Conformational Studies on Analogues of the Invertebrate Peptide pyroGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 using ^1H NMR

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The peptide pQDPFLRFamide is one of several closely related heptapeptides found in invertebrates, including molluscs. Electrophysiological findings and ligand binding studies have both suggested that these heptapeptides probably interact with receptors distinct from those that are activated by the related tetrapeptide FMRFamide (also found in the same group of invertebrates), despite the fact that some synthetic *N*-terminally extended analogues of the latter show marked tetrapeptide-like activity. We have carried out structural studies, using 1- and 2-dimensional ^1H NMR, on pQDPFLRFamide and some synthetic analogues in which Asp-2 was replaced by Asn and Pro-3 by either Aib (α-amino isobutyric acid) or by Gly. The results are consistent with the suggestion that pQDPFLRFamide can adopt a bent conformation, which might form the basis of the selectivity of this and related heptapeptides.

Two types of FMRFamide-related peptides (Farps), containing either four or seven amino acids have been found in several groups of invertebrates, including molluscs.[1,2] These peptides are thought to participate in a range of neuroregulatory functions. In the land snail *Helix aspersa*, the peptides include FXRFamide (X = M or L) and XDPFLRFamide (X = pQ, S or N).[3,4] Cloning of the gene that codes for the heptapeptides in this mollusc has revealed the presence of other, closely related heptapeptide sequences.[4] Electrophysiological studies in this species have indicated that these two groups of peptides probably act through different receptors.[5] Although ligand-binding studies have shown the presence of high and low affinity binding sites on membrane preparations from *Helix* tissues for the tetrapeptides and their analogues,[6–8] so far no specific high affinity binding site for the heptapeptides has been unequivocally demonstrated in such preparations.

High affinity binding at the tetrapeptide-binding site is not restricted to peptides containing four amino acids. For example, some *N*-terminal extensions of FMRFamide or its analogues also interact efficiently at this site, in some cases with an affinity better than that of the natural ligand.[6,7] These include *N*-3-(*p*-hydroxyphenyl)propanoyl-FMRFamide, YFMRFamide and YGGFMRFamide.

This is not the case for the naturally occurring heptapeptide Farps, which compete only weakly with tetrapeptides such as FMRFamide.[6–8] It is clear therefore that the particular *N*-terminal tripeptide sequence XDP in the heptapeptides must be crucial for discriminating between the tetrapeptide and putative heptapeptide receptors. One possible way in which this might happen is if the heptapeptides contained a turn in the region of the Asp–Pro sequence, since it has been shown that the sequence Asx–Pro is frequently found to occur at positions 1 and 2 of β-turns in proteins and peptides.[9,10] Such a conformation might also be stabilised by electrostatic interactions between the side chains of the Asp and Arg residues. We have previously shown that the effects on agonist activity and ligand binding of substitutions of Asp or Pro in pQDPFLRFamide are broadly in keeping with the presence of a bent conformation, though the electrostatic interaction may not be obligatory for biological activity.[11] Evidence that might support this viewpoint has been sought by an NMR study of the pQDPFLRamide and some analogues. A preliminary report of part of this work has already been published.[12]

It is unlikely that peptides such as those considered here will adopt a single conformation. All studies to date point to small linear peptides as possessing considerable conformational freedom and existing as an equilibrium over several conformations of similar energy.[13] The object of a study such as this must be to see if any conformation(s) or families of conformations exist with sufficient population to permit detection.[14] It is also relevant to consider the most appropriate solvent in which to study biomolecules. Water is often seen as the obvious choice,[15] but, as well as introducing some technical problems, a solution in water cannot really be considered typical of conditions prevailing *in vivo*, when many other ions and molecules will be present. Furthermore, we are more interested in predicting what may be the conformation of a peptide in a binding site, and such regions are usually assumed to be a lot less polar than bulk water. DMSO has often been used as a solvent not only because many peptides are readily soluble in it, but also with the hope that its polarity may be similar to that of the binding pocket in a receptor or enzyme. However, it has also been argued that, *in vivo*, layers of water near to a protein surface are more viscous than bulk water and that this increased viscosity, by reducing conformational mobility, may 'filter' an otherwise flexible peptide into a predominant conformation.[16] Mixtures of water and DMSO have higher viscosity than either solvent alone and have been suggested as an appropriate solvent for conformational studies.[15,16]

Experimental

Synthesis.—The following peptides used in this study were prepared by solid-phase synthesis, as described previously;[11] pQDPFLRFamide, pQNPFLRFamide, pQDAibFLRFamide (Aib = α-amino isobutyric acid), pQDGFLRFamide. As reported, all peptides were purified using reversed-phase C_{18} HPLC (μ-Bondapak, Waters), gave satisfactory amino acid analyses (Picotag Analyser, Waters) and the expected molecular ions were observed in FAB mass spectrometry (VG AutoSpec. instrument at the SERC Mass Spectrometry Service Centre). In addition, the Gly[3] analogue was subjected to additional analysis to eliminate the possibility that complexities observed in the NMR spectrum were due to impurities. Capillary electrophoresis of this analogue (Thermo Separation Products) at pH 3.0, 4.5 and 9.1 and in the presence of SDS (sodium

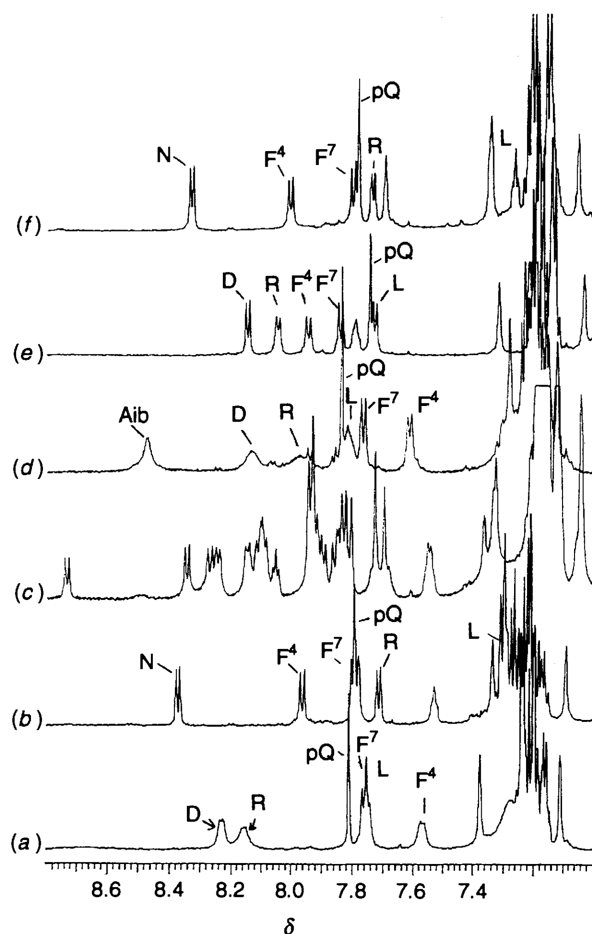

Fig. 1 Amide regions of the 600 MHz NMR spectra of pQDPFLRFamide (*a*), pQNPFLRFamide (*b*), pQDGFLRFamide (*c*) and pQDAibFLRFamide (*d*), all in [$^2H_6$]DMSO and pQDPFLRFamide (*e*) and pQNPFLRFamide (*f*) in [$^2H_6$]DMSO/H_2O (2:1)

dodecyl sulfate) at pH 9.0 showed only one major component with an integrated value of 93%. Similarly, electrospray mass spectrometry (VG Quattro instrument fitted with electrospray ionisation), which because it analyses the total sample is more likely than FABMS to detect all components present, identified one main component (m/z = 864.9; M_r 863.4) with other species present at levels less than 1%.

NMR Studies.—Experiments were carried out on samples prepared as follows. Approximately 5 mg of peptide was dissolved in deionised water and the pH adjusted to near 6.0. The solvent was removed under vacuum in a Speed VAC concentrator (Savant). The samples were further dried *in vacuo* over phosphorous pentoxide for 24 h. The peptide was then dissolved in [$^2H_6$]DMSO (0.5 cm^3) and in some cases this was further diluted with H_2O. In one case the solvent used was $^1H_2O/^2H_2O$ (9:1). Spectra were recorded on a Varian VXR-600S spectrometer at the Edinburgh University Ultra High Field NMR Service.

^{1}H NMR spectra were acquired with 32K data points and Fourier transformed with zero filling into 64K points. For some spectra, an exponential line broadening factor of 0.5 was applied. 2D spectra were obtained by the Hypercomplex method of States, Haberkorn and Reuben [17] as 512 FIDs of 2 K data points and transformed with zero filling into 1K × 2K points using either a sine bell or Gaussian window. Assignments of proton resonances in the peptides were made using several types of 2D spectra,[18a,19] *i.e.* DQF-COSY, TOCSY and ROESY. TOCSY spectra were acquired with a mixing time of 65 ms (for DMSO solution) or 80 ms (for DMSO/H_2O solution) and ROESY spectra with mixing times of 150 (DMSO and H_2O) or 200 ms (DMSO/H_2O). For ROESY spectra, the frequency offset was varied to ensure that the observed cross peaks did not arise from Hartman–Hahn transfer coupled with incoherent magnetisation transfer.[20]

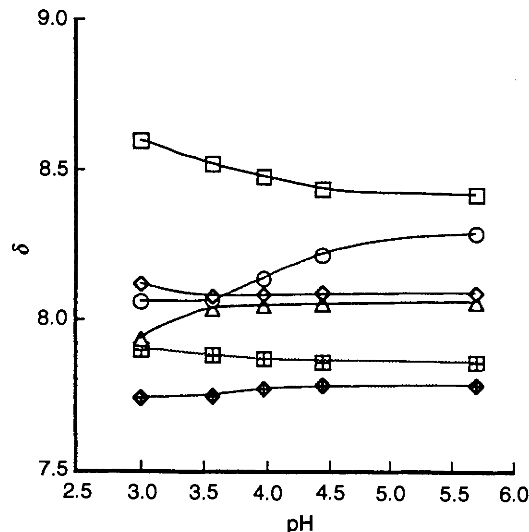

Fig. 2 Plot of the chemical shifts of the amide protons of pQDPFLRFamide against pH in $H_2O/^2H_2O$ (9:1). □: Asp-2; ○: Arg-6; ◇: Phe-7; △: Phe-4; ⊞: pGlu-1; ◈: Leu-5.

The temperature dependence of amide protons was determined from 1D spectra recorded at 5 °C intervals from 25 to 55 °C. In all cases the variation of the chemical shift of the amide proton with temperature was linear and the temperature coefficient was determined by linear regression analysis (correlation coefficients ⩾0.95).

Results and Discussion

^{1}H Resonance Assignments.—The amide regions of the 1D spectra of the peptides are shown in Fig. 1. Detailed analyses of the NMR spectra of the peptides, under a variety of conditions were made using DQF-COSY, TOCSY and ROESY experiments and are summarised for pQDPFLRFamide and the Asn^2 and Aib^3 analogues in Tables 1 to 3, respectively. The temperature coefficients of the amide protons of the peptides are listed in Table 4. The effects of pH on the amide protons of pQDPFLRFamide are shown in Fig. 2. The nuclear Overhauser connectivities observed in ROESY spectra are summarised in Table 5 and two examples of the spectra obtained are shown in Figs. 3 and 4. Moving the frequency offset in selected ROESY spectra by up to 300 Hz yielded essentially identical spectra.

Conformational Studies.—As mentioned above, the available evidence suggests that most small peptides exist as an ensemble of interconverting conformations. Inspection of the assignments reported in Tables 1 to 3 shows that considerable differences exist between the spectrum of pQDPFLRFamide and those of the Asn^2, Aib^3 and Gly^3 analogues and between the spectra of pQDPFLRFamide recorded under different conditions. At the very least, this points to the various conformations available to the peptides being populated to different extents for each peptide or set of conditions. Examination of all the observed NMR spectra (Tables 1 to 3 and Fig. 1), the amide proton temperature coefficients (Table 4) and the nuclear Overhauser connectivities (Table 5), allows certain conclusions to be drawn. These will now be presented for pQDPFLRFamide and the Asn^2 and Aib^3 analogues. The Gly^3 analogue, which displays rather different behaviour, will be discussed later.

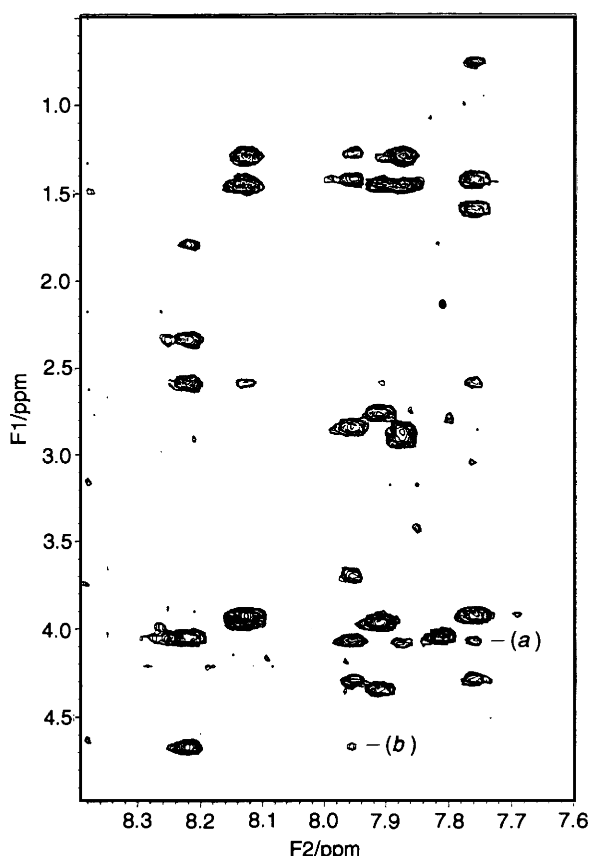

Fig. 3 Portion of the ROESY spectrum of pQDPFLRFamide in [$^2H_6$]DMSO/H_2O (2:1) indicating the crosspeaks between Leu NH and Pro α-proton (*a*) and between Phe-4 NH and Asp α-proton (*b*)

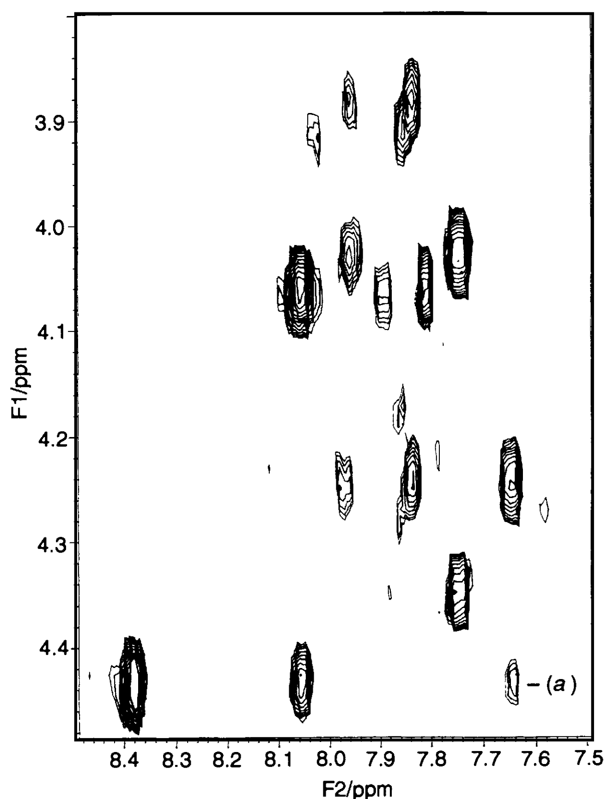

Fig. 4 Portion of the ROESY spectrum of pQDAibFLRFamide in [$^2H_6$]DMSO indicating the crosspeak between Phe-4 NH and Asp α-proton (*a*)

Configuration of the Asx-Pro peptide bond. Under all the conditions used, the ROESY spectra obtained for pQDPFLRFamide and the Asn[2] analogue display a strong $d_{\alpha\delta}(i,i+1)$ connectivity between Asx-2 and Pro-3, indicating that the peptide exists predominantly as the *trans* isomer of the Asx-Pro peptide bond.[18b] In several of the 1D spectra (*e.g.* pQDPFLRFamide in H_2O at pH 3.0 and the Asn[2] analogue in DMSO) a set of minor peaks is visible which could be due to a small fraction of molecules existing as the *cis* isomer, but no $d_{\alpha\alpha}(i,i+1)$ or of $d_{N\alpha}(i,i+1)$ connectivities between Asx-2 and Pro-3, characteristics of a *cis* peptide bond, were ever observed.

Existence of a salt bridge between Asp-2 and Arg-6. Several types of evidence suggest that such a salt bridge exists and has an influence on the conformation.

(*a*) *Appearance of amide proton resonances.* The amide proton resonances of the Asn[2] analogue in both DMSO and DMSO/H_2O are sharp, consistent with the averaging over the available conformations being essentially complete. However, for pQDPFLRFamide and the Aib[3] analogue in DMSO the amide resonances are broad. Again, this could be interpreted as a case of equilibria over an ensemble of conformers but now, because of the additional constraint of an internal salt bridge between the sidechains of Asp-2 and Arg-6, the rates of interconversion are slower and the averaging of the NMR signals is less complete than for the Asn[2] analogue.

(*b*) *Differences in chemical shift values.* Compared with the Asn[2] analogue in DMSO, the amide proton resonance of Phe-4 in pQDPFLRFamide in DMSO has moved from 7.97 to 7.59 and the amide and α proton resonances of Leu-5 have moved from 7.30 and 4.29, respectively, to 7.77 and 3.95 (Tables 1 and 2). The corresponding residues in the Aib[3] analogue, where a salt bridge is also possible, have chemical shifts close to those of pQDPFLRFamide (Table 3). It is difficult to account for differences between pQDPFLRFamide and the Asn[2] analogue other than by postulating a major conformational difference due to a salt bridge.

(*c*) *Variation with pH of the amide protons in pQDPFLRFamide.* Not only does the chemical shift of the Asp-2 amide proton vary as the pH is altered over a range covering the pK_a of the Asp sidechain carboxyl group, but the Phe-4 and Arg-6 amide resonances also change dramatically (Fig. 2). Such changes imply that a major alteration in conformation, affecting most of the peptide, occurs on ionisation of the Asp sidechain. It seems most likely that this involves an increase in number of those conformers which allow a salt bridge between the sidechains of Asp-2 and Arg-6. If such a salt bridge exists in aqueous solution, it is even more likely to exist in solvents of lower dielectric constant.

Flexibility of the peptides. Under all the conditions examined, at least some $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+1)$ connectivities were observed for all of the peptides. The former connectivity is characteristic of conformations with Φ and Ψ torsion angles near those found in α helices, while the latter is characteristic of extended conformations.[21] If both types of connectivity are found over an extended region of a peptide this is frequently taken as indicative of conformational averaging over both extended and 'bent' structures. Except for pQDPFLRFamide in DMSO, all possible $d_{\alpha N}(i,i+1)$ and most of the possible $d_{NN}(i,i+1)$ connectivities were observed, so that every residue spends time in both extended and 'bent' conformations. For pQDPFLRFamide in DMSO, $d_{\alpha N}(i,i+1)$ connectivities were only observed at the *N*- and *C*-termini, indicating perhaps that extended conformations are sparsely populated towards the centre of the peptide.

Further evidence for extensive flexibility comes from the $^3J_{\alpha NH}$ coupling constants reported in Tables 1 to 3. Coupling constants between the amide proton and the α proton of an amino acid ($^3J_{\alpha NH}$) are often not useful as indicators of

Table 1 Chemical shift assignments for pQDPFLRFamide[a]

	NH	αCH	βCH	γCH	δCH	Others	${}^3J_{\alpha NH}$[b]
pGlu[c]	7.82	4.03	2.22/1.82	2.14/2.08			
Asp[c]	8.24	4.82	2.70/2.39				5.98
Pro[c]	—	4.15	1.90/1.36	1.70/1.49	3.78/3.72		
Phe[c]	7.58	4.37	3.10/2.88			Ar ~ 7.23	6.68
Leu[c]	7.77	3.96	1.66/1.47	1.47	0.88/0.84		6.41
Arg[c]	8.17	4.08	1.58	1.44	3.11/2.94		5.98
Phe[c]	7.78	4.40	3.00/2.82			Ar ~ 7.20	7.91
NH_2[c]	7.38/7.13						
pGlu[d]	7.76	4.10	2.28/2.18	1.84			
Asp[d]	8.16	4.69	2.63/2.38				6.59
Pro[d]	—	4.15	1.99/1.30	1.71/1.44	3.71/3.67		
Phe[d]	7.96	4.32	3.05/2.85			Ar ~ 7.23	8.06
Leu[d]	7.74	3.93	1.78/1.43	1.43	0.89/0.75		6.77
Arg[d]	8.06	4.03	1.52	1.34	2.93	ωNH 7.90	6.96
Phe[d]	7.85	4.37	3.01/2.78			Ar ~ 7.18	8.06
NH_2[d]	7.40/7.14						
pGlu[e]	7.90	4.34	2.50/2.00	2.39			
Asp[e]	8.61	4.96	2.93/2.76				6.77
Pro[e]	—	4.34	2.14	1.91/1.66	3.74/3.66		
Phe[e]	7.94	4.54	3.20/3.03			Ar ~ 7.36	7.69
Leu[e]	7.76	4.25	1.56/1.49	1.49	0.94/0.88		6.94
Arg[e]	8.07	4.18	1.63	1.42	3.09	ωNH 7.14	7.32
Phe[e]	8.13	4.60	3.20/2.98			Ar ~ 7.28	6.96
NH_2[e]	7.50/7.09						

[a] Shifts relative to DMSO = 2.50 ppm. [b] Hz. [c] $[^2H_6]$DMSO. [d] $[^2H_6]$DMSO/^1H_2O (2:1). [e] $^1H_2O/^2H_2O$ (9:1).

Table 2 Chemical shift assignments for pQNPFLRFamide[a]

	NH	αCH	βCH	γCH	δCH	Others	${}^3J_{\alpha NH}$[b]
pGlu[c]	7.80	4.05	2.24/1.82	2.14/2.06			
Asn[c]	8.37	4.80	2.77/2.48			NH 7.80/7.28	7.91
Pro[c]	—	4.17	1.88/1.43	1.67/1.30	3.70/3.61		
Phe[c]	7.97	4.32	3.11/2.90			Ar ~ 7.40	8.33
Leu[c]	7.30	4.29	1.50	1.55	0.90/0.81		*e*
Arg[c]	7.71	4.11	1.60/1.36	1.36	3.03	ωNH 7.53	7.69
Phe[c]	7.80	4.40	3.00/2.80			Ar ~ 7.20	8.48
NH_2[c]	7.35/7.09						
pGlu[d]	7.78	4.06	2.24/1.78	2.24/2.12			
Asn[d]	8.33	4.72	2.71/2.53			NH 7.68/7.18	7.65
Pro[d]	—	4.14	1.93/1.40	1.70/1.32	3.64/3.54		
Phe[d]	8.04	4.30	3.08/2.84			Ar ~ 7.22	7.90
Leu[d]	7.35	4.10	1.29	1.43	0.84/0.75		*e*
Arg[d]	7.74	4.03	1.47/1.42	1.24	2.93	ωNH 7.25	7.05
Phe[d]	7.82	4.35	3.98/2.76			Ar ~ 7.15	8.12
NH_2[d]	7.35/7.04						

[a] Shifts relative to DMSO = 2.50 ppm. [b] Hz. [c] $[^2H_6]$DMSO. [d] $[^2H_6]$DMSO/^1H_2O (2:1). [e] Peak obscured by other signals in 1D spectrum.

Table 3 Chemical shift assignments for pQDAibFLRFamide in DMSO[a]

	NH	αCH	βCH	γCH	δCH	Others	${}^3J_{\alpha NH}$[b]
pGlu	7.84	4.09	2.18/1.83	2.18/2.08			
Asp	8.06	4.44	2.58				6.78
Aib	8.42	—	1.21[c]/0.98[d]				
Phe	7.65	4.25	3.19/2.87			Ar ~ 7.16	7.51
Leu	7.86	3.90	1.86/1.50	1.52	0.84/0.80		6.78
Arg	7.99	4.03	1.54/1.39	1.39	2.98/2.88		6.77
Phe	7.78	4.35	3.02/2.80			Ar ~ 7.19	8.24
NH_2	7.28/7.10						

[a] Shifts relative to DMSO = 2.50 ppm. [b] Hz. [c] Major conformer. [d] Minor conformer.

Table 4 Temperature coefficients[a] of NH chemical shifts for pQDPFLRFamide and the Asn^2 and Aib^3 analogues under the conditions shown

	pQDPFLRFamide		pQNPFLRFamide	pQDAibFLRFamide
Residue	DMSO	$DMSO/H_2O$	DMSO	DMSO
pGlu	−4.41	−5.41	−3.99	−4.55
Asp/Asn	−3.91	−6.98	−3.99	−1.82
Pro/Aib	—	—	—	−6.78
Phe	+0.563	−1.18	−2.09	−0.812
Leu	−2.72	−2.70	*c*	−2.94
Arg	−5.64	−7.21	−4.17	−5.43
Phe	−2.41	−6.84	−4.21	−4.13

[a] Temperature coefficients as 10^{-3} ppm K^{-1}. [b] Hz. [c] Peak obscured by other resonances.

Table 5 Nuclear Overhauser connectivities observed in ROESY spectra for pQDPFLRFamide and the Asn^2 and Aib^3 analogues under the conditions shown

	pQDPFLRFamide			pQNPFLRFamide		pQDAibFLRFamide
Connectivity	DMSO	$DMSO/H_2O$ (2:1)	H_2O (pH 3.0)	DMSO	$DMSO/H_2O$ (2:1)	DMSO
$d_{NN}(i,i+1)$	F^4-L	pQ-D; F^4-F^7	pQ-D; F^4-F^7	F^4-R	pQ-D; F^4-R	pQ-L; F^7-NH_2
$d_{\alpha N}(i,i)$	pQ; F^7	All possible	All possible	All possible	pQ; D; R	All possible
$d_{\alpha N}(i,i+1)$	pQ-D; R-F^7	pQ-D; P-F^7-NH_2	pQ-D; P-F^7-NH_2	pQ-N; P-F^7-NH_2	pQ-D; P-F^7	pQ-Aib; F^4-F^7-NH_2
$d_{\alpha N}(i,i+2)$		P-L; D-F^4 (vw)[a]		N-F^4	F^7-L	D-F^4
$d_{N\beta}$		D-pQ; F^4-P; L-F^4; L, R-D; F^7-R	L-F^4	pQ-N		L > R ≫ F^4-D; F^4-Aib (M^b and m); L-Aib (M)[b]
$d_{\alpha\beta}$		R-P				F^4-Aib (M^b and m)
Other	Dα-Pδ	Dα-Pδ; F^4NH-Pγ, Pδ; pQα, Pα-Lδ	Dα-Pδ; F^4NH-Pγ, Pδ	Dα-Pδ; RNH, F^7NH-Lδ	Dα-Pδ; RNH, F^7NH-Lδ	DNH-Lδ; pQα,Rα, F^7α-Lδ

[a] vw = very weak. [b] M = major peak, m = minor peak.

conformation as even a small contribution from extended conformations leads to $^3J_{\alpha NH}$ values of 7 to 8 (ref. 22). Except for pQDPFLRFamide in DMSO, most of the $^3J_{\alpha NH}$ coupling constants measured were >6.9 Hz, implying conformational averaging involving extended structures. In the case of pQDPFLRFamide in DMSO, all of the $^3J_{\alpha NH}$ values except that between Arg-6 and Phe-7, are at the low end of the normal range and may point to average conformations which are not extended, though they do not define a simple, single β-bend. It should be noted that evidence for conformational averaging is not inconsistent with evidence presented below that significant populations of specific conformations may occur.

Existence of populated bent conformations. Dyson and Wright[21] have summarised criteria for identifying bends in small peptides using NMR spectroscopy. For Type I or Type II β-bends these include: (1) a low temperature coefficient for the amide proton of residue 4 of the turn; (2) an NOE connectivity between the α-proton of residue 2 of the bend and the amide proton of residue 4 of the bend [a $d_{\alpha N}(2,4)$ connectivity]; (3) a $d_{NN}(3,4)$ connectivity [plus a $d_{NN}(2,3)$ connectivity for Type I bends]; (4) an appropriate $^3J_{\alpha NH}$ coupling constant. Satisfying these criteria does not mean the peptide exists solely in a conformation with this bend; merely that there exists a population of bent molecules great enough to be detected. In practice, as discussed above, criterion (4) is rarely met for linear peptides.

(*a*) *Temperature coefficients.* For pQDPFLRFamide and the Asn^2 and Aib^3 analogues in DMSO the amide proton of Phe-4 displays a low value. That for the Asn^2 analogue is probably on the borderline of significance, but the values for the other two peptides are very low and imply that for these peptides in DMSO solution, this proton is well shielded from exposure to solvent molecules, *e.g.* by being involved in a hydrogen bond.[23] If this is due to participation in a β-bend then Phe-4 must be residue 4 of the bend, which thus extends over the residues pGlu-Asx-(Pro/Aib)-Phe. The combination Asx-Pro occurs widely in β-bends in proteins, but usually at positions 1 and 2. Rarely, if at all, does it occur at positions 2 and 3. However, most β-bends in proteins are found on the surface, where they protrude into an aqueous environment. The data under discussion here were obtained in DMSO and this difference may account for this unusual feature. For pQDPFLRFamide in $DMSO/H_2O$ solution, the temperature coefficients of the amide protons of Phe-4 and of Leu-5 are low enough to imply shielding from solvent.[14] Discussion of this more complicated result is deferred until later.

(*b*) *NOE evidence for bent conformations.* The evidence is of two types. The existence of an isolated $d_{\alpha N}(2,4)$ connectivity defines the position of a possible β-bend while the presence of further connectivities between protons on residues which are spatially close together because of the β-bend provides additional supporting evidence.

The ROESY spectrum of pQDPFLRFamide displayed relatively few crosspeaks and, despite the exceptionally low value of the temperature coefficient for the amide proton of Phe-4 (Table 4), no $d_{\alpha N}(2,4)$ was seen between Asp-2 and Phe-4. The ROESY spectrum of the Asn^2 analogue did show a very weak $d_{\alpha N}(2,4)$ connectivity between Asn-2 and Phe-4. In the more viscous solvent $DMSO/H_2O$, no $d_{\alpha N}(i,i+2)$ could be seen between any residues in the Asn^2 analogue. It is not clear whether the paucity of crosspeaks found in these spectra is an inherent property of the molecules or means that the spectra were not obtained under ideal conditions. However, spectra with more crosspeaks were obtained under the same or similar conditions for the Aib^3 analogue in DMSO and for pQDPFLRFamide in $DMSO/H_2O$.

A clear $d_{\alpha N}(2,4)$ between Asp-2 and Phe-4 was seen in the ROESY spectrum of the Aib^3 analogue. This points to a β-bend over residues pGlu-Asp-Aib-Phe, consistent with the low value of the temperature coefficient reported for the amide proton of

Phe-4. In addition $d_{NN}(i,i+1)$ connectivities were seen over a range including Asp-Aib-Phe which is consistent with a Type I β-bend. Further medium range connectivities were seen across the β-bend, between amide protons of Leu > Arg ≫ Phe-4 and the β-protons of Asp-2 and between the amide proton of Asp-2 and the δ-protons of Leu-5.

With pQDPFLRFamide in DMSO/H_2O two $d_{\alpha N}(i,i+2)$ connectivities were seen; one, moderately intense, between Pro-3 and Leu-5, and a second, very weak, between Asp-2 and Phe-4. These connectivities can be interpreted as arising from two different conformations. One, sparsely populated, has a β-bend over the residues pGlu-Asp-Pro-Phe, a bend similar to that reported above for the peptides in DMSO solution, and another, more populated, a β-bend over the residues Asp-Pro-Phe-Leu, which is more akin to the situation found in proteins. Such bends would require the amide protons of both Phe-4 and Leu-5 to be involved in hydrogen bonds, which is consistent with the temperature coefficients reported in Table 4. No $d_{NN}(i,i+1)$ connectivities are possible to Pro-3 which precludes identification of the type of β-bend. Although all possible $d_{\alpha N}(i,i+1)$ and $d_{\alpha N}(i,i)$ connectivities were seen, the intensities of both types of crosspeak were greatest at the extremities of the peptide. In particular, the $d_{\alpha N}(i,i)$ crosspeak for Phe-4 is very weak compared to that of the other residues. This may imply that Phe-4 spends much of its time in a conformation with the maximum possible distance between the amide and the α proton. This corresponds to a Φ torsion angle of approximately −100° (ref. 18*c*) which is near the optimum value (−90°) for the third residue in a type I β-bend.[18d] Additional connectivities were seen between the amide protons of both Leu-5 and Arg-6 and one of the β-protons of Asp-2, and between the α-proton of Pro-3 and the δ-protons of Leu-5, confirming the existence of a bend over the intervening residues. A clear set of connectivities was seen between the amide proton of Phe-4 and one each of the β-, γ- and δ-protons of Pro-3. This implies that the amide proton is held rather rigidly on one side of the proline ring, between the proline and phenylalanine sidechains. In such a position it will be well shielded from solvent, whether or not it is involved in hydrogen bonding, consistent with the low value of the temperature coefficient.

Conformational studies on pQDGFLRFamide. The amide region of the 1D spectrum of this analogue is shown in Fig. 1(*c*). It is immediately obvious that there are many more than the expected number of resonances present. In the TOCSY spectrum between 10 and 12 amide–α-proton crosspeaks can be seen, rather than the expected 6 or 7. Possible explanations for this are that the peptide exists as two conformations (or conformational families) with approximately equal populations, which are in slow exchange, or that the peptide is impure. The latter is the simpler hypothesis but all analytical techniques used showed only one major component forming at least 93% of the total. Thus, the alternative hypothesis of slow exchange between conformers remains as the likely explanation. Heating the sample to 55 °C brought about changes in the spectrum with an apparent increase in the proportion of some resonances relative to others, consistent with the above hypothesis. It may be that, in DMSO at least, the constraint of an internal salt bridge, coupled with the flexibility of Gly, allows the peptide to populate, almost equally, two families of conformations, whereas the other analogues allow only one. Further studies to define the two conformations were not carried out, in part due to lack of sample.

Conformational conclusions. The studies described above indicate that all the peptides examined are flexible to a greater or lesser degree. Nonetheless, while they all spend some time in extended states, a significant population of conformers with a bend towards the *N*-terminus appears to exist and such a population may be important for biological activity. For pQDGFLRFamide in DMSO, however, two distinct conformations (or conformational families) are observed. This implies that the primary sequence, with a suitable conformationally constrained residue at position 3, is more important for the accumulation of a single population with an *N*-terminal bend than the possibility of a salt bridge between the sidechains of residues 2 and 6.

For pQDPFLRFamide in DMSO/H_2O, two conformations or conformational families with β-bends in slightly different positions appear to be present. It may be that this is an oversimplification and that a conformational equilibrium exists between several types of bend near the *N*-terminus, with the populations of the various conformers being solvent dependent. This could have implications for the conformation of the peptides when bound to the receptor, *i.e.* if the polarity of the surroundings has the largest influence, one might predict that the peptide would adopt a conformation with a β-bend over residues Asp-2 to Leu-5 when in free solution, but a β-bend over residues pGlu-1 to Phe-4 would be favoured at or near the receptor binding site.

Correlations with Biological Activity.—A final question is whether these conformational studies show any correlation with the observed biological properties of the peptides. There are two types of activity to be considered, at putative tetrapeptide and heptapeptide receptors. Both physiological testing and receptor-binding studies have been carried out with the peptides under discussion and are reported in detail elsewhere,[11] but a brief summary is as follows.

In competition with a radiolabelled ligand for the putative tetrapeptide receptor on membranes isolated from circumoesophageal ganglia of the common snail *Helix aspersa*, the following IC_{50} values were obtained: FMRFamide (0.22 μmol dm^{-3}), pQDGFLRFamide (0.8 μmol dm^{-3}), pQNPFLRFamide (1.6 μmol dm^{-3}), pQDAibFLRFamide (15.0 μmol dm^{-3}) and pQDPFLRFamide (24.0 μmol dm^{-3}). This gives a good inverse correlation with the propensity for bend formation deduced above. The most flexible peptide, pQDGFLRFamide displaced the radioligand most strongly, while pQDAibFLRFamide and pQDPFLRFamide, which both appear to have a significant population with an *N*-terminal bend, were approximately equally poor at displacing the label. Such results are consistent with our hypothesis that the binding site on the tetrapeptide receptor is a linear groove in the receptor surface and that *N*-terminally extended peptides with a bend towards the *N*-terminus cannot readily fit in and fulfil other requirements such as correct alignment of sidechains, *etc.* On the other hand, it is known that *N*-terminally extended peptides for which no *N*-terminal bend is predicted (and presumably also pQDGFLRFamide) can readily displace FMRFamide.[6,7]

The situation is more complex with the heptapeptide receptor, which is not so well characterised. At present, no radioligands exist for this receptor and comparisons are made of physiological response. The peptides have been tested on heart and tentacle retractor muscles from *Helix aspersa* and on the extensor-tibiae neuromuscular preparation of the locust *Schistocerca gregaria.*[11] In these assays, pQNPFLRFamide, pQDAibFLRFamide and pQDPFLRFamide all showed agonist activity, but pQNPFLRFamide was noticeably the most potent. The peptide pQDGFLRFamide was much less effective, and at higher concentrations displayed some of the characteristics of tetrapeptide ligands. Thus, as in the tetrapeptide binding studies mentioned above, the Gly^3 analogue does appear to show differences from the other three peptides in biological as well as in conformational studies. The activity of the Asn^2 analogue seems to rule out any requirement for an anionic side-chain at position 2, either as part of an intramolecular ion-pair or as a specificity requirement at the

receptor, despite the fact that such a side-chain is conserved in all known members of this family of peptides.

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