A Rapid Cold-Hardening Process in Insects

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was analyzed by adding proteinase K to the medium (outside of the cells). The precursor form was mainly protected from proteinase K by the plasma membrane (Fig. 4, lanes 1 to 3) and only digested after disruption of the membrane by detergent (Fig. 4, lane 4). Most of the precursor was therefore located in the cytoplasm indicating that translocation across the membrane, and not processing, is the rate-limiting step for coat production. The coat protein, however, was digested by proteinase K (Fig. 4, lanes 5 to 8) and had therefore been normally inserted into the inner membrane of *E. coli*. The proteolysis of OmpA was taken as an internal control (Fig. 4, lanes 9 to 12). The other cleavage product, consisting of the ribulokinase fragment fused to the leader peptide of procoat, was not accessible to the protease (Fig. 4, lanes 1 to 3). This result indicates that the NH_2-terminal part of the leader peptide remains in the cytoplasm during the membrane-insertion process in vivo. An alternative interpretation, assuming a portion of the ribulokinase-derived sequence enters the membrane, is unlikely since a cluster of 11 charged amino acids precedes the leader sequence.

A cleavable leader sequence therefore does not need to be placed at the NH_2-terminus of a protein to retain its function of initiating membrane insertion and subsequent cleavage. The reason why leader (signal) sequences are located at one end of the protein might simply be that this allows an easy removal of this hydrophobic region. Such a removal is probably often required since a leader region might interfere with the function or with the location of a protein (*13*). An NH_2-terminal position of the leader sequence might be preferred since it allows the protein to interact with the membrane rapidly, before its synthesis is completed or before its folding has become too compact (*8*). A crucial requirement for membrane insertion of a pre-protein is probably the conformational arrangement of the leader sequence so that it is exposed rather than buried in the folded pre-protein. Conformational protrusion is probably also required for uncleaved internal leader sequences. Both cleavable and uncleavable internal signal sequences share the basic mechanism of membrane insertion. The internal signal sequence of the asialoglycoprotein receptor can initiate membrane insertion of rat α-tubulin when placed at the NH_2-terminus (*14*). Dalbey *et al.* have recently shown that the internal signal sequence of leader peptidase can functionally replace the NH_2-terminal leader sequence of OmpA and M13 procoat (*15*). These results taken together show that internal signal sequences and cleavable leader sequences are functionally exchangeable and, in principle, not restricted to a definite location in the protein.

Earlier studies with pro-lipoprotein (*16*) have shown that a fusion of two signal peptides allows cleavage at both cleavage sites, suggesting that internalized signal peptides are still functional, although less efficient than the NH_2-terminal signal peptide. Although pro-lipoprotein differs from other pre-proteins in its insertion pathway and is cleaved by a different signal peptidase, the effect of an internal leader (signal) peptide on the translocation rate is similar to that of procoat. The internal location of a leader may slow membrane insertion if it is buried in the already folded NH_2-terminal part of the protein. In a similar study with a fusion protein of α-globin and pre-prolactin it was observed that both signal-sequence–flanking protein regions were secreted into dog pancreas microsomes (*17*). However, these investigators observed that only a portion of the signal peptide fusion had been sequestered into the microsomes, suggesting the possibility that proteins insert into the endoplasmic reticulum by two different mechanisms.

In support of a loop-like insertion mechanism Kuhn *et al.* have recently shown that the COOH-terminus of procoat is necessary for membrane insertion but remains in the cytoplasm (*4*). Similar to NH_2-terminal fusion, COOH-terminal fusion does not prevent membrane insertion or processing by leader peptidase. Protease mapping experiments led to the conclusion that the COOH-terminal fused peptide was located in the cytoplasm while the acidic coat region was translocated across the membrane. Taken together, these results are in agreement with the idea that M13 procoat, in vivo, initially inserts into the membrane as a loop structure, leaving both termini (NH_2 and COOH) in the cytoplasm.

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Traditionally studies of cold tolerance in insects have focused on seasonal adaptations related to overwintering that are observed after weeks or months of exposure to low temperature. In contrast, an extremely rapid cold-hardening response was observed in nonoverwintering stages that confers protection against injury due to cold shock at temperatures above the supercooling point. This response was observed in nondiapausing larvae and pharate adults of the flesh fly, *Sarcophaga crassipalpis*, nondiapausing adults of the elm leaf beetle, *Xanthogaleruca luteola*, and the milkweed bug, *Oncopeltus fasciatus*. The rapid hardening response is correlated with the accumulation of glycerol.

MANY INSECTS RESPOND TO THE approach of winter by entering a period of dormancy (diapause) and by making physiological adjustments that increase their ability to tolerate low temperatures. For the many species that cannot tolerate tissue freezing, preparation for winter usually involves a gradual accumula-

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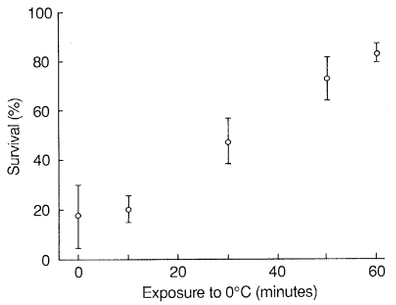

Fig. 1. Effect of the duration of chilling at 0°C before exposure to −10°C on the survival to adult ecdysis in *Sarcophaga crassipalpis*. Flies were reared at LD 15:9 and 25°C and tested 12 days after pupariation as pharate adults in the red-eye stage of development. Each value represents the mean (± SEM) survivorship of three replicate trials of ten flies each.

tion of a cryoprotectant such as glycerol and other low molecular weight polyhydric alcohols and sugars. We report a response operating even in nondiapausing species that provides a rapid protective mechanism against cold injury that may enable insects to respond to changing environmental temperatures on a daily or even an hourly basis.

Flesh flies (*Sarcophaga crassipalpis* and *Sarcophoga bullata*) develop without diapause when reared under long day lengths [light: dark cycle (LD) 15:9] and at 25°C (*1*). Generation time is 1 month. In contrast, flies reared at short daylength (LD 12:12) enter an overwintering pupal diapause that may persist for more than 100 days (*1*). These flies are freeze susceptible and do not tolerate tissue freezing in any stage of development (*2*). The supercooling point refers to the temperature at which spontaneous nucleation of body water occurs. Though both diapause and nondiapause pupae can supercool to −20°C or below, the supercooling point is not indicative of the lower lethal temperature: both types of pupae die at temperatures far above the supercooling point (*2*). This mortality occurring in the absence of tissue ice formation is known as cold shock, direct chilling injury or thermal shock, and is known from a variety of biological systems including bacteria, algae, protozoa, higher plants, and mammalian embryos, but is little known among the insects (*3*).

We found that most nondiapausing larvae and pharate adults of *Sarcophaga* do not survive even a 2-hour exposure to −10°C (Table 1). Survival was defined as successful adult emergence. For the unchilled control groups of larvae and pupae, emergence rates were always greater than 95%. The lethal effects of chilling were not immediately evident since flies often continued development for several days, but ultimately were unable to survive to adult emergence. In contrast, most flies survive if subzero exposure is preceded by a 2-hour period of chilling at 0°C. As little as 30 minutes of chilling at 0°C before exposure to −10°C doubled the rate of survival in *S. crassipalpis* (Fig. 1), whereas an hour of chilling resulted in a fourfold increase. Therefore short-term chilling results in protection against the cold shock injury that occurs at temperatures as much as 10°C above the supercooling point. Similar responses were obtained for nondiapausing adults of the elm leaf beetle, *Xanthogaleruca luteola*, and the milkweed bug, *Oncopeltus fasciatus* (Table 1).

This response is not restricted to nondiapausing stages. During the first month of diapause, pupae of *S. crassipalpis* gradually accumulate glycerol and concomitantly enhance their tolerance to low temperature. Thus, early in diapause pupae are still vulnerable to cold shock injury. Again, a brief cold pulse enhances survival. Three days after pupariation, diapausing pupae of *S. crassipalpis* were exposed to −17°C for 1 day: no individual (n = 30) survived direct exposure, whereas a 2-hour cold pulse (0°C) before subzero exposure yielded a survival rate of 91.1% (n = 45).

Rapid accumulation of glycerol may provide at least a partial basis for this cryoprotective response. Larvae and pharate adult flies rapidly accumulate glycerol in response to short-term chilling (*4*). After 2 hours of exposure to 0°C, wandering larvae have glycerol levels 2.4 times those of prechilled values of 18.2 m*M*, whereas glycerol levels in pharate adults increased nearly threefold to 81.4 m*M*.

Accumulation of cryoprotective compounds, particularly glycerol, is well known to be associated with cold-hardening in insects intolerant of freezing. These compounds are believed to confer protection by a number of colligative and noncolligative mechanisms including the depression of whole body supercooling points and hemolymph melting points, the stabilization of enzyme function at low temperature and protection against desiccation during winter (*5*). On the basis of the rapid acclimation response that we observed, we propose that glycerol may provide cryoprotection against injury due to cold shock in nonoverwintering insects, although this suggestion does not rule out the existence of other additional mechanisms. The observed increase in glycerol was not of a magnitude to change significantly colligative properties, however recent evidence (*6*) suggests that specific interactions between glycerol and other cellular components may be of critical importance. For example, glycerol may alter the nature of phase transitions of membrane lipids during cooling and thereby protect against cold shock (*6*).

The apparently widespread, but generally unrecognized, capacity for rapid accumulation of glycerol in response to low temperature exposure in a number of phylogenetically diverse insects lends additional support to this hypothesis. Exposure of insect fat body either in vivo or in vitro to cold causes the activation of phosphorylase to the *a* form which, in turn, catalyzes the rapid breakdown of glycogen and the accumulation of glycerol (*7*). Within 10 minutes after the transfer of silkmoth pupal fat body to 0° the percentage of phosphorylase *a* increases 2.3 times over initial levels to 30% (*7*). The rapidity of this response is consistent with that observed for the rate of hardening observed in this study (Fig. 1). Acclimation to warm temperatures reverses this process. Rapid cold activation of phosphorylase, 2.5

Table 1. Effect of short-term chilling at 0°C before exposure to subzero temperatures on the survival of various nondiapausing stages of insects. One group of flies (*Sarcophaga*) was transferred directly from 25°C to −10°C for an exposure period of 2 hours and returned to 25°C until adult emergence. A second group was chilled for 2 hours at 0°C immediately before exposure to −10°C. A similar protocol was used for the other species except that elm leaf beetles, *Xanthogaleruca luteola*, were chilled for 4 hours at 0°C followed by exposure to −7°C for 1 hour and milkweed bugs, *Oncopeltus fasciatus*, were chilled for 2 hours at 0°C followed by exposure to −10°C for 3 hours. All paired values show statistically significant differences ($P < 0.001$, z-test for two binomial proportions). Data represent mean values ± SEM.

Stage	Survival (%)	
	No chilling	Chilling
	Sarcophaga bullata	
Pupae	51.1 ± 7.5 (45)*	84.1 ± 5.5 (44)
Pharate adult, red-eye stage	0 (45)	91.1 ± 4.2 (45)
	Sarcophaga crassipalpis	
Third instar larvae	0 (45)	88.9 ± 4.6 (45)
Pharate adults, red-eye stage	5.0 ± 2.8 (60)	98.9 ± 1.3 (60)
	Xanthogaleruca luteola	
Adults, 2 days old	15.0 ± 7.9 (20)	90.0 ± 6.7 (20)
	Oncopeltus fasciatus	
Adults, mixed ages	33.3 ± 8.6 (30)	93.3 ± 4.5 (30)

*Numbers in parentheses are sample sizes.

to 7.5 times that of control values within 2 hours of transfer, has been reported for other active nonoverwintering insects including crickets and locusts (*7*) and for diapausing silkworm pupae (*8*). Thus, the cold activation of phosphorylase and the ensuing rapid accumulation of glycerol may be a general adaptation of insects for protection against cold shock injury.

The rapid cold-hardening capacity we describe may be of considerable ecological importance in early spring and late autumn. Our results suggest that many insects, even those in nondiapausing stages, have the ability to quickly enhance their cold tolerance in response to a rapid temperature drop. Though such a mechanism, in the absence of diapause, may not enable an insect to survive the prolonged cold exposure characteristic of winter, it should permit the insect to adapt to diurnal changes in temperature and enable the insect to survive brief periods of exposure to low temperature. Our observation that cold shock injury can be reduced by brief exposure to 0°C suggests that methods can be developed for long-term cryopreservation and storage of *Drosophila* and other nondiapausing insects.

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Similarity of Cruzin, an Inhibitor of *Trypanosoma cruzi* Neuraminidase, to High-Density Lipoprotein

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A specific inhibitor of the neuraminidase of the protozoan parasite *Trypanosoma cruzi* was isolated recently and named cruzin. It is now shown that cruzin is similar to high-density lipoprotein by amino acid homology, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, by immunoblot analysis, and by isoelectric focusing. Cruzin purified by ion exchange chromatography and high-density lipoprotein isolated by density gradient ultracentrifugation inhibited *Trypanosoma cruzi* neuraminidase to the same extent. Cruzin or high-density lipoprotein restores to normal the decreased multiplication rate of *Trypanosoma cruzi* epimastigotes grown in a medium depleted of lipoproteins, suggesting that it may be important for survival of the parasite in nature.

TRYPANOSOMA CRUZI, THE CAUSative agent of Chagas' disease, produces a developmentally regulated neuraminidase (E.C. 3.2.1.18) (*1*). The activity of the enzyme is greater in infective trypomastigotes than in epimastigotes (*1*), the parasite stage that multiplies in the gut of bloodsucking reduviid bugs. During infection in vitro, intact trypomastigotes desialylate erythrocytes and other blood cells (*1, 2*) as well as myocardial and endothelial cells (*3*). The neuraminidase is heterogeneously distributed among strains of *T. cruzi*, with some of the relatively low virulent ones possessing enzyme activity several orders of magnitude higher than the more virulent strains (*4*). Antibodies specific for *T. cruzi* neuraminidase augment parasite infection in vitro, and the enhancement is prevented by the addition of exogenous *Vibrio cholerae* neuraminidase (*5*). The *T. cruzi* neuraminidase may therefore participate in the association of *T. cruzi* with mammalian hosts through a negative control mechanism.

We recently isolated an inhibitor of the *T. cruzi* neuraminidase from human plasma and named it cruzin (*6, 7*). Purified cruzin is remarkably specific for *T. cruzi* neuraminidase, with 50% inhibition being reached at concentrations as low as $10^{-9}M$ (*6*). Cruzin inhibits trypanosome desialylation of cells, but not of soluble glycoconjugates; it is equally effective when the enzyme is present in a soluble form or on the outer membrane of living parasites (*6*). Molecular characterization of the purified material indicates that the native form has an M_r of 246,000 ± 20,000 with a major subunit of M_r 28,000 ± 2,000. Kinetics analysis of cruzin activity suggested a noncompetitive mechanism of inhibition (*6*).

Since many human plasma proteins have been identified and characterized, it was reasonable to expect that cruzin was already described in the literature. Initial attempts to match cruzin with a known human plasma component by using commercially available antisera to individual plasma proteins were unsuccessful. We therefore determined the sequence of the first 20 amino acids of the major protein component of cruzin and searched for matching sequences in a protein and nucleic acid sequence database. The sequence was identical in 18 of 20 positions with the amino terminus of human apolipoprotein A-I (apoA-I) (Fig. 1a), the major protein component of plasma high-density lipoprotein (HDL). The amino acids in positions 8 and 10 could not be positively identified during the sequencing of cruzin, because the former was obscured by diphenylurea, a by-product formed during the Edman degradation, and the latter was not present in sufficient amounts for detection.

This sequence similarity suggested that cruzin contained apoA-I and that it was HDL. This possibility was tested with the following results. In SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*8*) (Fig. 1b), both cruzin and HDL displayed one major band of M_r 28,000 (apoA-I) (lanes A and B, respectively) whereas other lipoprotein fractions (*9*) such as low-density lipoprotein (LDL) (lane C) and very low density lipoprotein (VLDL) (lane D) showed a major band of M_r >200,000 (apolipoprotein B). In immunoblot analysis (*10*) with a monospecific rabbit antibody to cruzin (*11*), the antibody recognized equally well a major band of M_r 28,000 in cruzin and HDL preparations (Fig. 1c, lanes A and B). No cross-reactivity was observed with apolipoprotein B of LDL (Fig. 1c, lane C). Low levels of apoA-I were detected in both VLDL and LDL fractions (lanes C and D), indicating a cross-contamination during the purification procedure that was not revealed by Coomassie blue staining of the gel (SDS-PAGE) (Fig. 1b, lanes C and D). A similar contamination was reported previously (*12*). These results demonstrated that antibody elicited to cruzin cross-reacted with apoA-I present in the various lipoprotein preparations tested.

Additional evidence for the identity of cruzin with HDL was provided by a com-

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