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Communication

Two disulphide bridges are present in juvenile hormone binding protein from *Galleria mellonella*✪

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The hemolymph juvenile hormone binding protein (JHBP) from *Galleria mellonella* contains two disulphide bridges/molecule and no free Cys residues. An alignment of primary structures of other Lepidopteran JHBPs indicates that Cys residues, equivalent to Cys^{10,17,151,195} in *G. mellonella* JHBP, may be involved in –S–S– bridge formation.

Juvenile hormone binding proteins (JHBPs) transport the juvenile hormone (JH) from the site of its synthesis (*corpora allata*) [1] to target tissues and serve as JH reservoir protecting the hormone from hydrolysis by non-specific esterases [2]. JHBPs have been isolated from several insect species, but only for three of JH carriers the number of Cys residues/protein molecule has been determined: *Bombyx mori* (5 Cys) [3], *Heliothis virescens* (5 Cys) [4], *Manducta sexta* (6 Cys) [5]. *Galleria mellonella* JHBP is a glycoprotein of molecular mass 25 880 Da [6]. JH binding to JHBP results in pronounced conformational transition as judged from sedimentation coefficient change and spectroscopic studies [7, 8]. Recently, in our laboratory the primary structure of *G. mellonella* JHBP was elucidated (AF4107772). It has been found that despite the substantial homology to the above men-

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Abbreviations: Cm, carboxymethyl residue; Cam, carbamoylmethyl residue; DTT, dithiothreitol; IAA, iodoacetate; IAM, iodoacetamide; JH, juvenile hormone; JHBP, juvenile hormone binding protein.

tioned JHBPs orthologs this protein possesses only four Cys residues. The previous reports indicated that a JHBP molecule from *M. sexta* [9] contains two disulphide bridges and two free cysteine residues, therefore it was interesting to find if it is an obligatory feature of other JHBPs to have disulphide bridges and free cysteine residues in the molecule. An analysis of cystine and cysteine residues in *G. mellonella* JHBP is presented in this communication.

MATERIALS AND METHODS

Reagents and chemicals. All chemicals were of analytical grade, commercially available. Their solutions were prepared in 100 mM Na-phosphate buffer, pH 7.2.

Protein purification. Homogenous JHBP from *G. mellonella* was isolated as previously described [10]. Protein samples were equilibrated by dialysis against 100 mM Na-phosphate buffer, pH 7.2, and concentrated to about 1.0 mg/mL using Millipore ultrafiltration membrane (PTGC-type, NMWL = 10 000; Sigma, Poland). JHBP concentration was determined spectrophotometrically at 280 nm. A solution of 1.0 mg of JHBP/mL has an $A_{1\ cm,\ 280}$ of 0.46 [8].

Polyacrylamide gel electrophoresis (PAGE) in 8 M urea. Noncontinuous gel system [11] was applied with 4% of stacking and 12% of running gel, containing 8 M urea. The electrode buffer (pH 8.4) contained 25 mM Tris and 192 mM glycine. Electrophoresis was performed using 5 mA/gel (height: 7.0 cm, width: 8.3 cm, thickness: 1.5 mm) for 10 h at room temperature. Gels were stained with Coomassie Blue [12].

Analysis of thiol residues. Free –SH groups and –S–S– bonds in the protein molecule were determined according to Creighton [13] and Hollecker [14]. Protein samples (0.16–0.38 mg/mL) were preincubated in 8 M urea overnight at 4°C in the presence or absence of 15 mM dithiothreitol (DTT). Then the samples were incubated with 50 mM iodoacetate (IAA), 50 mM iodoacetamide (IAM) or 50 mM IAA+IAM mixture for 30 min at room temperature. The resulting mixtures after chemical modification were dialysed against 8 M urea and subjected to urea/PAGE.

RESULTS AND DISCUSSION

To study the number of free thiols and disulphide bridges in JHBP molecule a simple procedure developed by Creighton and Hollecker [13, 14] was applied. It is based on chemical modification of cysteine residues of the analysed protein samples with IAA or IAM or the mixture of IAA+IAM. These reagents add negatively charged carboxymethyl (Cm) or inert carbamoylmethyl (Cam) groups to protein molecules, respectively [13]. Thus differently charged protein molecules can be separated by 8 M urea/PAGE and the number of Cm/protein molecule can be calculated. The chemical modification is performed on separate protein samples before and after reduction with DTT. To detect all possible combinations of modified protein molecules different ratios of IAA : IAM were used.

Theoretically, if one assumes that JHBP molecules contain four free Cys residues per molecule, or two free Cys and one disulphide bridge per molecule or two disulphide bridges per molecule, the electrophoretic pattern should show five, three or one distinct protein bands, respectively (Fig. 1A). Such protein samples treated with a reducing agent prior to chemical modification would yield five distinct bands, irrespective of the number (zero, one or two) of disulphide bridges per molecule (Fig. 1A). The postulated composition of Cm and Cam residues in JHBP molecules is shown in Fig. 1A.

Experiments presented in this paper show that *G. mellonella* JHBP samples treated with IAA and/or IAM, prior to reduction with DTT, yielded on PAGE one diffuse protein band in-

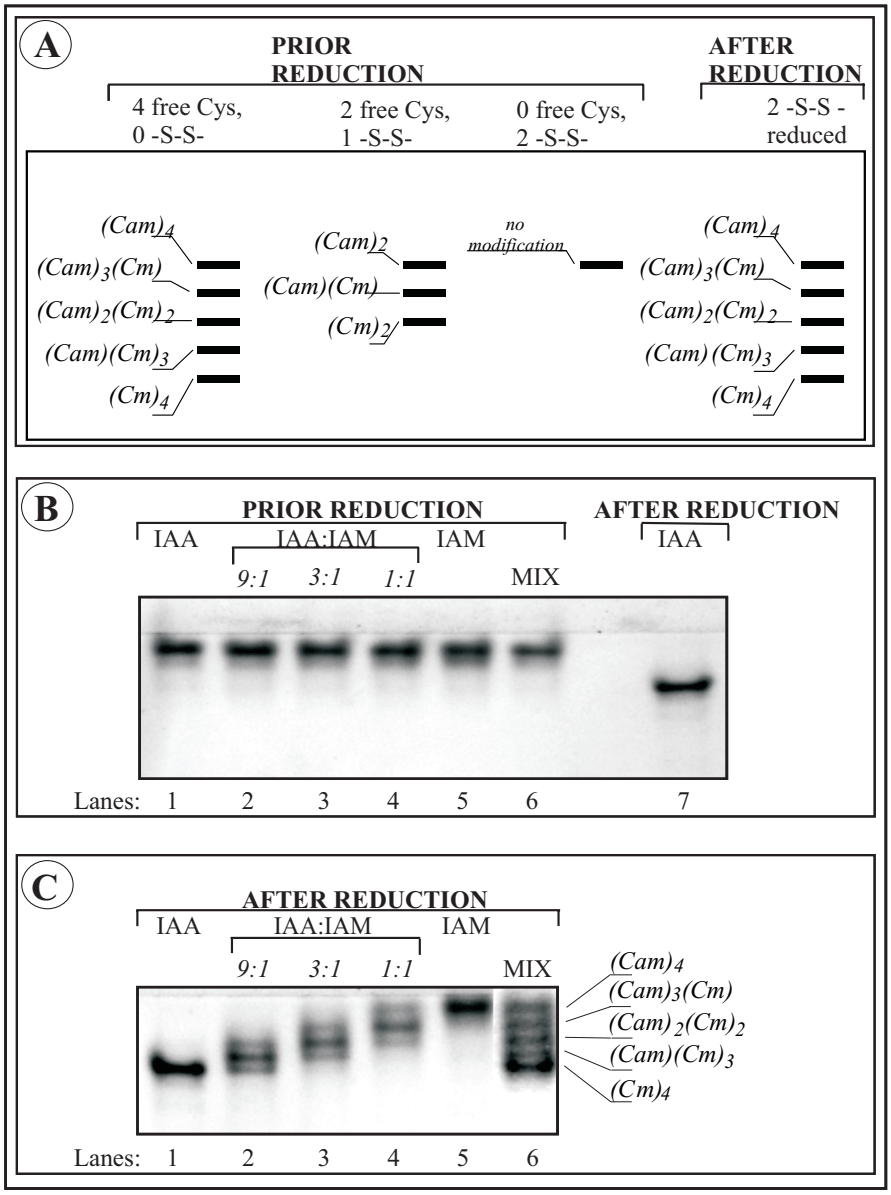

Figure 1. Determination of free thiols and disulphide bonds in JHBP.

Protein samples were preincubated overnight in 100 mM Na-phosphate buffer, pH 7.2, containing 8 M urea in the absence or presence of 15 mM DTT. The samples were then incubated with 50 mM iodoacetate (IAA), or 50 mM iodoacetamide (IAM) or 50 mM IAA : IAM (9:1, 3:1, 1:1) mixture for 30 min at room temperature. 6.4 μg of each protein sample was applied to 8 M urea/PAGE (lanes 1–5). Equal amounts of samples 1–5 were mixed (12.8 μg total protein) and subjected to 8 M urea/PAGE (lane 6). Electrophoresis was carried out for 10 h, 5 mA/gel at room temperature. Gels were stained for proteins with Coomassie Blue. The postulated composition of carbamoylmethyl (Cam) and carboxymethyl (Cm) residues in JHBP molecules is indicated. (A) A hypothetical distribution of protein molecules, containing 4 Cys/molecule or 2 Cys and 1 disulphide bridge/molecule, or 2 disulphide bridges/molecule, in 8 M urea/PAGE. (B) Electrophoretic mobilities of non-reduced JHBP molecules treated with IAA and/or IAM. (C) Electrophoretic mobilities of reduced JHBP molecules treated with IAA and/or IAM. For details see Materials and Methods.

dicating some heterogeneity (Fig. 1B, lanes 1–6). Most likely this heterogeneity was caused by modification of His residues with IAA [15] and less likely by preservation of free –SH groups (see below). To confirm this supposition JHBP was reduced with DTT and then treated with IAA. In this case one type of protein molecules of the highest electropho-

retic mobility was detected (Fig. 1B, lane 7). Reduction of JHBP and treatment with IAA, IAM or a mixture of IAA and IAM yielded five protein bands differing in electrophoretic mobility (Fig. 1C, lanes 1–5). The above results clearly indicate that *G. mellonella* JHBP contains two disulphide bridges and no free cysteine residues in the native state. Upon reduction those two disulphide bridges can be converted into four free –SH groups as judged from five-membered hybrid set (Cam_4, $CmCam_3$, Cm_2Cam_2, Cm_3Cam, Cm_4) on lane 6, produced by mixing equal portions of samples applied to lanes 1–5 (Fig. 1C).

Comparison of amino-acid sequences of JHBPs from *G. mellonella* ($Cys^{10,17,151,195}$) (AF4107772), *B. mori* ($Cys^{9,16,151,194,200}$) [3], *H. virescens* ($Cys^{9,16,29,150,194}$) [4] and *M. sexta* ($Cys^{9,16,29,151,195,201}$) [5] shows that all Cys residues present in *G. mellonella* are conserved in the alignment of the proteins mentioned above (J.M. Rodriguez Parkitna *et al.*, unpublished results). This strongly suggests that the two disulphide bridges reported previously for *M. sexta* JHBP should appear at $Cys^{9,16,151,195}$. Since this protein alignment has shown that $Cys^{9,16,151,194}$ in *B. mori* [3] and $Cys^{9,16,150,194}$ in *H. virescens* [4] are conserved, therefore it is reasonable to postulate that JHBPs from these species also contain two disulphide bridges, which may be required for stabilizing the protein fold. It remains to be elucidated what is the topology at the –S–S bridge formation.

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