Ligand Binding by a Recombinant Insect Juvenile Hormone Binding Protein†

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ABSTRACT: A cDNA for the hemolymph juvenile hormone binding protein (JHBP) of larval *Manduca sexta* has been isolated, sequenced, and expressed in an insect cell line. A recombinant baculovirus, containing the JHBP cDNA fused to the p10 promoter of *Autographa californica* nuclear polyhedrosis virus, was constructed. Insect cells (Sf9) infected with this virus secreted recombinant JHBP (rJHBP) into the medium (>50 μg/mL), and cotranslational removal of an 18 amino acid leader sequence was observed. rJHBP was cross-reactive with an antiserum prepared to the hemolymph JHBP and was specifically labeled by [^{3}H]EHDA, a photoaffinity analog of JH II, demonstrating that rJHBP was an isoform of the previously reported 32-kDa JHBP [Lerro, K. A., & Prestwich, G. D. (1990) *J. Biol. Chem. 265,* 19800–19806]. rJHBP was purified from insect cell medium to homogeneity by ion-exchange and gel-filtration chromatography. The purified rJHBP had a higher affinity (K_D = 11 nM for JH I and K_D = 42 nM for JH II) than that reported for crude hemolymph JHBP (K_D = 80 nM for JH I). The circular dichroism (CD) spectrum of purified rJHBP indicated 34% α-helix and 23% β-sheet. The CD spectra of rJHBP in the presence and absence of JH II were the same, indicating no change in secondary structure induced by ligand binding. Thus, the rJHBP expressed in insect cells binds JHs and is suitable for structural and functional analysis.

Juvenile hormone (JH)[1] regulates growth and development of immature insects by preventing metamorphosis (Riddiford, 1986) and regulates aspects of reproductive maturation in many adult insects (Koeppe et al., 1985). At the target cells, JH regulates gene expression through an unknown signal transduction pathway. Since JH can readily diffuse through cell membranes due to its lipophilicity, one model for JH signal transduction suggests that specific cellular receptors for JH mediate the process, resulting in stimulation of transcription in a manner analogous to that of steroid hormones (Yamamoto, 1985). In some insects, however, JH appears to interact with proteins at the membrane level (Ilenchuck & Davey, 1984), which leads to uptake of proteins such as vitellogenin (Ilenchuck & Davey, 1987). An unambiguous mechanism of JH signal transduction remains to be elucidated.

In insects, as well as in vertebrates, the circulatory system transports trace amounts of biologically-active compounds (hormones) from the site of synthesis to the target tissues. In hemolymph, JH secreted from the corpora allata binds to both low and higher affinity binding proteins (Goodman & Chang, 1985). These serve a transport function and may protect JH from nonspecific adsorption to lipid deposits and from nonspecific degradation by esterases (Sanburg et al., 1975). Among the high-affinity binding proteins ($K_D < 10^{-7}$ M) in insects, the *Manduca sexta* hemolymph JH binding protein (JHBP) (32 kDa) was the first to be identified, purified (Kramer et al., 1976), and sequenced (Lerro & Prestwich, 1990). The *M. sexta* JHBP does not have significant homology with other proteins (Lerro & Prestwich, 1990), but the N-terminal sequences from several lepidopteran JHBPs show significant similarity (Prestwich & Atkinson, 1990), indicating that JHBPs may comprise a new superfamily of proteins.

Certain biochemical and structural studies require relatively large amounts of homogeneous protein. One cDNA expression system that can express, process, modify, and target large quantities of foreign protein is the baculovirus system using insect cells (Summers & Smith, 1987; Miller, 1988; Maeda, 1989). The foreign gene incorporated into baculovirus DNA (*Autographa californica* nuclear polyhedrosis virus; AcNPV) can be expressed in biologically-active form at up to 50% of total cellular protein when under control of a strong promoter, e.g., the polyhedrin promoter or the p10 promoter (Miller, 1988; Weyer et al., 1990). These characteristics of the insect cell expression system led us to express the hemolymph JHBP for structural studies using the baculovirus expression system.

In this paper, we report the isolation of a JHBP cDNA containing the entire open reading frame and the expression of recombinant JHBP (rJHBP) using a baculovirus expression system. The binding constants for juvenile hormone affinity for the homogeneous rJHBP were determined, and the effects of freezing and thawing on the binding affinity of the purified rJHBP were measured. The CD spectra of homogeneous recombinant JHBP and of JH–JHBP complex were obtained to determine if any change in secondary structure occurred as a resulting of ligand binding.

MATERIALS AND METHODS

Chemicals and Materials. Restriction endonuclease *Eco*RI and Klenow fragment of DNA polymerase I were obtained from Bethesda Research Laboratories. Endonucleases *Afl*III

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[1] Abbreviations: JH, juvenile hormone; (r)JHBP, (recombinant) juvenile hormone binding protein; EHDA, epoxyhomofarnesyl diazoacetate; FDK, farnesyl diazoketone; PAGE, polyacrylamide gel electrophoresis; AcNPV, *Autographa californica* nuclear polyhedrosis virus; DCC, dextran-coated charcoal; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PVDF, poly(vinylidene difluoride).

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and *Bgl*II and T4 DNA ligase were obtained from U.S. Biochemical Corp. Endonucleases *Bam*HI and *Sal*I and phosphorylated *Bam*HI linkers were purchased from Stratagene. Commercial products used include [α-^{32}P]dCTP (Amersham), protein low molecular weight markers (Bio-Rad), DNA molecular weight markers (1-kb DNA ladder, Gibco-BRL), RNA molecular weight markers (0.24–9.5-kb RNA ladder, Gibco-BRL), nitrocellulose filters (0.45 μm) (Schleicher and Schuell), Plasmid-Quick columns (Stratagene), Sequenase T7 DNA polymerase and the Sequenase kit (U.S. Biochemical Corp.), glycan detection kit (Boehringer Mannheim Biochemicals), and Protoblot kit for immunostaining (Promega). Custom oligonucleotide primers, ranging from 17 to 25 bases in length, were purchased from Bio-Synthesis, Inc. The 745-bp *M. sexta* JHBP cDNA (cDNA 1), the fat body cDNA library from day 1 fifth-instar larval *M. sexta*, and the anti-JHBP antiserum were described previously (Lerro & Prestwich, 1990). Fat body RNA was provided by Dr. V. Soroker. Optically-active high specific activity [^{3}H]-(10*R*,11*S*)-JH I and II (58 Ci/mmol) (Prestwich & Wawrzenczyk, 1985) and the photoaffinity label [^{3}H]-EHDA ([^{3}H]epoxyhomofarnesyl diazoacetate; 58 Ci/mmol) (Ujváry et al., 1990) were prepared by Drs. I. Ujváry and W.-s. Eng. Optically-active (10*R*,11*S*)-JH I and II (>95% enantiomeric excess) were prepared by Dr. C. Wawrzenczyk. Polyhedrin negative virus DNA (AcRP8) (Matsuura et al., 1987), transfer vector (pAcUW21), *Spodoptera frugiperda* cells (IPLB-Sf21, Sf9) (Vaughn et al., 1977) and polyhedrin positive wild-type viruses (AcNPV C6) were obtained from Dr. R. D. Possee (NERC, IVEM, Oxford, U.K.). *M. sexta* eggs were provided by Dr. J. S. Buckner (U.S. Department of Agriculture), and fifth-instar larval hemolymph (day 1) was obtained as described elsewhere (Lerro & Prestwich, 1990).

Isolation and Sequencing of JHBP cDNA 2. The ~660-bp *Eco*RI/*Hin*cII fragment isolated from JHBP cDNA 1 (Lerro & Prestwich, 1990) by low melting point agarose gel electrophoresis was labeled with [^{32}P]dCTP using random hexanucleotide primers and Klenow DNA polymerase (Feinberg & Vogelstein, 1984). Approximately 10^5 recombinant plaques from the *M. sexta* day 1 fifth-instar fat body cDNA library were blotted onto nitrocellulose filter paper and hybridized to the [^{32}P]-labeled JHBP cDNA probe (Maniatis et al., 1982). The washed filters were exposed to X-ray film overnight, and the 22 positive plaques were picked from the plates and eluted into SM buffer containing a few drops of chloroform. The positive plaques were subjected to a second round of plaque purification.

The 14 isolated positive phages were subcloned into the *Eco*RI site of pBluescript by superinfection with R-408 helper phage according to the Stratagene protocol. The sizes of the cDNA inserts were determined by *Eco*RI digestion and agarose gel electrophoresis. The two largest cDNA clones were restriction-mapped with different combinations of *Hin*cII, *Ssp*I, and *Eco*RI. A 1.4-kb cDNA, termed JHBP cDNA 2, was subcloned into both orientations in the *Eco*RI site of pBluescript, and single-stranded DNA produced by infection of transformed XL1-Blue cells with VCS-M13 or R-408 helper phage was sequenced by the Sanger dideoxynucleotide chain-termination method (Sanger et al., 1977), using Sequenase T7 DNA polymerase with the vector T3 primer and a set of custom oligonucleotide primers that were complementary to previously sequenced regions (Sambrook et al., 1989).

Northern Blotting. Fat body tissue was dissected from 15 day 1 fifth-instar larvae, and total RNA was isolated by extraction with guanidine hydrochloride (Izumi et al., 1980). Aliquots of 20 μg/lane were denatured, separated on a 1.4% formaldehyde–agarose gel, and blotted onto nitrocellulose paper, which was probed with the [^{32}P]-labeled *Eco*RI/*Hin*cII fragment of JHBP cDNA 1 (see above) at 65 °C without formamide (Maniatis et al., 1982). After being rinsed, the blots were exposed to X-ray film for 36 h. The molecular weight of the RNA was interpolated from a log/linear plot of the migration of the molecular weight markers.

Construction of Plasmid Vector. The strategy for construction of the recombinant transfer vector is illustrated in Figure 2. An 840-bp DNA fragment encoding JHBP (Figure 2A) was excised from recombinant pBluescript with *Eco*RI and *Afl*III and was blunt-ended using the Klenow fragment of DNA polymerase I. This fragment was ligated to phosphorylated *Bam*HI linker DNA and was digested with *Bam*HI. The polyhedrin positive transfer vector, pAcUW21 (Figure 2B), was linearized with *Bgl*II and ligated to the prepared JHBP DNA fragment with T4 DNA ligase. The plasmids were introduced into competent HB101 cells (Novagene), which were spread onto LB plates. Colonies were grown overnight at 37 °C, transferred to nitrocellulose filters, and hybridized to the [^{32}P]-labeled JHBP cDNA probe as described above after lysis and fixation according to Sambrook et al. (1989). Well-isolated, positive colonies were picked and grown in LB medium overnight, and plasmid DNA was isolated by alkaline lysis (Sambrook et al., 1989). The insert orientation was determined by restriction enzyme mapping using *Sal*I. The plasmid containing correctly-oriented JHBP cDNA (pAcUW21.JHBP) (Figure 2C) was further amplified and isolated.

Transfection and Screening for Recombinant Viruses. The *S. frugiperda* cells (Sf21) were propagated in ExCell-400 medium (JRH Bioscience) containing 1% (v/v) penicillin/streptomycin solution (Sigma). The Sf21 cells were cotransfected with polyhedra negative virus DNA (AcRP8) and recombinant transfer vector (pAcUW21.JHBP) using Lipofectin transfection reagent (Gibco). The cotransfection supernatant was harvested 2 days postinfection and subjected to plaque purification by screening for viruses forming polyhedrin-positive plaques. The recombinant viruses isolated by the first plaque assay were purified by another three sequential plaque assays.

Western Blotting and Immunodetection of rJHBP. The Sf21 cells were infected with purified recombinant viruses. At 5 days postinfection, the culture media were collected and used for immunoblot analysis. Thus, the supernatants from wild-type virus and recombinant virus infected cells were boiled in SDS sample buffer and loaded on an SDS–12.5% polyacrylamide gel (Laemmli, 1970). The separated proteins were transferred to nitrocellulose by electroblotting for 2 h at 50 V and 0.25 A in 10 mM CAPS buffer (10% MeOH, pH 11.0) in a blotting cell (4 L). The blot was blocked with 1% gelatin in 10 mM Tris-HCl with 150 mM NaCl (pH 8.0) (TBS) for 30 min, incubated with rabbit anti-JHBP antiserum (1:1000 dilution) (Lerro & Prestwich, 1990) for 1 h, and washed with TBS. Immunostaining was performed using the Protoblot kit of secondary antibody–alkaline phosphatase conjugate as described by the supplier (Promega).

After proteins were transferred to nitrocellulose filters as described above, a glycan blot was performed using a glycan-staining kit (Boehringer Mannheim Biochemicals) following the instructions provided by the supplier.

Expression and Purification of rJHBP. For large-scale expression of rJHBP, Sf9 cells infected with the recombinant

virus (10 plaque-forming units (pfu)/cell) were inoculated in a spinner flask in ExCell-400 medium supplemented with 2.5% fetal bovine serum (FBS) (Gibco) for 3 days. Alternatively, Sf21 cells infected with the recombinant virus (0.1 pfu/cell) were inoculated for 7–10 days without 2.5% FBS. Cells were then removed from the medium by centrifugation at 3000*g* for 10 min. The supernatant was mixed with five volumes of 10 mM Tris-HCl buffer (pH 8.0).

The sample solution was mixed with DEAE-Sephacel (Pharmacia) (1 mL of DEAE/8 mL of sample solution) which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0), and then the solution mixture was agitated for 30 min on ice. The mixture was allowed to settle in a column, and unretained protein was passed through the column (column size 2.5 × 25 cm). The retained proteins including rJHBP were eluted with a stepwise gradient from 25 to 200 mM NaCl (10 mM Tris-HCl, pH 8.0) at a flow rate of 60 mL/h. The JH-binding activity was measured by the dextran-coated charcoal (DCC) assay using [^{3}H]JH II as described below. rJHBP was eluted from the column with 25–50 mM NaCl. Fractions containing binding activity were collected and concentrated by ultrafiltration using YM-10 membranes (Amicon).

Gel filtration of the concentrated sample was carried out using Sephacryl S-200 (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.4) or 10 mM phosphate buffer (pH 7.4) (flow rate 15 mL/h, column size 1.4 × 50 cm). Fractions containing binding activity were detected by the DCC assay. The purity of rJHBP was demonstrated on an SDS–12.5% polyacrylamide gel by Coomassie staining (Laemmli, 1970). The concentration of the purified protein was estimated by the A_{280} value.

Electroblotting and Sequencing of rJHBP. Purified rJHBP separated on an SDS–12.5% polyacrylamide gel was blotted onto a PVDF membrane (Bio-Rad TransBlot) in an electrotransfer cell (4 L) using 10 mM CAPS buffer (10% MeOH, pH 11.0) at 50 V and 0.25 A for 2 h. The membrane was stained briefly with 0.1% Coomassie Blue R-250 in 50% MeOH, destained with 50% MeOH, washed three times with water for 20 min, and air-dried. The rJHBP band was excised and sequenced by Edman degradation using an Applied Biosystems Model 475A pulsed liquid-phase sequencer.

Photoaffinity Labeling and Fluorography. A typical photoaffinity labeling experiment was described elsewhere (Prestwich et al., 1987). The purified rJHBP and *M. sexta* hemolymph JHBP were labeled with [^{3}H]EHDA (final concentration of radioligand 200 nM) in the presence or absence of a 100-fold excess of JH II. The labeled proteins were separated on an SDS–12.5% polyacrylamide gel, and the staining and fluorography were performed as described elsewhere (Mohamed et al., 1989).

Binding Assay for rJHBP. The dissociation constant (K_D) was determined by a modified method of a DCC assay as described by Goodman et al. (1976). The charcoal–dextran stock solution was made from 5% (w/v) activated Norit-A (Fisher) with 0.5% dextran T70 (Sigma), 1.5 mM EDTA, and 3 mM sodium azide in 10 mM Tris-HCl buffer (pH 7.3). To the sample solution was added various concentrations of [^{3}H]JH I or [^{3}H]JH II, and samples were incubated for 1 h at room temperature. At the end of the incubations, charcoal–dextran solution was added to a final charcoal concentration of 0.83% (w/v). The mixture was gently vortexed, incubated for 2 min, and centrifuged at 12000*g* for 4 min. The radioactivity of the supernatant was measured using a liquid scintillation counter (LKB) with Scintiverse II (Fisher). Nonspecific binding was measured by adding a 100-fold excess of radioinert JH I or JH II. The K_D value was determined from the slope of the Scatchard plot (Scatchard, 1949). The above experiments were replicated three times and the data were averaged. To check the effect of freeze–thawing, the partially purified sample was frozen in liquid nitrogen and thawed by hand. This process was repeated three times.

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                                                                  AflIII
-36                                  5'     TCTGCATTGTTGTTCTTCCCCAAAAACGTGTTC

-3   AAT ATG AAT GGT TTT AAA GTA TTT CTG TTT CTG CTA TTT GCA AAA TGT GTT TTA TCG
-18      MET Asn Gly Phe Lys Val Phe Leu Phe Leu Leu Phe Ala Lys Cys Val Leu Ser

55   GAT CAA GGG GCA CTC TTC GAA CCA TGC AGC ACA CAG GAC ATA GCC TGC TTG AGT AGA
1    Asp Gln Gly Ala Leu Phe Glu Pro Cys Ser Thr Gln Asp Ile Ala Cys Leu Ser Arg

112  GCA ACG CAG CAA TTC TTG GAG AAG GCC TGT CGA GGA GTC CCA GAA TAT GAC ATC AGG
20   Ala Thr Gln Gln Phe Leu Glu Lys Ala Cys Arg Gly Val Pro Glu Tyr Asp Ile Arg

169  CCT ATA GAT CCT TTA ATT ATA CCA TCT TTG GAC GTG GCT GCA TAT GAC GAC ATA GGC
39   Pro Ile Asp Pro Leu Ile Ile Pro Ser Leu Asp Val Ala Ala Tyr Asp Asp Ile Gly

226  TTA ATA TTC CAC TTT AAA AAC CTG AAC GTA ACA GGT TTG AAG AAT CAG AAG ATA TCG
58   Leu Ile Phe His Phe Lys Asn Leu Asn Val Thr Gly Leu Lys Asn Gln Lys Ile Ser

283  GAT TTC AGG ATG GAC ACA ACG AGG AAG TCT GTG CTG CTG AAG ACG CAA GCA GAC TTA
77   Asp Phe Arg Met Asp Thr Thr Arg Lys Ser Val Leu Leu Lys Thr Gln Ala Asp Leu

340  AAT GTG GTC GCA GAC GTC GTT ATA GAA TTG AGT AAA CAA TCA AAA TCG TTT GCA GGA
96   Asn Val Val Ala Asp Val Val Ile Glu Leu Ser Lys Gln Ser Lys Ser Phe Ala Gly

397  GTG ATG AAT ATC CAA GCA AGC ATA ATA GGA GGA GCG AAG TAT AGC TAT GAT TTG CAA
115  Val Met Asn Ile Gln Ala Ser Ile Ile Gly Gly Ala Lys Tyr Ser Tyr Asp Leu Gln

454  GAT GAT TCA AAA GGT GTG AAG CAT TTT GAG GTT GGC CAA GAG ACG ATT TCT TGC GAA
134  Asp Asp Ser Lys Gly Val Lys His Phe Glu Val Gly Gln Glu Thr Ile Ser Cys Glu

511  TCG ATA GGC GAG CCA GCA GTG AAT TTG AAT CCA GAA TTA GCG GAT GCT TTA TTG AAA
153  Ser Ile Gly Glu Pro Ala Val Asn Leu Asn Pro Glu Leu Ala Asp Ala Leu Leu Lys

568  GAT CCT GAT ACA ACA CAC TAC AGG AAG GAC TAC GAA GCT CAT GCG GTT GGT ATT CGA
172  Asp Pro Asp Thr Thr His Tyr Arg Lys Asp Tyr Glu Ala His Arg Val Gly Ile Arg

625  CAA AGA TCA TTG TGC AAG ATA GTT GAG CTA TGT TAT GTT GAT GTC GTA CAC AAC ATC
191  Gln Arg Ser Leu Cys Lys Ile Val Glu Leu Cys Tyr Val Asp Val Val His Asn Ile

682  AGA GCA GTT GCC AAG ATA TTG CCG TCG ACA GCA TTC TTC ACT GAT GTT AAT TAA TTT
210  Arg Ala Val Ala Lys Ile Leu Pro Ser Thr Ala Phe Phe Thr Asp Val Asn END

739  TCAAACTATTGACTTATTACAATAAACGTTTGTACAGATGTGTTTTTAATCTGCTACAATATAAACAACATAAAA

     AAAAAAAAAAAAAAA    3'
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FIGURE 1: Partial nucleotide sequence of JHBP cDNA 2 and deduced amino acid sequence of the JHBP. The signal peptide is shown in boldface type. The underlined bases and amino acids differ from JHBP cDNA 1 (9). Doubly-underlined regions represent possible polyadenylation signals. The 5′ end shows the 36 nucleotides upstream of the signal peptide and the *Afl*III site.

RESULTS

Isolation and Analysis of JHBP cDNA 2. Two cDNA clones in pBluescript (>1 kb) were observed out of 14 isolated positive clones. Restriction mapping revealed that a 1.4-kb cDNA was similar to the JHBP cDNA 1 (Lerro & Prestwich, 1990). This cDNA, termed JHBP cDNA 2, was sequenced in its entirety, and the nucleotide sequence of the relevant part of this clone is shown in Figure 1. The JHBP cDNA 2 described herein differed from JHBP cDNA 1 in several ways. It had an ATG start codon followed by a stretch of 18 hydrophobic amino acids that appeared to be the signal peptide. The CAATATG sequence at the initiation codon appeared to be a ribosome binding site consensus sequence (Cavener, 1987). Of the 13 nucleotide substitutions between cDNA 1 and 2, one was in the 3′ noncoding region and 12 were in the JHBP coding region (Figure 1, underlined residues); nine of the coding substitutions were silent, while three led to amino acid changes: Ser^{46} to Pro^{46}, Ile^{67} to Val^{67}, and Ser^{188} to Gly^{188}.

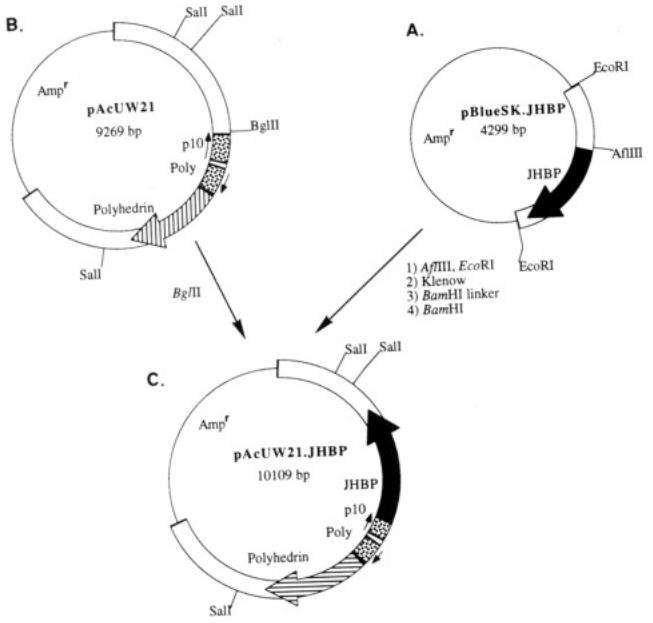

FIGURE 2: Construction of the transfer vector pAcUW21.JHBP. The JHBP cDNA was excised from the recombinant pBluescript (A), modified with *Bam*HI linkers, and inserted into the *Bgl*II cloning site of the transfer vector, pAcUW21 (B), resulting in the recombinant pAcUW21.JHBP (C).

The 3′ untranslated region of cDNA 2 was 38 nucleotides longer than that of cDNA 1, and the sequence AATATAAA located seven nucleotides upstream from the poly(A) tail appeared to contain an alternate polyadenylation signal (Proudfoot & Brownlee, 1976). The extent of the authentic 5′ end of cDNA 2 was not determined due to a cloning artifact (Lerro, 1991). That is, cDNA 2 contained a 5′ open reading frame (ORF) for a 114-residue protein fused via 86 bp to the start codon of the JHBP ORF. A Northern blot showed a 1.0-kb JHBP mRNA (data not shown).

Production of Recombinant Baculovirus. The strategy for subcloning the JHBP cDNA into the baculovirus transfer vector is shown in Figure 2. The recombinant pBluescript containing JHBP cDNA 2 contained an upstream open reading frame for an unknown protein, but the JHBP coding region was readily excised using *Afl*III. This JHBP cDNA fragment was inserted into an AcNPV transfer vector (pAcUW21) downstream from the p10 promoter and upstream from the polyhedrin gene, to produce the recombinant plasmid pAcUW12.JHBP (Figure 2C). The recombinant plasmid was isolated by hybridization to [^{32}P]-labeled JHBP cDNA, and restriction mapping confirmed that the JHBP cDNA was inserted in the correct orientation. Lipofectin-mediated cotransfection of cultured Sf21 cells with pAcUW21.JHBP and polyhedrin-negative AcNPV DNA produced the polyhedrin-positive recombinants, which were purified by four sequential plaque assays based on the presence of polyhedra.

Analysis of rJHBP Expression. Five days after infection of Sf21 cells with purified recombinant or wild-type viruses, the culture supernatants were harvested and assayed for the presence of rJHBP by immunoblot analysis and photoaffinity labeling. The presence of rJHBP expressed and secreted into the medium was confirmed by Coomassie Blue staining of SDS–polyacrylamide gels and immunostaining of protein blots. Figures 3 and 4A show the expression and secretion of immunoreactive rJHBP that comigrates with the 32-kDa hemolymph JHBP. According to immunoblot analysis, almost all of the expressed rJHBP was exported into the culture medium (data not shown) as expected from the 18 amino acid

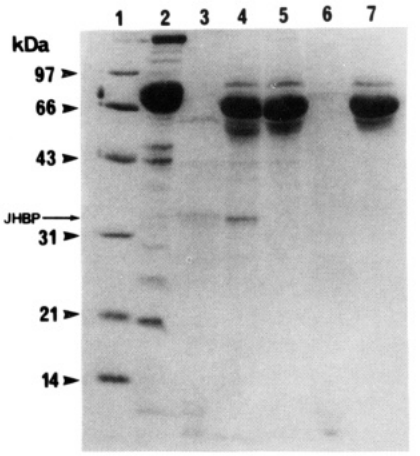

FIGURE 3: Expression of recombinant JHBP. The proteins were separated on an SDS–12.5% polyacrylamide gel and stained with Coomassie Blue. Lane 1, molecular weight markers; lane 2, larval *M. sexta* (fifth instar) crude hemolymph (3 μL); lane 3, 20-μL aliquot of recombinant virus infected Sf21 cell medium (0.1 pfu/cell) harvested at 8 days postinfection (dpi) in the absence of 2.5% fetal bovine serum (FBS); lane 4, 20-μL aliquot of recombinant virus infected Sf9 cell medium (10 pfu/cell) harvested at 3 dpi in the presence of 2.5% FBS; lane 5, 20-μL aliquot of wild-type virus infected Sf21 cell medium harvested at 5 dpi in the presence of 2.5% FBS; lane 6, Sf21 cell extract; and lane 7, 20-μL aliquot of ExCell-400 medium in the presence of 2.5% FBS.

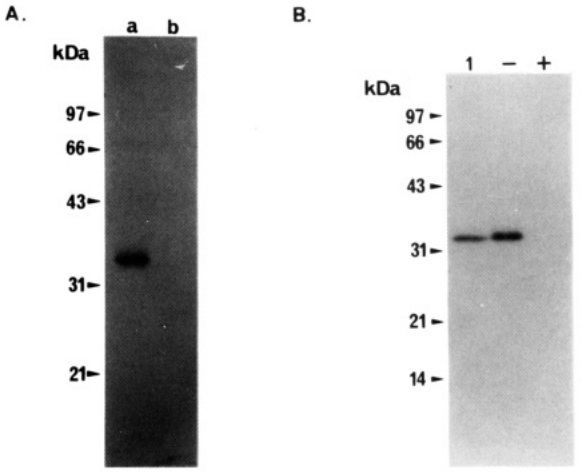

FIGURE 4: Immunoblot and photoaffinity labeling of recombinant JHBP expressed in Sf21 cells. Panel A: Aliquots (20 μL) of (a) recombinant virus infected and (b) wild-type virus infected Sf21 cell media were separated on an SDS–12.5% polyacrylamide gel, blotted onto nitrocellulose, and immunostained with anti-JHBP antiserum. Panel B: *M. sexta* crude hemolymph was photoaffinity-labeled with [^{3}H]EHDA (lane 1). The purified recombinant JHBP was photoaffinity-labeled with [^{3}H]EHDA in the presence (+) or absence (–) of a 100-fold excess of JH II.

leader sequence (Figure 1). N-Terminal sequence analysis revealed that secreted rJHBP was correctly posttranslationally cleaved. A total of 200 pmol of PVDF-immobilized rJHBP was sequenced to give the sequence Asp-Gln-Gly-Ala-Leu-Phe-Glu-Pro-X-Ser, identical to the first 10 amino acids of hemolymph JHBP. Importantly, the rJHBP expressed in Sf21 cells was specifically labeled with the JH II photoaffinity analog [^{3}H]EHDA, demonstrating that rJHBP was an isomer of the 32-kDa JHBP (Figure 4B). The labeling of rJHBP was completely displaced by an excess amount of JH II, demonstrating that rJHBP was expressed in a form in which binding affinity and specificity for JH were retained.

Purification of rJHBP. The secretion of rJHBP into the medium facilitated the purification of rJHBP. Because of

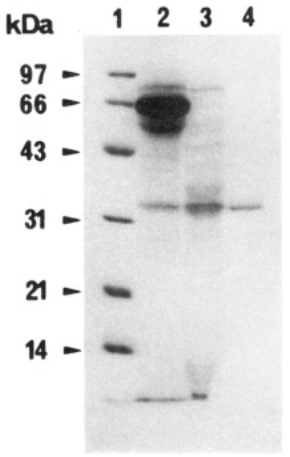

FIGURE 5: Purification of recombinant JHBP. The recombinant JHBP was purified from recombinant virus infected Sf9 cell medium by DEAE-Sephacel and Sephacryl S-200 chromatography. Lane 1, molecular weight markers; lane 2, crude medium; lane 3, purification by DEAE column; and lane 4, further purification by gel filtration.

the low pH (pH 6.5) and high salt concentration of the medium, adjustment of pH and dilution of culture medium were necessary for ion-exchange chromatography. Since JHBP is an acidic protein, the DEAE column separated JHBP from most other proteins in the medium. JHBP was eluted with 50–75 mM NaCl, which is also characteristic for the hemolymph JHBP (Goodman et al., 1978). The subsequent gel filtration resulted in >95% homogeneous rJHBP, based on SDS–PAGE analysis (Figure 5). This purified rJHBP was immunoreactive with the anti-JHBP antiserum raised against hemolymph JHBP (Lerro & Prestwich, 1990) and was specifically labeled by [^{3}H]EHDA (Figure 4B). Thus, purified rJHBP retained its specific binding affinity for JH.

Binding Assay. The dissociation constant of purified rJHBP was determined by the DCC assay (Goodman et al., 1976). Figure 6 shows the result of a binding assay in which the JH binding properties of rJHBP were characterized. The purified rJHBP bound (10*R*,11*S*)-JH I with K_D = 11 nM, and (10*R*,-11*S*)-JH II with K_D = 42 nM as revealed by a Scatchard plot (Scatchard, 1949), which was slightly lower than the previous value of K_D = 80 nM for (10*R*,11*S*)-JH I reported for crude *M. sexta* hemolymph JHBP (Prestwich et al., 1988). Together with the apparent saturation of binding (data not shown), rJHBP possessed properties consistent with those of hemolymph JHBP.

A Scatchard plot of binding data for a sample of rJHBP aged by repeated freeze–thaw cycles during ion-exchange and gel-filtration purifications showed a biphasic plot (K_D = 16 nM and 96 nM for JH I) (Figure 7A), suggesting that the higher affinity site was partially lost and was replaced by the lower affinity component. To rule out any possibility that high salt conditions were responsible for the appearance of biphasic binding curves, partially-purified rJHBP obtained directly from a DEAE column was utilized for the binding assay. The K_D value was 27 nM for the freshly purified rJHBP sample. This *same sample* was then subjected to three freeze (–196 °C)–thaw (30 °C) cycles and the binding activity was reassayed. A biphasic Scatchard plot with K_D values of 23 nM and 183 nM resulted (Figure 7B), confirming that freeze–thaw cycles adversely affect the ligand binding affinity of JHBP.

Circular Dichroism Spectra. The CD spectrum of rJHBP (Figure 8) revealed that JHBP had approximately 34% α-helix and 23% β-sheet. Table I shows the comparison of secondary structure by various prediction methods. The secondary structure prediction, especially by the Garnier method but also by the Chou–Fasman method, gave data consistent with CD analysis. The CD spectra of rJHBP in the presence and absence of JH II were superimposable (Figure 8), suggesting that there was no gross change in secondary structure induced by binding of JH.

Table I: Secondary Structure Analysis of JHBP

method	α-helix (%)	β-sheet (A + P)[a] (%)	others (T + O)[a] (%)
Chou–Fasman	31.0	32.3	36.7
		23.5[b]	45.5
Garnier et al.[c]	32.3	24.8	42.9
Garnier et al.[d]	26.1	23.0	50.9
CD	34.3	23.4	42.3[e]

[a] A = antiparallel and P = parallel; T = turns and O = others. [b] Percentage of only strong β-sheet prediction. [c] No decision constant. [d] The restriction that minimum length of α-helix is six and of β-sheet is four was added. [e] T = 12.0%, O = 30.3%.

DISCUSSION

The molecular action of JH has been extensively studied for many years, but the precise mechanism of JH action has yet to be fully elucidated. The model in which JH acts in a similar manner as steroid hormones (Yamamoto, 1985) has been proposed, since putative cytosolic and nuclear receptors were identified and characterized in a variety of insects (Engelmann et al., 1987; Chang et al., 1980; Roberts & Jefferies, 1986; Palli et al., 1991). An alternative signal transduction pathway through the recognition of JH–JHBP complex at the membrane level has also been proposed (Goodman & Chang, 1985). Juvenile hormone synthesized in the corpora allata is transported to the target tissues in the hemolymph. JH is susceptible to degradation by esterases and hydrolases and can be readily adsorbed into lipid depots nonspecifically. The JHBP concentration (1 μM) (Goodman et al., 1978), maximal hormone concentration (10 nM) (Peter et al., 1976), and association constant (K_a = 10^7 M^{-1}) (Goodman et al., 1978) indicate that all JH is bound to JHBP in hemolymph, suggesting that the high-affinity JHBP plays a role in JH transport, distribution, and protection (Sanburg et al., 1975). *M. sexta* JHBP (32 kDa) was the first insect JHBP to be cloned and sequenced (Lerro & Prestwich, 1990), revealing that JHBP may comprise a new superfamily of insect hormone binding proteins. Since the original *M. sexta* JHBP cDNA clone lacked a signal peptide and start codon (Lerro & Prestwich, 1990), we reexamined the cDNA library to obtain a full-length clone.

The newly isolated JHBP cDNA 2 differed by three amino acid substitutions among 12 nucleotide changes in the coding region from the first cDNA clone (JHBP cDNA 1). Since both cDNAs predict JHBPs having N-terminal sequences and amino acid composition consistent with those determined from direct analysis of the purified JHBP (Akamatsu et al., 1975; Kulcsár & Prestwich, 1988), the two cDNAs represent different isoforms of JHBP. These isoforms might be due to polymorphisms among individual insects, since the original cDNA library was constructed from the fat body of 15 insects. A Northern blot showed a 1.0-kb JHBP mRNA; this value replaces the previously reported size of 1.8 kb (Lerro & Prestwich, 1990), since the *Eco*RI/*Hin*cII fragment of JHBP cDNA 1, which lacks the 3′ noncoding region, was used as the probe in the experiments reported herein under the same stringency as the previous experiment.

Since hemolymph JHBP constitutes only 0.5–1% of total hemolymph protein (Prestwich et al., 1988), one aim of this

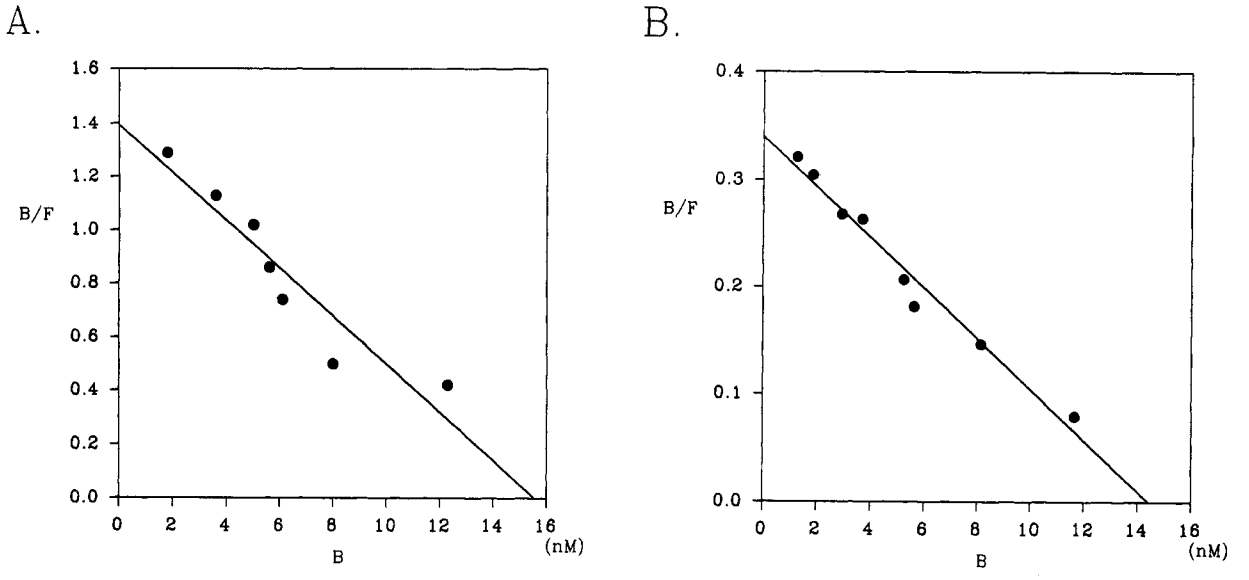

FIGURE 6: Binding of [^{3}H]JH to recombinant JHBP. The binding of JH I and II to purified rJHBP was analyzed by the DCC assay method. Scatchard analyses of specific binding data for JH I (panel A) and JH II (panel B) are shown. The abscissa and ordinate represent hormone bound [B (nM)] and bound/free (B/F), respectively. The K_D value was determined from the slope.

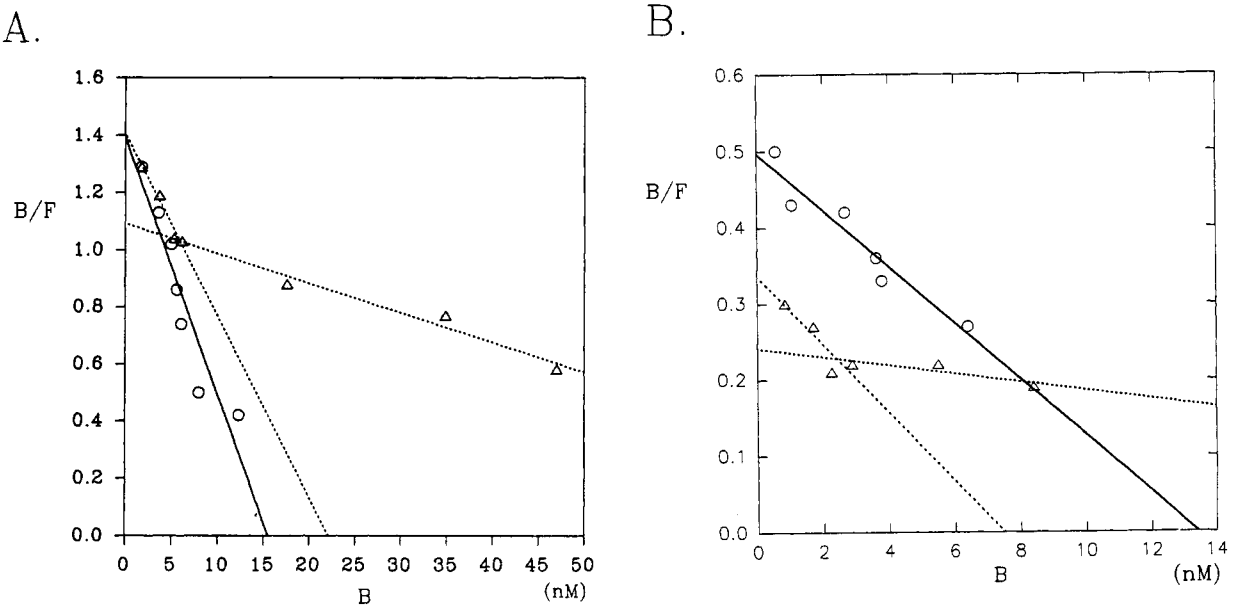

FIGURE 7: Effect of freezing and thawing on binding data for purified JHBP. Panel A: the K_D value for rJHBP freshly purified by DEAE column and gel filtration was 11 nM for JH I (circles). After prolonged storage at −20 °C, during which time partial thawing may have occurred, this sample of rJHBP showed the appearance of a lower affinity component (K_D = 96 nM) as seen on a biphasic Scatchard plot (triangles). Panel B: The degradation of rJHBP binding affinity can be accelerated by freeze–thaw cycles. Thus, the K_D values for rJHBP freshly purified by DEAE column was 27 nM for JH I (circles); after three freeze–thaw cycles, this sample showed a biphasic Scatchard plot (triangles). B (nM), bound JH I; F (nM), free JH I.

study was to obtain large quantities of homogeneous, active JHBP for physical analysis of the interaction of JH and JHBP as well as physiological studies to evaluate the significance of JHBP in vivo. Recombinant JHBP expressed in the baculovirus system was essentially indistinguishable from hemolymph JHBP with respect to size (SDS–PAGE), immunoreactivity, ligand binding affinity and specificity, chromatographic behavior, and N-terminal sequence. It was confirmed by N-terminal sequence analysis that rJHBP was secreted into the medium after correct posttranslational removal of the 18 amino acid signal sequence. The posttranslational modification and targeting of proteins is a major advantage of this eukaryotic expression system (Miller, 1988), which leads to a high level of expression and easier isolation of proteins from the culture medium. Immunoblot staining clearly demonstrated that virtually all rJHBP was present in the medium. Since the JHBP cDNA was inserted in the *Bgl*II site of the transfer vector, expression of rJHBP was under control of the strong p10 promoter. More than 10 mg of homogeneous rJHBP was obtained from 250 mL of culture medium of the recombinant virus infected Sf9 cells. Thus, we estimate that >50 mg/L rJHBP was present in the culture medium.

M. sexta high-affinity JHBPs have been known to consist of a heterogeneous population (Kramer et al., 1976; Akamatsu et al., 1975; Kulcsár & Prestwich, 1988). The 32-kDa JHBP in hemolymph was specifically labeled with [^{3}H]EHDA. JHBP expressed from the first clone (JHBP cDNA 1) as a β-galactosidase–JHBP fusion protein in *Escherichia coli* was specifically labeled with [^{3}H]EHDA (Lerro & Prestwich, 1990), demonstrating that it encoded a 32-kDa JHBP. The second clone, which had the entire open reading frame

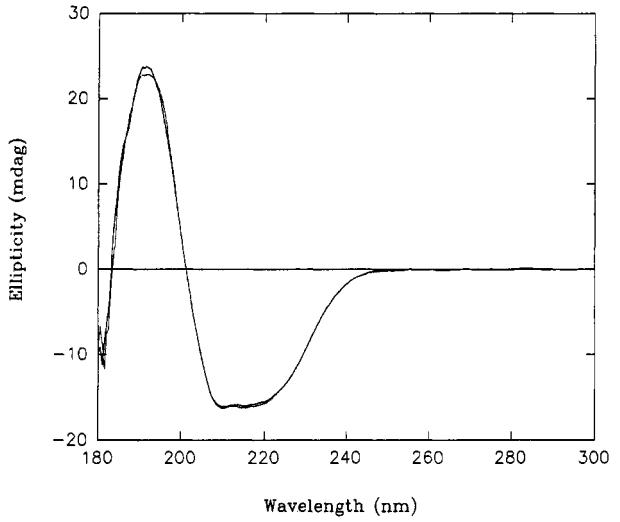

FIGURE 8: CD spectra of recombinant JHBP in the presence (···) or the absence (—) of JH II. The CD spectra for JHBP and JH–JHBP complex (14 μM) were measured in 10 mM phosphate buffer (pH 7.4) in the absence and presence of 25 μM JH II, respectively.

including the 18 amino acid signal peptide-like sequence (Figure 1; JHBP cDNA 2), was employed for the baculovirus expression studies. This clone, which had three amino acid substitutions (Ser^{46} to Pro^{46}, Ile^{67} to Val^{67}, and Ser^{188} to Gly^{188}), encodes a different 32-kDa JHBP isoform. This conclusion is based on three observations: (a) the rJHBP expressed in the baculovirus system was also specifically labeled with [^{3}H]-EHDA, (b) it had the same size as hemolymph 32-kDa JHBP on SDS–polyacrylamide gels, and (c) it had the same isoelectric point, p*I* 4.9, as the original 32-kDa JHBP (data not shown). Thus, these amino acid substitutions appeared not to be involved in JH recognition at the binding site.

Purification of hemolymph JHBP is commonly accomplished by column chromatography, isoelectric focusing, HPLC, or affinity chromatography (Goodman & Chang, 1985; Kramer et al., 1976). Specifically, separation by charge, with ion-exchange chromatogaphy and isoelectric focusing, gave efficient enrichment of JHBP (Prestwich & Atkinson, 1990; Prestwich, 1991). Thus, rJHBP has been purified to >95% homogeneity by ion-exchange and gel-filtration chromatography. The chromatographic mobility of rJHBP was the same as that of hemolymph JHBP. Furthermore, rJHBP had the same isoelectric point as hemolymph 32-kDa JHBP (p*I* 4.9) (data not shown). Purified rJHBP retained its binding affinity according to photoaffinity labeling experiments and its measured dissociation constants (K_D = 11 nM for JH I and K_D = 42 nM for JH II). The reported K_D value for crude *M. sexta* hemolymph JHBP was 80 nM for (10*R*,11*S*)-JH I using the DCC assay method (Prestwich et al., 1988). Partially-purified JHBP also had a similar value for racemic JH I [K_D = 93.5 nM (Goodman et al., 1976) and K_D = 88 nM (Koeppe et al., 1984)]. Other methods using hydroxyapatite or DEAE filters gave similar dissociation constants. The relative binding affinity for *M. sexta* JHBP decreases with increasing polarity in the order of JH 0 > JH I > JH II > JH III (Goodman et al., 1976). The dissociation constant for rJHBP was consistent with the previous report that the dissociation constant for JH II was a 3-fold higher value than that for JH I (Goodman et al., 1976). Purified rJHBP, however, had a higher affinity than the previously reported data on hemolymph JHBP. This could occur because of the absence of lower affinity binding proteins that might not be completely eliminated by partial purification of JHBP from hemolymph or because of the formation of lower affinity isoforms during the previously reported purification.

Experiments demonstrate that, during protein purification, freezing and thawing appear to cause the partial loss of the high-affinity binding site (16 nM for JH I) and the appearance of a lower affinity component (96 nM for JH I) as seen in a biphasic Scatchard plot (Figure 7). Thus, it is likely that previous binding studies on partially-purified JHBP from *M. sexta* hemolymph have characterized this lower affinity site. This lower affinity component was *not* due to a degradation product of JHBP, since there was no difference in the amount of degraded products (28 and 24 kDa on an SDS–polyacrylamide gel) among the various samples according to the immunoblot analysis (data not shown). The dissociation constant for rJHBP obtained in this study reflects the closest value for this single homogeneous isoform of the 32-kDa hemolymph JHBP. Thus, baculovirus-expressed rJHBP had chromatographic and biological characteristics consistent with those of hemolymph JHBP.

A minor immunoreactive band, which had slightly larger molecular size on SDS–polyacrylamide gels, was detected in the culture medium of the recombinant baculovirus-infected cells (Figure 4). Although hemolymph JHBP has been known to be a nonglycoprotein (Goodman et al., 1978), it had one consensus sequence for a glycosylation site (Asn^{66}-Val^{67}-Thr^{68}). The glycan blot, however, demonstrated that the minor immunoreactive band was not glycosylated JHBP. In addition, this minor protein was not labeled with [^{3}H]EHDA, suggesting the absence of JH-binding activity. Further characterization of this minor protein was therefore not pursued.

A model for JH–JHBP interaction has been proposed such that the lipophilic backbone of JH can be recognized by a hydrophobic region of JHBP, and the epoxide and ester moieties can interact with hydrophilic residues (Goodman & Chang, 1985). Hydrophobic β-sheet regions predicted by the Chou–Fasman algorithm included the sites covalently modified by a photoaffinity analog (Touhara & Prestwich, 1992). The CD spectra of rJHBP in the presence and absence of JH II (Figure 8) were indistinguishable, indicating that no measurable ligand-induced change in secondary structure occurs when JH binds to JHBP. In contrast, a conformational change of *Galleria mellonella* JHBP induced by JH binding was observed by measuring the shift of gel mobility and sedimentation coefficient (Wieczorek & Kochman, 1991). The absence of a conformational change upon JH binding is consistent with the hypothesis that JHBP functions to protect JH from nonspecific enzymatic degradation and nonspecific adsorption to lipid depots (Sanburg et al., 1975).

Preliminary results showed that infection of *Heliothis virescens* with this JHBP-encoding recombinant virus did not affect the phenotypic behavior in comparison with wild-type virus (B. C. Bonning, unpublished results). In contrast, it has been reported that infection by a JH esterase-encoding recombinant virus had significant biological effects (Hammock et al., 1990). Since JHBP exists in hemolymph of all life stages (Goodman, 1985), including eggs (K. Touhara, unpublished results) and hemolymph of black mutant *M. sexta* larvae (K. Touhara, K. A. Lerro, L. M. Riddiford, and G. D. Prestwich, unpublished results), this result was expected. Larval development is most likely regulated by the level of JH biosynthesis and JH degradation rather than the expression level of JHBP. Since virtually all of JH is present as a relatively stable JH–JHBP complex (Goodman & Chang, 1985), as is found for vertebrate steroid hormone–serum binding protein complexes (Westphal, 1978), it may be that the JH–JHBP

complex is necessary for recognition at the target cells. Further studies are in progress to evaluate the significance of JH–JHBP complex in this regard.

In summary, baculovirus-expressed rJHBP provided large amounts of homogeneous and binding-active JHBP for biochemical and structural studies. Expressed rJHBP can be utilized for physical and physiological studies to elucidate the mode of JH–JHBP interaction.

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