Affinity Purification and Binding Analysis of the Hemolymph Juvenile Hormone Binding Protein from *Manduca sexta*†

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ABSTRACT: A high-affinity juvenile hormone binding protein was purified from the hemolymph of the tobacco hornworm, *Manduca sexta*, employing ammonium sulfate precipitation and affinity and size-separation chromatography. The naturally occurring enantiomer of juvenile hormone III (10*R*) was converted to juvenile hormone III acid and then covalently attached to aminohexyl-Sepharose 4B. Hemolymph from early fifth stadium (60 h postecdysis) larvae was used as the source of hJHBP. The yield of hJHBP was approximately 25% of the starting material, with 3.5 mg of highly purified, biologically active hJHBP recovered from 100 mL of hemolymph. Binding parameters were examined using equilibrium dialysis and highly purified, enantiomerically correct juvenile hormone I and II and racemic JH III. The equilibrium dissociation constants for juvenile hormone I and II were approximately 6×10^{-10} M at 4 °C, while racemic juvenile hormone III displayed an equilibrium dissociation constant of 1.9×10^{-9} M. At 25 °C the equilibrium dissociation constant for juvenile hormone I was 1.6×10^{-9} M. Half-times of dissociation were also determined for the three homologs. The half-time of dissociation was 30 s for juvenile hormone I, 20 s for juvenile hormone II, and 13 s for juvenile hormone III at either 4 or 25 °C. Using the new equilibrium dissociation constants, we calculate that better than 99% of the circulating juvenile hormone titer may be bound to this hemolymph protein.

Lipophilic hormones in the circulatory system are often associated with specific, high-affinity binding proteins. These noncovalent complexes generate a dynamic binding equilibrium that adjusts to the prevailing hormone concentration in accordance with the law of mass action. As a result, the protein-bound hormone is readily distributed to peripheral target tissues. Other consequences of this interaction include a reduction in enzymatic metabolism and excretion and the storage of relatively large amounts of the hormone in an inactive form that serves as a readily available reservoir to target tissues (Westphal, 1986). In addition, the serum hormone binding proteins may facilitate the passage of newly synthesized hormone from the site of synthesis into the circulatory system (Ewing et al., 1976). Recent evidence suggests that certain hormone binding proteins may bind to target cell membranes (Khan et al., 1990; Porto et al., 1992) and increase intracellular cyclic nucleotide concentrations (Rosner, 1990).

One of the most lipophilic families of all eukaryotic hormones are the insect hormones termed the JHs.[1] These acyclic sesquiterpenoid hormones are involved in larval growth and development (Riddiford, 1985) and in the adult play a role in reproduction. Given the hormone's surface-active nature (Law, 1980) and lability to hemolymph enzymes (Hammock, 1985; Roe & Venkatesh, 1990), it is not surprising that virtually every insect studied displays some type of hemolymph JH transport protein. There appear to be two classes of high-affinity ($K_D < 10^{-7}$ M) hJHBPs: the low molecular mass proteins (<100 kDa) characteristic of the order Lepidoptera and the high molecular mass proteins found in most other insects. Considerable attention has centered on the high-affinity, low molecular weight protein from *Manduca sexta* [see Goodman (1990) for review]. Estimates of molecular mass for this hJHBP range from 25 kDa as determined by the cDNA-deduced amino acid composition (Lerro & Prestwich, 1990) to 32 kDa as obtained by SDS–PAGE. Previous binding analyses indicated that the K_D for the hormone–protein complex was approximately 1×10^{-7} M (Kramer et al., 1976a) using racemic JH I.

Although hJHBP from *M. sexta* was previously purified (Kramer et al., 1976a; Goodman et al., 1978a; Peterson et al., 1982; Prestwich, 1991), advances in protein purification technology and development of enantiomerically pure, high specific activity ligands (Prestwich and Wawrzenczyk, 1985) have greatly enhanced protein recovery and made assessment of binding kinetics more accurate. We here report two advances in the study of this important transport protein: (1) a rapid and efficient method for hJHBP purification exploiting affinity chromatography and (2) establishment of equilibrium

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[1] Abbreviations: association rate constant, k_1; dimethylaminopyridine, DMAP; ethylenediaminetetraacetic acid, EDTA; dissociation rate constant, k_{-1}; DL-dithiothreitol, DTT; enzyme immunoassay, EIA; 10,11-epoxyfarnesyl diazoacetate, EFDA; equilibrium dissociation constant, K_D; half-time of dissociation, $t_{1/2}$; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, EDAC; hemolymph juvenile hormone binding protein, hJHBP; high-performance liquid chromatography, HPLC; hydroxylapatite, HA; immunoglobulin G, IgG; juvenile hormone, JH; maximum number of binding sites, B_{max}; *N*-hydroxysuccinimide, NHS; 3-(octylthiol)-1,1,1-trifluoro-2-propanone, OTFP; phenylmethanesulfonyl fluoride, PMSF; 1-phenyl-2-thiourea, PTU; polyethylene glycol, M_r = 20 000, PEG; sodium dodecyl sulfate, SDS; sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS–PAGE; tris(hydroxymethyl)aminomethane, Tris; thin-layer chromatography, TLC.

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dissociation constants and dissociation rate constants for JH I, JH II, and JH III.

MATERIALS AND METHODS

Animals and Hemolymph Preparation. M. sexta were reared and staged as described by Goodman et al. (1985). Hemolymph for purification studies was collected from fifth-stadium larvae 60 h postecdysis, while hemolymph for binding studies was collected from first-gate, fourth-stadium, head capsule slippage larvae at white mandible stage. Animals were bled by puncturing a proleg and expressing the hemolymph into an ice-chilled tube containing a few granules of PTU. Hemolymph for hJHBP purification was centrifuged at 10500*g* for 30 min and frozen immediately at −20 °C. Hemolymph for binding analysis was centrifuged at 10500*g* for 5 min and passed through a pressure filtration unit fitted with a YM 100 filter (Amicon). The partially purified hJHBP contained in the filtrate was divided into aliquots and stored at −80 °C.

Chemicals and Materials. Enantiomerically pure JH I and II (10*R*,11*S*), the tritiated forms (42 Ci/mmol), and [^{3}H]EF-DA were a gift from Prof. G. D. Prestwich (SUNY—Stony Brook). Racemic [^{3}H]JH III (10 Ci/mmol) was purchased from NEN Research. Enantiomerically pure JH III (10*R*) was extracted from the plant *Cyperus* sp. (Toong et al., 1988). All radiolabeled and radioinert JH used in these studies was purified by HPLC as previously described (Goodman et al., 1990a). The esterase inhibitor OTFP was a gift from Prof. B. D. Hammock (UC—Davis). Phenylmethanesulfonyl fluoride, aprotinin, pepstatin, leupeptin, soybean trypsin inhibitor, PTU, DTT, PEG, Tris, Sephacryl S-200, Sepharose 4B, AH-Sepharose 4B, and molecular weight markers bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase were purchased from Sigma Chemical Company. *N*-Hydroxysuccinimide, DMAP, and ethylene glycol (spectrophotometric grade) were purchased from Aldrich Chemical Company, while EDAC was obtained from Calbiochem Corporation. Hydroxylapatite (HTP powder), Bradford reagent for protein determinations, and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Laboratories. Unless otherwise specified, all solvents were of HPLC quality. Dialysis tubing was purchased from Fisher Scientific Company. Thin-layer chromatographic plates (silica gel 60, without fluorescent indicator) were obtained from E. Merck. Immunochemical reagents were purchased from Kirkegaard and Perry Laboratories.

Synthesis of Affinity Resin. JH III (10*R*) (20 mg) was converted to JH acid by base hydrolysis and then purified by HPLC (Goodman & Adams, 1984). The affinity resin was prepared as described by Goodman and Goodman (1981). The resin (approximately 30 mL) was diluted with an equal portion of Sepharose 4B.

Purification of hJHBP. DTT was added to thawed hemolymph (100 mL) to effect a final concentration of 1 mM. Ammonium sulfate (solid) was added slowly with stirring to achieve 50% saturation. After the sample was mixed for 1 h, the hemolymph was centrifuged at 15000*g* for 30 min and the precipitate was discarded. Ammonium sulfate (solid) was added to the supernatant to achieve 75% saturation. After being mixed for 30 min, the sample was centrifuged at 15000*g* for 1 h. The precipitate was solubilized in BK buffer (sodium borate, 100 mM, and KCl, 100 mM, pH 8.0), and the volume was adjusted to 20 mL. The sample was brought to a final concentration of 1 mM EDTA, 1 mM PMSF, 1 μM OTFP, 0.3 μM aprotinin, 1 μM pepstatin, 1 μM leupeptin, and 20 μg/mL soybean trypsin inhibitor and incubated for 1 h. The sample was then incubated with [^{3}H]JH III (5×10^5 dpm) for at least 2 h. The sample was passed through a Sephacryl S-200 column (2.5 × 87 cm) equilibrated with BK buffer at a flow rate of 1 mL/min. Fractions were radioassayed, and the hJHBP peak was pooled. The pooled material (~100 mL) was passed through a pressure filtration unit fitted with a YM 100 membrane (Amicon). The retentate was washed three times, each time using an equal amount (100 mL) of BK buffer. The pooled filtrates, containing the hJHBP, were concentrated by pressure filtration using a YM 10 membrane (Amicon). The concentrate (~10 mL) was incubated with affinity resin (60 mL) in a sintered-glass funnel for 2 h with occasional mixing. The resin was then washed with BK buffer (~180 mL) until the A_{280} of the eluate was <0.005. hJHBP was eluted from the resin with 50 mL of BEG buffer (sodium borate, 100 mM, with 20% ethylene glycol, pH 7.2) containing JH III (10 mg of JH III in 100 μL of ethanol per 50 mL of BEG buffer) followed by 100 mL of BEG buffer without JH. The eluate (~150 mL) was concentrated by pressure filtration using a YM 10 membrane. Aliquots (750 μL) of the concentrate (2–3 mL) were injected onto a Toso-Haas TSK-GEL G3000SW size-exclusion HPLC column (21.5 mm × 60 cm). hJHBP was eluted with BEG buffer at a flow rate of 1.0 mL/min, and fractions were monitored spectrophotometrically at 220 nm and immunochemically by EIA (Goodman et al., 1990b). The hJHBP peaks from all runs were pooled and concentrated by pressure filtration using a YM 10 membrane. All steps excluding HPLC purification were conducted at 4 °C; HPLC was performed at room temperature.

The purification steps were monitored using an hJHBP EIA. Samples and standards were first diluted (at least 1:5, v/v) in phosphate-buffered saline containing 0.5% SDS. The EIA was then performed as described by Goodman et al. (1990b) using an hJHBP-directed monoclonal antibody (MAb 6) and a goat anti-mouse horseradish peroxidase-conjugated secondary antibody. Purification was also monitored by SDS–PAGE using 10% gels (Hames, 1981) stained with Coomassie Brilliant Blue. Protein concentrations were determined by a dye-binding assay (Bradford, 1976) using bovine IgG as the standard. Protein concentration of the purified hJHBP was determined using UV absorption at 205 nm (Scopes, 1974) as well.

Binding Studies: Equilibrium Dissociation Constants. (*A*) *Equilibrium Dialysis.* Vials (15 × 45 mm) were coated with PEG to minimize nonspecific binding (Goodman et al., 1978b). Dialysis tubing was prepared using the method of Klotz (1990). Partially purified hJHBP was incubated with OTFP for 4 h at 4 °C and then diluted appropriately (100- to 500-fold) with dialysis buffer (Tris, 100 mM, and KCl, 100 mM, pH 8 at 4 °C). The radiolabeled or radioinert ligand was placed into the PEG-treated vials, the carrier solvent was evaporated under vacuum, and 1 mL of dialysis buffer was added to each vial. One-milliliter aliquots of diluted OTFP-treated hJHBP were transferred to prepared dialysis bags, and the weight of each bag was recorded. Dialysis bags were incubated in capped vials on a rotary shaker for 16 h at either 4 or 25 °C. After incubation, the outside of each bag was quickly blotted dry, and the bag was reweighed to determine if a significant volume change had occurred. Dialysis bags displaying a volume change of more than 7% were discarded. Aliquots (400 μL) of the bag contents and the outside compartment were radioassayed by liquid scintillation counting. Recovery of radiolabeled ligand and assessment of hormone distribution

(no protein in dialysis bag) were monitored for each dialysis experiment. Ligand integrity was monitored upon completion of dialysis by partitioning the hormone into organic solvent and then analyzing the radiolabel by TLC (Goodman et al., 1990a). The quantity of JH bound was determined by subtracting the concentration of hormone in the outer compartment from that inside the dialysis bag. Data reduction was performed using the software Minitab and Ligand (Munson & Rodbard, 1980).

(*B*) *Hydroxylapatite Assay.* Analysis of JH–hJHBP interaction was also examined using the hydroxylapatite assay (Goodman et al., 1978b). Partially purified hemolymph (see above) was diluted 250-fold using HA assay buffer (Tris, 10 mM, and KCl, 10 mM, pH 7.5 at 4 °C). The protein was added to PEG-treated vials (15 × 45 mm) containing radiolabeled and radioinert JH. After incubation, HA slurry was added to the vial and incubated 45 min; the vial was then centrifuged (400*g*) to pellet the slurry. The supernatant was removed; the slurry was resuspended with HA buffer (1 mL) and recentrifuged. The process was performed five times to ensure a low background. Background was determined by adding excess (100-fold) radioinert JH to vials containing both radiolabeled hormone and protein. After the supernatant from the last wash was removed, scintillation fluid was added directly to the vial and the sample was radioassayed. Data reduction was performed as described above.

Binding Studies: Dissociation Rate Constants. (*A*) *Estimation of Dissociation Rate Constant Using Unbound Ligand.* Small glass columns (6 × 20 mm) were fabricated from Pasteur pipets by removing all but the top 20 mm of the pipet. Both ends were fired to reduce the openings to 1.5 mm. The column was treated with PEG as described above, and the bottom was plugged with glass wool to a height of 1 mm to prevent leakage of HA. The top of the column was connected to a peristaltic pump. HA (100 mg/mL) was equilibrated with HA assay buffer. Radiolabeled ligand (2.8×10^6 dpm) was placed into PEG-treated vials, and the solvent was removed under vacuum. Partially purified hJHBP pretreated with OTFP was added to the vials and incubated overnight at 4 °C. The sample (50 μL) was transferred to the HA minicolumn, and the complex was gently mixed for 15 s. Buffer overlaying the HA was quickly removed by vacuum (5 s). The column was then attached to the peristaltic pump, and the HA was washed with excess levels of radioinert hormone (1 μg/20 mL of buffer) at a rate of approximately 80 μL/s. Five drops of eluate was collected per vial. Although the eluate was collected for a 3-min period, collections from the first half-minute were discarded. To determine the dissociation rate constant at 25 °C, radiolabeled ligand and hJHBP were incubated at 4 °C overnight and then warmed to 25 °C and assayed at that temperature. To monitor hJHBP bleeding from the HA minicolumn, hJHBP was photoaffinity labeled with the JH III analog [^{3}H]EFDA as previously described (Koeppe et al., 1984), dialyzed to remove unbound label, and then incubated with HA. The HA was then washed with radioinert hormone, and the eluate was collected and assayed as described above.

(*B*) *Estimation of Dissociation Rate Constant Using Bound Ligand.* hJHBP was incubated with radiolabeled hormone as described above, and an aliquot (10 μL) was then transferred to the HA minicolumn. After a 15-s incubation, 100 μL of the radioinert hormone (50 ng) was added to the column and the buffer in the column was immediately removed by vacuum. The contents of the column were then transferred to a scintillation vial and radioassayed. The process was repeated using increasingly longer incubation periods with the radioinert hormone. As a control, elution of unbound radiolabeled JH was monitored.

Table I: Purification of Hemolymph Juvenile Hormone Binding Protein[a]

step	vol (mL)	total protein[b] (mg)	total hJHBP[c] (mg)	sp act. (hJHBP/ protein)	yield (%)	purification (fold)
hemolymph	105.0	2856.0	15.2	0.005	100	1.0
50% $(NH_4)_2SO_4$ precipitation	113.0	1989.0	15.0	0.008	98	1.4
75% $(NH_4)_2SO_4$ precipitation	22.0	1320.0	14.7	0.010	97	2.2
gel filtration S-200	123.0	172.0	12.7	0.074	83	14.0
pressure filtration	14.0	116.0	12.5	0.110	82	20.3
affinity resin	3.4	5.4	3.6	0.660	23	124.7
gel filtration TSK-GEL G3000SW	1.4	3.5	3.5	1.000	23	189.0

[a] Values for the first pass of the partially purified hJHBP. [b] Determined by dye-binding assay. [c] Determined by enzyme immunoassay.

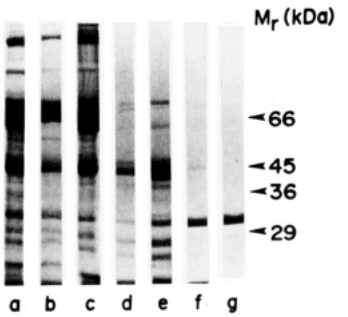

FIGURE 1: SDS–PAGE analysis of purification steps. Samples were separated on a 10% reducing SDS gel. Lane a, hemolymph (54 μg); lane b, supernatant from 50% ammonium sulfate treatment (35 μg); lane c, precipitate from 75% ammonium sulfate treatment (60 μg); lane d, hJHBP fraction from Sephacryl S-200 (35 μg); lane e, eluate from YM 100 ultrafiltration (80 μg); lane f, hJHBP fraction from affinity resin (32 μg); lane g, hJHBP fraction from TSK-GEL G3000SW (35 μg). Molecular weight markers are indicated by arrows.

RESULTS

Purification of hJHBP. Conventional isolation of hJHBP has been difficult due to its tendency to aggregate as purification proceeds and due to its apparent lability at lower pH. To maximize yield, we developed a protocol that was both rapid and nondenaturing, utilizing ammonium sulfate precipitation, size-exclusion chromatography, and affinity chromatography. Table I indicates that precipitation with 50% ammonium sulfate removed ~30% of the total protein, while precipitation of the supernatant with 75% ammonium sulfate eliminated an additional 35% of the contaminating protein. Loss of hJHBP during the salting-out procedure was less than 5%. Gel filtration on Sephacryl S-200 yielded three UV-absorbing peaks: arylphorins and lipophorins, the biliverdin-binding protein insecticyanin, and small peptides that precipitated with the 75% ammonium sulfate treatment. SDS–PAGE analysis of the hJHBP fraction (Figure 1) indicates that gel filtration greatly reduced the concentration of arylphorins and lipophorins but did not reduce the levels of a protein of ~40 kDa. hJHBP, when monitored using radiolabeled JH, did not correspond to any UV-absorbing (280 nm) peak; however, hJHBP can be visualized by SDS–PAGE (Figure 1). Unfortunately, size-exclusion chromatography does not completely remove the arylphorins. Since these proteins interact nonspecifically with JH, their concentration must be significantly reduced prior to affinity purification. Thus, the hJHBP fraction was subjected to

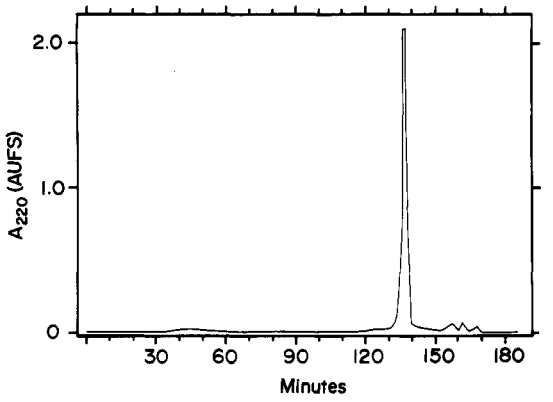

FIGURE 2: Size-exclusion chromatography of affinity-purified hJHBP. Sample eluted from the affinity resin with JH III was applied to a TSK-GEL G3000SW HPLC column. hJHBP was eluted with BEG buffer at a flow rate of 1 mL/min.

ultrafiltration through a YM 100 membrane, which removed most of the arylphorin component (Figure 1).

The presence of several hemolymph proteins closely related to hJHBP in size, charge, and hydrophobicity stimulated the search for alternative separation methods. Because purification based on charge initiates limited denaturing of hJHBP, affinity chromatography was used, exploiting the unique binding properties of the protein. To generate the affinity matrix, JH III was first converted to its acid by base hydrolysis and then purified using a LiChrosorb diol column (Goodman & Adams, 1984). The acid was covalently attached to AH-Sepharose 4B through the C-1 terminus, forming an amide bond. JH III (10*R*) was used as the affinity ligand. Although the dissociation rate constant for JH III is higher than for JH I or II, JH III was the only ligand readily available in sufficient quantity. The matrix generated for this study incorporated 10.8 mg of JH acid per 7.5 g of AH-Sepharose 4B. Although the resin has 6–10 μmol/mL coupling sites available, only 0.25 μmol/mL JH acid was attached as determined by radioassay. Despite the apparent low degree of substitution, the affinity resin bound hJHBP more efficiently when diluted 50% with unsubstituted Sepharose 4B.

Since holo-hJHBP (hJHBP loaded with JH) will not bind to the affinity resin, the protein must be either stripped of its endogenous hormone or collected at a time when JH titers are low. The collection of hemolymph at 60 h postecdysis, fifth stadium, obviates the need to strip the protein of endogenous JH. Due to the short half-life of the JH–hJHBP complex, the time needed to remove extraneous protein from the affinity matrix must be minimized. As a result, batch purification proved more efficient than column chromatography. Since ethylene glycol reduces the affinity constant, it was added only after the elution of nonspecific protein. Ethylene glycol was included in the elution buffer to eliminate aggregation of purified hJHBP. As a result, no high molecular weight aggregates were apparent (Figure 1). In a single pass, the resin yielded 3.50 mg of highly purified hJHBP. Although another 300 μg of hJHBP was recovered in the second pass through the affinity resin, the limited recovery does not justify the effort. To eliminate contaminating proteins that coeluted with hJHBP, size-exclusion HPLC was employed. HPLC purification (Figure 2) on a TSK-GEL G3000SW yielded one large peak with a retention time of 137 min and several minor contaminants that eluted at 45 and 150–170 min. The homogeneity of the hJHBP peak at 137 min was confirmed by SDS–PAGE (Figure 1). Binding analyses on the purified hJHBP indicated 0.85 to 0.95 hormone binding site per molecule of hJHBP (data not shown). That these values are less than 1 presumably stems from imprecision in accurately determining hJHBP concentration. Indeed, comparison of the dye-binding method (Bradford, 1976) and the UV-absorbance method (Scopes, 1974) indicated a surprising 20% difference between identical samples. All values reported here, *a priori*, are based on the dye-binding assay.

Equilibrium Dialysis. To ensure that dialysis conditions were optimized, several parameters were monitored. Equilibrium between the inside and outside compartments was attained in approximately 12 h; samples were radioassayed at 16 h. The volume of the dialysis bag dropped slightly (5 ± 2%) during the 16-h period. In the presence of the JH esterase inhibitor OTFP, ligand degradation was minimal (~4%) at either 4 or 25 °C; however, considerable catabolism (75% or greater) was observed in the absence of OTFP. After incubation, the hormone recovered from both the inside and the outside compartment accounted for only 75 ± 2% of the hormone originally added. The remaining 25% was associated with the dialysis membrane. Although it is generally assumed that steroid hormone association with dialysis membrane is weak (Mickelson & Westphal, 1980), the nature of the interaction between JH and the membrane was unknown. Dialysis bags were incubated with radiolabeled JH II for 16 h at 4 °C as described above. Without removal of the excess radiolabeled buffer on the outside of the bags, the bags were immediately transferred to vials containing 1 mL of the dialysis buffer (no hormone) and incubated for 3 min at 4 °C. After this process was repeated twice, the bags were immersed in liquid scintillation fluid and radioassayed. Less than 3% of the original hormone added to the vial was associated with the bag; the remaining hormone was found distributed in the three washes. As in the case of steroid hormones, the JH–dialysis bag interaction is weak and has little effect on JH–hJHBP binding. Dilution of the partially purified hJHBP (100- to 500-fold) did not alter the dissociation constant, indicating that endogenous hormone associated with the hemolymph did not affect the experimental procedure. Finally, the removal of high-capacity, low-affinity binding proteins, i.e., lipophorins and arylphorins, was critical for accurately assessing binding parameters. The single-pass partial purification using YM 100 ultrafiltration removed these interfering proteins rapidly, avoiding unwanted modifications (Park & Goodman, 1993); however, recovery was poor. This posed little problem since the partially purified protein was further diluted prior to dialysis.

Estimation of Dissociation Constants. Accurate estimates of the K_D require saturating concentrations of ligand (Klotz, 1990). A semilog saturation plot clearly indicates that JH I and II reached saturation but JH III did not (Figure 3, inset). Equilibrium dissociation constants for the three homologs were determined using modified Scatchard plots (Scatchard, 1949) (Figure 3). At 4 °C and pH 8, hJHBP displayed the highest affinity for JH I (0.67 nM), followed by JH II (0.74 nM) and JH III (1.86 nM; Table II). Although JH I and II displayed no significant differences in K_D, the K_D of the JH III–hJHBP complex was significantly different. It should be noted that the radiolabeled JH III used in these studies was racemic and may not reflect enantiomer binding.

Moderate changes in pH and temperature were tested for their influence on JH–hJHBP binding. Since the pH of *M. sexta* hemolymph is maintained between 6.6 and 6.8 during the larval feeding stages (Nowock et al., 1975), JH II–hJHBP interaction was monitored at pH 6.8 (Table II). No significant difference in affinity was observed. Although the pH used in

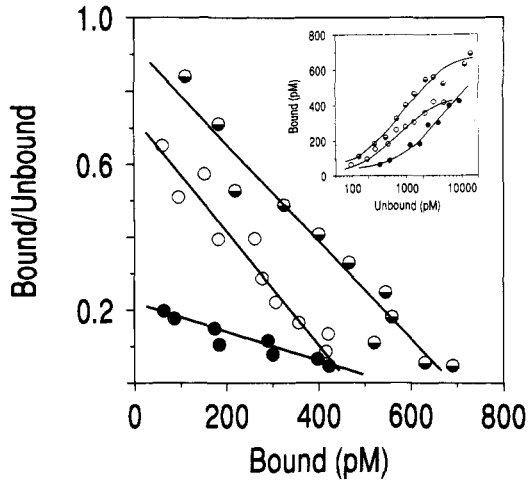

FIGURE 3: Saturation plot of hJHBP interaction with JH homologs. Equilibrium dialysis was performed with diluted hJHBP and radiolabeled and radioinert hormone. JH I (O) and JH II (◒) were enantiomerically pure (10*R*,11*S*); JH III (●) was racemic. hJHBP concentrations were adjusted so that saturating conditions were used for each homolog. The inset displays the same data using a semilog plot.

Table II: Summary of Equilibrium Dissociation Constants

homolog	K_D ± SE (nM)	method[a]	ligand[b]	temp (°C)	pH
JH I (10*R*,11*S*)	13.20 ± 1.00	HAA	inert/label	4	7.5
JH II (10*R*,11*S*)	12.50 ± 1.09	HAA	inert/label	4	7.5
JH I (10*R*,11*S*)	0.64 ± 0.06	ED	label	4	8.0
JH I (10*R*,11*S*)	0.67 ± 0.06	ED	inert/label	4	8.0
JH I (10*R*,11*S*)	1.57 ± 0.13	ED	inert/label	25	8.0
JH II (10*R*,11*S*)	0.74 ± 0.08	ED	inert/label	4	8.0
JH II (10*R*,11*S*)	0.87 ± 0.11	ED	inert/label	4	6.8
JH III (10*R*,*S*)	1.86 ± 0.35	ED	label	4	8.0

[a] HAA = hydroxylapatite assay; ED = equilibrium dialysis. [b] Binding assay was performed either with radiolabeled ligand only (label) or with both radioinert and radiolabeled hormone (inert/label).

these binding studies was somewhat higher than physiological, binding appeared unaffected by the moderate change in pH. Temperature differences led to a significant change in binding affinity. The binding affinity for JH I dropped nearly 3-fold when the temperature was increased to 25 °C. As a reference point, previous studies using the HA assay are included in Table II. There is a 20-fold difference in the affinities for each of the homologs using the HA assay.

Estimation of k_{-1}. There are a number of methods for determining k_{-1}; one of the most popular is the dextran-coated-charcoal assay. In our hands, the amount of charcoal necessary to adsorb all unbound ligand proved unworkable, as it bound hJHBP as well. Although HA was not a particularly good support matrix for the study of K_D (see Table II), it provided an excellent matrix for kinetic studies. When bound to HA, [^{3}H]EFDA-labeled hJHBP did not elute from the resin during a 3-min wash. Nonspecific binding of [^{3}H]JH was minimal, with background being achieved 20 s after addition of the unbound hormone.

Two approaches were used to estimate k_{-1}. The first approach initiated dissociation of the JH–hJHBP complex prior to immobilization on HA and monitored the amount of [^{3}H]JH–hJHBP bound to HA. In this case, a single measurement of the amount of radiolabeled hormone bound to the matrix was made, and the $t_{1/2}$ was calculated using the equations outlined by Schonbrunn and Tashijian (1978). The $t_{1/2}$ values for JH I, II, and III were 38, 27, and 19 s, respectively. The second method required the [^{3}H]JH–hJHBP

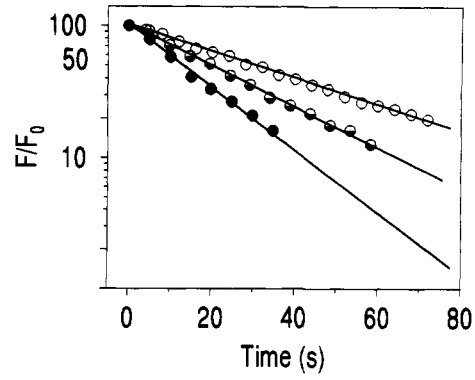

FIGURE 4: Half-times of dissociation for the JH–hJHBP complex. hJHBP was diluted and incubated with saturating amounts of radiolabeled JH I (O), JH II (◒) and JH III (●). The complex was then incubated with HA, quickly transferred to a minicolumn, and treated with a 500-fold excess of radioinert JH. Fractions were collected approximately every 5 s. F_0 represents the unbound hormone at time 0, while F represents the unbound hormone at successive intervals. F/F_0 is expressed as a percentage.

Table III: Summary of Association and Dissociation Rate Constants and $t_{1/2}$ Values

homolog	k_1[a] (M^{-1} s^{-1} × 10^7)	k_{-1} ± SE[b] (s^{-1})	$t_{1/2}$ (s)	temp (°C)	method[c]
JH I (10*R*,11*S*)	3.43	0.0230 ± 0.0003	30	4	U
JH II (10*R*,11*S*)	4.78	0.0354 ± 0.0006	20	4	U
JH III (10*R*,*S*)	2.82	0.0525 ± 0.0014	13	4	U
JH I (10*R*,11*S*)	1.54	0.0243 ± 0.0008	29	25	U
JH II (10*R*,11*S*)		0.0367 ± 0.0009	19	25	U
JH III (10*R*,*S*)		0.0537 ± 0.0026	13	25	U
JH I (10*R*,11*S*)	1.17	0.0183 ± 0.0036	38	25	B
JH II (10*R*,11*S*)		0.0257 ± 0.0017	27	25	B
JH III (10*R*,*S*)		0.0357 ± 0.0043	19	25	B

[a] Association rate constant determined by k_{-1}/K_D. [b] Average of three determinations. [c] Rate constant determined by monitoring either unbound (U) or bound (B) hormone.

to be bound to the HA matrix first and the [^{3}H]JH then to be displaced by the addition of radioinert hormone. In this case, it was the unbound hormone that was monitored (Figure 4). The data derived from monitoring the unbound hormone proved to be more consistent since multiple measurements were made on a single column. The correlation coefficient was excellent (r = 0.99) for each homolog. The dissociation and association rate constants for JH I, II, and III are shown in Table III. The $t_{1/2}$ values derived from measurement of unbound hormone are consistently shorter than the $t_{1/2}$ values derived from monitoring the bound hormone and may reflect the effect of hJHBP–HA interaction. Increasing the temperature from 4 to 25 °C had no effect on the dissociation rate constants.

DISCUSSION

Purification of hJHBP from *M. sexta* has been difficult due to its tendency to aggregate in the purified state. In previous studies the medium for the final purification step fortuitously stabilized the protein (Kramer et al., 1976a; Goodman et al., 1978a). A later purification method that employed ion exchange as a last step yielded partially inactivated hJHBP as determined by Scatchard plot analysis (Peterson et al., 1982). The recently generated recombinant hJHBP displays the same instability upon purification and storage (Touhara et al., 1993).

The present approach relies heavily on affinity chromatography due to the specific and gentle nature of this method.

Although the chemistry for the linkage of JH to a resin was worked out better than a decade ago (Goodman & Goodman, 1981), the method was impractical without adequate supplies of hormone and a means of detecting holo-hJHBP. With the development of monoclonal antibodies against hJHBP and a readily available source of enantiomerically pure JH III, the use of this technology became feasible. JH III, the ligand tethered to the resin, has the lowest avidity for hJHBP of the naturally occurring hormones. Although this characteristic decreases the binding capacity of the affinity column, a ligand with higher affinity would make hJHBP elution more difficult.

Attachment of JH acid to the matrix was made through the carboxyl end of the hormone for two reasons: first, hJHBP will recognize extensive modification at the C-1 terminus (Peterson, 1977) and will not recognize modification of the hormone's epoxide end (Kramer et al., 1976b); second, the chemistry of this reaction is reasonably simple and does not lead to unwanted modifications in the hormone. This linkage used the catalyst DMAP, which doubled the incorporation rate. In addition, the use of EDAC instead of dicyclohexylcarbodiimide enhanced the reaction 15-fold and eliminated an HPLC purification step. This two-step synthesis is a considerable improvement over past syntheses linking JH to amino groups (Lauer et al., 1974; Baehr et al., 1976) since it minimizes preparation time and avoids the loss of labile intermediate JH–NHS by eliminating the second HPLC purification.

The effectiveness of affinity purification depends upon the availability of an unliganded binding site. The approach taken in this study was to isolate hJHBP from animals with a very low JH titer but a reasonably high level of hJHBP. Hemolymph from fifth-stadium larvae (60 h postecdysis) fulfilled both these requirements (Baker et al., 1987; Goodman, 1985). Purification of hJHBP from animals with high JH titers poses little problem, since the highest physiological concentration of JH and the lowest concentration of hJHBP will yield only 2% holo-hJHBP. Moreover, dissociation of endogenous hormone during the first size-separation step further reduces the holo-hJHBP concentration.

In the initial steps of purification, stability of the hJHBP posed little problem; however, in the later stages, use of a stabilizing buffer proved critical. Twenty percent ethylene glycol prevented aggregation of 0.5 mg/mL hJHBP, the highest concentration tested. Moreover, it did not affect size-exclusion separation by HPLC or interfere with protein detection at low UV. The protein was active in BEG buffer at 4 °C for up to a month. Binding studies cannot be conducted in this buffer, as ethylene glycol reduces binding; however, the effect is reversible when the protein is diluted to low concentrations in BK buffer. The mechanism by which ethylene glycol minimizes aggregation of hJHBP is unknown.

A number of methods have been developed for determining equilibrium dissociation constants, but the simplest and most reliable has been equilibrium dialysis (Klotz, 1990). There are several limitations to this method. Stock radiolabeled JH decays into several radiolabeled contaminants that may bind with reduced affinity. Importantly, these contaminants cannot be distinguished from JH on TLC (Y. C. Park, unpublished observations) and must be removed by HPLC. Generation of contaminants during dialysis poses a second problem. Even though the hemolymph used in these studies was low in JH esterase activity, preincubation with esterase inhibitor was essential to maintain radiolabel integrity. The hJHBP may also undergo metabolism during dialysis, yielding multiple forms of the protein that display different kinetic properties. Comparing partially purified hJHBP samples that had been incubated with a cocktail of protease inhibitors with controls that were not treated indicated no differences in the K_D or B_{max} (data not shown), nor did the mathematical transforms indicate heterogeneity of binding sites (Werder et al., 1976).

Binding studies demonstrated that the K_D values for the *M. sexta* JH–hJHBP complex were nearly 2 orders of magnitude lower than previously reported (Kramer et al., 1976a; Goodman et al., 1978a,b; Prestwich et al., 1988). Much of the discrepancy can be attributed to ligand quality and the methods used to separate the bound and the unbound hormone. Repurification of the [^{3}H]JH just prior to analysis, combined with the use of enantiomerically pure radioligands of high specific activity (Prestwich & Wawrzenczyk, 1985), contributed significantly to the increase in affinity. Even when enantiomerically pure radioligand was used, the HA assay yielded K_D values that were an order of magnitude higher than those generated by equilibrium dialysis. Likewise, the B_{max} derived from the HA assay was lower than that observed in equilibrium dialysis, indicating that the HA matrix may sterically hinder binding or modify the binding domain by slightly altering the three-dimensional structure of the protein. The most plausible explanation for the difference between the two assays centers on the method by which bound and unbound hormone are separated. The HA assay requires five washes to remove unbound hormone, during which there is a continual shifting of the equilibrium that favors dissociation of holo-hJHBP. The result is a significant underestimation of the bound fraction. This study clearly indicates that estimation of equilibrium constants is a function of the method used to separate the bound and the unbound hormone.

A comparison of homolog K_D values suggests that the polarity of the JH homolog is not as important in binding as first reported (Goodman et al., 1978b). The polarity rule (Westphal, 1986) states that, within a series of small nonpolar homologous hormones, the least polar of the group will display the highest affinity. Although the present study indicates that JH I, the least polar of the homologs tested, is bound with the highest affinity, the affinity is not significantly different for the more polar JH II. The significantly higher K_D for JH III may reflect the use of a racemic hormone mixture rather than the decreased polarity of this homolog. Previous studies on binding of the JH III racemate support this premise (Schooley et al., 1978). In that study it was observed that the naturally occurring enantiomer, JH III (10*R*), bound 14-fold better than the 10*S* antipode.

Although the polarity rule may not apply to equilibrium constants, it is quite clear that the more polar the homolog, the shorter the $t_{1/2}$. Thus, JH I, the least polar of the homologs tested, has the longest half-life, while the most polar, JH III, has the shortest. Indeed, earlier studies on dissociation constants using nonequilibrium methods may have been unduly influenced by a rapid dissociation of the JH–hJHBP complex (Kramer et al., 1976a; Goodman et al., 1978a,b; Schooley et al., 1978; Peterson et al., 1982). Although a preliminary study claimed a $t_{1/2}$ of 15 min for the JH–hJHBP complex (Sanburg et al., 1975), the half-times of dissociation reported here are consistent with those of certain hormone transport proteins found in vertebrate serum, such as thyroxine binding globulin and sex hormone binding globulin (Mendel, 1989). That temperature has a minimal effect on the dissociation rate constants indicates that the forces maintaining the hormone–protein complex are hydrophobic in nature (Tanford, 1980) as previously suggested (Goodman et al., 1978b).

From a biological standpoint, target site delivery must take into account thermodynamic parameters as well as association and dissociation rate constants of the hormone–transport protein complex (Westphal, 1986; Ekins, 1990). Estimations of bound hormone using the newly derived equilibrium dissociation constants and the concentrations of JH and hJHBP indicate that better than 99% of the hormone is bound at any given time during the fourth larval stadium, a time when JH titers are at their highest levels (P. Hidayat and W. G. Goodman, unpublished observations). Likewise, an estimation of the average time interval (Ekins, 1990) during which JH I remains bound (41 s) or unbound (25 ms) suggests that very little free hormone is available to the target tissue. Indeed, the amount of hormone necessary in vitro to fully maintain the larval state is far greater than the amount of unbound hormone present at any given interval during development (Riddiford, 1976). At present, there is no direct experimental evidence to suggest that dissociation of the JH–hJHBP complex is the rate-limiting step in hormone action; however, evidence supports the hypothesis that the binding protein not only is important in hormone transport and passive maintenance of peripheral hormone titers but also may aid hormone interaction at the target site. The development of efficient purification and stabilization methods combined with the development of recombinant hJHBP technology (Touhara et al., 1993) will provide the necessary probes to resolve these questions.

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