

Structure–Activity Relationships of α_{S1} -Casomorphin Using AM1 Calculations and Molecular Dynamics Simulations

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This paper investigates the structure–activity relationships of α_{S1} -casomorphin (α_{S1} -CM) using AM1 calculations and molecular dynamics (MD) simulations. Previous studies have shown that this peptide has remarkable opioid actions, and not only has a high affinity toward all three subtypes (κ_1 – κ_3) of the κ -opioid sites, but also inhibits the proliferation of the T47D human breast cancer cell line. The systematic conformer search performed by the AM1 calculations is based on the torsional angles of the Val²-Pro³ (ω_2) and Phe⁴-Pro⁵ (ω_4) amide bonds. The AM1 results reveal that the α_{S1} -CM conformers strongly favor the cis/cis pair of the ω_2/ω_4 amide bonds in the minimized energy state. Furthermore, the picture of these stable conformers is found to be a strong interaction of the coulomb's force between two terminuses. MD simulations are performed to investigate the features of both the structural stability and pharmacological activity of α_{S1} -CM in aqueous solution. The simulation results reveal that the ω_2/ω_4 amide bonds favor the cis/cis status in the stable state. Furthermore, the pharmacophoric distance between two aromatic rings is found to be 5.0~5.4Å. The χ_1 rotamers of the Tyr and Phe residues show a preference for gauche (–) and trans, respectively. The side chain rotamers of α_{S1} -CM are competed to those of other opioid ligands with a known potency and selectivity for δ - and μ -opioid receptors. Finally, we address a likely κ pharmacophore model compared to the δ pharmacophore model.

Introduction

The opioid system modulates a range of physiological and behavioral processes, including those of pain perception, stress response, immune response, and neuroendocrine functions.¹ In addition to the pain relief effects of opioid peptides, a number of nonopioid actions have also been identified.² Researchers have reported that certain nonclassical opioid peptides affect many physiological processes, e.g., nociception, cell proliferation, and so forth. These peptides include hemorphins,^{3,4} exorphins,⁵ and casomorphins.^{6–9}

Opioid peptides and their receptors are found in primary human breast carcinomas,^{10,11} but are absent in normal breast tissue.^{12,13} Conventionally, the MCF-7 and T47D human breast cancer cell lines have been used as models to evaluate the effects of regulatory factors on cell growth and proliferation.^{14,15} MCF-7 cells possess a high affinity toward the μ - and κ -opioid receptor binding sites, but have a low affinity to the δ -opioid receptor.¹⁴ Meanwhile, T47D cells have a high affinity toward the κ - and δ -opioid receptor binding sites, but a low affinity to the μ -opioid receptor.¹⁵ Several studies have reported that opioid receptor agonists suppress cell proliferation in various systems, including breast cancer cells.^{14–16} However, other studies have suggested that opioid peptides have no effect in regulating the growth of tumor cells.^{17–19} Despite the conflicting findings of these studies, a number of opioid peptides are nevertheless known to suppress the growth of cancer cells.

Traditionally, the objective of research into opioid ligands and their receptors has been to develop strong analgesics with

no adverse side effects or potential for abuse. In contrast to μ opioid agonists, κ opioid agonists do not prompt respiratory depression, physical dependence, and strong addictive properties.^{20–22} Consequently, many researchers have investigated the feasibility of developing agonists for κ opioid receptor as peripheral analgesics.^{23–28} The presence of α_{S1} -casein in human milk has been identified.²⁹ A casein peptide fragment with five amino acids, i.e., α_{S1} -casomorphin (α_{S1} -CM) [H-Tyr-Val-Pro-Phe-Pro], has been isolated from the human α_{S1} -casein sequence [α_{S1} -casein-(158–162)].⁷ It has been shown that α_{S1} -CM binds with high affinity to all three subtypes (κ_1 – κ_3) of the κ opioid receptor. Furthermore, it has been reported that the α_{S1} -CM has a potent effect in inhibiting T47D cell proliferation.⁷ Therefore, it is desirable to develop a detailed understanding of the structure–activity relationships of α_{S1} -CM at the atomic level in order to facilitate the development of new therapeutic agents to suppress the proliferation of human breast cancer cells.

The α_{S1} -CM is a linear pentapeptide hampered with inherent flexibility, calculating its structural stability properties is challenging. Surprisingly, to the best of our knowledge, none of the experimental data depict the conformational characteristic for α_{S1} -CM. Therefore, the present study attempts to investigate the structure–activity relationships of α_{S1} -CM in vacuo and in aqueous solution using AM1 calculations and MD simulations, respectively. A likely κ pharmacophore model is addressed and compared to the δ pharmacophore model.³⁰

Theoretical Calculations

Structural Model. Figure 1 illustrates the geometric structure of α_{S1} -CM pentapeptide and labels its major parameters, including the rotamers of the backbone and side chain. The

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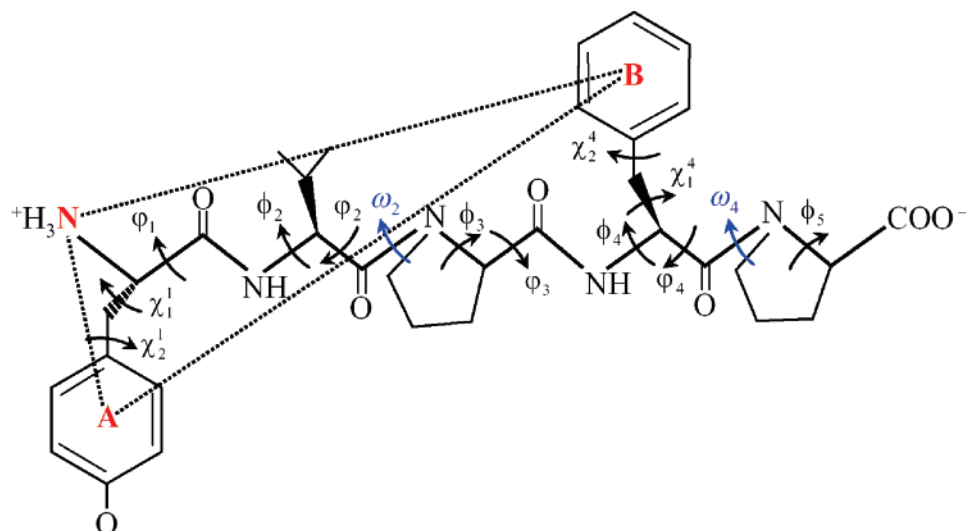


Figure 1. Schematic model of α_{S1} -casomorphin structure. The pharmacophoric elements (shown in red) include protonated nitrogen (N), centroid of phenolic group (A), and centroid of hydrophobic group (B). The key amide bonds (shown in blue) are ω_2 and ω_4 . The rotamers of the structural backbone and the side chain (Tyr¹ and Phe⁴) are marked.

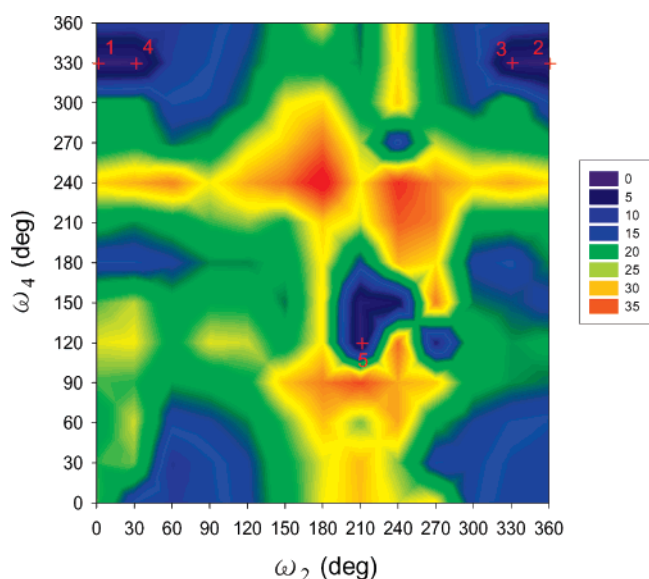


Figure 2. Conformer energy map of α_{S1} -casomorphin around the ω_2 and ω_4 torsional angles in vacuo using AM1 calculations. Torsional angles of ω_2 and ω_4 correspond to Val²-Pro³ and Phe⁴-Pro⁵ amide bonds, respectively. The red labels indicate the five lowest-energy conformers.

current analysis considers the torsional angles of the two amide bonds, i.e., ω_2 and ω_4 . It is generally accepted that the most important pharmacophoric parameters in opioid peptides include the distance from the protonated amine (N) to the Tyr aromatic ring (A), the distance from the protonated amine to a second hydrophobic center (B), and the distance between the Tyr ring and the hydrophobic center (AB).³¹

Systematic Conformer Search. The initial structure was constructed using the Spartan package from Wavefunction. The structural profile of α_{S1} -CM in vacuo ($\epsilon = 1$) was then examined

by conducting a systematic conformer analysis using Spartan program. Since the ω_2 and ω_4 amide bonds are significant for structural flexibility of α_{S1} -CM, the torsional angles of two bonds were selected as the criterion for calculating the conformer profiles. At the first step, the torsional angles of ω_2 and ω_4 were both specified as 0°, and other rotamers were free motions. The result shown that 2025 conformers were produced by Spartan/MM calculations. Only one of these conformers that with the lowest energy was selected then for calculating the ω_2/ω_4 energy map by AM1 calculations. Subsequently, the torsional angles of ω_2 and ω_4 were mutually rotated by increments of 30°, and repeat the first step. Finally, we can obtain the $13 \times 13 = 169$ conformers of minimized energy.

Molecular Dynamics Simulations. The current MD simulations were performed using the GROMACS simulation program (Version 3.2) with the GROMOS96 (ffG43a1) force field.^{32,33} In the simulations, the initial structure of α_{S1} -CM pentapeptide was specified in accordance with that of the lowest-energy conformer derived from the AM1 calculations. The simulations assumed the α_{S1} -CM to be surrounded by a total of 1205 water molecules. The simulation system was contained within a cubic box with sides of length ca. 34 Å and was subject to periodic boundary conditions. An energy minimization technique was then used to refine the initial structure of α_{S1} -CM in aqueous solution. The MD simulations assumed the atoms to have a random velocity consistent with a Maxwellian distribution. The positions and velocities of the atoms were integrated using the standard Verlet algorithm with a time step of $dt = 2$ fs. The system was initially equilibrated to a temperature of 300 K and a constant pressure of 1 atm using the Berendsen coupling scheme³⁴ with a time constant of 0.2. Following equilibration, the time evolutions of the quantities of interest were recorded over a duration of 20 ns. In the simulations, the particle-mesh-Ewald (PME) method³⁵ was applied to calculate the coulomb-

TABLE 1: Comparison of Five Lowest-Energy Conformers of α_{S1} -Casomorphin in Vacuo Using AM1 Calculations

no. of conformers		ω_2/ω_4	ΔE	r_{ee}	NA	NB	AB	χ_1^1	χ_1^4
1	cis/cis	360/330	0.000	3.09	3.896	9.022	12.086	33.25	32.89
2	cis/cis	0/330	0.001	3.10	3.894	9.023	12.078	33.16	32.86
3	cis/cis	330/330	0.610	3.11	3.928	8.897	12.257	35.02	30.87
4	cis/cis	30/330	5.986	2.86	4.137	7.552	9.392	57.72	40.88
5	trans/trans	210/150	16.017	3.04	4.182	9.279	11.899	56.65	-176.34

interactions and the nonbonded potentials were truncated using a cutoff radius of 14 Å. In our study, the formation of a hydrogen bond between an atomic pair was determined by a geometric criterion, i.e., a hydrogen bond forms if bond length (r_{HB}) $\leq$ 3.5 Å and the angle (θ_{HB}) $\leq$ 30° are fulfilled.

Results

Systematic Conformer Analysis In Vacuo. Figure 2 shows the conformational energy map of α_{S1} -CM calculated using the AM1 method. The five lowest-energy conformers of α_{S1} -CM are labeled as 1–5, respectively. It can be seen that the structural preferences of the ω_2/ω_4 pair are cis/cis > trans/trans > cis/trans > trans/cis. Moreover, the results show that the α_{S1} -CM conformers of lower minimized energy are restricted almost entirely to cis ($\sim 0^\circ$) and trans ($\sim \pm 180^\circ$) isomerization regarding the ω_2 and ω_4 torsional angles. Table 1 summarizes the structural features of conformers 1–5 in vacuo. For each conformer, the AM1 results include the end-to-end distance (r_{ee}), the pharmacophoric distances, i.e., **NA**, **NB**, and **AB**, and the key pharmacophoric rotamers, i.e., χ_1^1 and χ_1^4 . It is observed that r_{ee} is ca. 3 Å for each conformer, while **AB** has a minimum value of ca. 9 Å. Moreover, when the conformer transits from cis/cis to trans/trans, the χ_1^4 dihedral angle transits from 30–40° to trans.

Structural Dynamic Behavior in Aqueous Solution. Figure 3A plots the variation of the peptide potential energy of α_{S1} -CM as a function of time. The inset shows the time profile of the corresponding root-mean-square-deviation (rmsd) value of the C α atoms for α_{S1} -CM. It is observed that α_{S1} -CM becomes structurally stable at ca. 2.3 ns (simulation time), and subsequently remains stable until the end of the simulation. Figure 3B presents the variations of r_{ee} and the length of the unique intramolecular hydrogen bond binding the Val²-NH group and the Pro⁵ carboxyl ($r_{\text{Val:N} \cdots \text{Pro5:O}}$) over the course of the simulation. It can be seen that both r_{ee} and $r_{\text{Val:N} \cdots \text{Pro5:O}}$ stabilize at 2.3 ns, with average values of approximately 5.4 and 3.05 Å, respectively. Figure 3C presents the variations of the ω_2 and ω_4 torsional angles over the course of the simulation. As shown, the angles exhibit a cis conformation over the full 20 ns of the simulation.

Backbone and Side chain Angles. The dihedral angles of the backbone and side chain of α_{S1} -CM in aqueous solution were analyzed as a function of time. The corresponding results are presented in Figures 4 and 5, respectively. In Figure 4, the horizontal lines indicate the corresponding values computed by the AM1 method for conformer 1 (Table 1). It is noted that the φ angle of Tyr¹ transits from gauche (–) ($\sim -60^\circ$) in vacuo to ca. $+138^\circ$ in aqueous solution. This transition causes the intramolecular hydrogen bond (HB) observed between the two terminuses of α_{S1} -CM in vacuo to disappear. Figure 5 compares the torsional angles of the side chain of α_{S1} -CM in aqueous solution to those of conformer 1 in vacuo. It is apparent that the key bioactive rotamers of the side chain, i.e., χ_1^1 and χ_1^4 , vary significantly from the values obtained in vacuo. Furthermore, it is evident that these angles favor the gauche (–) and trans rotamers in the stable structure, respectively. Table 2 summarizes the bioactive structural requirements for Tyr¹ and Phe^{3(4)}} residues of opioid ligands with high affinity to the δ -, μ -, and κ -opioid receptor binding sites. It is observed that these key orientations have different preferences for those opioid receptors.

κ Pharmacophore Model. Figure 6 illustrates the different pharmacophore model between the κ - and δ -selective opioid ligands. The principal difference between the two models is the distance **AB**. In the stable α_{S1} -CM structure, characterized by

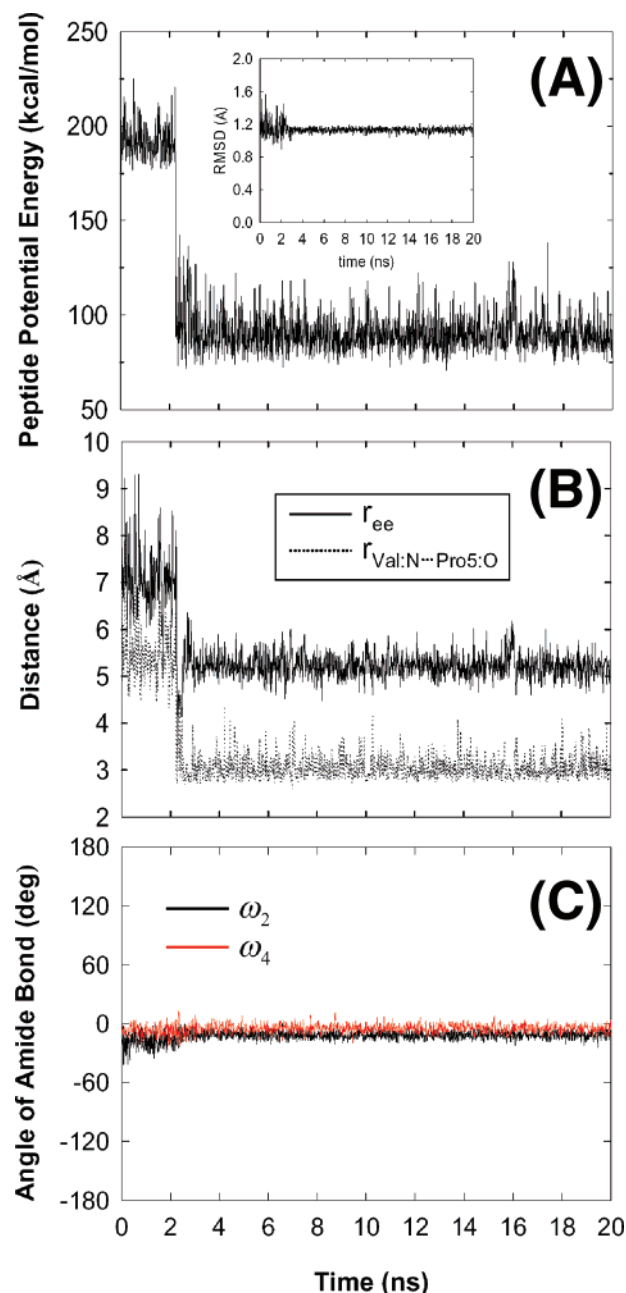


Figure 3. Molecular dynamics simulation results for dynamic behavior of α_{S1} -CM in aqueous solution. (A) Variation of peptide potential energy of α_{S1} -CM over time. Inset plots time profile of corresponding RMSD value of C α atoms. The structure attains structural stability at ca. 2.3 ns (simulation time) and subsequently remain stable until the end of the simulation. (B) Variations of end-to-end distance, r_{ee} , (solid line) and length of hydrogen bond, $r_{\text{Val:N} \cdots \text{Pro5:O}}$, (dotted line) over time. The r_{ee} and $r_{\text{Val:N} \cdots \text{Pro5:O}}$ distances both stabilize after 2.3 ns, attaining average values of approximately 5.4 and 3.05 Å, respectively. (C) Time evolution of torsional angles of amide bonds (ω_2 and ω_4). It is clear known that ω_2 and ω_4 exhibit cis rotamer preference over the full 20 ns of the simulation.

a high affinity to all three subtypes of the κ opioid receptor, the distance **AB** has a value of approximately 5.0–5.4 Å, while in the δ pharmacophore model, the distance **AB** is approximately 5.7–8.3 Å.

Discussion

In addition to the pain relief effects of classical opioid peptides, a number of nonopioid actions have also been

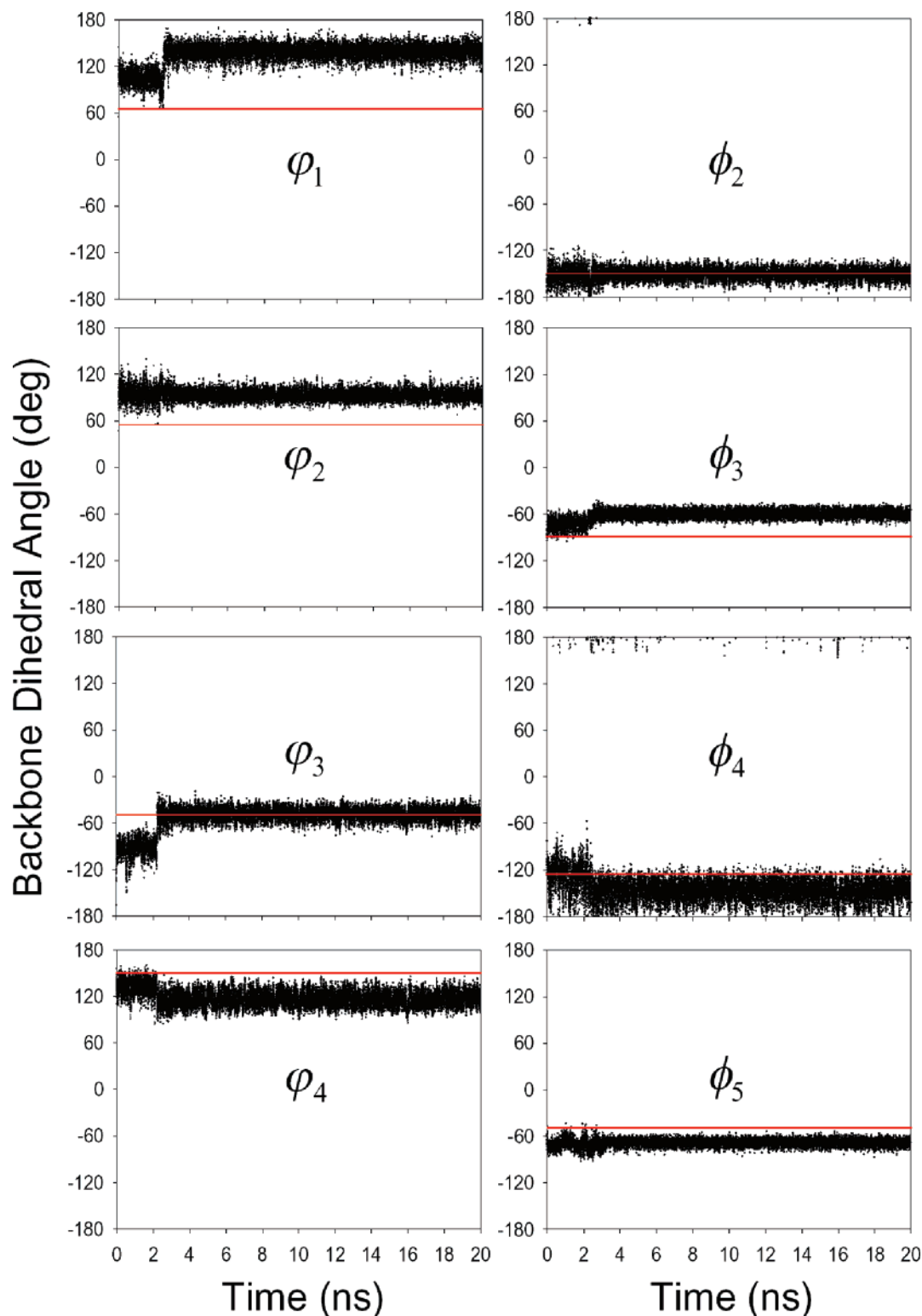


Figure 4. Time evolutions of backbone distributions expressed in terms of ϕ and φ angles for selected residues. Horizontal lines indicate corresponding values of conformer **1** as computed by AM1 method. The φ angle of Tyr¹ transits from gauche (−) in vacuo to ca. +138° in aqueous solution causing the intramolecular hydrogen bond observed between the two terminuses of α_{S1} -CM in vacuo to disappear.

identified.² The α_{S1} -CM pentapeptide has been isolated from the human α_{S1} -casein sequence.⁷ It has been shown that α_{S1} -CM not only binds with high affinity to κ -opioid receptor, but also inhibits the proliferation of T47D human breast cancer cells. Therefore, developing an improved understanding of the bioactive structure of α_{S1} -CM at the atomic level is fundamental in developing new therapeutic agents for regulating breast cancer cells.

In general, the cis/trans structural features of the amide bonds for the peptides and proteins are quite significant, not only affecting the conformations of the backbone, but also the steric arrangements of the side chain. Hence, the structural features of the amide bonds have a fundamental effect on the bioactive behavior of α_{S1} -CM, including the binding of the opioid ligands to the receptor sites. Since α_{S1} -CM has two primary proline residues in its sequence, the systematic structural search

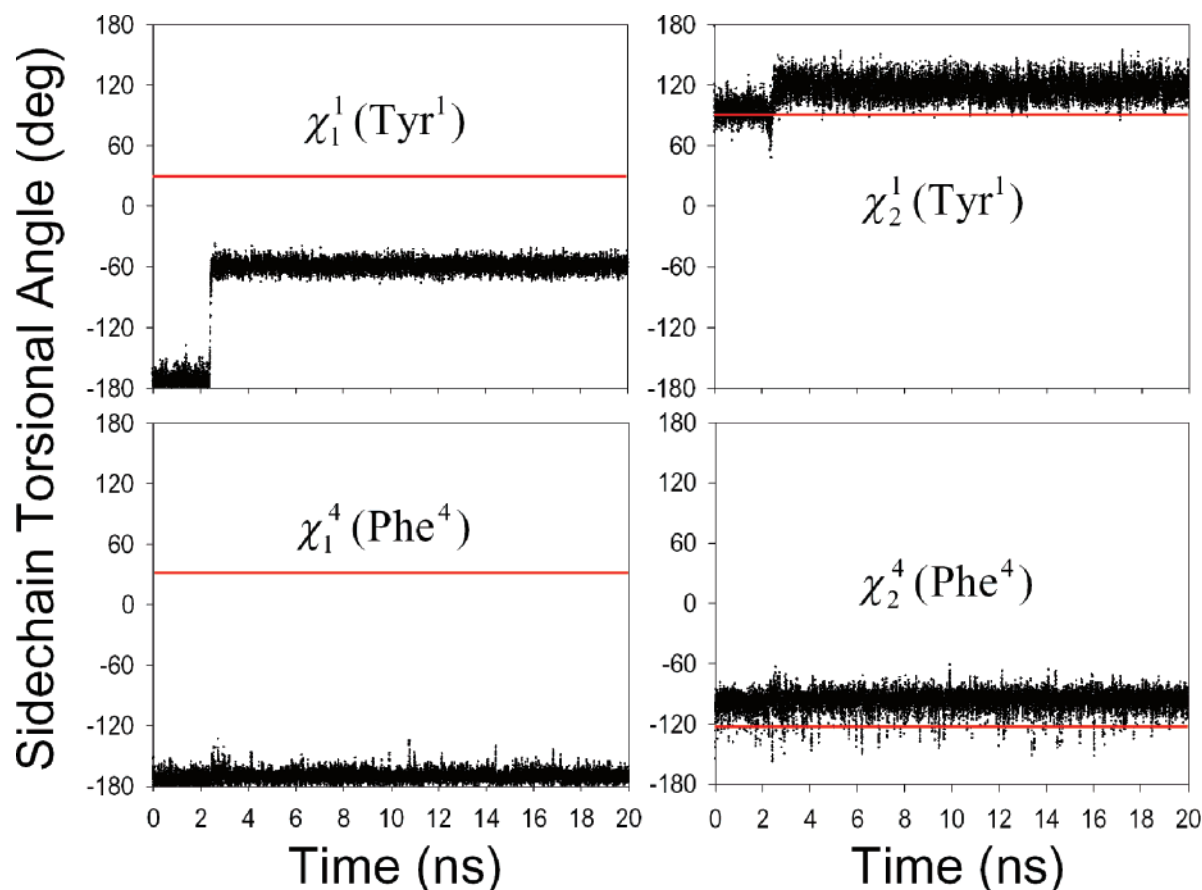


Figure 5. Time evolutions of side chain orientations expressed in terms of χ_1 and χ_2 angles for selected residues. Horizontal lines indicate corresponding values of conformer **1** as computed by AM1 method. It is seen that the χ_2 rotamers of Tyr¹ and Phe⁴ exhibit less variations than the χ_1 rotamers. Furthermore, the χ_1^1 and χ_1^4 angles favor the gauche (−) and trans rotamers in the stable structure, respectively.

TABLE 2: Bioactive Structural Requirements for Opioid Ligands with High Affinity Binding to δ -, μ -, and κ -Opioid Receptor, Respectively

opioid receptor (ligand)	side chain rotamer ($\sim \chi_1^\circ$)			
	Tyr ¹		Phe ³⁽⁴⁾	
δ (DPDPE)	180°	trans	−60°	gauche (−)
δ (JOM-13)	180°	trans	−60°	gauche (−)
μ (JOM-6)	180°	trans	180°	trans
κ (MP16)	^a	^a	180°	trans
κ (α_{S1} -CM)	−60.04°	gauche (−)	−169.97°	trans

^a Data not determined.

performed by the current AM1 calculations was based on the torsional angles of the ω_2 and ω_4 amide bonds. The resulting conformational energy map for α_{S1} -CM is presented in Figure 2. The results show that the α_{S1} -CM conformers strongly favor the cis/cis pair of the ω_2/ω_4 amide bonds in the minimized energy state (see Table 1). Although α_{S1} -CM is characterized by a high structural flexibility, the results presented in Table 1 confirm the ability of the AM1 computational approach to identify the structural characterization of α_{S1} -CM in vacuo.

MD simulation provides a powerful technique for investigating biomolecular dynamic behavior at the atomic level. Accordingly, this study employed the MD approach to calculate the bioactive structural features of α_{S1} -CM in aqueous solution. Since no experimental data were available to describe the structural features of α_{S1} -CM, the MD simulations specified the initial structure of α_{S1} -CM in accordance with that of conformer **1** derived from the AM1 calculations. The peptide energy and RMSD values presented in Figure 3A show that α_{S1} -CM attains structural stability at ca. 2.3 ns. Furthermore, the end-to-end distance, r_{ee} , and the length of the unique intramolecular HB

binding, $r_{Val:N\cdots Pro5:O}$, also stabilize at ca. 2.3 ns, as shown in Figure 3B. Nevertheless, the AM1 calculations have shown that the key feature of the stable conformers of α_{S1} -CM is the formation of the HB binding between the two terminuses as a result of a strong interaction of the coulomb's force. Many natural and synthetic opioid peptides show both a high affinity and a high specificity for the μ opioid receptor, including endomorphins³⁶ [endomorphin-1 (H-Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH₂)] and morphiceptin (H-Tyr-Pro-Phe-Pro-NH₂).³⁷ These peptides share a common sequence feature, namely a proline residue in position 2. Consequently, cis/trans isomerization occurs at the Tyr¹-Pro² amide bond. The cis/trans configuration around the Tyr¹-Pro² amide bond is of particular significance in determining the bioactivity of morphiceptin and endomorphins since this amide bond is located within the biologically important N-terminal tripeptide message sequence. By contrast, the structural features of α_{S1} -CM are similar to those of tetrapeptides, i.e., α_{S1} -CM contains a proline residue spacer between two aromatic rings bearing the pharmacological activity and a proline residue within

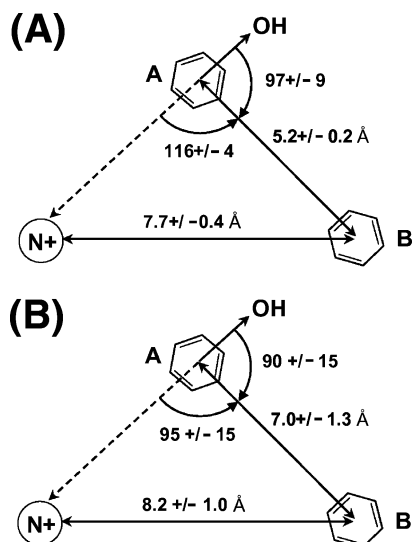


Figure 6. (A) 3D pharmacophore model for κ -selective endogenous pentapeptide of α_{S1} -CM. (B) 3D pharmacophore model for δ -selective ligand pharmacophore model. The major difference between the two models is the distance AB.

the address domain at the C-terminus. As a result, cis/trans isomerization of α_{S1} -CM can potentially occur in both the Val²-Pro³ (ω_2) and the Phe⁴-Pro⁵ (ω_4) amide bonds. Figure 3C illustrates the variation of the torsional angles of the ω_2 and ω_4 amide bonds. The results show that these angles exhibit a cis conformation over the full 20 ns of the simulation, and are, therefore, not only in accordance with conformer **1**, but also consistent with the findings reported for tetrapeptides,^{38,39} which demonstrate the existence of a cis amide bond in the bioactive conformation.

In general, the χ_1 rotamers of aromatic rings (e.g., Tyr and Phe) play an essential role in determining the pharmacological activity. As a result, even though the backbone distributions of α_{S1} -CM in aqueous solution are similar to those observed in vacuo (Figure 4), the χ_1^1 and χ_1^4 rotamers of the side chain are notably different (Figure 5). It has been shown experimentally that α_{S1} -CM has the highest specific affinity to the κ -opioid receptor.²⁹ Therefore, this study has compared the χ_1^1 and χ_1^4 rotamers of α_{S1} -CM to other opioid ligands with a known pharmacological potency to the δ - and μ -opioid receptor, respectively (Table 2). Previous studies have reported that restricting the Tyr¹ side chain in the trans rotamer is favorable in achieving a high binding affinity to both δ - and μ -opioid receptor, e.g., c[D-Pen²,d-Pen⁵]enkephalin (DPDPE) and Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13), meanwhile, restricting the Phe³⁽⁴⁾ side chain in the gauche (−) rotamer is favorable only in achieving a high binding affinity to the δ -opioid receptor.^{40–42} Finally, restricting the Phe³⁽⁴⁾ side chain in the trans rotamer provides a binding affinity to both the μ - and κ -opioid receptor, e.g., Tyr-c[D-Cys-Phe-D-Pen]NH₂ (Et) (JOM-6) and Tyr-c[D-Cys-Phe-D-Cys]NH₂ (MP16).^{41,42} In the case of α_{S1} -CM, Table 2 indicates that the orientation of the Phe³⁽⁴⁾ χ_1 favors trans rotamer and is, therefore, consistent with MP16, which is a κ -selective ligand. Although the favored orientation of Tyr¹ χ_1 of MP16 for the κ -selective ligand is not reported in the literature, Table 2 shows that the stable orientation of α_{S1} -CM favors gauche (−) rotamer. Therefore, it seems that the opioid ligands binding to δ -, μ -, and κ -opioid receptor, respectively, have different bioactive orientations of the key aromatic rings. Furthermore, although the δ pharmacophore model is relatively well understood, that of the κ pharmacophore model so far is still unclear, particularly in regard to the endogenous opioid

peptides, which specifically bind to the κ opioid receptor. Thus, we further analyzed, in detail, the likely κ pharmacophore model containing the pharmacophoric distances and relevant angles, as shown in Figure 6A. As a result, it is clearly shown that the pharmacophoric distance between the aromatic rings of α_{S1} -CM, i.e., the distance AB, differs significantly from that given by the δ pharmacophore model (see Figure 6B).

Conclusions

In recent years, intensive efforts have been made to develop κ -selective agonists as potent and effective analgesics. Additionally, the effect of endogenous opioid systems on the growth of normal and abnormal tissues and cells has attracted increasing interest. Previous studies have shown that α_{S1} -CM has remarkable opioid actions, and not only has high affinity with all three subtypes (κ_1 – κ_3) of the κ -opioid sites, but also inhibits the proliferation of the T47D human breast cancer cell line. Therefore, this study has employed AM1 calculations and MD simulations to investigate the structure–activity relationships of α_{S1} -CM. The results have shown that the pharmacophoric distance between two aromatic rings is 5.0–5.4 Å in the stable structure of α_{S1} -CM in aqueous solution. Furthermore, the χ_1 rotamers of the Tyr¹ and Phe⁴ are of preference for gauche (−) and trans, respectively. The results of this study have provided valuable information for the design of possible cancer therapeutic drugs and contributed further insights into the structure–activity relationship of the κ -opioid system. Although our MD simulations here have shown the pharmacophoric distance for the α_{S1} -CM, the ultimate confirmation of our simulation data is to compare it with experimental results, which to our best knowledge, are absent in the literature to date. Nonetheless, our theoretical work provides significant structural characteristics for the κ -selective peptide.

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