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CIRCULAR DICHROISM AND ABSORPTION STUDY OF THE STRUCTURE OF METHIONINE-ENKEPHALIN IN SOLUTION.

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SUMMARY: The circular dichroism and absorption spectra of methionine-enkephalin have been measured in aqueous solution, as functions of temperature and pH, and in 2,2,2-trifluoroethanol. Ranges covered were: 190-330 nm; 5-50°C; pH = 1-12. Absorption data provide no evidence for a strong intramolecular hydrogen bond involving the hydroxyl proton of the tyrosine residue. All data can be interpreted without assuming methionine-enkephalin preferentially occupies a single conformation when in dilute solution. The biologically important conformation is presumably one of many which are present to significant extent.

INTRODUCTION: Enkephalins are a series of pentapeptides that show opiate activity (1). In particular they bind to the same receptor sites in the brain as morphine and act as agonists or mixed agonist-antagonists in that regard (2). Two naturally occurring sequences have been found: Tyr-Gly-Gly-Phe-Met (Met^5-Enk) and Tyr-Gly-Gly-Phe-Leu (Leu^5-Enk) (3). The methionine-enkephalin is the more active of the two (1). Alterations in the structure modify the analgesic effect of these peptides (1). Among the functional groups essential for activity are the phenolic hydroxyl group, and the phenyl group.

Several attempts have been made to elucidate the conformational preferences of Met^5-Enk in solution. One ^{1}H NMR study (4), conducted in DMSO and in aqueous solution as a function of pH, concluded that the Gly-Gly-Phe-Met segment forms a β_I-bend. Side chains of the phenylalanyl and methionyl residues are oriented away from the backbone. The tyrosyl residue was near the methionyl residue, but the phenoxy group was free to rotate. A second NMR study measured ^{1}H and ^{13}C resonances and relaxation times for Met^5-Enk in DMSO, D_2O, and a D_2O-H_2O mixture

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(5). The D_2O solutions were acidic (pH 2.6-2.7). This study concluded that, while no hydrogen bonds existed, the motion of the tyrosyl residue was restricted so that the separation of the phenoxy group and amino terminus was the same as in morphine. Different conformations were proposed to exist in D_2O and DMSO, but in each case the phenylalanyl and methionyl side chains had significant internal rotational freedom.

A calculation (6) based on statistical studies proposed a β-turn involving Tyr-Gly-Gly-Phe. This conformation allows free rotation of all three side chains as well as the amino and carboxyl termini. A different conformational energy calculation (7) predicts a type II'-bend at the Gly^3-Phe^4 position and a strong intramolecular hydrogen bond between the tyrosyl hydroxyl group and the carbonyl group of the third or fourth residue.

The disperity of the experimental and theoretical results summarized above calls for the application of additional experimental techniques to elucidate the conformational characteristics of enkephalins in solution. Absorption spectroscopy and circular dichroism (CD) have been utilized in our experiments to this end. These properties have been measured in the low energy aromatic region (330-250 nm) and in the higher energy aromatic and peptide region (250-190 nm) as functions of temperature, pH, and solvent composition. It is not necessary to invoke the existence of ordered structures in order to rationalize the results. The most straight-forward interpretation is that Met^5-Enk in solution is a flexible molecule which has a large number of conformations accessible of approximately equal energy.

EXPERIMENTAL: Materials. Methionine enkephalin was prepared by a solid phase technique (8). The lyophilized pentapeptide was dissolved in deionized water or 2,2,2-trifluoroethanol (TFE) and, where necessary, the pH was adjusted using HCl or NaOH solutions. The pH of the solutions in the optical cell was measured before and after each spectrum using a Beckman 3500 digital pH meter.

Spectra. CD and absorption spectra were measured using a JASCO J-20 spectropolarimeter which has been modified to measure absorption spectra. All spectra were recorded at least twice, with noisy regions being run up to four times. Those spectra depicted are averages over all runs. Noise levels and spectral slit-widths were as shown. Wavelengths and wavelength differences are accurate to ± 0.5 nm. The latter error is due mainly to the breadth of the lines being measured. All spectra were recorded using solutions whose concentration was 0.5 mM or less. Spectra obeyed Beer's law in the ranges covered.

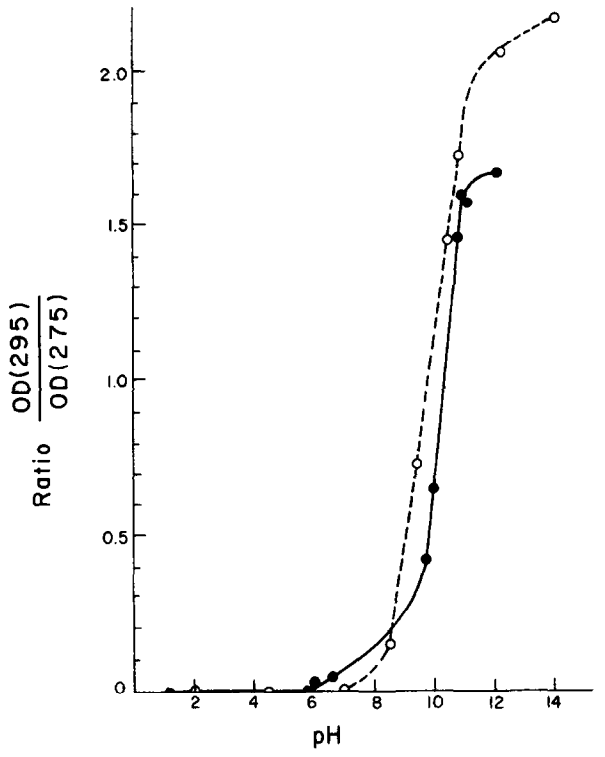
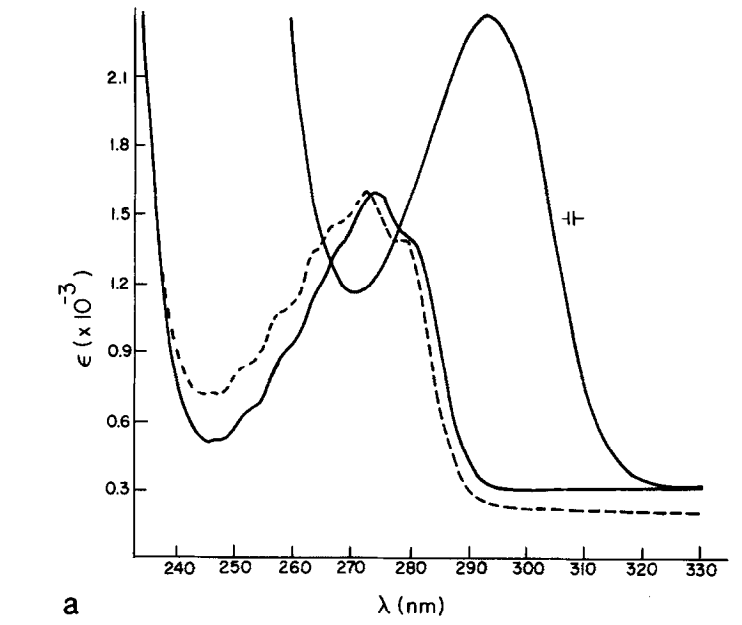


FIGURE 1: Plot of the ratio of absorbance at 295 nm to that at 275 nm in tyrosine ( ) and Met^5-Enk (•) vs. pH.

RESULTS: Absorption. The pK_a of the tyrosyl hydroxyl group was determined by an optical titration. The protonated form of the tyrosyl residue has a peak absorption at about 275 nm, while the deprotonated form absorbs maximally at about 295 nm (9). There is no significant absorption in this spectral region by the phenylalanyl residue (10). Ionization of the terminal amino and carboxyl groups produced no detectable effect on the tyrosyl absorption. Figure 1 depicts the ratio of absorption intensity at 295 nm to that at 275 nm for tyrosine and Met^5-Enk as a function of pH. This method of presentation eliminates any errors due to inaccuracies in concentrations and any absorption by other transitions. The pK_a for tyrosine, taken as the pH of half-height, was found to be 9.9 ± 0.2, which is in agreement with the reported value of 10.07 (11). A pK_a of 10.2 ± 0.2 was found for the tyrosyl hydroxyl group in Met^5-Enk.



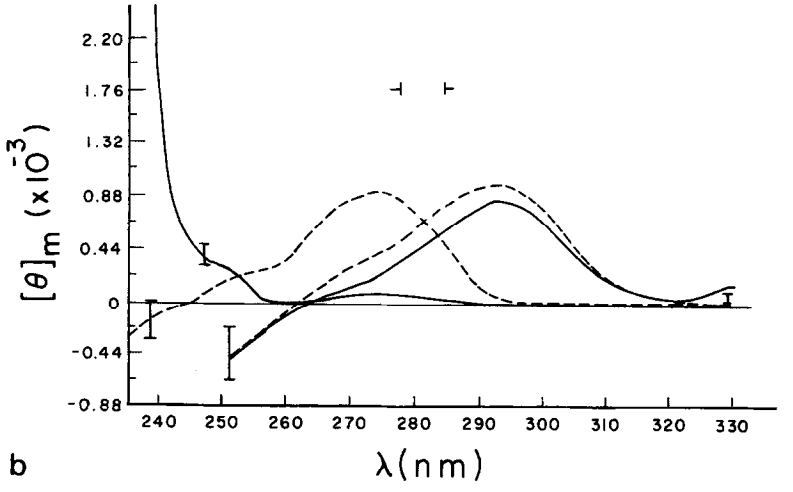


FIGURE 2a: Absorption spectrum of Met^5-Enk in the low energy aromatic region. Solid lines are in aqueous solution pH = 11.5 (peak at 295 nm) and pH = 5.75 (peak at 275 nm). Dashed line is in TFE. The slit-width is shown.

FIGURE 2b: CD spectrum of Met^5-Enk (solid lines) and an equimolar solution of tyrosine and phenylalanine (dashed lines) at pH = 11.5 (peak at 295 nm) and pH = 5.75 (peak at 275 nm). The slit width and noise levels are shown. There is a monotonic increase in noise as the wavelength decreases.

Figure 2a depicts absorption spectra of Met^5-Enk from 240-330 nm in TFE and water at various pH's. An equimolar solution of tyrosine and phenylalanine (Tyr+Phe) exhibits essentially the same absorption as Met^5-Enk at the same pH. On going from TFE to water (pH<6) there is a red shift of 1.4±0.5 nm. A small (2%) decrease in intensity occurs on raising the temperature of a pH 5.75 solution from 5° to 50°C, but the peak absorption wavelength is unaffected.

Circular dichroism. Figure 2b depicts CD spectra for the same spectral region covered by the absorption spectra in Figure 2a. For pH<6 the peak anisotropy (12), the ratio of CD to absorption, in Met^5-Enk is a factor of five smaller than in tyrosine (Tyr+Phe). For a pH near 11.5 the Met^5-Enk anisotropy is decreased by only 25% from that in tyrosine. Anisotropy of Met^5-Enk is the same in TFE as in water at pH<6. The anisotropy of Met^5-Enk at pH 5.75 shows no significant change between 5 and 50°C.

Figure 3 depicts the CD at 190-250 nm for Met^5-Enk, Tyr+Phe, and their difference. Only positive CD is obtained with Met^5-Enk. The CD is unaffected by changes in temperature (5-50°C) and by dropping the pH to zero. The spectrum in TFE is experimentally indistinguishable from that obtained in water at pH 5.75. Noise levels in these spectra are such that only the sign and general shape of the signal have significance.

DISCUSSION: The pK_a for the tyrosyl hydroxyl group in Met^5-Enk is not significantly different from that in tyrosine itself, and is less than the value (10.49) reported for Gly-Tyr (11). Absence of an appreciable decrease in the acidity of the tyrosyl hydroxyl group is incompatible with its participation in a strong intramolecular hydrogen bond (13). The most straight-forward explanation is that the tyrosyl hydroxyl group in Met^5-Enk is interacting with the aqueous environment essentially as it would in the free amino acid. Further support for this contention is provided by the similarity in the absorption spectra of Met^5-Enk and an equimolar mixture of tyrosine and phenylalanine.

Hydrogen bond formation by the phenolic hydroxyl is accompanied by a red shift in the absorption spectrum of tyrosine (14). The observed red shift in

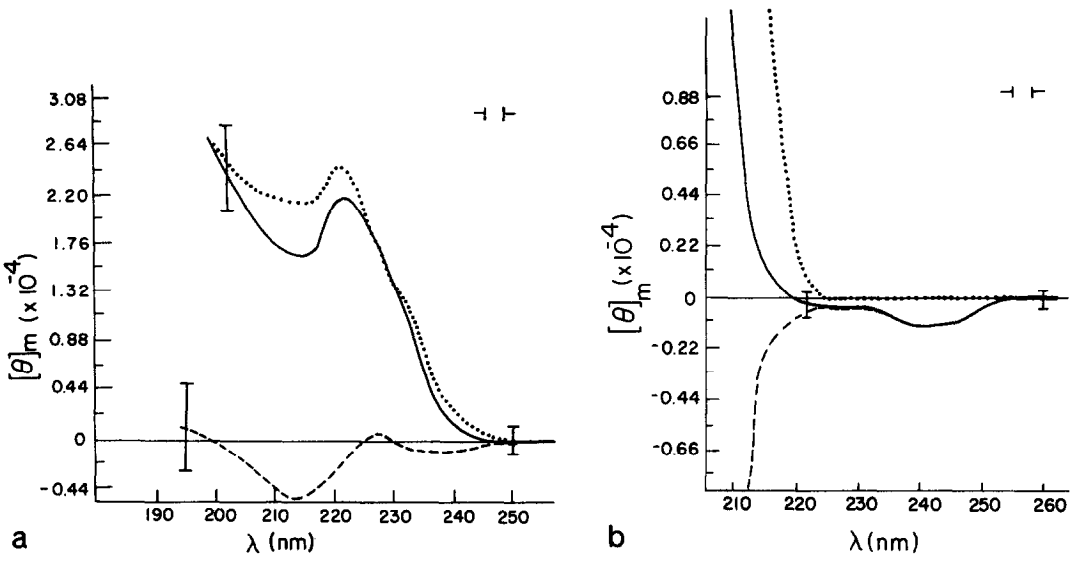


FIGURE 3a: CD spectrum at pH = 5.75 of Met^5-Enk (solid line), and equimolar solution of tyrosine and phenylalamine (dashed line) and their difference (circles) - the corrected peptide spectrum. The slit-width and noise levels are shown. There is a monotonic increase in noise as the wavelength decreases.

FIGURE 3b: Sames as Figure 3a for pH = 11.5

water compared to TFE is the result expected if the tyrosyl group participates in hydrogen bond formation with the solvent in each case, the stronger hydrogen bond being formed in aqueous media. This conclusion is compatible with that attained above.

Spectra should demonstrate a marked temperature dependence if a marginally stable ordered structure were present. Lack of any appreciable temperature effect on the aqueous CD and absorption spectra is consistent with the occupation of either a single exceptionally stable structure or a wide variety of conformations of nearly the same stability. Consistency with the foregoing dictates the latter alternative.

Disordered polypeptides generally exhibit weak CD above 210 nm. In most instances the absolute value of the maximum CD does not exceed about 6000 when expressed per mole of amino acid residue (15). Results reported here for Met^5-

Enk fall within this range. The tendency of the CD to remain positive below 210 nm is puzzling, as is the decrease in anistropy for the low energy tyrosyl transition in Met^5-Enk compared to tyrosine. A possible explanation may lie in a weak coupling of the electronic transitions on the aromatics, arising because the average separation of these two side chains is small. At high pH repulsion of the negatively charged tyrosyl and carboxyl groups would tend to increase the average separation of these groups, leading to the observed normalization of the anisotropy.

The most important feature of the above interpretation is that at no time have we been forced to invoke the presence of an ordered structure. The absence of order in Met^5-Enk may be attributed to its low molecular weight and to the large number of low energy conformations available to the Gly-Gly segment (16). Presumably the conformation which interacts with the biologically important receptor site is one of the many which constitute the ensemble of conformations present in solution.

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