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Long-term effects of the trehalase inhibitor trehazolin on trehalase activity in locust flight muscle

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SUMMARY

Trehalase (EC 3.2.1.28) hydrolyzes the main haemolymph sugar of insects, trehalose, into the essential cellular substrate glucose. Trehalase in locust flight muscle is bound to membranes that appear in the microsomal fraction upon tissue fractionation, but the exact location *in vivo* has remained elusive. Trehalase has been proposed to be regulated by a novel type of activity control that is based on the reversible transformation of a latent (inactive) form into an overt (active) form. Most trehalase activity from saline-injected controls was membrane-bound (95%) and comprised an overt form (~25%) and a latent form (75%). Latent trehalase could be assayed only after the integrity of membranes had been destroyed. Trehazolin, a potent tight-binding inhibitor of trehalase, is confined to the extracellular space and has been used as a tool to gather information on the relationship between latent and overt trehalase. Trehazolin was injected into the haemolymph of locusts, and the trehalase activity of the flight muscle was determined at different times over a 30-day period. Total trehalase activity in locust flight muscle was markedly inhibited during the first half of the interval, but reappeared during the second half. Inhibition of the overt form preceded inhibition of the latent form, and the time course suggested a reversible precursor–product relation (cycling) between the two forms. The results support the working hypothesis that trehalase functions as an ectoenzyme, the activity of which is regulated by reversible transformation of latent into overt trehalase.

Key words: trehalose, muscle fractionation, overt trehalase, latent trehalase, trehalase localization, insect, *Locusta migratoria*.

INTRODUCTION

Glucose is an essential substrate for energy metabolism in animals. In vertebrates, it is the principal sugar in the blood, which serves to distribute it to the various organs of the body. In most insects, however, glucose is a minor compound in the haemolymph, whereas the disaccharide trehalose is the main blood sugar (for review, see Wyatt, 1967; Steele, 1985; Becker et al., 1996; Candy et al., 1997; Elbein et al., 2003; Thompson, 2003). The rationale for this is as follows.

Insects have an open circulatory system, i.e. their organs are bathed in the haemolymph, from which substrates diffuse into the tissues. The efficacy of diffusion can be improved by increasing the concentration of substrates, and this seems necessary to meet metabolic demands, which can be extremely high in insects, especially when they engage in flight (for reviews, see Kammer and Heinrich, 1978; Wegener, 1996). High concentrations of haemolymph glucose would be critical because glucose is rather toxic, owing to its tendency to react non-enzymatically with proteins. Protein glycation is a major factor in the pathology of diabetes (Cohen, 1986). In insects, this problem does not arise because the two glucose units in trehalose are combined in an α-1,1-α-link, thus eliminating their reactive groups. Trehalose is produced from nutritional carbohydrate or stored glycogen by the fat body and released into the haemolymph. This metabolic detour is costly, as trehalose synthesis from glucose requires ATP and UTP (see Candy et al., 1997). Given the parsimony principle of nature, this again indicates the physiological significance of trehalose.

In locusts, haemolymph trehalose is a main store of carbohydrate and an important substrate for flight. Catabolism of trehalose is low in the flight muscle of resting locusts yet swiftly increased upon flight, and is again reduced when, during prolonged flight, the insects switch to oxidizing fat as the main substrate (for reviews, see Jutsum and Goldsworthy, 1976; van der Horst et al., 1978; Wegener, 1996; Mentel et al., 2003). To be used in muscle metabolism, trehalose must be hydrolyzed into glucose by trehalase (EC 3.2.1.28). The potential activity of trehalase in the flight muscle of locusts is high and the reaction is strongly exergonic, so that the activity of trehalase in flight muscle must be strictly controlled in a reversible manner, lest all available trehalose be split. How trehalase activity is regulated in locust flight muscle is still unknown, as none of the established mechanisms for the control of enzyme activities seems to operate (Worm, 1981; Vaandrager et al., 1989; Becker et al., 1996; Wegener et al., 2003; Liebl et al., 2010).

Trehalase in the flight muscle of locusts and other insects with synchronous flight muscles is bound to membranes that appear in the microsomal fraction upon tissue fractionation (Gussin and Wyatt, 1965; Worm, 1981; Vaandrager, 1989), but the exact cellular localization has not yet been established. However, it has been observed that trehalase activity in homogenates of flight muscles from cockroaches, moths and locusts (Zebe and McShan, 1959; Gussin and Wyatt, 1965; Gilby et al., 1967; Candy, 1974) is increased after the disruption of membrane integrity, e.g. by detergents. According to this observation, various forms of trehalase activity can be differentiated. Trehalase activity that can be measured directly in tissue homogenates by merely adding the substrate trehalose is designated as overt trehalase activity. Overt trehalase activity comprises soluble trehalase (i.e. trehalase activity in the supernatant after centrifugation at 100,000 ***g*** for 60 min) and bound (particulate) overt trehalase activity that is in the sediment

after 100,000***g*** centrifugation. Detergents have no effect on soluble trehalase activity, but bring about an increase in trehalase activity of the sediment. This additional activity is called latent trehalase activity. Bound overt trehalase plus latent trehalase make up total bound trehalase activity. The latter activity plus soluble activity accounts for total trehalase activity (for details see Materials and methods and Discussion). The sources and physiological functions of the different forms of trehalase activity remain unknown.

Interest in trehalase was boosted when very potent and highly specific competitive inhibitors of trehalase were discovered and applied to insects. These inhibitors, such as trehazolin (Ando et al., 1991) or validoxylamine A (Asano et al., 1990; Asano, 2003), are analogues of trehalose of bacterial origin that bind tightly to trehalase. When injected into insects, these inhibitors affect motor activity, feeding, metabolism, growth, development, reproduction and flight (Kono et al., 1993; Kono et al., 1994a; Kono et al., 1994b; Ando et al., 1995a; Tanaka et al., 1998), thus indicating that trehalose metabolism is involved in many aspects of insect physiology. The inhibitors interfere specifically with the hydrolysis of trehalose (Ando et al., 1995b) but apparently do not block its production and release into the haemolymph by the fat body (Kono et al., 1995; Wegener et al., 2003; Liebl et al., 2010). The prominent inhibitor-induced increase in haemolymph trehalose was noticed early and has been reported repeatedly (Kono et al., 1993; Kono et al., 1999). The precipitous decrease in haemolymph glucose was later discovered as a spectacular metabolic effect that appeared to be a crucial factor in trehazolin toxicity in locusts (Wegener et al., 2003).

Trehalase inhibitors seem to have long-lasting effects on metabolism, suggesting that trehalase activity might also be reduced for considerable time spans. This has recently been confirmed for total trehalase activity in locust flight muscle (Liebl et al., 2010). Because trehalase in locust flight muscle homogenates appears in different forms – soluble, overt and latent – with yet unknown locations and functions *in vivo*, a detailed study on the long-term effects of a trehalase inhibitor on the various forms of the enzyme seemed desirable. It was expected that the inhibitory effects on the trehalase system of flight muscle would indicate possible functions of the different forms of trehalase in intact locusts and also shed light on possible relationships between them. For these reasons, locusts were injected with a sublethal dose of trehazolin and the following questions were addressed: (1) How are the overt and latent forms of trehalase affected by trehazolin? (2) What are the time courses of inhibition and reappearance of total as well as overt and latent trehalase activities?

The results of the present study indicate that trehazolin irreversibly inhibits trehalase activity in locust flight muscle. They further suggest a reversible precursor–product relationship between latent and overt trehalase activity in locust flight muscle that could be pivotal in the regulation of trehalase activity *in vivo*. The mechanism of control appears to be based on reversibly sequestering the enzyme from its substrate. In essence, trehalase in locust flight muscle could represent a novel type of reversible regulation of enzyme activity.

MATERIALS AND METHODS

Treatment of locusts

Adult male locusts (*Locusta migratoria*, L.; Orthoptera), approximately 2 weeks after their final moult, were purchased from commercial suppliers and kept under crowded conditions as previously described (Wegener et al., 2003; Liebl et al., 2010). The haemolymph of the locusts was injected with 20 µg of trehazolin (experimental) in 5 µl of Hoyle's saline (130 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} $NaHCO_3$, 6 mmol l^{-1} Na_2HPO_4) (Hoyle, 1953) or with 5 µl saline (control). To this end, the needle of a 10 µl Hamilton syringe was inserted into the thoracic cavity through the soft membrane at the base of the right hind leg. The locusts were then kept under crowded conditions as before and provided with food and water *ad libitum*. Three trehazolin-injected experimental locusts were sacrificed and analyzed per day during the first 10 days of a 30-day period. Thereafter, analyses were performed at 13, 22, 24 and 30 days post-injection. Some of the injected locusts died during the course of the experiments, but mortality did not increase noticeably (compared with untreated locusts of the same batch).

Fractionation of locust flight muscle

In order to characterize the effects of trehazolin on the different forms of trehalase activity, locust flight muscle was fractionated by homogenization, centrifugation and treatment with detergent. Locusts were sacrificed by cutting off the tip of the abdomen and swiftly removing the head together with the adhering digestive tract. The thorax was then isolated by cutting off the abdomen, the legs and most of the wings. The thorax was cut into halves by median ventral and dorsal cuts. After removing large tracheae and air sacs as well as adhering parts of the fat body, the right half-thorax was tethered with a cotton thread to the stump of a wing and carefully washed in Hoyle's saline solution in a beaker on a magnetic stirrer at 4°C for 1 h (with one change of medium after 30 min) to remove adhering haemolymph and non-specifically bound trehazolin from the tissue. The half-thorax was carefully blotted with soft tissue to remove adhering solution, and muscle was removed and collected in Eppendorf tubes on ice. The flight muscles were fractionated as follows (see Fig. 1). The tissue was thoroughly homogenized in 9 parts (v/w) of homogenization buffer (50 mmol l^{-1} sodium maleate, pH 6.5) in a small hand-homogenizer (2 ml, glass in glass; Neolab, Heidelberg, Germany). After removing an aliquot from the homogenate [fraction 1 (F1)] to measure total overt trehalase activity, the homogenate was centrifuged in a Sorvall RC5C (40,000***g***, 4°C, 60 min; Thermo Fisher Scientific, Langenselbold, Germany) and the supernatant [fraction 2 (F2), containing soluble trehalase activity, a subfraction of the total overt trehalase] was stored on ice. The sediment was resuspended with 9 parts (v/w, minus the volume of the aliquot taken from fraction 1) of homogenization buffer with a small microhomogenizer and by repeated vigorous pipetting in and out of a 1000 µl pipette tip. This yielded fraction 3 (F3), from which an aliquot was removed to measure bound overt trehalase activity. Triton X-100 (final concentration 1%, v/v) was added to the remaining suspension, which was then sonicated (Sonifier, micro tip; Branson, Schwäbisch Gmünd, Germany), thus solubilizing bound trehalase [fraction 4 (F4), total bound trehalase activity].

Assay of trehalase activities

A discontinuous two-step assay was used that combined hydrolysis of trehalose at pH 6.5 and 30°C (step 1) and spectrophotometric measurement of the produced glucose at pH 7.6 and 25°C (step 2).

In step 1, two aliquots from each tissue fraction were pre-incubated for 20 min in Eppendorf reaction vessels in sodium acetate (120 mmol l^{-1}, pH 6.5). The trehalase reaction was started by adding trehalose at a final concentration of 20 mmol l^{-1} to one of the vessels and distilled water to the other (total volume 500 µl). After 20 min incubation, reactions were stopped with 50 µl of 15% perchloric acid. The assays were vortexed, neutralized with 10 µl of potassium

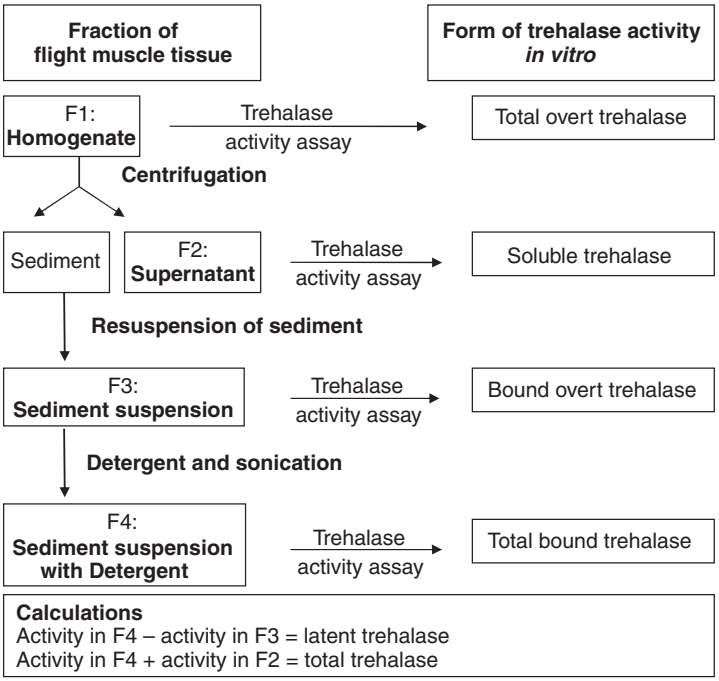

Fig. 1. Scheme of locust flight muscle fractionation to produce different fractions (F1–F4) of trehalase activity *in vitro* (for details see Fractionation of locust flight muscle). F1, homogenate, yielding total overt trehalase activity; F2, supernatant of F1, yielding soluble trehalase activity; F3, resuspended sediment of F1, yielding bound overt trehalase activity; F4, F3 plus Triton X-100 and sonication, yielding total bound trehalase activity (overt plus latent). Latent trehalase activity was calculated as the activity in F4 minus the activity in F3. Total trehalase activity was calculated as the activity in F4 plus the activity in F2.

carbonate (5 mol l^{-1} K_2CO_3) and centrifuged at 10,000 ***g*** for 5 min to precipitate the potassium perchlorate. The supernatants were kept at –20°C for measuring glucose in step 2 of the assay.

Step 2 of the assay used 96-well microtiter plates and the Multireader Ascent (Pharmacia, Freiburg, Germany) to enzymatically measure the glucose produced by trehalase in step 1. The assay was performed in a volume of 200 µl at 25°C and pH 7.6 and comprised 150 mmol l^{-1} triethanolamine, 10 mmol l^{-1} $MgCl_2$, 0.6 mmol l^{-1} $NADP^+$, 0.5 mmol l^{-1} ATP and either 10 or 20 µl sample from step 1. After 5 min, the assays were started by adding a mixture of hexokinase and glucose-6-phosphate dehydrogenase (0.21 and 0.33 U $well^{-1}$, respectively). The extinctions at 340 nm were read after 10 and 15 min to make sure all glucose had been transformed. The readings were corrected by subtracting the glucose present in the blank assays (without addition of trehalose, see above) to ensure only glucose produced by trehalase activity was taken into account. This glucose production (=trehalase activity) in step 1 has a linear relationship with incubation time and amount of tissue fraction.

Presentation of data and statistics

Trehalase activities are given as international units (U) per g fresh muscle and are means ± s.e.m. based on the number of locusts (*N*). One U is the activity hydrolyzing 1 µmol trehalose min^{-1} (at 30°C). The Mann–Whitney *U*-test was used to analyze data for statistical significance.

RESULTS

Effects of trehazolin on locust behaviour

Locusts injected with Hoyle's saline (control) behaved as usual whereas those injected with trehazolin in saline (experimental) showed reduced spontaneous motor activity and hardly jumped to escape when they were approached or picked up. Consumption of food was also reduced during the first third of the experiment. After ~10 days, the behaviour of trehazolin-injected locusts had returned to normal.

Demonstration of various forms of trehalase activity by flight muscle fractionation

Fractionation of flight muscle from control locusts (injected with 5 µl of Hoyle's saline) resulted in different forms of trehalase activity (see Fig. 1, Table 1). On average, ~25% of the total trehalase activity was measured in the homogenate (F1) by adding trehalose (at a final concentration of 20 mmol l^{-1}) to start the reaction. This trehalase activity, the total overt trehalase activity, was separated into two distinct forms of overt trehalase by centrifugation (see Table 1). Close to 20% of total overt trehalase remained in the supernatant after centrifugation at 40,000 ***g*** whereas more than 80% was in the sediment. Control experiments using ultra-centrifugation (100,000 ***g*** for 1 h in a Beckman centrifuge) gave very similar results. Therefore, trehalase in the supernatant (F2) was designated soluble trehalase activity. Soluble trehalase could not be activated by Triton X-100 or other detergents.

In order to analyze the trehalase in the sediment, the centrifugation pellets were resuspended and their trehalase activity was measured and characterized as bound overt trehalase activity (F3). Under ideal conditions, the sum of the soluble and bound overt trehalase activities should equal the total overt trehalase activity. In the present experiments, bound overt trehalase activity was usually higher than

Table 1. Forms of trehalase activity in fractions of locust flight muscle

	Trehalase activity (U g^{-1} muscle at 30°C)			
	Day 1	Day 10	Day 20	Day 30
Total overt (F1)	1.29±0.07	1.01±0.11	1.28±0.22	1.35±0.22
Soluble (F2)	0.20±0.03	0.21±0.0	0.32±0.01	0.24±0.01
Bound overt (F3)	1.14±0.04	1.14±0.05	1.21±0.13	0.98±0.13
Total bound (F4)	4.44±0.27	4.25±0.05	5.13±0.18	4.41±0.29
Latent (F4–F3)	3.29±0.28	3.12±0.05	3.92±0.31	3.43±0.27
Total activity (F4+F2)	4.64±0.30	4.46±0.04	5.45±0.17	4.65±0.28

Male locusts approximately 2 weeks after the final moult were injected with 5 µl of Hoyle's saline. The locusts were sacrificed on the days indicated, their flight muscles fractionated and the trehalase activity assayed (see Fig. 1). F1–F4, fractions of trehalase activity. Values are means ± s.e.m. (*N*=3).

expected and, in some cases, even exceeded total bound trehalase activity. These observations indicate that resuspension of the pellets transformed some latent trehalase activity into overt trehalase activity (see below and Discussion).

Addition of Triton X-100 (1% final concentration) to the resuspended pellets plus sonication (yielding F4) caused a marked increase in trehalase activity, and all trehalase in F4 was solubilized (i.e. could not be sedimented by ultra-centrifugation). This result was interpreted as evidence for the existence of an occluded form of trehalase (latent trehalase) in F3 that was bound to cellular membranes and inactive because it was sequestered from its substrate (see below and Discussion). The trehalase activity in F4 was hence regarded as representing total bound activity (total particulate trehalase activity), and its latent fraction was calculated as the trehalase activity in F4 minus that in F3 (see Fig. 1). Total trehalase activity was calculated as the trehalase activity in F4 plus that in F2.

Effects of trehazolin on total trehalase activity of locust flight muscle

Control locusts, injected with Hoyle's saline and analyzed after 1, 10, 20 and 30 days, had very similar trehalase activities in all fractions of flight muscle. In particular, the data do not indicate an effect of ageing on any form of trehalase over the 30-day period of the experiment (see Table 1).

Injection of trehazolin into locust haemolymph had dramatic effects on trehalase activity in fractions of flight muscle. The total activity of trehalase was reduced to $1.75\,U\,g^{-1}$ one day after the injection (compared with $4.64\,U\,g^{-1}$ in controls). It was decreased further on day 2 and reached very low values from day 4 to day 8, with activities between 0.50 (on day 6) and $0.70\,U\,g^{-1}$ (on day 8). The activities were hardly elevated until day 13, but had significantly increased to 2.8 and $3.0\,U\,g^{-1}$ on days 22 and 24, respectively (Fig. 2). When the experiment was finished at day 30, total trehalase activity had returned to control levels.

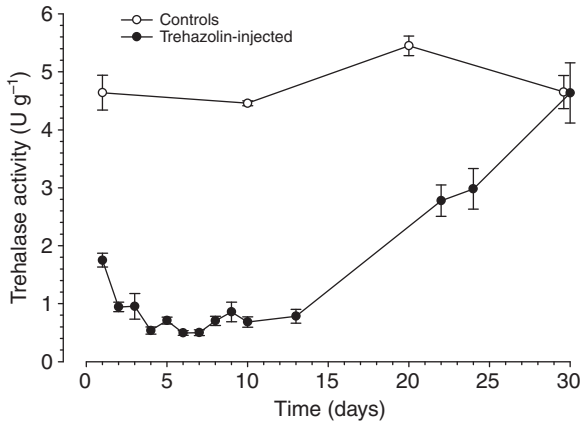

Fig. 2. Long-term effects of trehazolin on the total activity of trehalase in locust flight muscle. Male locusts, about 2 weeks after the final moult, were injected with 20 g of trehazolin in 5 µl Hoyle's saline and the total activity of trehalase was measured (see Materials and methods) at 30°C on the days indicated. Controls (open symbols) were injected with 5 µl of saline only. Activities are given as $U\,g^{-1}$ flight muscle and are means ± s.e.m. (N=3). Total trehalase activity was significantly reduced by trehazolin but reappeared in the second half of the 30-day experiment.

Effects of trehazolin on trehalase forms in locust flight muscle fractions

The changes in trehalase activity with time become more meaningful if the overt and latent trehalase that together make up the total activity are presented separately (see Fig. 3). The activity of total overt trehalase decreased rapidly and reached a minimum of $0.15\,U\,g^{-1}$ two days after the injection of trehazolin. Overt activity remained low until day 4, when it transiently increased to a plateau between days 5 and 9 (with trehalase activities ranging from 0.33 to $0.49\,U\,g^{-1}$). During the last 10 days of the experiment, overt activity returned to control levels (Fig. 3).

The activity of latent trehalase (i.e. the activity that appeared after destruction of the structural integrity of membranes by detergents) from the flight muscle of trehazolin-injected locusts initially followed a time course similar to that of overt trehalase, but lagged behind by 4 to 5 days. Overt trehalase had minimal activity from day 2 to day 4, whereas latent trehalase was minimal between days 6 and 8, when it fell to levels between 2.4% (at day 6) and 1.6% (at day 8) of the control values. Interestingly, during this minimum phase of latent activity, the activity of overt trehalase went through a transient maximum. The mirror-symmetry of the resulting curves suggests a relationship between the two forms of trehalase that is consistent with the view that latent trehalase was a precursor or a storage form of overt trehalase (see Fig. 3 and Discussion).

Analysis of data using a Mann–Whitney U-test revealed that the time courses of overt and latent trehalase activity were significantly different (for details see legend of Fig. 3).

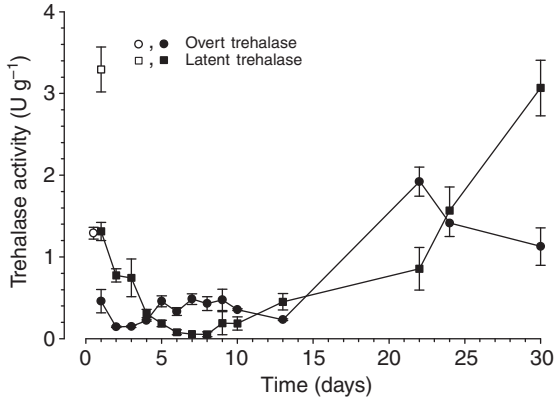

Fig. 3. Long-term effects of trehazolin on the overt and latent activity of trehalase in fractions of locust flight muscle. The data are based on the same locusts as shown in Fig. 2, but the overt and latent forms of trehalase are presented to demonstrate that trehazolin affects overt and latent trehalase differently, thus indicating a time-dependent relationship between the two forms (for details see Effects of trehazolin on trehalase forms). Activities are given as $U\,g^{-1}$ flight muscle and are means ± s.e.m. (N=3). Open symbols represent control locusts injected with Hoyle's saline. Statistics (Mann-Whitney U-test) proved the time courses of overt and latent trehalase activities to be significantly different. From day 1 to day 3 after injection of trehazolin, overt trehalase activity was significantly lower than the latent activity for each of the three days (P<0.05, N=3). When the overt activities of the three days were pooled they proved significantly lower than the pooled latent activities (P<0.001, N=9). The opposite relationship holds true during the interval from day 5 to day 10 post-injection when, with the exception of day 9, overt activity exceeded latent activity (P<0.05, N=3). When the data from day 5 to day 10 were pooled, the difference between overt and latent activities was highly significant (P<0.00001, N=18).

DISCUSSION

Effects of trehazolin on locust behaviour

Injection of 20 µg of trehazolin markedly reduced motor activity and food consumption in experimental locusts (see also Wegener et al., 2003; Liebl et al., 2010). This is regarded as a consequence of the fact that the poisoned locusts were unable to utilize carbohydrates properly. The inhibitor trehazolin suppresses the production of glucose from trehalose, and glucose seems to be vital for physiological functions and survival of locusts (see Wegener et al., 2003).

Forms of trehalase activity in locust flight muscle

Fractionation of flight muscle from locust revealed the existence of different forms of trehalase activity *in vitro*. Soluble trehalase activity comprised a minor subfraction of overt trehalase activity, ~5% of total trehalase activity, and part of the soluble trehalase could have been artificially detached from membranes during homogenization. Soluble trehalase does not seem to play a significant role in flight muscle because there is no conclusive proof for its existence in flight muscle *in vivo* and its activity in flight muscle fractions is minor and appears not to be regulated.

Latent trehalase, which is always associated with membranes, is the main and most interesting form of trehalase activity in homogenized flight muscle from resting locusts. The existence of bound (particulate) trehalase that could be activated by membrane destruction has been reported (Gussin and Wyatt, 1965; Candy, 1974; Worm, 1981; Vaandrager et al., 1989; Wegener et al., 2003), but its source and function have remained elusive. In the present study, latent trehalase activity was underestimated rather than overestimated because the resuspension of the membrane pellet produced more overt trehalase activity than expected (see Results and Table 1). This could be due to damage to membranes that (artificially) transformed latent into overt trehalase activity (see below).

Latent trehalase *in vitro* was inactive, even at high substrate concentrations, unless it was activated (i.e. became solubilized and, hence, overt) by the destruction of membranes with detergent. This indicates that inhibition of activity in latent trehalase does not act on the enzyme directly, but is due to a barrier that separates trehalase from its substrate. The barrier is obviously based on structurally intact membranes. This view is supported by the observation that all forms of trehalase that were active *in vitro* had high affinities for trehalose. Soluble trehalase (F2), the bound overt trehalase (F3) and solubilized (previously bound overt plus latent) trehalase (F4) showed Michaelis–Menten kinetics and K_m-values of ~1 mmol l^{-1} (P.S. and G.W., unpublished results). This strongly indicates that the substrate trehalose has easy access to the active site of the enzyme in active forms of trehalase but not in latent trehalase. Results of the present study support the view that there is a dynamic precursor–product relationship between the latent and overt forms of trehalase activity *in vivo*.

Trehazolin and total trehalase activity in locust flight muscle

Trehazolin is a potent tight-binding inhibitor of locust flight muscle trehalase *in vitro*, and also caused a marked long-term inhibition of total trehalase activity of flight muscle *in vivo*. Contrary to our expectation (see below), the inhibition was not complete, which prompted speculations about the compartmentation of trehazolin and trehalase in locust flight muscle. The remaining trehalase activity in trehazolin-injected locusts could not have been due to dissociation of the trehalase-trehazolin complex during washing of the flight muscles (60 min at 4°C) because trehalase inhibited by trehazolin could not be reactivated by extended dialysis under these conditions (C.M. and G.W., unpublished results). Ando et al. have reported the same result on trehalase from the *Bombyx* midgut (Ando et al., 1995b).

The concentration of trehazolin that causes 50% inhibition in purified flight muscle trehalase (IC_{50}) was ~10^{-7} mol l^{-1} (at 20 mmol l^{-1} trehalose without pre-incubation of enzyme and inhibitor) (see Wegener et al., 2003). Even if the 20 µg trehazolin (=54.6 nmol) injected into locust haemolymph had been evenly distributed in the total water space of a locust (~1 ml), the concentration of trehazolin would still be ≥50 µmol l^{-1}, i.e. more than 2 orders of magnitude higher than the IC_{50} *in vitro*. However, trehazolin obviously does not permeate intact cellular membranes, but seems confined to the haemolymph (extracellular space). Liebl et al. determined the haemolymph concentration of trehazolin as 142.3 µmol l^{-1} two hours after injection of 20 µg (Liebl et al., 2010), and this concentration accounted for almost all of the injected trehazolin. The concentration of trehazolin in the haemolymph at this time was hence about 1000-times the IC_{50}. Like trehazolin, trehalose is apparently not transported from the haemolymph into the muscle cells, as no transporter for trehalose has yet been reported in locusts. The observation that trehalase was only partially inhibited by trehazolin after 24 h incubation *in vivo* (when the haemolymph concentration was >10^{-4} mol l^{-1}) (see Liebl et al., 2010) is an indication that trehazolin had access to only a fraction of trehalase molecules of flight muscle *in vivo*. This indicates that another fraction of trehalase in locust flight muscle can (temporarily) be confined to a metabolic compartment not reached by trehazolin.

The effects of trehazolin on the different forms of trehalase activity led to a working hypothesis about the relationship between overt and latent trehalase and the mechanism by which trehalase might be controlled in locust flight muscle.

Trehazolin as a tool to study overt and latent trehalase activities and their relationship

The activity of all forms of trehalase in flight muscle from locusts injected with Hoyle's saline (controls) showed no age-related changes over the 30 days of the experiment (Table 1). The changes in trehalase activities in flight muscle from locusts injected with trehazolin must therefore be attributed to the trehalase inhibitor. These changes were dramatic, long lasting and indicated a dynamic relationship between the overt and latent forms of trehalase activity.

The time course of total trehalase activity after injection of trehazolin was represented by a simple and typical curve characterized by a precipitous initial decrease, followed by a prolonged phase of minimal activity and a slow recovery thereafter (Fig. 2). However, total trehalase activity is not a simple parameter, but the sum of overt and latent trehalase activities. When the components were displayed separately it became apparent that they followed significantly different time courses (for *P*-values, see legend of Fig. 3) and that the seemingly simple curve of total activity concealed a dynamic relationship between latent and overt trehalase. Overt trehalase dropped precipitously to an early minimum but then increased to a transient plateau (between days 5 and 10) when latent trehalase reached a corresponding minimum. These counterbalancing changes suggest that the transiently increased overt activity is brought about at the expense of latent activity. Latent activity could therefore be interpreted as a precursor or a storage form of trehalase activity that was not initially exposed to the inhibitor, but was later transformed into overt trehalase and then inhibited by trehazolin.

As a working hypothesis we interpret overt trehalase (*in vitro*) as originating from trehalase (*in vivo*) that was exposed to the inhibitor in the haemolymph, whereas the *in vitro* latent form of trehalase is derived from trehalase shielded from the inhibitor by membranes. Trehalase inhibitors such as trehazolin are structurally similar to trehalose and they act as competitive inhibitors with a high affinity for trehalase, probably by mimicking the transition state of trehalose in the trehalase reaction (Ando et al., 1995b). Two observations are pertinent to the current discussion and support the working hypothesis: (1) as mentioned before, trehazolin injected into locust haemolymph remains in this compartment, from which it is excreted within ~10 days (Liebl et al., 2010) and (2) trehalase from locust flight muscle is a mannose-rich, N-linked glycoprotein (P.S. and G.W., unpublished results). If bound to membranes, a glycoprotein of this type is orientated in such a way that the sugar moieties will be directed either towards the extracellular space (if the protein is localized in plasma membranes) or towards the lumen of intracellular membrane structures (e.g. endoplasmic reticulum, Golgi complex, vesicles). This fact is significant because no transporter for trehalose has been reported in locusts. If trehalose cannot permeate cell membranes, trehalase must function as an ectoenzyme as has been suggested (Wegener et al., 2003). Trehalose would hence be hydrolyzed extracellularly and the product glucose transported across the plasma membrane. The mechanism(s) that control the reversible transformation of the overt and latent forms of trehalase could be the basis of a novel type of reversible regulation of enzyme activity. This makes trehalase an intriguing enzyme beyond the field of insect physiology.

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REFERENCES

Ando, O., Satake, H., Itoi, K., Sato, A., Nakajima, M., Takahashi, S., Haruyama, H., Ohkuma, J., Kinoshita, I. and Enokita, R. (1991). Trehazolin, a new trehalase inhibitor. *J. Antibiot.* **4**, 1165-1168.

Ando, O., Kifune, M. and Nakajima, M. (1995a). Effects of trehazolin, a potent trehalase inhibitor on *Bombyx mori* and plant pathogenic fungi. *Biosci. Biotech. Biochem.* **59**, 711-712.

Ando, O., Nakajima, M., Kifune, M., Fang, H. and Tanzawa, K. (1995b). Trehazolin, a slow tight-binding inhibitor of silkworm trehalase. *Biochim. Biophys. Acta* **1244**, 295-302.

Asano, N. (2003). Glycosidase inhibitors: uptake and perspectives on practical use. *Glycobiology* **13**, 93R-104R.

Asano, N., Takeuchi, M., Kameda, Y., Matsui, K. and Kono, Y. (1990). Trehalase inhibitors, validoxylamine A and related compounds as insecticides. *J. Antibiot.* **43**, 722-726.

Becker, A., Schlöder, P., Steele, J. E. and Wegener, G. (1996). The regulation of trehalose metabolism in insects. *Experientia* **52**, 433-439.

Candy, D. J. (1974). The control of muscle trehalase activity during locust flight. *Biochem. Soc. Trans.* **2**, 1107-1109.

Candy, D. J., Becker, A. and Wegener, G. (1997). Coordination and integration of metabolism in insect flight. *Comp. Biochem. Physiol.* **117B**, 497-512.

Cohen, M. P. (1986). *Diabetes and Protein Glycosylation.* New York, Berlin: Springer.

Elbein, A. D., Pan, Y. T., Pastuszak, I. and Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. *Glycobiology* **13**, 17R-27R.

Gilby, A. R., Wyatt, S. S. and Wyatt, G. R. (1967). Trehalases from the cockroach, *Blaberus discoidalis:* activation, solubilization and properties of the muscle enzyme and some properties of the intestinal enzyme. *Acta Biochim. Pol.* **14**, 83-100.

Gussin, A. E. S. and Wyatt, G. R. (1965). Membrane-bound trehalase from cecropia silkmoth muscle. *Arch. Biochem. Biophys.* **112**, 626-634.

Hoyle, G. (1953). Potassium ions and insect nerve muscle. *J. Exp. Biol.* **30**, 121-135.

Jutsum, A. R. and Goldsworthy, G. J. (1976). Fuels for flight in *Locusta*. *J. Insect Physiol.* **22**, 243-249.

Kammer, A. E. and Heinrich, B. (1978). Insect flight metabolism. *Adv. Insect Physiol.* **13**, 133-228.

Kono, Y., Takeda, S., Kameda, Y., Takahashi, M., Matsushita, K., Nishina, M. and Hori, E. (1993). Lethal activity of a trehalase inhibitor, validoxylamine A, and its influence on the blood sugar level in *Bombyx mori* (Lepidoptera: Bombycidae). *App. Entomol. Zool.* **28**, 379-386.

Kono, Y., Takahashi, M., Matsushita, K., Nishina, M., Kameda, Y. and Hori, E. (1994a). Inhibition of flight in *Periplaneta americana* (Linn.) by a trehalase inhibitor, validoxylamine A. *J. Insect Physiol.* **40**, 455-461.

Kono, Y., Takeda, S. and Kameda, Y. (1994b). Lethal activity of a trehalase inhibitor, validoxylamine A, against *Mamestra brassicae* and *Spodoptera littura*. *J. Pesticide Sci.* **19**, 39-42.

Kono, Y., Takeda, S., Kameda, Y., Takahashi, M., Matsushita, K., Nishina, M. and Hori, E. (1995). NMR analysis of the effect of validoxylamine-A, a trehalase inhibitor, on the larvae of the cabbage army-worm. *J. Pesticide Sci.* **20**, 83-91.

Kono, Y., Tanaka, S., Hasegawa, E., Takahashi, M., Nishina, M. and Matsushita, K. (1999). The role of trehalose in lipid mobilization of *Locusta migratoria*: effects of a trehalase inhibitor, validoxylamine A, on flight activity and hemolymph trehalose and lipid concentrations. *Entomol. Sci.* **2**, 449-456.

Liebl, M., Nelius, V., Kamp, G., Ando, O. and Wegener, G. (2010). Fate and effects of the trehalase inhibitor trehazolin in the migratory locust (*Locusta migratoria*). *J. Insect Physiol.* **56**, 567-574.

Mentel, T., Duch, C., Stypa, H., Wegener, G., Müller, U. and Pflüger, H.-J. (2003). Central modulatory neurons control fuel selection in flight muscle of migratory locust. *J. Neurochem.* **15**, 1109-1113.

Steele, J. E. (1985). Control of metabolic processes. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 8 (ed. G. A. Kerkut and L. I. Gilbert), pp. 99-145. Oxford: Pergamon Press.

Tanaka, S., Okuda, T., Hasegawa, E. and Kono, Y. (1998). Suppression of oocyte development by a trehalase inhibitor, validoxylamine A, through inhibition of juvenile hormone biosynthesis and vitellogenesis in the migratory locust, *Locusta migratoria*, L. *Entomol. Sci.* **1**, 313-320.

Thompson, S. N. (2003). Trehalose – the insect blood sugar. *Adv. Insect Physiol.* **31**, 205-285.

Vaandrager, S. H., Haller, T. B., van Marrewijk, W. J. A. and Beenakkers, A. M. Th. (1989). Fractionation and kinetic properties of trehalase from flight muscle and hemolymph of the locust, *Locusta migratoria*. *Insect Biochem.* **19**, 89-94.

Van der Horst, D. J., Van Doorn, J. M. and Beenakkers, A. M. Th. (1978). Dynamics in the haemolymph trehalose pool during flight of the locust *Locusta migratoria*. *Insect Biochem.* **8**, 413-416.

Wegener, G. (1996). Flying insects: model systems in exercise physiology. *Experientia* **52**, 404-412.

Wegener, G., Tschiedel, V., Schlöder, P. and Ando, O. (2003). The toxic and lethal effects of the trehalase inhibitor trehazolin in locusts are caused by hypoglycaemia. *J. Exp. Biol.* **206**, 1233-1240.

Worm, R. A. A. (1981). Characterization of trehalase from locust flight muscle. *Comp. Biochem. Physiol.* **70B**, 509-514.

Wyatt, G. R. (1967). The biochemistry of sugars and polysaccharides in insects. *Adv. Insect Physiol.* **4**, 287-360.

Zebe, E. C. and McShan, W. H. (1959). Trehalase in thoracic muscle of the woodroach, *Leucophaea maderae*. *J. Cell. Comp. Physiol.* **53**, 21-29.