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Very high-density lipoprotein and vitellin as carriers of novel biliverdins IXα with a farnesyl side-chain presumably derived from heme A in *Spodoptera littoralis*

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ABSTRACT

Bilins in complex with specific proteins play key roles in many forms of life. Biliproteins have also been isolated from insects; however, structural details are rare and possible functions largely unknown. Recently, we identified a high-molecular weight biliprotein from a moth, *Cerura vinula*, as an arylphorin-type hexameric storage protein linked to a novel farnesyl biliverdin IXα; its unusual structure suggests formation by cleavage of mitochondrial heme A. In the present study of another moth, *Spodoptera littoralis*, we isolated two different biliproteins. These proteins were identified as a very high-density lipoprotein (VHDL) and as vitellin, respectively, by mass spectrometric sequencing. Both proteins are associated with three different farnesyl biliverdins IXα: the one bilin isolated from *C. vinula* and two new structurally closely related bilins, supposed to be intermediates of heme A degradation. The different bilin composition of the two biliproteins suggests that the presumed oxidations at the farnesyl side-chain take place mainly during egg development. The egg bilins are supposedly transferred from hemolymph VHDL to vitellin in the female. Both biliproteins show strong induced circular dichroism activity compatible with a predominance of the *M*-conformation of the bilins. This conformation is opposite to that of the arylphorin-type biliprotein from *C. vinula*. Electron microscopy of the VHDL-type biliprotein from *S. littoralis* provided a preliminary view of its structure as a homodimer and confirmed the biochemically determined molecular mass of ~350 kDa. Further, images of *S. littoralis* hexamerins revealed a 2 × 3 construction identical to that known from the hexamerin from *C. vinula*.

1. Introduction

Tetrapyrroles are essential cofactors in many diverse processes in all forms of life, acting as cyclic porphyrins or as open-chain bilins (Frankenberg and Lagarias, 2003). These bilins are derived from the IXα-isomer of biliverdin by modification of the chromophore and covalent binding to specific proteins. In vertebrates, bilins, commonly of the IXα-type, are produced by the degradation of heme from haemoglobin and various other heme proteins, and are finally destined for excretion. Insect bilins display more structural diversity, since either the α- or the γ-isomer of biliverdin is produced depending on the taxonomy of the species; the γ-isomer is almost exclusively present in Lepidoptera (Kayser, 1985). The known insect bilins are non-covalently associated with protein. Although a number of insect biliproteins have been purified, most from Lepidoptera, the proteins and the bilins in particular have only been firmly identified in a few cases, most of the pigments being tentatively described as 'biliverdin' or 'biliverdin-like', and their roles are largely subject to speculations (Kayser, 1985, 2005).

Recently, we isolated a high-molecular weight biliprotein from larval hemolymph of a moth, *Cerura vinula*, and identified the apoprotein as an arylphorin-type hexamerin specifically associated with a novel bilin (Kayser et al., 2009). It represents the first biliprotein among the arylphorin class of insect storage proteins.

Abbreviations: AS, ammonium sulphate; CD, circular dichroism; CV-bilin, bilin from *Cerura vinula*; EM, electron microscopy; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; STEM, scanning transmission EM; TEM, transmission EM; VHDL, very high-density lipoprotein..

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Arylphorins are produced in high quantities together with the methionine-rich storage proteins, another major class of the hexamerins, as reserves for developmental transformation (Burmester, 1999). The structure of this bilin, denominated CV-bilin, was fully elucidated using the most recent spectrometric methodology, and shown to be biliverdin IXα with a modified farnesyl side-chain and a hydroxymethyl group at one of the pyrrole rings (Kayser et al., 2014). The substituent pattern of this unique bilin suggests that it may result from α-cleavage of mitochondrial heme A (or its precursor, heme O), which is the only known tetrapyrrole carrying a farnesyl group (Brown et al., 2002). So, heme A cleavage, a conserved key step of heme degradation, is expected to give rise to a farnesyl bilin. By contrast to heme B, however, the degradation pathway of heme A is unknown to date (Hederstedt, 2012).

The presence of an α-isomer of biliverdin in *C. vinula*, a member of the Notodontid family, was unexpected because Lepidoptera are known to produce the γ-isomer, as mentioned above. This prompted us to study another species, *Spodoptera littoralis*, which is a member of a different moth family, the Noctuidae. Here, we isolated not only a bilin suggested to be identical with CV-bilin from all molecular data obtained, but also two other structurally closely related farnesyl biliverdins that are considered to be intermediates of the proposed transformation of heme A to CV-bilin. In *S. littoralis*, however, the three bilins are not associated with an arylphorin as in *C. vinula*, but with a very high-density lipoprotein (VHDL) in larval hemolymph and with vitellin in eggs. The bilins may be transferred from VDHL to vitellin, as bilin synthesis in ovaries has not yet been shown. The bilin composition changes with the *Cerura* bilin being predominant in the egg biliprotein, the two other bilins are mainly associated with the hemolymph VHDL. In addition, we studied the structure of the VHDL and of hexamerins by electron microscopy (EM). The images present a preliminary view on an insect VHDL and confirmed the conserved architecture of the hexamerin complexes.

2. Materials and methods

2.1. Chemicals and insects

Unless stated otherwise, chemicals were obtained from Merck or Sigma–Aldrich. Larvae and eggs of the cotton leaf worm, *S. littoralis* (Noctuidae), were from an established culture maintained on an artificial diet.

2.2. Protein purification

Hemolymph was collected in cold 0.05 M Tris/HCl, pH 7.4, containing 0.05 M NaCl, and processed as in previous work (Kayser et al., 2009). Briefly, the blue biliprotein was precipitated from centrifugation supernatants by the addition of solid ammonium sulphate (AS) in the saturation range of 45%–70% at 5 °C. Usually, narrower ranges of saturation were applied to improve purification (see 3.1.). The biliprotein was purified by gradient chromatography on DEAE-Sephacel (2.5 × 34 cm). Blue fractions were characterized by their UV–visible spectra and pooled according to purity (A662 nm/A278 nm). Frozen eggs, harvested 6–12 h after deposition, and newly hatched larvae, were homogenized first in cold 0.05 M Tris/HCl, pH 7.4, containing 0.05 M NaCl, and then in 0.05 M Tris/HCl, pH 7.4, containing 0.5 M NaCl. Egg extracts were fractionated by the addition of AS in the range of 45%–67% saturation. Samples were purified on a short column of DEAE-Sephacel (1.4 × 13 cm or 2 × 5 cm), which was step-eluted with 0.125 M and 0.25 M NaCl in 0.05 M Tris/HCl, pH 7.4.

2.3. Size-exclusion chromatography

Size-exclusion chromatography was performed on a TSK HW 55-S column (2.5 × 34 cm) (Merck) with 0.05 M Tris/HCl, pH 7.4, at a rate of 0.8 mL/min at room temperature. The high-molecular weight kit (GE Healthcare Life Sciences) of proteins was used for calibration.

2.4. Polyacrylamide gel electrophoresis

SDS-PAGE was performed with pre-cast NuPAGE Novex Bis-Tris 10% and 4–12% gels using either Mes or Mops buffer, as described (Kayser et al., 2009).

2.5. Density gradient centrifugation

Density gradients were generated with 46% KBr in 0.05 M Tris/HCl, pH 7.4, in Opti Seal tubes spun at 100,000 rpm (543,000 ***g***) using a Beckman Optima TLX ultracentrifuge with rotor TLA 100.4. The running time was 5 h at a set temperature of 20 °C. After termination, the tubes were punctured at the bottom and fractions were collected for gravimetric determination of density and for UV–visible spectroscopy. Alternatively, bands were collected from the top using a Pasteur pipette.

2.6. Protein identification

Proteins separated by SDS-PAGE, were identified by in-gel tryptic digestion of stained gel slices and sequencing of cleavage peptides by mass spectrometry (MS), as in previous work (Kayser et al., 2009). The (glyco-)peptides were analysed by electrospray ionization mass spectrometry (ESI-MS/MS) (Nimtz et al., 2004). An Orbitrap XL mass spectrometer (ThermoScientific, Bremen, Germany) equipped with a nanospray ion source was used. For collision-induced dissociation experiments, parent ions were selected in the ion trap and fragmented in the HCD cell, using nitrogen as collision gas. The resulting daughter ions were then separated in the Orbitrap analyser at a resolution of ca. 60,000. These spectra of tryptic peptides were used for manual *de novo* sequencing. If necessary for sequence assignments, MS3 experiments were performed. As Leu and Ile cannot be distinguished with this technique L always means L or I. Database searches against insect proteins were performed using the fasta 3 algorithm. For matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) peptide mass fingerprinting, protein bands were cut out from SDS-gels, reduced and carboxamidomethylated, and then subjected to in-gel tryptic digestion. The resulting peptides were extracted, desalted using ZipTip devices (Millipore) and analysed by MALDI-TOF-MS with alpha-cyano-4-hydroxycinnamic acid as matrix, using a Bruker Ultraflex TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The MASCOT search engine (http://www.matrixscience.com) was employed to identify proteins.

2.7. Bilin isolation and characterisation

As in previous work (Kayser et al., 2014), bilins were liberated by precipitation of the carrier protein with methanol, and isolated from the supernatant by solid-phase adsorption to a Sep-Pak C18 cartridge and elution with methanol, or by extraction into chloroform. Methylation, acetylation, micro degradation and reverse-phase HPLC of bilins were performed also as described (Kayser et al., 2014).