Biochimica et Biophysica Acta 1788 (2009) 1695–1705

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Juvenile hormone binding protein traffic — Interaction with ATP synthase and lipid transfer proteins

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ARTICLE INFO

Article history:
Received 21 August 2008
Received in revised form 19 April 2009
Accepted 29 April 2009
Available online 6 May 2009

Keywords:
Arylphorin
ATP synthase
Hexamerin
Juvenile hormone binding protein
Lipophorin

ABSTRACT

Juvenile hormone (JH) controls insect development, metamorphosis and reproduction. In insect hemolymph a significant proportion of JH is bound to juvenile hormone binding protein (JHBP), which serves as a carrier supplying the hormone to the target tissues. To shed some light on JHBP passage within insect tissues, the interaction of this carrier with other proteins from *Galleria mellonella* (Lepidoptera) was investigated. Our studies revealed the presence of JHBP within the tracheal epithelium and fat body cells in both the membrane and cytoplasmic sections. We found that the interaction between JHBP and membrane proteins occurs with saturation kinetics and is specific and reversible. ATP synthase was indicated as a JHBP membrane binding protein based upon SPR-BIA and MS analysis. It was found that in *G. mellonella* fat body, this enzyme is present in mitochondrial fraction, plasma membranes and cytosol as well. In the model system containing bovine F_1 ATP synthase and JHBP, the interaction between these two components occurs with $K_d = 0.86$ nM. In hemolymph we detected JHBP binding to apolipophorin, arylphorin and hexamerin. These results provide the first demonstration of the physical interaction of JHBP with membrane and hemolymph proteins which can be involved in JHBP molecule traffic.

1. Introduction

The term juvenile hormone (JH) refers to six sesquiterpene homologs, all of which contain an ester bond and epoxy bond that are required for hormone regulatory functions. JH regulates many processes, including the growth, development, metamorphosis and reproduction of insects [1,2]. The diversity of JH-mediated physiological effects suggests that target cells may respond to the hormone directly by gene expression and/or via a secondary messenger [3,4]. JH, secreted from corpora allata to hemolymph, binds to juvenile hormone binding protein (JHBP), which protects the hormone from the action of nonspecific esterases and JH epoxide hydrolase [1,5]. JHBP serves as a carrier protein to target cells as part of the hormonal signal transfer mechanisms.

Three types of JHBP have been described in insects: low molecular weight proteins of approximately 30 kDa, and two types of high molecular weight proteins: lipophorins and hexameric proteins. Lipophorins contain two or three apolipoproteins: apolipophorin I (apoLp-I), apolipophorin II (apoLp-II) and apolipophorin III (apoLp-III) with molecular weights of ~220–250, 80, 17–20 kDa, respectively [1,6]. The insect apolipoproteins belong to the family of large lipid transfer (LLT) proteins that includes vitellogenins, microsomal triglyceride transfer protein and mammalian apolipoprotein B. The second group of high molecular weight proteins binding JH belongs to the superfamily of hexameric larval hemolymph proteins (LHP), named according to their composition of six identical subunits of 74–82 kDa and in *G. mellonella*, four LHPs with numbers indicating their subunit molecular masses were identified: LHP 74, 76, 81, 82 [7]. In Lepidoptera only low molecular mass proteins (~30 kDa) have specific affinity to JH [8,9]. It was previously assumed that the lipophilic nature of JH allows it to diffuse through a cell membrane bilayer. However, more than 99% of the JH molecules in hemolymph are bound by JHBP [10].

JH binding to low molecular weight JHBP in *G. mellonella* (Greater Wax Moth) induces a profound conformational transition in the protein molecule reflected in the changes in the sedimentation coefficient, electrophoretic mobility and perturbation of Tyr residues and disulfide bridges [11,12]. This might be important in the transmission of hormone signals and the recognition of target cells. However, there is no information concerning the traffic of JHBP

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doi:10.1016/j.bbamem.2009.04.022

molecules from fat body cells, where they are expressed [13], to hemolymph and then to target cells. This passage proceeds in the presence of other macromolecules possibly interacting with each other. There are a vast number of transient protein–protein interactions, which in turn control a large number of cellular processes [14]. In order to understand the significance of various protein interactions we need to identify them, determine the extent to which they occur, and examine their consequences. Thus, a study of the JHBP interaction network with other protein(s) is a prerequisite to understanding the mechanism of delivering JH to target cell membranes.

In this report JHBP interaction with apolipophorin, arylphorin and hexamerin from *G. mellonella* hemolymph was shown. In fat body cell membranes, ATP synthase was identified as a JHBP-binding protein. This allowed us to postulate that ATP synthase participates in JHBP export, and JHBP in complex with hemolymph proteins take part in JH transport to target cells.

2. Materials and methods

2.1. Chemicals

Juvenile hormone III (10R,S-JH III) was purchased from Sigma. 10-[^{3}H]-labeled JH III and Na [^{125}I] were purchased from Polatom (Poland).

2.2. Insects

Galleria mellonella (Lepidoptera, Pyralidae) larvae were reared in constant darkness at 30 °C on a semi-artificial diet prepared as described by Sehnal and Slama [15].

The hemolymph from 4th day, VIIth instar larvae was collected into a plastic tube containing a few crystals of 1-phenyl-2-thiourea and stored at −20 °C.

2.3. Determination of protein concentrations

Protein concentrations were determined using the Bradford method [16]. For samples containing Triton X-100, the sample was diluted with a buffer to a detergent concentration of 0.1% before assay, and bovine serum albumin (BSA) in 0.1% Triton X-100 was used as a standard protein.

2.4. The purification of juvenile hormone binding protein (JHBP) and preparation of anti-JHBP antibodies

JHBP was purified from hemolymph by immunoaffinity chromatography [17]. The JH-binding activity was determined with a charcoal assay [18] in the presence of 0.1% gelatin [19]. Polyclonal antibodies against JHBP were obtained as previously described [13].

2.5. Immunostaining

Immunohistochemistry was performed on 4 μm thick sections, mounted on poly-L-lysine coated slides, using the avidin–biotin–peroxidase complex (ABC) technique with reagents supplied by DAKO. *G. mellonella* larvae were embedded in paraffin. Following deparaffination and rehydration, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide, followed by a non-immune swine serum for 30 min to block nonspecific binding. The sections were then incubated with primary polyclonal antibodies against JHBP at 4 °C overnight at a dilution of 1:1500. Control trials were incubated with phosphate-buffered saline (PBS) instead of the primary anti-JHBP antibodies. This was followed by incubation with biotinylated horse anti-rabbit IgG for 15 min and thereafter with the avidin–biotin–peroxidase complex (ABC reagent) for 15 min. Between each step sections were washed twice by PBS. The reaction product was visually observed using 3,3-diaminobenzidine tetrahydrochloride (DAB) solution. After counterstaining with haematoxylin, slides were dehydrated, cover slipped and examined using a light microscope supplied with a digital camera system.

2.6. Separation of hemolymph high molecular weight proteins from low molecular weight proteins

7 ml of hemolymph was applied onto a Sephadex G-200 column (K 16/100, Pharmacia) equilibrated with a 10 mM Tris buffer, 100 mM NaCl, 0.25 mM 1-phenyl-2-thiourea, pH 7.3. The flow rate was 17 ml/h and 7 ml fractions were collected. Fractions from the first absorption peak (A_{280}) [17,19], containing high molecular weight proteins (HMWP), were used for ligand blotting analysis.

2.7. SDS-PAGE electrophoresis and ligand blotting of hemolymph fractions

Proteins were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [20] under reducing or non-reducing conditions (we didn't boil the sample or add reducing agents to minimize irreversible protein denaturation). Gels consisted of a 4% stacking gel and a 12% resolving gel. Electrophoresis was carried out at 30 mA/slab gel until the dye front reached the bottom of the gels. Separated protein gels were stained with Coomassie Brilliant Blue R for visual observation. Proteins from unstained gels were transferred to nitrocellulose transfer paper [21] for 1 h at 150 V. The papers containing transferred proteins were first incubated in a buffer (10 mM Tris, 150 mM NaCl, pH 7.5, supplemented with 3% non-fat dry milk) for 1 h at 25 °C. After blocking, the papers were incubated with JHBP (1 mg/ml) or with JHBP (1 mg/ml) preincubated for 0.5 h at 4 °C with JH (20 μM) in polyethylene glycol coated tubes. A negative control was done with BSA (1 mg/ml) in a 10 mM MOPS, 100 mM NaCl pH 7.2 buffer. Next, the papers were washed three times with the buffer, treated with a 1:25 000 dilution of polyclonal anti-JHBP and washed again three times with the buffer. Then, the nitrocellulose papers were incubated with 1:5000 dilution of goat anti-rabbit polyclonal antibodies conjugated with horseradish peroxidase (HRP) (Sigma). The blot assay was developed using a peroxidase detection kit (ECL plus Western Blotting Detection System, Amersham).

2.8. Coupling of the CNBr-activated Sepharose beads with JHBP

JHBP was coupled to Sepharose beads according to the manufacturer's instructions (Sigma). Briefly, 1 mg (0.75 ml) of JHBP in 0.1 M $NaHCO_3$, pH 8.3, was mixed with 0.25 ml of CNBr-activated Sepharose beads in suspension and left overnight at 4 °C with gentle shaking. After washing with the buffer and blocking with ethanoloamine, pH 8.0, the beads were stored in 0.04% NaN_3 at 4 °C before use.

2.9. Binding hemolymph proteins to JHBP immobilized on Sepharose

Hemolymph proteins were separated on a Sephadex G-200 column [17], equilibrated with a 10 mM MOPS, 100 mM NaCl buffer, pH 7.2 and the fractions were combined from the first absorption peak (A_{280}), containing high molecular weight proteins (HMWP). 10 ml of HMWP (3 mg/ml) in the buffer was incubated with 0.2 ml of immobilized JHBP (0.6 mg). After incubation for 1 h at 4 °C the beads were washed with 6 volumes of the buffer and the bound protein complexes were eluted by competitive elution with JHBP (0.3 mg, 0.4 ml) or with aldolase (0.46 mg, 0.4 ml) as a control. Eluted proteins were electrophoresed and detected by Coomassie Blue staining. Protein bands were excised and identified using tandem mass spectrometry analyses (MS). MS experiments and

database searches using the Mascot program were performed by the Laboratory of Mass Spectrometry, Polish Academy of Sciences, Warsaw, Poland.

2.10. Radio-labeling of JHBP

JHBP was iodinated with Na [^{125}I] using an Iodogen reagent as an oxidizing agent (Sigma). 60 μg of JHBP (75 μl) in a 0.1 M sodium phosphate buffer, pH 7.2 was added to tubes coated with 10 μg of Iodogen and incubated with 0.5 mCi Na [^{125}I] (25 μl) for 10 min at 4 °C. Iodinated protein was separated from free iodine by gel filtration on a Sephadex G25 (PD-10, Amersham). The radioactive protein samples were combined. The specific activity of radio-iodinated protein was usually 2–2.5 × 10^3 cpm/ng JHBP. The iodinated JHBP activity was verified by the JH-binding activity test.

2.11. Isolation and solubilization of fat body membrane proteins

To obtain membrane fraction we adopted Ueno et al. procedure previously applied to preparation of membranes from fat body of *Sarcophaga peregrina* [22]. Briefly, fat body tissue was isolated from 4th day, VIIth instar larvae of *G. mellonella*, placed in a 50 mM HEPES, 0.1 mM $CaCl_2$ buffer, pH 8.5 and stored at −80 °C until used. Fat body was homogenized in the ice-cold buffer containing protease inhibitors: leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (1 μg/ml) and bestatin (1 μg/ml) using a glass-Teflon tissue grinder. The homogenate was centrifuged at 800 × *g* for 10 min at 4 °C to remove nuclei and the resulting supernatant was centrifuged at 10 000 × *g* for 20 min at 4 °C to collect membrane fraction [22]. The pellet was washed once with the buffer. Membrane protein suspension was used directly in binding ^{125}I-JHBP or was diluted with an equal volume of buffer containing 2% Triton X-100 for use in SPR analysis. After mixing for 3 h at 4 °C, insoluble material was removed by centrifugation at 15 000 × *g* for 20 min. To evaluate the quality of membrane protein preparation the phosphatase activity was examined.

As it will be shown later, we detected ATP synthase in the membrane fraction obtained according to the procedure described above. To show that *G. mellonella* fat body membranes indeed contain the ATP synthase and that its presence is not due to mitochondria, a second method of subcellular structure fractionation described by Hryb et al. [23] was also used. Briefly, freshly excised fat body tissue was placed in an isotonic buffer composed of 0.3 M sucrose, 25 mM Tris, pH 7.4 and protease inhibitors. The tissue was homogenized and the homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C to remove nuclei and the resulting supernatant was centrifuged at 15 000 × *g* for 20 min at 4 °C to collect some membrane sheets and mitochondria. The supernatant was centrifuged at 100 000 × *g* for 1 h at 4 °C to collect membranes. SDS-PAGE and western blotting with anti-ATPase was applied to analyze the obtained fractions.

2.12. SDS-PAGE electrophoresis of subcellular fractions and their analysis with anti-α chain of ATP synthase

Proteins were separated with SDS-PAGE under reducing conditions [20]. Separated protein bands were transferred to nitrocellulose papers [21] for 1 h at 150 V. The papers containing transferred proteins were first incubated in a blocking buffer (10 mM Tris, 150 mM NaCl, pH 7.5, supplemented with 5% non-fat dry milk) for 1 h at 25 °C. The nitrocellulose membranes after blocking were treated with monoclonal antibody to α chain of ATP synthase (Mitosciences) diluted 1:1000 in the buffer supplemented with 1% non-fat dry milk and washed three times with the buffer. Then, the nitrocellulose papers were incubated with 1:5000 dilution of horse anti-mouse antibodies, diluted in the buffer supplemented with 1% non-fat dry milk, conjugated with horseradish peroxidase (Vector Laboratories) and washed three times with the buffer. The blot assay was developed using a peroxidase detection kit (ECL plus Western Blotting Detection System, Amersham).

2.13. Phosphatase assay

Alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate as a substrate [24]. Briefly, *p*-nitrophenyl phosphate (25 mM) was dissolved in a 50 mM Tris buffer, pH 10.0 and membrane protein suspension was added. After incubation at 37 °C for 30 min the amount of product formed was calculated from the absorbance at 410 nm, with reference to a standard curve prepared using pure *p*-nitrophenol. One unit of enzyme activity corresponds to one μmole of *p*-nitrophenol produced per minute. The specific activity in the membrane protein fraction was usually 0.3 U/mg.

2.14. Binding ^{125}I-JHBP to fat body membrane proteins

The reaction mixture (0.2 ml) contained radio-iodinated JHBP (6 to 95 nM), membrane protein suspension (0.5 mg/ml) and BSA (10 mg/ml) in a 10 mM MOPS, 100 mM NaCl buffer, pH 7.2. ^{125}I-JHBP was incubated with membrane proteins in the presence or absence of a 100-fold excess of cold JHBP for 1 h at 37 °C. Following incubation, membrane proteins were separated by centrifugation at 10 000 × *g* for 10 min. After the supernatant was removed, membrane proteins were washed twice rapidly with 0.2 ml of the buffer and then suspended in 0.2 ml of this buffer. Radioactivity was measured with a γ-scintillation counter (Beckman, Tri-Carb 2700TR). The amount of bound JHBP was calculated from the radioactivity associated with the membrane proteins. Nonspecific binding was determined using a 100-fold excess of unlabeled JHBP. The specific ^{125}I-JHBP binding was obtained by subtracting the nonspecific binding from the total radio-ligand binding.

2.15. Limited trypsinization of fat body membrane proteins

Fat body membrane protein suspension (0.5 mg/ml) was incubated with trypsin (25 μg/ml) for 0–30 min at 37 °C in 10 mM MOPS, 100 mM NaCl, pH 7.2, followed by centrifugation (20 min, 10 000 × *g*). Subsequently, the membrane proteins were resuspended in the buffer and assayed for binding ^{125}I-JHBP under standard conditions.

2.16. Surface plasmon resonance-biomolecular interaction analysis (SPR-BIA)

SPR-BIA was performed with a Biacore 3000 instrument. An HBS buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 0.005% (w/v) polysorbate and 3 mM EDTA was used as a running buffer at 20 μl/min flow at 25 °C. JHBP-JH complex was prepared by incubating JHBP (1 mg/ml) with JH (20 μM) for 0.5 h at 4 °C in polyethylene glycol coated tubes. JHBP and JHBP-JH were covalently immobilized on the carboxymethyl dextran in the flow cells of a CM5 sensor (chip) surface using the standard amine-coupling method [25]. EDC/NHS (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride/*N*-hydroxysulfosuccinimide) was used to activate the carboxyl groups of the carboxymethyl surface. Then, 150 μl of JHBP (100 μg/ml) in an acetate buffer, pH 4.0, was injected over one flow cell. On the other flow cells, 150 μl of JHBP-JH (100 μg/ml) and BSA (200 μg/ml) were injected. Next, ethanolamine was added to block NHS esters which had not reacted. The SPR response measured at the end of EDC/NHS injection was subtracted from the final SPR response measured after ethanolamine injection to estimate the amount of protein immobilized on the cell surface.

Next, fat body membrane protein solution from *G. mellonella* was diluted to 0.47 μg/ml in an HBS buffer (at 0.2% final concentration of Triton X-100) and 180 μl aliquots were injected over JHBP and JHBP-JH-attached SPR solid support (sensor) surfaces as well as over empty flow cell.

2.17. Isolating JHBP-binding proteins using surface plasmon resonance and enzymatic digestion of recovered proteins

180 μl (0.47 μg/ml) of membrane protein solution was injected over a JHBP-attached sensor surface. After washing out the unbound fraction, the residual JHBP-binding proteins were eluted with 6 μl of 0.5 M urea/10 mM triethylamine [26] and collected in an Eppendorf tube according to the recovery procedure of the Biacore instrument. The recovery step was done using the full chip volume. Immediately after elution, triethylamine was rapidly evaporated under a vacuum. The proteins were digested by adding a solution of trypsin in 50 mM NH_4HCO_3 (20 ng/μl sequence grade, Promega). The mixture was incubated for 3 h at 37 °C.

2.18. Nanoelectrospray tandem mass spectrometry

Recovered peptides were subjected to nanoLC-MS/MS analysis on an ESI-Q-TOF mass spectrometer (QSTAR XL, Applied Biosystems) operating in the positive mode with a 2.1 kV spray voltage. Chromatographic separation was performed on a 75 μm ID × 15 cm PepMap C18 column (Dionex/LC Packings, USA) at a flow rate of 200 nl/minute using a linear gradient of increasing acetonitrile (ACN) in water (5–50%) over 40 min with 0.1% formic acid as the ion pairing agent. Data were acquired with Analyst QS (version 1.1, Applied Biosystems).

2.19. Direct binding assays by surface plasmon resonance

JHBP and BSA (as a control) were immobilized under the conditions described above, assuming that 1000 RU corresponds to 1 ng of immobilized protein. To measure binding interactions, 90 μl of F_1 ATP synthase, at different concentrations ranging from 0.75 nM to 25 nM in HBS, was passed over the immobilized protein surface at a flow rate of 30 μl/min. Next, the formed complexes were allowed to dissociate by injecting 120 μl of the HBS. After each binding assay flow cells were regenerated with short pulses of 0.005% SDS, at a flow rate of 30 μl/min and repeated washes with HBS. Results are represented as sensorgrams, expressed as the response in resonance units as a function of time in seconds. Rate constants were determined by using a global fitting routine provided by BIAcore (BIAevaluation 4.0.1). The measured association (k_a) and dissociation (k_d) rate constants allowed us to determine the equilibrium dissociation constant K_d, through the ratio k_d/k_a.

3. Results

3.1. The immunohistochemical localization of JHBP in the fat body cell membranes of G. mellonella

To show the tissue localization of JHBP, an immunohistochemical analysis was performed on paraffin embedded sections of *G. mellonella* larvae, using anti-JHBP polyclonal antibodies. Significant staining was observed in the fat body (both membrane and cytoplasmic reaction) (Fig. 1A–C) and in the tracheal epithelium (Fig. 1B). Scattered JHBP was also present in the cuticular epithelium (not shown). JHBP immunoreactivity was not detected in the muscles (Fig. 1A). The distribution of JHBP appeared to be divergent – not only histologically (Fig. 1A, B) – but also at the cellular level. At higher magnification, JHBP was detected on plasma membranes suggesting that it interacts with cell membrane protein(s) (Fig. 1C).

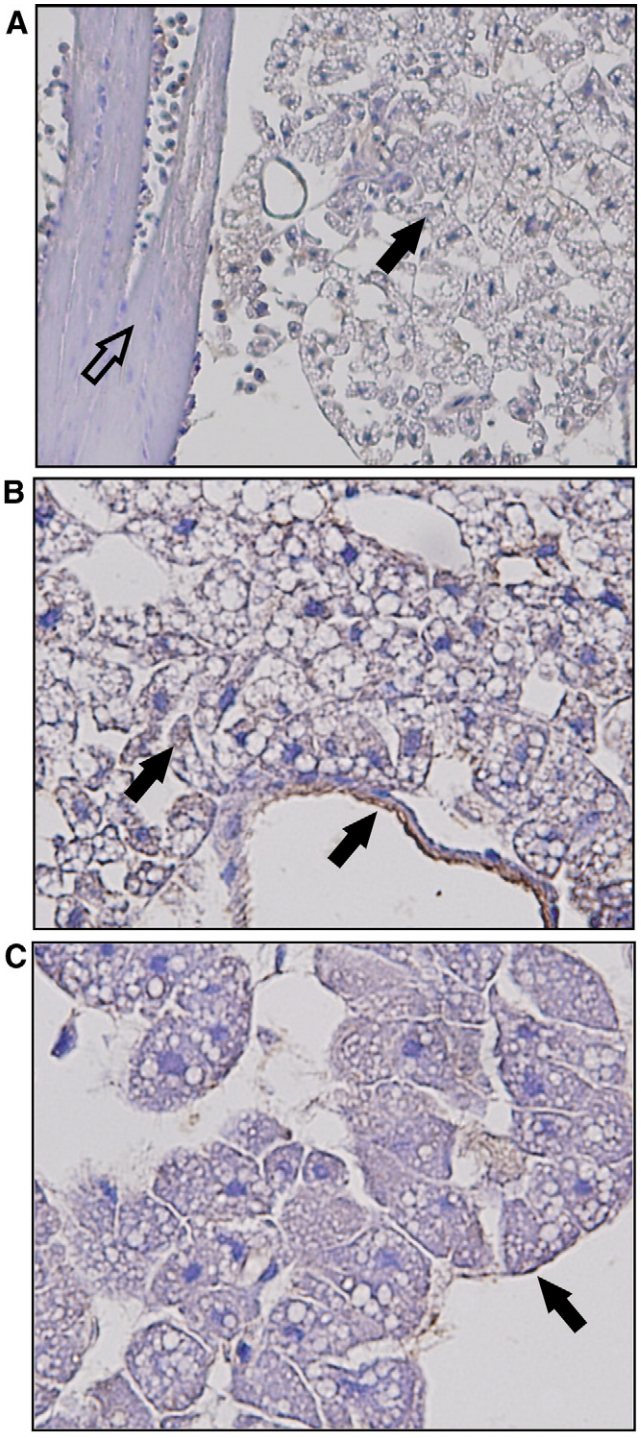


Fig. 1. Immunohistological staining for JHBP in the fat body, muscles and tracheal epithelium of the 4th day, VIIth instar larvae of *G. mellonella*. Anti-JHBP polyclonal antibodies were used for the immunoassay which gave a brown positive signal. Purple staining represents counterstaining with haematoxylin. (A) JHBP was found in the fat body cells (filled arrow) and muscles remain negative for JHBP (empty arrow) — magnification × 220. (B) JHBP is present in fat body cells (filled arrow) and also in the tracheal epithelium (filled arrow) — magnification × 550. (C) Localization of JHBP on the fat body cell membrane (filled arrow) — magnification × 660.

3.2. Do JHBP molecules interact with proteins present in hemolymph?

The concentration of hemolymph proteins is extremely high, approaching 150 mg/ml [19]. This raised two questions: in what form do JHBP molecules appear with such a high protein concentration, and

is JHBP preferentially bound to some proteins? To this end hemolymph proteins were fractionated on a Sephadex G-200 column and separated into two main A_{280} absorption peaks, the first containing high molecular weight proteins (HMWP) and the second low molecular weight proteins (LMWP) as shown by Ożyhar and Kochman [19] and Wieczorek et al. [17]. Proteins from the first peak were applied to SDS-PAGE. In order to determine whether JHBP interacts with hemolymph proteins, ligand blotting was performed [27]. Interaction between JHBP and hemolymph proteins was observed with HMWP containing mostly lipoproteins. In this fraction we found at least two protein bands of about 220 kDa and 120 kDa (Fig. 2, lane P_1) which interact with JHBP. An analogous experiment performed in the presence of JH revealed an increase in the intensity of the 220 kDa band in the HMWP fraction interacting with JHBP (Fig. 2, lane P_2). In the control experiment, where bovine serum albumin (BSA) was used instead of JHBP, no antibody binding to HMWP was observed (Fig. 2, lane K_1). For comparison, electrophoretic mobility of JHBP is shown in lane K_2. Its microheterogeneity is due to different extent of glycosylation of JHBP [28].

3.3. The identification of JHBP-binding proteins with affinity chromatography and mass spectrometry analysis

The affinity chromatography technique was used to confirm that hemolymph proteins preferentially bind JHBP. HMWP were applied on a JHBP-bound Sepharose column and after excessive washing with a buffer, the column was treated with free JHBP acting as a specific competitor or with aldolase as a control (nonspecific) ligand. The eluted protein fractions were separated on SDS-PAGE and analyzed with mass spectrometry. For control elution we chose aldolase because in SDS-PAGE this protein band is easily separated from high molecular weight proteins. In contrast, commercial BSA was heterogeneous in SDS-PAGE, showing protein bands partially overlapping with high molecular weight proteins (not shown). Additionally, muscle aldolase exhibits some affinity to lipids and is a basic protein with a similar p*I* as JHBP [29,30]. SDS-PAGE analysis revealed three proteins eluted by JHBP (Fig. 3, lane E_1). Their positions correspond to molecular masses: 75 kDa, 80 kDa and 220 kDa. In the fraction eluted by aldolase, 220 kDa protein band is also present but it is barely visible in comparison to JHBP eluted protein. Very weak additional bands (not marked) present in the fraction eluted by aldolase (Fig. 3, lane E_2) are also present in the lane with free aldolase (Fig. 3, lane P_2) and represent some impurities, and therefore cannot be hemolymph proteins. Protein bands corresponding to 220 kDa, 80 kDa and 75 kDa masses were subjected to ESI-FTICR-MS analysis and identified as apolipophorin, hexamerin and arylphorin, respectively (Table 1). Mass spectrometric analysis detected 623 of the 1515 amino acids of apolipophorin (41%), 159 of the 706 amino acids of hexamerin (22%) and 403 of the 702 amino acids of arylphorin (57%). Sequences of identified *G. mellonella* proteins, published earlier on the Expasy Proteomics Server, came from a conceptual translation of DNA, because these three proteins have not yet been purified and sequenced from *G. mellonella*. Interestingly, potential JHBP-binding proteins: apolipophorin, arylphorin and hexamerin are

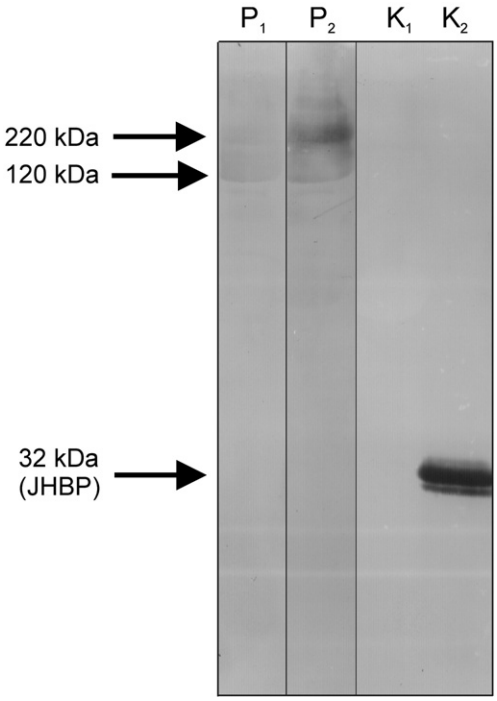


Fig. 2. Ligand blot analysis of JHBP or JHBP-JH complex binding to hemolymph HMWP. 40 μg of HMWP (lanes: P_1, P_2 and K_1) and 0.2 μg of JHBP (lane: K_2) were separated by SDS-PAGE under conditions designed to minimize irreversible protein denaturation and were transferred to nitrocellulose membrane. The binding of HMWP was performed in the absence (lane K_1) or presence of JHBP (lane P_1) or JHBP-JH complex (lane P_2) as ligands. The membrane after washing was subsequently treated with polyclonal antibodies against JHBP (1:25 000). Binding was visualized with HRP-goat anti-rabbit (1:5000) as a secondary antibody and a chemiluminescence system. The arrows indicate the positions of proteins involved in interaction with JHBP and/or JHBP-JH complex (220 and 120 kDa). For comparison the mobility of free JHBP molecules is indicated (32 kDa).

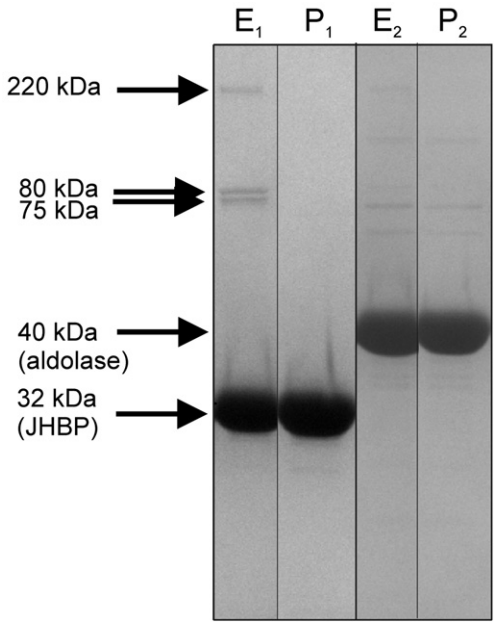


Fig. 3. Interaction between the HMWP and JHBP by affinity chromatography. 10 ml of HMWP (3 mg/ml) was incubated with 0.6 mg of JHBP immobilized on Sepharose beads (0.2 ml). After incubation the beads were washed. Proteins eluted by JHBP (0.3 mg, 0.4 ml) or by aldolase (0.46 mg, 0.4 ml) as specific and nonspecific competitors are shown in lanes E_1 and E_2, respectively. Eluates (28 μl) were analyzed by SDS-PAGE and stained with Coomassie Blue. Samples (28 μl) of JHBP (lane P_1) and aldolase (lane P_2) used for elution were electrophoresed as additional controls. The arrows indicate the positions of JHBP-binding proteins (220, 80, 75 kDa) and competitors, JHBP (32 kDa) and aldolase (40 kDa).

Table 1
JHBP binding hemolymph proteins found by affinity chromatography and identified by tandem mass spectrometry and Mascot database searches.

Molecular mass from SDS-PAGE [kDa]	Protein name (*Galleria mellonella*)	NCBI accession number	Calculated molecular mass [kDa]	No. of peptides identified	Sequence coverage [%]
220	Apolipophorin [fragment]	Q68YP1	168.3	61	41
80	Hexamerin	Q24997	81.4	17	22
75	Arylphorin	Q24995	83.7	55	57

high molecular weight proteins that have been shown to be involved in JH binding in insects [1].

3.4. Analysis of JHBP binding to fat body membranes

As was demonstrated above, immunohistochemical studies revealed the presence of JHBP in fat body cells and the tracheal epithelium (both in cytoplasm and in plasma membranes) (Fig. 1). To characterize the kinetics of interaction between JHBP and fat body membrane proteins, binding experiments were conducted in the presence of a fixed amount of fat body membrane protein suspension and increasing amounts of radio-iodinated JHBP (6 to 95 nM). The binding of radio-labeled JHBP to membrane proteins was proportional to the membrane protein concentration and maximum binding was obtained after 60 min at 37 °C (not shown). These conditions were used for all subsequent experiments. We observed a nonspecific binding which was dependent on the BSA concentration (1–10 mg/ml). Thus, further experiments were carried out in the presence of 10 mg/ml of BSA. The specific binding of JHBP to a fixed amount of membrane proteins increased with a rise in the dose of JHBP and reached a level of saturation. Fig. 4A shows the specific and nonspecific JHBP binding to fat body membrane proteins and shows that equilibrium was achieved. The equilibrium binding data were analyzed using the Scatchard equation (Fig. 4B). We noted that the plot of bound versus bound/free JHBP is linear, suggesting the presence of a single type of binding site. The estimated dissociation constant (K_d) of JHBP was 0.105 ± 0.04 μM. These results indicate that fat body membranes contain specific binding sites for JHBP. To find out whether JHBP binding to fat body membrane proteins occurs via proteins or via other components of the membranes, the fat body membrane protein suspension was treated with trypsin. It has been previously shown that JHBP is resistant to trypsinolysis [12]. As shown in Fig. 4C, JHBP binding decreased with an increase in the pretreatment time of fat body membrane proteins with trypsin. Thus, we conclude that JHBP binding to fat body membranes is sensitive to trypsin and that binding activity is due to the presence of proteins in the membrane.

3.5. Detection of JHBP binding to fat body membrane proteins by SPR-BIA

Surface Plasmon Resonance-Biomolecular Interaction Analysis (SPR-BIA) is a technique capable of real-time monitoring of the interactions between two partners without prior labeling of either of them [31]. The ability of fat body membrane proteins purified from *G. mellonella* to bind to immobilized JHBP and JHBP-JH was investigated using this technology.

JHBP and JHBP-JH were firstly immobilized as described in Materials and methods section (2.16). Assuming that 1000 Resonance Units (RU) correspond to 1 ng of protein attached to the sensor surface per mm^2, final density of JHBP immobilized onto the electrode surface was 2.3 ng/mm^2 (2300 RU) and final density of JHBP-JH was 2.4 ng/mm^2 (2400 RU). Solubilized membrane proteins in 0.2% Triton X-100 were injected over JHBP, JHBP-JH complex and over a reference cell surface (Fig. 5A). The reference cell surface represents a free cell which was activated and blocked without any protein. In pre-experiments we found that 0.2% Triton X-100 solution does not change the value of JHBP's response units (not shown). The differential responses describing the specific interaction between membrane proteins and JHBP were obtained by subtracting a nonspecific response signal generated by the reference cell from the signal observed with cells containing immobilized JHBP or JHBP-JH complex (Fig. 5B). At the end of a 4 min injection step, a specific interaction of 140 RU was reached over the surface coated with JHBP, whereas no binding was observed on the surface coated with the JHBP-JH (Fig. 5B). This indicates that when JHBP binds JH, it is incapable of protein–protein interactions in the above applied conditions.

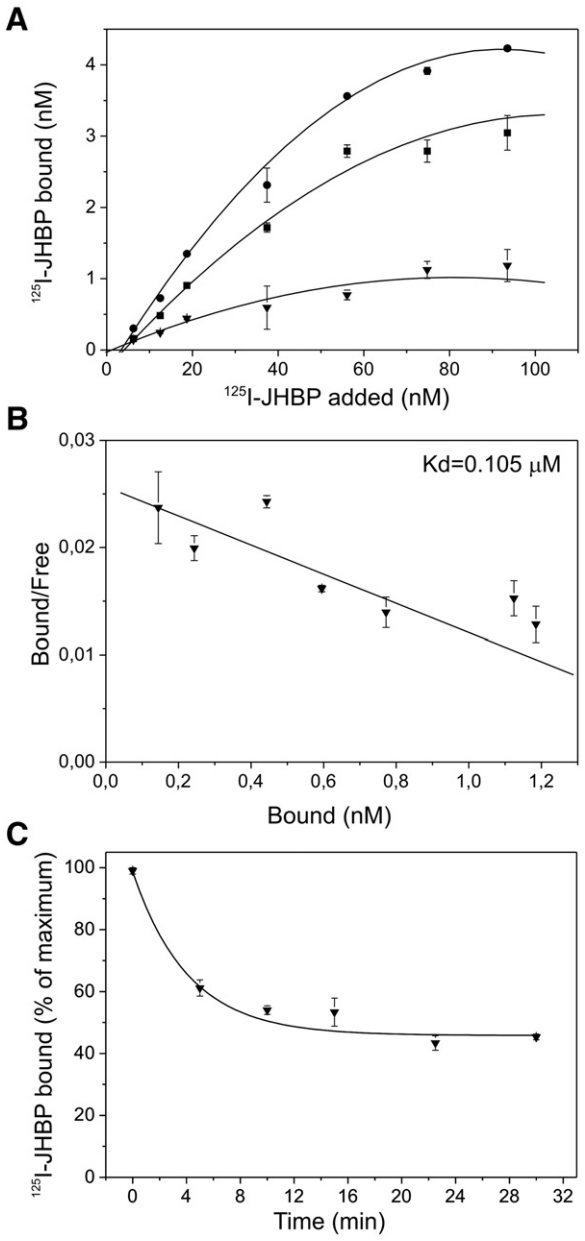


Fig. 4. JHBP binding to fat body membrane proteins from *G. mellonella*. (A) Equilibrium binding experiments were performed using increasing amounts of radio-labeled JHBP (6 to 95 nM). Fat body membrane protein suspension (100 μg) was incubated with ^{125}I-labeled JHBP alone or in the presence of a 100-fold excess of unlabeled JHBP. (●) — total binding; (■) — binding in the presence of cold JHBP (nonspecific binding); (▼) — specific binding (total minus nonspecific). (B) Scatchard analysis of the binding of ^{125}I-JHBP to membrane proteins. The analysis indicates that JHBP binds to a membrane ligand with $K_d = 0.105 \pm 0.04$ μM. (C) The effect of trypsin pretreatment of the fat body membrane proteins on JHBP binding. Membrane protein suspension (0.5 mg/ml) was preincubated for 0–30 min, with 25 μg/ml trypsin. The binding assay was performed with 100 nM ^{125}I-JHBP. Each point represents the mean ± S.D.

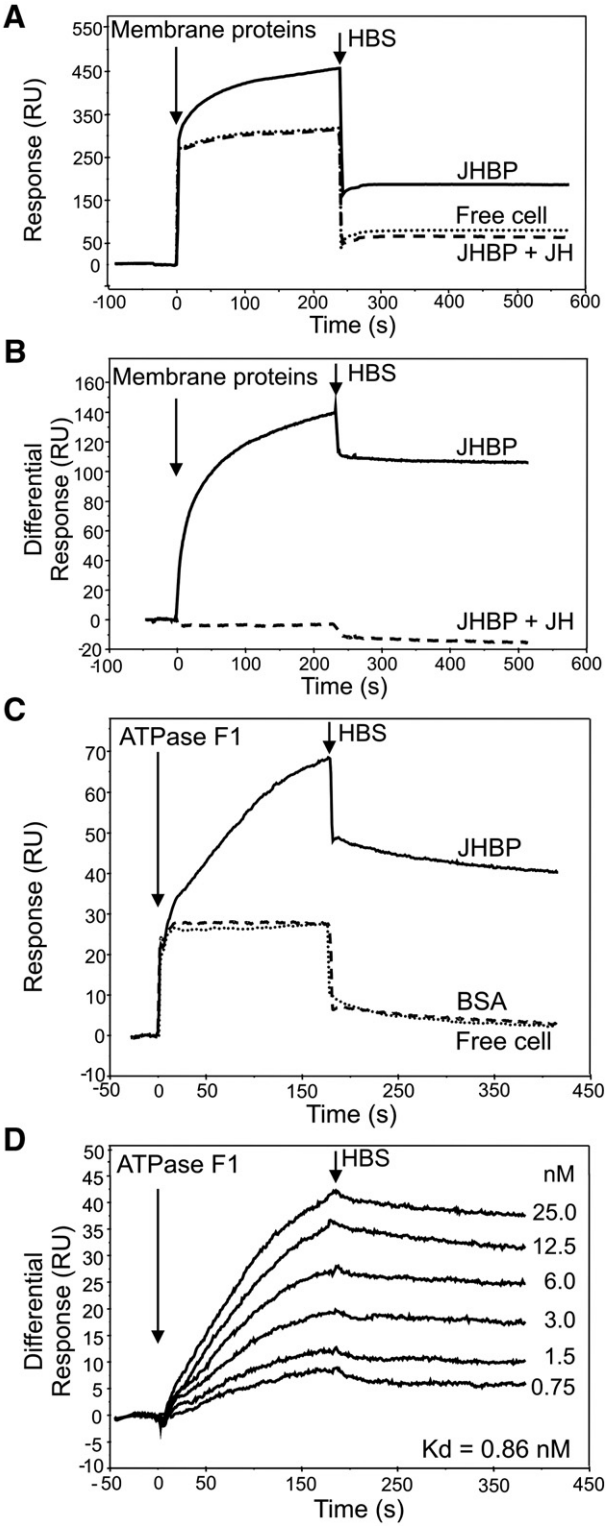
3.6. Identification of JHBP-binding proteins by SPR-BIA combined with mass spectrometry

Recently, researchers combined SPR-BIA and MS in a method that allowed selective retrieval of an analyte from a sensor chip [26]. Using this method, followed by mass spectrometry analysis of the elute from the sensor chip, we were able to identify JHBP-binding proteins. Protein identification was performed using ESI-QUAD-TOF-MS/MS together with database searching using the Mascot programme [32]. Identified proteins were grouped in Table 2 (using 5 peptides identified as a cut-off). Mass spectrometric analysis identified α and

β chains of ATP synthase as JHBP-binding proteins. Among the proteins bound on the chip we also found translocase, which probably associates with ATP synthase. This had been suggested earlier by Aggeler et al. [33]. Mass spectrometric analysis detected 113 of the 551 amino acids of the *Anopheles gambiae* ATP synthase α chain (21%), 102 of the 505 amino acids of the *Drosophila melanogaster* ATP synthase β chain (20%) and 46 of the 300 amino acids of *Bombyx mori* translocase (15%). In total, 11 peptides with amino acid sequences that matched the exact sequences of the ATP synthase α chain, 8 peptides of the ATP synthase β chain and 5 peptides of translocase were identified (Table 2).

Table 2
The potential JHBP-binding proteins identified in fat body membrane proteins eluted from the chip surface and identified by tandem mass spectrometry and Mascot database searches categorized according to their sequence coverage.

Protein name (organism identity)	NCBI accession number	Calculated molecular mass [kDa]	No. of peptides identified	Sequence coverage [%]
α chain ATP synthase (*Anopheles gambiae*)	Q7PHI8	59.4	11	21
β chain ATP synthase (*Drosophila melanogaster*)	Q05825	54.1	8	20
Translocase (*Bombyx mori*)	Q86PG2	32.9	5	15



Detection of ATP synthase in the membrane protein fraction could be due to the presence of some mitochondria remaining in the membrane sheets fraction obtained at 10 000 ×*g* centrifugation, see Materials and methods (2.11). To clarify this point, we subjected the 10 000 ×*g* supernatant to 100 000 ×*g* centrifugation. Protein samples were separated by SDS-PAGE and subjected to western blotting analysis using antibodies of broad specificity against both mammalian and insect ATP synthase. As expected, we detected ATP synthase in the pellet but positive staining was also observed with proteins present in 100 000 ×*g* supernatant (Fig. 6A). To verify that ATP synthase appears in the cell membrane faction too, we applied another procedure of mitochondria separation from cell membranes, namely the tissue homogenization was done in the isotonic 0.3 M sucrose solution and fractionated with centrifugation at 1000 ×*g*, 15 000 ×*g* and 100 000 ×*g*, as described by Hryb et al. [23]. Again ATP synthase was detected not only in mitochondrial fraction obtained at 15 000 ×*g* centrifugation but also in the pellet obtained at 100 000 ×*g* centrifugation and a relatively small amount is still present in the supernatant obtained after 100 000 ×*g* centrifugation (Fig. 6B). Therefore, we conclude that ATP synthase components appear not only in mitochondria but also in cell membrane and cytosolic fractions (see Discussion).

3.7. SPR analyses of F_1 ATP synthase binding to JHBP

The interaction of ATP synthase was analyzed to assess whether ATP synthase protein components directly bind to JHBP. Since this protein from *G. mellonella* has not been purified and its sequence is not yet known, we needed an alternative. Considering that sequences of α and β chains of F_1 portion of ATP synthase from bovine and fruit fly (*D. melanogaster*) have been conserved in 81.5% and 82.3%, respectively, we decided to use bovine F_1 ATP synthase in further experiments.

Fig. 5. Real-time binding of fat body membrane protein solution and F_1 ATP synthase to JHBP. (A) The analysis of binding fat body membrane protein solution to immobilized JHBP, JHBP-JH and a free cell. JHBP (2300 RU) and JHBP-JH (2400 RU) were immobilized on the dextran matrix of the SPR solid support (sensor) surface by the amine-coupling method. Binding is expressed in resonance units (RU) as a function of time in seconds. 150 μl of fat body membrane proteins from *G. mellonella* (0.047 mg/ml) was injected at 20 μl/min over the sensor surfaces containing JHBP, JHBP-JH and a free cell. Dissociation of the complexes was then performed in HBS. (B) Sensorgrams, for passing fat body membrane protein solution over immobilized JHBP and JHBP-JH, were obtained by subtracting the unspecific binding of membrane proteins to the sensor chip control cell. Approximately 140 RU of binding was detected on the JHBP immobilized sensor surface at 250 s after injection. No binding was detected on the JHBP-JH sensor surface. (C) The analysis of F_1 ATP synthase binding to immobilized JHBP. JHBP (4900 RU) and BSA (8400 RU) were immobilized on the dextran matrix of the sensor surface by the amine-coupling method. 90 μl of F_1 ATP synthase (25 nM) was injected at 30 μl/min over the sensor surfaces containing JHBP, BSA and a free cell. Dissociation of the complexes was then performed in HBS. (D) Sensorgrams, for passing F_1 ATP synthase at the indicated concentrations over immobilized JHBP, were obtained by subtracting the unspecific binding of F_1 ATP synthase to the sensor chip control cell (BSA). The kinetic constants of the interaction are $k_a = 9.3 \pm 0.096 \times 10^5$ $M^{-1}s^{-1}$, $k_d = 7.97 \pm 0.705 \times 10^{-4}$ s^{-1}, $K_d = 0.86 \pm 0.08$ nM.

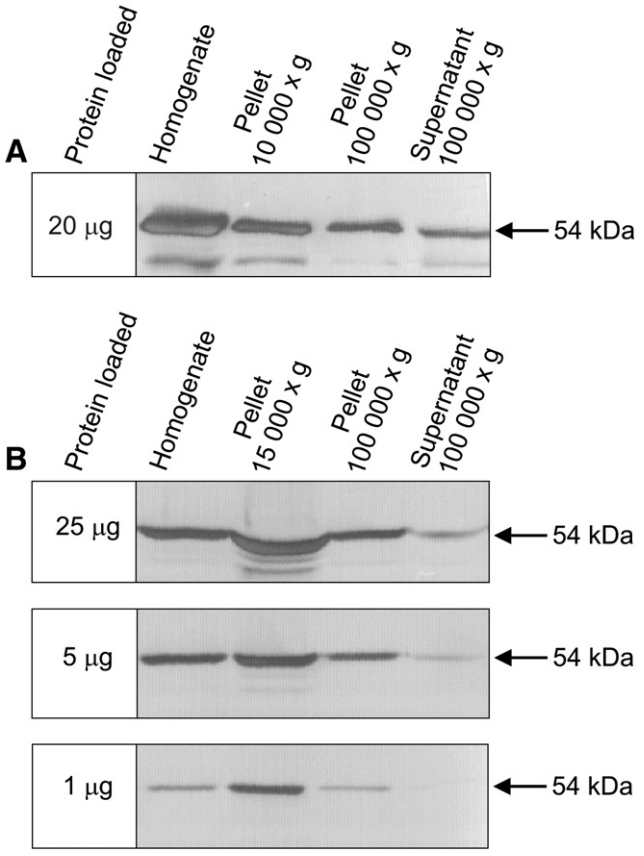


Fig. 6. Immunodetection of ATP synthase in the subcellular fractions of *G. mellonella* fat body. The fat body subcellular structure fractionations were performed according to Ueno et al. [22] (A) or Hryb et al. [23] (B). The indicated amounts of protein samples (1, 5, 20 or 25 µg) containing homogenate, pellets (obtained by centrifugation at 10 000 ×*g*, 15 000 ×*g* and 100 000 ×*g*) and supernatant (obtained after 100 000 ×*g* centrifugation) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes after blocking were treated with monoclonal antibody to α chain of ATP synthase (1:1000). Binding was visualized with HRP-horse anti-mouse (1:5000) as described in Materials and methods. Protein molecular weight markers (Fermentas) were used to deduce relative molecular mass of ATP synthase.

The ability of bovine F_1 ATP synthase to bind to immobilized JHBP and BSA as a control was investigated using SPR-BIA. JHBP and BSA were firstly immobilized as described in Materials and methods section (2.16) and a reference cell surface was activated and blocked without any protein. Approximately 40 RU of binding was detected after the injection of 25 nM F_1 ATP synthase over the JHBP surface, no detectable binding was observed over the reference surface or the BSA surface (Fig. 5C). A set of increasing dose dependent differential responses was obtained by injecting F_1 ATP synthase in the range 0.75 nM to 25 nM (Fig. 5D). Kinetic constants (k_a, k_d, K_d) were evaluated using the global fitting routine of the BIAevaluation 4.01 software provided by BIAcore using the Langmuir 1/1 model. Calculated constants were $k_a = 9.3 \pm 0.096 \times 10^5\ M^{-1}s^{-1}$, $k_d = 7.97 \pm 0.705 \times 10^{-4}\ s^{-1}$, with a calculated high affinity binding constant $K_d = 0.86 \pm 0.08$ nM.

4. Discussion

4.1. Interaction of JHBP with hemolymph proteins

This is the first report which shows that an insect JHBP interacts both with hemolymph and membrane proteins. Using mass spectrometry analysis, we identified three proteins in hemolymph that may directly or indirectly bind to JHBP: apolipophorin, arylphorin and hexamerin (Table 1). This finding suggests that JHBP and other hemolymph proteins form diverse multimeric complexes in hemolymph under native conditions.

Apolipophorin constitutes the major component of lipophorin, which mediates the transport of various types of lipids in hemolymph. It can also be involved in the transport of hydrophobic ligands like pheromones, hydrocarbons, carotenoids and juvenile hormones [34]. The insect apolipoproteins belong to the family of large lipid transfer (LLT) proteins that share a large N-terminal domain of about 900 amino acids containing a large lipid binding pocket [35]. Arylphorin (LHP 76) and hexamerin (LHP 82) belong to the superfamily of hexameric proteins that are structurally related to arthropod hemocyanins, but the organization of the genes that encode these proteins is different [36]. Surprisingly, the interacting proteins have remnant structural similarities to the *jhbp* gene sequence. Intron A of JHBP contains 51, 85, 25 and 39 bp fragments of 88%, 82%, 96% and 87% identity with 3′UTR of the hexamerin gene from *G. mellonella* [37]. Hexameric proteins are synthesized within the fat body of insects, secreted into the larval hemolymph and taken up by fat body cells before pupation and thus they function like storage proteins. In some species, hexamerins transport JH molecules [1] and recently, experiments suggesting that hexamerin molecules covalently bind JH were presented [38]. Thus, "could JHBP be binding JH that is covalently bound to hexamerin?" is an interesting question. The answer to this question may explain why JHBP exhibits some affinity to other JH binding proteins.

Just recently we were able to determine the 3D structure of *G. mellonella* JHBP [39]. The JHBP molecule has a unique fold, consisting of a long helix wrapped in a highly curved antiparallel β-sheet, resembling the folding motif found in some mammalian lipid binding proteins, namely: bactericidal permeability-increasing protein (BPI) and cholesteryl ester transfer protein (CETP) [40,41]. An important difference between JHBP and these proteins is that the boomerang-like lipid binding proteins are composed of two sequentially connected JHBP-like domains, whereas JHBP contains only one such domain. The role of CETP is to transfer the esterified form of cholesterol from high density lipoproteins (HDL) to triglyceride-rich lipoproteins and to transfer triglycerides in the opposite direction [42]. The concave surface of CETP matches the radius of curvature of HDL molecules, suggesting that this region makes contacts with the curved lipoprotein surface of HDL. It is possible that JHBP molecules may exploit the analogical interaction with hemolymph lipid binding proteins. It is noteworthy that two hydrophobic cavities named W and E, of about 632 $(Å)^3$ and 668 $(Å)^3$, respectively are present within the JHBP molecule located at the two poles of the molecule. The W binding pocket with a volume of 632 $(Å)^3$ appears to match the JH molecule volume. The second cavity contains one negatively charged (Glu166) and three positively charged (Lys170, Lys176, Arg190) residues at its entrance, which is 6 Å by 13 Å wide [39]. It is possible that this region of the JHBP molecule may compete for a phospholipid molecule bound to hemolymph lipid binding proteins forming a transient complex with these proteins. The profound conformational transition of the JHBP molecule caused by JH binding has been previously observed [11,12] and suggests that this protein contains a flexible part which may assume different conformations of higher or lower affinity to other proteins (ligands).

4.2. JHBP interaction with membrane proteins

JHBP binding to fat body membrane proteins from *G. mellonella* was analyzed using three different experimental methods. Immunohistological experiments revealed the presence of JHBP both in the fat body cell membrane and in the cytosol. It is not surprising that JHBP was found in the cytosol, because mRNA was observed in the tissue expression of JHBP [13]. Interestingly, the strong JHBP positive reaction has been detected in tracheal epithelium. However the

significance of this finding requires further study as it may result from the presence of a JHBP receptor in tracheal epithelium.

The second type of experiments was performed with iodinated JHBP, and showed that *G. mellonella* fat body membrane suspension from 4th day, VIIth instar larvae binds JHBP. The association of JHBP to the membrane was found to be concentration-dependent, specific, saturable and susceptible to proteolytic digestion, indicating its protein nature. The estimated dissociation constant (K_d) is 0.105 μM.

The third method applied the SPR technique for the analysis of JHBP binding to solubilized fat body cell membrane proteins. We found that membrane proteins clearly exhibit an affinity to JHBP but not to the JHBP-JH complex. We used mass spectrometric analysis of proteins captured on a sensor surface with immobilized JHBP, and ATP synthase (α and β chains) was identified as a JHBP-binding protein.

For a long time the ATP synthase multisubunit complex was considered to be present exclusively in mitochondria. Few years ago some structural subunits of mitochondrial F_0F_1 ATP synthase were detected on membranes of cultured cells from tumoral and/or proliferating cell lines, where this protein serves as a receptor for different ligands and participates in processes such as the regulation of lipoprotein metabolism and cholesterol uptake, control of the proliferation and differentiation of endothelial cells, angiogenesis and hypertension [43]. Recently, ATP synthase has been also found on the extracellular surface of isolated normal tissue cells such as rat hepatocytes [44]. Liver is functionally related to insect fat body. The results from two types of tissue homogenization and centrifugal fractionation applied in this communication clearly suggest that ATP synthase has apparently ubiquitous subcellular distribution in *G. mellonella* (mitochondria, plasma membranes and cytosol). This finding is in agreement with other laboratories notion that ATP synthase resides not only in mitochondria but also in cell membranes of several tissues [43–45]. The relative molecular mass of α chain of ATP synthase from *G. mellonella* fat body membranes, closely corresponds to 55 kDa mass of ATP synthase, previously detected on the surface of human endothelial cells [46]. As one may see, in fractions overloaded with ATP synthase some minor bands of lower molecular mass (<54 kDa) are also visible (Fig. 6A, B). As it was explained by antibody producer, α subunit of the ATP synthase is prone to degradation, which results in multiple immunoreactive products. Relatively higher percentage of ATP synthase in the 100 000 × *g* supernatant fraction obtained from fat body using method based on Ueno et al. [22] procedure indicates an increased degree of cell components fragmentation.

Surface plasmon resonance experiments revealed that not only ATP synthase interacts with JHBP, but also that solubilized fat body membrane proteins are not bound to immobilized JHBP on the sensor surface when JH was added. Since ATP synthase α and β chains and translocase (probably associated with ATP synthase) were detected, this may indicate that JH releases JHBP from the ATP synthase complex. SPR experiments showed that pure bovine ATP synthase binds to JHBP molecules with a dissociation constant of (K_d) 0.86 nM. However the K_d value for binding iodinated JHBP to fat body membrane suspension is 0.105 μM. At present we cannot explain the difference in these K_d values. It is possible that the affinity of free ATP synthase molecules to JHBP molecules is different when ATP synthase is embedded in the cell membrane and is engaged in interaction with many other proteins. In particular that plasma membrane ATP synthase appears to be a receptor not only for angiostatin, endothelial monocyte-activating polypeptide II, but also for apolipoprotein AI (apoA-I) [45–47].

Earlier studies found that a membrane ATP synthase complex binds to apoA-I, the main protein constituent of HDL, to induce endocytosis of HDL protein particles by a mechanism dependent on the generation of ADP by the ATP synthase [45]. Moreover, ATP synthase was also reported to interact with apolipophorin in *D. melanogaster* [48]. All these findings nicely correspond with our results which show that JHBP interacts with ATP synthase on fat body membrane and with apolipophorin in hemolymph.

4.3. A model of the involvement of ATP synthase and hemolymph proteins in JHBP molecule traffic

Our experiments show that: (1) ATP synthase interacts with JHBP with high affinity, (2) In the presence of hormone ATP synthase does not bind with JHBP. This suggests, that the JHBP-ATP synthase complex dissociates in the presence of JH, (3) Hemolymph's proteins (apolipophorin, arylphorin and hexamerin) bind JHBP, both free and in a complex with JH. However, this binding is stronger in the presence of JH. It has been shown in previous studies that the JHBP molecule undergoes a profound conformational transition upon binding to JH

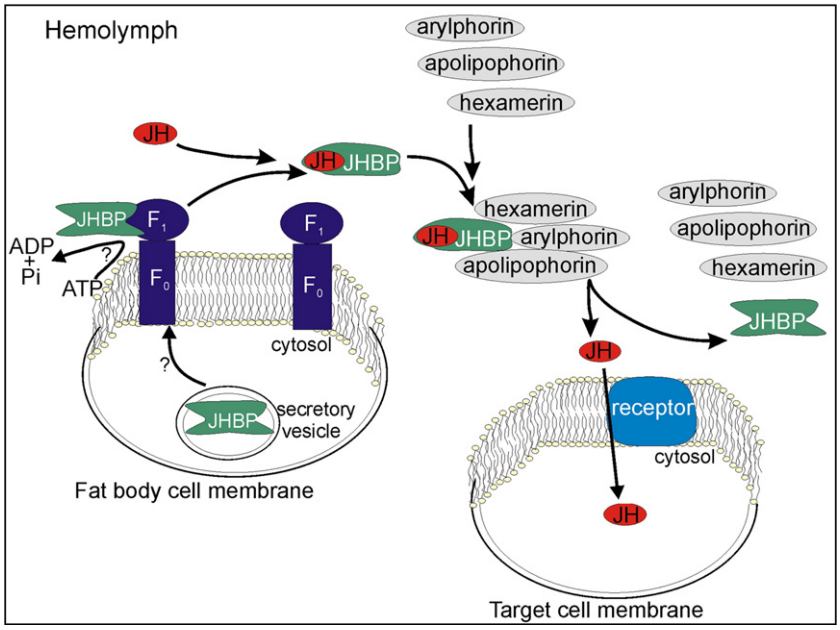


Fig. 7. The model for the mechanism by which ATP synthase is involved in the export of JHBP molecules from fat body cells, and apolipophorin, arylphorin and hexamerin participate in the JHBP-JH complex transport to a target cell membrane. F_0, F_1 — portions of ATP synthase.

[11,12]. Thus, it is reasonable to assume that such conformational change might have an impact on the affinity of JHBP to our studied proteins.

Based upon the above findings we propose the following working hypothesis of JHBP traffic (Fig. 7). The fat body membrane ATP synthase, perhaps with the use of ATP, takes part in the export of JHBP molecules from the fat body cells where JHBP is synthesized [13]. Although, the function of cell membrane ATP synthase in the export of proteins has not been shown directly, it has been shown that the overexpression of the ATP synthase β subunit in INS1 cells (rat insulinoma cell line) increased insulin secretion [49]. Once outside the cell the JHBP-ATP synthase complex is then released by JH with a concomitant conformational transition in JHBP molecules [11,12]. Then the JH-JHBP complex enters into a supramolecular complex with lipid binding proteins (apolipophorin, arylphorin and hexamerin) and travels to the target cell membrane receptor where JH is released and bound to higher-affinity JH cytosol binding proteins. The presence of these proteins was previously reported [50].

Thus, we postulate that ATP synthase participates in JHBP export from fat body cells and apolipophorin, arylphorin and hexamerin take part in JHBP-JH complex transport to target cells. Further studies are needed to examine the above hypothesis and determine the physiological roles of the interactions between JHBP and its binding proteins.

Acknowledgements

The authors gratefully thank Dr. Laurent Martinez (Institut National de la Santé et de la Recherche Médicale, Unité 563, Département Lipoprotéines, et Médiateurs Lipidiques, Toulouse, France) for a generous gift of F_1 ATP synthase and Msc. Barbara Buryło (Department of Biochemistry, Faculty of Chemistry, Wrocław University of Technology, Wrocław, Poland) for aldolase. We also thank Dr. Robert Kołodziejczyk (Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznań, Poland) for his help and support in preliminary experiments, Dr. Dariusz Rakus (Department of Animal Physiology, Institute of Zoology, Wroclaw University, Poland) for his help in ultracentrifugation experiments and anonymous reviewers for helpful comments and their highly stimulating suggestions.

This research was supported by a grant from the Polish Ministry of Education and Scientific Research.

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