Key Disulfide Bonds in an Insect Hormone Binding Protein: cDNA Cloning of a Juvenile Hormone Binding Protein of *Heliothis virescens* and Ligand Binding by Native and Mutant Forms[†,‡]

Hubert Wojtasek and Glenn D. Prestwich*

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400

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ABSTRACT: The hemolymph juvenile hormone binding protein (JHBP) from the early fifth instar larvae of *Heliothis virescens* (Lepidoptera, Noctuidae) has been purified, and three cDNA clones for this protein have been isolated from a fat body cDNA library constructed in bacteriophage λZAP XR. The deduced amino acid sequence of the full-length clone predicts a mature protein consisting of 224 residues, a molecular mass of 24 976 Da, and a p*I* of 5.29. Comparison of the amino acid sequence to that of the previously described JHBP from *Manduca sexta* shows 51% overall identity with highly conserved N- and C-terminal regions. One of the three clones bound photoactivatable analogs of juvenile hormones with much lower affinity than the other two. This clone had Phe^{150} in place of the expected Cys^{150} conserved in other JHBP clones. The F150C mutant of this clone regained native binding affinity. For native Hvir-JHBP, the affinity for [^{3}H]JH I was lower under reducing conditions (87 nM) relative to a 40 nM affinity under nonreducing conditions. The importance of pairs of Cys residues was addressed by preparing Cys to Ala mutants at each site. Expressed proteins were tested for binding affinity by photoaffinity labeling with tritium-labeled JH analogs and by binding assays using (10*R*,11*S*)-[^{3}H]JH I. Curiously, the C150A mutant retained full activity, implying that the aberrant C150F was dysfunctional due to steric hindrance rather than to a missing disulfide linkage. Likewise, C29A and C194A had binding affinities unchanged from that of the full-length wild-type clone. Two constructs, C9A and C16A, showed reduced binding affinity, suggesting that they form a disulfide bond important for ligand recognition.

Insect juvenile hormones (JHs)[1] regulate the growth and development of larvae by preventing metamorphosis (Riddiford, 1986) and are involved in reproductive maturation in adults (Koeppe et al., 1985). These lipophilic hormones require a hemolymph carrier protein that facilitates delivery of the hormone to target tissues, prevents adsorption to lipophilic sites in tissues, modulates nonspecific and JH-specific enzyme degradation (Sanburg et al., 1975; Touhara et al., 1995; Touhara & Prestwich, 1994), facilitates JH removal prior to pupation (Hammock, 1985; Touhara et al., 1995), and may interact with membrane proteins to deliver JH to target tissues (Hidayat & Goodman, 1994). High-affinity JH binding proteins (JHBPs) with molecular sizes of about 30 kDa have been partially characterized in six orders of the class Insecta using photoaffinity labels (Prestwich et al., 1994). Particular interest has centered on the hemolymph JHBPs (hJHBPs) from the lepidopterous larvae (Prestwich et al., 1987). The prototypical hJHBP from *Manduca sexta* larvae has been purified by several methods (Kramer et al., 1976a; Peterson et al., 1982; Lerro & Prestwich, 1990; Park et al., 1993), several cDNAs have been cloned (Lerro & Prestwich, 1990), and the recombinant protein has been expressed in a eukaryotic system (Touhara et al., 1993).

Although the physiological role of JHBP has been well-studied, details of the ligand−protein interaction have drawn less attention. Features of the binding pocket were probed with various structural isomers of natural and unnatural ligands (Peterson et al., 1977; Goodman et al., 1978; Prestwich et al., 1987). Regions involved in the recognition of the ester group were located by peptide mapping of the photoaffinity-labeled protein (Touhara & Prestwich, 1992). The role of several functional groups was suggested by chemical modifications (Peterson et al., 1982; Park & Goodman, 1993). However, no structural information is available about the amino acids that determine ligand binding specificity. The role of disulfide bonds, an important element in establishing protein tertiary structure and stability, has not yet been addressed, although a working model implicating disulfide(s) in maintaining a receptive JH binding cleft has been proposed (Touhara & Prestwich, 1992).

To address this question, a JHBP from a second species, the tobacco budworm *Heliothis virescens*, was isolated and several cDNAs were cloned and sequenced. Because the JH esterase from this insect has been cloned (Hanzlik et al., 1989) and shown to have potential applications in genetically engineered baculovirus for pest control (Hammock et al., 1990; Bonning et al., 1992), this species was a logical choice. We describe herein the purification of the hJHBP from larval *H. virescens* and the use of photoaffinity analogs of JH (Scheme 1) to determine binding affinity and selectivity. We

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[1] Abbreviations used: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; dH_2O, distilled, nanopure-filtered water; DCC, dextran-coated charcoal; DTT, dithiothreitol; EBDA, epoxybishomofarnesyldiazoacetate; EHDA, epoxyhomofarnesyldiazoacetate; IEF, isoelectric focusing; IPTG, isopropyl β-D-thiogalactopyranoside; JH, juvenile hormone; JHBP, JH binding protein; JHE, JH esterase; NBT, nitro blue tetrazolium; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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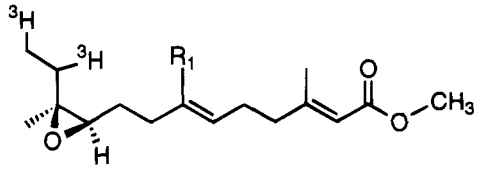
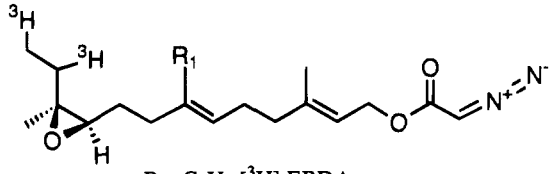
Scheme 1: Structures of Tritium-Labeled (10*R*,11*S*)-JH I, -JH II, and Their Photoaffinity Analogs Used in This Study



$R_1=C_2H_5$ [^{3}H] JH I
$R_1=CH_3$ [^{3}H] JH II



$R_1=C_2H_5$ [^{3}H] EBDA
$R_1=CH_3$ [^{3}H] EHDA

describe three cDNAs with differences in the deduced protein sequence at several positions, one of which significantly altered the binding affinity of the JHBP for JH. A curative mutation (F150C) in one dysfunctional cDNA identified Cys^{150} as being important in JH binding. Finally, we show that Cys^9 and Cys^{16} are the most important Cys residues for protein function and that they appear to form a disulfide bond that is important in ligand recognition.

MATERIALS AND METHODS

Insect Rearing and Hemolymph Collection. Eggs of *H. virescens* were provided by Mr. F. Adams (USDA, Gainesville, FL). Larvae were raised on an artificial diet (BioServ) (King & Hartley, 1985). Hemolymph was collected from the early fifth instar (day 1 or 2) in polypropylene tubes containing several crystals of phenylthiourea, as described for *M. sexta* (Kramer et al., 1974). After collection, the hemolymph was centrifuged (5000*g*, 30 min) to remove hemocytes and cellular debris; the clear supernatant was stored at −20 °C.

Photoaffinity Labeling and Fluorography. Photoaffinity labeling experiments were performed as described in detail previously (Prestwich et al., 1987). [12,13-$^3H_2$]Epoxyhomofarnesyl diazoacetate ([^{3}H]EHDA) and [12,13-$^3H_2$]-epoxybishomofarnesyldiazoacetate ([^{3}H]EBDA) (Ujváry et al., 1990) shown in Scheme 1 were synthesized or repurified by B. Latli and G. Dormán (SUNY at Stony Brook). The hJHBP and the recombinant proteins were labeled with either of these photolabels (50−200 nM final concentration) in the presence or absence of a 100-fold excess of (10*R*,11*S*)-JH I or -JH II. The labeled proteins were separated on 12% SDS−polyacrylamide gels, and the staining and fluorography were performed as described (Mohamed et al., 1989).

Protein Blotting and Immunodetection. In a routine blotting experiment, 40−60 μg of protein per lane was separated on a 12% SDS−polyacrylamide gel and electroblotted to a nitrocellulose membrane (BA 85, Schleicher & Schuell) using a Mini Trans-Blot transfer cell (Bio-Rad) for 1.5 h at 100 V in transfer buffer (10 mM CAPS (pH 11.0) and 10% methanol (v/v). Membranes were stained with Ponceau S (0.2% in 3% acetic acid (w/v) in water) to visualize proteins, washed with dH_2O and Tris-buffered saline (TBS: 10 mM Tris-HCl and 150 mM NaCl (pH 8.0), blocked for 30 min in 1% gelatin in TBS, incubated with rabbit anti-JHBP antiserum (1:500 in TBS:1% gelatin) for 1 h (as a control, preimmune serum was used), and washed with TBS. Protein−antibody complexes were visualized by a secondary antibody−alkaline phosphatase conjugate and chromogenic substrates (BCIP, NBT), as described by the supplier (Promega).

Purification of the Hemolymph JHBP. Juvenile hormone binding protein was purified by preparative isoelectric focusing (IEF) and anion exchange chromatography (Prestwich & Atkinson, 1990). Two slightly different modifications were used for analytical and semipreparative scales.

IEF of crude hemolymph was performed as follows. Four to six milliliter batches (ca. 50 mg/mL protein) were diluted with dH_2O containing 2% (w/v) Pharmalyte (pH 4.0−6.5) (Pharmacia) to 55 mL and loaded into a Rotofor cell (Bio-Rad), prepared according to the manufacturer's instructions. Focusing was carried out for 5 h at constant power (12 W). Fractions between pH 4.5 and 5.7 were combined, diluted with dH_2O to 55 mL, loaded into Rotofor, and refocused for 5 h. Solid NaCl was added to each fraction to 1 M final concentration, and fractions were dialyzed overnight (12−16 h) against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5 mM DTT. Aliquots of each fraction were labeled with [^{3}H]EHDA and analyzed by SDS−PAGE and fluorography. Fractions containing JHBP (pH 5.1−5.4) were combined and concentrated ca. 5-fold by ultrafiltration (YM-10, Amicon). The concentrated sample was labeled with [^{3}H]EHDA. Five volumes of 10 mM Tris-HCl (pH 8.0) were added, and the sample was ultrafiltered (YM-10, then Centricon-10) to ca. 3.0 mL and to ca. 500 μL.

Ion exchange chromatography was then performed as follows. The concentrated sample was loaded onto a MonoQ anion exchange column (Pharmacia) that had been preequilibrated with 10 mM Tris-HCl (pH 8.0), and proteins were eluted at 1.0 mL/min with a 0−200 mM linear gradient of NaCl using an FPLC system (Pharmacia-LKB). Aliquots (1%) of each fraction were assayed for radioactivity using a RackBeta 1218 liquid scintillation counter (LKB). Fractions containing major peaks of radioactivity (eluting at 50−90 mM NaCl) were analyzed by SDS−PAGE and fluorography. The fraction containing pure JHBP was concentrated ca. 5-fold (Centricon-10) and then lyophilized using a Speed-Vac. Protein was run on a 12% SDS−polyacrylamide gel and electroblotted to a poly(vinylidene difluoride) (PVDF) membrane. Protein sequencing was performed as described (Du et al., 1994).

Semipreparative purification samples were not photolabeled prior to ion exchange chromatography. Instead, small aliquots (20−50 μL) of fractions collected from the column (50−90 mM NaCl) were labeled with [^{3}H]EHDA and analyzed by SDS−PAGE and fluorography. All fractions containing JHBP (purity >95%) were combined, NaCl was added to a final concentration of 0.2 M, the sample was concentrated by ultrafiltration (YM-10) to 80 μg of protein/mL, divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C. Aliquots were later thawed and used to raise polyclonal antibodies against JHBP and for binding assays.

Molecular Cloning. The cDNA library was prepared as follows. Fat body was isolated from the early fifth instar larvae and immediately frozen in liquid nitrogen. Total RNA was isolated by a single-step, acid/guanidinium/phenol/

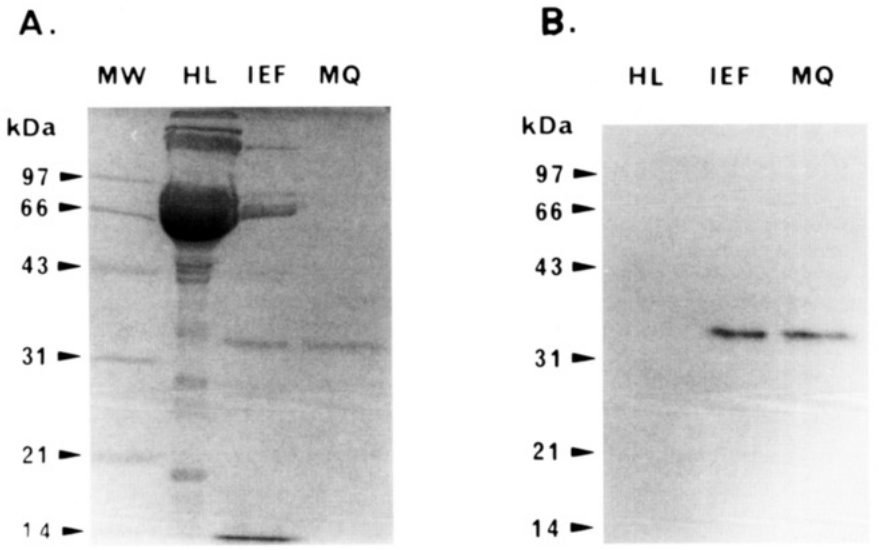


FIGURE 1: Purification of *H. virescens* JHBP. Proteins were separated on 12% SDS−PAGE, stained, and processed for fluorography. IEF fractions containing JHBP (pH 5.1−5.4) were pooled and labeled with [^{3}H]EHDA. Abbreviations: HL, crude hemolymph; IEF, pooled fractions from Rotofor; MQ, pure fraction obtained from a MonoQ column. Panel A: Coomassie Blue staining of an SDS−polyacrylamide gel. Panel B: Corresponding fluorogram.

chloroform extraction (Chomczynski & Sacchi, 1987). Poly(A) RNA was isolated using biotinylated oligo(dT) primers and streptavidin coupled to paramagnetic particles (PolyATtract, Promega). The library was constructed in a λZAP XR system (Stratagene) using cDNA synthesized (Gubler & Hoffman, 1983) from 8 μg of poly(A) RNA. cDNAs larger than 500 bp were ligated into the vector, the ligation product was packaged *in vitro,* and the phages were grown on the SURE strain of *Escherichia coli*. The resulting library contained about 3.5×10^5 recombinant vectors. The library was amplified once using SURE host cells.

An initial screening of 5×10^5 recombinant phages was performed with anti-JHBP antibody by inducing the protein expression with IPTG, transferring proteins from plates to nitrocellulose membranes (BA85, Schleicher & Schuell), and immunostaining as described earlier. Two positive clones (Hv-1 and Hv-2) were isolated by three rounds of plaque purification, amplified, and then subcloned *in vivo* into pBluescriptSK(−) and sequenced. Since neither of the first two clones contained the start codon, a 364 bp *Eco*RI fragment from Hv-1 was labeled with [^{32}P]dATP using random hexanucleotide primers and Klenow DNA polymerase I (Feinberg & Vogelstein, 1984). DNA was transferred to nylon membranes (Hybond N, Amersham) and hybridized to the radiolabeled probe (5× SSC, 5× Denhardt's reagent, 0.5% SDS, and 100 μg/mL salmon testes DNA) at 65 °C (Sambrook et al., 1989). Membranes were washed in 2× SSC and 0.1% SDS at room temperature (2 × 10 min) and then in 1× SSC and 0.1% SDS at 65 °C (15 min). Six clones giving the strongest signals were purified and subcloned *in vivo* into pBluescriptSK(−), and the size of the inserts was checked by restriction enzyme digestion. Clones longer than Hv-1 were partially sequenced from the 5′-end. A third positive clone, Hv-3, containing the starting codon was sequenced entirely.

Preparation of E. coli Protein Extracts. The expression of recombinant proteins was induced with 10 mM IPTG. Sodium deoxycholate and hypertonic buffer were used for the isolation of proteins from lysozyme-treated cells (Sharp et al., 1983). Cellular debris was removed by centrifugation (12000*g*, 5 min), and crude protein solutions were used for Western blotting. *H. virescens* hemolymph and *E. coli* XL1-Blue containing a nonrecombinant pBluescript were used as positive and negative controls, respectively. Before photoaffinity labeling, the protein concentration was measured in each extract, and samples were diluted with 10 mM Tris-HCl (pH 7.4) to a final protein concentration of 400 μg/mL, and labeled with [^{3}H]EHDA in the absence and presence of a 100-fold excess of JH II.

Site-Directed Mutagenesis. Mutagenesis was performed (Kunkel, 1985) using a Muta-Gene *in vitro* mutagenesis kit (Bio-Rad). To recover the binding affinity in the poorly photolabeled JHBP expressed from Hv-2, three amino acids were targeted, Phe^{150}, Glu^{186}, and Thr^{187}, the latter two being mutated simultaneously. Each amino acid was mutated to its equivalent in the Hv-1/Hv-3 clone: F150C, E186V, and T187A. The triple mutant was also obtained. For the functional analysis of cysteines, each of the five Cys residues in the full-length clone Hv-3 was mutated to Ala. Additional silent changes creating new restriction sites were introduced into the mutagenic primers to facilitate the identification of mutants. The integrity of the mutated cDNAs was confirmed by sequencing. All mutants, as well as the parental clones Hv-2 and Hv-3, were expressed in the MV1190 strain of *E. coli*. Crude protein extracts were isolated as described earlier, and the proteins were used for Western blotting and photoaffinity labeling with 100 nM [^{3}H]EBDA or were partially purified and used for binding assays.

Binding Assays. Dissociation constants were measured by a modification of the DCC assay (Kramer et al., 1976b; Prestwich et al., 1987) using (10*R*,11*S*)-[12,13-$^3H_2$]-JH I (Scheme 1) synthesized as described previously (Prestwich & Wawrzenczyk, 1985) by G. Dormán using precursors from B. Latli. Typically, a protein solution (20−40 nM, 100 μL) in 20 mM Tris-HCl (pH 7.4) was incubated with various concentrations of a radioactive ligand (3−200 nM of [^{3}H]-JH I) for 1 h at room temperature and then on ice an additional 15 min. The DCC solution was added, and the tubes were gently vortex mixed, incubated on ice for 5 min, and centrifuged for 2 min at 12000*g*. Radioactivity in the supernatant was measured using a liquid scintillation counter. Nonspecific binding was determined by adding a 100-fold

FIGURE 2: *H. virescens* JHBP cDNA sequences. Panel A: Deduced amino acid sequences of isolated clones and the sequence of the protein obtained from Edman degradation. Full-length Hv-3 and partial Hv-1 were exactly the same in the overlapped region; Hv-2 encoded four changes in the protein (Ile^{94} → Thr^{94}, Cys^{150} → Phe^{150}, Val^{186} → Glu^{186}, Ala^{187} → Thr^{187}). Differences between clones are underlined. Corresponding nucleotides for Hv-2 are given above the DNA sequence, and corresponding amino acids are below the deduced protein sequence. Phe^{150} and Glu^{186}Thr^{187} in the weakly active clone that were mutated to their equivalents in the active clones are in italics. Panel B: Comparison of *M. sexta* and *H. virescens* JHBPs. Alignment of proteins was performed by the GAP program from the GCG package. Proteins show 51% identity and 67% similarity. Conserved cysteine residues that were mutated to alanines are highlighted. Fragments identified as labeled in the peptide mapping experiment of *M. sexta* JHBP (Touhara & Prestwich, 1992) are underlined.

excess of radioinert (10*R*,11*S*)-JH I. For determination of the dissociation constant under reducing conditions, the hJHBP was incubated prior to the assay for 1 h in 20 mM Tris-HCl (pH 7.4) and 25 mM DDT under nitrogen at room temperature.

Cys mutant proteins were expressed in 500 mL cultures and partially purified by ion exchange chromatography. Crude extracts were applied to a DEAE column equilibrated with 10 mM Tris-HCl (pH 8.0), and proteins were eluted with a linear gradient (0−200 mM) of NaCl. Fractions were

analyzed by DCC assay and then photoaffinity-labeled; fractions containing recombinant JHBPs were pooled and frozen in liquid nitrogen. Binding assays were performed as described earlier, and dissociation constants were determined by Scatchard analysis. Assays for each protein (native, recombinant, or mutated) were performed in triplicate, and the results were averaged. Incubation of the purified hJHBP or bacterial cell extracts with 5 pmol of [^{3}H]-JH I (50 nM final concentration) for 3 h at 30 °C did not show any JH esterase activity. Binding assays were therefore performed without the addition of JHE inhibitors.

RESULTS

Determination of the protein sequence was accomplished by a standard multistage process: purification of the native protein, preparation of antisera, screening of an expression library, molecular cloning, cDNA sequencing, and authentication of the expressed protein from the single clone. For the first step, a simple, two-part purification procedure of the hJHBP gave material that was over 95% homogeneous as shown by SDS−PAGE (Figure 1A). This 32 kDa protein was specifically labeled with the photoaffinity analogs of JHs (Figures 1B and 3). Edman degradation of 10 μg of hJHBP provided the N-terminal 29 amino acids shown in Figure 2. This sequence showed significant homology to the N-terminal sequences of lepidopteran JHBPs described previously (Peterson et al., 1982; Lerro & Prestwich, 1990; Prestwich & Atkinson, 1990), especially to *M. sexta* (55% identity) and *Lymantria dispar* (47% identity).

Next, the purification was scaled up, and 500 μg of JHBP was obtained from 20 mL of hemolymph. This protein was used to raise anti-JHBP antibodies and determine dissociation constants. The antiserum was characterized by Western blotting of *H. virescens* hemolymph and purified JHBP. We did not notice any significant change in protein activity after prolonged storage at −80 °C.

Immunoscreening of a fat body cDNA library prepared in λZAP XR resulted in the isolation of two clones, Hv-1 and Hv-2, which expressed immunoreactive fusion proteins migrating at 37 kDa on SDS−PAGE. Sequencing of the cDNAs encoding these proteins revealed that they did not contain the entire open reading frame for JHBP. Hv-1 contained the sequence for 14 amino acids of the signal peptide, and Hv-2 was similarly truncated but was three nucleotides longer. The deduced N-terminal amino acid sequence of the mature protein differed at one position (Ser^{18} instead of Arg^{18}) from the sequence obtained by Edman degradation (Figure 2). More importantly, the nucleotide sequences of Hv-1 and Hv-2 differed in ten positions; five of these base changes encoded a total of four changes in the predicted amino acid sequence. The photoaffinity labeling of the Hv-1 and Hv-2 fusion proteins gave a surprising result. Although the Hv-1 protein was efficiently and specifically labeled by [^{3}H]EHDA, the Hv-2 protein was very weakly photolabeled (Figure 3) at an equivalent protein concentration. This result provided the impetus for the first mutagenesis experiments to determine which residues could account for the reduced JH affinity.

The full-length clone (Hv-3), obtained by rescreening of the cDNA library with a 364 bp fragment of Hv-1, contained the start codon and 18 upstream nucleotides; it had a sequence identical to that of Hv-1 in the overlapped region. The open reading frame (726 bp) encoded an 18 amino acid

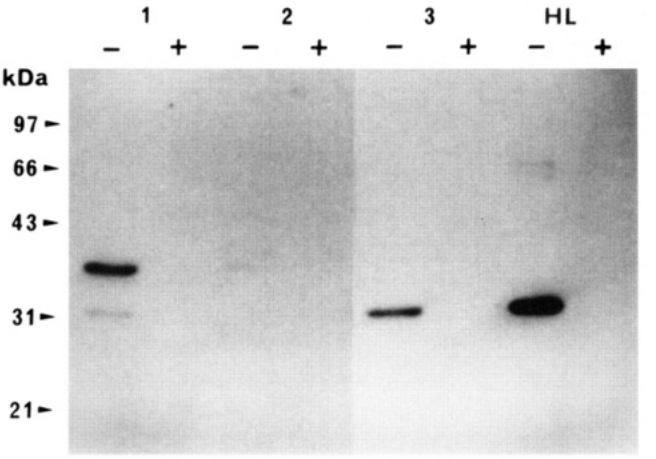


FIGURE 3: Photoaffinity labeling of recombinant JHBPs. Proteins were expressed in *E. coli* XL1-Blue, labeled with 100 nM [^{3}H]-EHDA, a photoactivatable analog of JH II, in the absence (−) or presence (+) of a 100-fold excess of JH II, separated on a 12% SDS−polyacrylamide gel, and exposed to X-ray film: (1) Hv-1; (2) Hv-2; (3) Hv-3; (HL) *Heliothis virescens* hemolymph taken as a control.

signal peptide and 224 amino acids of the mature protein. Expression of Hv-3 in *E. coli* XL1-Blue cells produced an immunoreactive protein of an apparent molecular mass of 32 kDa that was specifically labeled with [^{3}H]EHDA (Figure 3). The calculated molecular mass of the recombinant JHBP was 24 976 Da, and the calculated isoelectric point was 5.29. This latter value was in good agreement with the experimental data (Wing et al., 1984). Comparison of this cDNA with that of the *M. sexta* JHBP (Lerro & Prestwich, 1990; Touhara et al., 1993) showed 51% amino acid identity and 67% similarity. Importantly, five out of six cysteines, four of which are believed to be involved in disulfide bonds (Park & Goodman, 1993), were conserved; these Cys residues were clustered in the N- and C-terminal regions of the protein. Searches in the GenBank, EMBL, and SwissProt databases up to August, 1994, failed to reveal any other sequences with significant homology, although limited similarities were identified in short regions of several unrelated proteins, such as *M. sexta* arylphorin, human cholesterol 7α-hydroxylase, and dog cytochrome P-450.

Three mutant JHBP constructs were prepared to test the hypothesis that the substitution of a conserved Cys at residue 150 in Hv-1 by a Phe in Hv-2 was responsible for the abrogation of JH binding activity, as determined by photoaffinity labeling. Thus, the F150C mutant, the E186V, T187A mutant, and the triple mutant of Hv-2 were prepared. To evaluate the binding ability of fusion proteins expressed from mutants, approximately equal amounts of each protein (as determined by Western blotting) were labeled with [^{3}H]-EHDA. While the E186V,T187A mutation had little or no influence on the binding of photolabels, both F150C and triple mutant recovered full binding activity (Figure 4). Reproducible binding data could not be obtained for wild-type Hv-2, due to its much lower affinity and to the partial degradation of fusion proteins in bacterial cells (see Figures 3 and 4). Nonetheless, preliminary data show a K_D value greater than 500 nM.

Although chemical modification of thiols in *M. sexta* JHBP did not show significant influence on binding activity (Park & Goodman, 1993), Cys residues may affect protein structure via the formation of disulfide bonds. To test this possibility,

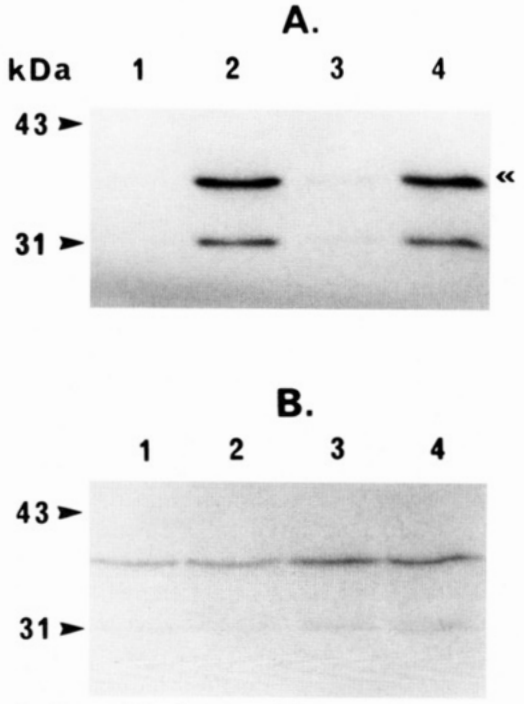


FIGURE 4: Photoaffinity labeling of fusion proteins expressed from mutants of weakly active clone Hv-2. Phe[150] and Glu[186]Thr[187] in the weakly active clone Hv-2 were mutated to their equivalents in the active clones by oligonucleotide-mediated mutagenesis. Equal amounts of each fusion protein from the expression in *E. coli* MV1190 were labeled with 100 nM [^{3}H]EBDA: lane 1, Hv-2; lane 2, F150C mutant; lane 3, E186V,T187A mutant; lane 4, triple mutant. Panel A: Fluorogram of labeled proteins separated by SDS−PAGE. Panel B: Corresponding Western blot. Fusion proteins are marked with a double arrow.

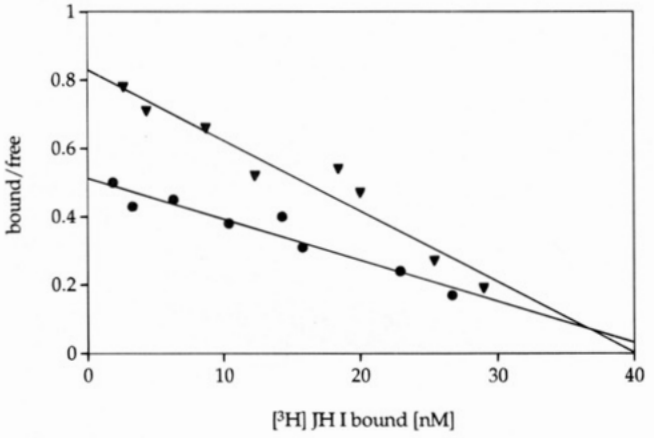


FIGURE 5: Scatchard plots for binding of [^{3}H]JH I to the hemolymph JHBP from larval *H. virescens*. Binding affinity of the purified protein was determined by the DDC assay: (▼) binding in the absence of a reducing agent; (●) binding in the presence of 25 mM DDT.

we compared the binding activity of native *H. virescens* JHBP under reducing and nonreducing conditions. The K_D value in the absence of a reducing agent was 40 ± 4.5 nM; in the presence of DTT, a value of 87 ± 3.7 nM was observed (Figure 5). This modest 2-fold difference in the binding affinity suggests that disulfide bridges may not be critical for the JHBP binding activity.

To explain the significant loss in activity of Hv-2 (Phe[150] instead of Cys[150]), and to disclose the role of the cysteines in this protein, each of the Cys residues was mutated to an Ala in five separate mutants. Two assays were performed on partially purified recombinant proteins to determine

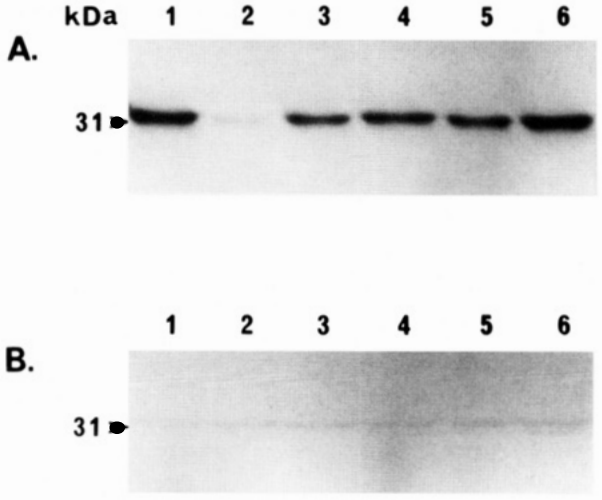


FIGURE 6: Photoaffinity labeling of proteins expressed from Cys mutants of Hv-3. All five Cys residues in *H. virescens* JHBP were sequentially mutated to Ala. Proteins were expressed in *E. coli* MV1190 cells. Approximately equal amounts of each protein were labeled with 100 nM [^{3}H]EBDA: lane 1, Hv-3; lane 2:, C9A; lane 3, C16A; lane 4, C29A; lane 5, C150A; lane 6, C194A. Panel A: Fluorogram. Panel B: Corresponding Western blot.

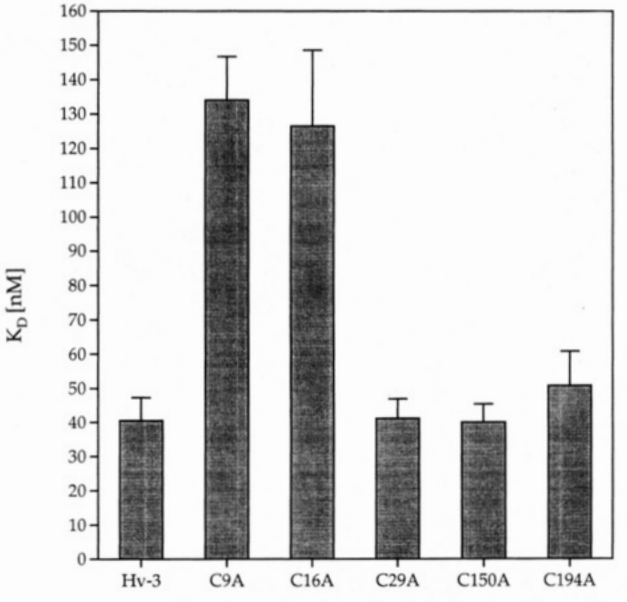


FIGURE 7: Dissociation constants for Cys mutants of Hv-3. Proteins were expressed in *E. coli* XL1-Blue and partially purified on a DEAE column. Binding was measured by the DCC assay. Dissociation constants were determined from Scatchard plots in triplicate and averaged. Error bars represent standard deviation for three independent measurements.

binding affinity: photoaffinity labeling with [^{3}H]EBDA and DCC binding assay with [^{3}H]JH I. Surprisingly, although C150F, the native state of Hv-2, was not photolabeled with [^{3}H]EBDA, C150A retained essentially full binding activity. Indeed, only one mutant (C9A) was photolabeled with significantly lower avidity (Figure 6) than the wild-type Hv-3 JHBP recombinant protein.

Since photoaffinity labeling cannot provide quantitative data, dissociation constants for all Cys mutants were determined. These experiments showed a lower binding affinity for C9A, as well as for a second mutant (C16A) (Figure 7). All other mutants (C29A, C150A, and C194A) had dissociation constants comparable to that of the wild-type Hv-3 JHBP recombinant protein. Importantly, each of these values was very close to the K_D value of the native

hJHBP (40 nM), indicating that the presence of the signal peptide in each of these constructs did not interfere with ligand binding (Figure 7).

DISCUSSION

H. virescens hJHBP was identified by Mumby and Hammock [cited in Kramer et al. (1976b)], and its K_D value of 610 nM and p*I* of 5.31 were first determined (Wing et al., 1984) over a decade ago. Although it is known that the *corpora allata* of *H. virescens* synthesize both JH I and JH II in a 1:3 ratio (Jennings et al., 1975), the larval titers have not been reported for this species. As described herein, binding affinities for JH I were obtained using 95% enantiomerically pure, high specific activity (10*R*,11*S*)-[^{3}H]JH I (Prestwich & Wawrzenczyk, 1985). The K_D values for the purified protein were consistently in the 40 nM range. It is worth noting that a K_D value of 11 nM was measured for purified *M. sexta* JHBP under similar conditions (Touhara et al., 1993). The estimation of equilibrium constants varies with the method used to separate bound and unbound hormone. Thus, DCC and hydroxylapatite assays overestimate the dissociation constants for hJHBP by an order of magnitude when compared to values obtained by equilibrium dialysis (Park et al., 1993). Nonetheless, consistent relative binding affinities for proteins from different species or for proteins altered by site-directed mutagenesis are readily obtained with these assays.

We employed tritium-labeled analogs of both JH I and JH II (i.e., [^{3}H]EBDA and [^{3}H]EHDA) in photoaffinity labeling experiments. The efficiency of labeling was essentially the same for both analogs. Freshly synthesized [^{3}H]-EBDA (a JH I analog) replaced [^{3}H]EHDA (a JH II analog) for the photoaffinity labeling of mutant proteins.

Our goals in conducting this study were twofold. First, we wished to sequence a full-length cDNA from a second lepidopteran larvae to determine conserved residues and regions likely to be important in hormone binding. Second, we wished to experimentally alter selected residues to test their role in determining JH affinity.

Previous studies had shown that Lys, Tyr, and Cys residues probably were not intimately involved in ligand recognition by JHBP (Peterson et al., 1982; Park & Goodman, 1993). These experiments did not, however, exclude the involvement of solvent-inaccessible residues located in a narrow, deep binding pocket. Moreover, previous work could not distinguish the effects of modifications of amino acid side chains at specific locations in the protein. To address the roles of individual residues, site-directed mutagenesis has become the method of choice. Specifically, the unexpected discovery of a native hJHBP cDNA encoding a low-affinity mutation (Cys^{150} to Phe^{150}) encouraged us to determine the role of each Cys in regulating JH binding affinity.

Although there is no evidence for the participation of free thiols in JH binding by *M. sexta* JHBP, the existence of two disulfide bonds has now been established (Park & Goodman, 1993). The involvement of N-terminal Cys residues in disulfide bonds was suggested when the N-terminus of the protein from *M. sexta* was first sequenced (Peterson et al., 1982). Such a bond would stabilize the flexible, terminal portion of the protein. *H. virescens* hJHBP shows 51% amino acid identity with the hJHBP of *M. sexta* (Lerro & Prestwich, 1990; Touhara et al., 1993). It is noteworthy that amino acid conservation is compartmentalized within the protein. That is, while the N-terminal residues 1−114 show 57% identity and the C-terminal residues 142−224 show 53% identity, the central residues 115−141 show only 24% identity. Most importantly, five conserved cysteines are located in highly conserved N- and C-terminal regions, suggesting the presence of conserved disulfide bridges.

Sequential replacement of each of the five Cys residues by Ala effectively removes zero or one disulfide bond. Evaluation of the binding affinities of each mutant *H. virescens* hJHBP suggested that only one disulfide bond could be moderately important for ligand binding. Because the JH binding affinities for C9A and C16A mutants were equal and 3-fold lower than C29A, C150A, and C194A, this suggests a Cys^9−Cys^{16} disulfide bond in the native JHBP. Partial purification of mutated proteins provided additional support for this hypothesis. Mutants C9A and C16A were eluted from the DEAE column at significantly higher salt concentrations (110−140 mM NaCl) than other mutants and the wild-type recombinant protein (80−110 mM). This suggested that an acidic residue was exposed to solvent and strengthened the interaction with the cationic resin; this could be either the cysteine released from the disulfide bond (pK_a of a sulfhydryl group equaled 8.3, while the pH of the elution buffer was 8.0) or a previously buried carboxylate. Note that, if a cysteine with a free thiol were mutated to Ala, one would predict a reduced, not increased, affinity for the anion exchange resin.

In the peptide mapping of *M. sexta* JHBP, both N-terminal (Asp^1−Glu^{34}) and C-terminal (Ala^{184}−Asn^{226}) portions of the protein were labeled by [^{3}H]EHDA, suggesting that both domains were involved in the recognition of the ester group (Touhara & Prestwich, 1992). A clam-shell architecture of the binding pocket was thus hypothesized in which these two domains were connected by disulfide bonds. However, it now appears that the disulfide bridges may have *intra*domain rather than *inter*domain character. Although the C-terminal fragment of *M. sexta* hJHBP was more intensely labeled at 4 °C, the N-terminal peptide fragment was more heavily labeled when the protein−[^{3}H]EHDA solution was frozen in liquid nitrogen prior to irradiation (Touhara & Prestwich, 1992). This result suggests higher flexibility for the N-terminal domain, consistent with the rigidifying role of a disulfide bond in this region of the protein.

Although juvenile hormones superficially resemble many lipophilic molecules (fatty acids, retinoids), the combination of the unsaturated methyl ester and epoxide functional groups is unique; both of these functionalities are critical for binding by JHBPs. Nonetheless, JHBP can tolerate the lack of the epoxide and can be efficiently and specifically labeled with [^{3}H]FDK, a photoaffinity analog of methyl farnesoate, or [^{3}H]MDK, a photoaffinity analog of methoprene (Touhara et al., 1994). The high binding affinity of [^{3}H]EBDA relative to JH I by caterpillar JHBPs (Prestwich et al., 1987) demonstrates that the orientation of the ester group can be reversed.

It is perhaps surprising that hemolymph JHBPs from two caterpillar larvae fail to show immunological cross-reactivity and exhibit only 51% amino acid identity. Nonetheless, the overall structure appears to be well-conserved. Prediction of the secondary structure with the Chou−Fasman algorithm showed essentially identical hydropathy profiles for the two JHBPs. Circular dichroism (Touhara et al., 1993) confirms that nearly half the protein is composed of α-helical elements. This contrasts markedly with the secondary and tertiary

structures of small lipid binding proteins (Benning et al., 1992; Cowan et al., 1993; Sacchettini & Gordon, 1993), which are composed primarily of eight or ten antiparallel β-strands. The highly α-helical character of the JHBPs does, however, have a strong precedent in the pheromone binding proteins (Du et al., 1994) of male moth antennae, which bind unsaturated fatty alcohol derivatives. That two helix-rich proteins have evolved for transportation and possibly transduction of intercellular lipid signals in insects is both noteworthy and provocative.

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