The Receptor Binding Conformation of Bombyxin Is Induced by Alanine(B15)†

George Fullbright and Erika E. Büllesbach*

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Avenue, P.O. Box 250509, Charleston, South Carolina 29425

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ABSTRACT: Bombyxin is an insect hormone with an insulin-like structure which affects the reduction of stored carbohydrates in the silkworm *Bombyx mori*. The receptor binding surface of bombyxin includes a trough on the interface between the B chain helix and the N-terminal A chain helix. Alanine(B15) is located on the edge of this feature, whereas the bottom is formed by hydrophobic core residues Ile(A2) and Leu(B14). Replacement of alanine(B15) with bulkier residues produces a negative steric effect on bombyxin receptor binding; α-aminobutyric acid reduced the affinity to 6.5%, valine to 1.1%, norvaline to 0.88%, and leucine to 0.05%. CD spectra of these analogues were indistinguishable from each other and identical to that of bombyxin. Changing the backbone structure by replacing alanine with glycine and α-aminoisobutyric acid resulted in analogues with activities of 3.7 and 1.4%, respectively, but also a disturbed structure as determined by CD spectroscopy. Replacement of other residues on the periphery of the trough, i.e., arginines at positions B12 and B16, also reduced the level of receptor binding but to a lesser extent than the replacement of alanine(B15). The level of receptor binding for citrulline(B12) bombyxin was 17% and for citrulline(B16) bombyxin was 45%. When it is considered that glycine(A1) is located on the edge of the same trough but across from Ala(B15) and is required for maintenance of the overall structure of bombyxin, it is proposed that the bombyxin receptor binding site forms a contiguous hydrophobic area consisting of residues Ile(A2), Leu(B14), and Ala(B15).

The insulin superfamily consists of a variety of functionally unrelated hormones and growth factors (*1*−*8*). The three-dimensional structures of several family members have been elucidated by either X-ray crystallography (*9*−*11*), NMR spectroscopy (*12*−*14*), or both. The similarity includes secondary structure elements and hydrophobic core formation so that the differences in biological activity must be due to the characteristics of accessible surface areas.

Bombyxin is a member of a family of hormones produced by four pairs of dorsal-medial neurosecretory cells of *Bombyx mori* brains, and is transported to the corpora allata where it is released into the hemolymph (*15*). Bombyxin levels vary during the life cycle of an insect, and they are low during the larval stage and high during the pupal stage (*16*), implying an effect in retrieval of stored energy, in sexual maturation, or in metamorphosis.

Bombyxin affects the release of stored energy by reducing major storage carbohydrates in the silkworm *B. mori*. The increasing level of trehalose hydrolysis in the hemolymph and glycogen breakdown in the fat body is paralleled by activation of trehalases and glycogen phosphorylases (*17*). The presence of bombyxin receptors on the fat body supports a role of bombyxin in regulating these functions (*18*). Bombyxin specific receptors are also abundant on the ovaries of *B. mori* (*18*) and on several insect ovarian cell lines (*18*−*20*), suggesting the potential involvement of bombyxin in ovarian development (*19*, *21*). This is supported by the observation that, in the pupal stage, bombyxin levels are significantly higher in the female than in the male (*16*).

We are interested in elucidating the active site of bombyxin, and in the study presented here, we investigated the role of alanine(B15) which is located in a prominent position at the rim of a putative binding pocket. By replacing alanine with other alkyl amino acids, we demonstrate that alanine in this position is required. All derivatives, except Gly(B15) bombyxin and Aib(B15) bombyxin, exhibited unchanged CD spectra but reduced the affinity for the receptor by up to 3 orders of magnitude. Since an alanine side chain has no functional group, the importance of alanine(B15) has to be solely steric, the provision of space and a restricted flexibility that is unique to alanine and not to other alkyl amino acids.

MATERIALS AND METHODS

Materials

Amino acid derivatives were purchased through Advanced Chemtech (Louisville, KY), Bachem Bioscience (King of Prussia, PA), and NovaBiochem (San Diego, CA). Chemicals for peptide synthesis were obtained from Perkin-Elmer Applied Biosystems. Solvents for peptide synthesis and chromatography were Burdick and Jackson high-purity solvents. The Sf9 cell line was obtained through the American Type Culture Collection and grown in SF-900 II SFM (Gibco-BRL) serum-free medium.

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* Corresponding author. Phone: (843) 792-9926. Fax: (843) 792-4322. E-mail: bullesee@musc.edu.

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Methods

Peptide Synthesis. Bombyxin II analogues were obtained by solid-phase peptide synthesis. Cit(B12) bombyxin and Cit-(B16) bombyxin were synthesized like bombyxin II as described previously (*22*). The A chain was synthesized by Fmoc chemistry, while the B chain was synthesized via Boc chemistry.

For bombyxin analogues with modifications at position B15, both chains were synthesized by Fmoc chemistry following by and large the procedure described by Maruama et al. (*23*). For the synthesis, the following side chain protecting groups were used: tertiary butyl ethers for serine, threonine, and tyrosine, tertiary butyl esters for aspartic acid and glutamic acid, triphenylmethyl groups for histidine, glutamine, and cysteines at positions A6, A11, and B22, tertiary butyl thioether for cysteine at position A20, the acetamidomethyl group for cysteines at positions A7 and B10, and 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl for arginine.

The Val(B15) B chain was synthesized on an automatic peptide synthesizer (ABI model 432A) on a 25 μmol scale. For the Gly(B15), Abu(B15), Aib(B15), Nva(B15), and Leu-(B15) B chains, the synthesis was started with 250 μmol of Fmoc-Asp(OBut) loaded on Wang resin. The sequence was assembled stepwise by conditioned Fmoc chemistry using an automatic peptide synthesizer (ABI 433A). After Arg-(B16) had been coupled, the Fmoc group was removed and the resin split into five equal portions. The first portion of the peptidyl resin was added to a reactor, and one Fmoc amino acid was reacted using the preprogrammed cycle for couplings to amide resin, which included the activation of the amino acid and its condensation to the amino group of the peptidyl resin and wash steps. The next portion of the peptidyl resin was added to the same reactor; a different Fmoc amino acid cartridge was used and the reaction performed as before. With the addition of the fifth and last portion, the mode of the synthesis was changed back to conditioned chemistry. The last of the five Fmoc amino acids was coupled; unreacted amino groups were acetylated, and subsequently, the 14 remaining residues were incorporated. To release all acid labile protecting groups, the peptidyl resin (50 mg/mL) was treated with a freshly prepared mixture of trifluoroacetic acid (TFA),[1] ethanedithiol, thioanisole, phenol, and water (10:0.25:0.5:0.75:0.5, v/v/v/w/v) for 2 h at room temperature (*24*). The resin was filtered off and the peptide precipitated with ether and centrifuged. The pellet was suspended in ether and collected by centrifugation three times. The peptide mixture was dried in vacuo and dissolved in 0.1% TFA in 84:16 (v/v) water/acetonitrile (~50 mL), and the chains were separated by preparative HPLC (Dynamax Rainin, C18, 41.4 mm × 25 cm) at a flow rate of 40 mL/min. The solvent system consisted of 0.1% TFA in water (A) and 0.1% TFA in 83.7% acetonitrile (B). The column was equilibrated at 20% B, and a linear gradient from 20 to 70% B was employed. The effluent was monitored at 280 nm. Fractions of 10 mL were collected and aliquots of 10 μL characterized by analytical HPLC (system 1). Fractions containing one compound were lyophilized, and fractions containing mixtures of two compounds were rechromatographed on Synchropak RP-P (10 mm × 250 mm) by loading 10−20 mg of peptide and eluting with a gradient from 20 to 50% B over the course of 30 min at a flow rate of 3 mL/min. Fractions of ~2 mL were manually collected, and 10 μL of each fraction was analyzed by analytical HPLC (system 1) for purity.

Yields: Abu(B15) B chain, 39.2 mg; Aib(B15) B chain, 22.5 mg; Gly(B15) B chain, 51.8 mg; Nva(B15) B chain, 40.5 mg; and Leu(B15) B chain, 46.4 mg.

Retention times of the B chains in system 1: bombyxin, 21.7 min; Gly(B15), 19.8 min; Abu(B15), 22.2 min; Aib-(B15), 24.4 min; Leu(B15), 24.9 min; Nva(B15), 23.5 min; and Val(B15), 22.5 min.

Chain Combination. The four cysteines of the A chain were protected as described in the literature (*23*). The intrachain disulfide bond was preformed by titration with iodine in 50% acetic acid. The tertiary butyl thioether in cysteine(A20) was removed and the sulfhydryl group activated as 2-pyridinesulfenyl, while cysteine in position A7 remained Acm-protected. The A chain was obtained in a pure form after HPLC purification on a Synchropak RP-P column (10 mm × 250 mm) using a linear gradient from 30 to 70% B over the course of 30 min.

Equimolar amounts of the A and B chains (2.2 μmol) were dissolved in 2 mL of pH 4.5 buffer [0.1 M acetic acid/NaOH (pH 4.5) containing 8 M guanidinium chloride] and reacted for 24 h at 37 °C. The mixture was separated on Sephadex G50-sf in 1 M acetic acid, and the product was lyophilized and further purified by HPLC on Synchropak RP-P using a linear gradient from 30 to 50% B over the course of 30 min. The main peak was collected and lyophilized (yield, 6−9.5 mg; 51.5−81.5%). The third disulfide bond was synthesized in 95% acetic acid using 25 equiv of iodine per Acm group (*25*). The reaction was performed for 1 h in the dark; excess iodine was reduced by addition of 1 M ascorbic acid in water and the polypeptide purified by gel filtration on Sephadex G25 in 1 M acetic acid followed by preparative HPLC on Synchropak RP-P using a linear gradient from 30 to 50% B over the course of 30 min (yield, 2.3−2.9 mg; 19.7−24.9%, based on A and B chains).

High-Performance Liquid Chromatography. Semipreparative HPLC was performed on a Waters dual-pump HPLC system fitted with a Rheodyne injector with a 2 mL sample loop. A Synchropak RP-P column (C_{18}, 10 mm × 250 mm) was used in combination with a solvent system consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in 83.7% acetonitrile (solvent B). The peptide (1−20 mg) was purified using linear gradients over the course of 30 min at a flow rate of 3 mL/min. Peptide containing fractions were identified by UV absorbance at 226 nm, manually collected, and lyophilized.

Analytical HPLC (system 1) was used to monitor purifications and to verify the homogeneity of bombyxin and bombyxin analogues and their synthetic intermediates. HPLC was performed on a Waters dual-pump HPLC system equipped with a Bakerbond wide-bored (C_{18}, 4.6 mm × 250 mm) column. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in 83.7% acetonitrile (solvent B). Samples (~10−30 μg) were injected and eluted

[1] Abbreviations: Abu, α-aminobutyric acid; Acm, acetamidomethyl; Aib, α-aminoisobutyric acid; Boc, *tert*-butyloxycarbonyl; But, *tert*-butyl; Cit, citrulline; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; Nva, norvaline; TFA, trifluoroacetic acid; Tris, tris(hydroxymethylamino)methane.

with a linear gradient from 20 to 60% B over the course of 30 min at a flow rate of 1 mL/min. The effluent was detected by UV absorbance at 220 nm.

Analytical HPLC (system 2) was used to analyze final products which were homogeneous in system 1. HPLC was performed on an ABI chromatograph (model 130A, Applied Biosystems) equipped with an Aquapore 300 column (C8, 2.1 mm × 30 mm). Approximately 1−2 μg of peptide was injected automatically and separated at a flow rate of 100 μL/min. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in 80% acetonitrile (solvent B). Usually, 60 min gradients from 25 to 45% B were employed, and the UV absorbance of the effluent was recorded at 230 nm.

Reduction and Chain Separation. The bombyxin analogues were dissolved in 1 mM HCl at a concentration of 1 μg/μL, and 10 μL was added to 10 μL of a freshly prepared 50 mM dithiothreitol solution in 0.2 M Tris-HCl (pH 8.6) containing 6 M guanidinium chloride. The reductions were performed for 60 min at 37 °C; the reaction mixtures were acidified with 5 μL of glacial acetic acid and separated by analytical HPLC (system 2).

Amino Acid Composition. Peptides were hydrolyzed in vapor phase 6 M HCl containing 0.1% phenol for 1 h at 150 °C, and the amino acids were modified with phenyl isothiocyanate and separated by HPLC (Pico·Tag system, Waters).

Sequence Analysis of the Gly(B15) B Chain. The B chain (20 pmol) was treated with pyroglutamate aminopeptidase (EC 3.4.19.3) (*26*) and the digest loaded into a Procise protein sequencer (Perkin-Elmer Applied Biosystems) connected to an in-line PTH analyzer. The corresponding phenylthiohydantoin of *S*-(acetamidomethyl)cysteine was identified at position B10, and free cysteine at position B22 appeared as a hole.

Protein determination was performed by UV spectroscopy using an Olis Cary-15 spectrophotometer conversion (On-Line Instrument Systems, Inc.). Bombyxin analogues (0.2−0.5 mg/mL) were dissolved in water or 10 mM HCl. The specific absorbance coefficient was calculated (*27*) for bombyxin II analogues which contain two tyrosines and one tryptophan (1.56 cm^{-2} mg^{-1}).

CD Spectroscopy. CD spectra were acquired using a Jasco J710 spectrapolarimeter at a resolution of 0.2 nm, with a bandwidth of 2 nm. Ten spectra were averaged. Bombyxin analogues were dissolved in 25 mM Tris-HCl (pH 7.5). For far-UV CD spectra, bombyxin analogues were used at a concentration of 0.15 mg/mL utilizing a cell with a path length of 0.1 cm. For near-UV CD spectra, bombyxin analogues were dissolved at a concentration of 0.375 mg/mL and measured in a cell with a path length of 1 cm. The molar ellipticity was calculated according to the literature (*28*) using a mean residual mass of 110.5 Da for bombyxin II.

Receptor Binding Assays. Crude membranes of Sf9 cells were prepared as previously described (*20*). Assays were performed in pH 7.5 buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 2.8 mM glucose, 25 μM $MgCl_2$, 1.5 mM $MnCl_2$, 1.6 mM $CaCl_2$ containing 1% bovine serum albumin, and 0.2 mM phenylmethanesulfonyl fluoride). [^{125}I]Bombyxin II (*20*) (100 000 cpm in 20 μL), various concentrations of bombyxin or bombyxin analogues (40 μL), and crude membranes of 400 000 cells (40 μL) were incubated for 1 h at room temperature. The suspension was diluted with 1 mL of ice-cold wash buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 1% bovine serum albumin, and 0.01% NaN_3) and centrifuged at 14 000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was discarded, and the tip of the vial was cut and the amount of radioactivity in the pellet counted in a γ-counter. Nonspecific binding was assessed in the presence of 380 nM bombyxin II (0.2 μg/assay). In a typical experiment, the total binding was 25% of the total amount of radioactivity added and the level of specific binding was 90−95% of the total binding. Assays in which the level of specific binding was less that 80% were discarded. Each analogue was measured in three independent experiments. Data were fitted as described by De Lean et al. (*29*) and evaluated and displayed using KaleidaGraph.

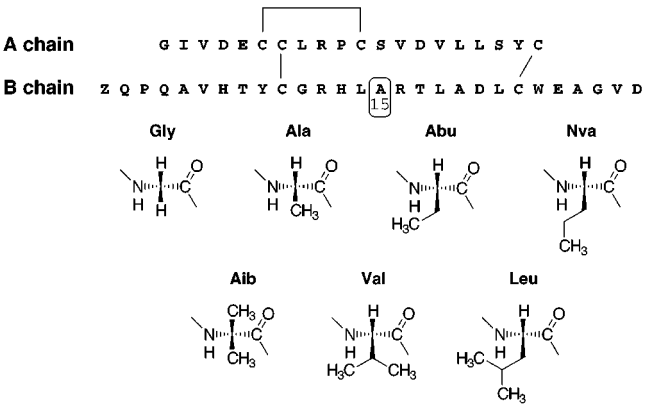

FIGURE 1: Primary structure of bombyxin II. The various amino acids introduced into position B15 are listed.

Molecular modeling was performed using the Sybyl65 software (Tripos Inc., St. Louis, MO). The NMR, averaged minimized structure of bombyxin II (*14*) (1bom), the NMR structure of insulin (*30*) (2hiu; structure **1**), and the X-ray structure of insulin (*10*) (4ins) were retrieved from the Brookhaven Data Bank. For display, the molecules were aligned on the B chain helix (B10−B22 of bombyxin = B7−B19 of insulin). Bombyxin II was coated with two layers of spc water in droplet form (660 water molecules were added). The amino acid at position B15 was mutated and the molecule energy minimized using the force field engine Tripos60 in combination with Gasteiger Huckel charges and the Powell minimization algorithm (100 iterations).

RESULTS AND DISCUSSION

Previously, we converted bombyxin II into a hybrid molecule that recognized the anti-relaxin antibody R6 by replacing four amino acid residues of bombyxin with the corresponding residues of relaxin (*22*). Later, we found that this relaxin−bombyxin hybrid did not bind to the bombyxin receptor and by subsequent single-amino acid replacements identified B15 as the most critical residue (unpublished results). Within all seven bombyxin families, position B15 is a conserved alanine (*31*−*34*), and when it is replaced with valine, the residue of porcine relaxin, the affinity for the bombyxin receptor was lost.

To further elucidate the role of Ala(B15), we incorporated alkyl groups with different sizes and shapes (Figure 1).

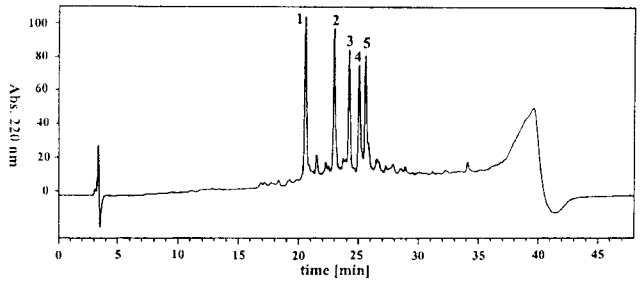

FIGURE 2: High-performance liquid chromatography of the mixture of deprotected synthetic B15-modified bombyxin B chains derived from the simultaneous synthesis: (1) Gly(B15), (2) Abu(B15), (3) Nva(B15), (4) Aib(B15), and (5) Leu(B15) bombyxin B chains. HPLC was performed on a Bakerbond wide-bored C18 column using a linear gradient from 20 to 60% B over the course of 30 min (solvent A being 0.1% trifluoroactetic acid in water and solvent B 0.1% TFA in 83.7% acetonitrile). The flow rate was 1 mL/min. UV absorbance was determined at 220 nm and displayed on a millivolt scale.

α-Aminobutyric acid (Abu) and α-aminovaleric acid (norvaline, Nva) were chosen to increase the length of the *n*-alkyl side chain of alanine by one and two methylene groups, respectively. Glycine was used to eliminate the methyl group, and branching of the side chain was introduced to increase the bulk. Branching was incorporated in the α- [Aib(B15) bombyxin], *β*- [Val(B15) bombyxin], and *γ*-position [Leu-(B15) bombyxin].

To increase the efficiency of the synthesis, five of the B chains were synthesized together. The identical region (B16−B28) was generated on a 0.25 mmol scale; the batch was split into five equal portions, and one portion was used to incorporate one of the variable residue. Thereafter, the peptides were pooled, and the synthesis was continued. The mixture was deprotected, and the different B chains were purified to homogeneity (Figure 2). The amino acid at position B15 was identified by amino acid analysis of the total hydrolysate. The structure was confirmed by sequence analysis of Gly(B15) bombyxin B chain. Each of the B chains carried an acetamidomethyl protecting group in the cysteine side chain B10 and a free thiol group at position B22. The monothiol B chain was reacted with an equimolar amount of thiol-activated A chain. The one intrachain and two interchain disulfide bonds were synthesized in individual chemical reactions as described previously (*22*, *25*, *35*). The purity of all bombyxin analogues was confirmed by HPLC in two independent systems and by amino acid analysis after total acid hydrolysis. The two-chain arrangement was verified by reduction and subsequent HPLC analysis.

Competitive binding of bombyxin analogues was assessed using crude membranes of Sf9 cells in combination with a ^{125}I-labeled bombyxin tracer (*20*) and compared with bombyxin II run in parallel. Figure 3 shows the relative potency of B15-modified bombyxins. While bombyxin bound to this receptor with an ED_{50} of 0.44 nM, all bombyxin analogues exhibited low affinities. Abu(B15) was the most potent analogue (6.8 nM, 6.5%), followed by Gly(B15) (11 nM, 2.1%), Aib(B15) (32 nM, 1.4%), Val(B15) (40 nM, 1.1%), and Nva(B15) (50 nM, 0.88%). The affinity dropped by another order of magnitude (900 nM, 0.05%) for Leu(B15) bombyxin which was as inactive as des-Gly(A1) bombyxin (*36*).

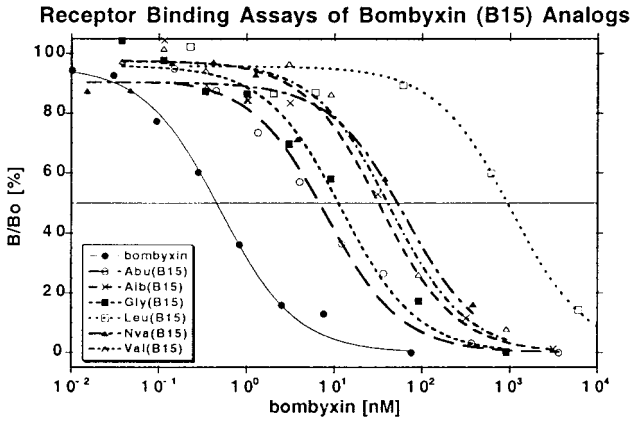

FIGURE 3: Receptor binding assays of B15-modified bombyxin analogues on crude Sf9 cell membranes. Each data point was determined in duplicate. At least three independent dose−response curves were acquired for each bombyxin analogue and compared with that of unmodified bombyxin run in parallel. One representative dose−response curve is shown for each analogue.

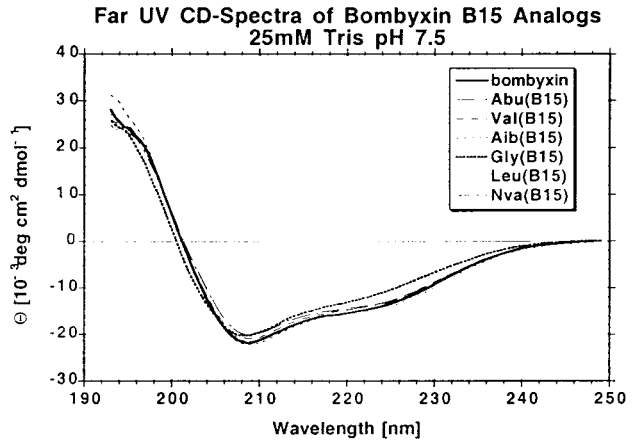

FIGURE 4: Far-UV CD spectra of bombyxin analogues modified at position B15. Bombyxin analogues at a concentration of 0.15 mg/mL were assessed in 25 mM Tris-HCl (pH 7.5) in a 0.1 cm cylindrical cell. Data were collected at a resolution of 0.2 nm and a bandwidth of 2 nm, and 10 spectra were averaged. The bold solid line is the spectrum of unmodified bombyxin, and the bold dotted line is the spectrum of Gly(B15) bombyxin.

CD spectroscopy indicated that bombyxin and all bombyxin analogues but glycine(B15) have identical CD spectra in the far UV (Figure 4). Alanine B15 is positioned on the second helical loop, and its side chain is exposed to solvent. Glycine is a known helix-breaking residue, while all other substituents promote helix formation (*37*). This overall effect is reflected in the CD spectra where in Gly(B15) bombyxin the intensity of the band at 222 nm is reduced and the crossover shifted to a lower wavelength.

Although the majority of the substituents do not disturb the α-helix, their structural preferences do vary; for instance, Aib forms only helices (*38*), and valine is a *β*-sheet promoter (*37*). The flexibilities of the backbone as reflected in the range of ϕ and ψ angles change with branching; i.e., Aib, the most restricted residue, has very narrow margins on the Ramachandran plot (*38*), and the range widens with the *β*-branched valine and widens even more with *γ*-branched or unbranched residues (*39*). Next to glycine, alanine has the highest backbone flexibility, and α-aminoisobutyric acid has the least. Near-UV CD spectroscopy (Figure 5) indicated that the spectra of both Gly(B15) and Aib(B15) bombyxin

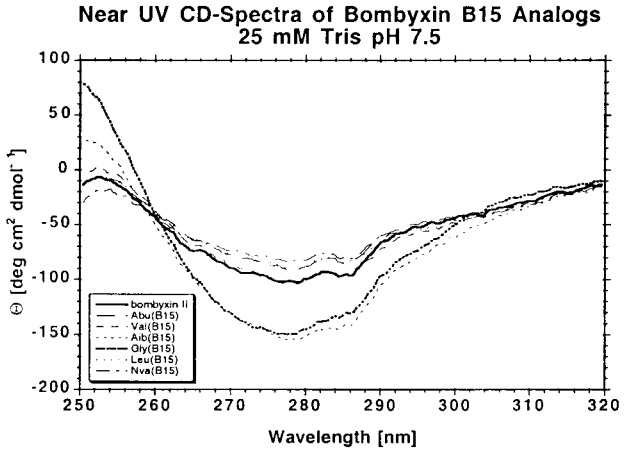

FIGURE 5: Near-UV CD spectra of bombyxin analogues modified at position B15. Bombyxin analogues at a concentration of 0.375 mg/mL were measured in 25 mM Tris-HCl (pH 7.5) in a 1 cm cylindrical cell. Data were collected at a resolution of 0.2 nm and a bandwidth of 2 nm, and 10 spectra were averaged. The bold solid line is the spectrum of unmodified bombyxin, and the bold dotted line is the spectrum of Gly(B15) bombyxin.

diverge from the bombyxin spectrum, implying a significant change in the environment of aromatic side chains.

Molecular modeling and energy minimization suggested that even the bulkiest alkyl group in position B15 can occupy the available space without disturbing the secondary structure and without interfering with other nearby side chains. The Connolly surface of the NMR-derived bombyxin structure (Figure 6, I) shows alanine(B15) on the edge of a trough which is about 2.5−3 Å deep and about 11 Å wide. Next to alanine is Cα of arginine(B12); across is residue Gly(A1), and the bottom of the cavity is formed by the side chains of Leu(B14) and Ile(A2) which are in hydrophobic contact. Although bulkier residues fit reasonably well, they disturb receptor contact. Their higher demand on space could cover other essential regions, or could restrict motions of the backbone or side chains such that bombyxin analogues with substitutions at position B15 cannot stabilize the active conformation. Alternatively, the steric hindrance could be inflicted by the receptor; however, there is too little known about the bombyxin receptor to evaluate such a possibility (*20*).

Initial molecular dynamic studies implied that in bombyxin the Arg(B12) side chain exhibits substantial flexibility and that this flexibility is restricted when larger groups are substituted for alanine. The replacement of either arginine with the isosteric and isoelectronic but uncharged citrulline lowered the affinity for the bombyxin receptor. The effect was more pronounced for Cit(B12) bombyxin (17%) then for Cit(B16) bombyxin (45%) (Figure 7) and might imply an ancillary role of Arg(B12) in receptor binding. Both analogues exhibited a far-UV CD spectrum identical to that of bombyxin, implying the integrity of the overall structure (Figure 8).

During our structure and function work on bombyxin, Gly-(A1) was identified as an essential structural component in

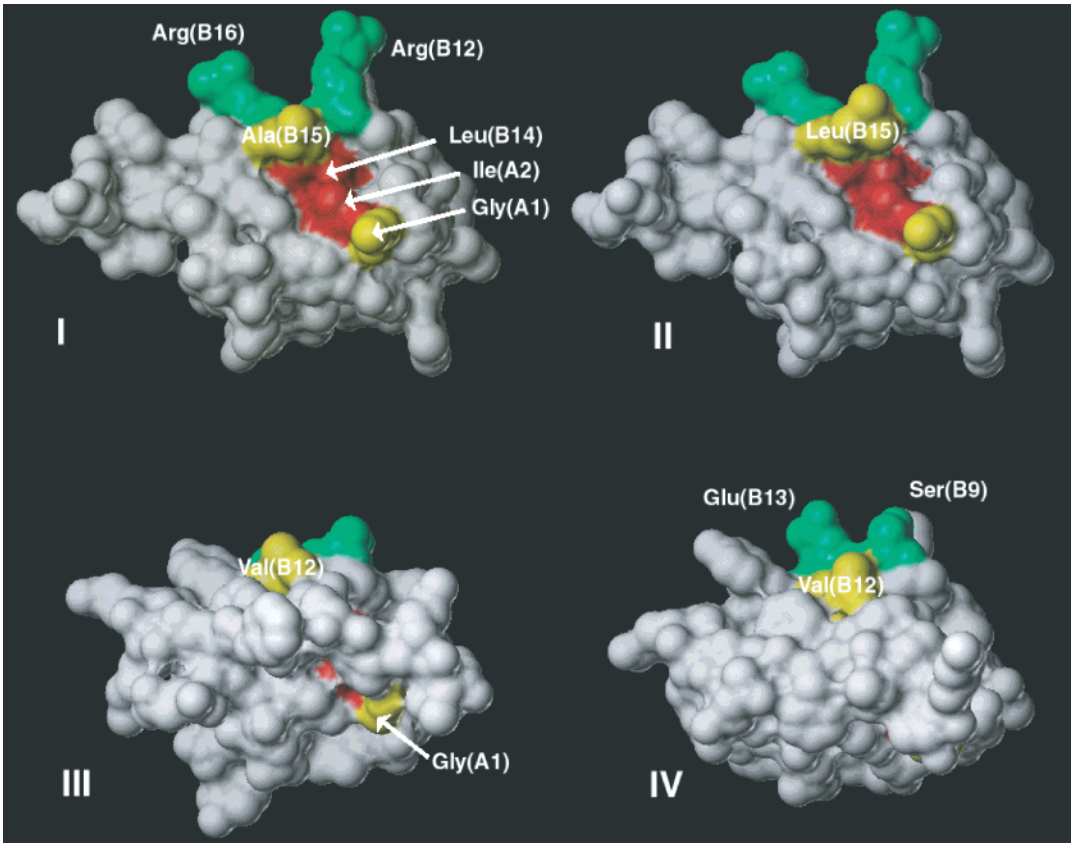

FIGURE 6: Proposed active site of bombyxin: (I) NMR structure of native bombyxin (1bom), (II) Leu(B15) bombyxin, (III) NMR structure of insulin (2hui), and (IV) crystal structure of insulin (4ins). Structures were aligned on the B chain helix (B10−B22 of bombyxin = B7−B19 of insulin). Bombyxin was coated with two layers of spc water in droplet form and energy minimized for 100 iterations using the force field engine Tripos60, Gasteiger Hückel charges, and the Powel algorithm: red, Leu(B14) and Ile(A2); yellow, B15 and Gly(A1); and green, Arg(B12) and Arg(B16). The corresponding residues in insulin are colored using the same code.

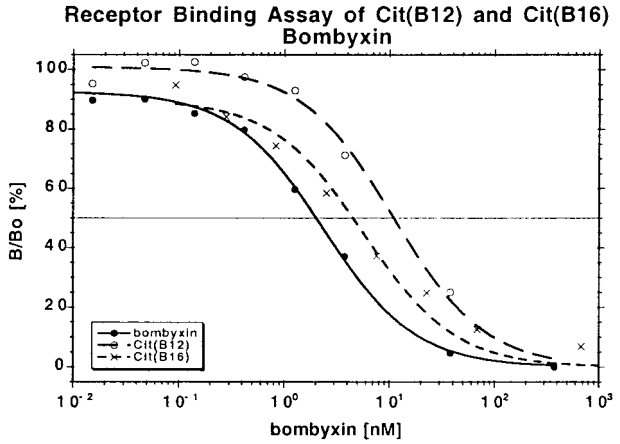

FIGURE 7: Receptor binding assays of Cit(B12) and Cit(B16) bombyxin on crude Sf9 cell membranes. Each data point was determined in duplicate. At least three independent dose−response curves were acquired for each analogue and compared with that of unmodified bombyxin run in parallel. One representative dose−response curve is shown for each analogue.

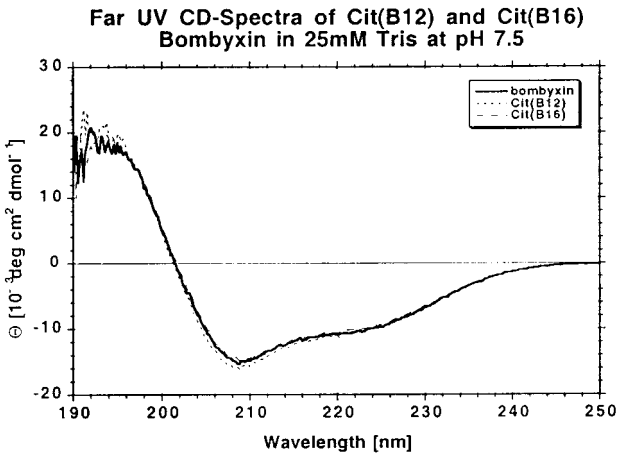

FIGURE 8: Far-UV CD spectra of Cit(B12) and Cit(B16) bombyxin run in parallel with bombyxin II. Bombyxin and bombyxin analogues were assessed at a concentration of 0.15 mg/mL in 25 mM Tris-HCl (pH 7.5) in a 0.1 cm cylindrical cell. Data were collected at a resolution of 0.2 nm and a bandwidth of 2 nm, and 10 spectra were averaged.

the bombyxin−receptor interaction. The removal of Gly(A1) reduced the affinity for the receptor by 3 orders of magnitude (*36*). On the basis of the fact that Ala(B15) and Gly(A1) are positioned relatively close to each other, the observed effects might be due to the cooperative action of the two residues. The bombyxin−receptor interaction site could involve the complete area between Gly(A1) and Ala(B15), including Ile(A2), and Leu(B14). Although both Ile(A2) and Leu(B14) are part of the hydrophobic core due to the openness of the bombyxin structure, they are accessible for receptor interaction. With the exception of Ala(B15), all other residues are identical in human insulin. In insulin, this area is covered by the C-terminus of the B chain (Figure 6, III and IV). This similarity in combination with the proposed flexibility of the solution structure of insulin (*30*) could explain the observed weak cross-reactivity between mammalian insulin and insect insulin-like receptors in the fruit fly *Drosophila melanogaster* (*40*) and the mosquito *Aedes aegypti* (*41*, *42*).

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