pH and Solvent Titrations of Enkephalins by Carbon-13 Nuclear Magnetic Resonance Spectroscopy: Complete Assignments of Resonances

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Carbon-13 magnetic resonances of two enkephalins, [Met^5]- and [Leu^5]-enkephalin, have been assigned in water, dimethyl sulphoxide, and trifluoroethanol, and assignments of [D-Ala^2]-enkephalin-amide have been made in water. The assignments of the Gly_2 carbon atoms have been made by comparison with [D-Ala^2]-enkephalin amide. pD Titrations were followed to assign all the carbon resonances in D_2O first, and then the assignments of resonances in other solvents were obtained by solvent–solvent titrations. An unusual titration behaviour of the Tyr_1 aromatic γ-atom at a higher pH was found, and this has been suggested to be due to a conformational change in the Tyr_1 C^α–C^β bond. Conformational features of enkephalins in solution are briefly considered in relation to previous studies.

Two pentapeptides with morphine-like activity have been shown to occur in the mammalian brain.[1] The amino-acid sequences of these peptides are H–L–Tyr_1–Gly_2–Gly_3–Phe_4–L–Met_5–OH and H–L–Tyr_1–Gly_2–Gly_3–L–Phe_4–L–Leu_5–OH, which are respectively called [Met^5]- and [Leu^5]-enkephalin (or M–E and L–E). Because of their opiate-like activity, it is likely that enkephalins bind to the same specific receptor site as does morphine. To understand the mechanism of their opiate-like activity at the receptor site, one needs to compare the three dimensional structure of enkephalins with morphines and their derivatives. The enkephalins, being small peptides, can adopt different conformations in solution while morphine has a rigid structure. Realizing this, several research groups [2-12] have investigated the conformational characteristics of enkephalin with the purpose of arriving at a structural framework which could provide the basis of competition with morphine for the brain opiate receptor.

Conformational models of enkephalin have been proposed on the basis of presumed structural similarities with morphine and related analgesics [2,3] of nuclear magnetic resonance studies,[4-10] and of conformational energy calculations.[11,12] Recently, Smith and Griffin [13] have solved the crystalline structure of [Leu^5]-enkephalin. In ali these studies (solutions, theoretical and crystallographic) there exist considerable differences in conformational features of enkephalin. In a recent communication,[14] we have demonstrated the occurrence of intermolecular association of these molecules which is dependent on solvent and concentration.

Assignment of resonances in n.m.r. spectroscopy is an essential initial step in obtaining conformational information in solution. We have, therefore, devoted a considerable effort to assigning the carbon-13 resonances of enkephalins in water by an extensive pH titration and in dimethyl sulphoxide and trifluoroethanol at low concentrations by following a solvent–solvent titration. An analogue of enkephalin, H–L–Tyr_1–D–Ala_2–Gly_3–L–Phe_4–L–Met_5 amide ([D-Ala^2]-enkephalin amide) has also been studied, which is of intrinsic interest because of its potency and which solves the problem of assignments of the resonances of two glycine residues in [Met^5]- and [Leu^5]-enkephalins.

Materials and Methods.—Both [Leu^5]- and [Met^5]-enkephalins (M–E and L–E) were purchased from Bachem, Inc. and were used without further purification. [D-Ala^2]-nekephalin amide was a gift from Dr. R. C. A. Frederickson of Eli Lilly Laboratories which is gratefully acknowledged. Carbon-13 spectral measurements were made on a JEOL PFT-100 spectrometer using a 1 s repetition time and a 40 μs pulse width for 90° tilt of the magnetization vector. The pD values were determined by means of a Model 25 Radiometer pH meter. The normal pD of M–E and L–E were found to be 4.72 and 4.55, respectively, in D_2O. The pD titration was carried out by adding 0.1N-DCl to the water solution of enkephalins stepwise to bring the pD down to zero. The pD range 0—12.5 was covered by the stepwise addition of 0.1N-NaOD. The titration points (in the pD range 0 to 5) obtained either by adding DCl or NaOD were found to fall on the same titration curve of each carbon resonance of the enkephalins. In water the concentration was less than 0.01M; in dimethyl sulphoxide (DMSO), 0.005M; and in trifluoroethanol (TFE), 0.05M.

RESULTS

Typical ^{1}C n.m.r. spectra of both [Met^5]- and [Leu^5]-enkephalins (M–E and L–E) in D_2O are shown in Figure 1. The spectra were obtained with respect to the internal reference hexamethyldisiloxane (HMDS) and the signals are grouped in A, B, and C regions. Region A contains the carbonyl and carboxy carbon resonances; B, the aromatic carbon resonances; and C, the α-carbons and aliphatic side-chain carbon resonances. All chemical shift data are listed in Table 1. Assignments of the chemical shifts to specific carbons were made on the basis of the pD titration behaviour of the spectral peaks (Figures 2, 3 and 4) and based on the literature data for the chemical shifts of the ^{13}C resonances of the amino-acids [15] and peptides.[16-19]

Typical pD titration curves of all the carbonyl carbon resonances (region A) are shown in Figure 2. Going from high pD to low pD, the lowest field signals at 179.5 p.p.m. for M–E and 178.9 p.p.m. for L–E showed upfield chemical-

shift changes (Figure 2a and 2b). The pK value of this change is 3.5 where the protonation of a carboxy-group occurs. Therefore, the signal at 179.5 p.p.m. for M–E is assigned to Met_5 C=O (Figure 1a), while the other at 178.9 p.p.m. for L–E is assigned to Leu_5 C=O (Figure 1b). As it has been previously observed [17,19] that the protonation of the C-terminal carboxy-group of a peptide produces a downfield shift of the carbonyl group of the preceding amino-acid residue, the downfield shift of the signals at 174.4 p.p.m. for M–E (Figure 2a) and at 175.1 p.p.m. for L–E (Figure 2b) with a pK of 3.5 are, therefore, assigned to the Phe_4 C=O (Figure 1). A rapid downfield chemical

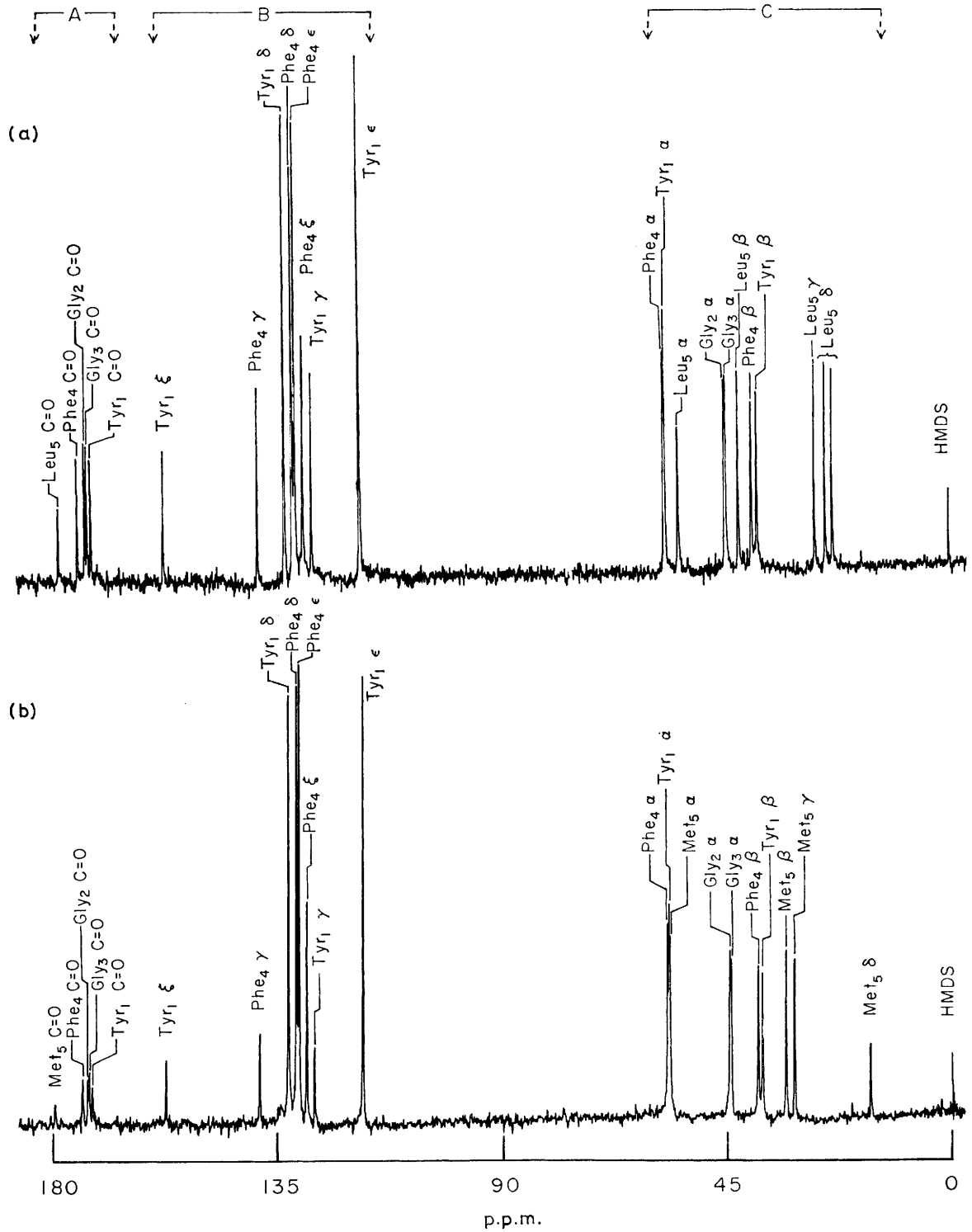

FIGURE 1 25.15 MHz Carbon-13 n.m.r. spectra. (a) [Leu^5]-enkephalin in D_2O at normal pD (4.55), (b) [Met^5]-enkephalin in D_2O at normal pD (4.72)

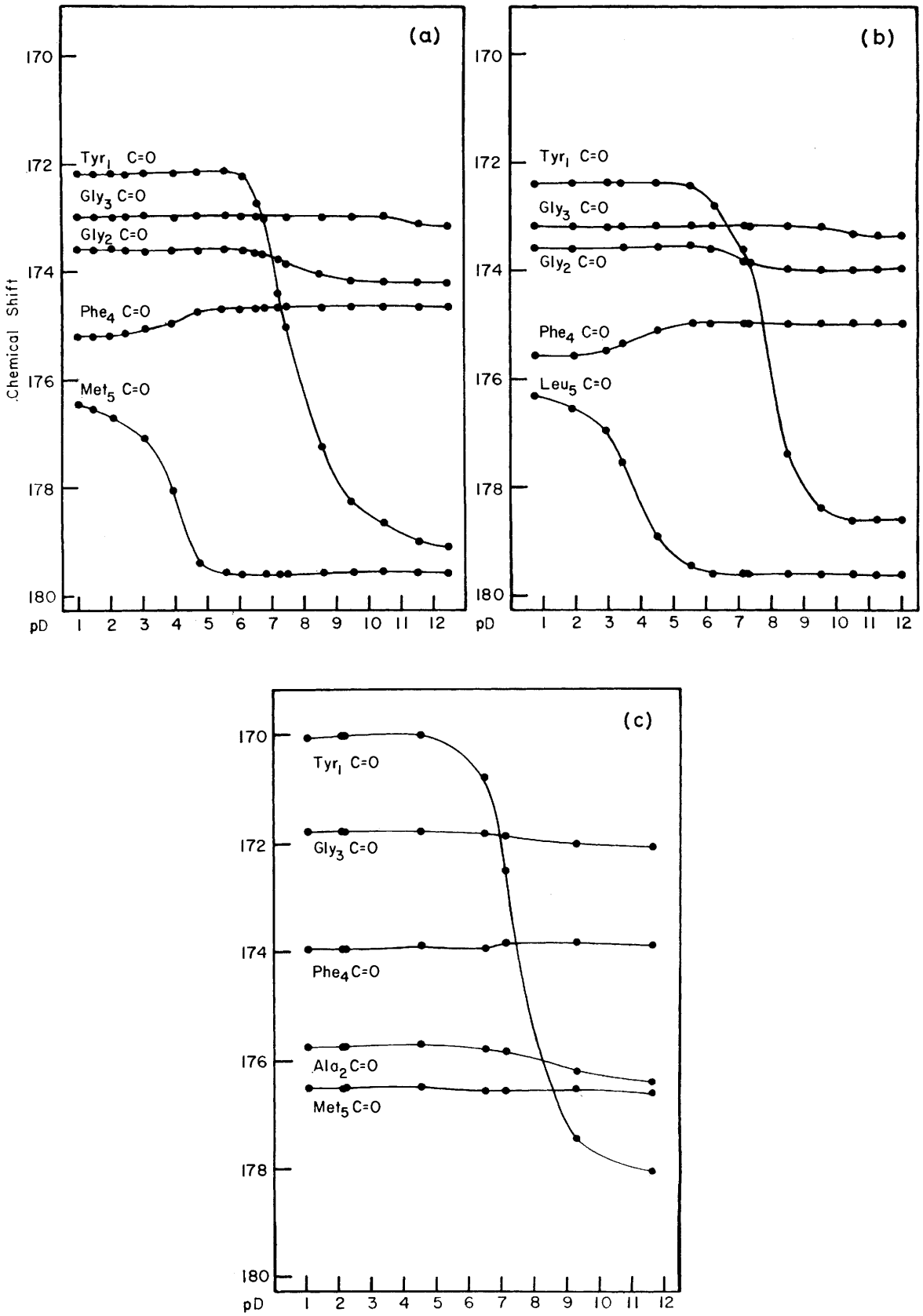

FIGURE 2 pD titration curves of carbonyl carbon resonances. (a) [Met^5]-enkephalin, (b) [Leu^5]-enkephalin, and (c) [D-Ala^2]-enkephalin amide

TABLE 1

^{13}C Chemical shifts of enkephalins in D_2O expressed in p.p.m. from hexamethyldisiloxane (HMDS)

Residues	Carbon atoms [a]	Enkephalin [b] [Met^5]	Enkephalin [c] [Leu^5]	D-Ala$_2$ Enkephalin [d] [Met^5-amide]
Tyr$_1$	α	56.9	56.8	56.06
	β	38.3	38.5	37.66
	γ	127.6	128.0	126.34
	δ	133.0	133.4	131.63
	ε	118.1	118.4	116.63
	ξ	157.4	157.7	155.95
	C=O	172.2	172.4	170.02
Gly$_2$	α	44.8	44.0	
	C=O	173.3	173.6	
D-Ala$_2$	α			50.67
	β			14.95
	C=O			175.65
Gly$_3$	α	44 6	44.5	43.05
	C=O	173.0	173.2	171.77
Phe$_4$	α	57.2	57.3	53.10
	β	39.3	39.7	36.94
	γ	138.8	138.8	136.77
	δ	131.4	131.8	130.02
	ε	130.9	131.8	129.69
	ξ	129.3	129.7	128.14
	C=O	174.4	175.1	173.86
Met$_5$/Leu$_5$	α	56.6	54.5	55.43
	β	33.5	42.3	30.87
	γ	31.9	26.9	30.14
	δ	16.5	23.3, 24.8	17.04
	C=O	179.5	178.9	176.43

[a] For designation of aromatic carbons, see Figure 3. [b] For a pD of 4.72. [c] For a pD of 4.55. [d] For a pD of 4.52.

shift of the signal in Figure 2 near 172 p.p.m. for M–E and for L–E on-going from pD 7 to 9 (pK 8) indicates the protonation of the α-amino-group; and, therefore, this resonance was assigned to Tyr$_1$ *C*=O (Figure 1). The assignment of the Gly$_2$ *C*=O resonance is suggested by observing the titration behaviour of the curve, which shifted downfield with a pK of *ca.* 8 due to the amino-group of the preceding residue, Tyr$_1$. To confirm this assignment, the pD titration curve (Figure 2c) of [D-Ala^2], [Met^5]-enkephalin amide (where Gly$_2$ is replaced by D–Ala and where the terminal carboxy-group is converted into the amide) was considered. It can be seen that the Ala$_2$ *C*=O at 175.7 p.p.m. shows a similar pD titration behaviour to a C=O resonance at 173.3 p.p.m. for M–E and at 173.6 for L–E. Therefore, this resonance was assigned to Gly$_2$ *C*=O, while the resonance at 173.0 p.p.m. for M–E and 173.2 p.p.m. for L–E was, by elimination, assigned to the Gly$_3$ *C*=O. It can be noted here that this Gly$_3$ *C*=O resonance (Figure 2a) shows a small downfield shift following the pK (10.2) of the Tyr aromatic OH group.

Region B, which contains the resonances of the aromatic carbons of the Tyr$_1$ and Phe$_4$ side-chains, is shown in Figure 3 as a function of pD. The assignments of the resonances to the individual aromatic carbon atom were made in accordance to the literature values [15] and with the findings of Christl and Roberts.[19] For the sake of clarity, the designation of all aromatic carbon atoms is shown at the top of Figure 3. It can be seen in this figure that within the experimental error there is no dependence of the Phe$_4$ aromatic carbon resonances on pD, while the Tyr$_1$ aromatic carbon resonances show chemical shifts which are dependent on pD. Of interest is the change occurring in the γ-carbon of Tyr$_1$ aromatic side-chain. This will be considered in the discussion.

The pD titration curves of all the α- and aliphatic side-chain carbon resonances (region C in Figure 1) are shown in Figure 4. The spectral behaviours of the α-carbon resonances are quite similar to their carbonyl carbon resonances (compare Figures 2 and 4). Identical behaviour of α-carbon resonances was also observed by previous authors; [16-19] and, therefore, assignments of α-carbon resonances were made by comparison with their carbonyl carbon resonances. The effect of deprotonation of the Tyr$_1$ amino-group and protonation of the Met$_5$/Leu$_5$ carboxy-group is also seen in their respective β-carbon resonances (Figure 4).

After having assigned all the resonances to each individual carbon atom (Figure 1) in D_2O from their pD titration behaviour, the DMSO–D_2O titration of the carbonyl region was followed in order to assign these resonances in DMSO. The solvent titration curves for both M–E and L–E are given in Figure 5. It can be seen in this figure that the Tyr$_1$ *C*=O resonance of both compounds shifted upfield rapidly with increased water content, while other signals shifted downfield. This effect could be attributed to the protonation of the Tyr$_1$ amino-group. It should also be noted here (Figure 5) that the Gly$_3$ *C*=O resonance shifts downfield less when compared to the Gly$_2$ *C*=O and Phe$_4$ *C*=O resonances.

The assignments of the carbonyl resonances in TFE were also obtained by the solvent titration of all carbonyl groups from DMSO to TFE. The results are summarized graphically in Figure 6 for both M–E and L–E. Similar to the above observation, it is also evident in this figure that the Gly$_3$ *C*=O is less perturbed (shifts downfield less) than all other C=O groups except Tyr$_2$ *C*=O. The relative upfield positioning of the Tyr$_1$ *C*=O with added TFE is not as significant as observed in the case of DMSO–D_2O titration (compare Figures 5 and 6) and is a consequence of the initial protonation of the Tyr$_1$ amino-group.

DISCUSSION

Since the pD dependence of ^{13}C resonances has been used to distinguish between NH_2-terminal and CO_2H-terminal, they may be sensitive to conformational equilibria [20,21] as well. Conformational changes can be considered when the shifts behave differently from what is normally expected from a pD titration. The normal pK value of a Tyr α-amino-group is 9.4. The deprotonation occurring at a lower pK (pK 8 instead of 9.4) perhaps is an indication of the proximity of a donor proton such as that of the carboxy-terminus. The large and steep chemical-shift change of the Tyr$_1$ *C*=O group of the NH_2-terminal residue on raising the pD in enkephalins is also unusual.[15-22] α-Amino-protons on interacting with a carboxy-group can make deprotonation of the α-amino-nitrogen easier. This seems to fit reasonably well with a head-to-tail interaction, which was suggested by Anteunis *et al.*[8] in a proton magnetic resonance study of [Met^5]-enkephalin during an acid-base titration.

The chemical shift changes of the aromatic carbon resonances of Tyr$_1$ are of interest (see Figure 3). It was pointed out earlier (see the Results section) that the γ-carbon resonance shows a differential movement in the pD range from 7 to 12. Its downfield change in the pD range 7 to 9 (pK 8) could be correlated with the deproton-

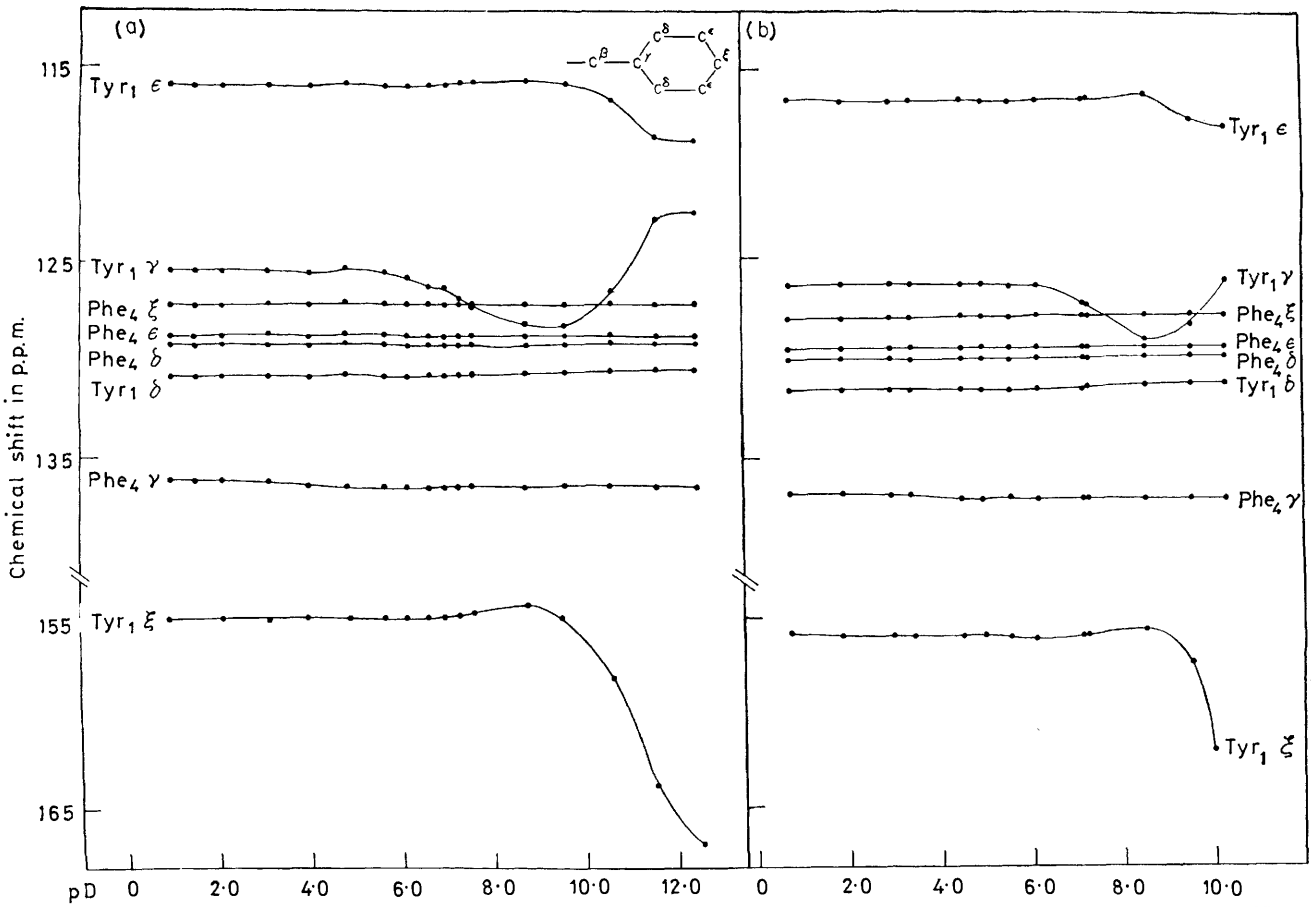

FIGURE 3 Tyr_1 and Phe_4 aromatic carbon resonances as function of pD: (a) [Met^5]-enkephalin and (b) [Leu^5]-enkephalin. Note atop the designation of aromatic carbon atoms (see text for discussion)

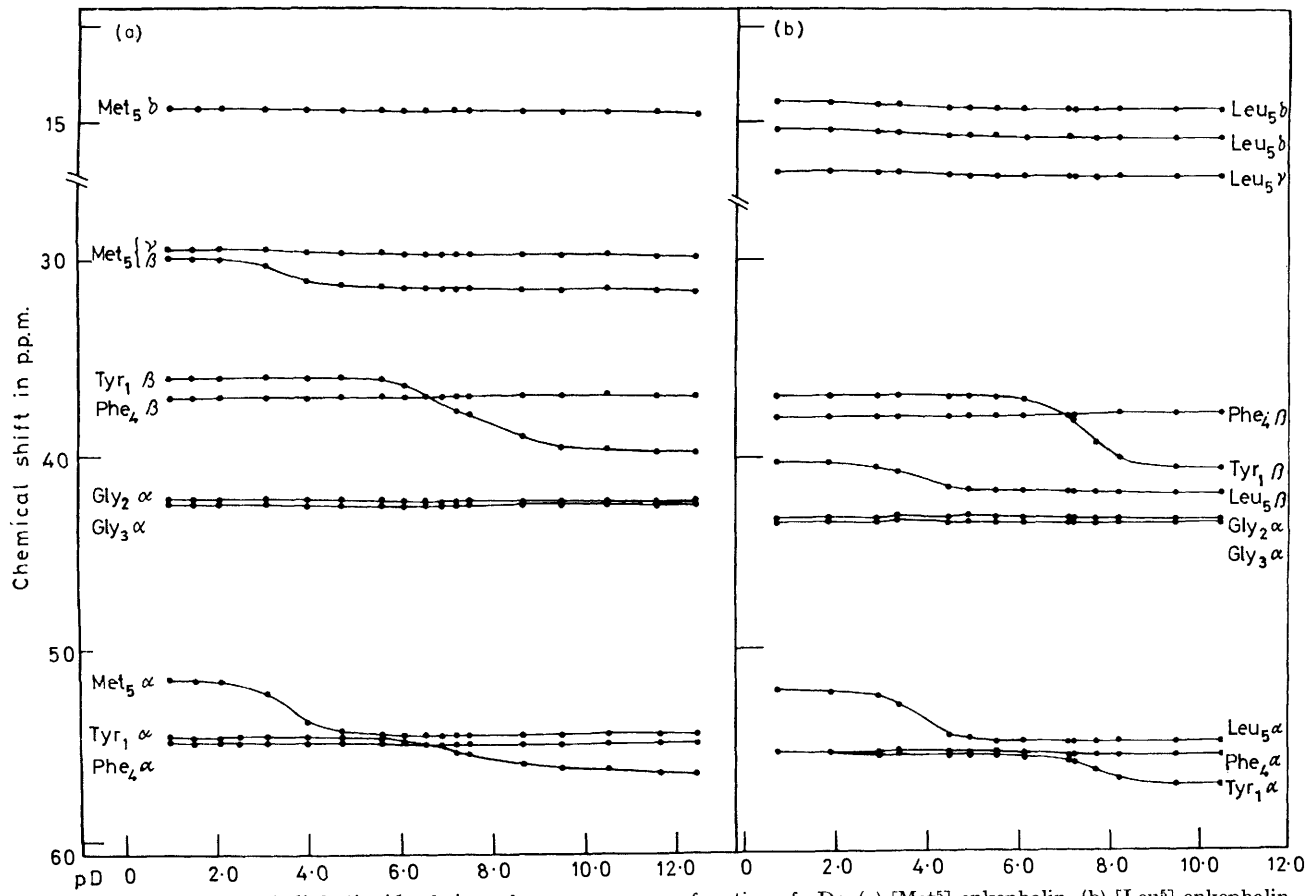

FIGURE 4 α-Carbon and aliphatic side-chain carbon resonances as function of pD: (a) [Met^5]-enkephalin, (b) [Leu^5]-enkephalin

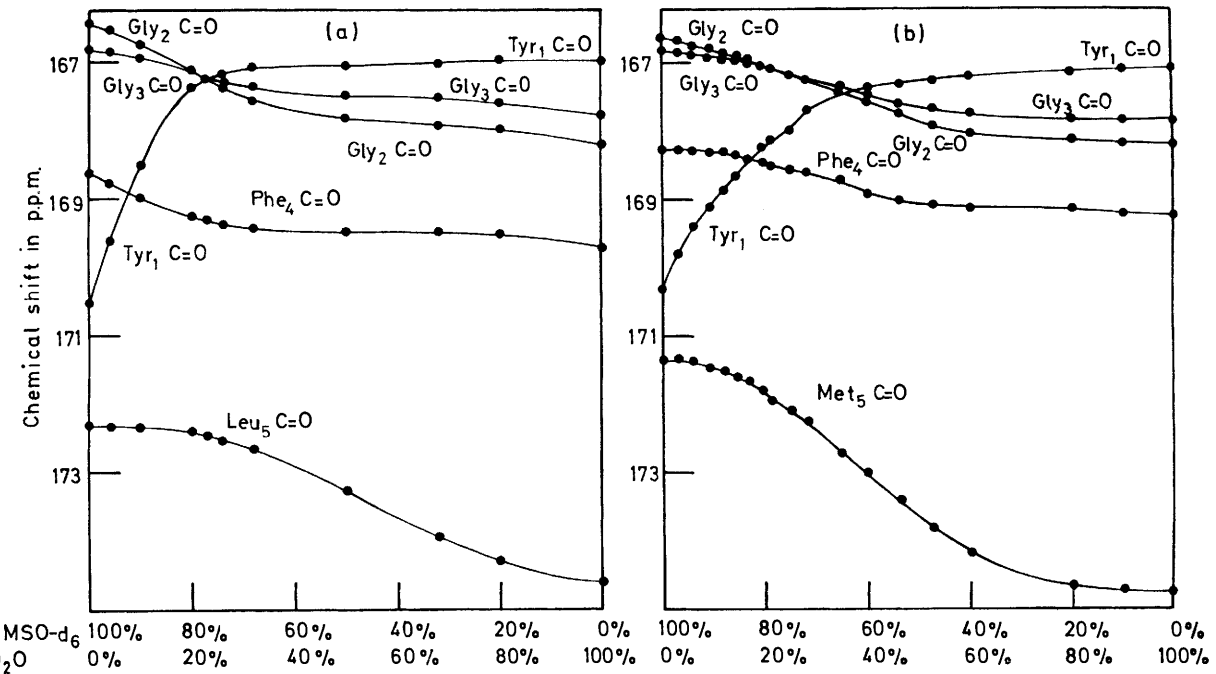

FIGURE 5 Dimethyl sulphoxide–water titration of carbonyl carbon resonances: (a) [Leu^5]-enkephalin and (b) [Met^5]-enkephalin. [Chemical shifts were converted to the TSP scale by using the formala δ(TSP) = δ(TMS) + 1.7 p.p.m.]

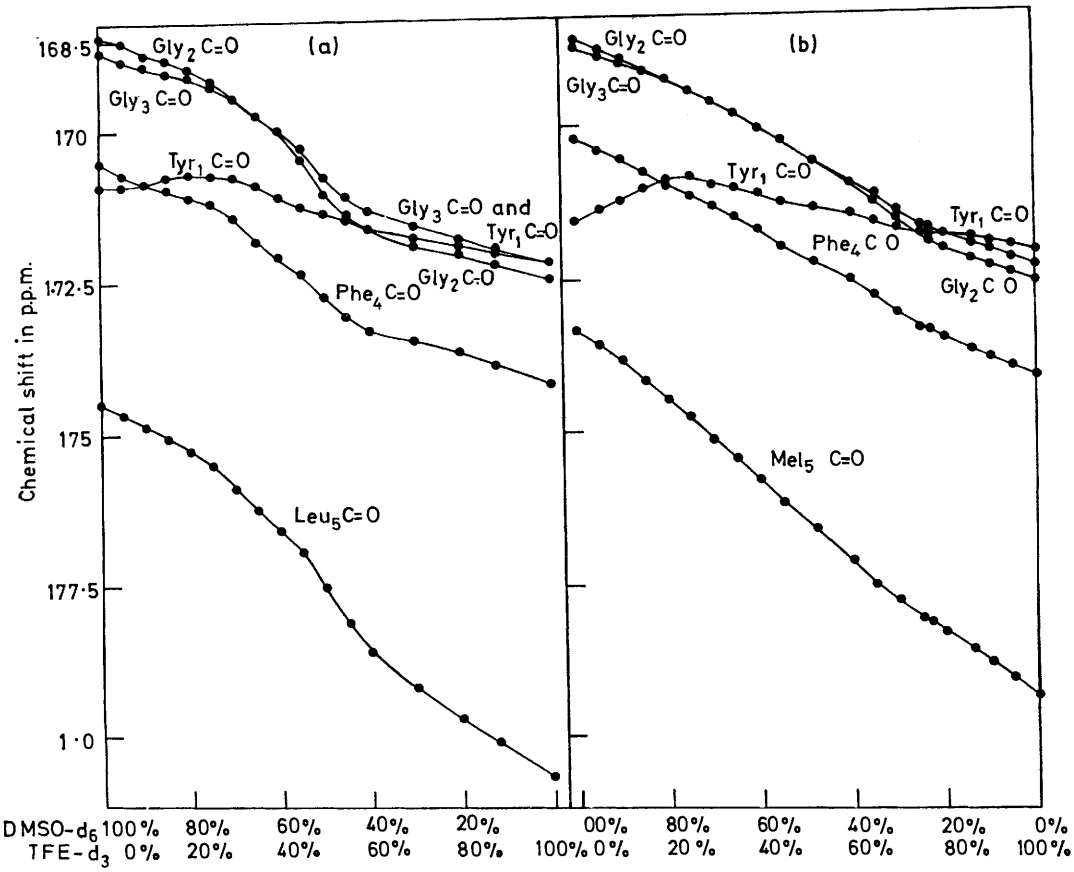

FIGURE 6 Dimethyl sulphoxide–trifluoroethanol titration of carbonyl carbon resonances. (a) [Leu^5]-enkephalin and (b) [Met^5]-enkephalin. (TMS was used as an internal standard)

ation of the Tyr_1 α-amino-group, while its upfield shift in the pD range 9 to 12 obviously requires additional explanation. This type of pD effect on the γ-carbon atom of tyrosine in some oligopeptides,[15-19] and in biologically active peptides, such as bovine pancreatic trypsin inhibitor [22] and angiotensin II,[18] has not been found, that is, in these systems no upfield shift is observed. It is reasonable to consider that the unusual behaviour of the γ-carbon atom of Tyr_1 could arise due to conformational changes occurring at the Tyr_1 C^{α}–C^{β} bond on deprotonation of the phenolic group. The deprotonation effect of the Tyr_1 phenolic group in the pD range 9—12 (pK 10.5) is observed in the ε and ξ-carbon resonances (Figure 3). As seen in Figure 2a, the Gly_3 C=O shows a slight downfield shift at the pK of the Tyr_1 phenolic group (10.5). This correlation suggests a weak H-bond between the Tyr_1 OH proton and the Gly_3 C=O group [14]. Formation of a weak H-bond of the Tyr OH with other functional groups has been suggested previously.[23,24]

Considering the pH titration behaviour of these enkephalins in water, it appears, however, that there could exist several preferred conformations in equilibrium. In addition to a monomeric crystalline form,[13] an associated form of enkephalin [14] has also been observed in crystalline state.[25] Another possibility is the formation of a turn of β-helix [26] which could be anticipated from the inversion of the pleat at the central Gly_3 α-carbon. Since these molecules behave differently in different solvents and at different concentrations, it would be speculative to attempt a direct correlation of solution conformational data with the receptor conformation. However, comparison of possible solution and crystal conformations with the rigid morphine structure may prove informative.[27]

Note added in proof: Very recently Blundell *et al.* in *X*-ray diffraction studies (*Science*, 1979, **205**, 220) have reported distortion at the Tyr_1 side chain and the occurrence of four different conformational states of [Leu^5]-enkephalin noting intermolecular hydrogen bonding.

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