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Farnesyl biliverdins IXα are novel ligands of biliproteins from moths of the Noctuoidea superfamily: A chemosystematic view of the Lepidoptera

Hartmut Kayser [a, *], Manfred Nimtz [b]

[a] Institute of General Zoology and Endocrinology, Ulm University, Helmholtzstrasse 8/1, 89081, Ulm, Germany
[b] Helmholtz Centre for Infection Disease, Cellular Proteome Research, Inhoffenstrasse 7, 38124, Braunschweig, Germany

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ABSTRACT

Bilins, derived from biliverdin IXα, are known from animals, plants and microorganisms, where they play vital roles as light-absorbing pigments. Bilins occur also in many insects. Recently, we discovered in insects a novel structural type of bilins with a farnesyl substituent at pyrrole ring A of biliverdin IXα. The first of these unusual bilins with a molecular mass of 852 ($C_{48}H_{60}O_{10}N_4$) was identified in *Cerura vinula*, subsequently in *Spodoptera littoralis*; both species are members of the Noctuoidea superfamily of moths. From an evolutionary point of view, it was of interest to examine other species and families of this monophyletic clade. Here, we show that other moths species in this clade (three Notodontidae species, one Erebidae species, and one Noctuidae species) have farnesylated biliverdins IXα that are present as a mixture of three bilins, differing by the number of oxygen atoms (O_{8-10}). These bilins are associated with typical hemolymph storage proteins, which were identified by mass spectroscopic sequencing of tryptic peptides as arylphorins (a class of 500-kDa hexamerins) in the Notodontidae and Erebidae families, and as 350-kDa very high-density lipoproteins in the Noctuidae family. Circular dichroism spectroscopy revealed that the bilins adopt opposite conformations in complex with the two different classes of proteins. At present, farnesylated biliverdins and IXα-isomers of bilins in general are known only from species of the Noctuoidea clade; the sister clades of Bombycoidea and Papilionoidea synthesise the IXγ-isomer of biliverdin and derivatives thereof.

1. Introduction

Bilins are non-cyclic tetrapyrroles that are derived from cyclic tetrapyrroles, the porphyrins, by an evolutionary conserved way of ring cleavage by heme oxygenases (Kikuchi et al., 2005; Wilks and Heinzl, 2014). Representatives of both types of tetrapyrroles play key roles in respiration, photosynthesis, accessory light harvesting, plant development, and pigmentation in many forms of life (Frankenberg and Lagarias, 2003). In vertebrates, heme B, the iron complex of protoporphyrin IX, is degraded to biliverdin, mainly to the α-isomer, and is further transformed to bilirubin for excretion. In insects, the type of biliverdin isomer varies depending on taxonomy. While the α-isomer has been identified in several hemimetabolous species, such as locusts, phasmids and mantids, the γ-isomer has been mainly found in lepidopteran insects (Kayser, 1985). Two recent reports on blood-sucking insects show that the bilin isomer produced is not strictly taxon-specific: while the blood-feeding bug *Rhodnius prolixus* detoxifies ingested heme by γ-cleavage, the dipteran *Aedes aegypti* produces the α-isomer of biliverdin (Paiva-Silva et al., 2006; Pereira et al., 2007). In both species, these biliverdin isomers are conjugated to form hydrophilic products for excretion, comparable to bilirubin in vertebrates.

Recently, we reported a novel type of a bilin, identified by chemical, biochemical and spectroscopic methods as biliverdin IXα substituted with an epoxi-dihydroxyethylfarnesyl substituent at pyrrole ring A and a hydroxymethyl substituent at pyrrole ring D, reminiscent to the structure of heme A (Fig. 1) (Kayser et al., 2014). This farnesyl bilin was isolated from the notodontid moth *Cerura vinula*, where it is present in high concentration in larval hemolymph associated with an arylphorin, a hexamerin-type storage protein (Kayser et al., 2009). From an evolutionary point of view, it

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

* Corresponding author.
E-mail address: hartmut.kayser@uni-ulm.de (H. Kayser).

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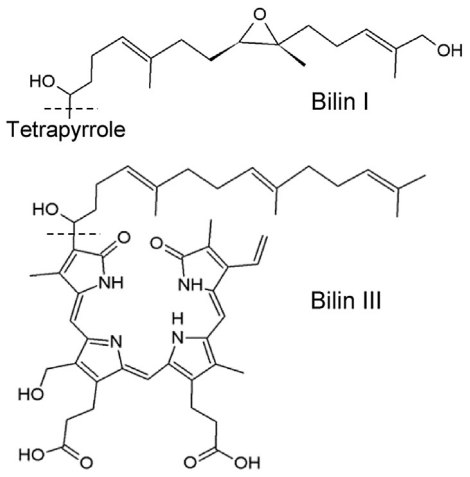

Fig. 1. Structures of farnesyl biliverdins IXα from Noctuoidea moths. Data suggest that bilin III is transformed to bilin I with bilin II (not shown) as an intermediate having either the epoxide or the farnesyl terminal hydroxyl group (the two isomers could not be discriminated).

was reasonable to assume that taxonomically related species may harbour the same or a structurally closely related form of this unusual bilin. We then studied *Spodoptera littoralis* from the noctuid family that is also a member of the Noctuoidea superfamily, like *C. vinula*. In fact, we identified not only the same IXα-type farnesyl bilin as in *C. vinula*, but in addition two other bilins, which are structurally even more close to heme A, the proposed precursor of these farnesyl biliverdins (Fig. 1) (Kayser et al., 2016).

In the present study, we extended the range of species representing three families from the Noctuoidea superfamily. In all examined species, three bilins of the IXα structural type were identified, as known from *S. littoralis* and *C. vinula*. All three bilins were associated with specific larval hemolymph proteins, identified as arylphorins in two families and as very high-density lipoprotein (VHDL) in one family. The conformation of the bound bilins differed between the two classes of proteins. Overall, our present results suggest that formation of farnesyl biliverdins IXα may be a specific feature of members of the monophyletic clade of Noctuoidea moths. With this in mind, it is of interest to state that the IXγ-isomer of biliverdin is widely present in species from the Bombycoidea (moths) and Papilionoidea (butterflies) superfamilies, which are sisters to the Noctuoidea clade.

2. Materials and methods

2.1. Chemicals and insects

Chemicals were obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany). The examined lepidopteran species represented three families from the Noctuoidea superfamily. These families were (taxonomy according to Fauna Europaea at http://www:faunaeur.org): i, the Notodontidae, represented by *Thaumetopoea pityocampa* (pine processionary); *Peridea anceps* (great prominent); *Phalera bucephala*) (buff-tip); ii, the Erebidae, represented by *Lymantria dispar* (gypsy moth); iii, the Noctuidae, represented by *Heliothis virescens* (tobacco budworm). Most species were obtained as final instar larvae *via* the entomology platform ACTIAS (http://www.actias.de.) and eventually fed the plant material recommended by the suppliers. *T. pityocampa* was received in soil at a stage around pupation from the INRA Centre de Biologie pour la Gestion des Populations (Montferrier-sur-Lez, France). *H. virescens* was from a stock maintained on an artificial diet by Syngenta Crop Protection (Basel, Switzerland). Hemolymph was collected from late last instar larvae by incising the first pair of abdominal legs. Fresh pupae were homogenized *in toto*, and the extracts were processed like hemolymph.

2.2. Protein purification

The procedures of biliprotein isolation were the same as in foregoing work (Kayser et al., 2016). The major steps were fractionation of hemolymph and pupal extracts, respectively, both diluted with standard buffer (0.05 M Tris/HCl buffer, pH 7.4), by addition of solid ammonium sulphate (AS) in ranges typically between 45% and 75% saturation at 5 °C. The dialyzed 10,000 ***g***-supernatants were subjected to chromatography on DEAE-Sephacel; elution was performed by a NaCl gradient (0.03–0.35 M) in standard buffer, or by a step-wise increase in NaCl in case of short columns. Yellow proteins, identified as carotenoproteins by their main absorption maximum around 450 nm, were separated from the biliproteins on the DEAE-column. Size-exclusion chromatography was performed on a TSK-HW 55S column with standard buffer. The blue protein fractions were further studied by UV–visible and circular dichroism (CD) spectroscopy, and by polyacrylamide gel electrophoresis (PAGE). Density gradient centrifugation of biliproteins was performed with 46% KBr in standard buffer using Opti Seal tubes spun at 100,000 rpm (543,000 ***g***) for 5 h at 20 °C in a Beckman Optima TLX ultracentrifuge with rotor TLA 100.4.

2.3. Protein identification

As in previous studies (Kayser et al., 2009, 2016), SDS-PAGE was performed with pre-cast NuPAGE Novex Bis-Tris 10% and 4–12% gels, using either Mes or Mops buffer. Stained protein bands of interest were excised from the gel and subjected to in-gel tryptic digestion. The cleavage peptides were isolated and sequenced by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Proteins were identified using the MASCOT search engine (http://www.matrixscience.com). Glycopeptides were analysed by electrospray ionization mass spectrometry (ESI-MS/MS). *UniProt* codes were used throughout.

2.4. Isolation and analysis of bilins

As in foregoing work (Kayser et al., 2014, 2016), bilins were extracted from the biliproteins with methanol and isolated by solid-phase adsorption and elution with methanol, or, preferentially, by phase-transfer to chloroform. UV–visible spectra were recorded on a Perkin Elmer Lambda 15 spectrophotometer. CD spectra together with UV–visible spectra were obtained with an Applied Photophysics Chirascan photometer. Reverse-phase HPLC was performed on a C18-column, coupled to a Shimadzu gradient system, and run with an acetonitrile/water gradient (25%–80% acetonitrile in 25 min) containing 0.1% trifluoroacetic acid; elution was monitored at 373 nm and operated at a flow rate of 1.8 mL/min. High resolution ESI MS was performed with a Thermo Science LTQ Orbitrap mass spectrometer with an accuracy of ± 3 ppm. The relative proportion of the three bilins in total isolates was estimated from the intensities of the molecular ions and, if present, specific derivatives (e.g., loss of water).

2.5. Chemistry

Oxidative micro degradation of bilins with chromate and chromic acid, respectively, combined with chromatography on silica gel was performed according to Rüdiger (1969). Methyl esters were