Specificity of the juvenile hormone binding protein: The geometrical isomers of juvenile hormone I

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ABSTRACT The binding of the geometrical isomers (2:99% pure) of juvenile hormone I to the hemolymph juvenile hormone binding protein of Manduca sexta (Lepidoptera, Sphingidae) was analyzed. A technique is described for isomer separation by micro preparative high-resolution liquid chromatography. Analysis of competition was performed by using a "batch adsorption" hydrosylapatite binding assay. Competition studies indicate that the naturally occurring isomer, 2E,6E,10cis, is bound with the highest affinity. Optimal binding appears to depend most heavily upon the configuration of the 2,3 double bond. Juvenile hormone binding protein shows a higher affinity for the 2E than for the 2Z configuration. The 6,7 double bond is of less importance in determining binding activity, and isomerism about the epoxide appears least important in conferring binding activity. The binding site may be a groove along the surface of the binding protein interacting with the side chains of juvenile hormone, including the ester methyl group. The grouping of the side chains and the ester methyl group thus constitutes a distinct hydrophobic face, and the hydrophobic interactions are essential in maintenance of the bound ligand.

Recognition and subsequent binding to specific macromolecules has been assumed to be prerequisite for the biological activity of the insect juvenile hormones, a hormone class responsible in large measure for controlling both morphogenesis and vitellogenesis in insects (1). Until recently, this hypothesis was based primarily on evidence drawn from vertebrate serum hormone binding proteins and hormone cellular receptors. With the discovery of a specific juvenile hormone binding protein (JHBP) in the hemolymph of Manduca sexta larvae, correlations between juvenile hormone biological activity and specific macromolecular binding could be examined more closely (2, 3).

Binding studies have shown that the JHBP exhibits a relatively high affinity for the homologous juvenile hormones (C18, C17, C16). The basic requirements for binding to the specific site include the ester methyl function, the epoxide, and the aliphatic side chains (4–6). It was previously observed that a mixture of geometrical isomers of C18 juvenile hormone (JH I) displayed decreased binding activity when compared to the natural isomer (2E,6E,10cis), but the optimal geometrical arrangement around the double bonds and epoxide function was unclear (4). Earlier evidence based on bioassay data supports the conclusion that some of the isomers, particularly the 2Z isomers, are biologically inactive (7–10). The present investigation was designed to evaluate the possible isomeric configurations of JH I that could be accepted into the specific JH binding site on the binding protein. It should be noted that JH I is the predominant juvenile hormone in the hemolymph of Manduca larvae (11) and Tor that reason was used in the present study.

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MATERIALS AND METHODS

Binding Protein Preparation. JHBP was obtained from the hemolymph of fourth instar M. sexta larvae and prepared as described (4). After partial purification by gel filtration (Sephadex G-150) and ion exchange chromatography (CM-cellulose), the active fraction was incubated with 1 mM disopropylfluorophosphate to ensure inactivation of the remaining esterase. This inhibitor does not influence the binding activity of the JHBP (2, 5). The preparation was then divided into small aliquots and stored at −80°C until needed. Immediately prior to the assay, fractions were thawed and delipidated in ethanol/ether, 1:2 (vol/vol), at 4°C (4). The delipidated protein was centrifuged and the pellet was washed in the delipidation solvent. The resulting protein was redissolved in a Tris/KCl buffer (0.01 M Tris/0.01 M KCl, pH 7.5) and diluted to the concentration appropriate for optimal binding activity. This buffer was used throughout this study. Protein determination was performed by a modified Lowry procedure (12).

Hormone and Isomer Purification. [3H]JH I [methyl 2E,6E-3,11-dimethyl-7-ethyl-cis-10,11-epoxytrideca-2,6-dienoate; 12.9 Ci/mmol; radio purity, 98%] was purchased from New England Nuclear Corp. Unlabeled JH I (2E,6E,10cis; Fig 1) was purchased from Calbiochem and used without further purification. The seven other racemates were a gift of Karl Trautmann (Hoffmann-LaRoche) and were prepared by the "Roche synthesis" [see Pfiffner (13) for details]. Final characterization consisted of analysis by gas/liquid chromatography (GLC) and nuclear magnetic resonance, infrared, and mass spectrometry (13).

Upon receipt of the samples, each geometrical isomer was purified by micro preparative high-resolution liquid chromatography (HRLC) to remove other isomers or unrelated impurities. The chromatographic system consisted of a DuPont model 860 liquid chromatograph fitted with a low dead volume Valco loop injector. The microparticulate silica column (Zorbax-SIL, DuPont, 22 × 0.46 cm) was eluted with 6% diethyl ether in n-pentane at a flow rate of 2.0 ml/min. Solvents (Mallinkrodt AR ether; Phillips n-pentane) were distilled in a Pyrex apparatus, and the mixed solvent was 50% saturated with water before use. A sample of approximately 150–200 µg of each isomer was chromatographed and a heart cut of the main peak was collected for subsequent binding assay. At these sample loadings, column efficiency was still quite good (4700–4900 theoretical plates), allowing high-resolution separation from minor closely eluting impurities.

Certain isomers were analyzed by GLC following micro preparative HRLC. The instruments used were Hewlett-

Abbreviations: JHBP, juvenile hormone binding protein; JH I, C18 juvenile hormone; HRLC, high-resolution liquid chromatography; GLC, gas/liquid chromatography; HTP, hydroxylapatite.

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Packard model 402 or 5711 gas chromatographs with flame ionization detectors. Glass columns (2 m × 2 mm internal diameter) were packed with 2% SP-2250 (Supelco, equivalent to OV-17) plus 0.2% Carbowax 20 M coated on 100–120 mesh Chromosorb W, acid-washed and dimethylchlorosilane-treated. Helium carrier gas was used at 40 mL/min. For analysis, about 1 μg of purified JH I isomer was injected at an initial column temperature of 150°C, followed by immediate temperature programming at 4°C/min. With a properly packed and conditioned column, no thermal rearrangement or decomposition products of juvenile hormone are detectable, but, after several months’ use of this type of column, such artifacts of analysis are observed.

Determination of Extinction Coefficients. The extinction coefficients of the 2E,6E,10trans and 2Z,6E,10trans isomers were determined, because chromatographic differences between the conjugated 2E and 2Z series were expected. A Perkin-Elmer model 124 spectrophotometer was purged with N2, and the absorbance of solutions (12.0 μg/mL) of the two isomers in spectroscopy grade methanol was determined. The 2E isomer showed A = 0.602 at λmax ≈ 217 nm, or ε = 14,770; the 2Z isomer showed A = 0.512 at λmax ≈ 215 nm, or ε = 12,560. In all cases, JH I mass was determined from the extinction coefficient.

Binding Assay. Isomers at concentrations from 0 to 200 ng were added to polyethylene glycol-coated assay tubes [6 × 50 mm, three tubes per concentration; (4)]. The hormone was evaporated under N2 and redissolved in absolute ethanol (5 μL), and the solution then was brought to a final volume of 50 μL with the Tris/KCl buffer. After addition of the buffer, 50 μL of the [3H]JH I stock solution was added. The tritiated hormone stock contained 54,000 dpm/50 μL and was prepared as described (4).

A 100-μL aliquot of the binding protein solution was added to the assay tube, vortex mixed, and incubated overnight at 4°C.

Hydroxylapatite Preparation. Analytical grade hydroxyapatite (HTP, Bio-Rad Laboratories) was equilibrated in the Tris/KCl buffer overnight and washed twice with this buffer. The resuspended HTP was brought to a final concentration of 0.1 g/mL in the buffer and stirred for approximately 30 min to produce a homogeneous slurry. A 300-μL aliquot of the HTP suspension was introduced into the assay mixture and incubated for 45 min. After incubation, the tubes were centrifuged at 2800 × g. The supernatant was removed and the pellet was resuspended in 300 μL of the Tris/KCl buffer. Washing of the pellet was repeated five times. Upon completion of the last wash, the pellet was vortex mixed and 600 μL of liquid scintillation fluid (Aquasol; New England Nuclear Corp.) was added. The tube was again vortex mixed to disperse the HTP pellet evenly throughout the scintillation fluid. Radioactivity was assayed by inserting the 6 × 50 mm assay tube into a 4-ml glass vial that, in turn, was placed into the 20-ml glass liquid scintillation counting vial. All radioassays utilized a Packard liquid scintillation spectrometer (model 3380), and efficiency of counting (95–40%) was monitored by use of internally quenched standards.

RESULTS

Purification and Analysis of Isomers. Analytical HRLC of isomer mixtures (Fig. 2) showed that this technique would result in total separation of each of the following JH I geometrical isomers from any other: 2Z,6Z,10trans; 2Z,6Z,10cis; 2Z,6E,10trans; and 2E,6E,10trans. These four isomers were considered to be totally freed of isomeric contamination by the micropreparative HRLC purification. The following JH I isomers could be freed of all other isomers by HRLC except as indicated: 2Z,6E,10cis and 2E,6Z,10trans were partially separable from one another; 2E,6Z,10cis and 2E,6E,10cis were quite inseparable. After micropreparative HRLC separation of these samples, GLC analysis was performed under conditions that afforded baseline resolution of the isomer pairs 2Z,6E,10cis versus 2E,6Z,10trans and 2E,6Z,10cis versus 2E,6E,10cis. The HRLC-purified sample of 2Z,6E,10cis was found to contain 0.8% of the 2E,6Z,10trans isomer by GLC analysis; all other HRLC-purified isomers analyzed were found to contain <0.5% isomeric impurities.

Assay Conditions. A fundamental requirement for the ligand binding studies is the separation of the protein-bound ligand from the unbound ligand. Uncomplexed JH I could be effectively removed from the HTP matrix by repeated washing: five washes were sufficient to decrease the unbound JH I to less than 1% of the concentration originally introduced into the assay tube (Fig. 3). Despite five washes, perturbation of the equilibrium between JH I and JHBP was minimal—that is, no significant amount of bound JH I was lost after five washes and less than 15% was lost after nine washes. Other studies demonstrated that JHBP is adsorbed to HTP (W. Goodman, P. A. O’Hern, R. Zaugg, and L. I. Gilbert, unpublished data); however, modification of the batch adsorption technique required that time for development of the adsorption complex be optimized. Adsorption of the protein appeared to be rapid and stable within 20 min. Therefore, assays were routinely incubated for 45 min (Fig. 4).

The small amount of HTP used in the assay made it important that efficient adsorption of the protein was being observed. Fig. 5 indicates that up to 7 μg of protein could be adsorbed by the HTP. In this study, approximately 3 μg of protein was routinely used for optimal competition. Ethanol extraction of the HTP to remove the protein-bound JH I for radioassay was
attempted initially, but this method increased variability in the replicates. Addition of scintillation fluid directly to the assay tube eliminated the need for extraction and transfer of the bound JH I. The presence of HTP in the scintillation fluid did not significantly influence the counting efficiency. Up to 500 µl of the HTP slurry was added to the tubes without a reduction in efficiency; thus, self-absorption by the HTP is minimal (Table 1). The geometry and position of the assay tube within the large (20 ml) scintillation vials was initially believed to impose problems during the process of counting, but repeated changes in the position of the assay tube led to no significant differences in the counts (P < 0.01).

To ensure that the binding observed was indeed that of the high-affinity JHBP, partial purification was performed to remove other possible binding proteins. Scatchard plot analysis indicated only one class of binding sites present in the preparation (K_D = 0.95–1.5 x 10^{-11} M) with a dextran-coated charcoal assay (4). Comparison between highly purified JHBP (W. Goodman, P. A. O’Hern, R. Zaugg, and L. I. Gilbert, unpublished data) and the preparation used in this experiment indicated that both preparations had the same affinity for the naturally occurring isomer. Thus, the preparation used in this study serves as an adequate model for studying the binding of JH I isomers to the high-affinity protein.

**Isomer Binding to JHBP.** Geometrical isomers of JH I displayed wide variation in their ability to displace labeled 2E,6E,10cis isomer from the JHBP (Fig. 6). The competition studies indicate that the 2,3 double bond is important in conferring the optimal isomeric configuration for binding. The E configuration at this position appears to promote increased binding to the JHBP whereas isomers having a 2Z configuration show significantly decreased binding relative to the naturally occurring isomer.

Within the 2E isomer group, the all-E configuration approached the binding activity of the naturally occurring 2E,6E,10cis isomer (Table 2) whereas the isomers 2E,6Z,10cis and 2E,6Z,10trans exhibited decreased binding activity. Replacement of the 2E configuration by the 2Z led to a marked reduction in relative binding activity. Although the 2Z,6E,10cis isomer had the greatest affinity among the 2Z isomers, the activity of this compound was only 8% of that of the 2E isomer. The E configuration of the 6,7 double bond influences binding activity, as demonstrated by the increased affinity of the 6Z isomer over the 6Z isomer in both the 2E and 2Z isomer groups. However, the relative importance is secondary to the marked differences in binding activity observed between the 2E and 2Z isomers. Although replacement of the naturally occurring

**FIG. 3.** Retention of unbound juvenile hormone by HTP. Equal aliquots of [PH]JH I were incubated with HTP minus protein at 4° for 45 min. After the wash, the pellet was assayed for the [PH]JH I remaining. Vertical lines represent SD; n = 4.

**FIG. 4.** Adsorption time for binding of JHBP to HTP. The JHBP-[PH]JH I complex was incubated with HTP for varying periods of time at 4° and the adsorbed JHBP complex was resuspended and washed five times and radioassayed for bound JH I. Vertical lines represent SD; n = 4.

**FIG. 5.** Adsorption of protein by HTP. Increasing amounts of JHBP-[PH]JH I complex were added to assay tubes to determine the amount of protein that could be efficiently adsorbed by HTP. Routinely, 3.0 µg of protein was used in this study. Vertical lines represent SD; n = 4; correlation coefficient = 0.97.
cis epoxide ring with the trans geometry at the 10,11 position resulted in a modest decrease in binding activity (except for the 2E,6Z isomer pair), the rearrangement at this position had the least influence of the three possible positions on the relative affinity.

**DISCUSSION**

To our knowledge, the eight geometrical isomers of JH I cannot be totally resolved on any HRLC or packed-column GLC system. Although all eight isomers are separable by glass capillary column GLC (15), this technique is suitable only for microanalysis. Fortunately, the selectivity of HRLC and GLC for these isomers is quite different. The 10,11 cis and trans epoxide isomer pairs are poorly resolved on GLC regardless of the double bond geometry at positions 2,3 and 6,7, with the trans epoxide always eluting slightly after the cis epoxide (10, 13). However, on HRLC, each trans epoxide elutes before the corresponding cis epoxide, and baseline resolution is achievable. Conversely, we found that two pairs of isomers were not totally separated by HRLC, but GLC analysis was able to resolve these pairs. Thus, four of the isomers after HRLC purification should be devoid of any other isomer; GLC analysis of the other three isomers gave the following results: 2Z,6E,10cis contained 0.8% 2E,6E,10trans; 2E,6Z,10trans contained 0.03% 2Z,6E,10cis; 2E,6Z,10cis contained 0.03% 2E,6E,10cis. Before HRLC analysis, the samples appeared to have a chemical purity of 92-97% as determined by GLC.

HTP was shown to be effective in adsorbing the JHBP-JH I complex but allowing the complete removal of the unbound ligand (W. Goodman, P. A. O’Hern, R. Zaugg, and L. I. Gilbert, unpublished data). Organic solvent extraction of the bound complex from the HTP for radioassay, as described (16), was not necessary in this study because JH I and protein are soluble in scintillation fluid and HTP does not interfere with the scintillation counting process. The rapid and simple nature of this assay lends itself well to conditions in which large numbers of tubes must be analyzed for binding activity. Due to the presence of a large amount of nonspecifically binding lipoprotein in the hemolymph, the advantages of this approach extend only to the purified or partially purified JHBP. Initial studies showed that low-affinity binding proteins, such as bovine serum albumin, could not be used as a nonspecific protein control because they bound significant amounts of JH I. This observation is in contrast to data derived from a dextran-coated charcoal assay in which the unbound as well as nonspecifically bound hormone is removed (4). The relative minor disturbance of equilibrium by the HTP batch method, when compared to the dextran-coated charcoal system, makes the HTP adsorption technique more applicable to conditions in which the protein is in low concentration and in a pure or partially purified state.

Previous evidence has demonstrated that binding activity is based on the presence of several functional determinants—specifically, the ester methyl group, the side chains, and the epoxide function (4-6). Discrimination between the juvenile hormone homologs on the basis of binding activity depends on the length and number of carbon units on the side chains. Addition of a methylene group in the side chain brings about an overall increase in hydrophobicity and subsequent binding activity (4-6). The polarity of the homolog, as well as its overall configuration, therefore regulates the relative binding affinity between the hormone and JHBP. The polarity rule, which in large measure governs steroid hormone−serum binding protein interactions (17), can therefore be extended to this particular situation as well.

Based on previous evidence as well as on data presented in this study, the hypothetical binding site can be envisioned as a groove along the surface of the binding protein with interactions occurring between the binding site and the side chains of the JH I molecule, including the methyl of the ester group. The grouping of the alkyl side chains, including the methyl group, thus constitutes a distinct hydrophobic face (Fig. 7). Predictions based on this model suggest that the 2,3 double bond in the Z configuration would lead to decreased binding affinity. An impressive accumulation of bioassay data (7−10) as well as binding studies on the geometrical isomers of JH III (18) confirm this observation. One possible 2Z conformation would direct the carboxy carbonyl group toward the hydrophobic site, removing the methyl group from the immediate area. The loss of the hydrophobic interactions between the methyl group and the binding site, and the possible introduction of the electrophilic carboxy carbonyl group into the binding site, would severely decrease the binding activity of the 2Z isomers. A similar situation has been observed between JHBP and juvenile hormone acid, the latter being the product of the intracellular cleavage of the hormone. The loss of the ester methyl group results in a carboxylate anion (at hemolymph pH); this ionic group would have little attraction for a hydrophobic site, accounting for the severe decrease in binding activity (4-6). Conversely, the addition of an extra carbon atom at the 3' position significantly enhances the relative binding activity (4-6). This indicates that hydrophobic interactions in this region of the binding site are somewhat flexible (in that they can accept an additional methyl group) and are critical in maintaining the bound ligand.

**Table 2. Ratio of association constants for the isomers of JH I**

<table>
<thead>
<tr>
<th>Isomer</th>
<th>Ratio of association constants*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2E,6E,10cis</td>
<td>1.00</td>
</tr>
<tr>
<td>2E,6E,10trans</td>
<td>0.64</td>
</tr>
<tr>
<td>2E,6Z,10trans</td>
<td>0.27</td>
</tr>
<tr>
<td>2E,6Z,10cis</td>
<td>0.12</td>
</tr>
<tr>
<td>2Z,6E,10cis</td>
<td>0.08</td>
</tr>
<tr>
<td>2Z,6E,10trans</td>
<td>0.03</td>
</tr>
<tr>
<td>2Z,6Z,10cis</td>
<td>0.02</td>
</tr>
<tr>
<td>2Z,6Z,10trans</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Calculated as described by Korenman (14).
metrical isomers indicate an appreciable shortening of the distance between the polar epoxide and carboxemethoxy functions (a decrease from ~1.4 nm to ~1.1 nm) when the hormone isomers are examined in an extended conformation. Because it would appear that there are distinct recognition sites for the epoxide ring and the carboxemethoxy group conjugated to the 2E double bond, the binding data regarding the 6Z isomers may imply that JH I bound to the protein is not in a maximally extended conformation. If it were, then changing to a 6Z configuration might be expected to have a much more adverse effect on binding than was observed.

Alternatively, the Z configuration of the 6,7 position may retain the extended conformation and thus the hydrophobic face of the molecule; distortions in intramolecular distances between functional substituents may decrease the binding relative to the naturally occurring isomer. It is unclear how these distortions may affect binding because fewer modifications have been made in this region of the molecule.

Changing from the natural cis-10 epoxide configuration to a trans-10 results in a modest diminution in binding activity. Prior data (4-6) indicate that the epoxide function is absolutely necessary for binding, perhaps implying hydrogen bonding between the oxygen of the epoxide and the binding site. Recent data on the stereoisomers of JH III indicate a relative binding activity of 10R > 10R,S > 10S (D. A. Schooley, B. J. Bergot, W. Goodman, and L. I. Gilbert, unpublished data), further emphasizing the importance of the epoxide ring for binding. Because preparations used in the present study were racemic, firm statements regarding the importance of the 10,11 geometry cannot be made. Perhaps the ethyl side chain is more accessible for hydrophobic bonding in the cis-10 configuration than in the trans-10.

As in the case of the more thoroughly studied neutral steroids (17), the predominant binding forces maintaining the JHBP–JH I complex are assumed to be hydrogen bonds and hydrophobic interactions, including van der Waal’s forces. The concept that binding is primarily hydrophobic is supported by the facile dissociation of the complex, the relatively high amount of hydrophobic amino acids, and the previous competition studies (W. Goodman, P. A. O’Hern, R. Zaugg, and L. I. Gilbert, unpublished data). The distances between interacting hydrophobic groups are critical; thus, for an efficient interaction, a close fit of the ligand and the protein surface is required. Although no conformational data are yet available, measurement of space-filling molecular models of JH I in an extended conformation suggest that the dimensions of the naturally occurring isomer are about 1.7 × 0.7 × 0.5 nm. [The estimated Stokes radius for JHBP as determined by gel filtration is approximately 2.5, R(W. Goodman, P. A. O’Hern, R. Zaugg, and L. I. Gilbert, unpublished data.) One assumes that, once bound, JH I is in the minimum energy form and that the process of binding does not radically distort the conformation of either the hormone or the protein. Thus, alterations in the length and width of the JH I molecule by configurational changes in the double bonds, as well as changes in its polarity by side chain and ester group modification, are important considerations for future studies on JHBP–JH I interactions.

We are most grateful to K. Trautmann and A. Pfiffner (Hoffmann-LaRoche & Co.) for supplying us with the isomers of JH I that were originally synthesized by Dr. Pfiffner. Without their splendid cooperation, this project could not have been undertaken. We thank G. B. Harding for drawing the space-filling model (Fig. 7), M. A. Ratcliff (Zoeccon) for suggesting the use of the SP-2520/Carbowax GLC column for analysis, and Professors U. Westphal (University of Louisville) and I. Klotz and J. Marshall (Northwestern University) for their critical reading of this manuscript. This research was supported by Grants AM-02818 from the National Institutes of Health and PCM 76-03620 from the National Science Foundation.