Crystal Structures of Peptide Enantiomers and Racemates: Probing Conformational Diversity in Heterochiral Pro-Pro Sequences

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Received 19 December 2007; revised 27 February 2008; accepted 27 February 2008

Published online 11 March 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.20982

ABSTRACT:

Multiple conformational states in heterochiral diproline sequences have been characterized in the solid state by the determination of the crystal structures of seven tripeptides in enantiomeric and racemic forms. The sequences of the type Piv-^DPro-^LPro-^DXxx-NHMe (D-L-D) [^DXxx = ^DVal 1, ^DLeu 3, and ^DPhe 5] and their corresponding enantiomeric L-D-L sequences [^LXxx = ^LVal 2, ^LLeu 4, and ^LPhe 6] have been investigated. Single crystals have been obtained for the pure enantiomers 1, 2, 3, 4 and for the racemates 1/2, 3/4, and 5/6. For Xxx = Leu, mirror image conformations (type II/II' β -turns) at Pro-Leu segment are obtained. For Xxx = Val, a ^LPro-^DPro type II β -turn in 2 and an open/extended structure is obtained in the solvated form of the enantiomer 1. For Xxx = Phe, suitable crystals could not be obtained for enantiomeric peptides. The racemate 5/6 revealed a cis peptide bond between the diproline segment with the absence of any intramolecular hydrogen bonds. Crystal structures of enantiomers and racemates prove useful in characterizing the multiple conformational states that are accessible to Pro-Pro segments. © 2008 Wiley Periodicals, Inc. *Biopolymers (Pept Sci)* 90: 537–543, 2008.

Keywords: diproline peptides; peptide enantiomers; peptide racemates; molecular conformation; conformational variability; X-ray diffraction

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

INTRODUCTION

In 1989 Alan Mackay made the intriguing suggestion that racemic proteins should crystallize in centro-symmetric fashion, resulting in a simplification of the problem of phase determination. Mackay noted "that the outcome of the Second World War turned on this issue, as the German Enigma coding machine was made centro-symmetrical for administrative convenience," a weakness that allowed "an entry to the decipherment of Enigma, which perhaps ultimately led to the military defeat of the Axis." He added insightfully that "Probably no crystallographers were involved on either side."¹ Subsequently, several reports have described the structure determination of racemic forms of proteins and peptides produced by chemical synthesis.² In exploring multiple conformational states of oligopeptides we have often encountered difficulties in crystallization of flexible peptides. The task of characterizing different conformational states of peptides in crystals is also facilitated by exploring conditions under which polymorphic forms are generated. Ideally, distinctly different crystal forms may provide an opportunity for capturing widely different structural states (conformational polymorphism). The definition of conformational polymorphism can be ambiguous. In the case of oligopeptides, molecules can crystallize in different crystal forms with similar backbone conformations over

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Contract grant sponsors: Council of Scientific and Industrial Research and Program Support; IRHPA Department of Biotechnology, India
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much of the peptide chain. Structural variations are often observed at the termini and in side-chain conformations. Examples of dramatic backbone conformational differences in polymorphic crystal forms are rare.^{3–5} It is in this context that we have turned our attention to the study of peptide racemates.

Di-proline peptides have attracted considerable recent attention because of their ability to nucleate stable secondary structures in short peptides. Homochiral (^LPro-^LPro) sequences have been advanced as helix forming templates,⁶ whereas heterochiral (^DPro-^LPro) sequences have been shown to be strong β -hairpin nucleators.⁷ Even though proline is the most conformationally-restricted residue in proteins, multiple states corresponding to different values of the torsion angle ψ are available to the proline residue.⁸ In ^LPro residues, two major conformational states have been widely observed: the polyproline-II (P_{II}) conformation ($\phi = -60^\circ$, $\psi = 120^\circ$) and the left-handed helical conformation ($\phi = -60^\circ$, $\psi = -30^\circ$). In addition, the C₇ or γ -turn conformation ($\phi = -70^\circ$, $\psi = 70^\circ$) has also been observed. In Xxx-Pro sequences the occurrence of *cis* peptide bonds further enhances the range of energetically accessible conformations. Pro-Pro peptides are therefore capable of exhibiting significant conformational diversity. We have employed the device of studying pure enantiomers and racemates to probe the structure space of tripeptides of the type Piv-^DPro-^LPro-^DXxx-NHMe/Piv-^LPro-^DPro-^LXxx-NHMe. Recent interest in the conformational properties of ^DPro-^LPro-^DXxx sequences have been stimulated by the demonstration that β -hairpins can be nucleated by a three residue connecting loop when Xxx = ^DAla.⁹

Six peptides with Xxx = ^DVal 1, ^LVal 2, ^DLeu 3, ^LLeu 4, ^DPhe 5, and ^LPhe 6 have been investigated. Crystallization attempts were made with the pure enantiomers *D-L-D* (peptides 1, 3, 5) and *L-D-L* (peptides 2, 4, 6). In addition, the racemates 1/2, 3/4, and 5/6 were prepared by mixing equimolar mixtures of the two individual enantiomers.

EXPERIMENTAL SECTION

Peptide Synthesis

Peptides were synthesized by conventional solution phase methods using a fragment condensation strategy. The pivaloyl (Piv) group was used for the N-terminus, while the C-terminus was blocked as *N*-methylamide. Couplings were mediated by dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBT). The synthesis of peptides was achieved by the condensation of Piv-^DPro-^LPro-OH/Piv-^LPro-^DPro-OH with fragment Xxx-OMe (Xxx = ^DVal 1, ^LVal 2, ^DLeu 3, ^LLeu 4, ^DPhe 5, and ^LPhe 6). All the six tripeptide methyl esters were converted to their respective *N*-methylamides by saturat-

ing the methanolic solution of the tripeptide esters with methylamine gas. The final peptide products were purified by silica-gel column chromatography followed by HPLC (C₁₈, 5–10 μ), employing methanol-water gradients. The homogeneity of the purified peptides was ascertained by analytical HPLC. Optical rotation parameters and the melting point of the peptides reported in this article are as follows:

Piv-^DPro-^LPro-^DVal-NHMe 1 $[\alpha]^{24}_D = -62.5$ ($c = 0.88$, CH₃OH); mp: 100.2°C, Piv-^LPro-^DPro-^LVal-NHMe 2 $[\alpha]^{25}_D = +62.3$ ($c = 1.46$, CH₃OH); mp: 99.3°C, Piv-^DPro-^LPro-^DLeu-NHMe 3 $[\alpha]^{23}_D = -40.0$ ($c = 1.00$, CH₃OH); mp: 106.4°C, Piv-^LPro-^DPro-^LLeu-NHMe 4 $[\alpha]^{24}_D = +52.0$ ($c = 1.5$, CH₃OH); mp: 105.9°C, Piv-^DPro-^LPro-^DPhe-NHMe 5 $[\alpha]^{23}_D = -34.0$ ($c = 0.8$, CH₃OH); gummy, Piv-^LPro-^DPro-^LPhe-NHMe 6 $[\alpha]^{23}_D = +39.0$ ($c = 1.00$, CH₃OH); gummy, Racemic mixture of Piv-^DPro-^LPro-^DVal-NHMe + Piv-^LPro-^DPro-^LVal-NHMe 1/2 mp: 150.2°C, Racemic mixture of Piv-^DPro-^LPro-^DLeu-NHMe + Piv-^LPro-^DPro-^LLeu-NHMe 3/4 mp: 130.4°C, Racemic mixture of Piv-^DPro-^LPro-^DPhe-NHMe + Piv-^LPro-^DPro-^LPhe-NHMe 5/6 mp: 115.2°C.

Single crystals were obtained by slow evaporation of pure enantiomers or racemic mixtures from petroleum ether/ethyl acetate, methanol/water and CHCl₃-DMSO.

X-Ray Diffraction

Single crystals suitable for X-ray diffraction were obtained in the cases of peptides 1, 2, 3, 4, 1/2, 3/4, and 5/6. In the case of Xxx = Phe (5, 6), the pure enantiomers failed to yield crystals. X-ray diffraction data were collected at room temperature on a Bruker AXS SMART APEX CCD diffractometer using Mo K α radiation ($\lambda = 0.71073$ Å). The ω -scan type was used. All the structures were solved by direct methods using SHELXS-97^{10a} and refined against F^2 , with full-matrix least-squares methods using SHELXL-97.^{10b} Nonhydrogen atoms were refined isotropically followed by full-matrix anisotropic least squares refinement. The crystal and diffraction parameters for the peptides solved are given in Table I. In the crystal structure of peptide 1, valine and the C-terminal blocking group *N*-methylamide (NHMe) are disordered over two positions with an occupancy of 0.42 in one site (Chain A: atoms C3A, C3', O3, N4, C1M, C3B, C3G, and C3D) and an occupancy of 0.58 in the other site (Chain B: atoms C33A, C33', O33, N41, C11M, C33B, C33G, and C33D). However, as both the chains were unacceptably close, refinement was effected using occupancy at a single site only. Notably, a water molecule with an occupancy of 0.42 is associated with only chain A of the molecule and not chain B. In the racemic mixture 1/2, a similar method was adopted for the refinement of a water molecule with an occupancy of 0.74 where symmetry related water molecules were unacceptably close. In loosely packed crystals, solvent (water occupancies) at available sites are often fractional. Orientational disorder can also result in the inability to locate definitive positions. In peptide 2, the hydrogen atom (H) bonded to Pro C1A; Pro C2A and NHMe N4 were located from difference Fourier map. In peptide 3/4, the hydrogen atom (H) bonded to Pro C1A; Pro C2A; Leu C3A; Leu N3; Leu C3B; and Leu C3G were located from difference Fourier map. The remaining hydrogens in all the other peptide structures were fixed geometrically using the riding atom model in the idealized positions and refined in the final cycle as riding over the heavier atom to which they were bonded. Crystallographic data (excluding structure factors) have been deposited at

Table I Crystal and Diffraction Parameters for Peptides 1, 2, 1/2, 3, 4, 3/4, and 5/6

	Peptide 1	Peptide 2	Peptide 1/2	Peptide 3	Peptide 4	Peptide 3/4	Peptide 5/6
Empirical formula	C ₂₁ H ₃₆ N ₄ O ₄ · 1.43H ₂ O	C ₂₁ H ₃₆ N ₄ O ₄	C ₂₁ H ₃₆ N ₄ O ₄ · 0.74H ₂ O	C ₂₂ H ₃₈ N ₄ O ₄ · H ₂ O	C ₂₂ H ₃₈ N ₄ O ₄ · H ₂ O	C ₂₂ H ₃₈ N ₄ O ₄ · H ₂ O	C ₂₅ H ₃₆ N ₄ O ₄ · 2 H ₂ O
Crystal habit	Clear, Rod	Clear, Rod	Clear, Thin Plates	Clear, Block	Clear, Thin needles	Clear, Rod	Translucent, Thin Plates
Crystal size (mm)	0.50 × 0.15 × 0.07	0.5 × 0.10 × 0.06	0.25 × 0.16 × 0.04	0.50 × 0.20 × 0.30	0.57 × 0.05 × 0.01	0.47 × 0.12 × 0.07	0.26 × 0.17 × 0.05
Crystallizing solvent	Ethylacetate/ petroleum ether	Ethylacetate/ petroleum ether	Ethylacetate/ petroleum ether	CHCl ₃ -DMSO	Ethylacetate/ petroleum ether	Ethylacetate/ petroleum ether	Methanol/water
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	C2/c	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ /c	P2 ₁ /c
a (Å)	8.744 (1)	9.743 (1)	36.667 (17)	10.531 (3)	10.462 (4)	10.555 (1)	8.883 (5)
b (Å)	11.609 (1)	11.423 (2)	10.092 (5)	11.659 (3)	11.572 (4)	11.687 (1)	18.811 (10)
c (Å)	23.577 (2)	21.664 (3)	13.846 (6)	20.425 (6)	20.262 (7)	20.108 (2)	16.033 (9)
α (deg)	90.0	90.0	90.0	90.0	90.0	90.000	90.0
β (deg)	90.0	90.0	107.3 (2)	90.0	90.0	95.5 (1)	96.3 (1)
γ (deg)	90.0	90.0	90.0	90.0	90.0	90.0	90.0
Volume (Å ³)	2393.5 (4)	2411.2(6)	4893.0 (4)	2507.9 (12)	2453.1 (15)	2469.2 (5)	2663.0 (3)
Z	4	4	8	4	4	4	4
Molecules/asymm. unit	1	1	1	1	1	1	1
Cocrystallized solvent	H ₂ O	None	H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O
Molecular weight	431.34	408.54	420.42	438.56	438.56	438.56	488.58
Final R (%) / wR2 (%)	6.25/18.56	8.03/18.99	13.17/24.06	4.44/12.39	9.68/24.18	7.61/17.83	12.18/22.66
Goodness of fit (S)	1.197	1.345	1.071	1.107	1.316	1.042	1.073
F (000)	934	888	1824	952	952	952	1048
2θ (max.)	53.58	53.94	46.50	53.64	53.60	53.54	46.50
Independent reflections	4739	4810	3497	4894	4805	4824	3805
Observed reflections	2364	2463	1238	2680	2230	3097	1597
[F > 4σ (F)]

FIGURE 1 Asymmetric unit and molecular conformation of a) Piv-^DPro-^LPro-^DVal-NHMe. b) Piv-^LPro-^DPro-^LVal-NHMe. c) Racemic mixture of Piv-^DPro-^LPro-^DVal-NHMe + Piv-^LPro-^DPro-^LVal-NHMe. (Only the *L-D-L* enantiomer has been shown). Hydrogen bonds are shown as dotted lines.

the Cambridge Crystallographic Data Centre with deposition numbers: 1 CCDC 611007; 2 CCDC 611008; 1/2 CCDC 611009; 3 CCDC 611010; 4 CCDC 611011; 3/4 CCDC 611012; 5/6 CCDC 611013. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB21EZ, U.K., Telephone: (+44)1223-762-910, Fax: (+44)1223-336-033 or via the website www.ccdc.cam.ac.uk/conts/retrieving.html (e-mail: deposit@ccdc.cam.ac.uk).

RESULTS AND DISCUSSION

Conformational Analysis of Peptides 1, 2, and 1/2

Figure 1 shows the comparison of the molecular conformations observed in the crystals of the enantiomers 1 and 2 and the racemate 1/2 (Xxx = Val). 1 crystallized as the hydrate while 2 lacks any co-crystallized water molecule. Notably, considerable differences are observed in the backbone conformations of the two enantiomers presumably facilitated by intermolecular hydrogen bonding of the central peptide unit

FIGURE 2 Asymmetric unit and molecular conformation of a) Piv-^DPro-^LPro-^DLeu-NHMe. b) Piv-^LPro-^DPro-^LLeu-NHMe. c) Racemic mixture of Piv-^DPro-^LPro-^DLeu-NHMe + Piv-^LPro-^DPro-^LLeu-NHMe. (Only the *L-D-L* enantiomer has been shown). Hydrogen bonds are shown as dotted lines.

FIGURE 3 Asymmetric unit and molecular conformation of racemic mixture of Piv-^DPro-^LPro-^DPhe-NHMe + Piv-^LPro-^DPro-^LPhe-NHMe. (Only the *L-D-L* enantiomer has been shown). Hydrogen bonds are shown as dotted lines.

Table II Hydrogen Bond Parameters for the Peptides 1, 2, 1/2, 3, 4, 3/4, and 5/6

		N/C/O...O					
Donor	Acceptor	Donor/ Acceptor (Å)	H...O (Å)	C=O..H (deg)	C=O...N (deg)	O...H—N (deg)	C—H...O (deg)
Piv- ^D Pro- ^L Pro- ^D Val NHMe (1)							
Intermolecular							
N(3)	O1w	3.023	2.100			163.89	
N(4)	O2w	2.782	1.995			151.82	
O1w	O(0) ^a	2.940					
O1w	O(1) ^b	2.821					
O2w	O1w ^c	3.368					
Piv- ^L Pro- ^D Pro- ^L Val-NHMe (2)							
Intramolecular							
4→1 N(3)	O(0)	3.388	2.648	142.32	138.71	144.93	
Intermolecular							
N(4)	O(2) ^d	2.833	2.062	132.33	135.11	164.51	
Racemic mixture of Piv- ^D Pro- ^L Pro- ^D Val-NHMe + Piv- ^L Pro- ^D Pro- ^L Val-NHMe (1/2)							
Intramolecular							
4→1 N(3)	O(0)	3.279	2.490	141.64	137.93	156.45	
Intermolecular							
N(4)	O(1) ^e	2.883	2.026	149.43	150.98	174.71	
O1w	O(2) ^f	2.954					
Piv- ^D Pro- ^L Pro- ^D Leu-NHMe (3)							
Intramolecular							
4→1 N(4)	O(1)	3.184	2.411	139.17	139.89	149.90	
Intermolecular							
N(3)	O1w	2.857	2.011			167.99	
O1w	O(0) ^g	2.769					
O1w	O(2) ^g	2.764					
Piv- ^L Pro- ^D Pro- ^L Leu-NMe (4)							
Intramolecular							
4→1 N(4)	O(1)	3.175	2.405	139.76	140.07	149.30	
Intermolecular							
N(3)	O1w	2.881	1.982			168.96	
O1w	O(0) ^h	2.790					
O1w	O(2) ^h	2.764					
Racemic mixture of Piv- ^D Pro- ^L Pro- ^D Leu-NHMe + Piv- ^L Pro- ^D Pro- ^L Leu-NHMe (3/4)							
Intramolecular							
4→1 N(4)	O(1)	3.087	2.316	139.98	141.18	149.36	
Intermolecular							
N(3)	O1w	2.865	2.025			165.46	
O1w	O(0) ⁱ	2.767					
O1w	O(2) ⁱ	2.792					
Racemic mixture of Piv- ^D Pro- ^L Pro- ^D Phe-NHMe + Piv- ^L Pro- ^D Pro- ^L Phe-NHMe (5/6)							
Intramolecular							
4→1 N(3)	O(0)	3.144	2.382	138.20	132.01	147.97	
4→1 N(4)	O(1)	3.109	2.285	120.93	126.06	160.37	
Intermolecular							
O1w	O2w ^j	2.930					
O1w	O(2)	2.731					
O2w	O(3)	3.148					
O1w	O2w	3.351					

^a Related by symmetry $x + 1/2, -y + 1/2, -z$.

^b Related by symmetry $x + 1, y, z$.

^c Symmetry related by $-x + 1, y - 1/2, -z + 1/2 + 1$.

^d Related by symmetry $x + 1/2, -y + 1/2, -z + 2$.

^e Symmetry related by $x, -y, z + 1/2$.

^f Symmetry related by $-x, y, -z + 1/2$.

^g Related by symmetry $-x, y - 1/2, -z + 1/2$.

^h Related by symmetry $-x + 1, y - 1/2, -z + 1/2 + 1$.

ⁱ Symmetry related by $-x, y - 1/2, -z + 1/2$.

^j Symmetry related by $x + 1/2, -y + 1/2 + 1, -z + 1$.

of **1** to the water molecule O1w. In the *D-L-D* peptide, the ^DPro-Pro peptide bond adopts a *cis* ($\omega = 8.4^\circ$) geometry and both the proline residues adopt a semi-extended conformation, with the molecule lacking any intramolecular hydrogen bond. In contrast, in the *L-D-L* enantiomer, the ^LPro-^DPro segment forms a type II β -turn conformation with an intramolecular 4 to 1 hydrogen bond between the pivaloyl C=O and the valine NH groups. In principle, the enantiomers might be expected to yield mirror image crystals, formed by molecules with identical conformations. The crystals of **1** and **2** illustrate conformational variability which may be observed when comparing solvent free crystals with solvates ("pseudo polymorphism").¹¹ Figure 1c shows the molecular conformation of the tripeptide in the racemic crystals of **1/2**. The asymmetric unit reveals a peptide folded into a type II β -turn conformation. This is almost a mirror image to the structure observed in the *L-D-L* enantiomer **2**.

Conformational Analysis of Peptides **3**, **4**, and **3/4**

The structures of the enantiomers **3** and **4** and the racemate **3/4** (Xxx = Leu) are shown in Figure 2. In the case of peptide **3**, the ^DPro residue adopts a semi-extended conformation with the formation of a type II β -turn at the ^LPro-^DLeu segment. Investigation of the backbone conformations for peptide **4** reveals that it adopts a nearly identical mirror image structure to that of peptide **3**. An interesting feature in both the structures is that the heterochiral Pro-Pro segment is semi-extended while type II (**3**) or II' (**4**) β -turn conformations are observed at the Pro-Leu segment. The central peptide unit in the β -turn is hydrated in both the peptides, a commonly observed feature in proteins and peptides.¹² In principle, in the *D-L-D/L-D-L* sequences, isolated β -turns of the *DL* or *LD* types are possible.¹³ While one type has been established for Xxx = Val, the other is observed for Xxx = Leu. The crystal structure of the racemate **3/4** reveals a molecular conformation that is almost identical to that observed in the enantiomers.

Conformational Analysis of Peptide **5/6**

In the case of Xxx = Phe, crystals could only be obtained for the racemate **5/6**. The asymmetric unit contains a single peptide molecule which adopts an open structure lacking any intramolecular hydrogen bond. Both the prolines adopt a semi-extended backbone conformation with the Phe residue adopting an extended conformation as shown in Figure 3. Both the water molecules are intermolecularly hydrogen bonded to C=O belonging to Piv group and Pro(**1**). Notably, this structure is remarkably similar to that of peptide **1** in its backbone conformation. A list of all intra- and inter-

molecular hydrogen bonds in all the peptides that have been discussed in this paper are listed in Table II.

CONCLUSIONS

This study was undertaken to explore the possibility of trapping distinct conformational segments of the diproline segments in crystals by generating racemic centro-symmetric crystals in which packing effects may be appreciably different from those observed in the crystals of individual pure enantiomeric peptides. In the case of the peptide Piv-Pro-Pro-Leu-NHMe, both the enantiomers and the racemate yielded mirror image conformations. In contrast, in the related sequence Piv-Pro-Pro-Val-NHMe, the two enantiomeric peptides crystallized in two different polymorphic forms. In one case an anhydrous crystal was obtained while in the other case the crystal structure contains trapped water molecules. The crystal structures of the peptides **1** and **2** may be considered as an example of "pseudo polymorphism" resulting from solvation. This example illustrates the difficulties in controlling crystallization conditions precisely when multiple modes of crystal nucleation are possible. The case of the peptides **5** and **6** is an interesting example where the individual enantiomers were recalcitrant to crystallization, while single crystals were readily obtained in the case of the racemate **5/6**. The ability of the racemates to pack more effectively than the pure enantiomers is a feature that has been recognized even in Kitaigorodski's classic analysis of molecular crystals.¹⁴ The conformational diversity of heterochiral Pro-Pro sequences, is clearly demonstrated by the examples discussed above. A *D-L-D* sequence (Xxx = ^DAla) has been used as a linking three residue loop in a designed β -hairpin peptide.⁹ In the present study, conformational variations are observed for different Xxx groups. Even more importantly, conformational differences are established for enantiomeric peptides and racemates. These results suggest that the energetic differences between these states is small. Conformational choice can therefore be readily influenced by environmental and sequence effects.

B. Chatterjee was supported by a Senior Research Fellowship of the University Grants Commission.

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