Recent studies have shown that trehalose plays a protective role in yeast in a variety of stresses, including heat, freezing and thawing, dehydration, hyperosmotic shock, and oxidant injury. Because (a) heat shock and anoxia share mechanisms that allow organisms to survive, (b) Drosophila melanogaster is tolerant to anoxia, and (c) trehalose is present in flies and is metabolically active, we asked whether trehalose can protect against anoxic stress. Here we report on a new role of trehalose in anoxia resistance in Drosophila. We first cloned the gene trehalose-6-phosphate synthase (tps1), which synthesizes trehalose, and examined the effect of tps1 over-expression as well as mutation on the resistance of Drosophila to anoxia. Upon induction of tps1, trehalose increased, and this was associated with increased tolerance to anoxia. Furthermore, in vitro experiments showed that trehalose reduced protein aggregation caused by anoxia. Homozygous tps1 mutant (P-element insertion into the third intron of the gene) leads to lethality at an early larval stage, and excision of the P-element rescues totally the phenotype. We conclude that trehalose contributes to anoxia tolerance in flies; this protection is likely to be due to a reduction of protein aggregation.

The ability of organisms to sustain O2 deprivation is limited. Irreversible injury may occur to mammalian tissues within 5–10 min of severe hypoxia or ischemia. Nervous tissue is particularly vulnerable to hypoxia (1). The range of tolerance of animals to hypoxia is wide, and in contrast to mammalian sensitive tissues, there are certain vertebrate or invertebrate animals that have an amazing resistance to anoxic injury (2). For example, we and others (3–5) have shown that the brain tissue of the turtle Pseudemys scripta elegans is exceedingly resistant to anoxia, surviving hours of experimental O2 deprivation with neurons continuing to fire with very little change in their electrophysiologic properties. Another example is the invertebrate Drosophila melanogaster: although its response to O2 deprivation is different from that of the turtle, the Drosophila also shows an extreme resistance to anoxia as it recovers from hours of anoxia without evidence of cell damage (6, 7).

The mechanisms underlying the susceptibility or tolerance to O2 deprivation are not well understood. Clinical trials using a variety of agents to treat cardiac or brain ischemic injury have not been successful so far (8). Indeed, several questions still need to be addressed: 1) What mechanisms promote anoxia resistance in the turtle or fruit fly? 2) If metabolic energy (ATP) is depleted during anoxia, what mechanisms prevent protein degradation in the flies after hours of anoxia? and 3) Do similar mechanisms operate in other stress conditions, e.g. desiccation, heat, freezing, or oxidant injury? Mechanisms that protect protein integrity must exist and are likely to be operative during anoxia since the post-stress behavior of this organism appears to be normal.

It is well known that there are at least two major types of substances that play an important role in preserving proteins during heat shock: heat shock proteins and organic compounds such as disaccharides. For example, trehalose is a non-reducing disaccharide and is found in diverse organisms such as bacteria, fungus, algae, and insects. It has been shown to play a protective role in yeast in a variety of stress conditions such as heat, freezing and thawing, dehydration, hyperosmotic shock, and oxidant injury (9–11). Singer and Lindquist (12) have demonstrated that trehalose reduces protein aggregation and maintains proteins in a partially folded state, facilitating their refolding by cellular chaperones at a later stage, possibly after the stress condition is terminated. Though trehalose is absent in mammals, it has been shown to improve the tolerance of human cells to desiccation (13) and tissue tolerance to cryopreservation (14).

The possible involvement of trehalose in cellular protection against anoxic damage has not been studied. Four reasons have led us to hypothesize that trehalose may do so: 1) Anoxia, like heat stress, induces an up-regulation of heat shock proteins; hence, anoxia may share similar types of mechanisms that allow organisms to survive. 2) We and others have shown that flies tolerate long term anoxia. 3) Flies synthesize and catalyze trehalose. and 4) Trehalase has been shown (as indicated above) to protect against heat shock.

The present studies were undertaken to investigate whether trehalose plays an important role in protecting flies against anoxic stress. We have tested whether overexpression or mutation of trehalose-6-phosphate synthase or tps11 gene, a gene that synthesizes trehalose in flies, increases or decreases tolerance to anoxia respectively, and if so, whether trehalose can reduce protein aggregation caused by anoxia in D. melanogaster.

The abbreviations used are: tps1, trehalose-6-phosphate synthase; tps2, trehalose-6-phosphate phosphatase; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid).
**Experimental Procedures**

*D. melanogaster* Stocks—Flies were maintained on standard *Drosophila* medium at temperatures of 24–25 °C or as otherwise specified. The following GALA fly lines were used: 32B-GALA (3rd) for constitutive general expression, pGMR-GALA (2nd) for specific eye tissue expression, and the heat shock inducible GAL4 line, Hs-GAL4 (3rd). Fly stocks with balancer chromosomes were used as Adv/CyO and Adv/CyO; Str/TM6B.

Cloning of *Drosophila tps1* cDNA—When yeast tps1 cDNA was used to blast the *Drosophila* data base, a gene with a 2427 bp open reading frame was found. The *Drosophila* gene was found to be 30% similar to yeast tps1. Two primers in a conserved domain (sense: 5′-cagcactac- cactcagct-3′ and antisense: 5′-ccgagtcgctcagct-3′) were designed and used. PCR was performed, and its product, a 710-bp segment, was cloned into pCR-TOPO vector (Invitrogen) and used as a probe. A *Drosophila* cDNA library, which was constructed in our laboratory, was screened using the probe labeled with 32P (a random primer labeling method). PCR was performed, and its product, a 710-bp segment, was cloned into pCR-TOPO vector (Invitrogen) and used as a probe. A *Drosophila* cDNA library, which was constructed in our laboratory, was screened using the probe labeled with 32P (a random primer labeling method). Library screening yielded three positive clones of about 3000 bp, which were digested with EcoRI, subcloned into pCR2.1, and sequenced.

**Generation of Transgenic Fly Lines and Overexpression of tps1 in Flies**—Fly tps1 cDNA was digested with AsclI and EcoRV, blunted, and ligated into pUASt (digested with NotI and blunted). The right orientation was confirmed by sequencing. The recombinant construct (UAS-tps1) was purified and microinjected into w1118 embryos (concentration of 1 µg/µl) to generate transgenic lines. pUASt-tps1 (2nd) and pUASt-tps1 (3rd) were crossed to 32B-GALA (3rd) and pGMR-GALA (2nd). Cultures were maintained at 24–25 °C when crossed with hs-GAL4 (3rd). Flies were allowed to recover at 25 °C for 12 h, and cultures were maintained at 24 °C or as otherwise specified. Five cultures were required for some cultures, as detailed below.

**Confirmation of P-element Location**—Genomic DNA was extracted from F838/CyO flies, digested with MspI, self-ligated, and PCR-amplified with primer Ppy4: 5′-ccagacttcagctcagct-3′ and Ppy5: 5′-gtgagcgtcagctcagct-3′ (15). The PCR product was recovered from an agarose gel and cloned into the pCR-TOPO vector and sequenced with primers SpeI-specific primer. The resulting sequence was used to blast the *Drosophila* genomic DNA data base.

**Anoxia Test**—The procedure for inducing anoxia and measuring recovery time has been described previously in detail (16). Briefly, groups of 10–12 adult flies, age 3–5 days, were placed in a specially designed chamber and exposed to anoxia (O2 concentration = 0% with administration of 100% N2) for 5 min prior to recovery in room air (O2 concentration, 20.8%). After the introduction of N2 in the chamber, flies became unconscious within ~30 s (when O2 concentration was about 1%), resulting in their fall to the bottom of the chamber, where they remained motionless for the rest of the anoxic period. Recovery time was measured as the latency between the end of a 5-min anoxia treatment and the time point when flies recovered, which was usually a very discrete event.

**Measurement of Trehalose Concentration in Flies**—30 mg of fly tissues or fly heads were homogenized with a plastic pestle in 0.25 ml of 12C or 13C (2H, 3H) water at 95 °C for 20 min. The rest of the anoxic period. Recovery time was measured as the latency between the end of a 5-min anoxia treatment and the time point when flies recovered, which was usually a very discrete event.

**NMR Measurement of Perchloric Acid-extracted Flies**—To determine whether trehalose is in a metabolically active pool in the fly, we resorted to NMR measurement of trehalose after [1-13C]glucose feeding. Tissues or fly heads were homogenized with a plastic pestle in 0.25 ml of 12C or 13C (3H) water at 95 °C for 20 min. The rest of the anoxic period. Recovery time was measured as the latency between the end of a 5-min anoxia treatment and the time point when flies recovered, which was usually a very discrete event.

**Western Blot**—Protein determinations were carried out in duplicate in each sample with the bicinechonic acid protein assay kit (Sigma) using bovine serum albumin as a standard. Protein samples (5 µg) were electrophoresed through 4–10% Novex bis-Tris denaturing gel (Invitrogen) and electrophoretically transferred to nitrocellulose membrane. Nonspecific binding sites were blocked, and the membranes were incubated for 1 h with primary antibodies (anti-β tubulin and anti-Na+/K+/ATPase, Jackson ImmunoResearch Laboratories, Inc.) and detected using horseradish peroxidase-conjugated secondary antibody with enhanced chemiluminescence (ECL; Amersham Biosciences, Inc.).

**RESULTS**

*Trehalose Is Metabolically Active in Wild Type Flies*—To assess whether trehalose is metabolically active in wild type flies, trehalose 13C isotopic labeling was assessed in 13C-edited, 2H NMR spectra of acid extracts of overnight fasted flies following a 2-h exposure to a solution of [1-13C]glucose (Fig. 1). The 1H1a doublet resonance of trehalose was visible at 5.2 ppm, whereas no resonance was detected for H1b, consistent with the assignment of trehalose and indicating that free glucose (1-13C)-labeled or unlabeled was not observed. Substantial 13C labeling of trehalose C1a was observed (40.4%). Thus, the results indicate that wild type flies have significant levels of trehalose and that the trehalose pool is metabolically active.

**Cloning of *Drosophila tps1* cDNA**—A total of 200,000 plaque-forming units from a primary fly head cDNA library were screened with the probe described above. Four positive clones were isolated, subcloned, and sequenced. Of these clones, three gave full-length cDNA of the *tps1* gene. This gene encodes a transits, 16,000 data points, and fully relaxed (repetition time/ transient of 21 s). A low power presaturation pulse was used to suppress the residual HDO resonance. Free-induction decays were processed using an exponential filter (0.5 Hz), zero-filled to 32,000 data points, and Fourier-transformed. The fractional 13C enrichment of the trehalose signals has been described previously in detail (16). Briefly, groups of 10–12 adult flies, age 3–5 days, were placed in a specially designed chamber and exposed to anoxia (O2 concentration = 0% with administration of 100% N2) for 5 min prior to recovery in room air (O2 concentration, 20.8%). After the introduction of N2 in the chamber, flies became unconscious within ~30 s (when O2 concentration was about 1%), resulting in their fall to the bottom of the chamber, where they remained motionless for the rest of the anoxic period. Recovery time was measured as the latency between the end of a 5-min anoxia treatment and the time point when flies recovered, which was usually a very discrete event.

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protein of 809 amino acids and contains both conserved domains of yeast tps1 and tps2 (Fig. 2). Using Flybase and Gadfly, we found that this gene is located on chromosome 2L at 24F01.

Overexpression of tps1 in Flies—To study the function of tps1, we overexpressed the tps1 gene using the UAS-GAL4 system (15). We first cloned tps1 cDNA into pUAST vector and generated a number of transgenic lines carrying the UAS-tps1 transgene on each of the three main chromosomes of the fly. When each of these specific transgenic lines were crossed with 32B-GAL4 for constitutive generalized overexpression (UAS-tps1/UAS-tps1/H11003/32B-GAL4), all larvae died at the stage of the first instar. Specific tissue overexpression of tps1, using pGMR-GAL4, resulted in a rough eye phenotype (Fig. 3C). The ommatidia were smaller and disorganized. However, the number of receptor cells in each ommatidia was normal with larger spaces present between ommatidia (Fig. 3D). When we overexpressed tps1 with the heat shock-inducible promoter Hs-GAL4 at either 24 °C or 18 °C, flies died at a late pupa stage (Fig. 3E); however, animals survived and developed into the adult stage when raised at 15 °C.

Overexpression of tps1 in Adult Flies: Trehalose Levels and Tolerance to Anoxia—Adult flies, grown at 15 °C, were used. After hatching, these flies were first transferred to and kept at 18 °C for 2 consecutive days before they were tested for their tolerance to anoxia. We used the same phenotypic assay we had validated previously and used extensively (16). We tested flies with tps1 overexpression and controls. Three kinds of controls were used: w1118, UAS-tps1/H11003, and w1118, Hs-GAL4/+. These were also grown at 15 °C. As in our previous studies, an

FIG. 2. Alignment of Drosophila TPS1 amino acid sequence to Saccharomyces cerevisiae TPS1, TPS2, and Selaginella lepidophylla TPS1. Drosophila TPS1 shows 29.7% identity to S. cerevisiae TPS1 and 17.4 and 22.5% identity to S. cerevisiae TPS2 and S. lepidophylla TPS1, respectively. (S. c TPS1, S. cerevisiae TPS1; S. c TPS2, S. cerevisiae TPS2; S. l TPS1, S. lepidophylla TPS1; D TPS1, Drosophila TPS1.)

FIG. 3. Overexpression of tps1 in flies. A, wild-type eye; B, wild-type eye section (×160); C, tps1-pGMR eye; D, tps1-pGMR eye section (×160). Compared with wild-type eye, the number of receptor cells in tps1-pGMR ommatidia is the same, but the size of the ommatidia is smaller, and larger spaces are present between ommatidia. E, overexpression of tps1 with Hs-GAL4 at 24 °C and 18 °C causes lethality at late pupa stage.
FIG. 4. Overexpression of tps1 in adult flies increases trehalose level and increases tolerance to anoxia. Flies with overexpression of tps1 have a shorter recovery time after being exposed to 5 min of anoxia (A). W1118, W1118; UAS-tps1/ (3rd), W1118; Hs-GAL4/+ (3rd), UAS-tps1/Hs-GAL4 (3rd), UAS-tps1/+ (2nd); Hs-GAL4/+ (3rd) were all raised at 15 °C. Subsequently, 1-day-old adult flies were collected and placed at 18 °C for 2 days. Anoxia tests were performed at that time. The recovery times (means ± S.E.) after 5 min anoxia were shorter in UAS-tps1/Hs-GAL4 (3rd), UAS-tps1/+ (2nd); Hs-GAL4/+ (3rd) than in W1118, W1118; UAS-tps1/+ (3rd), W1118; Hs-GAL4/+ (3rd). *, p < 0.005 as compared with controls. Flies with overexpression of tps1 contain more trehalose than controls (B). W1118, W1118; UAS-tps1/+ (3rd), W1118; Hs-GAL4/+ (3rd), UAS-tps1/Hs-GAL4 (3rd), UAS-tps1/+ (2nd); Hs-GAL4/+ (3rd) were treated in the same way as in the previous experiment. Total trehalose (means ± S.E. glucose equivalents in 30 mg of flies) in UAS-tps1/Hs-GAL4 (3rd), UAS-tps1/+ (2nd); Hs-GAL4/+ (3rd) almost doubled over that in W1118, W1118; UAS-tps1/+ (3rd), W1118; Hs-GAL4/+ (3rd). *, p < 0.005 as compared with controls.

anoxia period of 5 min was used to determine the recovery latency and tolerance to \(O_2\) deprivation. Overexpression of tps1 in adult flies reduced the recovery time and rendered flies even more tolerant to anoxia than the wild type flies. The recovery times from anoxia were all significantly longer in the controls than in the transgenic flies expressing tps1 and Hs-GAL4 (Fig. 4A). Parallel to the shortened recovery times in the transgenic flies were the trehalose levels, which were much higher in those flies containing Hs-GAL4 and tps1 than in the control flies (Fig. 4B).

Mutation of tps1 in Flies: Lethality in the First Instar Larval Stage—To further understand the tps1 function, we needed to analyze the tps1 mutant. We used a homozygous lethal line, P838/CyO, with a P-element in the tps1 gene from the fly stock center. The location of the P-element was in the 3rd intron of the tps1 gene, and this was confirmed as detailed under “Experimental Procedures.” In order to ascertain that the lethality is caused by the P-element insertion, we first crossed the P838/CyO to two other separate lines with deficiencies. The first deficiency was a deletion between cytological locations 24F04 and 25A01–04, and the second was a deletion between 24C03–05 and 25A02–03. We found that there was complementation with the first deletion but not with the second. In addition, excision of the P-element by jumping the P838 P-element restores the viability of the fly, indicating that lethality is due to P-element insertion into the tps1 gene. The lethality occurred in the first instar larval stage because about 1/4 of the larvae died in this stage when virgin females P838/CyO were crossed to a male P838/Adv.

Long Term Anoxia and Protein Aggregation in Flies: Can Trehalose Reduce Aggregation or Prevent Na\(^+\)/K\(^-\) ATPase from Aggregating?—The data presented above showed that overexpression of tps1 in flies almost doubled the trehalose level and increased the tolerance to anoxia. To further explore the mechanisms underlying the effect of trehalose, we investigated 1) whether anoxia causes protein aggregation in flies and 2) whether trehalose helps to reduce protein aggregation caused by anoxia. Wild type flies (w1118) were treated with \(N_2\) for 0, 1, 3, 4 h, put on dry ice immediately, and stored in −80 °C. Of 18 mg of total protein extracted from flies, we found increased amounts of aggregated proteins in flies after 3 h but especially after 4 h of anoxia (mean ± S.E.: 1931 ± 336 S.E.) as compared with flies that were not exposed to anoxia (415 ± 21 mg, Fig. 5A). Protein aggregation in samples from flies subjected to 4 h of anoxia was reduced with increasing trehalose concentration (Fig. 5B). Interestingly, protein aggregation was also decreased with other disaccharides, such as 0.5 M sucrose (765 ± 41 mg), although not to the same degree as with trehalose (470 ± 36 mg). However, glycerol at a similar concentration did not seem to have any effect on the solubility of proteins.

To determine the fate of specific proteins, we examined the solubility of Na\(^+\)/K\(^-\) ATPase, which is known to unfold and aggregate in low \(O_2\) conditions (19). This was examined in flies...
Trehalose is beneficial and can protect against anoxia. Second, trehalose markedly reduced protein aggregation seen after 4 h of anoxia. In this regard, we tested whether this reduction in protein aggregation could be seen with some important proteins that have been shown in mammalian tissue to be protected during anoxic stress (19). Indeed, we found that the aggregation of the Na+/K+ ATPase protein decreases in a major way with increasing amounts of trehalose when trehalose was added to tissues taken from flies exposed to 4 h of anoxia. Third, although not necessarily related to anoxic stress directly, when the gene that encodes for trehalose synthesis was mutated with a P-element insertion, flies did not develop, and they died early in the larval stage. This indicates that tps1/trehalose are important as cell protectants in early life such as during the process of burrowing into food when larvae are constantly or intermittently exposed to anoxia. Alternatively, it is possible that the lack of tps1/trehalose is deleterious in early fly development because of their involvement in functions that are not related to protection from anoxia.

Although we show in this work that trehalose prevents anoxia-induced aggregation, this does not argue against a similar function of other disaccharides or small molecules such as glycerol. Indeed, we have shown that sucrose does help against aggregation but to a lesser degree. Interestingly, glycerol did not help in this activity.

Another important issue is the concentration of trehalose. The overall average concentration of trehalose that we have measured in flies is much less than 500 mM, but this does not imply that concentrations of that high magnitude cannot be reached in specific locations of the cytosol. Major intracytosolic gradients have been shown to exist, such as for ATP and Ca2+ concentrations.

How does trehalose protect cells? Is it possible that trehalose is used as another carbon source that enhances the metabolic machinery in cells? We doubt that trehalose acts mainly by providing an alternative energy source for at least three reasons. (i) Studies from yeast have shown that trehalose is not produced when nutrients are abundant but that it is produced even in the absence of glucose in the medium and when glycerol is metabolized (21). Furthermore, trehalose is not used until all glycogen stores are depleted and yeast is close to cell death (22). (ii) Trehalose has been found to suppress the aggregation of denatured proteins and maintain them in a semifused state through hydrogen bond stabilization promoting refolding and reattainment of bioactivity (23, 24). This stabilizing effect of trehalose is accounted for by the change in the transfer of free energy on unfolding; the interaction between trehalose and proteins is less favorable with the denatured than with the native protein (25). Whether trehalose protects proteins by interacting with these proteins or whether there are intermediary components is not clear from this work. However, it is possible that trehalose “prepares” proteins for the chaperones to act upon and rescue them (26). (iii) Our results show that protein aggregation, including the Na+/K+ ATPase, is reduced considerably and in a dose-response fashion when trehalose is added with these proteins. Thus, the results from the literature and our current study would strongly suggest that this disaccharide has a function that can help in the refolding of proteins when cells and tissues are exposed to stresses such as O2 deprivation, although we cannot rule out totally the idea that trehalose may contribute metabolically as a carbon source in extraordinary circumstances of energy depletion.

If trehalose can assist in refolding proteins and maintenance of protein integrity during stress, why is it that overexpression of tps1 and trehalose at a relatively high temperature (at 18° and 24 °C) leads to lethality, and why is it that trehalose leads to grossly abnormal eye development when overexpressed with...
pGMR? The answers are not totally apparent at present but may lie in the molecular interactions between trehalose, heat shock proteins, and proteins that are being refolded. Normally, trehalose suppresses the aggregation of protein and prevents native protein from unfolding (12), thus promoting interactions of the target protein with heat shock proteins to enhance proper refolding to the native state. If trehalose is present in large quantities during stress (or in the absence of stress), it is believed that it would interfere with refolding of the targeted proteins by heat shock proteins. Whether this is the reason for larval lethality or the grossly abnormal eyes with higher expression of trehalose is not known, but this is a possible explanation. It is interesting to note that survival until first instar larva or late pupae stage depends on the level of overexpression. It is interesting to note that survival until first instar larva or late pupae stage depends on the level of overexpression. With the 32B-GAL4 promoter at 24 °C, early larvae died; with Hs-GAL4 at 18 °C and 24 °C, late pupae did hatch but died shortly after (Fig. 4E). It was only when the temperature was rather low during development (15 °C) that we could obtain adult flies and test them.

In summary, we have shown that constitutive overexpression of tsp1 enhances anoxia tolerance. Furthermore, tsp1 and trehalose seem to play a critical role during development, and a mutation in the gene leads to early larval lethality. We hypothesize that enhanced anoxia tolerance shown following tsp1 and trehalose overexpression is due to a reduction of protein aggregation and the likelihood of protein denaturation during O2 deprivation.

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Role of Trehalose Phosphate Synthase in Anoxia Tolerance and Development in
*Drosophila melanogaster*

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