X-ray Structure of [D-Pen^2,D-Pen^5]enkephalin, a Highly Potent, δ Opioid Receptor-Selective Compound: Comparisons with Proposed Solution Conformations

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Abstract: [D-Pen^2, D-Pen^5]enkephalin (DPDPE), a cyclic, constrained, highly potent, and δ opioid receptor-selective analogue of enkephalin, has been obtained from an aqueous solution in a crystalline form suitable for X-ray analysis. It crystallizes in the triclinic space group $P1$. The unit cell contains three conformationally distinct molecules of DPDPE which are located with approximate 3-fold symmetry about a water channel made up of approximately 24 disordered and one ordered water molecules. There are also 13 ordered water molecules which form an intricate network of hydrogen bonds which hold the peptide molecules together in the crystal. The conformation of the 14-membered ring is essentially identical for all three molecules; however, the Tyr-1 residue is conformationally different in each case. Comparison of the conformations found in the crystal with those previously determined by NMR methods in conjunction with energy calculations indicates that the most favorable conformation of the 14-membered ring in aqueous solution is similar to that in the crystal. This was interpreted to be due to the cyclic constraint in DPDPE and the high degree of solvation in the crystal structure. In addition, low-energy conformations previously determined by computational methods in attempts to determine the binding conformations of DPDPE gave conformations of the 14-membered rings which were generally similar to those found in the crystal structure. These results and previous structure–activity relationships suggest that the solid-state conformations are a useful starting point for understanding the bioactive conformation important for biological activity and δ receptor selectivity of cyclic enkephalin analogues.

Introduction

A central goal of modern structure–biological activity studies of peptide and protein hormones, neurotransmitters, growth factors, and other such chemical messengers is the development of analogues with high potency and selectivity for a particular receptor of these ligands.[1-4] Often the native peptide or protein is not very selective, and hence changes in the structure and especially the conformation and/or topography are necessary to obtain highly selective and potent analogues.[2-6]

A case in point is the opioid peptides and their receptors. It was postulated many years ago that there are multiple opioid receptors of which the μ, δ, κ, and ε receptors are the most commonly accepted.[7-10] In general, the endogenous mammalian opioid peptides including [Met^5]enkephalin, [Leu^5]enkephalin, dynorphin, β-endorphin, and related peptides are not very selective (<30-fold) at any of the opioid receptors.[11] Hence systematic, rational approaches for the design of potent and selective analogues are needed. For this purpose, the application of conformational and more recently topographical constraints has become of central importance,[6,12,13] and numerous successful applications of this approach have been reported. One of the early successes of this approach was the development of the highly potent and δ opioid receptor-selective enkephalin analogue [D-Pen^2,D-Pen^5]enkephalin (H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (**1**), DPDPE, Figure 1; Pen = penicillamine, β,β-dimethylcysteine).[14] In this analogue, the Gly^2 and Met^5 (or Leu^5) residues of the native enkephalin were replaced by the bulky β,β-disubstituted residue penicillamine, and the 14-membered highly constrained ring system was formed. For high potency and selectivity, it is important to note that replacement of Gly^2 with a D-amino acid residue was necessary, whereas in the 5 position both the L and D amino acid could be used, though the D-Pen^5 analogues were found to be more selective than the L-Pen^5 analogues.[14-16] Since this discovery, more potent

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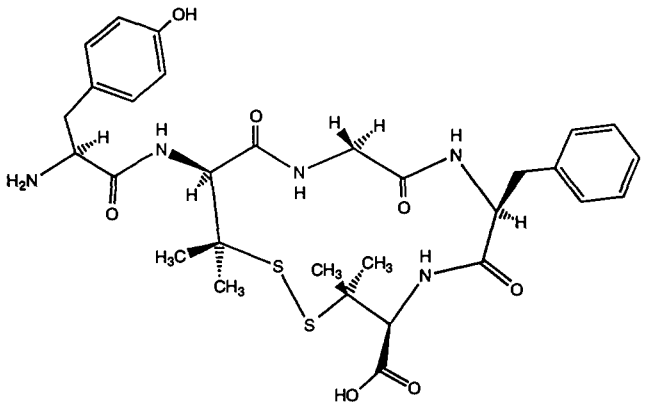

H - Tyr - D-Pen - Gly - Phe - D-Pen - OH

DPDPE 1

Figure 1. Structure of [D-Pen^2,D-Pen^5]enkephalin.

and selective cyclic enkephalin analogues have been designed (e.g., see refs 17–20). In addition, the discovery of the highly δ selective linear deltorphins[21] (e.g., H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 = deltorphin I, DTI) and dermenkephalin[22] (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2) have been reported, and it has been shown recently that DPDPE and DTI each interact with a unique functional subtype of δ receptor (δ_1 and δ_2).[23,24]

Several efforts have been made to determine the conformation of DPDPE in solution,[25-29] but thus far no consensus conformation has been reached, though all suggested conformations have common features. We report here the X-ray crystal structure of the highly potent and selective δ opioid receptor ligand [D-Pen^2, D-Pen^5]enkephalin and compare and contrast the three different conformations found in the crystal with the conformations previously suggested in the literature for the solution conformation and for the "bioactive conformation".

Methods

General. DPDPE was obtained as a lyophilized powder from Multiple Peptide Systems (San Diego, CA) or was synthesized and purified as previously reported.[14] Purity was assessed using high-pressure liquid chromatography and thin-layer chromatography as previously reported.[14]

X-ray Analysis. A stock solution of DPDPE was prepared by dissolving 5 mg of the peptide in 0.25 mL of deionized water. Crystallization trials, using hanging drop vapor diffusion techniques, were then performed utilizing a commercial screening kit (Crystal Screen, Hampton Research, Riverside, CA). After a period of several weeks, small crystals appeared in the drop containing a 1:1 mixture of the stock peptide solution and the crystal screen reagent containing 0.2 M $(NH_4)_2SO_4$, 0.1 M sodium cacodylate (pH 6.5), and 20% PEG 8000. Several macroseeding experiments (using a 1:1 40-μL sitting drop) were then set up using varying PEG concentrations. The crystals used for data collection grew over a period of approximately 30 days in the drop containing 25% w/v PEG 8000. The data crystal was transferred from the sitting drop into high-viscosity microscope oil (Type NVH, Cargille). It was then mounted on a glass rod coated with stopcock grease (Lubriseal, Thomas Scientific) while still in the oil and transferred immediately to the cold stream (–60 °C) of an automatic 4-circle Siemens R3m/V diffractometer for data collection. The cell dimensions, given in Table 1 together with other relevant crystal data, were determined from a least-squares refinement of the angular positions for 32 reflections with 2θ values ranging from 40.0 to 78.0°. The diffractometer, equipped with a graphite monochromator, was used in the θ/2θ scan mode with a constant 2θ scan speed of 30 deg/min for data out to $2\theta_{max}$ of 100° and 15 deg/min for data from 100 to 120°. Three standard reflections repeated after every 97 reflections showed a variance of ±2.5%, indicating that the crystal did not deteriorate during data collection. The data were corrected for Lorentz and polarization effects. Empirical absorption corrections were not applied due to the fact that the low-temperature apparatus severely limited the amount of accessible absorption data.

Table 1. Summary of Crystal Data Parameters

molecular formula (DPDPE)	$C_{30}H_{39}N_5O_7S_2$
unit cell contents	$3(C_{30}H_{39}N_5O_7S_2)\cdot 14H_2O\cdot 23.5H_2O$
crystal size, mm	0.55 × 0.35 × 0.65
crystal system	triclinic
space group	$P\bar{1}$
a, Å	12.768(5)
b, Å	17.777(5)
c, Å	18.122(6)
α, deg	107.58(2)
β, deg	104.92(3)
γ, deg	107.44(2)
cell volume, Å^3	3456.5(2)
density (calcd), g/mL	1.25
absorption coefficient, mm^{-1}	1.69
$F(000)$	1401
radiation	Cu Kα (λ = 1.541 78 Å)
θ range for data collection, deg	2.8–59.33
resolution, Å	0.9
reflections collected	10 496
independent reflections	10 144 (R_{int} = 0.023)
no. of parameters refined	1885
no. of restraints	366
final R indices for 9037 reflections with $(I > 2\sigma I)$	$R1$ = 0.085 w$R2$ = 0.219
final R indices (all data – 9802 reflections)	$R1$ = 0.092 w$R2$ = 0.231
final R_{free} (338 reflections)	$R1$ = 0.114 w$R2$ = 0.285
goodness-of-fit on F^2	1.03
largest difference peak and hole	0.84 and –0.64

Structure Solution and Refinement. The structure was solved by direct methods using the program SHELX86.[30] All non-hydrogen atoms in the three independent peptides molecules and several of the ordered water molecules were apparent in the E map. There are 14 ordered (full occupancy) waters in the asymmetric unit, 13 of which are associated with the peptide molecules. The remaining water molecules lie in a channel which is surrounded by but does not interact with either the peptide molecules or their associated water molecules. All the waters in the channel except one are disordered. Fifty-seven disordered solvent positions were identified within the channel. The full occupancy water sits in the middle of the channel where it can form hydrogen bonds to several of the disordered waters. The structure was refined using full-matrix blocked least-squares on F^2 values using the program SHELXLS93[31] on the full set of 9802 independent reflections. Coordinates and anisotropic thermal parameters were refined for the three peptide molecules and the 14 ordered (full occupancy) water molecules. For the disordered water molecules, coordinates, occupancies, and anisotropic thermal parameters were refined;

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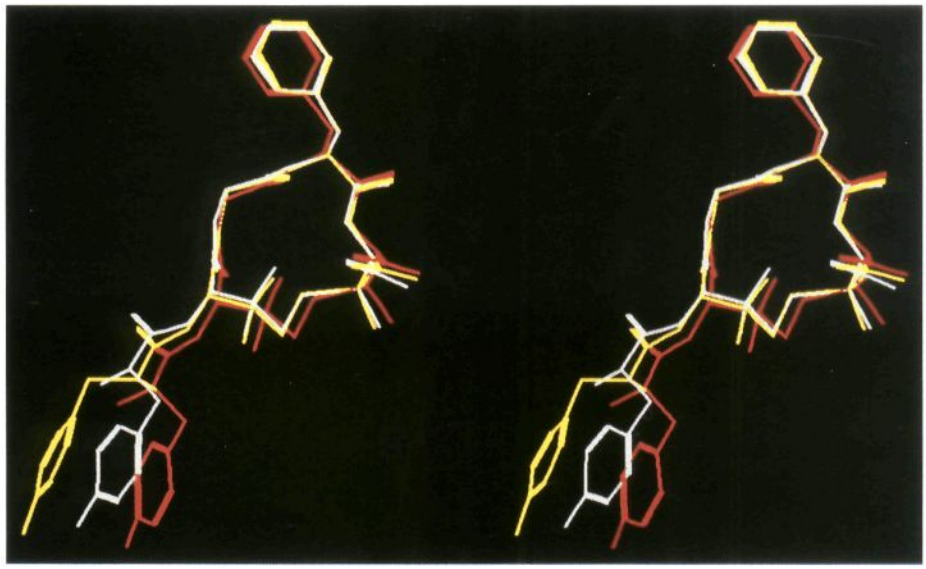

Figure 2. Comparison of the three independent peptide conformations found in the X-ray study of DPDPE. Molecule 1, red; molecule 2, yellow; molecule 3, white. A least-squares fit for the 14 ring atoms gave an average root-mean-square deviation of 0.088 Å for molecule 1 fitted to molecule 2, 0.076 Å for molecule 1 fitted to molecule 3, and 0.093 Å for molecule 2 fitted to molecule 3.

however, the thermal parameters were constrained to be as spherical as possible. The aromatic rings of the Tyr¹ and Phe⁴ residues were restrained to be planar. Hydrogen atoms on the peptide molecules were put in at calculated positions and were allowed to ride on their covalently bonded atoms (C–H = 0.98, N–H = 0.89, and O–H = 0.82 Å). Isotropic hydrogen thermal parameters were reset at the end of each refinement cycle to be equal to 1.1 × the U_{eq} value of their covalently bonded atoms ($1.2U_{eq}$ values for methyl hydrogens). Tables of atomic coordinates for all atoms and bond lengths, bond angles, and anisotropic thermal parameters for non-hydrogen atoms have been deposited as supplementary material.

The presence of a water channel which accounts for ~16% (by weight) of the unit cell contents is somewhat unusual in a small molecule structure, and there are no well-established procedures for handling least-squares refinement of the water molecules within the channel. For this structure, water molecules were added at positions indicated by the highest difference map peaks, and both their occupancies and thermal parameters were allowed to vary, at times together in the same refinement cycle and at times in alternating cycles. Water molecules were eliminated if their occupancy went too low (<0.15) or if their thermal parameters went to high (U_{eq} > 0.25). New waters were then added from subsequent difference maps, and the procedure was continued until it was not possible to add new water molecules which would refine to reasonable values. At this point the highest difference peaks were not in the channel but were close to sulfur atoms in the DPDPE molecules. Two tests were performed to assess the reasonableness of the solvent channel model. To assure that the sum of occupancies contributing to each water site was not greater than 1.0, the atoms within the van der Waals radius of each water site were identified and their overlapping volumes calculated. The occupancy at any given site was then calculated by summing the fractional volume of the "main" water molecule and the overlapping volumes of the "satellite" water molecules, which were weighted by their respective occupancy values. No sites in the final model exceeded full occupancy. In addition, all the disordered water was omitted from the channel, and a void volume analysis was performed using the program PLATON,[32] which indicated space for approximately 24 water molecules in addition to the 14 fully occupied sites. The sum of the occupancies for the disordered water molecules in the channel is 23.5. As a further check on the reasonableness of the overall refinement, an *R*-free was calculated after each refinement cycle. *R*-free is the *R*-factor for a set of 338 reflections (every 30th one in the data set) which were omitted from the refinement. The final *R*-factors are given in Table 1.

Results and Discussion

Conformational Comparisons. The three X-ray conformations of DPDPE reported here were compared with previous literature conformations of DPDPE found by NMR in aqueous and DMSO solution and by theoretical calculations (see Results and Discussion section for literature citations). All DPDPE conformations considered were converted into MacroModel[33] file format using the in-house (UofA) software INTMMOD, which translates internal coordinate input (generally dihedral angles) into MacroModel formatted Cartesian coordinates. The X-ray and literature DPDPE conformations were then visualized and compared interactively by atomic root-mean-square superimposition in MacroModel v. 3.6 on Iris 4D/20G+ workstation. Multiple structure comparison was also achieved by linear least-squares fitting of atomic superimposition using the rapid convergence algorithm of Sippl and Stegbucher,[34] which was adapted for MacroModel file format.

Peptide Conformation. There are three independent molecules of DPDPE in the asymmetric unit. The conformation of the 14-membered ring and the orientation of the Phe⁴ side chain is essentially the same in all three molecules (see Figures 2 and 3 and Table 2). The largest differences between the three molecules lie in the orientation of the Tyr¹ residue. None of the atoms in Tyr¹ lie over or are bonded to the 14-membered ring. Therefore, it has much greater torsional freedom than the remaining four residues, all of which are constrained to a considerable degree by the ring closure.

The amide bond of Tyr¹ (Clα–Cl′–N2–C2α) is *trans* in all three molecules, but the rotation about the Clα–Cl′ bond (described by the ψ torsion) is –157, +9, and –175° for molecules 1, 2, and 3, respectively (Table 2). The difference in rotation of 18° between molecules 1 and 3 does not significantly affect the overall conformation of the peptide molecule itself. However, in molecule 2, the ψ torsion is much different than that found in the other two molecules, and N1 is *trans* to the carbonyl oxygen (C=O¹) and *cis* with respect to N2 such that the N···O and N···N distances are the reverse of what they are for all the other residues (N··O = 3.61 Å). The close proximity of the Tyr¹ NH_3^+ moiety and the D-Pen² peptide amide NH group, as well as the fact the NH_3^+ group is directed in toward a predominantly hydrophobic portion of the molecule, may explain why only one of the four polar hydrogens involved participates in a hydrogen bond. The distance between the centers of the aromatic rings is 15.0 Å for molecules 1 and 3 and 15.9 Å for molecule 2. In proteins, the torsion angle about the S–S bond in a Cys-Cys bridge is found in both the + and – chirality and usually within the range

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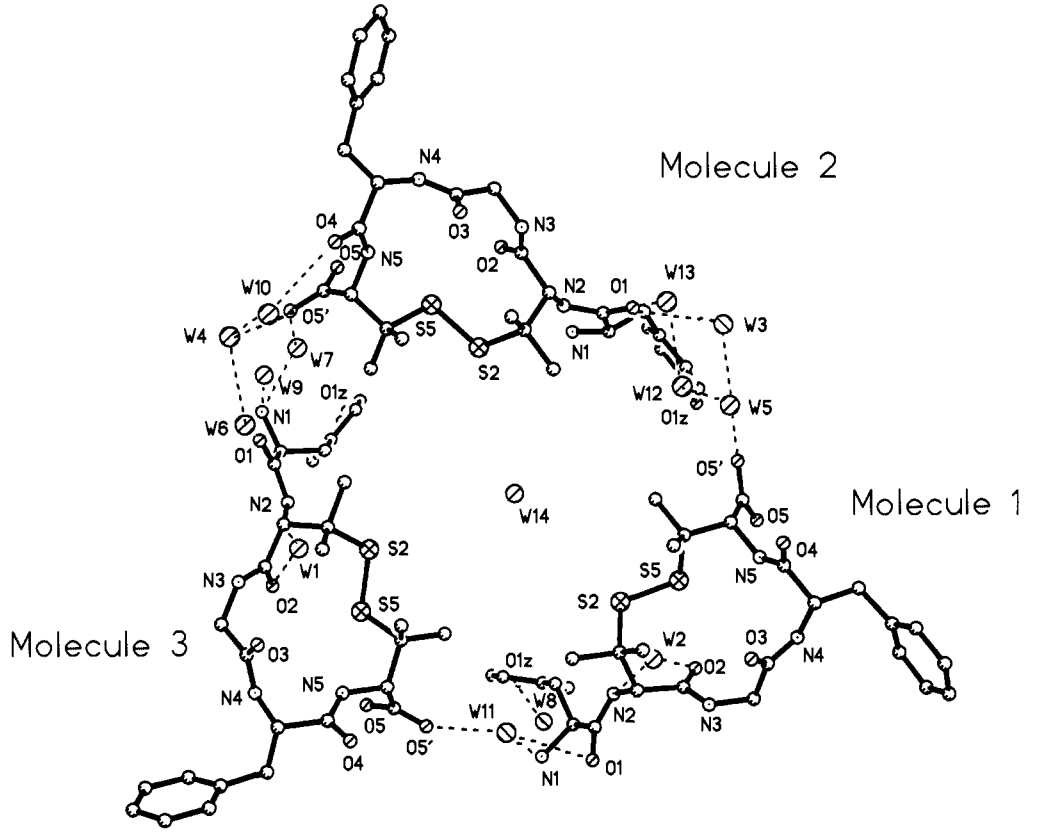

Figure 3. Three independent molecules of DPDPE and the ordered water molecules. The figure was drawn from the experimentally determined coordinates and shows the peptide–solvent hydrogen bonds that link the peptide molecules. W14 is the only fully occupied water site in the solvent channel.

Table 2. Experimentally Determined Structures of DPDPE: Torsion Angles Found for the X-ray Crystal Structure in the Three Molecules of DPDPE in the Unit Cell and for Proposed Conformations in Water Based on Aqueous NMR and Energy Calculations in Previous Studies

residue	torsion angle	molecule 1	molecule 2	molecule 3	Nikiforovich et al.[a]	Hruby et al.[b]	Mosberg et al.[c]
Tyr[1]	ψ	−157	9	−175	149	164	163
	ω	−179	172	−175	−175	175	−177
	χ^1	−68	−71	−61		163	−173
	χ^2	118	99	127		51	−115
D-Pen[2]	ϕ	110	129	129	135	111	149
	ψ	−147	−152	−145	−143	14	−153
	ω	−171	−175	−173	−177	173	−175
Gly[3]	ϕ	98	107	99	78	−75	78
	ψ	−141	−138	−138	−72	−41	−111
	ω	−178	−180	−178	−170	−177	−164
Phe[4]	ϕ	−74	−76	−81	−67	−94	−85
	ψ	−36	−30	−18	−53	−50	38
	ω	−175	−175	−170	−169	−177	172
	χ^1	−67	−67	−69		−60	−64
	χ^2	−85	−80	−85		102	105
D-Pen[5]	ϕ	126	116	106	127	87	61
S–S bridge	χ^1	−58	−59	−54		−180	−78
	χ^2	−73	−73	−74		143	178
	S–S	−105	−108	−104		110	110
	χ^2	174	176	168		119	60
	χ^1	−51	−46	−52		−70	−87

[a] From ref 27, Table 3, conformer 4. [b] From ref 25, Table 6, conformer 2′. [c] From ref 26, Table 8, conformer iii.

of 80–100°.[32] Values found in cyclic cystine peptides have ranged from −87 to −101° and from +82 to +100°.[35,37] In the two cyclic Cys-Cys peptides with 14-membered rings reported previously,[38,39] the S–S torsion angle was 82°. In this study, the first in which the S–S bridge is formed by two D-Pen residues, the S–S torsions are −105, −108, and −104° for the three molecules in the asymmetric unit (Table 2), which is greater than the expected ±90(10)°.[40]

Packing of the DPDPE Molecules in the Crystal. The 14-membered rings of the three molecules of DPDPE which are found in the asymmetric unit are related by a pseudo-3-fold axis which disposes the molecules "symmetrically" about the water channel (Figure 4). The plane which passes through the centers of the three peptide rings can be used as a reference point to describe the crystal packing. The peptide rings are tilted

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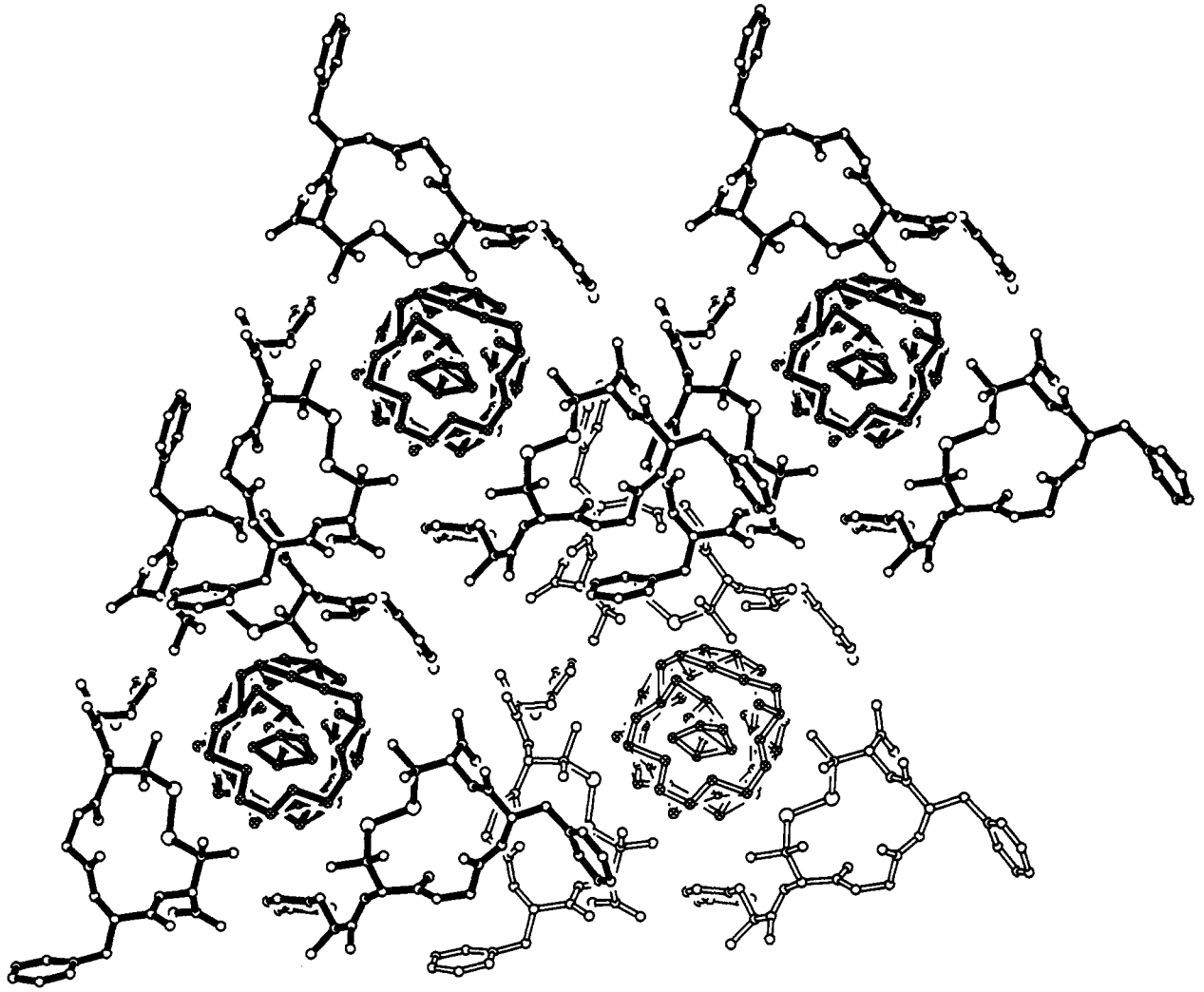

Figure 4. Packing of DPDPE molecules around the solvent channel. Four units cells, viewed looking down the *a* axis of the unit cell, are shown. The asymmetric unit is shown with hollow bonds, and the other cells are generated by unit cell translations along either the *b* or the *c* axis.

approximately 20° out of the orientation plane, and the centers of the rings range from 7.0 to 8.1 Å from the center of the channel. The hydrophobic sulfur-containing bridges lie above the plane and face inward toward the channel, forming the narrow part of the channel boundary (distances across the channel in this area range from approximately 9.5 to 11.0 Å). The closest intermolecular approaches around the edges of the channel are between the D-Pen methyl groups, with approaches of 3.7 Å between molecules 2 and 3, 4.3 Å between molecules 1 and 3, and 5.6 Å between molecules 1 and 2. The channel boundary is completed by the Tyr¹ side-chain groups which extend up and away from the plane and are approximately perpendicular to it (angles between the planes of the aromatic rings and the orientation plane are 77, 96, and 92° for molecules 1, 2, and 3, respectively). This is also the widest part of the channel, with distances across the channel of up to 14 Å. The Gly³-Phe⁴ edge of the peptide ring lies below the orientation plane and is directed away from the water channel. The Phe⁴ side-chain groups also are extended away from the peptide rings and the water channel.

There are no intramolecular hydrogen bonds in the DPDPE molecules nor are there any peptide–peptide hydrogen bonds connecting those molecules chosen as the asymmetric unit which are linked to one another only by water molecules (Table 3, Figure 3). Molecules 1 and 3 are linked by a single water molecule, W11, which donates to the CO^- of molecule 3 and the $C{=}O^1$ of molecule 1 and accepts a hydrogen from the NH_3^+ of molecule 1 (Table 3). Molecules 2 and 3 also are linked by a single water molecule, W7, which donates to the CO^- of molecule 2, and accepts from the NH_3^+ of molecule 3. There also is a three-water bridge linking the $C{=}O^4$ of molecule 2 and the $C{=}O^1$ of molecule 3 ($C{=}O$–W10–W4–W6–$C{=}O$). Molecules 1 and 3 are linked by a two-water and a three-water bridge, both of which link CO^- of molecule 1 to $C{=}O^1$ of molecule 3 (CO^-—W5—W3—$C{=}O$ and CO^-–W5–W12–W13–$C{=}O$). In space group *P*1, the only symmetry operations allowed are unit cell translations. The water channel boundary is fully defined when the asymmetric "layers" are translated along the *a* cell direction to form columns (Figure 5). The latter are linked in this direction through water bridges joining each Tyr OH to two independent DPDPE molecules in the layer above it. The OH of molecule 1 links to the NH_3^+ (molecule 1′) through a W12–W5 bridge and to the $C{=}O^1$ (molecule 2′) through a W12–W13 bridge; and the OH of molecule 3 links to the $C{=}O^1$ (molecule 3′) through W6 and to the $C{=}O^5$ (molecule 2′) through a W6–W4 bridge.

The peptide/water columns translate along both the *b* and *c* cell directions to complete the packing model (Figure 4). It is only between these columns that any peptide–peptide hydrogen bonds are found. There are five NH···O=C bonds among the 19 intermolecular hydrogen bonds that link the columns into a full three-dimensional entity (Table 3). None of the N5 hydrogens participate in hydrogen bonds interconnecting the DPDPE molecules and the ordered water system. Griffin and Smith have reported[41] that there seem to be some common patterns of solvation in the solid state enkephalin structures. They found that the CO^- and OH always bind to water and that $C{=}O^4$ quite often does. The pattern continues in this structure with only the $C{=}O^4$ of

(41) Griffin, J.; Smith, G. D. *Opiod Peptides: An Update*; NIDA Research Monograph 87; National Institute of Drug Abuse: Rockville, MD, 1988; pp 48–56.

Table 3. Hydrogen Bonds Found in the Crystal Structure of DPDPE

donor	acceptor	symmetry of acceptor	distance (Å)
		Peptide–Peptide	
N1(2)	C=O⁴(3)	$x-1, y-1, z$	3.08
N3(2)	C=O⁵(3)	$x, y-1, z-1$	2.73
N4(2)	C=O³(1)	$x-1, y-1, z-1$	2.86
N1(3)	OH(2)	$x, y, z-1$	2.81
N3(3)	C=O⁵(1)	$x, y, z-1$	2.75
N4(3)	C=O³(2)	$x, y+1, z$	2.89
		Peptide–Solvent	
N1(1)	W3	$x-1, y+1, z$	3.00
N1(1)	W9	$x+1, y+1, z+1$	2.87
N1(1)	W11		2.80
N1(1)	W13	$x, y+1, z$	3.21
N2(1)	W2		2.97
N3(1)	C=O⁵(2)	$x+1, y+1, z+1$	2.76
N4(1)	C=O³(3)	$x, y, z+1$	2.85
N1(3)	W7		2.68
N1(3)	W9		2.86
N2(3)	W1		2.82
OH(1)	W8		2.71
OH(2)	W12	$x-1, y, z$	2.63
OH(3)	W6	$x-1, y, z$	2.74
W1	C=O⁴(1)	$x-1, y, z-1$	2.75
W1	C=O²(3)		2.73
W2	C=O²(1)		2.72
W2	C=O⁴(2)	$x, y+1, z+1$	2.83
W3	C=O¹(2)		2.95
W4	CO⁻(2)		2.81
W5	CO⁻(1)		2.70
W6	CO⁻(1)	$x, y-1, z$	2.67
W6	C=O¹(3)		2.84
W7	C=O¹(1)	$x-1, y-1, z-1$	2.77
W7	CO⁻(2)		2.83
W8	CO⁻(2)	$x+1, y+1, z+1$	2.88
W10	C=O⁴(2)		3.03
W11	C=O¹(1)		2.94
W11	CO⁻(3)		2.63
W13	C=O¹(2)		2.78
W13	CO⁻(3)	$x, y+1, z+1$	2.67
		Solvent–Solvent	
W3	W4	$x, y+1, z+1$	2.86
W4	W6		2.81
W4	W10		2.82
W5	W3		2.79
W8	W11	$x-1, y, z$	3.20
W9	W5	$x-1, y, z$	2.77
W9	W10	$x-1, y, z$	3.03
W12	W5		2.84
W12	W13		2.78

molecule 3 not hydrogen bonded to a water molecule. It is involved in the only hydrogen bond formed by the NH_3^+ moiety of molecule 2.

W14, the only fully occupied water site in the channel, sits in the middle of the channel at the center of the centroids of the three Tyr aromatic rings. Forty-five of the 57 remaining water in the channel form a "sphere" surrounding W14. There are nine sites on the inside "surface" of the sphere that are less than 3.0 Å from W14 and an additional six sites that lie within 3.0–3.2 Å from W14. There are many different ways to construct hydrogen bonding patterns involving W14 and the waters on the sphere. The narrow portion of the solvent channel passes through that part of the channel defined by the S–S bridge side chains. The water channel propagates through the cell by hydrogen bonds joining the bottom of the channel neck to the top of the solvent sphere in the unit cell below it, giving the overall appearance of an extended dumbbell. There are no contacts less than van der Waals approaches between any of the water sites in the solvent channel and either the peptides molecules or their associated water molecules (Figures 4 and 5).

Comparison of the X-ray Structures of DPDPE with Other Proposed Conformations for DPDPE. As already mentioned, the conformations of all three independent molecules in the X-ray structure of DPDPE are quite similar. Figure 2 shows the conformations of the 14-membered ring, and the orientation of the Phe⁴ side chain is essentially the same in all three molecules. The only significant difference is the orientation of the Tyr¹ residue in molecule 2. Due to a rotation of approximately 180° around the C1$^\alpha$–C1′ bond, the ψ^1 torsion angle is 9° in molecule 2 as opposed to –157° and –175° respectively for molecules 1 and 3.

Three studies have used a combination of NMR parameters and conformational calculations to arrive at suggested low-energy

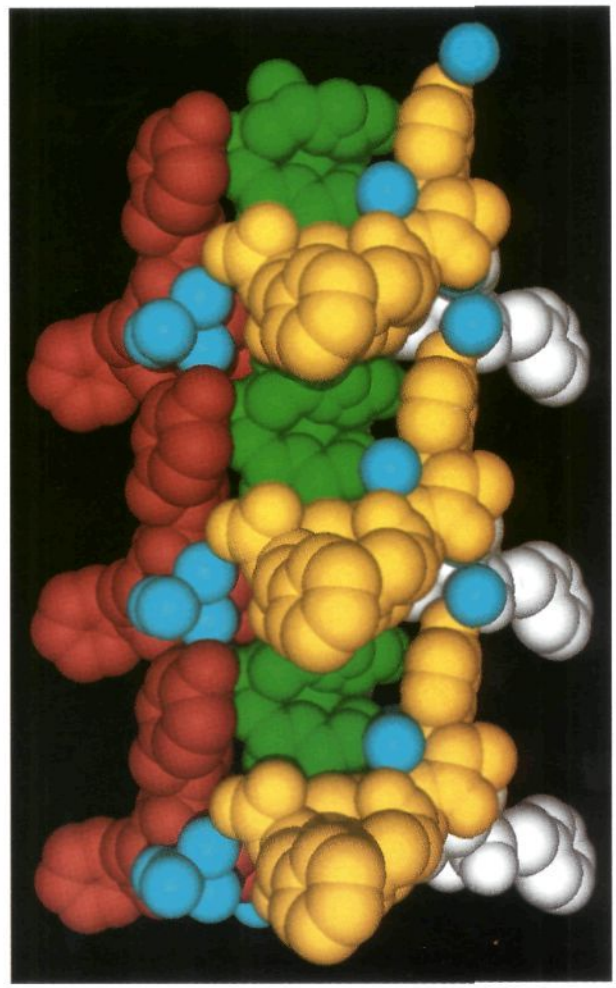

Figure 5. Stacking of DPDPE molecules around the solvent channel. This view is perpendicular to that shown in Figure 4 and represents three unit cells generated by translating the asymmetric unit along the *a* axis. The disordered waters are shown in green, and ordered waters are blue. The three independent peptide molecules are shown in red, white, and yellow.

Table 4. RMS (Å) Deviation of Three X-ray Crystal Structures of DPDPE Compared with Several Proposed Conformations of DPDPE Found by Solution NMR[a]

	X-ray structures		
source of DPDPE conformer	1	2	3
Nikiforovich et al.[27]			
structure 4	1.26	1.23	1.23
Hruby et al.[25]			
structure 1	1.47	1.47	1.46
structure 1′	1.91	1.92	1.93
structure 2	1.43	1.46	1.44
structure 2′	2.06	2.07	2.08
Mosberg et al.[26]			
structure i	1.07	1.83	1.05
structure ii	1.70	2.35	1.71
structure iiia	0.509	1.40	0.495
structure iiib	0.770	1.52	0.770

[a] RMS overlay achieved using all α and β carbon atoms.

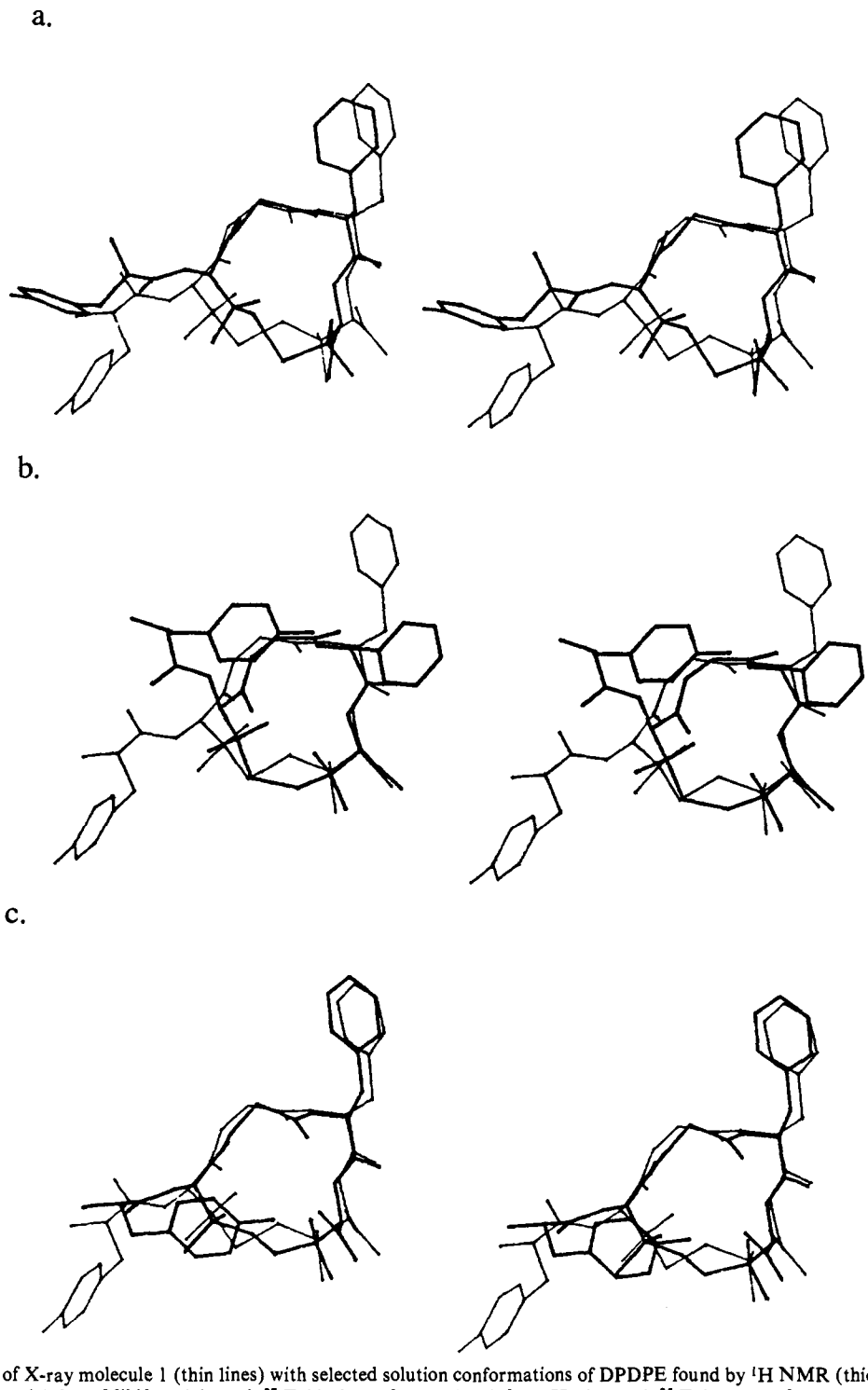

Figure 6. Comparison of X-ray molecule 1 (thin lines) with selected solution conformations of DPDPE found by ^{1}H NMR (thick lines). The solution NMR conformations are (a) from Nikiforovich et al.,[27] Table 3, conformer 4; (b) from Hruby et al.,[25] Table 6, conformer 2; and (c) from Mosberg et al.,[26] Table 8 conformer iiib. RMS deviations were determined by a fit of the corresponding α and β carbon atoms of the ring residues and are given in Table 4.

conformations for DPDPE in aqueous solution[25,26,27] and in DMSO solution.[27] Only the latter study considered all relevant NOEs, coupling constants, and other parameters for backbone conformations. All studies concentrated primarily on the conformation of the 14-membered ring. The relationship of the Phe⁴ residue side chain to the 14-membered ring was also considered in detail by Nikiforovich and co-workers.[27] Given the highly solvated nature of the molecules in the X-ray structure, it can be assumed that they provide a reasonable benchmark by which to compare the solution conformations, particularly for the 14-membered ring. As can be seen in Table 4, the root-mean-square deviations of the NMR-derived structures relative to X-ray structures were generally between 0.5 and 2.0 Å for the studies of Hruby et al.[25] and Mosberg et al.[26] This indicates that there are some differences in the conformations of the 14-membered ring of DPDPE among the solution conformations suggested previously, but some similarities as well. Nikiforovich et al.[27] found the most similar solution structures to the X-ray results. This is clearly demonstrated by comparison of the backbone ϕ, ψ, and ω torsional angles shown in Table 2 for the X-ray structure of 1 and conformation 4 of Nikiforovich et al.[27] Figure 6 illustrates, using stereo structures, comparison of the X-ray structure 1 and the three NMR conformations given in Table 2. Differences exist in the side-chain conformations of Tyr¹ and Phe⁴, and in the disulfide region as well.

Comparison with the X-ray conformations demonstrates the

Table 5. Torsion Angles for the X-ray Crystal Structure of Two Conformations of DPDPE and Low-Energy Conformations Suggested from Conformational Calculations

residue	torsion angle	molecule 1	molecule 2	Chew et al.[a]	Wilkes et al.[b]	Froimowitz[c]	Nikiforovich et al.[d]
Tyr[1]	ψ	−157	9	−53	−55	−57	142
	ω	−178	172	179	−176	176	−179
	χ^1	−68	−71	176	164	56	−180
	χ^2	118	99	−117	45	105	62
D-Pen[2]	ϕ	110	129	65	63	62	80
	ψ	−147	−152	−153	−150	39	−145
	ω	−171	−175	−172	179	179	174
Gly[3]	ϕ	98	107	84	85	−94	66
	ψ	−141	−138	55	−142	−77	27
	ω	−178	−180	180	−177	−172	175
Phe[4]	ϕ	−74	−76	−66	−65	−86	−157
	ψ	−36	−30	−65	−39	−47	−57
	ω	−175	−175	−176	−176	−171	179
	χ^1	−67	−67	−57	67	57	−75
	χ^2	−85	−80	116	83	93	−64
D-Pen[5]	ϕ	126	116	57	142	130	126
	χ^1	−58	−59	−177	−48	−63	−66
	χ^2	−75	−73	−152	−55	179	
S–S bridge	S–S	−105	−108	−100	−117	112	−146
	χ^2	174	176	−48	−179	71	
	χ^1	−51	−46	68	−58	−76	173

[a] From ref 28, Table 2. [b] From ref 45, Table 1, conformer DK 11.85. [c] From ref 43, Table 7, conformer 1. [d] From ref 42, Table 7, conformer 1.

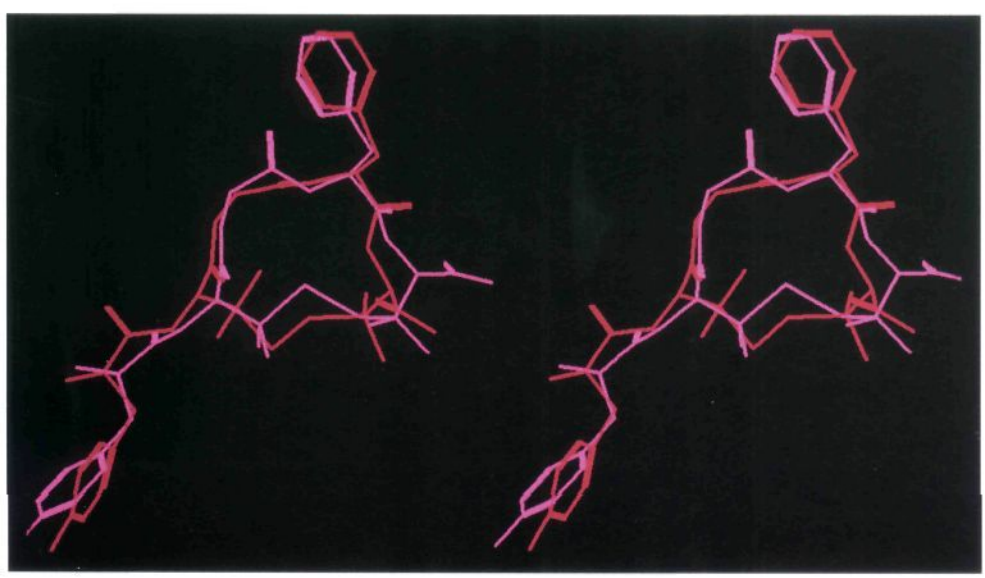

Figure 7. Comparison of the pharmacophores of X-ray conformation 1 (red) with the putative δ-receptor binding conformation of DPDPE (purple) predicted by Nikiforovich et al.[42] (see Table 5). The RMS deviations of the binding conformation when fitted to the three X-ray molecules are given in Table 6.

accuracy of the combination of NMR with energy calculations in the elucidation of the conformations of small peptide systems which are inherently flexible. The conformation ensemble in solution can produce the observation of weak or no NOEs or conformationally conflicting NOEs which are due to less populated, folded structures. This is demonstrated by the conformations found in solution for Tyr[1] by Hruby et al.[25] and Mosberg et al.[26] (Figures 6b and 6c, respectively). In each case, the Tyr[1] side chain lies over the D-Pen[2] methyl groups. Hruby et al.[25] found this interaction due to the observation of weak NOEs between these side chains, and in both cases subsequent gas-phase energy minimization may have overexaggerated intramolecular contacts such as this. In either case, the Tyr[1] residue in solution is probably highly flexible and can adopt many different conformations, as indicated by the X-ray results.

Some regions of similarity are clear between the X-ray and NMR structures. The Phe[4] side chain is located in the gauche (−) orientation in all of the solution structures, as was found for the X-ray results (Table 2, Figure 6). Given the flexibility of the 14-membered ring, the NMR and X-ray structures exhibit general conformational similarities, particularly about the Gly[3]-Phe[4]-D-Pen[5] region. However, comparison of the disulfide regions in all cases shows considerable differences (Table 2, Figure 6).

Several attempts have been made to arrive at the likely conformational properties of DPDPE using various computational energy calculation methods without inclusion of experimental constraints.[28,42-45] The availability of an X-ray structure provides an opportunity to compare conformations suggested from such calculations with the X-ray crystal structure conformations. In Tables 5 and 6, molecules 1 and 2 (Table 2) of the X-ray structure are compared with low-energy conformations of DPDPE obtained by various search strategies in the gas phase. All of the calculated

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(45) Wilkes, B.-C.; Schiller, P. W. *J. Comput.-Aided Mol. Des.* **1991**, *5*, 293–302.

Table 6. Root-Mean-Square (Å) Deviations of Proposed Pharmacophore Structure Components of DPDPE with Those Found in the X-ray Analysis[a]

	X-ray Structures		
source of DPDPE conformer[a]	1	2	3
Chew et al.[28] Table 2, DPDPE	2.48	0.85	2.52
Wilkes et al.[45] Table 1, conformer DK 11.85	2.13	2.42	2.13
Froimowitz[43] Table 7, conformer I	4.30	1.16	4.39
Nikiforovich et al.[42] Table 7, conformer I	0.791	1.55	0.792

[a] The pharmacophore components for DPDPE are the Tyr¹ Nα Tyr and Phe⁴ aromatics and *D*-Pen² Cα.

compounds except that of Froimowitz (Table 6) have similar ring conformations, while most of them differ significantly from the Tyr¹ residue found in the X-ray crystal structure. The backbone conformation for the Phe⁴ residue is similar in all of the calculated conformations and compares favorably with those found by X-ray (Table 6, Table 2). In addition, except for Wilkes et al.[45] and Froimowitz,[43] who find the Phe⁴ side-chain conformation to be gauche (+), all of the other studies found the Phe⁴ side chain to have a gauche (−) conformation, as observed in the X-ray structures. Interestingly, Smith and Pettitt[46] have done extensive calculations of potentials of mean force (pmfs) for rotation about the χ_1 torsional angle of the aromatic side chains. Their calculations suggest[46] that, whereas in aqueous solution both Tyr¹ and Phe⁴ prefer gauche (−) conformations for χ_1, in saline solution both prefer a *trans* conformation at χ_1. From these calculations and from previous molecular dynamics simulations, Smith and Pettitt concluded that the gauche (+) conformation is unlikely to be a preferred conformation for the aromatic residues in DPDPE. This conclusion is also consistent with previous NMR studies in aqueous solution[25,26] and from structure–activity studies using β-MePhe⁴-substituted analogues of DPDPE.[20,47]

Of the gas-phase conformations considered in Table 6, the study of Nikiforovich et al.[42] was unique in that it not only analyzed the backbone conformation but also investigated the topographical properties of DPDPE. In this case, the topographical properties were considered by examining the relative arrangement of the pharmacophores of DPDPE, which were taken to be the α-amine of Tyr¹ and the Tyr¹ and Phe⁴ aromatics and the Cα atom of the D-penicillamine in position 2. By comparison of the relative positions of these pharmacophores in low-energy structures of DPDPE with the corresponding arrangements of these groups in other δ-selective peptides, putative δ receptor bound conformations of DPDPE were proposed.[42] The dihedral angles of one of these binding conformations are shown in Table 5, and a root-mean-square superimposition of the pharmacophores of this structure with X-ray structure 1 (Figure 7) demonstrates a remarkable similarity in both topography and conformation.

This similarity indicates an interesting correlation between the solution and δ-receptor bound conformation(s) of DPDPE. As discussed above, the aqueous solution conformations determined by NMR might be considered to exhibit similar backbone structure, within experimental error, to that observed by the X-ray analysis presented here. Notably, the crystal structure shows limited peptide–peptide interactions, and the peptide molecules are largely surrounded by solvent water. One may thus assume that these X-ray structures are in fact good approximations to the aqueous solution conformation of DPDPE. However, theoretical calculations *also* indicate that the δ-receptor bound conformation of DPDPE[42] shows high topographical and conformational homology with the X-ray structures (Figure 7), but some differences do exist in the disulfide bridge. These observations suggest that the solution and receptor bound conformations of DPDPE are actually very close or are the same.

Conclusions

[D-Pen²,D-Pen⁵]enkephalin (DPDPE), a cyclic, constrained, highly potent and δ opioid receptor-selective analogue of enkephalin, has been obtained from an aqueous solution in a crystalline form suitable for X-ray analysis. The unit cell contains three conformationally distinct molecules of DPDPE, 14 full occupancy water molecules, and approximately 24 additional water molecules which form a disordered solvent channel (Figures 2 and 3, Table 2). The peptide molecules are located "symmetrically" about the disordered water channel and are held together in the crystal by an intricate network of hydrogen bonds involving 13 of the 14 ordered water molecules. The conformation of the 14-membered ring was essentially identical for all three molecules; however, the Tyr¹ residue was conformationally different in each case. Comparison of the conformations found in the crystal with those previously determined by NMR methods in conjunction with energy calculations indicated that the conformation of the 14-membered ring in aqueous solution was similar to that in the crystal structure. This was interpreted to be due to the cyclic constraint in DPDPE and the high degree of solvation in the crystal structure. In addition, low-energy conformations previously determined by computational methods in attempts to determine the binding conformations of DPDPE gave conformations of the 14-membered rings which were generally similar to those found in the crystal structure. These results and previous structure–activity relationships suggest that the crystal structure(s) is a useful starting point for understanding the bioactive conformation important for biological activity and δ receptor selectivity of cyclic enkephalin analogues.

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Supplementary Material Available: Tables listing atomic coordinates, hydrogen coordinates, isotropic and anisotropic displacement parameters, and bond lengths and angles for DPDPE (16 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information. Atomic coordinates will also be deposited with Cambridge Structural Database.

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