

Further studies on the structural requirements for mast cell degranulating (MCD) peptide-mediated histamine release☆

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Abstract

Mast cell degranulating (MCD) peptide was modified in its two disulfide bridges and in the two arginine residues in order to measure the ability of these analogs to induce histamine release from mast cells in vitro. Analogs prepared were [Ala^{3,15}]MCD, [Ala^{5,19}]MCD, [Orn¹⁶]MCD, and [Orn^{7,16}]MCD. Their histamine-releasing activity was determined spectrofluorometrically with peritoneal mast cells. The monocyclic analogs in which the cysteine residues were replaced pairwise with alanine residues showed three- to ten-fold diminished histamine-releasing activity respectively, compared with the parent MCD peptide. Substantial increases in activity were observed where arginine residues were replaced by ornithines. The ornithine-mono substituted analog showed an almost six-fold increase and the ornithine-doubly substituted analog three-fold increase in histamine-releasing activity compared with the parent MCD peptide. The structural changes associated with these activities were followed by circular dichroism (CD) spectroscopy. Changes in the shape and ellipticity of the CD spectra reflected a role for the disulfide bonds and the two arginine residues in the overall conformation and biological activity of the molecule. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

Among the biologically active peptides isolated from bee venom, mast cell degranulating (MCD) peptide is of special interest [4]. It has activities related to allergy, which affects nearly 20% of the population [24], and it is therefore important to understand its relevance and role. This peptide has intriguing biological properties. It releases histamine at very low concentrations [12] and is one of the most potent natural histamine secretagogues known [18]. On the other hand this peptide has been found to inhibit mast cell degranulation at concentrations higher than those which release histamine [1,13]. This may be due to its interaction with the IgE molecule which is associated with allergic reactions. It has been postulated that disulfide exchange between IgE and MCD peptide in high doses on the mast

cell surface may inhibit histamine release, allowing the MCD peptide to act as an anti-allergic agent [2]. The primary structure of MCD peptide: H-Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-Asn-NH₂ indicates that it is strongly basic and stabilized in its structure by two disulfide bonds. These two properties are thought to be essential for its biological activities. Previous syntheses of MCD peptide with different methods were not quite satisfactory with respect to racemate free peptide or to histamine-releasing activity [3,14]. Structure-activity studies (SAR), therefore, with synthetic analogs necessary for understanding its mechanism of action, have not been undertaken. Consequently, in our initial studies with MCD peptide we established a fast and convenient solid phase protocol and obtained synthetic MCD peptide with the same biological activity as the natural molecule [5]. This opened the way to synthesizing the first MCD peptide analogs. Because the positive charges in the MCD peptide molecule are distributed throughout the peptide chain, we synthesized several truncated analogs to determine in which part of the molecule the basic amino acids are most crucial for histamine release [6–8]. These studies showed that the

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loss of histamine-releasing activity was greater when positive charges were removed from the C-end of the peptide. This part of the sequence, where the MCD peptide molecule has a helical part between His¹³ and Asn²² [15,16,23], proved to be critical for this activity [7,8]. These results, however, did not reveal the role of individual basic residues or the influence of the disulfide bonds in structure and activity.

In this continuing study with MCD peptide we made the first single amino acid substitutions in the peptide chain by replacing the most basic arginine residues with one and/or two ornithine residues. These substitutions at the same time modify the basicity and the length between the peptide backbone and the side chain guanidinium groups of the arginine residues. In addition, because of the histamine-releasing or non-releasing action of MCD peptide on mast cells, subtle changes in basicity, as introduced with the ornithine substitutions, might increase our understanding as to how to separate these two activities.

We also prepared the first monocyclic analogs of MCD peptide by replacing the cysteine residues of the peptide pairwise with alanine residues thus disrupting the backbone conformation of MCD peptide. Here we report the structural characteristics and biological activity of these analogs.

2. Methods

2.1. Peptide synthesis

The peptides were synthesized by solid phase on a p-methylbenzhydrylamine (MBHA) resin with standard Boc/benzyl methodology, and purified and analyzed as described with other MCD peptide analogs [5,6,7,8]. The N^α-Boc-N^δ-(2-chloro-Z) protecting groups were used for L-ornithine (Fluka).

2.2. Histamine assay

The histamine-releasing activity of the peptides was determined by a newly developed method in which histamine release and histamine assay were carried out on 96-well microplates [20]. Briefly, rat peritoneal mast cells (from 300–400 g Sprague-Dawley rats of mixed sexes and ages) were lavaged into modified Tyrode's buffer (without carbonate and bivalent cations) buffered with 15 mM HEPES (pH 7.2) and 0.1% bovine serum albumin (BSA) and isolated on an Accudenz gradient (Accurate Chemicals, Westbury, N. Y.) as previously described [21]. Prior to use the cells were counted by toluidine blue staining and their viability determined using Trypan blue exclusion. Each well containing 25 μ l peptide plus 100 μ l prewarmed cells ($1-2 \times 10^3$ per well) on prewarmed plates was treated for 20 min. at 37°C. Cells were washed with modified Tyrode's buffer (without bicarbonate) and centrifuged in a microplate centrifuge (ICE centrifuge, Needham, MA). Cell-associated

Table 1

Histamine releasing activity of MCD peptide analogs

Peptide	Histamine release ED ₅₀ (10 ⁻⁵ M) $\pm$ SEM [†]
[C ^{3,15,5,19}] MCD (standard)	1.1 $\pm$ 0.10 ⁺
[Ala ^{3,15} ,C ^{5,19}] MCD	3.1 $\pm$ 0.20 ⁺
[C ^{3,15} ,Ala ^{5,19}] MCD	10.3 $\pm$ 2.20 ⁺
[C ^{3,15} ,C(Acm) ^{5,19}] MCD*	4.2 $\pm$ 0.16 ⁺
[C ^{3,15,5,19} ,Orn ¹⁶] MCD	0.2 $\pm$ 0.25 ⁺
[C ^{3,15,5,19} ,Orn ^{7,16}] MCD	0.3 $\pm$ 0.40 ⁺

[†] ED₅₀ is the effective concentration for half-maximal response (n = 6).

* Data value from Ref. 6.

⁺ p < 0.05 vs potency of the analog.

histamine was released on the same plates with 100 μ l of 0.1% Triton X-100 for 20 min at 37°C followed by 100 μ l of 14% trichloroacetic acid and then kept in the cold over night. The plate contents were centrifuged and aliquots of the supernatants were assayed for histamine. Samples were done in replicate (n = 6) on microplates and the histamine release was detected spectrofluorometrically after addition of 2N NaOH and phthalaldehyde by a slightly modified protocol after Shore [22]. Statistical analysis of the correlation of the groups of data were evaluated by nonlinear regression done with Sigma Plot®.

P values less than < 0.05 were considered statistically significant. P values of all analogs were between < 0.0001–0.03.

2.3. Circular dichroism (CD) spectroscopy

CD spectra were recorded with a Jasco J-810 spectropolarimeter in a range between 190–260 nm in 0.1 cm path length cells at room temperature. The concentration of the samples determined by amino acid analysis was 100 μ M dissolved in 1.5 ml of water or 50% trifluoroethanol. Simple algorithm calculation [9] allowed the estimation of the helical content of the peptides.

3. Results

3.1. Histamine-releasing activity of monocyclic MCD peptide analogs

Two monocyclic analogs replacing the two cysteine residues in position 3,15 and 5,19 respectively, have been synthesized. As Table 1 shows, these alanine substitutions produce analogs with decreased histamine-releasing activity compared to that of MCD peptide. The activity in the analog which has the alanine residues in positions 5,19, was 10-fold less than that of MCD peptide. The alanine 3,15 analog is about 3-fold more active than its counterpart peptide and is almost equipotent in histamine-releasing activity with a previously synthesized monocyclic analog. In this analog

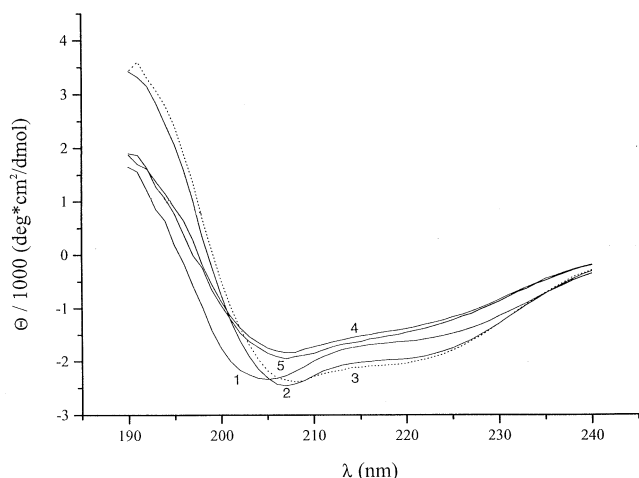


Fig. 1. Circular dichroism spectra of: MCD peptide 1; [Ala^{3,15}, C^{5,19}] MCD 2; [C^{3,15}, Ala^{5,19}] MCD 3; [C^{3,15,5,19}, Orn¹⁶] MCD 4; [C^{3,15,5,19}, Orn^{7,19}] MCD 5, in 50% TFE (100 μ M/1.5 ml, 25°C).

the two cysteine residues in positions 5 and 19 were protected by the acetamidomethyl (Acm) group [6]. Like the 3,15 alanine analog this analog shows almost the same loss of activity compared with the MCD peptide. However, the analog with two alanines at positions 5,19 and the same ring opening as the di(Acm) cysteine analog is almost three-fold weaker in histamine release compared with the latter peptide. Generally, all monocyclic analogs show a decrease in releasing activity within one order of magnitude compared with the parent MCD peptide.

3.2. Histamine-releasing activity of ornithine-substituted analogs of MCD peptide

Since nothing is known about the importance of individual amino acids in the MCD peptide sequence for biological activity, we decided to start with basic amino acids substitutions which are dominant in this sequence. For this purpose we synthesized two analogs modified in the two arginine residues, Arg⁷ and Arg¹⁶ of MCD peptide, by substituting one and two ornithine residues for these two arginines. As shown in Table 1, the histamine-releasing activity of the two analogs was significantly higher than that of the native peptide. The Orn¹⁶ analog was about six-fold more potent than MCD peptide. The bisubstituted Orn^{7,16} analog was one and a half-fold less potent than the Orn¹⁶ analog and showed a four-fold higher histamine-releasing activity than MCD peptide.

3.3. Circular dichroism (CD)

Fig. 1 shows the CD spectra of the four synthesized analogs along with those of MCD peptide taken in 50% trifluoroethanol (TFE). Although the MCD peptide has a nascent helix it does not display this structure in aqueous solution. In TFE the helix is stabilized through intrapeptide

hydrogen bonding and gains sufficient populations of α -helical secondary structure to be observed by CD. The CD spectra of the ornithine analogs show patterns similar to each other as do the monocyclic analogs. There are certain differences in shape and molar ellipticity between these spectra and that of the MCD peptide. Although they have different intensities they all yield a negative peak between 203–207 nm and a small deflection around 220 nm suggesting that the analogs form small α -helical structures. The calculated helix content for MCD peptide was 4.9%, for Ala^{3,15} 5.9%, for Ala^{5,19} 6.12%, for Orn¹⁶ 4.06%, and for Orn^{7,16} 4.3%. These values are in the same range as previously calculated for MCD peptide and analogs using the protein SSE-338 secondary structure estimation program from Jasco Spectroscopic Co. [6]. Using more sensitive instrumentation, however, and a VARSELEC algorithm the real α -helix content of MCD peptides was calculated to be as high as 30–35% [7].

4. Discussion

The first phase of structure-activity studies for MCD peptide included truncated analogs in order to determine segments of the sequence important for histamine-releasing activity. In addition it was shown that there is a correlation between helicity and biological activity of the synthesized analogs [7,8]. In this study we tried to address the role of the two disulfide bridges of MCD peptide and to assess the importance of the two most basic amino acids in its sequence, namely the arginine residues in position 7 and 16 of the molecule.

The CD spectra of the two monocyclic analogs compared with the CD of MCD peptide show visual differences as well as higher intensities in the $n-\pi^*$ transition at 222 nm and the $\pi-\pi^*$ transition at 203–208 nm, i.e. in helical content (Fig. 1). The CD spectra of these analogs, although not superimposable, show similar general features. In contrast with their CD spectra these two analogs show decreased histamine-releasing activity compared with MCD peptide and with each other.

The slight increase in helical content of the monocyclic analogs where alanine residues replace the cysteine residues is consistent with the known helix-promoting properties of alanine (10), especially in analog 3 with alanine located in the center of the helix. On the other hand the loss of the disulfide bonds results in a certain loss of conformational stability of the molecule. This flexibility may influence the backbone conformation and therefore the correct spatial orientation of the C-terminal helix to the N-terminus of MCD peptide, which is essential for full biological activity [7]. This effect and the loss of activity are more pronounced in the Alanine^{5,19} analog, which loses the stabilizing impact of the disulfide bond located at the core of the helix, whereas the disulfide bond in the Alanine^{3,15} analog is located more toward the N-terminus of the peptide and in

this respect is less affected. Interestingly, in the analog with protected cysteines in positions 5,19, the helix seems to be already folding in the right orientation. This analog shows a three-fold increase in activity compared with the Alanine^{5,19} analog.

Since little is known about the contribution of individual charged residues in the MCD peptide sequence, we initiated the substitution of Arg¹⁶ and Arg^{7,16} with ornithine residues. The high histamine-releasing potency of the two ornithine analogs, despite the loss of the two guanidinium groups is at first a somehow surprising result. Earlier studies, however, showed that analogs with chemically modified arginine residues, despite the loss of the positive charges in these positions, still retained MCD peptide-like histamine-releasing activity [11]. The CD spectra of the ornithine analogs in Fig. 1 show lower intensity and helical content than those of MCD peptide but have a shape similar to each other. These differences in helicity probably confer a conformational, i.e. a helix flexibility, effect. It has been experimentally shown that the closer a charge group of a basic amino acid is to the peptide backbone the greater is the helix-destabilizing effect. For this reason, shortening of the side chain of a basic amino acid decreases its helical propensity [19]. In other words, the helical propensity of ornithine is smaller than that of arginine. Contrary to the loss of helicity of the analogs, the increase in histamine release was substantial. It is reasonable to assume that the increase in activity is not the result of the base strength of the positive charges because ornithine is less basic than arginine. Since the net positive charge of the molecule remained intact and since at physiological pH all basic residues are similarly protonated, the activity differences must be of other origin. In these analogs ornithine residues replaced arginine, an amino acid having the lowest hydrophobicity values of all the essential amino acid residues [17]. Therefore, it appears that the change in activity is probably due to ornithine's nonspecific side chain hydrophobic interactions with the mast cell membrane receptor. The results show that these hydrophobic interactions are important for activity complementing those of an ionic nature. Such a hydrophobic-hydrophilic balance may also be one reason for the difference in activity between the mono-Orn¹⁶ and the analog with the two ornithine substitutions, one located in the C-terminal helical part (position 16) and one in the N-terminus of MCD peptide (position 7).

Also this is the first evidence that the length of the side chains at least in positions 7 and 16 of MCD peptide are of great importance for histamine release.

In conclusion two types of modifications intended to address two important features of MCD peptide were made. The monocyclic analogs led to a decrease in histamine release and indicate that the bicyclic nature of MCD peptide must be retained in order to test the hypothesis of MCD/IgE disulfide exchange interaction. The substitutions with ornithines resulted in novel, potent (superagonist) analogs. These results clearly show the impact of a single unnatural

amino acid on secondary structure, activity, and probably other tertiary structural parameters in the peptide chain. Such findings must be kept in mind for further SAR studies and for the design of antagonists of MCD peptide.

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