J Biochem. **130**, 575–580 (2001)

Efficient Folding of the Insect Neuropeptide Eclosion Hormone by Protein Disulfide Isomerase[1]

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Received July 11, 2001, accepted August 6, 2001

Eclosion hormone is an insect neuropeptide that consists of 62 amino acid residues including three disulfide bonds. We have previously reported its hypothetical 3D structure consisting mainly of three α-helices. In this paper, we report the effects of chaperone proteins on the refolding of denatured eclosion hormone in a redox buffer containing reduced and oxidized glutathione. Urea-denatured eclosion hormone was spontaneously reactivated within 1 min with a yield of more than 90%, while β-mercaptoethanol-denatured eclosion hormone was reactivated in a few minutes with a yield of 75%. Under the same experimental conditions, eclosion hormone treated with β-mercaptoethanol and urea was reactivated slowly with a yield of 47% over a period of 2 h. Protein disulfide isomerase, a eucaryotic chaperone protein, markedly increased the reactivation yield and rate of the totally denatured hormone. GroE oligomers slightly improved the reactivation yield but peptidyl prolyl isomerase had no influence on yield or rate. We propose that the folding pathway of eclosion hormone involves at least two rate-limiting steps, and that protein disulfide isomerase is likely to be involved in the folding in insect neuronal cells.

Key words: eclosion hormone, folding, GroE, molecule chaperone, protein disulfide isomerase.

Eclosion hormone is an insect neurosecretory peptide that plays crucial roles in ecdyses and eclosion behaviour in an inositoltrisphosphate-dependent manner (*1–3*). The primary structure of eclosion hormone from silkworm brain consists of 62 amino acid residues including six cysteines (*4*). The map of three disulfide bonds formed by these cysteine residues has been elucidated (*5*).

In previous papers, we have reported that the eclosion hormone contains three α-helices linked by three disulfide bonds in the molecule (*6, 7*). The helices are indispensable for maintaining the active peptide structure. Moreover, we found residues Met24, Phe25, Phe29, Pro47, Ile55, Phe58, and Leu59 important for the activity of silkworm eclosion hormone (*8, 9*). In order to understand these results stereochemically, we constructed a 3D structure using computer aided molecular modeling (*6*). The hypothetical 3D model showed that Phe25 and Phe58 interact in a hydrophobic manner to keep a globular form Phe29, Pro47, Ile55, and Leu59 are exposed to the solvent to cause a hydrophobic interaction with an eclosion hormone receptor

Although the structure of eclosion hormone has been elucidated as mentioned above, there is no knowledge of the folding pathway. Umemura *et al.* have reported that several hours are required for the spontaneous folding of synthetic eclosion hormone *in vitro* (*10*). However, the biosynthesis and release of eclosion hormone are controlled precisely by circadian rhythm, requiring both rapid folding and a precise control of gene-expression.

Within a cell, there exists a series of proteins called chaperones, such as GroE, protein disulfide isomerase (PDI) and peptidyl prolyl isomerase (PPI). These proteins may make the folding of newborn polypeptides within a cell more efficient and quicker than *in vitro* folding.

X-ray crystal structure analyses have shown that the *Escherichia coli* chaperone GroEL oligomer is composed of 14 identical 57 kDa subunits arranged in two heptameric rings stacked back to back (*11*). Each ring defines a central cavity 45 Å in diameter. The GroES oligomer is composed of seven 10 kDa subunits arranged in a heptameric ring, which binds to a GroEL-ring to promote the folding of non-native polypeptides with the aid of ATP (*12*). It remains unclear what kinds of structural features of polypeptides are recognized by the GroEL oligomer. Several different proposals, from a fully unfolded conformation to a native-like one, have been described (*13–16*).

On the other hand, PDI, with a molecular mass of 55 kDa, is localized as a homodimer in the lumen of the endoplasmic reticulum. PDI catalyzes the formation of disulfide linkages of non-native polypeptides (*17*). Interestingly, Song *et al.* have reported that PDI accelerates the folding of a protein that does not contain cysteine residues, demonstrating that PDI can act as a chaperone (*18, 19*). Recently, PDI was found to be a heterodimer with PPI (*20, 21*). PPI catalyzes the *cis-trans* isomerization of non-native polypeptides, so that protein folding is fully facilitated (*22*). How-

[1] This work was supported, in part, by Grants-in-Aid for millennium projects from the Ministry of Education, Science, Sports and Culture of Japan

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Abbreviations: EH, eclosion hormone, ME, β-mercaptoethanol.

ever, the functional role of the heterodimer has not yet been elucidated.

In this paper we report that the chaperone-like protein PDI is likely to be involved in the folding of an insect neuropeptide, eclosion hormone.

MATERIALS AND METHODS

GroEL, GroES, and bovine PDI were purchased from Takara. Bovine PPI was kindly donated by Miss C. Yosho in the laboratory of molecular biology, Ritsumeikan University. β-Mercaptoethanol, urea, acetonitrile were from Nacalai Tesque. Bovine serum albumin (BSA) was from Sigma. All other reagents were of the highest grade commercially available.

Preparation of Recombinant Eclosion Hormone—We prepared *Bombyx* eclosion hormone from recombinant *E. coli* as previously reported (*8*). Briefly, the plasmid pPL-MEH containing the silkworm eclosion hormone gene was expressed in a strain, LC137. After cultivation, bacterial cells were lysed by sonication with a Sonifier II (BRANSON). Inclusion bodies were washed with buffer A (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 3 M urea) and solubilized in buffer B (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 7 M urea) for 1 h (*23*). After subsequent centrifugation, the crude extract was subjected to precipitation with 50% ammonium sulfate. The pellet was extracted with 40% acetonitrile in 0.1% TFA and the extract was further purified by RP-HPLC (C18 column; YMC) using 34% acetonitrile as the eluant.

Circular Dichroism (CD) Measurements—CD spectra of the native and denatured eclosion hormones were measured with a JASCO J600 spectropolarimeter at room temperature. A quartz cell with a path length of 0.1 cm was used in the far ultraviolet regions (195–260 nm). The peptide samples were desolved in phosphate buffer. The molar ellipticity of the peptide in the solutions was calculated from the equation of $[\theta] = \theta/(10\ lcn)$, where θ is the observed ellipticity in millidegrees.

Denaturation and Reactivation of Eclosion Hormone—Eclosion hormone prepared in 50 mM sodium phosphate buffer, pH 8.0, at a concentration of 140 μg/ml, was reacted with various concentrations of β-mercaptoethanol at 25°C for 1 h. Subsequently, each solution was boiled for 10 min. Then, urea was added to the solutions at a final concentration of 8 M in order to complete denaturation. Eclosion hormone, reacted with 50 mM β-mercaptoethanol for 1 h and boiled for 10 min, was used as a β-mercaptoethanol-denatured eclosion hormone (ME-denatured eclosion hormone). The ME-denatured eclosion hormone was further treated with 8 M urea to be used as an urea and ME-denatured eclosion hormone.

Reactivation was initiated by diluting the denatured eclosion hormones in reactivating buffer and incubating at 25°C for 1 min to 2 h. The reactivating buffer consisted of 50 mM Tris-HCl (pH 7.8), 1 mM reduced glutathione, 0.5 mM oxidized glutathione, 10 mM $Mg(CH_3COO)_2$, and various concentrations of chaperone proteins or BSA (0 1 mg/ml). The concentrations of reduced and oxidized glutathione used have already been determined to be optimum for the folding of synthetic eclosion hormone (*10*). Aliquots of the reaction mixture were withdrawn at time intervals and assayed for eclosion activity using pharate adults (see "Bioassay"). Reactivation yield was determined as the percentage of hormonal units relative to the native hormone. The concentrations of GroEL and GroES that refer to the 14-mer and 7-mer, respectively, were calculated as one molecule.

Bioassay of Native and Reactivated Eclosion Hormones—The 5th instar larvae of silkworm, *Bombyx mori*, were purchased from Katakura Industrial (Saitama). The larvae were reared at 25°C with a 16-h-light/8-h-dark photoperiod. Pharate adults were selected by the developmental marker antennae appearing on the 9th day (male) or 11th day (female) after pupation. Bioassays were conducted under a prolonged light period 2–4 h before natural eclosion (*24, 25*). The amount of eclosion hormone in each assay was quantified before dilution by the Bradford method using BSA as a molecular weight standard. Statistical analyses were made by Student's t test with $p < 0.05$ regarded as significant.

RESULTS

The ED_{50} value of the purified eclosion hormone for bioactivity was 0.2 ng, which is the same value as the native eclosion hormone purified from *Bombyx*, suggesting that the eclosion hormone purified from recombinant *E. coli* forms complete disulfide linkages.

The assays showed the eclosion hormone to be active even if allowed to react with 25 mM β-mercaptoethanol for 1 h at 25°C, while subsequent boiling for 10 min resulted in a complete loss of bioactivity (Fig. 1). The CD spectrum of 25 mM β-mercaptoethanol–treated eclosion hormone was very similar to that of the native eclosion hormone. The spectra showed strong α-helical patterns, namely negative Cotton bands at 208 and 222 nm (Fig. 2). However, subsequent boiling resulted in a decrease in the absorption at 208 and 222 nm to 65% This value indicates that about two-thirds of the α-helices remained in the ME-denatured eclosion hormone, but the hormonal activity was totally lost. Furthermore, the spectrum of urea and ME-denatured eclosion hormone showed that it contain no marked secondary structure (Fig. 2) Treatment of intact eclosion hormone with 8 M urea caused complete inactivation, and the CD

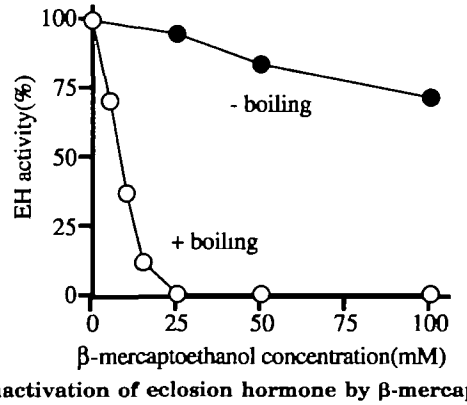

Fig 1 **Inactivation of eclosion hormone by β-mercaptoethanol.** EH prepared in 50 mM sodium phosphate buffer, pH 8 0, at a concentration of 140 μg/ml was reacted with various concentrations of β-mercaptoethanol at 25°C for 1 h (●) Subsequently, the solutions were boiled for 10 min to complete inactivation (○) Bioassays were used to monitor the effect of β-mercaptoethanol and boiling treatments. Data points are the means of two independent experiments

spectrum of this inactivated hormone was similar to that of the urea and ME-denatured eclosion hormone (data not shown).

The reactivation of denatured eclosion hormones was initiated by 40-fold dilution in the reactivating buffer. The hormonal activity of the urea and ME-denatured eclosion hormone was spontaneously restored with a yield of 47% over a period of 2 h. The $t_{1/2}$ for reactivation was about 20 min (Fig. 3). However, under the same experimental conditions, ME-denatured eclosion hormone showed a fast reactivation with a yield of 75%. The $t_{1/2}$ for the reactivation was about 1 min (Fig. 3). Moreover, the dilution of 8 M urea-denatured eclosion hormone led to an immediate reactivation within 1 min with a yield of more than 90% (Fig. 3). The $t_{1/2}$ for the immediate reactivation could not be calculated accurately.

In the presence of a 5-fold molar excess of GroE 21-mer oligomers over the urea and ME-denatured eclosion hormone, the reactivation yield improved slightly (about 10%), while the reactivation rate did not improve significantly

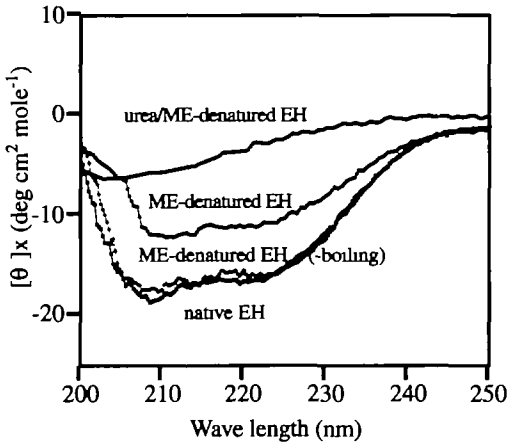

Fig 2 **CD spectra of native and denatured eclosion hormones.** β-Mercaptoethanol–treated eclosion hormones with and without boiling were prepared as in Fig 1 Urea and ME-denatured EH was prepared by adding urea at a final concentration of 8 M to the solution of ME-denatured EH Samples were subjected to the CD spectra measurements at room temperature.

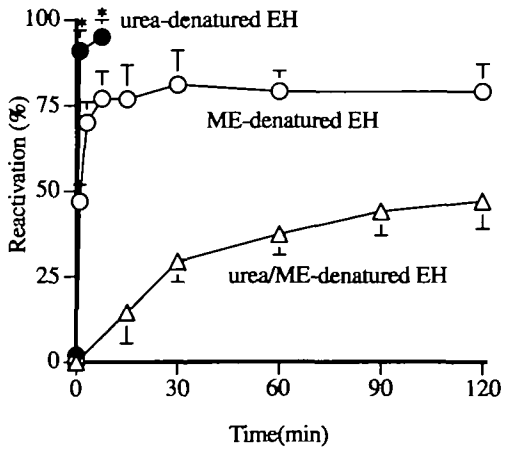

Fig 3. **The time courses of spontaneous reactivation of denatured eclosion hormones.** Reactivation was achieved by rapid dilution in reactivation buffer in the presence of 0 1 mg/ml BSA. (△) urea and ME-denatured EH, (○) ME-denatured EH, (●) urea-denatured EH. Each data point represents the mean ± SD of three or more independent assays All the results obtained with ME-denatured EH were significantly different from those obtained with urea and ME-denatured EH * $p < 0.01$ compared to the value obtained with ME-denatured EH

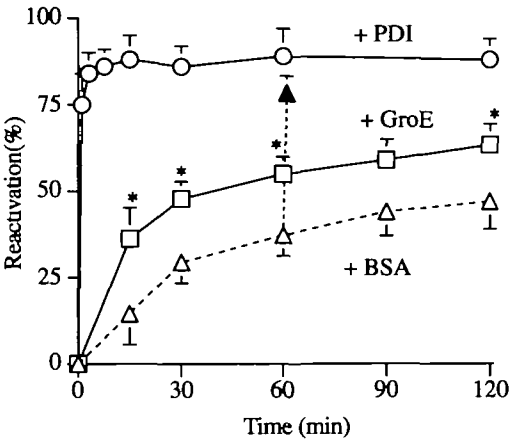

Fig 4 **Effects of GroE oligomers or PDI on the time course of reactivation of urea and ME-denatured EH.** Reactivation was achieved by rapid dilution in reactivation buffer in the presence of 0 1 mg/ml BSA (△), a 5-fold molar excess of GroE oligomers + 2 mM ATP (□), or a 5-fold molar excess of PDI (○). The data shown by open triangles are from Fig 3 A 5-fold molar excess of PDI was added to the reactivating mixture in the presence of BSA 60 min after dilution and the reaction was continued for 1 min (▲) Each data point represents the mean ± SD of three or more independent assays All the results obtained with PDI were significantly different from those obtained with BSA. * $p < 0.01$ compared to the control value obtained with BSA.

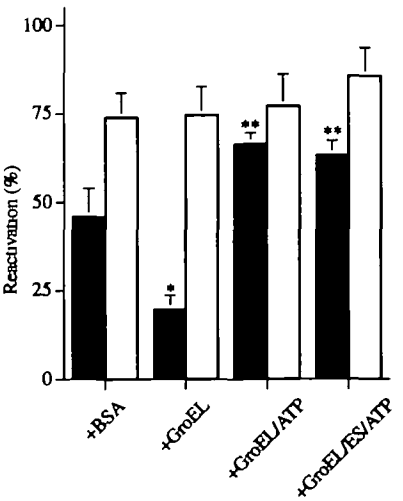

Fig. 5. **Effect of GroEL oligomers on the reactivation of ME- or urea and ME-denatured EH.** Reactivation was achieved by rapid dilution in reactivation buffer and incubation for 2 h in the presence of 0.1 mg/ml BSA, a 5-fold molar excess of GroEL oligomers, a 5-fold molar excess of GroEL oligomers + 2 mM ATP, or a 5-fold molar excess of GroE oligomers + 2 mM ATP The white bars and black bars show the results for ME-denatured and urea and ME-denatured EH, respectively Each bar is the mean ± SD of three or more independent experiments. * $p < 0.01$, ** $p < 0.05$ compared with the control value obtained with BSA.

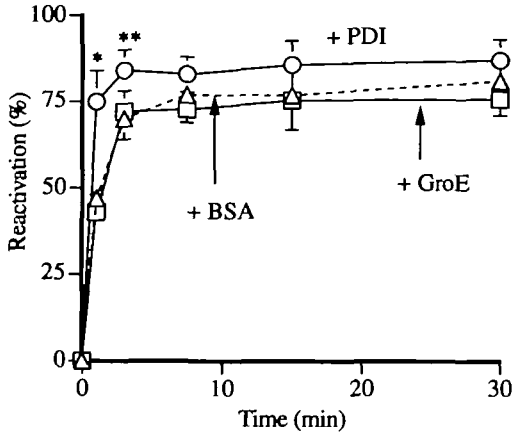

Fig. 6 **Effects of GroE oligomers or PDI on the time course of reactivation of ME-denatured EH.** Reactivation was achieved by rapid dilution in reactivation buffer in the presence of 0.1 mg/ml BSA (△), a 5-fold molar excess of GroE oligomers + 2 mM ATP (□), or a 5-fold molar excess of PDI (○) The data shown by open triangles are from Fig 3 Each data point represents the mean ± SD of four independent assays * $p < 0.01$, ** $p < 0.05$ compared with the control value obtained with BSA.

(Fig. 4). On the other hand, a higher recovery of hormonal activity (approximately 80% of the native peptide) was observed if PDI was present at a 5-fold molar excess. Furthermore, PDI markedly accelerated the reactivation rate. The $t_{1/2}$ was less than 1 min (Fig. 4). A further increase in PDI concentration had no effect on either the rate or extent of recovery of hormonal activity (data not shown). Delaying the addition of PDI for up to 1 h after dilution of the urea and ME-denatured eclosion hormone resulted in a fast reactivation with a final yield of about 80% (Fig. 4), suggesting that the miss-folded eclosion hormone may be acting as a substrate for PDI.

Reactivation experiments in the presence of only GroEL 14-mer oligomers were also performed. A 5-fold molar excess of GroEL oligomers suppressed the reactivation of urea and ME-denatured eclosion hormone. However, with the addition of 2 mM ATP to the reaction mixture, the activity resumed with a yield of 64%, similar to the value obtained in the presence of GroE oligomers (Fig. 5). When ATP was added to the mixture 1 h after the commencement of refolding of the denatured hormone with GroEL oligomers, the suppressed activity was recovered within 15 min with a final yield of about 65%. In contrast to the urea and ME-denatured eclosion hormone, the ME-denatured eclosion hormone was not influenced by GroEL or GroE oligomers (Figs. 5 and 6).

Figure 6 shows the time courses of the ME-denatured eclosion hormone in the presence of GroE oligomers or PDI. The hormonal activity recovered spontaneously during the first 3 min with a yield of 72%. The addition of a 5-fold molar excess of GroE oligomers over ME-denatured eclosion hormone had a negligible effect on the reactivation. On the other hand, the same amount of PDI slightly but significantly facilitated the reactivation rate. However, the assay method does not result in a precise time course on the order of seconds.

In order to test whether PPI acts as a chaperone-like protein in the reactivation of urea and ME-denatured eclosion hormone, we performed the reactivation assay in the presence of excess amounts of PPI. PPI was shown to have no effect on the reactivation even at a 10-fold molar excess (Table I).

TABLE I **The degree of reactivation of the urea and ME-denatured eclosion hormone in the presence of various concentrations of PPI.** Reactivation was achieved by rapid dilution in reactivation buffer in the presence of BSA (0 1 mg/ml) or various concentrations of PPI, and incubation for 30 min Values are means ± SD for the numbers of experiments shown in parentheses

BSA or PPI (molar ratio)	% of reactivation	(# of experiments)
BSA	33 ± 5	(6)
PPI (1 1)	28 ± 7	(3)
PPI (5.1)	36 ± 6	(5)
PPI (10 1)	40 ± 7	(3)

DISCUSSION

We first examined the conditions needed to denature eclosion hormone with β-mercaptoethanol and urea. β-Mercaptoethanol is generally used as a protecting agent of SH groups in proteins at the final concentration of 0.1–10 mM. β-Mercaptoethanol at a concentration higher than 10 mM brings about the cleavage of disulfide bonds in proteins. As shown in Fig. 1, treatment with 100 mM β-mercaptoethanol at 25°C was not sufficient to inactivate eclosion hormone. Therefore, we tested the effect of boiling the β-mercaptoethanol–treated eclosion hormone for 10 min. Boiling at 100°C for 10 min was found to be sufficient to cause the complete inactivation of eclosion hormone treated with 25 mM or more β-mercaptoethanol prior to boiling. As the denatured eclosion hormone obtained in this way did not show any eclosion activity at a dose of 10 μg (5,000 times excess of the ED_{50} value of the native hormone), we conclude all three disulfide bonds in the ME-denatured eclosion hormone were cleaved (*10*).

The CD spectrum of ME-denatured eclosion hormone showed that about 65% of the α-helices remained (Fig. 2). As is apparent from the results, the degree of helical formation does not correspond well to the extent of biological activity: the loss of one third of the helices produced by boiling the 25 mM β-mercaptoethanol-treated eclosion hormone resulted in a complete loss of eclosion activity. Probably, the heat treatment broke hydrophobic interactions necessary for maintaining the active globular structure including the α-helices. This scenario is supported by our previous report that the partial α-helix near the N-terminal is maintained without any disulfide bond in the synthetic partial eclosion hormone (34 residues from the N-terminal). This peptide does not show any bioactivity (*7*). Furthermore, the retention time of the ME-denatured eclosion hormone in C_{18} column chromatography was prolonged in a manner similar to that of the full length synthetic eclosion hormone in which all the cysteine residues were blocked with acetamidomethyl groups (*10*).

It is likely that the ME-denatured eclosion hormone was totally inactive but retained some partial secondary structure. In order to break the remaining secondary structure in the ME-denatured eclosion hormone, we treated it with 8 M urea. As a result, there was no profile characteristic of an α-helix in the CD spectrum of the urea and ME-denatured eclosion hormone. Because the secondary structure

was broken and the solubility of the hydrophobic amino acid residues was elevated by urea, the urea and ME-denatured eclosion hormone is considered to have become completely unfolded.

The spontaneous reactivation of the ME-denatured eclosion hormone occurred within a few minutes (Fig. 3) It is reported that the folding of peptides with a molecular mass of 10,000 Da or less is very rapid. For example, barstar, with 89 residues, folds rapidly *in vitro* in the millisecond range (*26*) However, the apparent $t_{1/2}$ for the reactivation of the ME-denatured eclosion hormone was about 1 min, suggesting that disulfide-bond formation is one of the rate limiting steps for the refolding of eclosion hormone. This assumption is supported by the immediate reactivation of urea-denatured eclosion hormone, which retains three disulfide bonds (Fig. 3). Furthermore, the reactivation of the urea and ME-denatured eclosion hormone was very slow ($t_{1/2}$ > 20 min) and the yield was significantly low (15% during the first 3 min) compared to the urea-denatured and ME-denatured eclosion hormones. Generally, the formation of secondary structures such as an α-helices progresses in the millisecond or even microsecond range (*13*). Supposing that the formation of α-helices in the eclosion hormone proceeds in the microsecond or millisecond range and that disulfide-bond formation is the only rate-limiting step in folding, the reactivation time courses of the ME-denatured and urea and ME-denatured eclosion hormones should be similar. However the spontaneous reactivation of the ME-denatured eclosion hormone ($t_{1/2}$ = 1 min) was much faster than that of the urea and ME-denatured eclosion hormone ($t_{1/2}$ > 20 min). Therefore, there seems to be another rate-limiting step prior to disulfide bond formation in the folding pathway of eclosion hormone

The reactivation of the urea and ME-denatured eclosion hormone was promoted in the presence of GroE oligomers (Fig. 4). Interestingly, the urea and ME-denatured eclosion hormone was arrested significantly in GroEL oligomers (Fig. 5). In the experiments using denatured enolase, GroEL oligomers trapped the enolase and did not release it so that the enzymatic activity was not recovered (*27*). By adding ATP to the reaction mixture, the enolase was released and the activity restored (*28*). Similarly, the reactivation of the urea and ME-denatured eclosion hormone was apparently decreased by GroEL oligomers, and was restored by adding 2 mM ATP to the reaction mixture (Fig. 5). On the other hand, the reactivation of ME-denatured eclosion hormone was not influenced by GroEL oligomers at all (Fig 5). There are some reports that GroEL interacts with unfolded proteins without any secondary structure. For example, α-lactalbumin with four disulfide bonds has been reported to interact with GroEL in the totally unfolded form rather than in the molten globule condition (*16, 29*). From these results, it is conceivable that the urea and ME-denatured eclosion hormone is caught by GroEL oligomers, and that refolding is promoted in the oligomers. Probably, an eclosion hormone with a molecular mass of about 6,800 Da is able to be a substrate, although peptides with a molecular mass of 10,000 Da or more are normal substrates that bond to GroEL oligomers. Furthermore, eclosion hormone does not require the presence of the co-chaperonine GroES for GroEL-supported refolding.

In comparison with urea-denatured eclosion hormone, the reactivation yields were significantly low for the ME-denatured and urea and ME-denatured eclosion hormones (Fig. 3). Therefore, we examined the effect of PDI on the reactivation of the ME-denatured and urea and ME-denatured eclosion hormones. As shown in Fig. 4, the reactivation of the urea and ME-denatured eclosion hormone was markedly promoted and accelerated by PDI. The final yield in the presence of PDI was higher than in the presence of GroE oligomers. Moreover, the apparent $t_{1/2}$ for reactivation in the presence of PDI became less than 1 min, a smaller value (by a factor of 10) than in the presence of GroE oligomers. The accelerative effect of PDI was also observed in the reactivation assay of the ME-denatured eclosion hormone (Fig. 6). These results may be due to the ability of PDI to promote disulfide-bond formation. However, since PDI can promote the folding of denatured proteins (*18, 30*), and the effect may be due to a chaperone-like activity, especially in the reactivation of the urea and ME-denatured eclosion hormone.

Other possible rate limiting steps, such as the isomerization of proline, also need to be considered, since one of the rate-limiting steps in the folding of denatured protein is the isomerization of disulfide bonds and/or proline imide (*31, 32*). These isomerizations *in vitro* require times in the range of minutes to hours. We have already reported that the activity of eclosion hormone decreases to 1/34 that of the wild type when the highly conserved residue Pro47 is replaced by glycine (*8*). Although the ratio of the *cis*- and *trans*-forms of Pro47 are not obvious in the urea and ME-denatured eclosion hormone, Pro47 in the non-native peptide is thought to contain both the *cis*- and *trans*-forms, unlike the native peptide. Therefore, the isomerization of proline can be a rate-limiting step in the refolding of the urea-denatured eclosion hormone. However, as shown in Table I, PPI did not facilitate the reactivation at all. This suggests that prolyl isomerization is not involved as a rate limiting step. While PPI did not show any direct effect on refolding, the significance of the co-existence of PDI may be related to the mechanism of the PDI-dependent folding of eclosion hormone. Further investigations will elucidate the relationship.

In conclusion, *de novo* synthesized eclosion hormone immediately after translation probably has a structure similar to the urea and ME-denatured form. Although GroE oligomers promote the reactivation of the urea and ME-denatured eclosion hormone, PDI facilitates the folding more efficiently. Recently, the existence of PDI in Drosophilae has been reported (*33*). Therefore, the folding of the newly synthesized eclosion hormone is considered to progress in the endoplasmic reticulum in a PDI-dependent manner. Although eclosion hormone is a small peptide, it is predicted to have three helices linked by three disulfide bonds. Therefore, multiple rate limiting steps in the folding pathway may exist. We speculate that one step is disulfide bond formation and another step is the formation of hydrophobic interactions prior to disulfide bond formation. PDI seems to act at both steps very efficiently to facilitate the folding of *de novo* synthesized eclosion hormone.

We are very grateful to E. Cooper (Ritsumeikan University) for helpful comments on the manuscript. We also thank C Yosho (Ritsumeikan University) for PPI preparation.

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