Acetaldehyde–Enkephalins: Structure Proof and Some Conformational Deductions from One- and Two-Dimensional Proton Nuclear Magnetic Resonance Spectra†

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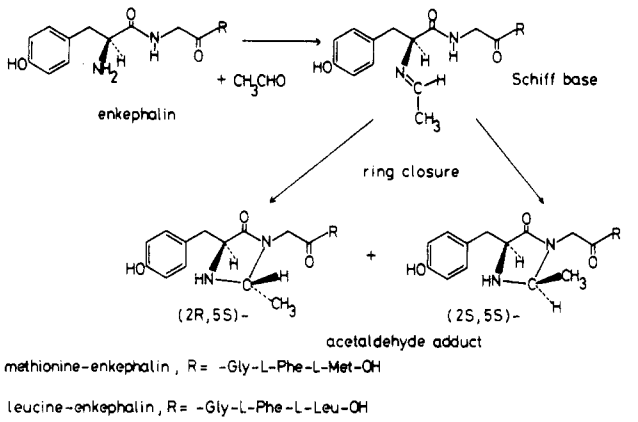
ABSTRACT: The structure of the adduct formed by reaction of acetaldehyde and Met^5-enkephalin has been determined by analysis of 400-MHz proton spectra: two-dimensional *J* spectroscopy was used to resolve and measure virtually all the overlapping resonances, and decoupling difference spectroscopy was used to assign the resonances. Suitable manipulation of the two-dimensional data allowed analysis of α-CH resonances which were completely buried under a water signal and of amide NH resonances which overlapped in both dimensions. The adduct was shown to be a mixture of two diastereoisomers, each containing a 2-methylimidazolidin-4-one ring formed by condensation of an acetaldehyde molecule with the N-terminal amino group and Gly^2 amide nitrogen. Analysis of the NMR data suggests that the folded conformation characteristic of native enkephalins in dimethyl-d_6 sulfoxide is not important in these derivatives.

The opioid pentapeptides Met^5-enkephalin (**1**) and Leu^5-enkephalin (**2**) react rapidly with aqueous solutions of acetaldehyde to yield adducts with much reduced opiate activity (Summers & Hayes, 1980), and three of us have recently presented some 100-MHz proton NMR evidence that these adducts have the 2-methylimidazolidin-4-one structure shown in Scheme I, each adduct being formed as a mixture of the 2*R*,5*S* and 2*S*,5*S* diastereoisomers (Summers et al., 1980). However, the complexity of the spectra at 100 MHz precluded any detailed analysis of structure or stereochemistry. In this paper, we show that a virtually complete analysis of the 400-MHz spectrum of the adduct of **1** in dimethyl-d_6 sulfoxide (Me_2SO-d_6)[1] fully confirms all our previous spectroscopic and chemical conclusions and throws some light on the conformation of these modified enkephalins.

The proton spectra of native enkephalins in Me_2SO-d_6 have been analyzed previously by a combination of classical spin-decoupling and spectral simulation (Garbay-Jaureguiberry et al., 1976, 1977; Jones et al., 1976; Higashijima et al., 1979), but in this study, the presence of two diastereoisomers rendered the α-CH and particularly the NH region of the spectrum so complex that such an approach appeared rather difficult. We chose therefore to exploit a strategy (Hall & Sanders, 1980) which uses two-dimensional *J* spectroscopy to resolve all the proton resonances in the spectrum and one-dimensional decoupling difference and NOE difference spectroscopy to assign them. In essence, the two-dimensional *J* experiment consists of a series of spin-echo spectra further processed to give a complete separation of chemical shifts and homonuclear coupling *along different frequency axes*, provided only that all the spin systems are weakly coupled. It is possible, therefore, to obtain a "proton-decoupled" spectrum displaying only singlets at each chemical shift and "partial *J* spectra"

Scheme I



of each multiplet even when these multiplets are severely overlapping and congested in the normal one-dimensional spectrum. Two-dimensional *J* spectroscopy was first developed in the mid-1970's (Aue et al., 1976) and has been used recently to unravel the otherwise intractable spectra of polypeptides (Nagayama et al., 1978), oligosaccharides (Hall et al., 1980), steroids (Hall & Sanders, 1980, 1981), and alkaloids (L. D. Hall, B. K. Hunter, and J. K. M. Sanders, unpublished results). In decoupling difference spectroscopy, a control spectrum is subtracted from a decoupled one so that only resonances which are affected by the irradiation appear in the difference spectrum (Barry et al., 1973; Kuo & Gibbons, 1979; Hall & Sanders, 1980, 1981); this makes it a powerful method for locating otherwise hidden resonances. Similarly, in NOE difference spectroscopy, only enhanced resonances appear.

Experimental Procedures

Materials. Acetaldehyde (BDH Chemicals) was stored at −20 °C as an aqueous solution, ~2.5 M, and replaced every 2 weeks. Solutions were assayed by measuring the yeast alcohol dehydrogenase catalyzed oxidation of NADH; the assay volume was 2.0 mL of 0.1 M phosphate buffer, pH 7.2, con-

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[1] Abbreviations used: Me_2SO-d_6, dimethyl-d_6 sulfoxide; FID, free induction decay; Leu, Leucine; Met, methionine; NADH, nicotinamide adenine dinucleotide (reduced form); NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ppm, chemical shift in parts per million from tetramethylsilane; TLC, thin-layer chromatography.

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taining yeast alcohol dehydrogenase (100 units, Sigma), NADH (0.05 mL, 10 mg/mL), and dithiothreitol (0.8 μmol). Pyridine was dried over CaH_2 and distilled from α-naphthyl isocyanate.

TLC was carried out on precoated silica gel GF_{254} aluminium-backed plates (Merck, Darmstadt, West Germany) with solvent system A, chloroform/methanol/acetic acid (9:2:1), B, 1-butanol/acetic acid/water (3:1:1), or C, ethyl acetate/pyridine/acetic acid/water (60:20:6:11).

Synthesis of Acetaldehyde–Met⁵-enkephalin. Met⁵-enkephalin (24 μmol, Miles Laboratories) and acetaldehyde (320 μmol) were dissolved in 0.05 M phosphate buffer (KH_2PO_4/Na_2HPO_4, 4.0 mL), pH 7.0, at room temperature. The course of the reaction was followed by analytical TLC until there was no longer any ninhydrin-positive starting material, usually about 4 h. The reaction mixture was diluted with water, 6.0 mL, and passed down a short column of Dowex-50 (H^+ form, 4% cross-linked, 1 × 25 cm). The column was washed extensively with water, and the product was eluted with 0.1 M aqueous pyridine. Fractions containing peptide were detected by the Folin–Lowry test (Lowry et al., 1951), freeze-dried, and stored anhydrous at −20 °C.

The chromatographic R_f values for Met⁵-enkephalin and its acetaldehyde adduct in solvents A, B, and C, respectively, are (enkephalin) 0.1, 0.55, and 0.61 and (adduct) 0.35, 0.64, and 0.75.

NMR Spectroscopy. Except where otherwise stated, all results reported here were obtained at ~30 °C on a Bruker WH 400 instrument in Vancouver operating at 400 MHz under ASPECT 2000 control. Earlier experiments were carried out at 100 (Summers et al., 1980) and 270 MHz (J. K. M. Sanders, unpublished results). The same sample (8 mg/mL in Me_2SO-d_6) was used throughout, and no attempt was made to remove the prominent water signal.

Two-dimensional *J* spectra were obtained with a prototype automated Bruker program. In the chemical shift direction (f_2), 4096 data points were acquired with a spectral width of 2700 Hz, giving a digital resolution of 1.32 Hz. For improvement of digital resolution, the spectrum offset was arranged so that the NH region around 8 ppm was folded over. The aromatic region folded upon itself and carried no useful information. For suppression of interference from the broad water signal and suppression of "tails" from intense singlets, sine bell resolution enhancement (De Marco & Wüthrich, 1976) was used in the chemical shift dimension (Hall & Sanders, 1980, 1981). In the *J* (f_1) dimension, after zero filling, 128 points were used over 42.2 Hz, giving a digital resolution of 0.33 Hz. Some resolution enhancement was also applied in this dimension. A total of 96 transients was acquired for each of the 64 incremental spectra, giving a total experimental acquisition time of 3.6 h. The resulting two-dimensional *J* spectrum is essentially noiseless except in the NH region.[2]

One-dimensional spectra were acquired with 8K or 16K data points, depending on the experiment; some were run with the same offset and spectral width as the two-dimensional spectra for comparison purposes, but generally foldover was avoided. Decoupling difference and NOE difference spectra were obtained by using automated programs which allow the concurrent (i.e., "simultaneous") acquisition of many difference spectra served by the same control spectrum (Hall & Sanders, 1981).

[2] The apparent intensity of the folded NH proton resonances was diminished both by their broadness and by an automatic filter which was set at 1.25 × spectral width.

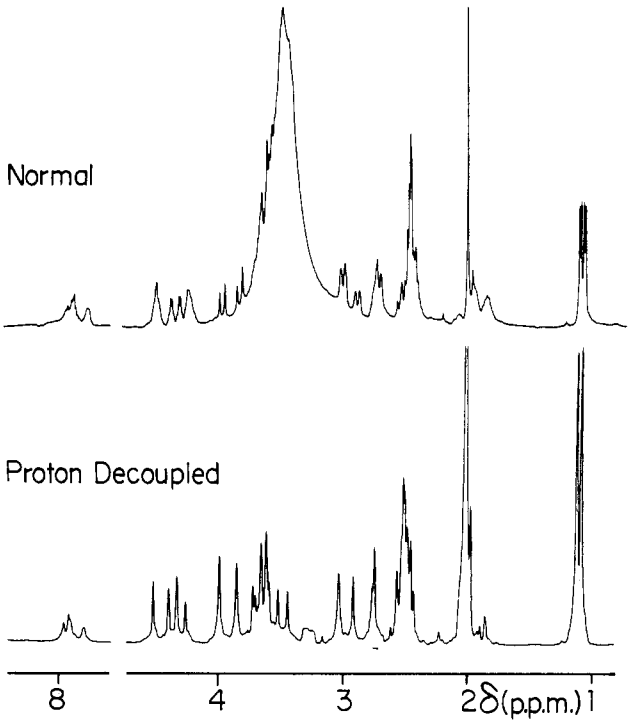

FIGURE 1: Partial 400-MHz ^1H NMR spectra of acetaldehyde–Met⁵-enkephalin with the amide NH region folded over. (Upper trace) One-dimensional spectrum. (Lower trace) "Proton-decoupled" spectrum obtained from the two-dimensional *J* spectrum by projecting onto the chemical shift (f_2) axis.

Results

Figure 1 shows the "proton-decoupled" spectrum of acetaldehyde–Met⁵-enkephalin obtained by projecting the two-dimensional *J* spectrum onto the chemical shift dimension and, for comparison, the one-dimensional spectrum obtained with the same offset and spectral width. It is immediately apparent that the dominant water signal which obscures most resonances between 3 and 4 ppm in the one-dimensional spectrum is totally absent from the two-dimensional spectrum of the same sample.[3] This is a useful consequence of using sine bell resolution enhancement and will be dealt with at greater length under Discussion. It is clear from the normal one-dimensional spectrum that our earlier deduction that *two* compounds are formed in equal amounts in the reaction with acetaldehyde is correct: there are two CH_3CH systems apparent at ca. 1.1 and 4.4 ppm and several resonances which integrate for just 0.5 proton each. From the proton-decoupled spectrum it was possible to obtain precise chemical shifts for all carbon-bound protons except Met γ and β which are both strongly coupled *and* adjacent to solvent and the Met methyl signals, respectively. The partial *J* spectra corresponding to each multiplet yielded all coupling constants (except those involving Met β and γ), but this information alone did not provide complete assignments, as there are several very similar sets of couplings.

The NH region was less clear, even in two dimensions (Figure 2B). In the stacked plot, two doublets (J = 7.7 Hz) are seen at 8.24 and 8.25 ppm, but the 8.10–8.15 region is crowded. In addition, the noise "tails" are not parallel to the f_1 (J) axis and distract the eye from the true multiplets which are parallel to f_1. The proton-decoupled spectrum (Figure 2A) is little help, but partial *J* spectra indicate the presence of a triplet at 8.08 ppm (J = 5.6 Hz), somewhat overlapping doublets at 8.12 and 8.14 (J = 8.0 Hz), and a triplet at 8.13

[3] This is only true if sine bell is used.

Table I: Chemical Shifts and Coupling Constants in Acetaldehyde-Met[5]-enkephalin[a]

	chemical shifts (ppm ± 0.01)						coupling constants (Hz ± 0.4)					
residue	H_α	$H_{\alpha'}$	H_β	$H_{\beta'}$	NH	other	$^2J_{\alpha\alpha'}$	$^3J_{\alpha\beta}$	$^3J_{\alpha\beta'}$	$^2J_{\beta\beta'}$	$^3J_{NH,H_\alpha}$	other
Tyr^1	3.46		2.94	2.6[b]		aromatics: 7.04, 7.05, 6.66, 6.67		3.6	8.9	14.0		aromatics: $J_{o,m}$ = 8.4
Tyr^{1*}	3.54		2.8[b]	2.6[b]				4.2	9.2	[c]		
Gly^2	4.01	3.64					16.6					
Gly^{2*}	3.87	3.68					16.4					
Gly^3	3.74	3.61			8.08, 8.13		16.5				5.6	
Phe^4	4.54		3.05	2.8[b]	8.12, 8.14	aromatics: 7.2–7.3		4.2	9.9	13.8	8.0	
Met^5	4.28		~2.0	~2.0	8.24, 8.25	H_γ 2.4[b], CH_3 2.05		[d]	[d]	[c]	7.7	

[a] In Me_2SO-d_6, 30 °C; the protons of the methylimidazolidinone ring resonate at 1.10 (CH_3) and 4.42 (CH) ppm in one diastereoisomer and at 1.14 and 4.32 ppm in the other. [b] Approximate due to signal overlap. [c] Not measured. [d] $J_{\alpha\beta} + J_{\alpha\beta'}$ = 13.7 Hz. Apparent splittings are 4.5 and 9.2 Hz, but near-equivalence of H_β protons may render this appearance deceptive.

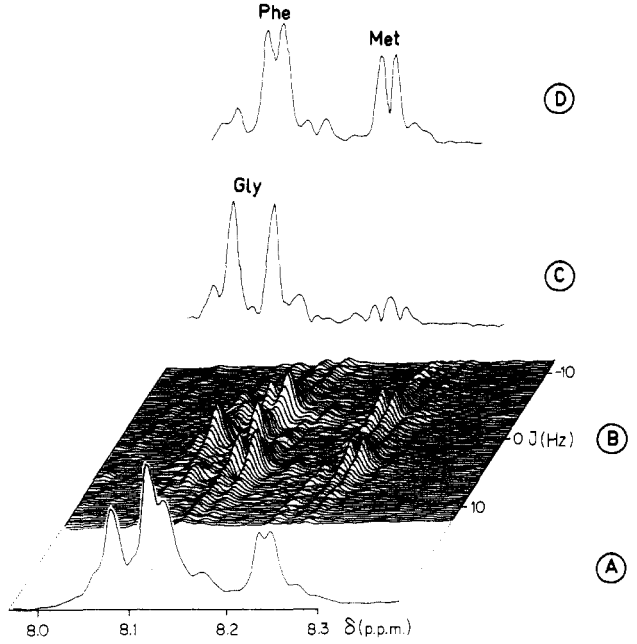

FIGURE 2: Partial two-dimensional J spectra of the amide NH region. (A) Proton-decoupled spectrum, (B) stacked plot, (C) center row of stacked plot (f_1 = 0 Hz), and (D) eleventh row from center of stacked plot (f_1 = 3.65 Hz).

(J = 5.6 Hz). These various components are most easily distinguished by plotting individual rows from the stacked plot. Figure 2C shows the central row corresponding to 0 Hz in the f_1 dimension; it contains only the central line from the triplets at δ 8.08 and 8.13. Figure 2D, the 11th row from the center corresponding to 3.65 Hz displays signals only from the doublets.[4]

Assignments to particular peptide residues were made by inspection where possible, but all were confirmed by spin-decoupling and decoupling difference spectroscopy. Figure 3 shows how decoupling difference spectra were used to confirm the assignment of the glycine protons coupled to the Gly^3 NH triplet and to pair the two sets of signals from Gly^2 residue. It proved more difficult to determine which set of signals originated from which diastereoisomer; however, NOE difference spectroscopy did establish that the methylimidazolidinone resonances at 1.10 and 4.42 ppm were in the same molecule as the Gly^2 protons at 3.64 and 4.01. No other interresidue NOE's were detected. All the NMR data and assignments are collected in Table I. Variable temperature experiments showed that all amide NH protons, including that

[4] The 12th row (4 Hz) is essentially identical.

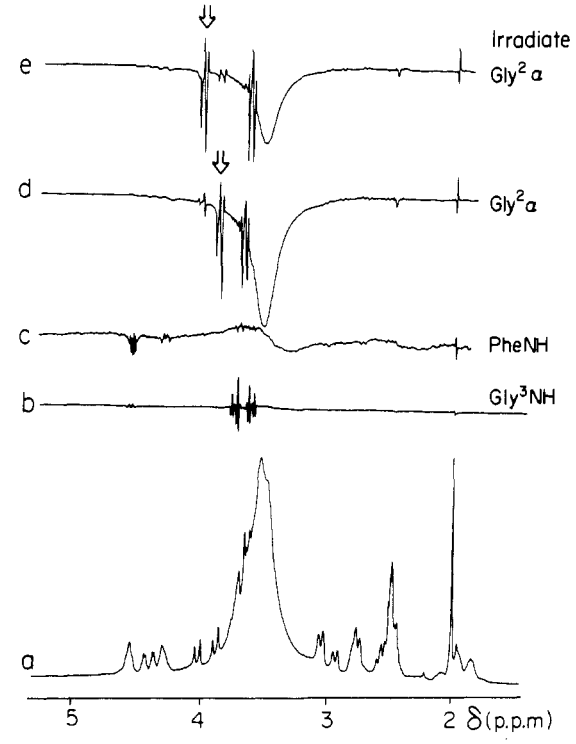

FIGURE 3: Partial decoupling difference spectra. The irradiated proton is indicated at the right of each trace and, in two cases, by arrows on the spectra. In the top two traces, saturation transfer from the wings to the center of the broad water resonance is visible.

of Met^5, shift upfield with essentially the same temperature coefficient (ca. 0.005 ppm/°C).

The adduct structure shown in Scheme I was proposed (Summers et al., 1980) on the basis that Gly^2 NH (which is generally rather broad) was missing, and on the basis of the appearance of new resonances which were *deduced* to arise from CH_3CH fragments. The results presented here directly confirm the presence of these fragments; they also show doubling of the entire spin systems of Tyr^1 and Gly^2 and of the NH signals of all the remaining residues. Another structure in which cyclization has occurred to Gly^3 instead of Gly^2 may be ruled out by several lines of reasoning, none of which is individually conclusive but the sum of which is reasonably conclusive: the remaining Gly NH is sharp, the Phe spin system is not doubled, and a 5-membered ring is more likely than an 8-membered ring.

Discussion

Spectroscopy. Two-dimensional J spectroscopy greatly facilitated the successful outcome of this study; although it

was probably not essential, it gave a complete and rapid separation of all multiplets in the α-CH region, but it very nearly did the same for the NH region. The almost total absence of interfering water in the two-dimensional spectrum is due primarily to the use of sine bell resolution enhancement. Broad signals in the frequency spectrum correspond to very rapidly decaying components in the FID, and sine bell removes them by effectively zeroing the first few data points in that FID (De Marco & Wüthrich, 1976). *This is equally true for one-dimensional spectra* which also show little interference. In this work, of course, the interfering water could, and perhaps should, have been removed chemically rather than spectroscopically, but these experiments do establish that it is possible to isolate sharp signals from broad in a very simple way [see also Hall & Sukumar (1980) and Rabenstein et al. (1979)]. The decoupling difference spectra also show that "interfering" resonances need not actually interfere.

The ability of individual rows from the two-dimensional spectrum to identify all the resonances with a particular coupling constant (Figure 2C,D)[5] promises to be useful when resonance line widths are comparable with or greater than digitization in the chemical shift dimension, as was the case here with the NH protons. Individual cross sections corresponding to the Phe NH protons and the Gly NH at 8.13 ppm suffered sufficient overlap of signal as to be difficult to interpret, but parts C and D of Figure 2 are quite unequivocal.

The lack of interresidue NOE's is probably a consequence of the particular combination of frequency and correlation time (Niccolai et al., 1980).

Conformation. It is noteworthy that nonequivalence between the amide protons in the two diastereoisomers is in the order $Gly^3 \gg Phe^4 > Met^5$ and that there is any nonequivalence observable at all for Met^5.

Comparison of the data in Table I with previously published data for the zwitterionic form of Met^5-enkephalin (Garbay-Jaureguiberry et al., 1976; Jones et al., 1976; Higashijima et al., 1979) shows that most of the chemical shifts in the adduct are within 0.2 ppm of the native peptide and that virtually all the coupling constants are identical within experimental error. The only exception—apart from the Tyr^1 α-CH which is expected to be different—is the Met^5 amide NH which is 0.35 ppm downfield from the corresponding proton in the natural enkephalin.

The temperature dependence of the chemical shift of the Met^5 amide NH, around 0.005 ppm/°C, is also in marked contrast to the near temperature independence of the corresponding proton in the natural enkephalin. This temperature independence has been interpreted in terms of a specific hydrogen bond or general solvent inaccessibility for that proton in a predominantly folded conformation involving a "head to tail" interaction between the charged amine and carboxylate groups. The loss of temperature independence for the Met^5 amide NH chemical shift suggests that derivatization of Met^5-enkephalin with acetaldehyde destabilizes the favored folded conformation in Me_2SO-d_6 solution: each of the amide protons is apparently freely accessible to solvent. In view of the lack of observable intramolecular interaction[6] and the similarity of the observed chemical shifts and coupling constants with those expected for a random coil conformation (Bundi et al., 1975), it is not unreasonable to assume that the observed NMR parameters correspond to the weighted average of a number of unfolded solution conformations. However, without a knowledge of the individual contributory structures, the detailed conformational information which could be derived from the data presented is probably physically meaningless (Jardetzky, 1980). In view of the similarity of chemical shifts and coupling constants in natural and acetaldehyde-derivatized enkephalins, it would also appear that these parameters are actually somewhat insensitive to conformational changes.

[5] Suggested by Dr. B. K. Hunter, Queen's University, Kingston, Ontario.

[6] At the concentrations used in this work, aggregation is unimportant (Higashijima et al., 1979).

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