Structure−Activity Relationship of Crustacean Molt-Inhibiting Hormone from the Kuruma Prawn *Marsupenaeus japonicus*†

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ABSTRACT: In crustaceans, molt-inhibiting hormone (MIH) controls molting by suppressing the synthesis and/or secretion of molting hormone. In our previous study, which determined the solution structure of MIH by NMR, we hypothesized that the peptide's functional site spanned the region encompassing the N-terminal α-helix and a portion of the C-terminus, both of which are located sterically close to each other [Katayama et al. (2003) *J. Biol. Chem. 278*, 9620−9623]. To confirm this hypothesis, various mutants of MIH were prepared and their molt-inhibiting activities were assessed. All peptides mutated at the putative functional site exhibited circular dichroism spectra similar to the natural MIH, suggesting that the mutants retained their natural conformation regardless of the mutations. As expected, a majority of the mutants, except for Δ12 (a deletion mutant of Gly^{12}) and Δ75−77 (a deletion mutant of the last three residues of the C-terminus), were less active than the natural MIH. In particular, I72G exhibited no molt-inhibiting activity even at 200 nM, while N13A and S71Y exhibited low activity at the same concentration. In contrast, the natural and recombinant MIHs exhibited full inhibitory activity at 20 nM. All these results indicate that the functional site of MIH is located in the region containing the C-terminal ends of the N- and C-terminal α-helices, and that Asn^{13}, Ser^{71}, and Ile^{72} are especially significant for conferring molt-inhibiting activity. Furthermore, these findings agree with the results and the proposed hypothesis presented in previous studies on the structure−activity relationship of MIH and its related peptides.

Molting in crustaceans is promoted by ecdysteroids, collectively known as molting hormone. Synthesis of ecdysteroids by the Y-organ is suppressed by molt-inhibiting hormone (MIH)[1] (*1*). Up to now, MIHs have been isolated and characterized from many crustacean species and consist of 75−79 amino acid residues. MIHs have similar amino acid sequences to crustacean hyperglycemic hormones (CHHs); consequently, MIHs and CHHs have been grouped together to form a peptide family referred to as the CHH family (*2*). Six cysteine residues are conserved in all CHH family peptides that form three intramolecular disulfide bonds.

CHH family peptides are classified, depending on the primary structures of the mature hormones, as either type I or type II (*3*, *4*). All CHHs are classified as type I, are comprised of 72, or in an exception 73, amino acid residues, and have an amidated C-terminus. On the other hand, most MIHs are classified as type II and contain a nonamidated C-terminus; exceptions to the later include MIHs from the crayfish *Procambarus clarkii* (*5*) and the spiny lobster *Jasus lalandii* (*6*). Moreover, in type II peptides, when compared to type I peptides, a glycine residue has inserted between positions 11 and 12 of type I peptides, resulting in an extension of the sequence by one residue between the first and second cysteine residues than type I peptides.

To date, there have been no systematic studies on the structure−activity relationship of the CHH family peptides, although preliminary evidence has been obtained from previous studies. For example, the C-terminal amide moiety of CHH is significant for conferring hyperglycemic activity (*7*), while extension of the N-terminus by a few residues has no effect on the activities of the CHH family peptides (*7*−*11*). When compared with the amino acid sequences of CHHs, MIHs have low sequence similarity in the C-terminal 20 residues. On the basis of these observations, it was presumed that the C-terminal region and the glycine residue at position 12 in MIHs were responsible for conferring biological activity to the CHH-family peptides. However, there are some exceptions with respect to this structure−activity relationship; the MIH of the Mexican crayfish, *Procambarus bouvieri*, although classified as type I, exhibited MIH activity but not CHH activity (*12*), while the CHH

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[1] Abbreviations: CD, circular dichroism; CHH, crustacean hyperglycemic hormone; MALDI-TOF, matrix assisted laser desorption ionization-time-of-flight; MIH, molt-inhibiting hormone; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; RP-HPLC, reverse-phase high performance liquid chromatography.

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Table 1: Nucleotide Sequences of Primers Used in This Study

primer	sequence
F1	CGTCCCTACGCTTTTACACT
F2	CCATGGCAAGCTTCATAGACAACACCTG
R1	GACAGCAGATTAGTGGGAAT
R2	GAATTCTCACTGTCCGGCGTTCAGGA
Δ1−5-F	CATATGACCTGTAGGGGCGTGATGGG
Δ12-R	GTCACGATTCATCACGCCCCTACAGGT
Δ12-F	GGCGTGATGAATCGTGACATTTACAAG
N13A-R	AAATGTCACGAGCACCCATCAC
N13A-F	GTGATGGGTGCTCGTGACATTT
R14A-R	GTAAATGTCAGCATTACCCATC
R14A-F	GATGGGTAATGCTGACATTTAC
W69A-R	CAGGATGCTGATCGCCACTCTGAA
W69A-F	TTCAGAGTGGCGATCAGCATCCTG
S71Y-R	GTTCAGGATGTAGATCCACACT
S71Y-F	AGTGTGGATCTACATCCTGAAC
I72G-R	GGCGTTCAGGCCGCTGATCCAC
I72G-F	GTGGATCAGCGGCCTGAACGCC
E48A-R	CAGGAACCATGCGTTGTAGAAG
E48A-F	CTTCTACAACGCATGGTTCCTG
A56Y-R	CCTGTTGGCGTACTTCAAGCAA
A56Y-F	TTGCTTGAAGTACGCCAACAGG
Δ77-R	GAATTCTCATCCGGCGTTCAGGATGCTGA
Δ76−77-R	GAATTCTTAGGCGTTCAGGATGCTGATCC
Δ75−77-R	GAATTCTTAGTTCAGGATGCTGATCCACA

of the American lobster, *Homarus americanus*, classified as type I, exhibits both CHH and MIH activities (*13*).

Recently, the solution structure of the MIH from the kuruma prawn, *Marsupenaeus japonicus*, was determined by NMR and the tertiary structure of the CHH was modeled based on the MIH structure (*14*). MIH is composed of five α-helices and does not contain β-structure. By comparing the tertiary and surface structures of MIH and CHH, we hypothesized that the functional site of MIH is located in the region encompassing the N-terminal helix (α1 helix) and the C-terminus (*14*). In this study, we sought to confirm this hypothesis by examining the biological activities of various MIH analogues that contained mutations of the putative functional site.

MATERIALS AND METHODS

Construction of Expression Plasmids. Various oligonucleotide primers were designed based on the nucleotide sequence of the cDNA encoding MIH (Table 1).

For construction of an expression plasmid containing an insert encoding MIH lacking the first five amino acid residues (Δ1−5), PCR was conducted with a set of primers, Δ1−5-F and R2, and plasmid containing MIH cDNA to serve as template. The amplified PCR product was subcloned into the pCR2.1 plasmid (Invitrogen, USA) and the nucleotide sequence was checked. Subsequently, the Δ1−5 insert was released from the pCR2.1 plasmid by digestion with *Nde* I/*Eco*R I, and then ligated into the *Nde* I/*Eco*R I site of the pET-32a expression plasmid (Novagen, USA).

For construction of an expression plasmid with an insert encoding MIH lacking the terminal three amino acid residues (Δ75−77), PCR was conducted with a set of primers, F2 and Δ75−77-R, and a plasmid containing MIH cDNA to serve as template. The amplified PCR product was processed in the same way as in Δ1−5 except the restriction enzyme pair used was *Nco* I/*Eco*R I and the expression vector was pET-28b (Novagen).

For construction of expression plasmids containing an insert encoding single amino acid mutation of MIH (Δ12, N13A, R14A, E48A, A56Y, W69A, S71Y, and I72G), three rounds of PCR were conducted. First, two rounds of PCR were conducted with the primer sets, F1 and (mutant)-R, and (mutant)-F and R1. The PCR products were separated on a 2% agarose gel and extracted using the Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, USA) according to the manufacturer's instructions. The full-length mutants were PCR amplified using the primers, F2 and R2, both of the PCR products from the previous reaction and a thermal cycler program consisting of 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The amplified PCR products were subcloned into pCR2.1 plasmids. Expression plasmids were constructed in the same way as described for the construction of the Δ75−77 expression plasmid.

Expression of Mutant Peptides. Escherichia coli BL21-Codonplus(DE3)-RIL competent cells (Stratagene, USA) were transformed with each expression plasmid containing the insert and selected on LB plates containing kanamycin (30 μg/mL) or ampicillin (50 μg/mL). Bacterial cells from a single colony were grown at 37 °C overnight in LB medium containing the same antibiotic and then diluted 50-fold with the same medium. The diluted medium was incubated at 37 °C for 2 h, and then isopropyl-β-D-thiogalactoside (IPTG) was added to the culture to a final concentration of 1 mM. After incubation for another 2 h, bacterial cells were harvested by centrifugation and suspended in 1/20 culture volume of phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.5) containing 0.02% (w/v) lysozyme (Wako, Japan). The cells were disrupted by sonication and centrifuged at 10000*g* for 10 min. The supernatant was removed, and the pellet was suspended in PBS. Both the supernatant and the suspended insoluble material were subjected to 15% SDS−polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions.

Refolding Reaction and Purification of Mutant Peptides. The insoluble material obtained after cell breakage was solubilized in 6 M guanidine-HCl/10 mM Tris-HCl (pH 8.0). The resulting solution was applied to a Sep-Pak C_{18} Cartridge (Waters, USA), and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid (TFA). The eluate was lyophilized, and dissolved in 8 M urea in 0.5 M Tris-HCl (pH 8.0) at a concentration of 40 μg/mL. To this solution, five volumes of dilution buffer (0.5 M Tris-HCl (pH 8.0) containing 9.6% glycerol, 12% acetonitrile, and 2.4 mM reduced form of glutathione) chilled on ice were added. The resulting solution was stirred gently for 10 min at 4 °C. Then, an oxidized form of glutathione was added to this solution to a final concentration of 1 mM and stirred gently for 40 h at 4 °C. The resulting peptide was purified by reverse-phase (RP)-HPLC on a Shodex Asahipak ODP-50 column (10 × 250 mm, Showa Denko, Tokyo). Separation was performed with an initial 5-min isocratic step consisting of 20% acetonitrile in 0.05% TFA, followed by a 30-min linear gradient of 20−60% acetonitrile in 0.05% TFA at a flow rate of 3 mL/min. The elution was monitored by measuring the absorbance at 280 nm.

Expression and Purification of the Recombinant MIH. The recombinant MIH was expressed in *E. coli*, refolded, and purified according to the method described previously (*8*, *15*).

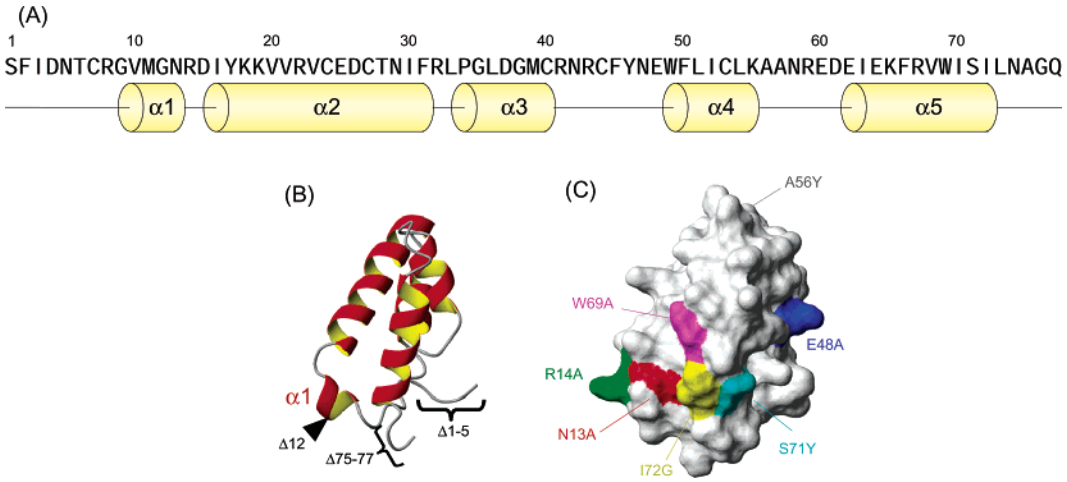

FIGURE 1: (A) Primary and secondary structures of the MIH from the prawn, *M. japonicus*. (B) Ribbon model of the MIH. Mutated sites of deletion mutants are indicated. (C) Surface structure of the MIH. Colors indicate the sites of various mutations.

Amino Acid Sequence Analysis. N-terminal amino acid sequences of peptides were analyzed on an Applied Biosystems model 476A protein sequencer in the pulsed-liquid mode.

Mass Spectral Analysis. Mass spectra were measured on a matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE STR, Applied Biosystems) with 3,5-dimethoxy-4-hydroxycinnamic acid or α-cyano-4-hydroxycinnamic acid as a matrix in the positive ion mode.

Circular Dichroism Spectral Analysis. Recombinant peptides (about 15 μg of each) were separately dissolved in 250 μL of PBS. Circular dichroism (CD) spectra were recorded from 200 to 260 nm on a JASCO J-720 spectropolarimeter at room temperature with a 1-mm path length cell.

Trypsin Digestion. Each 10 μg of the recombinant MIH and I72G was dissolved in 500 μL of 25 mM HEPES buffer (pH 6.8). To this solution, 1 μL of modified trypsin (Promega, USA) solution (1 mg/mL) was added, and the mixture was incubated at 37 °C for 20 h. The reaction was stopped by the addition of 1 μL of TFA. The tryptic digests were separated by RP-HPLC on a Capcell-Pak C_{18} column (2.0 × 250 mm, Shiseido, Japan) with a 30-min linear gradient of 0−40% acetonitrile in 0.05% TFA, and a 5-min linear gradient of 40−80% acetonitrile in 0.05% TFA at a flow rate of 0.2 mL/min.

Bioassay for Molt-Inhibiting Activity. Molt-inhibiting activity was assessed with an in vitro culture of the Y-organ of the crayfish *Procambarus clarkii* and an ecdysteroid radioimmunoassay (RIA) as reported previously (*16*, *17*).

RESULTS

Expression, Refolding Reaction, and Purification of Mutant Peptides. To identify MIH residues essential for activity, the amino acids to be mutated were selected according to the following two criteria; first, residues must reside in a region containing the α1 helix and C-terminus which was previously presumed to be the functional site (*14*); second, residues must have a side chain exposed at the molecular surface. Thus, seven mutants, N13A (a substitution mutant at position 13 from Asn to Ala), R14A, W69A, S71Y, I72G, Δ12 (a deletion mutant of Gly[12]), and Δ75−77 (a mutant truncated

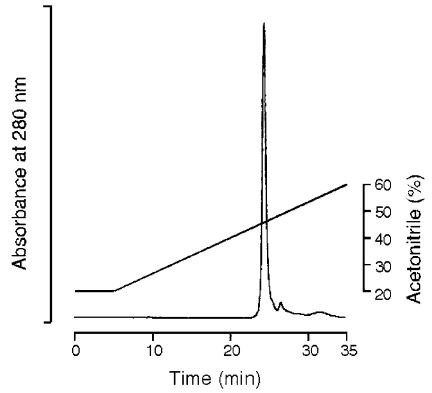

FIGURE 2: RP-HPLC elution profile of samples after refolding reaction of Δ1−5. Separation was performed on an Asahipak ODP-50 column (10 × 250 mm) with a 5-min hold at 20% acetonitrile in 0.05% TFA, and a 30-min linear gradient of 20−60% acetonitrile in 0.05% TFA at a flow rate of 3 mL/min. The concentration of acetonitrile is indicated by the solid line.

three residues at the C-terminus) were designed. In addition, E48A, A56Y, and Δ1−5 (a mutant truncated five residues at the N-terminus) were also designed to examine the influence of the putative nonfunctional position (Figure 1).

These mutant peptides were expressed in *E. coli*, which were then harvested, suspended in PBS, and sonicated. Soluble and insoluble fractions obtained by centrifugation were subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE), and heavily stained bands corresponding to recombinant mutant peptide candidates were detected only in the insoluble fractions of all mutants (data not shown). These results indicated that all of the mutants were expressed in *E. coli* cells as inclusion bodies. Therefore, refolding was considered to be necessary to obtain mutant peptides with the native conformation.

The RP-HPLC elution profile of Δ1−5 after refolding is shown in Figure 2. All mutants were obtained as a single peak on RP-HPLC after refolding. MALDI-TOF mass spectral and N-terminal amino acid sequence analyses of each peak material showed the expected single molecular ion peak (data not shown) and N-terminal sequence, respectively, except for Δ1−5. The N-terminal sequence analysis of Δ1−5 gave the sequence, Met/Thr-Thr/Xaa-Xaa/Arg-Arg/Gly-Gly/Val-, indicating that the N-terminal residue had been partially

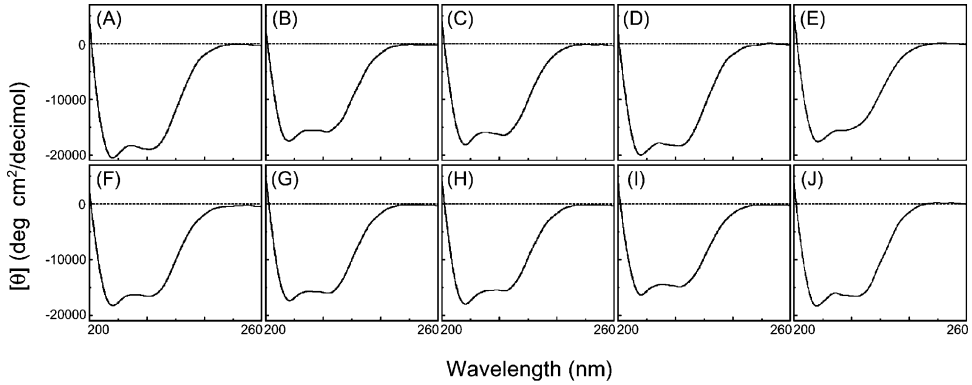

FIGURE 3: Circular dichroism spectra of Δ12 (A), N13A (B), R14A (C), Δ75−77 (D), W69A (E), I72G (F), Δ1−5 (G), E48A (H), A56Y (I), and the recombinant MIH (J). The spectrum of the recombinant MIH was redrawn from Katayama et al. (*15*).

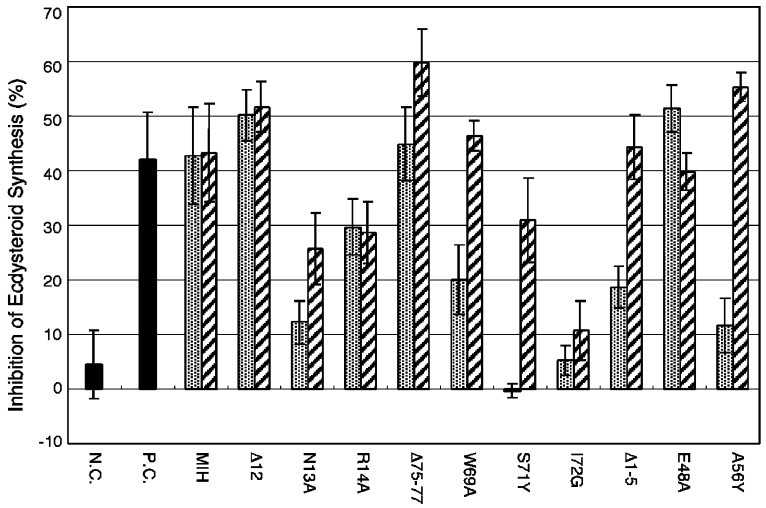

FIGURE 4: Molt-inhibiting activity of various mutants, controls, and the recombinant MIH. N.C., no sample was added as negative control; P.C., sinus gland extract of the crayfish *Procambarus clarkii* (0.5 sinus gland equivalent) as positive control. Mutants and the recombinant MIH were tested at 20 nM (shaded columns) and 200 nM (hatched columns). Results are expressed as the mean ± SE ($N = 3-9$).

removed by methionine aminopeptidase in *E. coli*. Since these two peptides could not be separated by HPLC, we used a mixture of the two Δ1−5 peptides in the following experiments. The N-terminal sequence of the other mutants, ASFIDNTXRG-, was identical to the natural peptide except for the N-terminal alanine residue that was derived from the expression vector.

Conformational Analysis. To confirm that the mutant peptides possessed the same conformation as the natural peptide, the CD spectra of the peptides in PBS were recorded at room temperature (Figure 3). Because the solubility of S71Y in PBS was very low, the CD spectrum of this mutant failed to be recorded. The CD spectra of the other mutants showed a pattern typical of α-helical proteins and were very similar to the spectra of the natural and recombinant molecules of MIH. Therefore, we concluded that the mutant peptides retained their natural conformation regardless of the mutations.

Biological Activity. The dose−response relationship of the recombinant MIH has been reported previously (*8*). Inhibition of ecdysteroid synthesis in vitro was observed at a concentration of 2 nM with maximum inhibition observed at 20 nM or higher. Consequently, we assessed the molt-inhibiting activity of each mutant peptide at two concentrations, 20 and 200 nM.

The results of the bioassays are shown in Figure 4. Three mutants, Δ12, Δ75−77, and E48A, exhibited high molt-inhibiting activity comparable to that of the recombinant MIH at 20 and 200 nM. W69A, A56Y, and Δ1−5 showed high activity at 200 nM, but significantly lower activity than the recombinant MIH at 20 nM, indicating that the mutations in these three peptides brought about a decrease in activity by approximately 1 order of magnitude. Neither N13A nor S71Y exhibited activity at 20 nM with low activity at 200 nM, indicating that N13A and S71Y are less active than the recombinant MIH by more than 1 order of magnitude. I72G showed no activity at the two concentrations tested, indicating that the activity of I72G is lower than that of the recombinant MIH by at least 2 orders of magnitude.

To confirm that the loss of activity in I72G was not due to improper folding, the arrangement of the disulfide bonds in I72G was analyzed. The recombinant MIH and I72G were separately digested with trypsin, and the digests were separated by RP-HPLC (Figure 5). The digests from these two peptides showed similar elution patterns except that peak 1 in MIH moved to peak 1′ in I72G. The peak 1 peptide was identified as the C-terminal fragment, $Val^{68}-Gln^{77}$, by MALDI-TOF mass spectral and amino acid sequence analyses. The peak 1′ peptide was found to be identical with peak 1 except that the Ile^{72} in peak 1 peptide was replaced

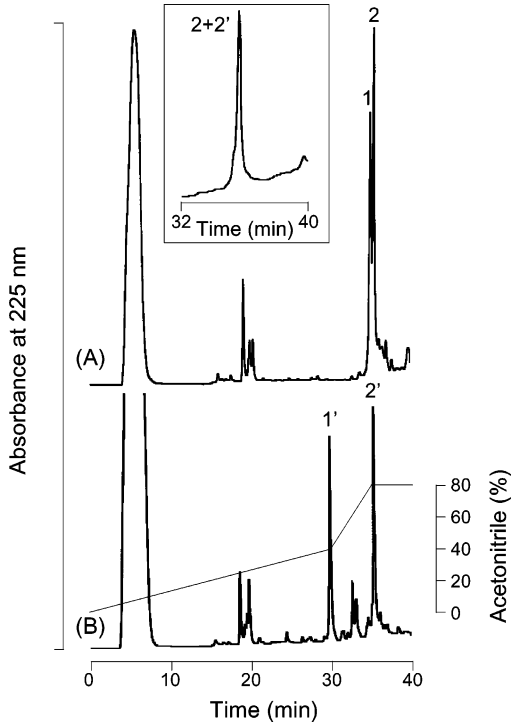

FIGURE 5: RP-HPLC elution profiles of tryptic digests of the recombinant MIH (A) and I72G (B). Separation was performed with a Capcell-pak C_{18} column (2.0 × 250 mm) using a 30-min linear gradient of 0−40% acetonitrile in 0.05% TFA, a 5-min linear gradient of 40−80% acetonitrile in 0.05% TFA, and a 5-min hold at 80% acetonitrile in 0.05% TFA at a flow rate of 0.2 mL/min. Inset, a coelution profile of nearly equal amounts of fragment-2 and fragment-2′ under the same conditions as described above.

by Gly^{72}. Mass spectral and amino acid sequence analyses of peaks 2 and 2′ indicated that they were the same peptide consisting of four peptide chains connected by three disulfide bonds. These two peptide fragments coeluted on RP-HPLC (Figure 5), strongly suggesting that the disulfide arrangement of I72G was the same as that of the recombinant MIH.

DISCUSSION

In this study, to confirm the hypothesis previously proposed that the functional site of MIH is located in the region encompassing the N-terminal helix (α1 helix) and the C-terminal part (*14*), various mutant molecules of MIH were prepared, and their molt-inhibiting activities were assessed. We selected residues exposed to the molecular surface for mutation, partly because the surface residues would directly interact with the receptor, and partly because conformational changes due to the mutations would be minimized. As expected, all the CD spectra of the mutants showed similar patterns to that of the recombinant MIH, indicating that the mutations did not cause significant conformational changes, although local conformational changes may have occurred.

N13A and R14A, mutated about the α1 helix, exhibited lower activities than the recombinant MIH, although Δ12, expected to lack the helical structure of the α1 helix due to the shortened peptide chain, retained activity comparable to that of the recombinant MIH. These results suggest that the amino acid residues located at the C-terminal end of the α1 helix, but not the helical structure itself, are significant for conferring molt-inhibiting activity, which is not inconceivable because some type I peptides in the CHH family show molt-inhibiting activity (*12*, *13*, *18*).

W69A, S71Y and I72G, mutated around the C-terminus, showed lower activities than the recombinant MIH, whereas Δ75−77, lacking the three C-terminal amino acid residues, was as active as the recombinant MIH. These results suggest that the residues located at the C-terminal side of the C-terminal helix (α5 helix), not including the terminal three residues, are significant for conferring molt-inhibiting activity. The C-termini of MIHs from many crustaceans show various structures; some peptides have a shortened, amidated C-terminus (*5*, *6*), while others possess an extended C-terminal sequence (*19*, *20*). Taken together, it is likely that a few residues at the C-terminus of MIH are not recognized by its receptor.

Among the many mutants, a notable decrease in molt-inhibiting activity was observed in I72G. The loss of activity in I72G cannot be attributed to improper folding and is, therefore, likely due to the one-residue mutation from Ile to Gly at position 72, because the disulfide bond arrangement of I72G was strongly suggested to be the same as that of the recombinant MIH. In addition, in the NOESY spectrum of the recombinant MIH, NOEs were not detected between the side chain of Ile^{72} and those of any other residues except for the typical NOEs inherent to α-helical structure (data not shown). Therefore, it is likely that I72G has the same conformation as the recombinant MIH, and that the Ile^{72} mutation caused a decrease in molt-inhibiting activity by means of reduced binding ability to its receptor but not through a conformational change in the peptide. The bulky, hydrophobic side chain of Ile^{72} may be essential for receptor binding. N13A and S71Y also exhibited very low activity. The changes in hydrophobicity in N13A and in the bulkiness and aromaticity in S71Y could affect their activities. All these results suggest that Ile^{72} is the most significant residue of the residues mutated in this study for conferring biological activity, and that Asn^{13} and Ser^{71}, both of which are sterically close to Ile^{72}, are also very important for activity, which is consistent with the previously proposed hypothesis (*14*).

On the other hand, it was unexpected that the A56Y mutation within the putative nonfunctional region of the peptide would exhibit lower molt-inhibiting activity than that of the recombinant MIH by approximately 1 order of magnitude. Two reasons are considered for this contradiction. First, a functionally important site may also be located around the Ala^{56} residue, and the decrease in activity may be because the aromatic ring of Tyr directly inhibits receptor−hormone binding; second, a slight conformational change which was not detectable in the CD spectrum may have been caused by this mutation, and as a result the binding affinity for the hormone receptor may have been reduced. In the NOESY spectrum of the recombinant MIH, NOEs were detected between the side chain of the Ala^{56} residue and those of the Val^{23} and the Ile^{30} residues (data not shown). Therefore, it is likely that Tyr^{56} in the A56Y mutation could not fit into the space due to its bulky side chain, causing weaker hydrophobic interactions.

The hypothesis was likewise contradicted with the decreased molt-inhibiting activity of the Δ1−5 mutation. In the crayfish *P. clarkii*, two CHHs, CHH-I and CHH-II, have been isolated from the sinus gland (*18*). These two CHHs have identical amino acid sequences except that CHH-II

has a D-Phe, which is L-Phe in CHH-I, at the third position from the N-terminus. These peptides have both CHH and MIH activities. However, CHH-I exhibits hyperglycemic activity as high as CHH-II, but displays lower molt-inhibiting activity than CHH-II by approximately 1 order of magnitude (*18*). These results, combined with our observations, suggest that the considerable structural change in the N-terminal region of CHH family peptides may not affect hyperglycemic activity but does cause a reduction in molt-inhibiting activity.

In CHHs, it has been reported that the C-terminal amide moiety is significant for conferring hyperglycemic activity (*7*, *11*). However, truncation of the three C-terminal amino acid residues did not affect molt-inhibiting activity in this study. All CHHs isolated from decapod crustacean species consist of 72 amino acid residues, while the MIH of *M. japonicus* consists of 77 amino acid residues (*21*). When these sequences are compared with each other, position 72 of the CHHs corresponds to position 73 in MIH. Therefore, some of the C-terminal residues in MIH may not be significant for conferring molt-inhibiting activity. Indeed, the crayfish MIH consisting of 75 amino acid residues lacks the two C-terminal residues (*5*).

Interestingly, Chan et al. proposed that the molecular evolution of the CHH family was similar to that of the vertebrate growth hormone (GH) family containing GH and prolactin (PRL) (*22*). GH and PRL are structurally very similar, but functionally completely different. However, this is also true for the CHH-family peptides but with some exceptions; CHHs of the lobster *H. americanus* and the crayfish *P. clarkii* have both CHH and MIH activities (*13*, *18*). Therefore, it is considered that molecular evolution of the CHH family is still on its way to attaining complete functional diversity. Since the C-terminal amide moiety of CHH is significant for conferring hyperglycemic activity (*7*), it is likely that the C-terminus of CHH is recognized strictly by its receptor. However, as described above, the C-terminus of MIH may not be important for receptor binding. Receptors may also change their structures in correspondence with the structural changes in hormones, and eventually may transduce the distinct signals leading to the different functions. Thus, the hormone/receptor system may also be moving toward increased diversity.

In conclusion, we have demonstrated that the functional site of crustacean MIH is located in the region containing the C-terminal side of the α1 helix and the C-terminal side of α5 helix. Residues, Asn^{13}, Ser^{71}, and Ile^{72} are especially significant residues for conferring molt-inhibiting activity. This is the first report of studies on the structure−activity relationship of the CHH family peptides. We hope that the present results will offer new insights into not only crustacean endocrinology, but also to the molecular evolution of the CHH family peptides.

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