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Butterfly wings, a new site of porphyrin synthesis and cleavage: Studies on the expression of the lipocalin bilin-binding protein in *Pieris brassicae*

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Abstract

The bilin-binding protein (BBP), a member of the lipocalin protein superfamily, is synthesized mainly in last instar larvae and in late pupae and newly emerged adults of *Pieris brassicae*, as previously reported. Here we present results from Northern blot analysis of the BBP gene transcript and from in vitro studies of holo-BBP biosynthesis with isolated wings using $[^{14}C]$5-aminolevulinic acid as a precursor to the bilin ligand, $[^{14}C]$-amino acids to label the apo-protein and inhibitors for both processes. Our combined data clearly demonstrate that BBP, which accumulates around pupa-adult transformation, is produced as holoprotein in the developing wings, while the BBP gene transcript is no longer detected in the rest of the body. Forewings and hind wings behave markedly different as the latter represent the major site of BBP synthesis, in agreement with the unequal distribution of BBP in the wings. The presence of an active pathway of porphyrin synthesis and cleavage in insect wings, shown here for the first time, and the role of the biliprotein during wing development remains an enigma so far. As part of this work sequences of fragments of the genes for actin and glyceraldehyde-3-phosphate dehydrogenase were obtained and examined as reference house-keeping genes in the expression studies.
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1. Introduction

The bilin-binding protein (BBP) from *Pieris brassicae* was recognised from its crystal structure (Huber et al., 1987) as one of the first representatives of a since rapidly growing protein superfamily called lipocalins (Flower et al., 2000). Soon after BBP, insecticyanin from *Manduca sexta* was found to display the same conserved protein fold (Holden et al., 1987). Members of the lipocalin family have in common a size of ∼20 kDa and a topology characterised by eight antiparallel β-strands that form a barrel or calyx-like structure encompassing a cavity for binding of a specific, typically lipophilic ligand. Lipocalins have been found in all phyla suggesting basic roles of these proteins since the origin of life. During evolution, the number of

lipocalin genes increased tremendously by duplications, which provided the basis for adaptations to a wide range of functions, as recently reviewed for the arthropods (Kayser, 2005; Ganfornina et al., 2006).

BBP and insecticyanin are the only insect biliproteins so far identified as lipocalins on the basis of their crystal structures. While present in insects from a number of orders, most biliproteins have been isolated and characterised from lepidopteran species (Kayser, 2005). The specific ligand of these blue proteins typically is biliverdin-IXγ, an isomer that is almost exclusively found in lepidopteran insects (Kayser, 1985). Representing intensely coloured chromoproteins the insect biliproteins are commonly thought to play a role in camouflage. While this is obviously true in certain cases (e.g. in plant-eating larvae), the presence of isoforms, tissue-specific and developmentally regulated expression, as best studied in *M. sexta* (Li and Riddiford, 1994), strongly suggest that biliproteins

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primarily serve other yet enigmatic functions (for a discussion, see Kayser, 2005).

In former studies (Kayser, 1984; Kayser and Krull-Savage, 1984), we found two major periods of BBP synthesis as revealed by radiolabelling of both the bilin ligand and the apo-protein. These periods were in the last larval instar and in the late pupa up to the young adult insect. This developmental pattern has been confirmed by Northern blot analysis of the BBP transcript, performed by others (Schmidt and Skerra, 1994). Since formation of BBP requires both synthesis of the apoprotein and synthesis of the bilin ligand, we recently focused on tetrapyrrole synthesis by studying the activity of porphobilinogen synthase (PBGS) that catalyses the first committed step of the porphyrin biosynthetic pathway (Kayser et al., 2005). While the developmental pattern of PBGS activity was in agreement with that of BBP synthesis in whole insects, we unexpectedly found fairly high PBGS activity in the developing wings during the period of BBP synthesis in the late pupa. This observation gave rise to the question whether the tetrapyrrole ligand of BBP that is accumulated in the wings is also produced at this tissue. If so, we speculated that also the apo-protein of BBP may be formed in the wings.

In the present work, we demonstrate by Northern blot analysis and radiolabelling experiments mainly performed with isolated wings in vitro that, in fact, both constituents of BBP, the bilin and the apo-protein, are synthesized in the wings in the last days of adult development in the pupa. Moreover, the hind wings turned out to be the major site of BBP synthesis. Concomitantly, BBP expression is shut down in the residual body.

2. Materials and methods

2.1. Insects

Rearing of *P. brassicae* L. was performed essentially as described (Kayser, 1984). Adult insects were maintained at 20–25 °C under ambient light supplemented with artificial light for 16 h. The larvae were fed leaves of cabbage (*Brassica oleracea* var.). Last instar larvae were synchronised at the wandering stage and kept at constant 22 °C under a 16 h light: 8 h dark regime (to prevent diapause) until adult eclosion. Pupation was monitored at 12 h intervals. The pupal instar lasted 10.5 ± 0.5 days.

2.2. RNA isolation

Total RNA was isolated either from whole pupae between day 0 and day 10 after pupation (P0–P10) or from isolated hind wings, forewings, and the rest of the pupal body (i.e. the developing adult insect) between day 5 and day 10 (P5–P10) by the guanidinium-isothiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987) using the RNA clean reagent (AGS, Heidelberg). Poly(A)$^+$ RNA was prepared by the method of Sambrook

et al. (1989) using poly(A)quik oligo(dT) cellulose columns (Stratagene). The quality of the RNA was controlled by electrophoresis on 1% agarose gels buffered with 89 mM Tris/borate, 0.1 mM EDTA (pH 8.3). All samples of denatured RNA showed a characteristic band at 1.8 kb representing the small ("18S") rRNA plus fragments of the thermolabile large ("28S") rRNA (cf. Ishikawa et al., 1981).

2.3. RT-PCR

Synthesis of cDNA was carried out with 2 μg poly(A)$^+$ RNA in a reaction volume of 25 μL containing 1x RT buffer (Life Technologies), 10 mM DTT, 1 unit/μL RNAsin (Promega), 1 mM dNTP, 24 ng/μL oligo-dT primers as well as 1 unit/μL M-MLV reverse transcriptase (Life Technologies). Initially, the RNA was denatured at 65 °C for 2 min, and then the reaction mixture was added. Reverse transcription (RT) was performed for 60 min at 37 °C and subsequently for 15 min at 42 °C. The reaction was stopped by adding three volumes of 20 mM Tris-HCl, 0.1 mM EDTA (pH 8.0), and aliquots were stored at −20 °C.

Polymerase chain reaction (PCR) was set up in a reaction volume of 50 μL containing 1–2 μL cDNA, 1x Q solution, 200 μM dNTP, 1x reaction buffer, 2 ng/μL of each oligo primer, and five units Taq polymerase (all reagents from Qiagen). PCR was performed in a PTC-200 (MJ Research, Watertown, MA) using the touchdown strategy (Don et al., 1991). For the BBP probe the following protocol was used (TD1): after initial full denaturation for 2 min at 93 °C, in each subsequent cycle denaturation was performed for 15 s at 93 °C and elongation for 60 s at 72 °C. Annealing was performed for 30 s in each cycle, and the temperature was 57 °C for the first two cycles, it was then lowered for every second cycle by 2 °C until an annealing temperature of 51 °C was reached followed by 30 cycles at 57 °C. An elongation step of 5 min at 72 °C finalised the reaction. The same denaturation and elongation conditions were used to amplify several genes considered as possible references in the expression studies. These genes were those for actin, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase, NAD-dehydrogenase subunit 1 and the large subunit ribosomal RNA, using the primers listed in Table 1. When degenerate primers were used, the annealing temperature was lowered five times by 2 °C for every second cycle from 58 °C (TD2) to 48 °C followed by 30 cycles at 58 °C.

2.4. cDNA cloning

Amplicon DNA ligated into pMOS*blue* T vectors (Amersham) was cloned into Ca^{2+}-competent *E. coli* DH5α cells, as described by Sambrook et al. (1989). Positive clones were identified by a blue-white selection and plasmid DNA was isolated using the Wizard *Plus* system (Promega), following the instructions of the manufacturer.