Vitrification is essential for anhydrobiosis in an African chironomid, *Polypedilum vanderplanki*

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Anhydrobiosis is an extremely dehydrated state in which organisms show no detectable metabolism but retain the ability to revive after rehydration. Thus far, two hypotheses have been proposed to explain how cells are protected during dehydration: (*i*) water replacement by compatible solutes and (*ii*) vitrification. The present study provides direct physiological and physicochemical evidence for these hypotheses in an African chironomid, *Polypedilum vanderplanki*, which is the largest multicellular animal capable of anhydrobiosis. Differential scanning calorimetry measurements and Fourier-transform infrared (FTIR) analyses indicated that the anhydrobiotic larvae were in a glassy state up to as high as 65°C. Changing from the glassy to the rubbery state by either heating or allowing slight moisture uptake greatly decreased the survival rate of dehydrated larvae. In addition, FTIR spectra showed that sugars formed hydrogen bonds with phospholipids and that membranes remained in the liquid-crystalline state in the anhydrobiotic larvae. These results indicate that larvae of *P. vanderplanki* survive extreme dehydration by replacing the normal intracellular medium with a biological glass. When entering anhydrobiosis, *P. vanderplanki* accumulated nonreducing disaccharide trehalose that was uniformly distributed throughout the dehydrated body by FTIR microscopic mapping image. Therefore, we assume that trehalose plays important roles in water replacement and intracellular glass formation, although other compounds are surely involved in these phenomena.

trehalose | water replacement | Fourier-transform infrared microspectroscopy | biological glass | cryptobiosis

Some organisms can survive adverse environments such as drought and low temperature through various physiological and biochemical adaptations. An ultimate strategy for the survival of drought is anhydrobiosis, in which an organism loses virtually all of its free intracellular water and ceases metabolism but remains capable of revival after rehydration (1). Anhydrobiosis has been found in various unicellular organisms, invertebrates, and plants (2–9). Based on studies on plant seeds and *in vitro* experiments, two mutually compatible hypotheses have been proposed. The vitrification hypothesis proposes that mixtures of accumulated nonreducing sugars and highly hydrophilic proteins enter a glassy state during dehydration and thereby immobilize membranes and macromolecules in the cytoplasm, protecting them from denaturation, coagulation, and disintegration (10–13). The water-replacement hypothesis holds that the hydrophilic molecules afford a similar protection by directly interacting with macromolecules, mainly through hydrogen bonds, and thus take the place of water (14–17). However, no physiological or physicochemical evidence for either hypothesis has been reported in whole anhydrobiotic animals.

The larva of the sleeping chironomid, *Polypedilum vanderplanki*, is the largest multicellular animal capable of anhydrobiosis (18, 19). The larvae dwell in temporary rock pools in semiarid regions in Africa (18). When these small, shallow pools dry up, the larvae desiccate. When rain refills the pools, the larvae rehydrate and revive. According to one report, larvae of *P. vanderplanki* can recover from desiccation of up to 17 years (20). Recently, we have succeeded in inducing *P. vanderplanki* larvae to enter anhydrobiosis under laboratory conditions and found that larvae synthesize and accumulate high levels of trehalose, ≈20% of the dry body mass, as they dehydrate (21, 22). Concurrently, late embryonic abundant (LEA) proteins increase in quantity (23). Trehalose and a highly hydrophilic LEA protein are found in many anhydrobiotic microbes and animals (1–8, 24–27) and are assumed to be involved in desiccation tolerance. Combination of these factors may contribute to the building of stable intracellular glasses (28–31).

In the present study, we assessed both vitrification and water-replacement hypotheses in *P. vanderplanki*. Results from Fourier-transform infrared (FTIR) analysis and differential scanning calorimetry (DSC) demonstrated glasses in the anhydrobiotic larvae and strongly suggested that cell membranes were protected by replacement of water by sugars. When desiccated larvae were made to change from the glassy to the rubbery state through either heat or humid treatments, their ability to recover upon rehydration was greatly decreased, indicating that vitrification is required for successful anhydrobiosis of the sleeping chironomid.

Results

Glass Transition in the Anhydrobiotic Larva. To see whether biological glasses are formed in dehydrated *P. vanderplanki*, we obtained two kinds of samples with very different desiccation tolerance. Larvae with an ability to recover from almost complete desiccation were obtained by slow dehydration over a period of 72 h (21, 22), while those without such ability were obtained by fast dehydration within several hours. We will refer to these two types of larvae as "slow or slowly" and "quick or quickly" dehydrated for the sake of brevity. The most conspicuous difference between the two samples was trehalose content, which was 14-fold larger in the slow sample than the quick sample (Fig. 1). No difference was found in the contents of total protein, triacylglycerol, or water in the two types of larvae (Fig. 1), although their protein profiles and probably also their lipid profiles differed (M. Fujita, T.K., and T.O., unpublished data).

Heat absorption of the slowly and quickly dehydrated larvae was analyzed by DSC (Fig. 2*A*). In quickly dehydrated larvae, no baseline shift or peak in absorption was observed as larvae were heated. In contrast, slowly dehydrated larvae exhibited a clear baseline shift in a stepwise manner, indicating a glass transition. The onset, middle, and end glass transition temperatures (*Tg*) were

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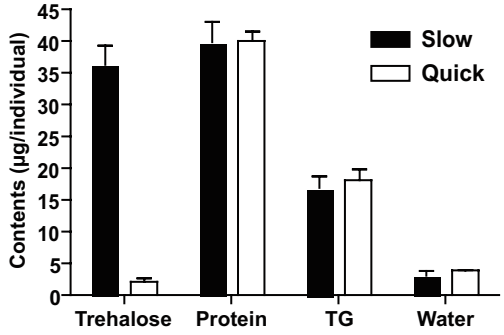

Fig. 1. Contents of trehalose, protein, triacylglyceride, and water in quickly and slowly dehydrated larvae of *P. vanderplanki*.

62°C, 65°C, and 71°C, respectively, meaning that larval cytoplasm was in a glassy state at <62°C and in a rubbery state at >71°C.

The glass transition phenomena were also assessed by FTIR analysis. FTIR spectra of the slow and quick samples showed obvious differences at 992 cm^{-1} and a region from 3,800 to 3,000 cm^{-1} (Fig. 3*A*). Because the slow sample contained a large amount of trehalose, which is a nonreducing disaccharide consisting of two D-glucose molecules joined by an α,α-1,1 linkage, a stretching vibration band of the linkage was observed at ≈992 cm^{-1} (red arrow). This band becomes clear when trehalose exists in a glassy state (32), and spectra thus indicated that trehalose was vitrified in the slowly dehydrated larvae.

In addition, in the high wavenumber region (3,800–3,000 cm^{-1}), where mainly the O-H and N-H stretching vibration bands appear

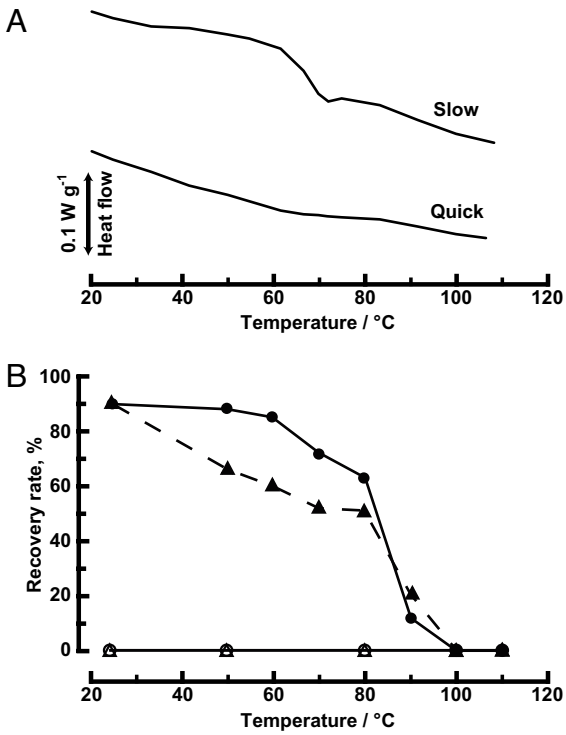

Fig. 2. Glass in anhydrobiotic larvae and their recovery after heat treatments. (*A*) DSC thermograms for slowly and quickly dehydrated larvae. A baseline shift of ≈60–70°C in the slowly dehydrated sample indicates the phase transition. (*B*) Dependence of the recovery rate after rehydration on exposure to high temperatures in slowly (filled symbols) and quickly (open symbols) dehydrated larvae. Circles and triangles show recovery after exposure to high temperature for 5 min and 1 h, respectively.

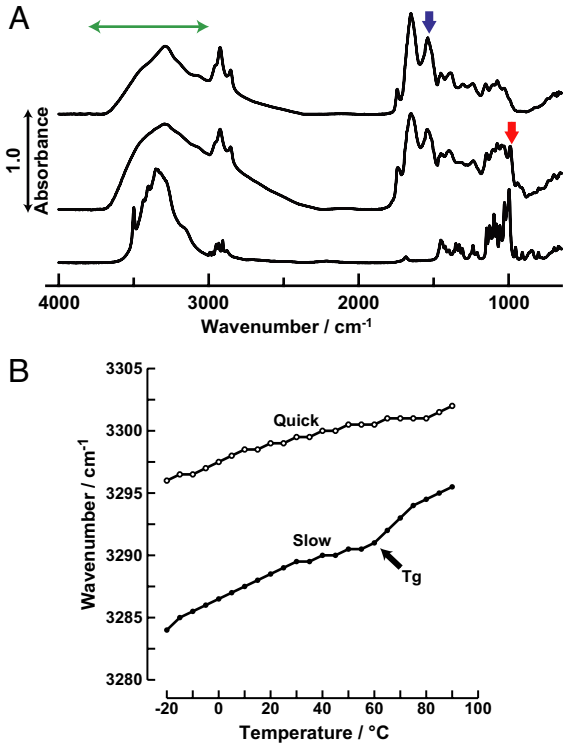

Fig. 3. FTIR analysis of desiccated *P. vanderplanki*. (*A*) FTIR spectra of anhydrous glassy trehalose (bottom), a slowly dehydrated larva (middle), and a quickly dehydrated larva (top). Red and blue arrows indicate the characteristic 992- and 1,540-cm^{-1} peaks of trehalose and the amide II band of total protein, respectively. A green line indicates a region (3,800–3,000 cm^{-1}) of O–H and N–H stretching vibration bands. (*B*) Temperature dependence of the maximal peak position in the region 3,800–3,000 cm^{-1}. An inflection point (*Tg*) was observed in the spectrum of the slowly dehydrated larva.

(33–35), a clear inflection point near 65°C was observed in the spectrum of the slowly dehydrated larvae but not the quickly dehydrated larvae when the maximal peak position in this region was plotted against temperature (Fig. 3*B*). These temperature-dependent behaviors of the IR band were consistent with the results from DSC analysis.

Although the above analysis is accepted as a method for determining the glass transition temperatures of sugars and intact cells (33–35), it does not necessarily indicate the exact compounds responsible for the phase transition. For this, we conducted a principal-component analysis (36). The IR band of the slowly (Fig. 4*A*) and quickly (Fig. 4*B*) dehydrated larvae could be decomposed into two major components (P1 and P2 for the former and P1′ and P2′ for the latter). When the value of each principal component was given as a function of temperature, P1 showed a peak at 65°C, which was close to the glass transition temperature, whereas P2 and P2′ had no clear peak or inflection point (Fig. 4 *C* and *D*). The P1 peak position is close to that of the characteristic shoulder observed in the FTIR spectrum of the slowly dehydrated larvae (Fig. 3*A*, middle) and also assumed to be composed of a rather uniform molecule in large quantity in the slowly dehydrated larvae. Among the likely candidates for such a compound are trehalose and desiccation-inducible proteins such as LEA proteins, which are strongly associated with anhydrobiosis (21–23).

Trehalose Distribution in Desiccated Larvae. The slowly dehydrated larvae have concentrations of trehalose of up to 18% of their dry body weight and are able to revive upon rehydration, suggesting that a high content of trehalose contributes to successful induction of their anhydrobiosis (21). To visualize their internal distribution of

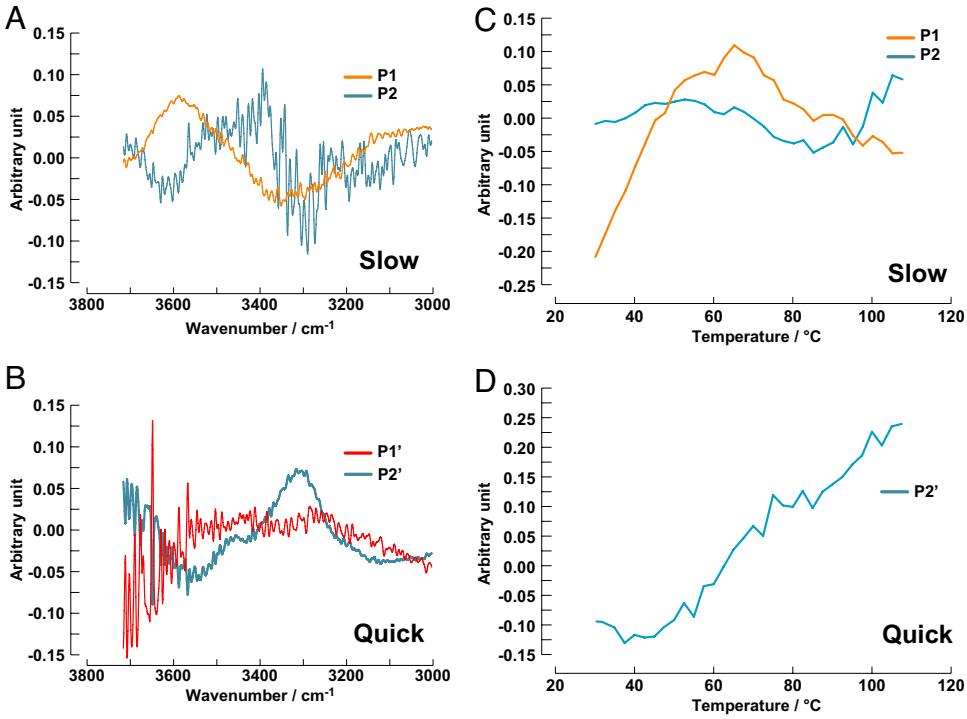

Fig. 4. Principal-component analysis of dehydrated *P. vanderplanki*. (*A* and *B*) FTIR spectra were decomposed into two components: P1 and P2 in slowly dehydrated larvae (*A*) and P1′ and P2′ in quickly dehydrated larvae (*B*). Shown is a region between 3,800 and 3,000 cm^{-1} (Fig. 3*A*). P1′ is likely to be a noise. (*C* and *D*) Temperature-dependent change of the score value for each principal component.

trehalose, we measured FTIR microscopic mapping images using the peak at 992 cm^{-1} of an α,α-1,1 linkage as an indicator for trehalose; no other biological disaccharide has this linkage. The images showed that large amounts of trehalose were distributed widely within a slowly dehydrated larva and that little trehalose was present in a quickly dehydrated one (Fig. 5). The raw images showed especially high amounts of trehalose in the central regions of the slowly dehydrated larva. To test whether this was an artifact of greater thickness in the central regions, we normalized the peak intensity at 992 cm^{-1} with the peak of amide II at 1,540 cm^{-1}, representing total proteins, which are uniformly distributed in the larva. The normalized trehalose distribution clearly showed that trehalose was almost uniformly distributed through the larval body (Fig. 5), at least at this level of resolution.

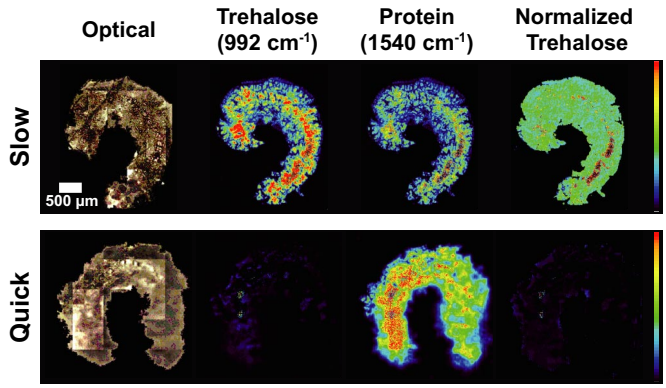

Fig. 5. Optical and FTIR imaging data for a slowly dehydrated larva and a quickly dehydrated larva. Mapped were intensities of the characteristic 992-cm^{-1} peak corresponding to trehalose and 1,540-cm^{-1} peak corresponding to the amide II of proteins. Unequal apparent trehalose distribution due to variation in thickness of the larvae was normalized by dividing the intensity of the peak at 992 cm^{-1} by that of the amide II band. Spatial resolution is 12.5 × 12.5 μm. Warm colors indicate higher intensity—i.e., larger amounts of the molecule. (Scale bar: 500 μm.)

Vitrification Is Essential for Anhydrobiosis. To evaluate the effect of biological glasses in dehydrated *P. vanderplanki*, we then examined whether the slowly dehydrated larvae can tolerate high temperatures at which glasses in the body convert to rubbers. Measured 48 h after rehydration, recovery from exposure to temperatures of up to 60°C while dehydrated was >80% after exposure for 5 min and >60% after exposure for 1 h (Fig. 2*B*). Recovery dropped sharply after exposures to temperatures >80°C. Because the larval cytoplasm was probably in a glassy state at 60°C and below, and in a rubbery one at 80°C and above (Fig. 2*A*), these results were consistent with promotion of recovery from dehydration by retention of the glassy state.

To test this further, we investigated the effect of humidity on anhydrobiosis because water is a good plasticizer of sugar glass (37). When slowly dehydrated larvae were exposed to 38% or 60% relative humidity (RH) for either 5 or 15 days, their water content slightly increased, and trehalose contents remained almost the same as the control at 5% RH (Table 1). The glass transition was still observed after such minor uptake of moisture, although the glass transition temperature ranges were much lower than in larvae kept at 5% RH [Table 1 and supporting information (SI) Fig. S1]. The slowly dehydrated larvae remained in a glassy state because the midpoint transition temperature was higher than the ambient temperature, and recovery was still high (Table 1). In contrast, when larvae were exposed to 93% RH or 98% RH, considerable uptake of moisture was followed by loss of the glassy state (Figs. S1 and S2) and large decreases in recovery rate (Table 1).

Involvement of the Water-Replacement Mechanism. Generally, *in vivo* IR spectra include significant information on ultramicroscopic states of biological tissues, which helps us to get deep insight into the mechanism of successful anhydrobiosis. To seek evidence for the water-replacement hypothesis in dehydrated *P. vanderplanki*, we examined the IR spectral regions between 1,280 and 1,200 cm^{-1}, where the main spectral contribution comes from the asymmetric stretching vibration of the P═O atomic groups and has been often used to investigate the interactions between the headgroup of phospholipids and sugars for both biological and biomimetic mem-

Table 1. Effects of humidity on the water and trehalose contents, recovery rate, and physicochemical properties of slowly dehydrated larvae

Condition	Water % by mass	Trehalose, μg/mg (larval dry weight)	Recovery rate,* %	*Tg* (onset), °C	*Tg* (midpoint), °C	*Tg* (end), °C
Slow	3	276.7	91	62	65	71
Quick	3	4.2	0	nd	nd	nd
5-day treatment						
38% RH	7	244.2	90	24	35	45
60% RH	10	246.2	93	19	32	43
93% RH	31	246.3	50	nd	nd	nd
98% RH	36	285.1	0	nd	nd	nd
15-day treatment†						
38% RH	7	248.7	90	23	31	42
60% RH	11	286.0	78	14	28	43

nd, not detected.

*Survival was examined 48 h after rehydration.

†Samples incubated for 15 days at 93% RH and 98% RH were spoiled.

branes (38–42). The peak position of this region, ν[P═O] cm^{-1}, was slightly lower in slowly than in quickly dehydrated larvae (Fig. 6*A*). This suggests that hydrogen bonds formed between the polar headgroups of phospholipids and sugars (40–42), although compounds other than phospholipids, such as DNA and RNA, also contain the P═O atomic group and could also cause differences in the peak position of the region.

To further test whether the effect was caused by phospholipids, we focused on the IR spectral regions between 2,856 and 2,849 cm^{-1}, where the absorption band appears due to the symmetric CH_2 stretching vibration. This region has been widely used to determine the gel-to-liquid crystalline temperature (T_m) in both biomimetic and cellular membranes (40–46). T_m is usually defined as the midpoint of the temperature range in which the peak position, ν[CH_2-sym], shifts from 2,850 to 2,854 cm^{-1} in a temperature-dependent manner (40–46). As shown in Fig. 6*B*, the transition curve of the slowly dehydrated larvae was shifted to lower temperatures than that of the quickly dehydrated larvae. This result suggests that the gel-to-liquid crystalline temperature of the former was lowered by forming hydrogen bonds between the polar headgroups of phospholipids and sugars (40–42). Taken together, these results indicate that water replacement and vitrification are both involved in anhydrobiosis in *P. vanderplanki*.

Discussion

P. vanderplanki is useful for biophysical and physiological analysis because of its relatively large body size; other anhydrobiotic animals are microscopic or nearly so (47). Taking advantage of this fact, we demonstrated that in the dehydrated larvae that had entered anhydrobiosis, biomembranes were protected by hydrogen-bonding with sugars and biological glasses were formed. Because a large amount of trehalose is distributed throughout the body of the anhydrobiotic larva, in which neither other sugars nor polyols were detected (21), we assume that trehalose plays an important role in anhydrobiosis in *P. vanderplanki*.

Although trehalose may be one of the key components of the intracellular glass formation in the slowly desiccated larvae of *P. vanderplanki*, its physical state could be influenced by other components. If anhydrous glassy trehalose without additives is exposed to relative humidity of >44%, it converts to dihydrate and crystallizes (48). However, x-ray diffraction measurements detected no crystal formation in the slowly dried larvae even after exposure to higher relative humidity (T.F. and M.S., unpublished data), indicating that the crystallization of endogenous glassy trehalose was hindered by the presence of cytoplasmic solutes such as proteins (49).

The glass transition temperatures of the slowly dehydrated larvae of *P. vanderplanki*, *Tg*[slow], shifted less with an increase in water content than expected from theoretical values that can be estimated as *Tg* of a binary mixture of pure trehalose in water using the Gordon–Taylor equation (50), $Tg = (w_1Tg_1 + kw_2Tg_2)/(w_1 + kw_2)$, where Tg_1 and Tg_2 represent the glass transition temperatures of pure sugar and water, respectively, and w_1 and w_2 are their weight fractions with $w_1 + w_2 = 1$. The onset values of Tg_1 and Tg_2 are taken to be 113.9°C (51) and −135°C (52) for trehalose and water, respectively, and k to be 7.5 (51).¶ As shown in Table 1, the onset value of *Tg*[slow] decreased from 62°C to 14°C as body water content increased from 3% to 11%, whereas the corresponding theoretical *Tg* value decreases more steeply, from 67°C to −6°C. This may imply the involvement of some factors other than trehalose when the vitreous state was formed in the body of *P. vanderplanki*. For plant anhydrobiotes, the possibility has been reported that proteins as well as soluble carbohydrates are vitrified in the cytoplasmic glass (29, 30). We obtained no evidence that proteins undergo vitrification when the larvae of *P. vanderplanki* enter anhydrobiosis. However, our results do not exclude the possibility that highly hydrophilic LEA proteins may also be responsible for intracellular glasses in *P. vanderplanki* (23). LEA proteins occur in various anhydrobiotic organisms (1, 24–27) and have been suggested to reinforce biological glasses (28), although no increased desiccation tolerance was found in human cells when an LEA protein from an anhydrobiotic nematode, *Aphelenchus avenae*, was introduced (53). Actual functions of LEA proteins are still controversial.

Based on the results of the current work and our previous studies (21–23, 54–57), we propose the following scheme of induction of anhydrobiosis in *P. vanderplanki.* When the body water content becomes <75%, changes in internal ion balance trigger several physiological events that induce anhydrobiosis (22). For example, many genes are up-regulated, including those coding for LEA proteins (23), a facilitated trehalose transporter (55), an aquaporin (57), and trehalose synthesis enzymes (K. Mitsumasu, T.K., and T.O., unpublished data). Trehalose is synthesized in the fat body (21, 22) and carried via the hemolymph to other cells and tissues (54). As water content decreases further, the intracellular medium may change from a water-dominated one to a trehalose-dominated one, resulting in a uniform distribution of trehalose in the body (Fig.

¶In recent years, values of ≈115 ± 2°C have been widely accepted as the onset or midpoint value of the glass-transition temperature of anhydrous trehalose, Tg_1 (51, 59–62), though observed value have ranged from 73°C (10) to 117°C (60, 61). Lower values could be due to the effects of residual water or impurities. There has been more consistent agreement between measurements of the glass-transition temperature of pure water (Tg_2), giving −135°C as the onset value (52, 59). The value of the parameter, k, in the Gordon–Taylor equation is sensitive to the values of Tg_1 and Tg_2, which should be determined by fitting onset glass transition temperatures measured at various water contents, with careful choice of the value of Tg_1. The Gordon–Taylor equation parameterized in ref. 51 satisfies these requirements for confidence.

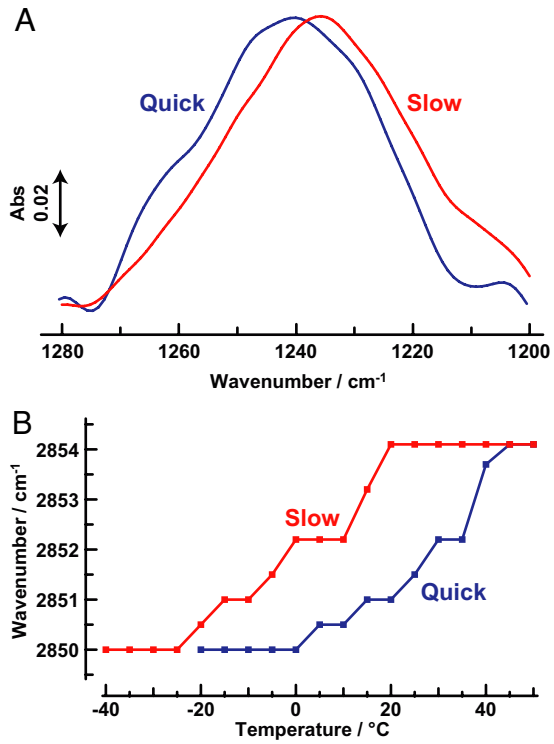

Fig. 6. FTIR analysis for interaction between cell membrane and sugars. (*A*) Slowly and quickly dehydrated larvae were measured by FTIR at 30°C. In the region 1,280–1,200 cm^{-1}, which shows asymmetric stretching vibration of P═O atomic groups, the peak position of the each band remained almost constant within the range of measured temperatures. (*B*) Slowly and quickly dehydrated larvae were measured by FTIR between −40°C and 50°C. In the region 2,849–2,856 cm^{-1}, which shows symmetric CH_2 stretching vibration, the peak position of the each band shifted in a temperature-dependent manner.

5*B*). In a highly dehydrated state, such as at 3% water content, the bound water molecules surrounding proteins and membranes are replaced by trehalose and highly hydrophilic proteins, which may form hydrogen bonds with them (Fig. 6*A*) and thereby possibly keep membranes in the liquid crystalline states at room temperature (Fig. 6*B*). Furthermore, the mixture of trehalose and protein is vitrified and embeds all of the macromolecules such as enzymes, DNA, and membrane lipids. The immobilized cellular components can escape physical and chemical destruction during the ametabolic state characteristic to anhydrobiosis. In other words, the successful anhydrobiotic larva is just like a substance assembled mainly with biological organic molecules, with the spatial arrangements required for normal physiology largely maintained by immobilization in the biological glasses.

Physical change of the larval body to its rubbery state by either heat or moisture absorption clearly damaged the desiccated larvae. Because rubbery states are $\approx 10^{-14}$ times as viscous as their corresponding glassy states (37), change to a rubbery state may allow spatial disarrangements of cellular components. It should be noted that uptake of water vapor is quite different from rehydration of larvae of *P. vanderplanki* in liquid water, in which rehydration very rapidly increases water content in larval bodies, such that the glassy matrix dissolves in water without passing into a rubbery state.

In nature, *P. vanderplanki* larvae can survive 8 months of the dry season in dried mud on rocks, despite the fact that surface temperatures of the rocks can reach 60°C at midday (according to our field survey). The dehydrated larvae should be able to maintain a glassy state under these conditions because their *Tg* (onset *Tg* = 62°C) is higher than the temperature on the rock surface. In the present study, heat treatment at 80°C for less than an hour did not kill all anhydrobiotic larvae, even though they probably passed from a glassy to a rubbery state. We assumed that damaging actions were delayed by high viscosity in the rubbery state, thus the slowly dehydrated larvae could escape from death in a brief period.

We conclude that vitrification is a prerequisite for successful anhydrobiosis in *P. vanderplanki*. One open question is whether this conclusion applies to other anhydrobiotic animals. Because anhydrobiosis is found in various taxonomic groups, it is thought that acquisition of this trait has taken place several times during their evolution (47). Therefore, it is possible that *P. vanderplanki* has developed a different mechanism from those of other organisms. For example, some anhydrobiotic rotifers lack any nonreducing sugars in dehydrated state (58) and probably lack sugar glasses. This suggests multiple strategies to form intracellular glasses (30) or mechanisms for desiccation tolerance without vitrification. In addition, we stress that anhydrobiosis is never achieved by only vitrification of the cellular matrix. Many other factors, such as chemical chaperones, antioxidants, and damage repair systems may contribute to desiccation tolerance in this chironomid (56).

The mechanisms of desiccation tolerance unveiled in this study might provide important hints for developing the long-term storage of a variety of cells, tissues, and possibly even organs in a dry state. Indeed, efforts are underway to confer desiccation tolerance on nonanhydrobiotic organisms by introducing large amounts of trehalose into target cells via a facilitated trehalose transporter (55) and by engineering concomitant functions necessary for protective responses to desiccation stress.

Materials and Methods

Sample Preparation. A laboratory colony was established from anhydrobiotic larvae of *P. vanderplanki* that were collected from rock pools in Nigeria (21). Larvae were reared through several generations under controlled light (13 h light:11 h dark) and temperature (27°C). Last-instar larvae ≈1 mg in body mass were used for the experiments. Groups of 8–10 larvae were put into a plastic Petri dish (50 mm in diameter, 8 mm high). Slowly dehydrated samples were prepared by incubating larvae at 100% RH for 1 day, 76% RH for a second day, and 5% for a third day (56). Quickly dehydrated samples are desiccated within a half of a day at 5% RH. Both types of samples were kept at 5% RH until experiments were performed.

Water Content of Larvae. The water content of larvae was determined by thermogravimetrical analysis with an ultramicrobalance (SE2; Sartorius); 10–15 larvae were heated at 120°C on an open aluminum pan for at least 15 min, after which the weight of the sample was found to level off.

Quantification of Biomolecules. Trehalose content in a larva was determined with a Shimadzu HPLC system (LC-10A system; Shimadzu) equipped with a reflective index detector (RID-6A; Shimadzu), following a previous study (21). To measure triacylglyceride, a larva was homogenized in 1 ml of chloroform/methanol (vol/vol = 2:1) with 1 mg of cholesterol acetate as an internal standard. After centrifugation at 1,000 × *g* for 10 min, triacylglyceride in the supernatant was determined using an Iatroscan TLC/FID analyzer (Iatroscan New MK-5; Mitsubishi Kagaku Iatron). Total protein was quantified with a Protein Assay Kit II (Bio-Rad), according to the instruction manual.

Heat Treatment. Slowly dehydrated larvae were transferred from the Petri dish into a glass tube (15 ml). Tubes containing 20–30 larvae were exposed to temperatures ranging from 25°C to 110°C for 5 min or 1 h. After cooling for several minutes at room temperature (24–26°C), larvae were submerged in distilled water and their recovery-checked 48 h after rehydration. A larva was judged to have survived if it could repeatedly contract its abdomen.

Absorption of Moisture. The slowly dehydrated larvae were allowed to absorb moisture at various relative humidities at ambient temperature (≈25°C) for 5 or 15 days. Humidity in the containers (≈2,000 ml) was controlled with saturated aqueous solutions of $MgCl_2$, $Mg(NO_3)_2$, KNO_3, or K_2SO_4, which provide 38% RH, 60% RH, 93% RH, and 98% RH, respectively. Dry air (5% RH) was provided in a plastic container (20 × 20 × 20 cm) with 1 kg of silica gel. Relative humidity in the containers was monitored with a temperature and humidity recorder (RT-11; Tabai Espec).

FTIR Measurements. The whole body of a desiccated larva was sandwiched between two KBr plates. Lattice mapping spectra in the 4,000–750 cm^{-1} range were collected by an infrared microscope (IMV-4000 with FT/IR-6200 spectrometer; JASCO) equipped with a liquid nitrogen-cooled, mercury-cadmium-telluride, 16-element, linear array detector. A screen image recorder camera attached to the microscope enabled the acquisition of a photomicrograph of the investigated area. Sequential spectra were collected at 128,000 points (320 × 400 points) in the specimen. The area of spectral acquisition was 20 mm^2 (4 × 5 mm). For each spectrum, 32 interferograms were collected, signal-averaged, and Fourier-transformed to generate spectra with a spectral resolution of 8 cm^{-1} and a spatial resolution of 12.5 μm in the transmission mode. The temperature of the sample was controlled with an LK-600 (Linkam Scientific Instruments) mounted on the stage of the above infrared microscope, while flowing a dry nitrogen gas inside the cell where the sample was placed.

DSC Measurements. DSC measurements were performed with a DSC-2920 and Q-100 (TA Instruments), calibrated with indium. Five to six intact bodies of dried larvae or one body after humid treatments were placed on a hermetically sealed aluminum pan with a pin hole. An empty pan was used as a reference. All measurements were carried out at a heating rate of 5°C/min, with the calorimeter head under a stream of dry nitrogen as the purge gas.

Data Analysis. IR spectral analyses were carried out using Spectral Manager (Version 2) and PCA Analysis (Version 2.02) (supplied by JASCO). The glass transition temperatures were read using Universal Analysis software (TA Instruments). The onset and end-point temperatures were taken to be the intersections of the extrapolated baseline with the tangent at the midpoint in the stepwise change in heat capacity. The midpoint glass transition temperature was taken to be the temperature at half-height of the heat capacity change.

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