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ORIGINAL PAPER

A molecular dynamics study on opioid activities of biphalin molecule

Jin-Yuan Hsieh · Tzen-Yuh Chiang · Jun-Liang Chen · Yun-Wen Chen · Hong-Chang Lin · Chi-Chuan Hwang

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Abstract Molecular dynamics simulations of the biphalin molecule, $(Tyr\text{-}D\text{-}Ala\text{-}Gly\text{-}Phe\text{-}NH)_2$, and the active tetrapeptide hydrazide, $Tyr\text{-}D\text{-}Ala\text{-}Gly\text{-}Phe\text{-}NH\text{-}NH_2$ were performed to investigate the cause of the increased μ and δ receptor binding affinities of the former over the latter. The simulation results demonstrate that the acylation of the two equal tetrapeptide fragments of biphalin produces the constrained hydrazide bridges $C_4^{\alpha} - C_4{}' - N_9 - N_{10}$ and $N_9 - N_{10} - C_5{}' - C_5^{\alpha}$, which in turn increase the opportunity of conformations for binding to μ or δ receptors. Meanwhile, the connection of the two active tetrapeptide fragments of biphalin also results in the constrained side chain torsion angle χ^2 at one of the two residues Phe. This constrained side chain torsion angle not only significantly increases the δ receptor binding affinity but also makes most of the δ receptor binding conformations of biphalin bind to the δ receptor through the fragment containing the mentioned residue Phe.

Keywords Biphalin · Molecular dynamics · Opioid activities

J.-Y. Hsieh
Department of Mechanical Engineering,
Ming Hsin University of Science and Technology,
Hsinchu 30401, Taiwan

J.-Y. Hsieh
Institute of Precision Mechatronic Engineering,
Ming Hsin University of Science and Technology,
Hsinchu 30401, Taiwan

T.-Y. Chiang
Department of Life Sciences, National Cheng Kung University,
Tainan 701, Taiwan

J.-L. Chen
Department of Mechanical Engineering,
Wu Feng Institute of Technology,
Minsyong,
Chiayi 62153, Taiwan

Y.-W. Chen · H.-C. Lin · C.-C. Hwang (✉)
Department of Engineering Science,
National Cheng Kung University,
Tainan 701, Taiwan
e-mail: chchwang@mail.ncku.edu.tw

Introduction

Opioid analgesic drugs are the most effective therapeutic agents currently available for the treatment of pains. As an agonist ligand, these opioid analgesics exhibit their analgesic activity via activating the opioid μ, δ, or κ receptors, which initiate a series of events modulating pains. However, the μ opioid agonists, such as morphine, provide the highest potency in pain relief but result in significant side-effects such as respiratory depression, constipation, development of drug tolerance and physical dependence, and addiction potential. Therefore one approach in developing new analgesic drugs is to limit the μ-receptor-mediated side-effects by selectively targeting the δ and κ opioid receptors. The use of ligands selective for δ and κ opioid receptors, however, has only limited success. The δ opioid agonists have a reduced addiction potential but a lower efficacy in pain relief, while the κ receptor agonists are limited to their analgesic effects only in peripheral tissues.

A contradictory approach to opioid analgesic development is to search for drugs that have mixed opioid activity at the different opioid receptors, so that the net result of the biological action of such drugs is synergistic. Egan and North [1] have demonstrated the existence of both μ and δ receptors within the same neuron in pain-modulating regions of the central nervous system. Several previous

studies have suggested the physical and functional interactions between the opioid receptors, particularly between the μ and δ receptors [2–5]. Therefore, opioid ligands with mixed opioid receptor interactions have become the promising candidate for novel analgesics. Biphalin, a compound first synthesized by Lipkowski et al. [6], is one of the bivalent ligands of opioid analogs containing two active fragments in one molecule and is no doubt the most successful example of this type of approach [7–9].

Biphalin, (Tyr-D-Ala-Gly-Phe-NH)$_2$, is a highly potent dimeric analog of enkephalin. It binds to all the μ, δ, and κ opioid receptors with nearly equal high affinity [10, 11] and is an extremely potent analgesic in in vivo tests [12, 13]. Most explanations for high potency of biphalin focused on the presence of two pharmacophores in one molecule and on the possible synergistic interactions between the μ and δ receptors. In particular, both the tetrapeptides of the biphalin molecule act as the N-terminal message sequence, which is composed of two pharmacophoric amino acid residues, Tyr and Phe, joined by two spacer residues, D-Ala and Gly. The amine and phenolic groups of Tyr and the aromatic group of Phe are required for opioid receptor recognition. Yamazaki et al. [14] have explained the biological activities of the vast majority of the μ-selective morphiceptin analogs using a pharmacophore model established by them. This model proposed that the characteristic distances with the three pharmacophore groups, d_1 (Tyr N-Tyr OH), d_2 (Tyr N-the center of the aromatic ring of residue Phe), and d_3 (Tyr OH-the center of the aromatic ring of residue Phe) are ~8, ~7, and ~11-13 Å, respectively. The model also requires the side chains in a *trans*-conformation (χ^1=180°) for the Tyr and Phe residues. Shenderovich et al. [15], by performing a comparative molecular modeling study of δ-opioid ligands, presented a three dimensional model of pharmacophore groups on binding to the δ-receptor. The pharmacophore model for δ-opioid agonists proposes a characteristic distance of 7.0± 1.3 Å between the two aromatic rings and of 8.2±1.0 Å between the nitrogen and phenyl ring. This model requires the conformer with a *trans*-rotamer of Tyr and a *gauche*-rotamer of Phe. According to these pharmacophore models, the combination of the two active fragments of the enkephalin analog in biphalin will lead to more probabilities in binding to both the μ and δ receptors, as compared to the single tetrapeptide of the enkephalin analog.

For a better understanding of the protein function of biphalin on the atomic level, this work performed molecular dynamics (MD) simulations of biphalin molecule (Fig. 1a) and one single fragment of enkephalin analog, the tetrapeptide hydrazide Tyr-D-Ala-Gly-Phe-NH-NH$_2$ (Fig. 1b), in aqueous solution, respectively. The biological activities of biphalin and the tetrapeptide hydrazide were compared based on the pharmacophore models mentioned above. This work focused on the forming probabilities of the characteristic distance between Tyr OH and the center of the aromatic ring of residue Phe that is required for binding to the μ receptor and the characteristic distance between the centers of the rings residues of Tyr and Phe that is required for binding to the δ receptor. The cause of the increased μ and δ receptor binding affinities of biphalin over the tetrapeptide hydrazide will then be investigated. The simulation results will demonstrate how the acylation

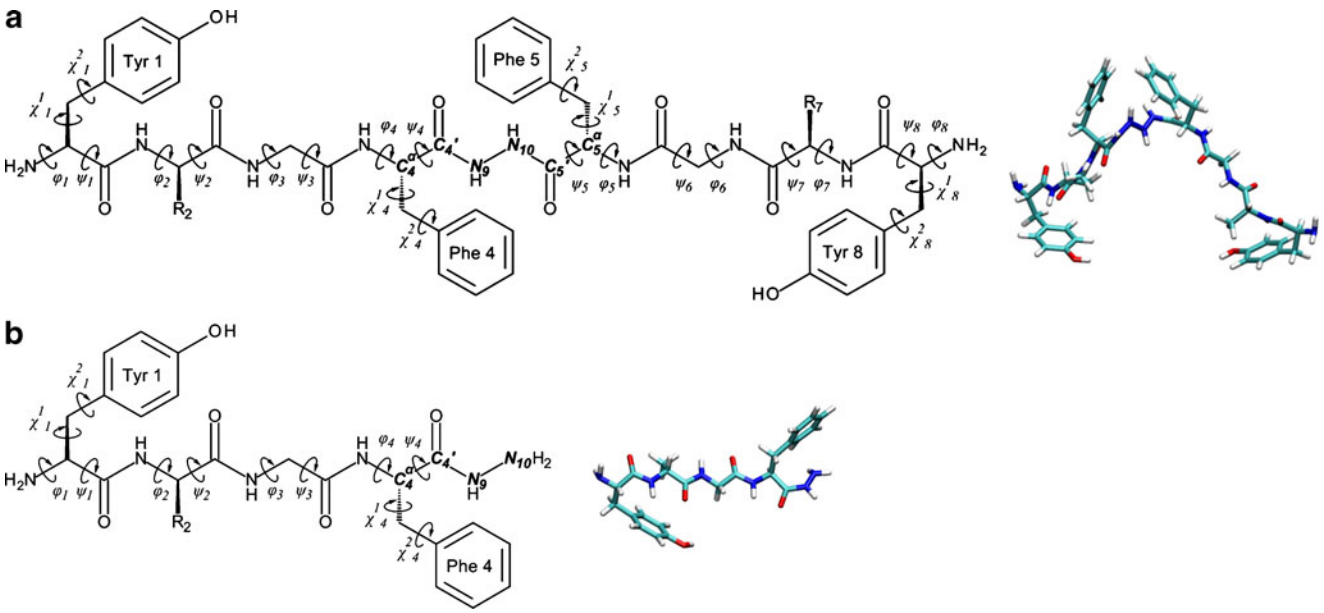

Fig. 1 The atomic configurations of (**a**) the biphalin molecule and (**b**) the tetrapeptide hydrazide Tyr-D-Ala-Gly-Phe-NH-NH$_2$. All the torsion angles discussed in this study are shown herein

of the two equal tetrapeptide fragments of biphalin constrains the torsion angles of the hydrazide bridges formed between the two fragments. Especially, the significant increase of the δ receptor binding affinities of biphalin will be highlighted by comparing to the weaker δ receptor binding affinities of the tetrapeptide hydrazide.

Methods

The MD simulations performed in this study were carried out using the GROMACS version 3.3.3 simulation program with the GROMOS96 (ffG43a1) force field [16, 17]. Biphalin molecule and the bioactive tetrapeptide hydrazide were simulated to be surrounded by a total of 4,539 water molecules, respectively. The initial structure of each peptide

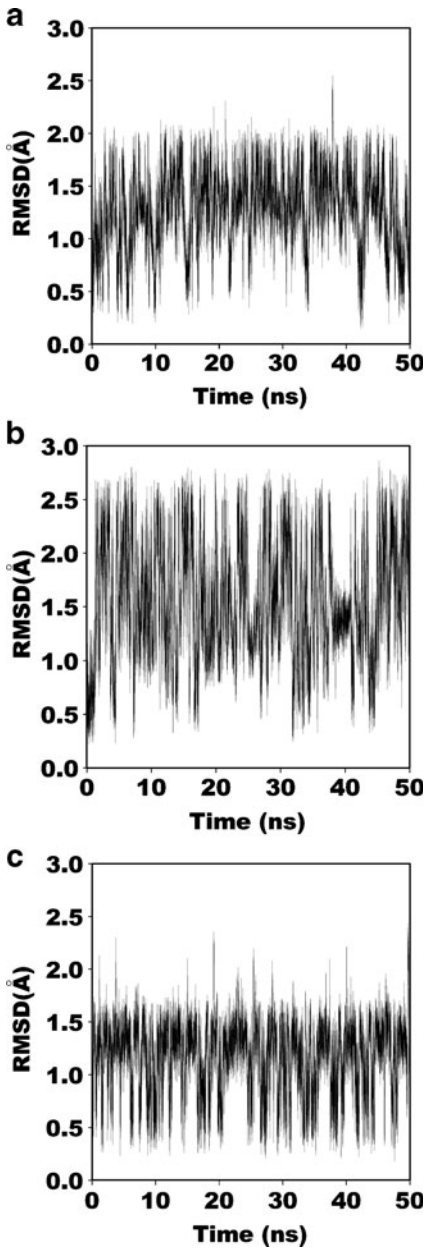

Fig. 2 The RMSD trajectories of the backbone carbon atoms in (**a**) tetrapeptide 1-4 and (**b**) tetrapeptide 5-8 of biphalin molecule and (**c**) the tetrapeptide hydrazide

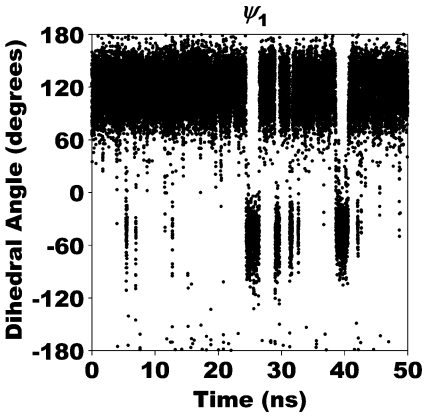

Fig. 3 Time evolution of the backbone torsion angle ψ_1 in the fragment 1-4 of biphalin. Evolutions of ψ_8 in the fragment 5-8 of biphalin and ψ_1 in the tetrapeptide hydrazide are similar to that in the fragment 1-4 of biphalin and are not shown here. For the same reason, only the evolution of the various torsion angles in the fragment 1-4 is shown in Figs. 4-6

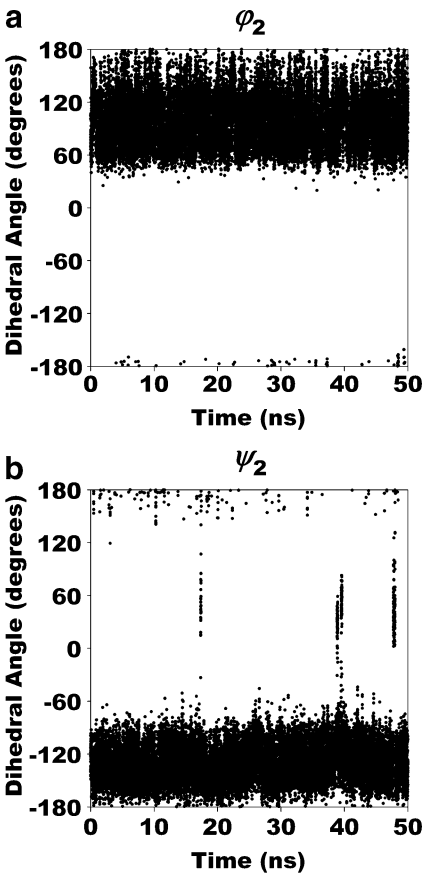

Fig. 4 Time evolutions of the backbone torsion angles (**a**) φ_2 and (**b**) ψ_2 in the fragment 1-4 of biphalin

was prepared according to the one constructed using the X-ray crystal analysis of biphalin [18]. The whole system of the aqueous solution was contained within a cubic box with a side of ca. 52 Å and periodic boundary conditions, so the average density of the solution was kept at ca. 1 g/cm^3. The MD simulations were begun assuming random velocity of the atoms that followed a Maxwellian distribution at 300 K. The atom positions and velocities were integrated according to the standard Verlet algorithm. The integration time step was chosen as Δt=2 fs. The system was first equilibrated at 300 K and the constant pressure relevant to the density of 1 g/cm^3, using the Berendsen algorithm [19]. After the equilibrium was achieved, the time evolutions of all quantities considered in this study were recorded per 2 ps, and the MD simulations were continued for a simulating time domain of 50 ns. In the MD simulations, long-range Coulomb interactions were calculated using the Particle

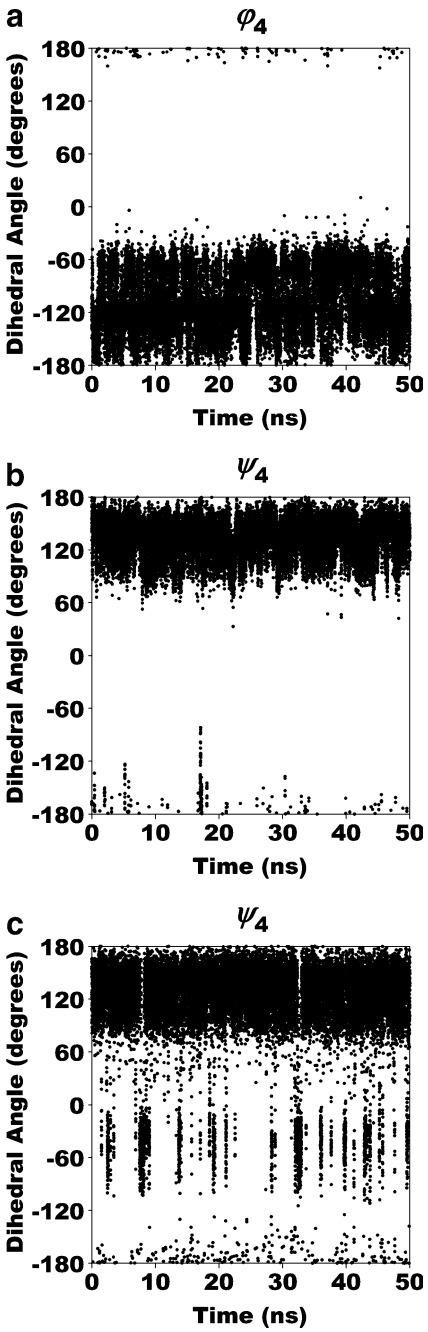

Fig. 5 Time evolutions of the backbone torsion angles (**a**) φ_4 and (**b**) ψ_4 in the biphalin molecule, and (**c**) ψ_4 in the tetrapeptide hydrazide

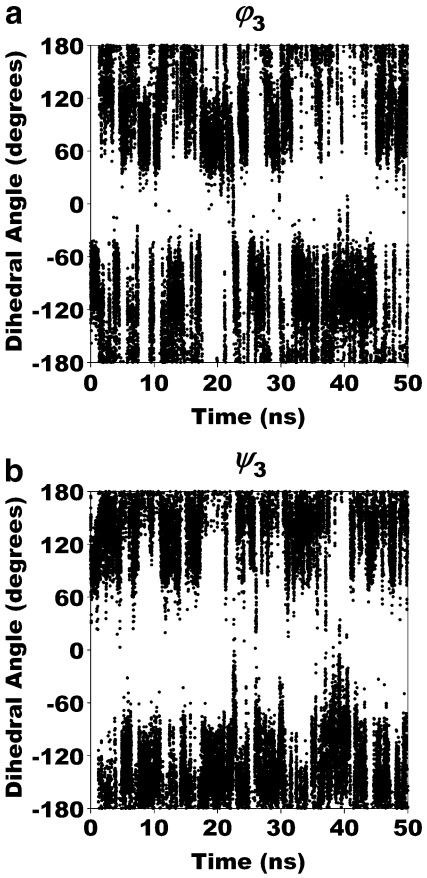

Fig. 6 Time evolutions of the backbone torsion angles (**a**) φ_3 and (**b**) ψ_3 in the biphalin molecule

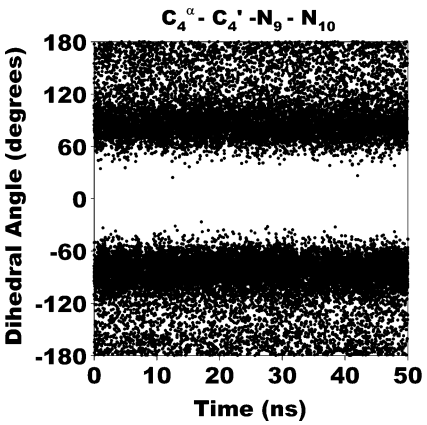

Fig. 7 Time evolution of the torsion angle of the N-N bridge $C_4^{\alpha} - C_4' - N_9 - N_{10}$ in the tetrapeptide hydrazide

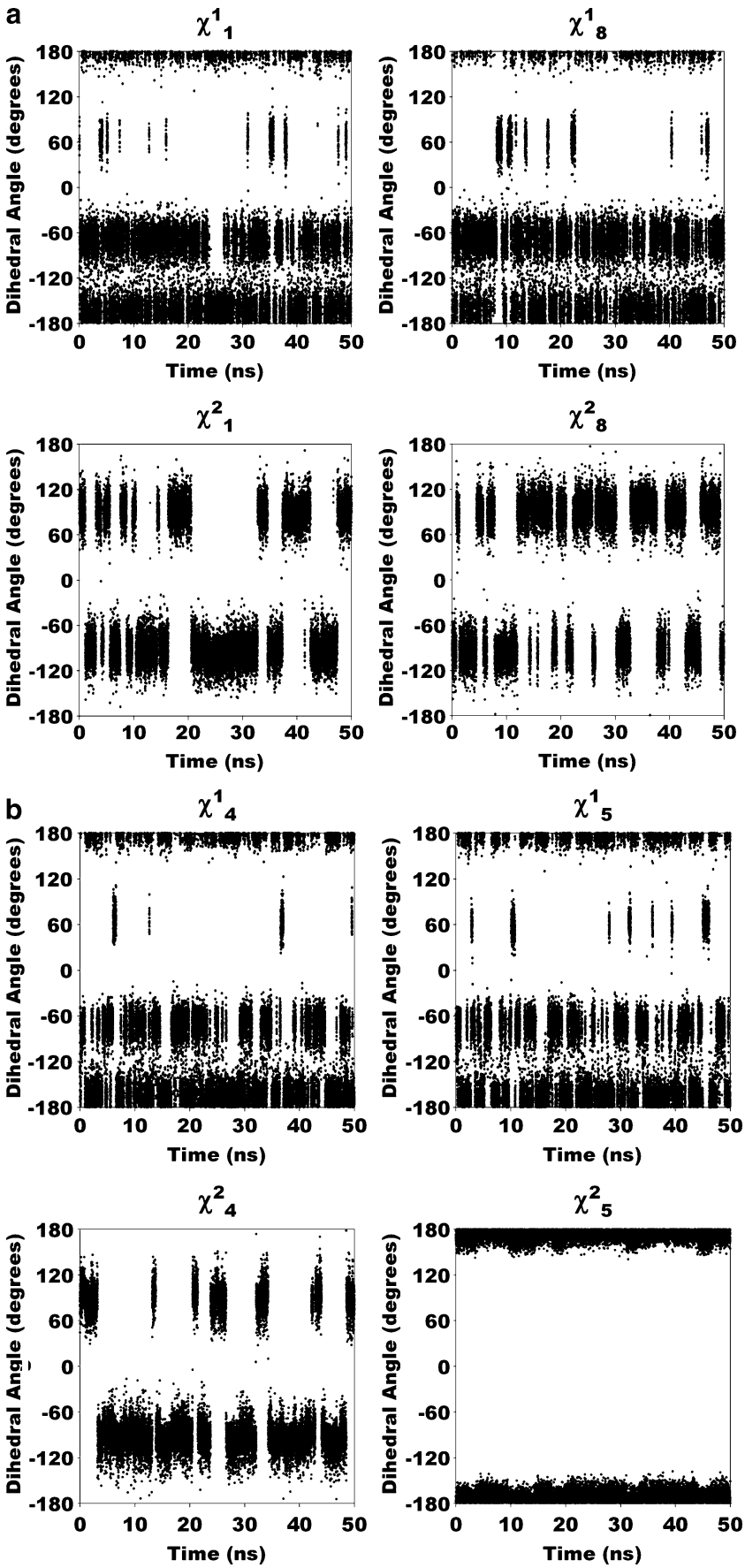

Fig. 8 Time evolutions of the torsion angles for the side chains of residues (**a**) Tyr and (**b**) Phe. Notice that the time evolution of χ^2_5 is rather different from those of χ^2_4 in the biphalin and χ^2_1 in the tetrapeptide hydrazide

Mesh Ewald (PME) method. All the non-bonded potentials were truncated with a cutoff radius of 14 Å, which is appropriate in avoiding severe artifacts in the simulations, as suggested by Darden et al. [20].

Results

Each MD simulation performed in this study gives a total of 25,001 samples of conformational configuration used as the datum base for evaluating all quantities needed in further analyses. As shown in Fig. 2, for each of the two tetrapeptide fragments in the biphalin molecule, and for the active tetrapeptide hydrazide as well, the root mean square deviation (RMSD) of the position of backbone carbon atoms related to the starting backbone structure exhibits fluctuation with ca. 1 Å amplitude. This figure shows the intrinsic conformational mobility of the peptides, which is controlled by the various backbone and side chain torsion angles within the molecule. Since the three-dimensional array of the pharmacophoric groups are defined by a set of conformational states around all the rotatable bonds in the peptide, it is therefore possible to estimate the bioactivity of the peptide by identifying the specific bioactive conformation states. Meanwhile, one of the pharmacophoric distances, either between the basic nitrogen and the center of the aromatic ring of tyrosine or between the basic nitrogen and the phenolic group of tyrosine, is determined by only the side chain torsion angle χ^1 of tyrosine, and according to Yamazaki et al. [14], the bioactive conformations for binding to μ receptor require the side chain of tyrosine to be in *trans* conformation (χ^1= 180°). In the present analysis, the population of the *trans*-conformations in percentage is ca. 50% (either $\chi_1^1 = 180°$ or $\chi_8^1 = 180°$, the subscript represents the residue position) in biphalin molecule and also ca. 50% in the tetrapeptide hydrazide, respectively. Note that since the backbone torsion angles at each of the various residues of the two fragments of the biphalin molecule and the single fragment of the tetrapeptide hydrazide exhibit rather similar time evolutions, in what follows, only those evolutions of the backbone torsion angles in the tetrapeptide 1-4 of the biphalin molecule are demonstrated in Figs. 3, 4, 5, 6. The present results display that the backbone torsion angle ψ at residue Tyr mostly is constrained in the range ψ=60°~ 180°, for the fragments in biphalin ($\psi_{1/8}$) and the tetrapeptide hydrazide (ψ_1) (Fig. 3). At residue D-Ala, the backbone torsion angles φ and ψ for the fragments in biphalin ($\varphi_{2/7}$ and $\psi_{2/7}$) and the tetrapeptide hydrazide (φ_2 and ψ_2) almost are constrained in the ranges φ=60°~180° and ψ=-90°~-180° (Fig. 4). Meanwhile, at residue Phe, the backbone torsion angle φ ($\varphi_{4/5}$ for biphalin and φ_4 for the tetrapeptide hydrazide) is almost constrained in φ=-60°~

Table 1 The torsion angles in the conformers of biphalin having the largest combination populations of dihedral angle states that are bioactive for binding to μ or δ receptors

Conformer	Torsion angles unit: degrees													Receptor	Samples
	ψ_1	χ_1^1	φ_3	ψ_3	φ_4	ψ_4	χ_4^1	ψ_8	χ_8^1	φ_6	ψ_6	φ_5	ψ_5	χ_5^1		
1								117.42± 24.074	-65.72± 12.177 g-	-95.53± 21.443	126.20± 23.650	-125.08± 14.948	131.41± 24.472	-69.02± 10.885 g-	δ	1043
2								117.39± 25.229	-65.83± 12.436 g-	-81.47± 25.306	99.29± 12.961	-123.72± 19.624	135.61 19.531	-170.70± 11.454 trans	δ	328
3								121.56± 21.799	-168.08± 10.489 trans	-98.84± 19.789	143.99± 14.331	-122.90± 15.463	128.16± 29.131	-68.79± 10.545 g-	δ/μ	311
4	120.12± 21.139	-169.20± 10.854 trans	-93.31± 23.291	121.21± 27.301	-133.46± 16.687	126.90± 26.021	-69.06± 11.093 g-								μ	928
5								121.22± 23.205 trans	-168.71± 10.744	-90.73± 26.521	145.85± 14.909	-91.07± 31.552	134.40± 18.761 trans	-171.55± 11.448	μ	789

Table 2 The torsion angles in the conformers of the tetrapeptide hydrazide having the largest combination populations of dihedral angle states that are bioactive for binding to μ or δ receptors

Conformer		Torsion angles unit: degrees							Receptor	Samples
	N-N bridge	ψ_1	χ_1^1	φ_3	ψ_3	φ_4	ψ_4	χ_4^1		
1	85.52±13.788	111.96±24.789	-64.69±13.002 g-	-79.36±28.187	101.29±12.854	-125.02±20.662	112.36±16.126	-170.42±10.996 trans	δ	114
2	-177.16±61.219	113.36±24.673	-67.20±10.108 g-	-81.21±27.803	100.09±13.534	-120.95±19.798	123.40±24.375	-171.07±9.522 trans	δ	73
3	-79.70±9.185	116.43±19.760	-170.26±9.820 trans	-178.37±14.423	95.59±12.520	-135.69±15.928	129.37±24.898	-66.90±12.549 g-	δ	34
4	-83.13±13.320	121.60±22.361	-167.05±10.599 trans	-99.11±28.604	147.49±14.828	-122.33±22.875	133.03±20.457	-168.54±10.824 trans	μ	208
5	-85.57±14.322	116.10±22.637	-168.23±10.590 trans	-93.33±22.622	147.17±14.777	-128.58±17.961	132.07±23.523	-70.63±10.580 g-	μ	177
6	86.21±14.147	116.56±21.510	-168.06±10.549 trans	-98.86±28.616	146.46±15.066	-123.83±21.253	111.85±18.871	-169.87±11.281 trans	μ	139

Table 3 Pharmacophoric distances in the various conformers listed in Table 1

Conformer	δ Pharmacophoric distances (Tyr-Phe:5.7-8.3; N-Phe:7.2-9.2) unit: Å												Receptor	Samples
	Tyr1-Phe4	Tyr8-Phe5	Tyr1-Phe5	Tyr8-Phe4	Tyr1-N1	Tyr8-N2	Tyr1-N2	Tyr8-N1	N1-Phe4	N2-Phe5	N1-Phe5	N2-Phe4		
1	12.3±2.81	8.5±0.87	16.1±3.73	17.9±1.76	4.5±0.49	4.0±0.18	18.7±4.54	20.7±3.51	10.0±3.10	7.2±0.58	14.7±2.61	14.6±1.53	δ	1043
2	13.0±2.72	9.0±2.15	16.0±2.57	14.6±2.45	4.5±0.49	4.0±0.18	19.7±3.19	21.0±2.67	10.6±2.85	6.2±1.03	14.0±2.10	11.9±2.07	δ	328
3	12.4±2.52	8.8±1.30	161±3.17	18.1±1.95	4.4±0.50	5.0±0.08	18.4±4.26	19.3±3.86	9.8±2.63	7.4±0.63	14.4±2.92	15.1±1.74	δ/μ	311
	μ Pharmacophoric distances ($O_{H\text{-}Tyr}$-Phe:11-13; $O_{H\text{-}Tyr}$-N:~8; N-Phe:~7) unit: Å													
	$O_{H\text{-}Tyr1}$-Phe4	$O_{H\text{-}Tyr8}$-Phe5	$O_{H\text{-}Tyr1}$-Phe5	$O_{H\text{-}Tyr8}$-Phe4	$O_{H\text{-}Tyr1}$-N1	$O_{H\text{-}Tyr8}$-N2	$O_{H\text{-}Tyr1}$-N2	$O_{H\text{-}Tyr8}$-N1	N1-Phe4	N2-Phe5	N1-Phe5	N2-Phe4		
3	14.3±2.84	10.9±1.57	17.5±3.80	19.8±2.33	6.9±0.64	7.7±0.12	19.5±4.98	20.7±4.12	-	-	-	-	δ/μ	311
4	11.2±1.09	14.3±2.96	19.6±2.17	16.6±5.03	7.6±0.13	7.1±0.62	19.7±3.46	18.2±5.63	5.5±1.41	8.2±1.03	14.7±1.82	14.7±2.10	μ	928
5	15.7±2.99	13.8±2.32	17.2±3.43	17.8±2.25	7.0±0.63	7.7±0.13	16.5±6.43	17.4±6.01	11.3±2.59	7.5±0.74	13.2±2.57	13.7±1.65	μ	789

-180°, and the backbone torsion angle ψ ($\psi_{4/5}$ for biphalin and ψ_4 for the tetrapeptide hydrazide) is mostly constrained in ψ=90°~180°(Fig. 5a and b). Nevertheless, the backbone torsion angle ψ_4 in the tetrapeptide hydrazide can occasionally be found in the range ψ=0°~-90° (Fig. 5c). As shown in Figs. 4 and 5, at residues D-Ala and Phe, the variable range for torsion angle φ is clearly somewhat wider than for torsion angle ψ. At residue Gly, however, both the backbone torsion angles φ and ψ vary in two rather large ranges, respectively, as shown in Fig. 6. Although not shown, all the torsion angles of the nitrogen-nitrogen bridge in the biphalin molecule, namely $C_4^{\alpha} - C_4' - N_9 - N_{10}$, $C_4' - N_9 - N_{10} - C_5'$, and $N_9 - N_{10} - C_5' - C_5^{\alpha}$, are demonstrated all in *trans*-conformations, but the torsion angle of the N-N bridge $C_4^{\alpha} - C_4' - N_9 - N_{10}$ in the tetrapeptide hydrazide can be in either *trans* or *gauche* conformations (Fig. 7). Without consideration of the mentioned constrained backbone torsion angles, on one hand, the bioactive conformations of the biphalin molecule can be identified through the remaining conformational states of the rotatable bonds, namely the $\chi^1_{1/8}$, $\chi^2_{1/8}$, $\varphi_{3/6}$, $\psi_{3/6}$, $\chi^1_{4/5}$, and $\chi^2_{4/5}$ torsion angles, which are demonstrated accounting for the conformational flexibilities of the two peptides considered in this work. On the other hand, the bioactive conformations of the tetrapeptide hydrazide are identified through the torsion angles χ^1_1, χ^2_1, φ_3, ψ_3, ψ_4, χ^1_4, and χ^2_4 and the torsion angle of the N-N bridge $C_4^{\alpha} - C_4' - N_9 - N_{10}$. Figure 8 demonstrates the time evolutions of the side chain torsion angles, χ^1, and χ^2, at residues Tyr and Phe for the two fragments of biphalin and the tetrapeptide hydrazide, respectively. As shown, each of these side chain torsion angles varies in two ranges (for χ^2) or three (for χ^1), except the side chain torsion angle χ^2_5 at residue Phe_5 in the biphalin molecule, which is only in *trans*-conformations. In general, it is clearly known that bioactive opioid peptide chooses opioid receptor depends on pharmacophoric distances and pharmacophoric torsion angle of side chains. Thus, the conformation samples obtained in the simulations can be divided into a finite number of conformers by the combination of all the specific torsion angles. Subsequently, the conformers suitable for binding to the μ or δ opioid receptors were selected by the larger probability of matching pharmacophoric distances according to the models proposed by Yamazaki et al. [14] and Shenderovich et al. [15], respectively. Simulation results show that, for the tetrapeptide hydrazide, there are totally 2985 and 540 samples of the conformation candidate for binding to μ and δ receptors, respectively. For the biphalin molecule, instead, the simulation results show totally 6833 and 3727 conformation candidates for binding to μ and δ receptors, respectively. Such results indicate that the affinity of the biphalin molecule for the μ receptor is more than two times of that of the tetrapeptide hydrazide, but the affinity of the biphalin molecule for the δ receptor is significantly greater than that of the tetrapeptide hydrazide. The present findings resemble the result of the experimental work of Lipkowski et al. [21], which demonstrated that the tetrapeptide hydrazide considered herein has good affinity for μ receptors, similar to the affinity of biphalin, but rather poor affinity for δ receptors, unlike the affinity of biphalin. Furthermore, conformers having the largest combination populations of dihedral angle states that are assumed to be the potential candidates for binding to μ or δ receptors were defined in Table 1 for biphalin and in Table 2 for the tetrapeptide hydrazide, respectively. Subsequently, all the pharmacophoric distances in these conformers were listed in Tables 3 and 4. Clearly, the top three popular μ receptor binding conformers of the biphalin molecule have a total population of conformation samples two to three-times larger than the top three popular conformers of the tetrapeptide hydrazide

Table 4 Pharmacophoric distances in the various conformers listed in Table 2

Conformer	δ Pharmacophoric distances (Tyr-Phe:5.7-8.3; N-Phe:7.2-9.2) unit: Å			Receptor	Samples
	Tyr1-Phe4	Tyr1-N1	N1-Phe4		
1	9.8±2.19	4.0±0.19	7.0±1.82	δ	114
2	10.1±2.24	4.1±0.16	7.3±1.82	δ	73
3	8.9±1.14	5.0±0.06	8.5±1.21	δ	34
	μ Pharmacophoric distances ($O_{H\text{-}Tyr}$-Phe:11-13; $O_{H\text{-}Tyr}$-N:~8; N-Phe:~7) unit: Å				
	$O_{H\text{-}Tyr1}$-Phe4	$O_{H\text{-}Tyr1}$-N1	N1-Phe4		
4	14.2±1.48	7.6±0.12	8.9±1.26	μ	208
5	11.3±1.33	7.7±0.12	5.5±1.39	μ	177
6	13.9±1.72	7.7±0.12	8.8±1.28	μ	139

do. As can be seen in the tables, all the μ receptor binding conformers have their side chain torsion angle pairs (χ^1, χ^1) at residues Tyr and Phe in either (*trans*, *trans*) or (*trans*, *gauche*(-)) conformations. For biphalin, the satisfactory pharmacophoric distances for the μ binding affinity of the (*trans*, *trans*) conformers are found in the tetrapeptide fragment 5- 8. For the (*trans*, *gauche*(-)) conformers of biphalin, the satisfactory μ binding pharmacophoric distances can occur in both the tetrapeptide fragments. Moreover, the top three conformers of the biphalin molecule for binding to δ receptors have populations of conformation samples significantly greater than those of the tetrapeptide hydrazide do. In particular, for the top three popular δ binding conformers of biphalin, more than half of the conformations have the (*gauche*(-), *gauche*(-)) pair for the side chain torsion angles of residues Tyr and Phe. Besides, the (*gauche*(-), *trans*) and (*trans*, *gauche*(-)) conformers of biphalin can also be the candidates for binding to the δ receptor. The present result supports that one of the causes of the increased populations of the conformer candidates of the biphalin molecule, and therefore the increased affinity for binding to μ or δ receptors, is the constrained torsion angles of the N-N bridges $C_4^{\alpha} - C_4{}' - N_9 - N_{10}$ and $N_9 - N_{10} - C_5{}' - C_5^{\alpha}$, which are only in *trans* conformation, while in the tetrapeptide hydrazide, the same torsion angle can vary among *trans*, *gauche*(+), and *gauche*(-) conformations. Another cause of the significantly increased δ receptor binding affinity for biphalin clearly is the constrained side chain torsion angle χ_5^2 at residue Phe_5 in the tetrapeptide fragment 5-8 of biphalin. Especially, the top three popular δ receptor binding conformers of biphalin have the satisfactory pharmacophoric distances occurring in the tetrapeptide fragment 5-8, i. e., most of the δ receptor binding conformations of biphalin bind to the δ receptor through the tetrapeptide fragment 5-8.

Conclusions

MD simulations of the biphalin molecule and the active single tetrapeptide hydrazide considered in this work were performed to investigate the cause of the increased μ and δ receptor binding affinities of the former over the latter. The simulation results demonstrate that the acylation of the two equal tetrapeptide fragments of biphalin produces the constrained hydrazide bridges $C_4^{\alpha} - C_4{}' - N_9 - N_{10}$ and $N_9 - N_{10} - C_5{}' - C_5^{\alpha}$, which in turn increase the opportunity of the conformations for binding to μ or δ receptors. Meanwhile, the connection of the two active tetrapeptide fragments of biphalin also results in the constrained side chain torsion angle χ_5^2. The constrained side chain torsion angle not only significantly increases the δ receptor binding affinity but also makes most of the δ receptor binding conformations of biphalin bind to the δ receptor through the fragment 5-8.

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