Int. J. Peptide Protein Res. **29**, 1987, 282–288

^{1}H-n.m.r. investigation of conformational features of cyclic, penicillamine-containing enkephalin analogs

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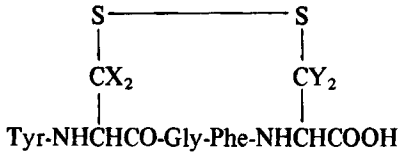
Received 9 June, accepted for publication 2 July 1986

Conformational features of a series of cyclic, penicillamine-containing enkephalin analogs, all of which display selectivity for the delta opioid receptor, were studied by ^{1}H n.m.r. in aqueous solution. Comparison of chemical shifts, coupling constants, and temperature dependence of amide proton chemical shifts suggests different conformational features among the analogs, some of which can be related to the different primary sequences of these peptides. The observation that some of the analogs display disparate individual conformational features while exhibiting similar opioid potency and receptor selectivity suggests that such analogs may share a similar overall topography or at the least maintain the same relative orientations of key portions of the molecule.

Key words: conformation; enkephalins; ^{1}H n.m.r.; opioids; peptides

Many endogenous peptide hormones and neurotransmitters are relatively small, flexible molecules that can utilize their inherent flexibility to interact with different subclasses of receptors which mediate different physiological events and which presumably place different conformational requirements upon the peptide ligand. In order to elucidate the molecular mechanism of action of a particular peptide hormone or neurotransmitter it is necessary to unravel the distinct actions mediated by the individual receptor subclasses and to determine the bioactive conformation of the peptide ligand at each of these receptors. A particularly useful approach toward these ends is the design and synthesis of analogs of the native peptide into which conformational restrictions are incorporated. One benefit of this approach is that the proper choice of conformational restriction can result in an analog able to assume the conformational requirements for interaction with one subclass of receptor but not other subclasses. Such highly receptor selective analogs can then be used to determine the physiological actions mediated by distinct receptor subclasses. An additional benefit of conformationally restricted analogs is that while the conformational analysis of flexible peptides is hampered by dynamic averaging of conformation dependent spectroscopic parameters, leading to the determination of an average solution conformation of dubious physical or biological significance, more rigid analogs do not share this liability. Thus a conformationally restricted analog can be expected to assume a more well defined solution conformation and allow a more reliable extrapolation to the active, receptor bound conformation. For conformationally restricted, receptor selective analogs this allows the determination of the bioactive conformation at *specific* receptor subclasses.

Analogs of the enkephalins, one class of endogenous opioid peptides, provide an example of the benefits of the approach outline above. Opioid receptor heterogeneity has been amply demonstrated and it is now clear that at least three distinct opioid receptor subtypes, designated μ, δ, and κ, exist (1, 2). A number of enkephalin analogs have been reported which display enhanced receptor selectivity relative to the native ligands. Among these highly selective analogs are a conformationally restricted cyclic tetrapeptide, Tyr-D-Orn-Phe-AspNH$_2$ reported by Schiller and coworkers (3) to have extremely high selectivity for the μ opioid receptor and a series of cyclic penicillamine-containing enkephalins that we have previously described (4–7) and which include analogs with the highest reported selectivity for the δ opioid receptor. We report here the results of initial ^{1}H n.m.r. studies on this latter series of analogs consisting of the cyclic pentapeptides, [D-Pen^2, L-Cys^5] enkephalin, [D-Pen^2, D-Cys^5] enkephalin, [D-Cys^2, L-Pen^5] enkephalin, [D-Cys^2, D-Pen^5] enkephalin, [D-Pen^2, L-Pen^5] enkephalin, and [D-Pen^2, D-Pen^5] enkephalin where Pen = penicillamine is β,β dimethyl cysteine. These cyclic, disulfide-containing enkephalin analogs have the general structure:



where X = H, CH_3; Y = H,CH_3.

MATERIALS AND METHODS

The cyclic, penicillamine-containing enkephalin analogs were synthesized as reported previously (4–7). Purification was effected as described therein or by semipreparative high performance liquid chromatography (HPLC) on a Vydac 218TP C-18 column (2.5 cm x 22 cm) using the solvent system 0.1% trifluoroacetic acid in H_2O/0.1% trifluoroacetic acid in acetonitrile (75/25). Purity of all analogs was greater than 98% as assessed by analytical HPLC monitored at both 280 nm and 230 nm.

^{1}H n.m.r. experiments were performed on Bruker WM 250 and IBM WP270SY spectrometers operating at 250 MHz and 270 MHz, respectively. Samples for n.m.r. experiments were prepared by dissolving the appropriate peptide in 100.0% D_2O (low in paramagnetic impurities, Aldrich, Milwaukee, WI) or in 90% H_2O/10% D_2O and adjusting the pH to 3.0 (uncorrected meter reading) with CD_3COOD. Samples used for experiments in 100.0% D_2O were previously dissolved in D_2O and lyophilized to replace exchangeable protons with deuterons. Final concentrations of peptides were approximately 10 mM. N.m.r. spectra of D_2O solutions were obtained either via accumulations of one-pulse experiments, utilizing a 60° pulse, or by the use of the 180°-τ-90°, WEFT (8) pulse sequence to minimize the residual HDO resonance. Experiments involving samples in H_2O/D_2O mixtures, designed to observe amide proton resonances, employed either the Redfield 2-1-4 pulse sequence (9) or presaturation of the water resonance. Computer simulations of strongly coupled spin systems were performed on an Aspect 2000A computer and best fit parameters for these are reported in the tables. Resonances arising from individual residues in each peptide were determined by homonuclear decoupling experiments.

RESULTS

Chemical shifts and J*NHαCH* values for all the residues in the cyclic enkephalin analogs and $J\alpha\alpha'$ values for the glycine α protons are listed in Table 1. Assignments for the mono penicillamine-containing analogs were done by inspection for the glycine, penicillamine, and aromatic resonances. The amino terminal tyrosine C^α and C^β protons were assigned by observing that irradiation of this C^α resonance in H_2O/D_2O solution led to no change in the amide region of the spectrum while irradiation of each remaining C^α proton resulted in a decoupled N^α amide proton resonance. Assignments of phenylalanine and cysteine resonances were accomplished by long range decoupling of the phenylalanine C^β proton-aromatic proton interactions as previously described (10). Tentative, differential assignments of Pen^2 and Pen^5 resonances in [D-Pen^2, D-Pen^5]

enkephalin and [D-Pen^2, L-Pen^5] enkephalin were made by comparison with the mono penicillamine analogs and were confirmed by examination of the ^{1}H n.m.r. spectra of bis penicillamine analogs in which the Pen^5 residue was deuterium labeled ($^2H_6$) in the β methyl groups. The chemical shift of the D-Pen^5 amide resonance of [D-Pen^2, D-Pen^5] enkephalin, which is obscured by the Phe aromatic resonances, was determined from difference decoupling spectra resulting from experiments in which the decoupler was alternately set on the D-Pen^5 C$^\alpha$ proton resonance and in a blank region of the spectrum.

Comparison of the chemical shift values observed for the set of enkephalin analogs summarized in Table 1 yields several interesting features. Chemical shift values for all tyrosine resonances are similar throughout the series as are the chemical shift values for like residues in position 2 of the peptides. The large chemical shift differences for the C$^\alpha$ protons of Cys^2 vs. Pen^2 residues are to be expected and result from the shielding effect of the Pen β,β dimethyl

TABLE 1

Proton chemical shifts and coupling constants for cyclic enkephalin analogs

Residue	$\delta\alpha$	$\delta\beta$	δ_{NH}	δ other	$J_{NH\alpha CH}$ (Hz)
Tyr^1	a. 4.36	3.18; 3.03	–	Arom = 7.16; 6.87	–
	b. 4.45	3.21; 3.04	–	7.18; 6.87	–
	c. 4.32	3.27; 3.01	–	7.13; 6.86	–
	d. 4.31	3.21; 3.05	–	7.14; 6.87	–
	e. 4.41	3.16; 3.06	–	7.17; 6.87	–
	f. 4.39	3.20; 3.08	–	7.16; 6.87	–
D-X^2	4.18	–	8.24	Methyl = 1.40; 0.83	7.9
	4.22	–	7.98	1.43; 0.88	7.9
	4.67	3.09; 2.69	8.16	–	7.4
	4.72	3.04; 2.82	8.26	–	7.3
	4.14	–	8.10	1.44; 0.86	8.1
	4.18	–	8.20	1.48; 0.84	7.8
Gly^3	4.22; 3.42	–	8.80	J$\alpha\alpha'$ (Hz) = 14.8	7.3; 7.9
	4.02; 3.52	–	8.80	14.8	5.5; 6.1
	4.14; 3.51	–	8.60	15.2	4.4; 7.4
	4.32; 3.53	–	8.69	15.5	2.9; 8.1
	4.18; 3.45	–	8.59	15.1	6.2; 6.9
	4.35; 3.54	–	8.52	15.4	4.3; 8.4
Phe^4	4.78	3.14; 3.01	8.37	Arom = 7.30	8.5
	4.59	3.32; 3.00	8.60	7.34	7.3
	4.74	3.20; 2.97	8.55	7.29	6.6
	4.50	3.18; 3.05	8.43	7.31	5.8
	4.83	3.15; 2.99	8.47	7.30	8.8
	4.52	3.20; 3.06	8.47	7.32	6.0
Y^5	4.62	3.21; 3.09	8.22	Methyl = –	7.3
	4.30	3.38; 3.30	8.04	–	7.3
	4.40	–	7.93	1.41; 1.35	8.8
	4.49	–	7.53	1.43; 1.39	8.8
	4.46	–	8.08	1.29; 1.27	9.5
	4.38	–	7.40	1.34; 1.29	8.6

Values: a. [D-Pen^2, L-Cys^5] enkephalin, b. [D-Pen^2, D-Cys^5] enkephalin, c. [D-Cys^2, L-Pen^5] enkephalin, d. [D-Cys^2, D-Pen^5] enkephalin, e. [D-Pen^2, L-Pen^5] enkephalin, and f. [D-Pen^2, D-Pen^5] enkephalin.

groups on the C^{α} proton resonance, which gives rise to the upfield shift of the Pen C^{α} proton relative to the Cys C^{α} proton. The chemical shift differences between the two penicillamine methyl groups in each Pen^2 analog are due to a ring current effect of the tyrosine aromatic moiety, which leads to an upfield shift for one methyl group in each pair as was previously observed for related analogs (11). Chemical shift variations of the Gly^3 C^{α} proton resonances are somewhat greater than for the preceding residues. A trend is apparent for these resonances in the analogs containing penicillamine in position 5. For these peptides, the Gly^3 C^{α} proton chemical shifts are similar for pairs of analogs with the same enantiomer of penicillamine in position 5. Thus [D-Cys^2, L-Pen^5]- and [D-Pen^2, L-Pen^5] enkephalin exhibit similar Gly^3 C^{α} proton chemical shifts (4.14, 3.51 and 4.18, 3.45, respectively) which differ from those for [D-Cys^2, D-Pen^5]- and [D-Pen^2, D-Pen^5] enkephalin (4.32; 3.53 and 4.35; 3.54, respectively). Chemical shift differences for Phe^4 resonances can also be related to the nature of the residue in position 5. For the Phe^4 C^{α} proton resonance, the chemical shifts of all analogs with a D amino acid in position 5 are similar and are approximately 0.25 p.p.m. upfield of the corresponding chemical shift of analogs with a carboxy terminal L-amino acid. Chemical shifts of the Phe^4 NH proton resonance for analogs with a carboxy terminal penicillamine residue are all similar and differ slightly from those with a cysteine residue in position 5. By contrast, chemical shifts for the Phe^4 C^{β} proton and aromatic resonances are similar throughout the series. Chemical shifts of the carboxy terminal amino acid also display differences related to the nature of this residue. As was observed for the Pen^2 resonances, the Pen^5 C^{α} proton resonances are all similar in chemical shift but are less shielded (farther downfield) than for Pen^2 C^{α} proton resonances. The Cys^5 C^{α} proton resonance of [D-Pen^2, L-Cys^5] enkephalin is similar to the Cys^2 chemical shift of [D-Cys^2, L-Pen^5]- and [D-Cys^2, D-Pen^5] enkephalin, while the C^{α} proton resonance of [D-Pen^2, D-Cys^5] enkephalin is approximately 0.3 p.p.m. upfield of these. Pen^5 amide proton chemical shifts for the four penicillamine terminal analogs correlate with the enantiomeric nature of this residue, chemical shifts for the D-Pen^5 analogs being approximately 0.5 p.p.m. upfield of those for L-Pen^5 analogs.

Table 1 also lists values of JN*H*α*CH*, the coupling constant between the amide and C^{α} protons for each residue other than the amino terminal tyrosine. This parameter can be related to the backbone dihedral angle, ϕ, about the N^{α}-C^{α} bond (12) and thus provides conformation information. As can be seen from the data in Table 1, only a small variation in JN*H*α*H* for residue 2 is observed throughout the series. By contrast, values of this parameter for Gly^3 vary greatly through the series in a manner which does not allow facile correlation with the linear sequence of analogs in the series. Considerable variation of the Phe^4 JN*H*α*CH* values is also observed. Within the series there is some similarity in this parameter between the [D-Pen^2, Y^5] analogs and between the [D-Pen^2, D-Y^5] analogs. However, since the Phe^4 JN*H*α*CH* values for both D-Cys^2 analogs are also similar to the latter it is unclear whether this correlation is of any real significance. Values of JN*H*α*CH* for the carboxy terminal residue of analogs in this series are identical for the two Cys^5 analogs while the four Pen^5 analogs display similar values, different from these.

Table 2 presents rotamer populations about the C^{α}–C^{β} bond for side chains of individual residues of the six analogs in this study. The rotamers listed are the low energy, staggered rotamers a, b, and c, which correspond to $\chi^1 -60°$; $\pm 180°$; $+60°$ respectively and the populations listed are calculated from the coupling constants $J_{\alpha\beta}$ and $J_{\alpha\beta'}$, by the method of Pachler (13). Since stereospecific assignment of the C^{β} protons of Tyr, Phe, and Cys residues is not possible from these experiments, the rotamer populations P(a) and P(b) for any given residue may be reversed. This ambiguity notwithstanding, similar fractional populations for a given residue throughout the series suggest similar side chain conformations. As can be seen in Table 2, rotamer populations for the Tyr^1 residue are virtually identical throughout the series. Similarly, rotamer populations for

TABLE 2

Calculated rotamer populations about C_α-C_β for cyclic enkephalin analogs

Analog	Tyr[1] P(a)	P(b)	P(c)	Cys[2] P(a)	P(b)	P(c)	Phe[4] P(a)	P(b)	P(c)	Cys[5] P(a)	P(b)	P(c)
[D-Pen[2], L-Cys[5]]-enkephalin	0.62	0.35	0.03		–		0.52	0.34	0.14	0.61	0.05	0.34
[D-Pen[2], D-Cys[5]]-enkephalin	0.65	0.33	0.02		–		0.70	0.24	0.06	0.73	0.05	0.22
[D-Cys[2], L-Pen[5]]-enkephalin	0.62	0.32	0.06	0.31	0	0.69	0.59	0.32	0.09		–	
[D-Cys[2], D-Pen[5]]-enkephalin	0.60	0.35	0.05	0.28	0	0.72	0.67	0.21	0.12		–	
[D-Pen[2], L-Pen[5]]-enkephalin	0.62	0.35	0.03		–		0.45	0.45	0.10		–	
[D-Pen[2], D-Pen[5]]-enkephalin	0.64	0.34	0.02		–		0.56	0.29	0.15		–	

the D-Cys[2] residue for the two analogs with this substitution are in excellent agreement and the rotamer populations of the Cys[5] residue for the two analogs containing this substitution are quite similar. By contrast, there is considerable variation in rotamer populations of the Phe[4] residue. Due to the lack of stereospecific assignment of the Phe[4] C^β protons, it is possible that the dissimilarity of rotamer populations for this residue may in fact be greater than indicated in Table 2, since P(a) and P(b) may be reversed in some analogs and not in others.

Temperature dependence of amide proton chemical shifts in H_2O solution for the series of analogs is summarized in Table 3. Generally, large values for this parameter ($> 6 \times 10^{-3}$ p.p.m./°C) are indicative of exposure to and exchange with solvent protons while small values ($< 2 \times 10^{-3}$ p.p.m./°C) suggest inaccessibility to the solvent or participation in intramolecular hydrogen bonding. As can be seen from Table 3, most amide hydrogens of all analogs are exposed to solvent with the only clear exceptions being the D-Pen[5] amide protons of [D-Cys[2], D-Pen[5]] enkephalin and [D-Pen[2], D-Pen[5]]-enkephalin.

DISCUSSION

While the data presented in Tables 1–3 are insufficient to allow the proposal of detailed

TABLE 3

Temperature dependence of amide proton chemical shifts

Analog	$(-10^3)d\delta/dT$ (p.p.m./°C) X[a]	Gly[3]	Phe[4]	Y[5]
[D-Pen[2], L-Cys[5]]enkephalin	6.4	6.0	8.4	5.7
[D-Pen[2], D-Cys[5]]enkephalin	7.9	6.5	9.0	3.5
[D-Cys[2], L-Pen[5]]enkephalin	7.0	5.0	7.9	3.9
[D-Cys[2], D-Pen[5]]enkephalin	6.5	4.8	5.8	1.8
[D-Pen[2], L-Pen[5]]enkephalin	5.5	5.3	8.3	5.5
[D-Pen[2], D-Pen[5]]enkephalin	6.6	5.2	5.2	0.9

conformational models for the analogs studied, certain conclusions may be drawn. The observed similarity of ^{1}H n.m.r. parameters for the Tyr^1 residue throughout the series suggests that the conformation of this residue is constant throughout the series. It could be argued that this amino terminal residue would be expected to possess more motional freedom than the more constrained cyclic portion of the peptide and that the similarity of the ^{1}H n.m.r. parameters for this residue reflects such dynamic averaging. However, the observation that the Tyr^1 aromatic side chain induces an upfield shift of one Pen^2 methyl proton resonance in all D-Pen^2 analogs (and by analogy may be responsible for the upfield shift of D-Cys^2 C^β proton resonances relative to Cys^5 C^β proton resonances) and the finding that the side chain rotamer population, P(c), for $\chi^1 = +60°$ is essentially zero, strongly suggest that the orientation of the Tyr^1 residue relative to the cyclic portion of the peptide is fairly fixed and is similar throughout the series.

It appears that D-Pen^2 vs. D-Cys^2 substitution has little effect on conformation of the X^2 residue as can be seen from the relatively minor variation in conformation dependent parameters for this residue. By contrast, Pen vs. Cys substitution in residue 5 does appear to alter the conformation of the Y^5 residue as is reflected by the dependence of JN*HαCH* values on the nature of this residue. Further, D-Pen^5 vs. L-Pen^5 alters the conformation as well, as is indicated by the temperature dependence of the Pen^5 amide chemical shifts, which suggest the participation of D-Pen^5 but not L-Pen^5 amide protons in intramolecular hydrogen bonding. This hydrogen bond is likely to be of little significance as a requirement for high delta opioid receptor selectivity, however, since [D-Pen^2, L-Pen^5]- and [D-Pen^2, D-Pen^5] enkephalin display very similar potency and delta receptor selectivity (7). D-Pen^5 vs. L-Pen^5 substitution also results in different chemical shifts for the Gly^3 α and Phe^4 α protons. These effects may reflect a different orientation of the Phe^4 aromatic ring resulting in a slight ring current effect. The observed rather large variation in JN*HαCH* and smaller but significant variation in χ^1 rotamer populations for Phe^4 are consistent with this interpretation.

The largest observed variation of a conformationally informative parameter is for JN*HαCH* of the Gly^3 residue. This variation reflects significantly different values for the Gly^3 ϕ and perhaps ψ backbone dihedral angles of this inherently more flexible residue. It seems reasonable to suggest that the Gly^3 residue plays a pivotal role by adopting a conformation in a particular analog which results in a similar gross topography for many or all of the analogs of the series. Such conformational compensation could result in similar relative orientations of portions of the molecule essential for a particular bioactivity while allowing for differences in conformation of other portions of the molecule. As an example, this could account for the similar opioid potencies and delta receptor selectivities of [D-Pen^2, L-Cys^5]- and [D-Cys^2, D-Pen^5] enkephalin (5), analogs which, as can be seen from Table 1–3, display multiple differences in conformationally related parameters.

It should be noted that there is very good agreement of JN*HαCH*, side chain rotamer populations, and amide proton chemical shift temperature dependence values between [D-Cys^2, D-Pen^5] enkephalin and [D-Pen^2, D-Pen^5] enkephalin. While this agreement suggests very similar conformations for the two analogs, the former is 17- and 100-fold more potent than the latter in the mouse vas deferens bioassay (delta opioid receptor potency) and guinea pig ileum bioassay (mu opioid receptor potency), respectively (5), and as a result [D-Cys^2, D-Pen^5] enkephalin is 6-fold *less* selective for the delta receptor than is [D-Pen^2, D-Pen^5] enkephalin. These findings suggest that the gem dimethyl groups of the D-Pen^2 residue of [D-Pen^2, D-Pen^5] enkephalin may cause an unfavorable steric interaction at both mu and delta opioid receptors, leading to a decrease in potency, and that this steric effect is more deleterious for binding to the mu opioid receptor.

Finally, it should be pointed out that the data for [D-Pen^2, L-Cys^5]- and [D-Pen^2, D-Cys^5] enkephalin are virtually indistinguishable from those for the corresponding enkephalinamide analogs reported previously

(11). Since all the experiments were performed on acidic solutions (pH $\simeq$ 3) in which the protonated form of the carboxy terminal of each enkephalin analog is expected to dominate, this result is not surprising. However, re-examination of these parameters for [D-Pen^2, L-Cys^5]- and [D-Pen^2, D-Cys^5] enkephalin at higher pH values (pH 4.5–6) revealed no large changes in measurable parameters. Unfortunately, increased amide proton exchange rates made it impossible to measure reliable values for Pen^2 and Gly^3 JN*H*α*CH* under these conditions. Nonetheless, the apparent lack of effect of pH on measurable parameters suggests similar conformations for corresponding penicillamine-containing enkephalin and enkephalinamide analogs.

ACKNOWLEDGMENTS

This work was supported by USPHS grants NS20428 and DA03910. The author wishes to thank Prof. R. W. Woodard for kindly providing samples $^2H_6$ (β,β CD_3) D, L penicillamine.

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