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The structure of an endomorphin analogue incorporating 1-aminocyclohexane-1-carboxylic acid for proline is similar to the β-turn of Leu-enkephalin

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Abstract

Endomorphin (EM2, Tyr–Pro–Phe–Phe–NH_2) can assume various conformations related to *cis*/*trans*-rotamers of the amide linkage of Tyr–Pro. To control isomerization, restricted or flexible components have been introduced at the Pro position. We focused on [Chx^2]EM2, an EM2 analogue substituting 1-aminocyclohexane-1-carboxylic acid (Chx) for Pro. X-ray diffraction analysis revealed that [Chx^2]EM2 is folded into the *trans*-form of Tyr–Chx. The manner of folding resembled that seen in D-TIPP, an EM analogue incorporating tetrahydroisoquinoline carboxylic acid, as well as the β-turn of Leu-enkephalin. Selectivity for the opioid μ-receptor was fairly well conserved by [Chx^2]EM, suggesting that the folded form is important for μ-selectivity.

Keywords: Crystal structure; Endomorphin; Enkephalin; Folding; Morphiceptin; Opioid receptor; Proline isomer

Endomorphin-1 (EM1, Tyr–Pro–Trp–Phe–NH_2) and endomorphin-2 (EM2, Tyr–Pro–Phe–Phe–NH_2) were originally isolated from mammalian brain cortex as endogenous μ-selective opioid peptides [1]. The *cis*/*trans*-rotamers of the amide linkage at Tyr–Pro allow EMs to exist as *cis*- and *trans*-isomers. Such isomerization underlies much of the conformational variation seen in EMs [2], though amidation at the C-terminus provides EMs with additional conformational flexibility [3]. Similar proline isomers have also been observed with morphiceptin (Tyr–Pro–Phe–Pro–NH_2) [4,5] and its Val^4 analogue (Tyr–Pro–Phe–Val–NH_2), which are related μ-selective opioid peptides isolated from milk protein [6,7]. Conformational variation in EMs has even been observed in a membrane-mimetic environment [8]. To control this isomerization, various peptidomimetic components have been incorporated into EMs. One way to restrict the conformational changes around proline is to insert tetrahydroisoquinoline carboxylic acid (Tic) [9–14]. In some instances, incorporation of Tic alters the opioid receptor selectivity of the modified agonist [10,11] and the structures of Tic-incorporated EM analogues and their relationship to receptor selectivity have been extensively studied [12–16]. Conversely, attempts have also been made to increase the conformational flexibility by incorporating β-amino acid or 2-aminocyclopentane carboxylic acid [7,17,18] and Yamada et al. [19] recently replaced the proline of EM2 with 1-aminocyclopentane-1-carboxylic acid (Cpn) or 1-aminocyclohexane-1-carboxylic acid (Chx).

Cpn and Chx are achiral analogues of Cα,Cα-disubstituted glycine (Fig. 1). There is no rotational limitation on the N–Cα bond of Cpn or Chx and the bulkiness of their aliphatic side chain is similar to that of proline. Moreover, binding assays showed opioid activities were fairly well conserved: IC_{50}(μ-receptor) of [Cpn^2]EM2 and [Chx^2]EM2 are 8.31 ± 2.13 and 47.1 ± 2.30 nM, respectively, and the IC_{50}(δ-receptor) of [Cpn^2]EM2 and [Chx^2]EM2 are 3470 ± 1650 and 2250 ± 1060 nM, respectively. Thus, [Cpn^2]- and [Chx^2]EM2s would seem to be excellent probes with which to characterize the structural behaviour of EM. Here, we report on the crystal structure of [Chx^2]EM2 hydrochloride.

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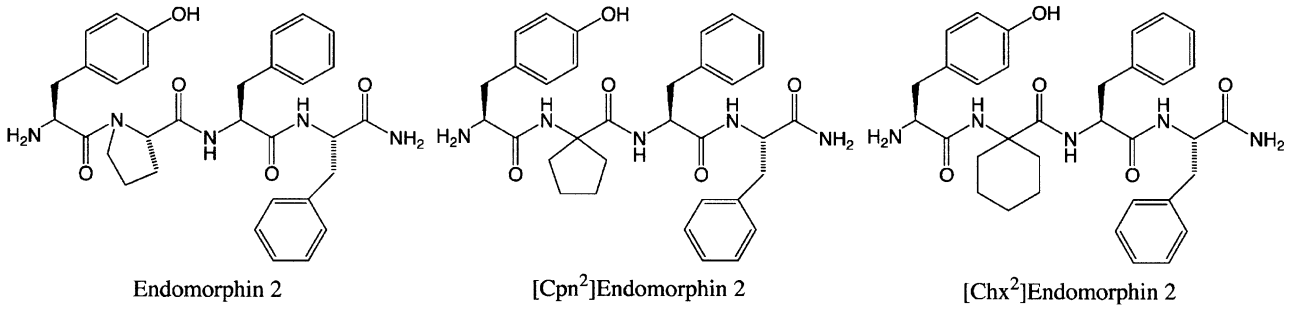

Fig. 1. Structures of endomorphin and its analogues.

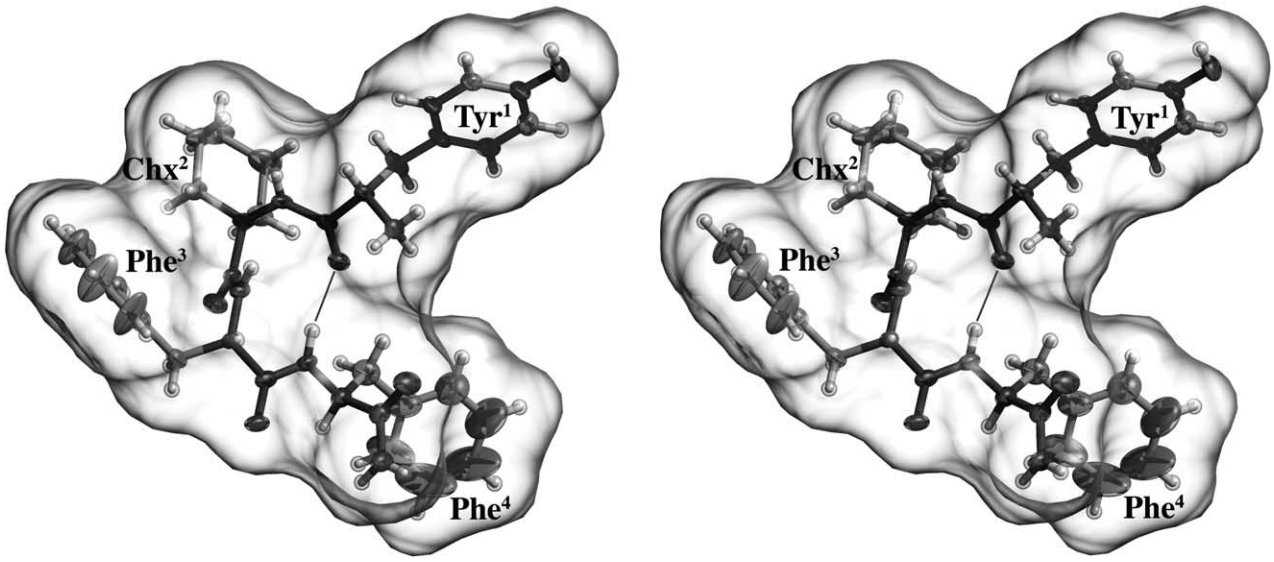

Fig. 2. Stereo view of [Chx^2]EM. The accessible surface is depicted as a ball-and-stick drawing to show the molecular appearance. Displacement ellipsoids are drawn at the 80% probability level. Thin lines represent hydrogen bonds. The figure was drawn using the Raster3D package [33] and the accessible surface was calculated with MSMS [34].

Materials and methods

Materials. [Chx^2]EM2 was synthesized using a conventional liquid-phase method. *tert*-Butyloxycarbonyl (Boc)-chemistry was employed for peptide elongation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 1-hydroxybenzotriazole. Initially, phenylalanine benzyl ester (Phe-OBzl) was coupled with Boc-Phe. The protected dipeptide obtained was treated with trifluoroacetic acid (TFA) and then coupled to Boc-1-aminocyclohexane-1-carboxlylic acid (Boc–Chx). The resultant protected tripeptide was treated with TFA and then coupled with Boc-Tyr, after which the protected tetrapeptide was saponified with 1 M NaOH and then amidated by coupling with 1-hydroxybenzotriazole ammonium salt. The resultant Boc-tetrapeptide was purified by silica gel-column chromatography, after which the purified peptide was treated with 4 M HCl/dioxane, and HCl · [Chx^2]EM2 was recrystallized from methanol–ethyl acetate solution. Single crystals were grown from acetonitrile (MeCN)–water solution using the vapour-diffusion method at room temperature.

Data collection and structure determination. A crystal of HCl · [Chx^2]EM2 was mounted on a nylon loop (Hampton Research, USA) with glycerol and then flash-frozen under a nitrogen stream (100 K). Data collection was performed on a CCD diffractometer (Bruker AXS SMART APEX). Crystal data: formula = $C_{34}H_{41}N_5O_5 \cdot HCl \cdot 2(CH_3CN) \cdot 2(H_2O)$, $M_r = 754.32$, orthorhombic, $P2_12_12_1$, $a = 10.2876(10)$ Å, $b = 16.3534(15)$ Å, $c = 25.815(2)$ Å, $V = 4343.1(7)$ Å^3, $Z = 4$, $F(000) = 1608$, μ(Mo Cα) = 0.139 mm^{-1}, number of observed reflections = 34,041, number of reflections used for refinement = 10,158, $R_{int} = 0.0358$, Flack × perimeter = 0.06(11), number of parameters = 496, $R = 0.0923$, $wR = 0.2548$, $(\Delta/\sigma)_{max} = 0.008$, $\Delta\rho_{max} = 1.733$ e Å^{-3}, and $\Delta\rho_{min} = -0.475$ e Å^{-3}. The structure was solved using the dual-spacing recycling method with SHELXD [20] and refined

Table 1
Torsion angles (°) of [Chz^2]EM and D-TIPP

Angle	[Chx^2]EM	D-TIPP[a]	Angle	[Chz^2]EM	D-TIPP[a]
	Tyr^1	Tyr^1		Phe^3	Phe^3
ψ_1	142.4(3)	156/140	ϕ_3	68.4(5)	−58/−88
ω_1	171.1(3)	167/−177	ψ_3	12.8(5)	−49/−16
χ_1	−54.3(4)	−74/−174	ω_3	−178.0(3)	−163/−179
	Chx^2	D-Tic^2	χ_3	−47.7(4)	−67/−59
ϕ_2	57.4(5)	62/65		Phe^4	Phe^4
ψ_2	27.6(5)	−146/−160	ϕ_4	−87.4(4)	−129/−109
ω_2	179.5(3)	−168/167	ψ_4	120.3(4)	3/−18
χ_{21}[b]	69.9(4)	47/−48	χ_4	178.3(4)	−61/−57
χ_{22}[b]	−65.8(4)				

[a] Two independent molecules exist in the symmetric unit of D-TIPP crystal [15].

[b] The Chx residue has two carbons at β-position.

using SHELXL97 [21]. In the refinement, the solvent molecules were found using a differential Fourier map and two MeCN and two water molecules were included for the structure refinements.

Results and discussion

The structure of [Chx^2]EM2 is shown in Fig. 2. The peptide is folded at the Chx–Phe moiety with an intermolecular hydrogen bond. The peptide bond of the peptidomimetic substituted for proline (Tyr^1–Chx^2) is in the *trans*-form (Table 1). It appears that the folding pattern of Chx^2–Phe^3 makes a 10-membered ring that resembles a β-turn, though the torsion angles do not readily fit into any class of β-turn. Solvent molecules and a counterion that interact with the peptide in various ways were also found in the [Chx^2]EM2 crystal. A Cl^- ion located in the bay of the folded peptide (Fig. 3A) makes contact with Tyr^1 NH_3, Phe^3 NH, Phe^4

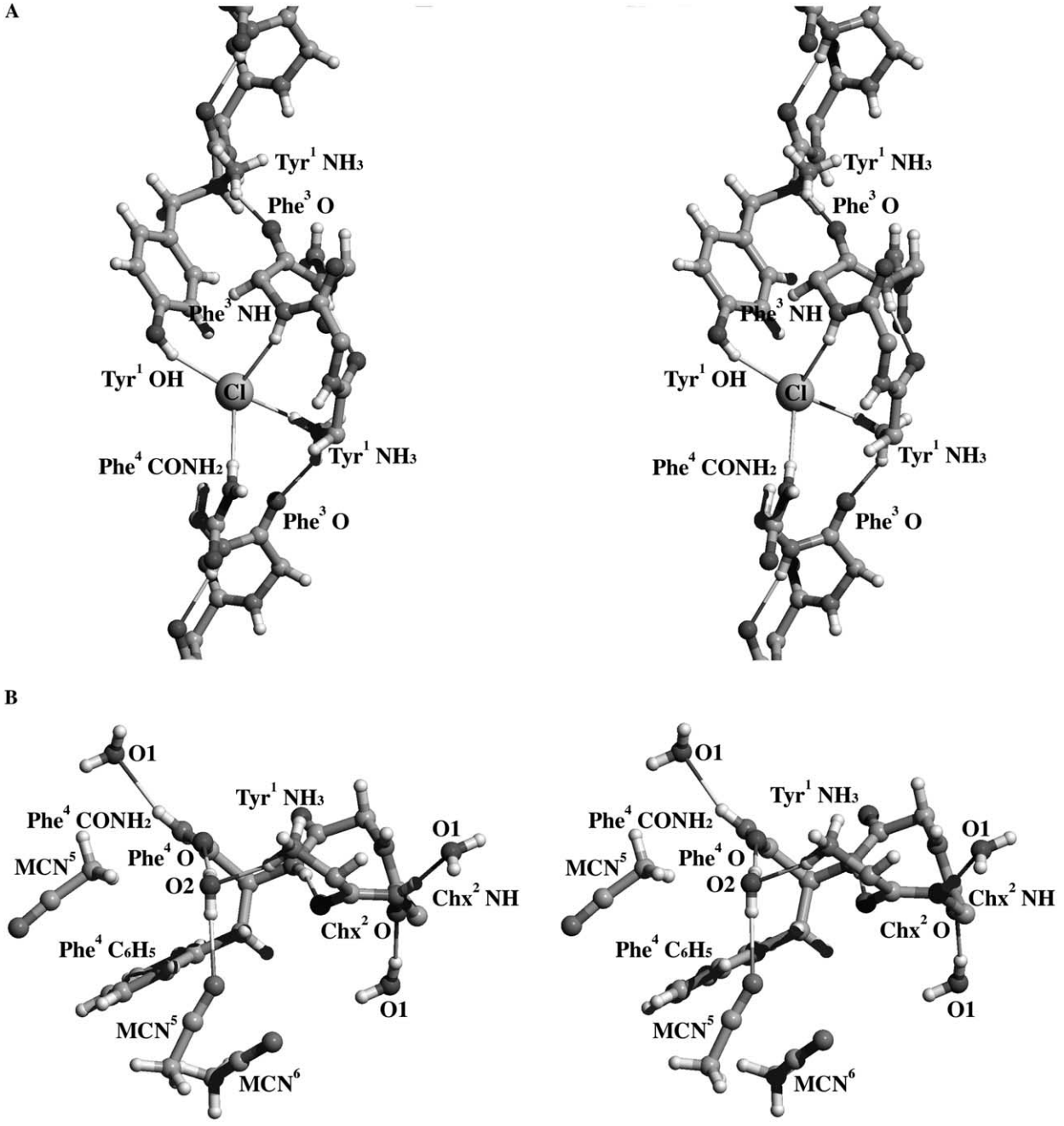

Fig. 3. Stereo views of interactions in the [Chx^2]EM crystal. (A) Interactions between Cl^- and the peptide; (B) interactions between solvent molecules and the peptide. Thin lines represent coordinations and hydrogen bonds. Some side chains are omitted for clarity. The figure was produced with RasMol [35] and POV-Ray [36].

Table 2
Hydrogen bonds and coordinations found in the [Chx^2]EM2 crystal

D	A	D···A (Å)	H···A (Å)	∠D–H···A (°)	Symmetry code
Phe^{4}NH	Tyr^{1}O	2.901(4)	2.036	167.4	
Tyr^{1}NH$_3$	Phe^{3}O	2.752(4)	1.943	147.2	$1-x,\ y+1/2,\ 3/2-z$
Tyr^{1}NH$_3$	Cl	3.098(3)	2.265	151.8	
Phe^{3}NH	Cl	3.282(3)	2.536	143.0	
Tyr^{1}OH	Cl	3.052(3)	2.275	154.2	$1-x,\ y+1/2,\ 3/2-z$
Phe^4 CONH$_2$	Cl	3.275(3)	2.412	166.9	$1-x,\ y-1/2,\ 3/2-z$
Tyr^{1}NH$_3$	O2[a]	2.742(5)	1.861	161.9	
Chx^{2}NH	O1[a]	3.065(5)	2.195	170.2	$-x,\ y+1/2,\ 3/2-z$
Phe^{4}CONH$_2$	O1[a]	2.986(4)	2.233	143.4	$x+1,\ y,\ z$
O1[a] H	Chx^2 O	2.741(5)	1.890	170.3	
O2[a] H	Phe^4 O	2.754(5)	1.867	167.9	
O2[a] H	MCN^5 N	2.921(15)	2.120	169.8	$x-1/2,\ 1/2-y,\ 1-z$

[a] O1 and O2 are oxygen atoms within water molecules.

CONH$_2$, and Tyr^1 OH at distances of 3.05–3.28 Å (Table 2). These interactions differ from the strong coordinated bonds between metal and ligands, but the Cl$^-$ ion does weakly coordinate to the peptide. A water molecule at O1 is hydrogen-bonded to Chx^2 NH, Phe^4 CONH$_2$, and Chx^2 O. Direct peptide–peptide interaction is only observed between Tyr^1 NH$_3$ and Phe^3 O, though hydrogen bonds related to the O1 atom contribute to the intermolecular interaction between the peptides (Fig. 3B). A water molecule at O2 interacts with Tyr^1 NH$_3$ and Phe^4 O. These hydrogen bonds make a 16-membered ring in the folded peptide. The O2 atom mediates the interaction between the N- and C-termini, and stabilizes the peptide folding. Acetonitrile molecules, MCN^5 and MCN^6, are positioned beside the phenyl ring of Phe^4 due to hydrophobic effects and the symmetry-translated MCN^5 is hydrogen-bonded to the water O2 molecule.

Flippen-Anderson et al. [15] described the folded structure of Tic-incorporated EM analogues (D-TIPP) as unique. Within [Chx^2]EM2, an intramolecular hydrogen bond is formed between Phe^4 NH and Tyr^1 O (Fig. 1 and Table 2). Similarly, D-TIPP forms an intramolecular hydrogen bond, but the hydrogen donor is Phe^4 CONH$_2$. There are also conformational differences in the ψ_2, ϕ_3, and ψ_3 angles that are related to the peptidomimetic residue (Table 1), but the two structures nevertheless appear similar in drawings (no coordinates of D-TIPP are deposited). It seems that the degree of restriction or flexibility of the N–Cα bond at the second residue results in only slight differences between the structures of [Chx^2]EM2 and D-TIPP.

Leu-enkephalin (LENK) is a pentapeptide (Tyr–Gly–Gly–Phe–Leu) with opioid activity [22] that exhibits folded, extended and other structures in crystal [23–30]. It is noteworthy that the β-turn of LENK resembles the structure of [Chx^2]EM2 and that the two molecules are comparable when their Cα atoms are fitted using the method of least squares (Fig. 4). Four independent molecules exist in the asymmetric unit of the LENK crystal. Although [Chx^2]EM2 is shorter, the backbones of [Chx^2]EM2 and LENK are similar, with an RMSD of 0.48–0.50 Å. LENK is selective for both the δ and μ-receptor subtypes, and its molecular folding is considered to be important for μ-selectivity [23,24]. On the other hand, molecular folding is also observed with δ-selective enkephalin analogues [31,32], and other exceptional structures have been reported [26,27]. Still, the μ-selectivity of the parent peptide is fairly well conserved in [Chx^2]EM2 [19] and we therefore believe that conformational similarities within the folded structures of EM analogues should be of interest when considering their opioid receptor selectivity.

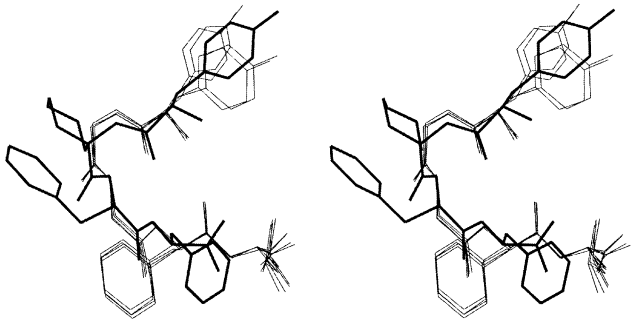

Fig. 4. Stereo view showing superimposition of [Chx^2]EM2 and [Leu]enkephalins, which are represented by thick and thin lines, respectively. Four independent molecules exist in the asymmetric unit of the [Leu]enkephalin crystal. The figure was drawn with Swiss-Pdb-Viewer [37].

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