Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 1245–1247

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Structure–Activity Relationships for Mini Atrial Natriuretic Peptide by Proline-Scanning Mutagenesis and Shortening of Peptide Backbone

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Received 31 January 2002; accepted 27 February 2002

Abstract—MiniANP is a synthetic pentadecapeptide analogue of atrial natriuretic polypeptide (ANP). We have used the proline-scanning mutagenesis and the analogue peptides with shorter backbones to characterize the turn-like conformation at residue 6–9 and an extended structure of Gly^5-Gly^6 as the receptor-bound structure of miniANP. A docking study of miniANP at the binding site of the type A natriuretic peptide receptor (NPR-A) supported the deduced conformation in the receptor-bound structure.

Mini atrial natriuretic peptide [miniANP (**1**)],[1] designed by alanine-scanning mutagenesis and monovalent phage display, is a cyclic pentadecapeptide analogue of ANP (Fig. 1). MiniANP (**1**) exhibits ANP-related activities such as regulation of fluid balance, electrolyte balance, blood pressure, and the renin–angiotensin system.[2] MiniANP (**1**) possesses high biological activity (one seventh that of ANP) while the size of the polypeptide is about half of ANP. Alanine-scanning mutagenesis studies revealed that Phe^4, Met^8, and Ile^{11} are the most important determinants.[1] MiniANP (**1**) specifically binds to the type A natriuretic peptide receptor (NPR-A), which is a transmembrane protein composed of approximately 1060-residues. NPR-A consists of an extracellular ligand-binding domain, a single transmembrane domain, an intracellular kinase-homologous domain, and a guanylate cyclase domain. Binding of miniANP (**1**) to the extracellular domain of NPR-A activates synthesis of cGMP (cyclic guanosine 5′-monophosphate).[3] Most of the physiological activity may be attributed to this activation.

MiniANP (**1**) is the smallest of the ANP-related peptides, and residues 4–11 of miniANP (**1**) are equal to the region of 8–15 of ANP (Fig. 1) which is a critical portion of ANP.[4] Therefore, the roles of the residues of miniANP (**1**) will be applicable to those of ANP and be utilized for designing smaller non-peptide ligands. Our previous study suggested that a positive dihedral angle ϕ for Gly^5 is characteristic for the receptor-bound structure since the substitution of D-Ala for Gly^5 led to a more potent analogue than miniANP (**1**). It is worthy to note that the positive dihedral angle ϕ is typical for D-amino acids.[5] In addition, structural analysis by NMR and restrained molecular dynamics (rMD) suggested that the receptor-bound structure has a proximate pair formed by the side chains of Phe^4 and Ile^{11} with a turn-like conformation at residues 6–9.

Figure 1. Sequence alignment of miniANP (**1**) and ANP. The consensus sequence is indicated by boldfaced type.

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0960-894X/02/$ - see front matter
PII: S0960-894X(02)00148-8

Proline is frequently found at the $i+1$ position of a turn in many proteins.[6] Furthermore, proline-scanning mutagenesis has been used to map secondary structures in the Alzheimer's peptide β/A4 structure[7] or the α-lactalbumin molten globule[8] by breaking secondary structures. Thus, substitution of proline can be used for searching not only turn-like structures, but also another secondary structure such as extended conformations.

In this report, we have applied the proline-scanning mutagenesis to examine the putative receptor-bound structure of miniANP (**1**). In addition, the Gly-Gly-mimicking amino acids with shorter backbones were substituted for the Gly-Gly fragment to elucidate the conformation of the dipeptide fragment. The docking study of miniANP (**1**) to the binding site in the crystal structure of the extracellular domain of NPR-A[9] was also performed for understanding the structure–activity relationships of the miniANP analogues.

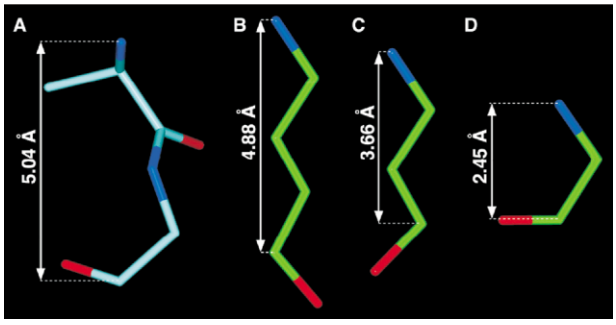

Figure 2. Comparison of the length between the amide nitrogen and the carbonyl carbon of D-Ala[5]-Gly[6] in [D-Ala[5]]miniANP (A), Gaba (B), β-Ala (C), and Gly (D).

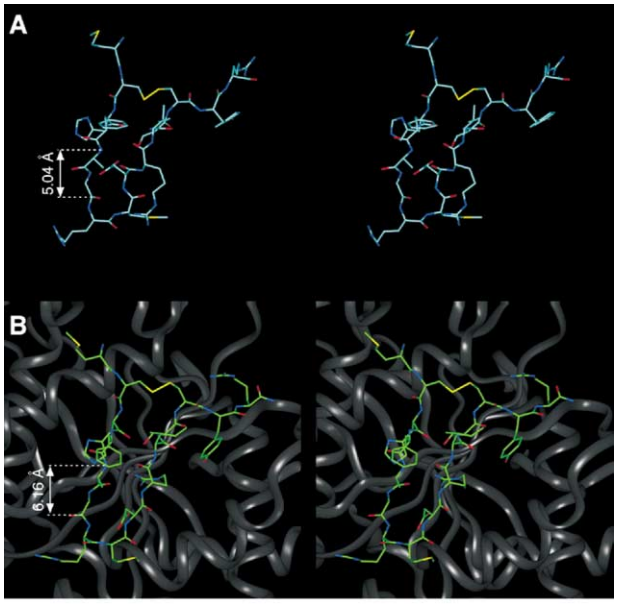

Figure 3. Stereo view of the solution structure (A) and the docked structure (B) of [D-Ala[5]]miniANP. The gray ribbon represents the backbone of the NPR-A structure. The length between the amide nitrogen in D-Ala[5] and the carbonyl carbon in Gly[6] is measured in both structures.

All peptides were synthesized using FastMoc chemistry by the standard solid-phase peptide synthesis protocol. Each residue from Gly[5] to Asp[9] was substituted with proline, that is [Pro[5]]miniANP (**2**), [Pro[6]]miniANP (**3**), [Pro[7]]miniANP (**4**), [Pro[8]]miniANP (**5**), and [Pro[9]]miniANP (**6**). Since the proline-substituted analogues lose the functional groups of the side chains, [Ala[7]]miniANP (**7**) and [Ala[8]]miniANP (**8**) were also synthesized to examine the influence of their side chains on the biological activity. These two residues are located at the center of the turn-like conformation in the putative receptor-bound structure.[5] It has been shown that the D-Ala[5]-Gly[6] dipeptide fragment of the solution structure of the [D-Ala[5]]miniANP analogue has a folded rather than an extended conformation as shown in Figures 2A and 3A. The backbone of the dipeptide fragment had a similar length to that of γ-aminobutyric acid (Gaba). Thus, des-Gly[6]-[Gaba[5]]miniANP (**9**), des-Gly[6]-[β-Ala[5]]miniANP (**10**), and des-Gly[6]-miniANP (**11**) were synthesized to examine the contribution of the folded conformation of the Gly[5]-Gly[6] fragment to the biological activity. The backbones of these analogues are shorter than that of miniANP (**1**) by one, two or three covalent bonds, respectively. Figure 2 represents the comparison of the length between the amide nitrogen and the carbonyl carbon of D-Ala[5]-Gly[6] in the solution structure of [D-Ala[5]]miniANP and the Gly-Gly-mimicking amino acids, which are shown as extended conformations. These analogues may afford the relationship between the length of the backbone and the biological activity.

Production of cGMP in CHO cells expressing NPR-A in response to the peptides was measured, as described in the previous report.[5] The activity of [Pro[7]]miniANP (**4**) was highest of the proline-substituted analogues (Table 1). While [Pro[7]]miniANP (**4**) was 7 times less active than miniANP, the activity of [Ala[7]]miniANP (**7**) was 6 times lower than that of [Pro[7]]miniANP (**4**). Thus, a bent conformation at the position should be important for the activity. The side chain of Arg[7] would be important to induce the turn-like conformation through the interaction with the receptor, since miniANP (**1**) shows no explicit conformation in the aqueous solution. For Met[8], the side chain appears to be important for the biological activity. However, since [Pro[8]]miniANP (**5**)

Table 1. Biological activity of miniANP (**1**) and analogue peptides

Peptide	Biological activity[a]	Relative biological activity
	EC_{50} (nmol)	EC_{50} analogue/EC_{50} miniANP
1	0.68±0.12	1.0
2	107.95±46.74	158.8
3	46.74±8.03	68.7
4	4.94±4.94	7.3
5	65.85±8.32	96.8
6	>10,000	>10,000
7	30.11±11.30	44.3
8	198.41±22.49	291.8
9	8.28±3.37	12.2
10	176.99±38.37	260.3
11	215.24±23.98	316.5

[a]The mean values of over two independent experiments.

showed about 3 times higher potency than [Ala^8]miniANP (**8**), the interaction of Met^8 with the receptor may be involved in the induction of the turn structure. Thus, the retained activity of [Pro^7]miniANP (**4**) suggests that Arg^7 of miniANP (**1**) corresponds to the $i+1$ position and the turn-like structure exists at residues 6–9 in the receptor-bound structure. The putative turn does not conflict with the presence of the bend at the eighth residue and is consistent with the structural analysis by NMR.[5]

As for the analogues with shorter backbones, miniANP (**1**) showed the most potent cGMP activity and the activity dropped with the shortening of the backbone (Table 1). Therefore, the shortening of the backbone of Gly^5-Gly^6 is unfavorable for binding to the receptor, even though the backbone of Gaba has a similar length with the D-Ala^5-Gly^6 fragment in the solution structure. Combining the lower activity of [Pro^5]miniANP (**2**) and [Pro^6]miniANP (**3**), the perturbation of the backbone of Gly^5-Gly^6 may bring disadvantage in the biological activity.

Docking of [D-Ala^5]miniANP into the ligand-binding site of NPR-A was carried out with the program Affinity (Accelrys, San Diego, CA, USA). It has been shown by the photo-affinity labeling study that ANP binds to near Met^{173} and His^{195} of NPR-A.[9] Thus, residues within 5 Å from the fragment Met^{173}-His^{195} were defined as a ligand-binding site. In the initial Monte Carlo docking simulation, the side chains of [D-Ala^5]-miniANP and the residues at the binding site were treated moveable and the other regions of the receptor were held rigid to generate 100 structures of [D-Ala^5]miniANP at the ligand-binding site. Then, the ligand and the ligand-binding region were further refined by simulated annealing without structural constraints.[10,11]

Figure 3B shows the docked structure with the lowest energy, in which the side chains of Arg^7, Arg^{10}, and Arg^{15} of [D-Ala^5]miniANP form hydrogen bonds with those of Gln^{149}, Asn^{184}, and Glu^{187} of NPR-A, respectively, and Asp^9 of [D-Ala^5]miniANP with Asn^{184} and Lys^{206} of NPR-A. The backbone of D-Ala^5-Gly^6 [corresponding to Gly^5-Gly^6 in miniANP (**1**)] is more extended than that in the solution structure. The length between the amide nitrogen in D-Ala^5 and the carbonyl carbon in Gly^6 became longer by 1.02 Å. This extended conformation gives an account for the reduced activity of the Gly^5-Gly^6 mimicking peptides. The fragment Asp^9-Arg^{10} also adopt an extended conformation. Contrary to [Pro^7]miniANP (**4**) and [Pro^8]miniANP (**5**), [Pro^9]miniANP (**6**) completely lost the activity. Thus, the substitution of Pro for Asp^9 may break the extended conformation and may consequently disturb the formation of the proximate Phe^4-Ile^{11} pair which may be an indispensable structural requirement for the activation of NPR-A.[5] These results indicate that the turn-like conformation at the residues 6–9 induced by the binding of the Arg^7 residue to the receptor and the extended structures at the residues 5–6 and 9–10 may play important roles for the formation of the receptor-bound structure of miniANP (**1**).

In this report, we have demonstrated that the proline-scanning mutagenesis can be applicable to the identification of the functional bend at Arg^7 on binding to the receptor and of the extended conformations at Gly^5-Gly^6 and Asp^9-Arg^{10}. The analogues having the amino acids with the shorter backbone structure supported the extended backbone of the Gly^5-Gly^6 region. The docking study provided the receptor-bound structure of miniANP (**1**), in which the turn structure and the extended conformations existed at the same regions. Thus, the characteristic conformations of residues at 5 through 9 lead to the proximity of Phe^4 and Ile^{11}, which are important for the expression of the biological activity.

Acknowledgements

This research was supported by the Special Coordination Fund for the Promotion of Science and Technology from The Science and Technology Agency of Japan. We thank Ms. Masako Sugiyama (Suntory Biomedical Research Limited) for measuring the cGMP activity of the analogue peptides.

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