, 1973; Crowe and Madin, 1974; Priestley, 1986; Wright, 1991; Crowe et al., 1992; Tomos, 1992; Potts, 1994; Sømme, 1995, 1996; Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Ostran, 1998; Ricci, 1998; Chandler and Bartels, 1999; Potts, 1999; Seckbach, 1999; Alpert, 2000; Scott, 2000; Wharton, 2002b; Watanabe et al., 2004). One of the common features of these anhydrobiotic stages is limited size, and most of the invertebrates are less than 1 mm. As an exception, larvae of *Po. vanderplanki* are large, and last-instar larvae which enter anhydrobiosis reach 7–8 mm in body length. No anhydrobiotic species have been found in vertebrates.

Crowe (1971, 1975) divided anhydrobiotic organisms into two major groups, based on the developmental stage in which cryptobiosis occurs. Members of one group enter anhydrobiosis only at an early ontogenetic stage (bacterial and fungal spores, seeds, eggs and larvae) and members of the other do so at any stage in their life cycle (tardigrades and rotifers etc.). This basic division of anhydrobiotic organisms appears to be generally accepted (Womersley, 1981). Anhydrobiotic organisms except for unicellular organisms can also be divided into two categories: in cysts of *Artemia*, for example, female parents produce either dormant cysts or free-swimming nauplii in response to the environmental conditions such as photoperiod and temperatures (Nambu et al., 2004), and to some extent prepare the molecules necessary for anhydrobiosis in the dormant cysts they deposit (Hochachka and Guppy, 1987). The dormant cysts can not enter anhydrobiosis again after embryogenesis proceeds (Hochachka and Guppy, 1987). In contrast, in larvae of a chironomid and in nymphs

and adults of tardigrades, nematodes and rotifers, the parents do not affect induction of anhydrobiosis in their progenies at all, but individual progeny may reversibly switch their physiology and biochemistry between active (developmental) and anhydrobiotic phases.

LONGEVITY AND TOLERANCE TO EXTREME CONDITIONS

Many anhydrobiotic animals can maintain viability for an extended period. Theoretically, metabolism and all chemical reactions should almost completely shut down during anhydrobiosis under conditions without humidity and oxygen. Anhydrobiotic tardigrades and rotifers recovered from a dry moss sample taken from an Italian museum after 120 years of preservation, although the individuals

underwent ‘quivers in several zones of its body’ (Franceschi, 1948; Jönsson and Bertolani, 2001). Viable embryos of copepods were isolated from anoxic marine sediments 40 years old (Marcus et al., 1994) and from anoxic freshwater sediments after 332 years (Hairston et al., 1995). These resting embryos are assumed to be the state of anoxybiosis, a kind of cryptobiosis (Clegg, 1997).

More reliable demonstrations of the longevity of anhydrobiotic invertebrates after laboratory preservation are listed in Table 1. Steiner and Albin (1946) reported 39-year-survival in a nematode, *Tylenchus polyhynchus*. The longest records of recovery from the anhydrobiotic state are 17 years in insects, 16 years in crustaceans and 9 years in tardigrades and rotifers (Baumann, 1922; Clegg, 1967; Adams, 1985; Guidetti and Jönsson, 2002). The revival rate of a nematode, *Anguillulina tritici*,

Table 1. Longevity records in anhydrobiotic invertebrates

Taxa	Species	Anhydrobiotic longevity	References
Insecta	<i>Polypedilum vanderplanki</i>	17 years	Adams, 1985
Crustacea	<i>Branchinecta packardii</i>	16 years	Dexter, 1973
Crustacea	<i>Artemia franciscana</i>	15 years	Clegg, 1967
Crustacea	<i>Streptocephalus sealii</i>	15 years	Moore, 1979
Crustacea	<i>Branchinecta mackini</i>	14 years	Dexter, 1973
Crustacea	<i>Thamnocephalus platyurus</i>	13 years	Dexter, 1973
Crustacea	<i>Streptocephalus proboscideus</i>	6 years	Brendonck and Persoone, 1993
Crustacea	<i>Macrobiotus</i> sp.	7 years	Baumann, 1922
Crustacea	<i>Branchinecta lindahli</i>	4 years	Donald, 1983
Collembola	<i>Folsomides variabilis</i>	several months	Poinsot, 1968
Tardigrade	<i>Ramazzottius oberhaeuseri</i>	9 years (eggs)	Guidetti and Jönsson, 2002
Tardigrade	<i>Ramazzottius oberhaeuseri</i>	4.4 years	Bertolani et al., 2004
Tardigrade	<i>Echiniscus</i> spp.	3 years	Bertolani et al., 2004
Tardigrade	<i>Adorybiotus coronifer</i>	8 months	Westh and Ramløv, 1991
Rotifer	<i>Mniobia</i> sp.	9 years (eggs)	Guidetti and Jönsson, 2002
Rotifer	<i>Mniobia magna</i>	2.5 years	Rahm, 1923
Rotifer	<i>Mniobia russeola</i>	2.5 years	Rahm, 1923
Rotifer	<i>Macrotrachela musculosa</i>	2–3 months	Rahm, 1923
Nematode	<i>Tylenchus polyhynchus</i>	39 years	Steiner and Albin, 1946
Nematode	<i>Anguina tritici</i>	32 years	Fielding, 1951; Norton, 1978; Womersley, 1980
Nematode	<i>Globodera rostochiensis</i>	25 years	Norton, 1978
Nematode	<i>Ditylenchus dipsaci</i>	23 years	Perry, 1977; Norton, 1978; Wharton, 1996
Nematode	<i>Panagrolaimus</i> sp.	8 years	Aroian et al., 1993
Nematode	<i>Heterodera glycines</i>	6 years	Norton, 1978
Nematode	<i>Heterodera avenae</i>	5.5 years	Norton, 1978
Nematode	<i>Anguina agrostis</i>	4 years	Norton, 1978; Preston and Bird, 1987
Nematode	<i>Ditylenchus trifurmis</i>	2.5 years	Norton, 1978
Nematode	<i>Aphelenchus avenae</i>	2.2 years	Crowe and Madin, 1974; Higa and Womersley, 1993
Nematode	<i>Acrobeloides nanus</i>	1 year	Nicholas and Stewart, 1989
Nematode	<i>Panagrellus silusiae</i>	1 year	Lees, 1953; Womersley et al., 1998
Nematode	<i>Pratylenchus penetrans</i>	11 months	Norton, 1978

gradually decreases with increase in the anhydrobiotic period: the maximum period for which the species can survive may be 10 years (Keilin, 1959). Many anhydrobiotic organisms can survive for more than a year, whereas many species of nematodes can stand only 1 or several days at 0% R.H. Both groups could be also regarded as anhydrobiosis from Keilin's definition (metabolism is almost completely ceased even transiently). The variation in survival period between anhydrobiotic animals seems to depend more on the rate of water loss they can tolerate than on the absolute degree of desiccation stress they can survive (Wharton, 2002a).

Anhydrobiotic organisms can express extremely high tolerances against various kinds of stresses. Gavaret (1859) showed that rotifers and tardigrades could survive being kept in a vacuum. *Artemia* cysts are usually preserved by vacuum and nitrogen gas pack to maintain high recovery rates. Anhydrobiotic tardigrades can tolerate extreme temperatures ranging from -270 to $+151^{\circ}\text{C}$, immersion in saturated brine and organic solvents and extremely high hydrostatic pressure (600 MPa) (Doyère, 1842; Rahm, 1923, 1937; Becquerel, 1950; Keilin, 1959; Seki and Toyoshima, 1998). The anhydrobiotic chironomid, *Po. vanderplanki*, also revives after exposure to -270 to $+103^{\circ}\text{C}$ and submersion in pure ethanol and glycerol (Hinton, 1951, 1960a, b, 1968).

A tardigrade, *Macrobiotus areolatus*, tolerates exposure to 570,000 R (≈ 5.5 kGy) of X-ray (May et al., 1964) and 50% of *Artemia* cysts can hatch after irradiation at 500 krad (≈ 5 kGy) of ^{60}Co γ -ray (Iwasaki, 1964). Anhydrobiotic larvae of *Po. vanderplanki* can recover even after 9 kGy of ^{60}Co γ -ray (Watanabe et al., 2006). The LD_{50} values for such anhydrobiotic invertebrates are extremely high in comparison with those for vertebrates such as humans and mice (less than 7 Gy) (Hirano, 1964; National Astronomical Observatory, 2004). Furthermore, cell lines from lepidopteran insects that do not have desiccation tolerance express much higher resistance to radioactivity than those from mammals: Sf9 and TN368 lepidopteran cell lines can proliferate after 200 Gy of gamma irradiation and can survive 18 to 20 days after 800 Gy of X-ray irradiation, respectively (Koval, 1984; Chandna et al., 2004). The physiological reasons for large difference of the resistance between inver-

tebrates and vertebrates remains unclear, although several hypotheses have been proposed, for example, small sizes of body, cells, chromosomes, and genome, composition and structure of membranes, a protective role of cuticle and large amounts of radio-protective chemicals such as cysteine and glutathione in the hemolymph (Hirano, 1964; Koyama, 2001).

ADAPTATIONS CONTROLLING RATE OF WATER LOSS

Many anhydrobiotic animals must slow down and control the rates of evaporative water loss. Anhydrobiotic nematodes can be divided into two groups: fast-dehydration and slow-dehydration strategists (Womersley, 1987; Wharton, 2002a). The fast-dehydration strategists can survive relatively high rates of dehydration or possess adaptations that slow down the rate of water loss. Only a few species of nematodes including *Ditylenchus phyllobius*, *Di. dipsaci*, *Anguina tritici* and *Plectus* sp. are classified in the former group. The majority of anhydrobiotic animals including both fast and slow-dehydration strategists have evolved behavioral, morphological, physiological and physical adaptations to reduce water loss.

Anhydrobiotic tardigrades always contract into a structure resembling a small "tun" when dehydrated (Sømme, 1995; Wright, 2001). The most conspicuous morphological changes are longitudinal contraction of the trunk and invagination of the legs and intersegmental cuticle. Similar behaviors are also observed in dehydrating bdelloid rotifers (Womersley, 1988). The rates of water loss and transpiration gradually decrease as the surface area is reduced during tun formation (Wright et al., 1992). Both rates rapidly decline just after completion of tun formation, finally to an undetectable level. When tardigrades are desiccated at a low relative humidity or under anoxia they cannot form tun and be revived (Crowe, 1972). Thus, tun formation is important for the successful induction of anhydrobiosis in tardigrades.

Nematodes have several behavioral adaptations for induction of anhydrobiosis (Wharton, 2002a). The most common behavioral strategies for decreasing the rate of water loss are coiling and clumping (aggregation). Coiling in *Aphelenchus avenae* decreases the rate of water loss by decreas-

ing the external surface of the body, enabling these nematodes to enter anhydrobiosis (Crowe and Madin, 1974). A relationship between coiling and dehydrated quiescent survival has been shown in a desiccation-tolerant nematode, *R. reniformis*, that is not a true anhydrobiosis (Womersley and Ching, 1989). Forms of aggregation by nematodes also decrease water loss. Juvenile stage 2 (J2s) of *An. tritici* and *An. agrostis* form a tightly packed large aggregation, without coiling, inside a gall because of space restrictions (Womersley et al., 1998). All anhydrobiotic juveniles and adults undergo coiling in a large aggregation in the gall-forming nematode, *An. amsinckiae* (Womersley et al., 1998). The rate of successful induction for anhydrobiosis is high in nematodes in the center of the aggregation in *Di. dipsaci* (Ellenby, 1969): the dead bodies of juvenile stage 4 (J4s) on the outside provide a barrier against the environment.

Larvae of the chironomid, *P. vanderplanki*, usually live in tubular soil nests in rock pools (Hinton, 1951). When the pools dry up, the larvae gradually dry out and their bodies fold in the middle inside the soil tubes which they construct from soil and detritus with their saliva. The tubes contribute to decrease the rate of loss of water from the larvae (Kikawada et al., 2005). Body folding adaptations inside the larval tubes in chironomids may also give mechanical protection for freezing (Danks, 1971) and probably for avoiding the mechanical damages in long-dormant dehydrated larvae of *P. vanderplanki* (Hinton, 1951, 1968). However, the folded larval form is not essential at least for induction of anhydrobiosis in this insect, because larvae dehydrated on wet filter paper in a glass Petri dish are often unfolded, and can recover after rehydration (Watanabe et al., 2002).

One physiological strategy for decreasing the rate of water loss is to change the permeability of the body surface (Wharton, 2002a). *Di. dipsaci* decreases water loss by rapidly lowering body surface permeability 2 min after the onset of desiccation (Wharton, 1996). This occurs via a phase change in the composition of the epicuticle or a decrease in the thickness of the cuticle (Wharton and Lemmon, 1998). A similar adaptation for decreasing water loss is also suggested for J2s of *An. tritici* (Ellenby, 1969). Unhatched J2s of *G. rostochiensis* lose water more slowly than other stages of eggs because of lower permeability of the eggshells (El-

lenby, 1969).

Physical adaptations to control evaporative water loss are the most powerful and critical means for successful induction of anhydrobiosis. Free-living nematodes and free-living stages of plant-parasitic nematodes show little adaptation against water loss, because slow dehydration is assured by the physical nature of the soil in most cases (Womersley et al., 1998). The lower soil layer represents a stable environment, whereas the rate of water loss depends upon the characteristics in the upper soil layer. A tubular nest made by soil and detritus decreases the rate of larval water loss during desiccation in *Po. vanderplanki*, which assures successful induction of anhydrobiosis (Kikawada et al., 2005). Many plant-parasitic nematodes enter anhydrobiosis within the host-plant tissues such as shoots and galls, which provide a physical barrier for delaying water loss. Retention of moulted juvenile cuticles slows the rate of water loss in a plant-parasitic nematode, *Rotylenchulus reniformis*, and in animal-parasitic nematodes at infective stages (Evans and Perry, 1976; Gaur and Perry, 1991). Eggshell, cysts and a gelatinous matrix allow slow dehydration in eggs and unhatched J2s of cyst nematodes (Ellenby, 1969). The gelatinous matrix or the egg sac probably decreases water loss from eggs of many root-knot nematodes. An extracuticular subcrystalline layer may play a role in decreasing water loss in *M. charis* (Demeure and Freckman, 1981).

ACCUMULATION OF TREHALOSE AND ITS PROTECTIVE ROLES

Trehalose is known as a common compatible solute accumulating in anhydrobiotic organisms, such as unicellular organisms (bacteria, yeast and spores of fungi), invertebrates (chironomids, tardigrades, nematodes and encysted *Artemia*) and resurrection plants (Yancey et al., 1982; Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999; Clegg, 2001; Watanabe et al., 2002; Tunnacliffe and Lapinski, 2003), whereas other disaccharides, mainly sucrose, are present in seeds and pollen grains of higher plants (Crowe and Clegg, 1973; Elbein, 1974; Ingram and Bartels, 1996; Majara et al., 1996; Behm, 1997; Crowe et al., 1997; Goddijin and van Dun, 1999; Alpert, 2000). Most of these organisms accumulate quite a large amount of trehalose during dehydration,

about 10–20% of the dry body weight. For example, nematodes of *Ap. avenae* and *An. tritici* accumulate 11–13% trehalose/5% glycerol and 9% trehalose/0.8% inositol, respectively (Madin and Crowe, 1975; Womersley and Smith, 1981). Encysted embryos of *Ar. salina* also accumulate 15–18% trehalose and 2% glycerol (Clegg, 1962, 1965, 1997). A chironomid, *Po. vanderplanki*, increases trehalose content from 0.5% to 20% of dry body weight during desiccation over 48 h (Watanabe et al., 2003). A tardigrade, *Adorybiotus coronifer*, accumulates trehalose from 0.1% up to 2.3% within 7 h of desiccation treatment (Westh and Ramløv, 1991). On the other hand, both growing and desiccated plants of *Selaginella lepidophylla* (resurrection plants) contain a large amount of trehalose with a small amount of sucrose (Adams et al., 1990). There was a close positive correlation between the trehalose content and viability after rehydration in yeast (Sakurai, 2001).

The characteristics and properties of trehalose and its possible protective roles in anhydrobiosis listed in Table 2 may explain why trehalose is used as the compatible solute in anhydrobiotic organisms ranging from unicellular organisms to higher invertebrates and plants (Ring and Danks, 1998). Trehalose has high solubility, low reactivity, and low tendency to crystallize. It is a non-reducing sugar, and so is less harmful to cells and tissues than reducing sugars such as glucose even at extremely high concentrations. Among sugars and polyols, trehalose provides the most effective protection against desiccation because of its high ability for water-replacement and glass formation (vitrification) (Burke, 1986; Crowe et al., 1987, 1998; Green and Angell, 1989; Franks et al., 1991; Levine and Slade, 1992; Sano et al., 1998): it substitutes for bound and free water, and so maintains the structures of cell membranes and proteins. Structuring of intracellular water induced by trehalose and/or heat shock proteins is essential for high resistance to water stress in yeast cells (Sakurai et al., 1999). The glassy state of trehalose may fill the spaces in tissues during dehydration and allow the orderly packing of body components, which prevents structural damage efficiently, and inhibits aggregation of biological molecules and increase in solute concentration. High and stable viscosity of trehalose glasses also stops all chemical reactions that require molecular diffusion. Tre-

Table 2. Superior characteristics and properties of trehalose

Non-reducing activity
Low tendency to crystallize
Stable glass formation
High vitrification temperature
High ability of water replacement
Structuring activity of intracellular water with HSP
Stabilization of dry membranes
Antioxidant activity of protein and fatty acids
Free-radical scavenger

Some possible properties are added to the table in Ring and Danks (1998).

halose has a higher glass transition temperature (T_g) than other monosaccharides and disaccharides. High T_g would be important for stabilizing the glass and hence maintaining a stable anhydrobiotic state. For example, anhydrobiotic larvae of *Po. vanderplanki* spend the dry season in dry mud and detritus in transitional rock pools in Africa and might be exposed to 60 to 70°C during the day (Hinton, 1951).

Trehalose stabilizes biological membranes and liposomes. In membranes dried with trehalose at concentrations near those found in anhydrobiotic organisms, morphological damage including vesicle fusion is completely inhibited during drying (Mouradian et al., 1984). Trehalose is the most efficient molecule for dry preservation of membranes, because trehalose acts as a protectant for dry preservation at lower concentrations than do other disaccharides (Rudolph and Cliff, 1990; Rudolph et al., 1990; Crowe et al., 1992). The stabilizing effect of trehalose on phospholipid bilayers might be the result of direct interaction between –OH groups on the trehalose and the phosphate of membrane phospholipids (Crowe et al., 1987, 1988, 1989; Quinn, 1989; Tsvetkov et al., 1989). Crowe et al. (1992) emphasized the importance of temperature-dependent inhibition of phase transition of dry phospholipid bilayers by trehalose. Phospholipid bilayers are in liquid crystalline phase at room temperature when the lipids are fully hydrated. Lipids dehydrated with trehalose keep the liquid crystalline phase, but those dehydrated without trehalose change into the gel phase. The dry bilayers in the gel phase would leak during rehydration. Thus, trehalose would prevent this leakage by depressing melting point (T_m) of the dry lipids (Crowe et al., 1986; Crowe and Crowe,

1988). These properties could keep cell membranes stable during dehydration-rehydration events.

Trehalose also stabilizes labile proteins during dehydration (Carpenter et al., 1987; Carpenter and Crowe, 1989). It is effective at stabilizing the protein such as phosphofructokinase (PFK) during extreme dehydration (Crowe et al., 1992), whereas other non-reducing compounds such as glycerol, and reducing sugars such as glucose, are not in themselves sufficient for stabilizing the protein (Crowe et al., 1990). Trehalose appears to interact directly with the dry protein, probably by hydrogen bonding of –OH groups to polar residues in the protein (Carpenter and Crowe, 1989). Trehalose also could efficiently inhibit harmful oxidation of proteins and unsaturated fatty acids in the dry state (Benaroudj et al., 2001; Oku et al., 2003, 2005).

Thus, trehalose may have various protective roles for stabilizing proteins and membranes, and the ability may be important for stable long-term maintenance of anhydrobiosis. However, much of the protective roles have been still theoretical, and the various theories should be clearly demonstrated *in vivo* in anhydrobiotic organisms.

On the other hand, Higa and Womersley (1993) reported that the accumulation of trehalose in itself is not sufficient for the induction of anhydrobiosis in *Ap. avenae*. Individuals with a wide variation of trehalose content ranging from 3% to 16% per dry weight cannot survive direct exposure to low humidity in *Di. myceliophagus* (Womersley and Higa, 1998). In *Po. vanderplanki*, all or most larvae with a relatively large amount of trehalose produced after quick desiccation for 0.5 to 1.5 day can not recover after rehydration (Watanabe et al., 2003; Watanabe, unpublished data). These results suggest that trehalose accumulation may be important for preliminary preparation for anhydrobiotic survival, but is not the only factor associated with induction of cryptobiosis. In fact, anhydrobiotic rotifers, *Philodina roseola* and *Adineta vaga*, do not accumulate trehalose nor any other low-molecular weight carbohydrates during dehydration (Lapinski and Tunnacliffe, 2003).

OTHER MOLECULES POTENTIALLY INVOLVED IN INDUCTION OF ANHYDROBIOSIS

Several families of heat shock proteins (HSP) serve as molecular chaperones. They participate in unfolding and relocalization of proteins damaged by stresses, assist the folding of newly synthesized proteins, protect them from denaturation and aggregation and aid in their renaturation, and influence the final intracellular location of mature proteins (Jakob et al., 1993; Parsell and Lindquist, 1993; Ellis and Hartl, 1999; Feder and Hoffmann, 1999; Ellis, 2000; MacRae, 2000). Because HSPs are involved in tolerance against various stresses such as low and high temperatures, oxidation, anoxia and heavy metals, they have been thought to have some protective roles in anhydrobiotic organisms. In fact, kinds of small HSP, p26 and artemin, may be involved in stress resistance in *Artemia* cysts (Willsie and Clegg, 2001, 2002; Chen et al., 2003; Collins and Clegg, 2004; Qiu et al., 2004; Warner et al., 2004). p26 appears to act synergistically with trehalose *in vitro* (Viner and Clegg, 2001).

Accumulation of late-embryogenesis-abundant (LEA) proteins was often reported in plant seeds and tissues of resurrection plants, associated with acquisition of desiccation tolerance during maturation (Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999; Cuming, 1999; Scott, 2000; Clegg, 2001). Expression of LEA is induced by drought, low temperature and high salt (Ingram and Bartels, 1996; Cuming, 1999), and confers increased osmotic or freezing tolerance in yeast, *Saccharomyces cerevisiae* (Imai et al., 1996; Honjoh et al., 1999; Zhang et al., 2000). A barley LEA improves drought tolerance in transgenic plants including rice and wheat (Xu et al., 1996; Sivamani et al., 2000). LEA-like protein (HSP12) is also suggested to be involved in protection of membrane proteins (Sales et al., 2000). Group 3 LEA proteins homologues were found in the nematodes, *Caenorhabditis elegans* and *Steinernema feltiae*, the anhydrobiotic nematode, *A. avenae*, and the prokaryotes *Deinococcus radiodurans*, *Bacillus subtilis* and *Haemophilus influenzae* (Solomon et al., 2000; Dure, 2001; Browne et al., 2002).

All groups of LEA proteins (1, 2, 3 and 6) have

been thought to act as molecular chaperones (Imai et al., 1996; Wise and Tunnacliffe, 2004). Wise and Tunnacliffe (2004) proposed a possible function of LEA proteins (group 3) during induction of anhydrobiosis; natively unfolded LEA proteins exhibit a strong α -helical component during desiccation. The superhelical structures form coils and filaments associated with the cytoskeleton, and finally generate intracellular filament networks within the dehydrating cells. This configuration might increase mechanical strength. LEA proteins also act synergistically with trehalose as an aggregation protectant for several kinds of enzymes (Goyal et al., 2005).

HSPs and LEA proteins have been found in broad taxa of anhydrobiotic organisms from plants to invertebrates, and seem to have important roles on induction and maintenance of anhydrobiosis. However, the protective roles have not been demonstrated in anhydrobiotic organisms including invertebrates. Further analysis of the behavior of proteins during water stress is required to elucidate the function of LEA proteins *in vivo* during anhydrobiosis.

MECHANISM OF INDUCTION OF ANHYDROBIOSIS

Many temperate insects enter diapause in various developmental stages in order to survive adverse conditions. The brain is the common prime regulator of larval, pupal and adult diapause (Denlinger, 1985). On the other hand, anhydrobiosis in *Po. vanderplanki* larvae occurs without the brain: larvae without a head accumulate relatively large amounts of trehalose during desiccation and recover after rehydration like intact larvae (Fig. 2) (Watanabe et al., 2002). Furthermore, body parts mainly consisting of fat body tissues can synthesize a large amount of trehalose during desiccation, and the fat body can recover after long-term dry-preservation (Fig. 3) (Watanabe et al., 2005). Therefore, the central nervous system is not involved in the induction of anhydrobiosis, and individual cells and tissues themselves could enter the anhydrobiotic state in this chironomid.

Trehalose content in anhydrobiotic organisms ranges from 2% in tardigrades to 40% in yeast. Trehalose cannot easily enter cells and tissues because of the relatively large molecular size of this

disaccharide. Importance of intracellular trehalose during dehydration has been suggested, but internal location of trehalose in anhydrobiotic invertebrates remains unclear. Recently it has been shown that the presence of intracellular trehalose at high level contributes to increase of desiccation tolerance in vertebrate cells: human primary fibroblasts intracellularly producing trehalose, due to a recombinant adenovirus vector, could be maintained in a completely dry state, although the period for dry preservation did not exceed 3 days (Guo et al., 2000). These cells accumulated only 1 mM trehalose intracellularly. Freeze-dried human and mammalian platelets (apryene) could recover after rehydration only when they were loaded intracellularly with a relatively high concentration of trehalose (20 mM) before freeze-drying (Wolkers et al., 2001, 2002). On the other hand, mouse cells containing 10% trehalose, through expressing trehalose phosphate synthase (TPS) intracellularly, cannot survive complete desiccation, although they have increased tolerance of high osmolarity (Garcia de Castro and Tunnacliffe, 2000). It is not still determined whether the importance of intracellular trehalose reported in vertebrate cells is applicable in cells, tissues and individuals of invertebrates at the anhydrobiotic state.

Different kinds of cells and tissues in larvae of *Po. vanderplanki* do not all behave the same during induction of anhydrobiosis. The larvae have a large amount of glycogen in their fat body in the hydrated state and a large amount of trehalose in their hemolymph just prior to complete dehydration (Watanabe et al., unpublished data). Because a large amount of intracellular trehalose may be important for induction of anhydrobiosis at the cellular level, all the tissues except for the fat body, including brain, neurons, muscles, alimentary canal and hormone-producing organs such as prothoracic glands and corpora allata, would need a mechanism for rapid intake of trehalose from the hemolymph. The mechanisms for rapid intake of trehalose remain unknown, although sugar transporters can move sugars from the outside to the inside of cells and vice versa (Ehrmann et al., 1998; Jespersen et al., 1999; Truernit, 2001; Guo et al., 2005). Study of the location of intra- and extracellular trehalose in anhydrobiotic larvae would provide important information for induction of anhydrobiosis in higher invertebrates.

Fig. 2. Recovery and trehalose content of decapitated anhydrobiotic larva of *Po. vanderplanki*. (A) Decapitated anhydrobiotic larva. (B) Decapitated larva after rehydration. (C) Change of trehalose level during desiccation. Intact, dry: dehydrated intact larvae, -Head, dry: dehydrated decapitated larvae, -Head, wet: decapitated larvae in water. Photographs and data from Watanabe et al. (2002).

Carbon sources for the solutes that protect against desiccation are consistent in unicellular organisms and plants, i.e., mainly glucose in the former and sucrose in the latter (Ingram and Bartels, 1996). By contrast, in insects, glycogen is the main source, and it is stored in the fat body (Storey and Storey, 1991). The distribution of newly synthesized trehalose to all cells and tissues, probably

Fig. 3. Fluorescence micrographs and trehalose content of fat body and alimentary canal tissue from *Po. vanderplanki* larva. The tissue samples were doubly stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and propidium iodide (PI). Tissue from an intact live larva (FB-live, AC-live); tissue from a dead larva (FB-dead, AC-dead); fat body and alimentary canal desiccated over 2 days (FB, AC) and quickly desiccated within 1 day (FB-Q, AC-Q). Reproduced from Watanabe et al. (2005) with permission of Elsevier.

from the fat body via the hemolymph, is likely to be important for dehydrating larvae.

What is the initial signal involved in induction of trehalose synthesis? Organisms usually lose water

Fig. 4. Trehalose content of *Po. vanderplanki* larvae incubated for 1 day in various solutions. (A) NaCl, (B) mannitol, (C) glycerol and (D) DMSO solutions at various osmotic pressures. (E) various salt solutions of the same osmotic pressure (342 mOs). Data from Watanabe et al. (2003).

under dry conditions. Such water loss causes an increase of internal osmolarity in multicellular organisms, exposing cells and tissues to high extracellular osmolarity. Decrease of water content therefore has been thought to be the initial signal that switches on the signal transduction pathways leading to the synthesis of trehalose and other compatible substances. Decrease of water content as the first signal is common among anhydrobiotic organisms from plants to invertebrates, but the following responsive molecules may be different. In resurrection plants, cessation of water supply to the root system induces increase of abscisic acid (ABA) (Scott, 2000). Abscissic acid seems to have a major role in initiating signal transduction for the stress response in plants (Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999). Signal transduction pathways for synthesis of compatible solutes in response to desiccation and high osmotic stresses have been demonstrated in the yeast, *S. cerevisiae*; exposure to high extracellular osmolarity induced a two-component osmosensor (Sln1 and Sho1) to activate the high osmolarity glycerol (HOG) and mitogen-activated protein (MAP) kinase cascades, and finally caused accumulation of glycerol (Maeda et al., 1994, 1995; Posas et al., 1996; Posas and Saito, 1997; Raitt et al., 2000). This activation response was induced by high osmolarity regardless of the kinds of solute (Maeda, 1999). The homologous

osmosensor and the following signal transduction pathways were also found in a higher plant, *Arabidopsis thaliana* (Shinozaki and Yamaguchi-Shinozaki, 1997; Miyata et al., 1998; Mizoguchi et al., 1998; Urano et al., 1998, 1999). Because MAP kinase cascades are genetically conserved in unicellular organisms, plants, invertebrates and vertebrates, the similar signal transduction in response to desiccation may be also common in invertebrates entering anhydrobiosis. However, the mechanisms for sensing stress in an anhydrobiotic chironomid, *P. vanderplanki*, may be different from the osmosensor found in yeast and plants (*Ar. thaliana*): the explosive production of trehalose occurred mainly in high concentration NaCl solutions, but not in the solutions of dimethyl sulfoxide (DMSO), glycerol and mannitol (Fig. 4) (Watanabe et al., 2003). Various salt solutions can trigger trehalose synthesis, and the amount of trehalose production depends on the kinds of cations in solution. These results suggest that increase of internal ion concentrations, probably associated with water loss, activates the following stress response cascades and finally causes accumulation of trehalose in this chironomid. The physiological and molecular basis for sensing internal ion stress remains unknown.

Fig. 5. Biological events occurring during induction of and recovery from anhydrobiosis.

CONCLUSION

Anhydrobiosis is a particularly stable biological state giving extremely high stress tolerance. Figure 5 summarizes biological events occurring between the normal and the anhydrobiotic states in invertebrates. Organisms require complex behavioral, morphological, physiological and biochemical changes during a relatively short period for successful induction of and recovery from anhydrobiosis, although the adaptations necessary for anhydrobiosis varies among taxa. Unlike unicellular organisms and plants, invertebrates have highly differentiated cells and tissues. In higher invertebrates like *Po. vanderplanki*, all tissues including the central nervous system can be preserved inside the body in an anhydrobiotic state for at least 17 years.

The group of invertebrates has the largest number of anhydrobiotic species. Each taxa of invertebrates has developed a wide variety of property in anhydrobiosis: *Artemia* cysts can enter anhydrobiosis only at the embryonic stage, and *Po. vanderplanki* can do only at the larval stages, whereas tardigrades and rotifers can do at all developmental stages including embryos, juveniles and adults. Behavioral, morphological, physiological and biochemical adaptations are different among anhydrobiotic invertebrates, i.e. a large amount of trehalose at anhydrobiotic state is not found in tardigrades

(not large, only 2% of the dry body weight) and rotifers (undetected level). The period necessary for induction of anhydrobiosis also differs among taxa: tardigrades with low level of trehalose and rotifers without trehalose can enter anhydrobiosis within 1 hour, whereas nematodes and chironomid with a large amount of trehalose take at least 2 days to succeed in entering anhydrobiosis. Understanding the diversity of anhydrobiosis in invertebrates would be a key to elucidate evolutionary scenarios in anhydrobiosis.

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