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A Proton Magnetic Resonance Study of the Conformation of Methionine-Enkephalin as a Function of pH†

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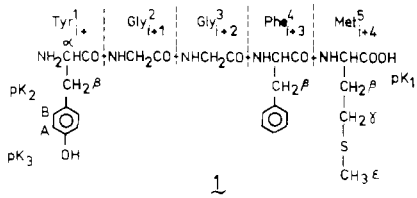
ABSTRACT: It is found that methionine-enkephalin has at least two different conformations in aqueous solution, one at low and one at high pH. From inspection of titration curves and coupling constant values, it seems reasonable to conclude that these conformations are characterized by a folding so as to bring the two ends of the molecule in close proximity. This behavior parallels that found recently in $(CD_3)_2SO$ as the solvent. It follows that the Phe-Met region of the molecule constitutes a relatively rigid region, but that the chain possesses flexibility around the first Gly residue. Possible implications of this behavior with respect to the receptor site are discussed.

It has been reported (Hughes et al., 1975) that two pentapeptides (enkephalins) act as endogenous agonists of opiates in brain. A recent report (Bradbury et al., 1976a) on binding of peptide fragments of lipotropin, which includes the Met-enkephalin (Met-E, **1**),[1] to crude opiate receptor preparations



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has further led to the suggestion that these peptides are the natural substrate for opiate receptor. As a result of the general belief that the conformation of a hormone may be related to its biological activity (Bumpus et al., 1961) and the known activity of opiates, attempts have been made to predict the conformation of Met-E and relate it to the structure and conformation of opiates (Bradbury et al., 1976b; Horn and Rodgers, 1976). The conformation of Met-E proposed by Bradbury et al. (1976b) on the basis of statistical studies (Chou and Fasman, 1974a,b) or theoretical calculations (Lewis et al., 1971) is characterized by a β turn involving the residues Tyr-Gly-Gly-Phe and a hydrogen bond between the CO of Tyr and the NH of Phe. It should be pointed out here that the theoretical calculations used in the above mentioned proposal are based on the x-ray data of proteins and enzymes and thus do not involve solvent effects. Therefore the conformation of Met-E in solution can differ from the one proposed above. However, it will be interesting to see if the conformation determined by x-ray is similar to the one predicted by these calculations or not. Horn and Rodgers (1976) have tried to predict the conformation of the tyrosine portion of enkephalins at the receptor. It is argued that, as opiates are fairly rigid molecules, the conformation obtained by x-ray data can be reasonably assumed to be similar to the one at the receptor and thus a structural and conformational correlation between the opiates and the enkephalins can reflect the conformation of the latter at the receptor. Recently we have advanced evidence (Roques et al., 1976a,b) for a β_I-bend conformation (Lewis et al., 1973) of Met-E, involving the sequence Gly-Gly-Phe-Met and a

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[1] Abbreviations used: Met-E, methionine-enkephalin; (Tyr-Gly-Gly-Phe-Met); $(CD_3)_2SO$, dimethyl-d_6 sulfoxide; NMR, nuclear magnetic resonance; Unc, uncorrected. The nomenclature used in this paper follows the rules suggested by the IUPAC-IUB Commission on Biochemical Nomenclature ((1972), *Biochemistry 11,* 1726). All optically active amino acids are in the L configuration.

hydrogen bond between the NH of Met_{i+4} and the CO of Gly_{i+1} residue, in dimethyl-d_6 sulfoxide ($(CD_3)_2SO$).

It has been suggested earlier, e.g., in the case of the luteinizing hormone releasing hormone (Deslauriers et al., 1975), that oligopeptide hormones may have a different conformation on binding to the receptor from that in the unbound state, the latter being the easiest to study in view of the methodologies involved. Nevertheless, an insight into peptide hormone–receptor interaction may be obtained by studying their relative flexibility, e.g., how sensitive the conformational preferences are to changes of the environment,[2] e.g., solvent, pH, temperature, etc. If the picture obtained can be related to a structure–activity profile, where other acceptor molecules have been used, one might hope to obtain a better recognition of the isosteric and allosteric structural fragments.

We have recently shown (Roques et al., 1976a,b) that the conformation of Met-E in $(CD_3)_2SO$ is a rather well-defined one (or a well-defined mixture of two forms, that is, situated in a relatively deep energy well of the ϕ,Ψ-conformational space) as was concluded from the low sensitivity of the spectral parameters to temperature variations. We have now undertaken an ^1H NMR study of Met-E (**1**) in aqueous medium as a function of pH. Similar studies in terms of conformational changes as a function of pH in peptides have been reported recently, e.g., angiotensin II (Asn^1-Val^5) (Glickson et al., 1973), Tyr-Gly-Gly, and Phe-Gly-Gly (Lala et al., 1976). From the present findings it follows that the behavior in aqueous medium parallels that found previously in $(CD_3)_2SO$ and that mainly two conformations have to be taken into account.

Materials and Methods

Met-E was prepared by liquid-phase synthesis, details of which will be reported elsewhere (Roques et al., 1976b). A 300-MHz Varian HR-300 spectrometer was used (in continuous wave mode), equipped with a SC 8525-2 decoupler unit and variable-temperature unit. The temperature was measured with an ethylene glycol thermometer. Because of solubility problems, we took $(CD_3)_2SO$–H_2O (34:66) as the most appropriate medium to mimic physiological conditions. Thirty milligrams of Met-E was dissolved in 200 μL of $(CD_3)_2SO$, to which 400 μL of H_2O was added. The spectra were taken at 35 °C without addition of a further internal standard and without lock, the peak of the residual partly deuterated dimethyl sulfoxide serving as the internal reference. The latter was found in a separate measurement to be invariant with pH changes or water content and was 2.51 ppm vs. internal Me_4Si. Double-resonance experiments could be performed in the pH 2–8 region, but leakage between the transmitter and receiver coils could not be compensated above this region. The uncorrected pH at dissolution was 5.8, which was then adjusted using a micropipet first with ca. 40% aqueous H_3PO_4 followed by the use of ca. 40% aqueous NaOH (total amount of additional dilution being ca. 50 μL of water). The pH was measured directly in the NMR sample tube with the aid of a micro-glass pD electrode (Ingold, Type HA 405-M3).

It is not possible to use the solvent suppression technique on our apparatus. Therefore, no spectral details could be obtained around δ 4, but we could extract all the H_α signals of the Gly moieties and even of Phe and Met at moderately high pH values (e.g., Phe-H_α at 4.1 ppm (pH 5.8)).

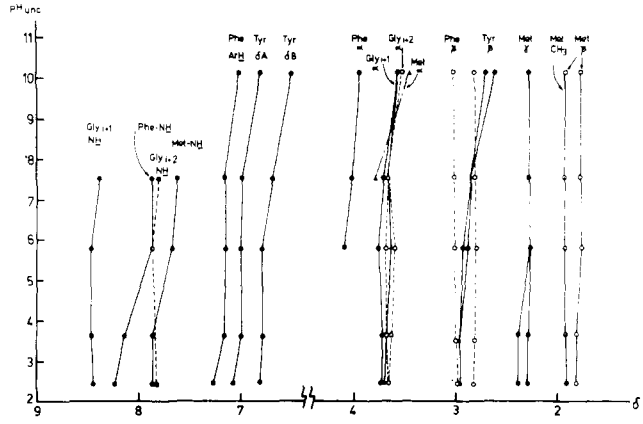

FIGURE 1: Chemical shift variations of proton resonances in Met-E as a function of pH.

Results

Spectral Assignments. The shifts of hydrogen signals obtained as a function of pH are reproduced in Figure 1. Shift data for NH protons above pH_{unc} 7.6 were not traceable because of extensive line broadening, and those of Phe-H_α and Met-H_α could not be obtained below pH 5.8 and 7.5, respectively, as they were buried under the solvent peak. The assignments were achieved by correlation through double irradiation (except at pH_{unc} 10.1), or were straightforward from the multiplet appearance in the spectrum. Thus, the assignment of the Met-NH was obtained by double irradiation experiments performed at pH 7.6, where Met-H_α could be correlated with the identifiable Met-β protons, and the former in turn with the relatively sharp doublet of the Met-NH. Finally, the choice between Met-NH and Phe-NH, and also especially the distinction between the Gly-NH patterns, could be made by close inspection of variation of line widths with pH. If the lifetime associated with the exchange of peptidic NH protons with protons of water becomes comparable to their mutual chemical shifts difference, the NH signals broaden out and disappear in the noise of the spectrum. Broadening of NH signals (and disappearance of accompanying coupling constants) is observed on heating or raising pH, the extent of the effect being related to the position of the amino residue in the peptide backbone (Berger et al., 1959; cf. Glickson et al., 1973). Thus the peptidic NH signal of a second residue (here Gly_{i+1}) is the first to disappear on raising the pH, while the NH signal of the last residue (Met_{i+4}) only broadens out at relatively high pH (ca. 10). Figure 2 shows the expected order of line broadening, allowing through concomitant and consecutive double irradiation experiments the assignments of the remaining patterns in the spectrum.

Titration Curves. It is noticeable that shift displacements toward higher fields around pK_1(COOH) are only appreciable for the Phe-NH and Met-NH, though somewhat less pronounced upfield shifts are also observed for Tyr-H_β (especially β_B) and Met-γ_A protons.[3] The upfield shifts of Met-NH occur as a result of its proximity to the site of ionization. Similar shift displacements around pK_2 (NH_2) and pK_3 (phenolic), as ex-

[2] This approach, which in fact is a search for the steepness of a conformational energy profile, has also been recently followed in theoretical calculations where a computer search for energy minima is made using ϕ, Ψ input data, deliberately chosen somewhat different from what might be expected (Tanaka and Scheraga, 1976). In following the rapidity of convergence and the detection of several minima during that process, one gets an idea of the flexibility of a molecule, e.g., the adaptability that it may have when put in an interacting medium.

[3] The subscripts A and B denote the proton of a CH_2 grouping found at respectively low and high field.

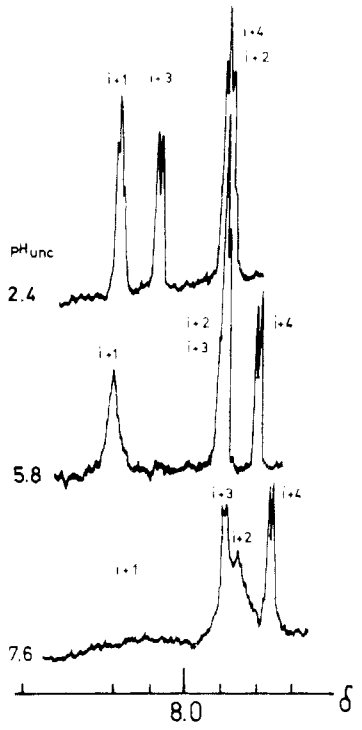

FIGURE 2: Specific line broadening of peptidic protons with variable pH allowing assignments to specific residues in Met-E.

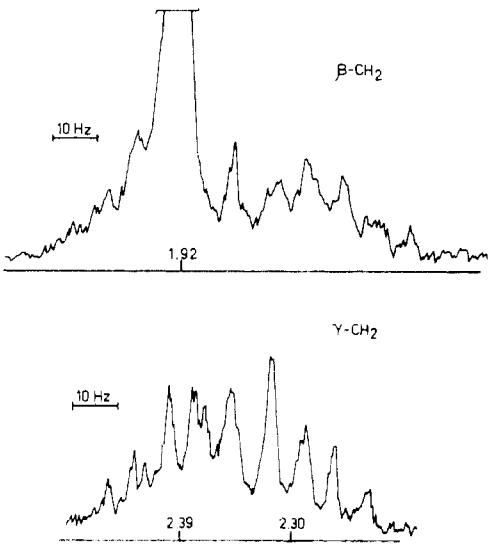

FIGURE 3: The β-CH_2 and γ-CH_2 proton resonances of Met in Met-E at pH_{unc} 2.45.

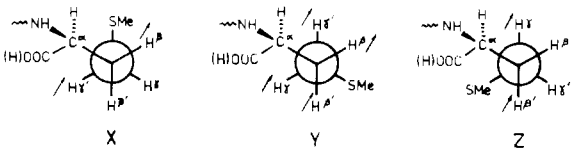

FIGURE 4: Newman projections around the C_β–C_γ bond for the three rotamers of Met in Met-E.

pected, are observed for Tyr-H_β and Tyr aromatic protons, but rather unexpected upfield shifts are also observed for Phe-H_α and Phe aromatic protons and quite interestingly also for the Met-α proton. We will come back to these features in the Discussion section.

Coupling Constants. The interproton coupling constants around the peptide backbone ($^3J(NH, C\text{-}\alpha H)$) were extracted from the NH and the Gly-CH_2 signals. These coupling constants allow the computation of some ϕ parameters (Cung et al., 1974). It is stressed here that $^3J(NH, C\text{-}\alpha H)$'s are not obtainable under conditions of rapid exchange of NH protons (cf. Roberts and Jardetzky, 1970). Thus the splittings in the H_α patterns due to coupling with the peptidic protons were not observable above the specific pH values where these NH protons broadened out (H_{α_i} above pH ~1; $H_{\alpha_{i+1}}$ above pH ~6; $H_{\alpha_{i+2}}$ above pH ~7.6 and the others above pH ~10). The side chain coupling constants ($^3J(C\text{-}\alpha H, C\text{-}\beta H)$) for the aromatic side chains were extracted from the Tyr-β and Phe-β proton signals. From these coupling constants the rotamer populations around the C_α–C_β bonds were calculated (Pachler, 1964). These data are given in Table I. For Met, $^3J(C\text{-}\alpha H, C\text{-}\beta H)$'s were extracted from the Met-H_α pattern only at pH 7.6, under which conditions it is not buried under the solvent peak. The observed value of 6.4 Hz indicates free rotation around the C_α–C_β bond of Met. Unfortunately, the spin system displayed by Met-H_β and -H_γ is poorly resolved and thus $^3J(C\text{-}\beta H, C\text{-}\gamma H)$ could not be extracted. This problem is further complicated by the fact that H_{β_A}[3] remains buried under the *S*-methyl signal in the entire pH range and that the γ protons are isochronous and thus appear as a triplet with apparent coupling constants of 7.5 Hz. Some information could be extracted at lower pH. At pH 2–4, the γ protons display shift nonequivalence (anisochronism) (Figure 3) and thus the following $^3J(C\text{-}\beta H, C\text{-}\gamma H)$ values[3] could be extracted, $^3J(\gamma_A, \beta_A)$ = 5.4, $^3J(\gamma_A, \beta_B)$ = 8.6, $^3J(\gamma_B, \beta_A)$ = 7.8, and $^3J(\alpha_B, \beta_B)$ = 7.2 Hz. From the predicted ^3J values of the three rotamers around C_β–C_γ bond (Figure 4 and Table II), it is clear that rotamer *y* has a higher contribution in the side chain rotation, as three out of the four experimental ^3J values are large. We do not expect rotamer *z* to have a high population for steric reasons, because model considerations of a folded backbone (see Discussion) with a head-to-tail interaction indicate strong steric interactions with the head (amino terminal) of Met-E. Nevertheless rotamer *z* has a contribution, though small, as illustrated by the fact that the smallest experimental coupling is 5.4 Hz, which can only result from a mixture of all the three rotamers. Further from the predicted and experimental ^3J values in Table II, four equations involving three variables $|x|$, $|y|$, and $|z|$, each representing the population of the corresponding rotamer, could be written. Thus for $|x| + |y| + |z| = 1$, the solution for these equations gave $|x| < 0.4$, $|y| > 0.5$ and $|z| \sim 0.1$. It is possible that rotational behavior of the Met side chain around C_β–C_γ remains unchanged in the entire pH range (2.45–10.10), as the mean value of $^3J(\beta, \gamma)$ extracted each time is 7.5 Hz. Thus the information on rotamer population of the Met side chain derived at pH 2.45 is likely to be similar around physiological pH.

Discussion

The pH dependences of the location of proton signals are not always reliable for the identification of the residue to which they belong, because they seem to be sensitive to both ionic and conformational equilibria (Glickson et al., 1973; Lala et al., 1976) and perturbations are found for resonances of protons both near to and remote from the ionization site. On the other hand conformational changes may be indicated if shifts behave differently from what might be expected from the location of the proton with respect to the titration site. It is, however, difficult to decide whether such conformational changes are at the origin of an anomalous shift behavior or if a proximity

TABLE I: Coupling Constants[a] in Hertz and Extracted Conformational Parameters as a Function of pH[b] in Met-Enkephalin (in $(CD_3)_2SO$–H_2O, 65:35 at 30 °C).

	Tyr_i		Gly_{i+1}			Gly_{i+2}			Phe_{i+3}				Met_{i+4}			
		^3J		^3J (NH, C$^\alpha$H)			^3J (NH, C$^\alpha$H)			^3J (NH, C$^\alpha$H)			^3J (NH, C$^\alpha$H)		^3J	
pH_{unc}[c]	$^2J\beta$	(α, β)[c]	^2J		Φ^i (deg)	^2J		Φ^i	$^2J\beta$		Φ	(α, β)[c]		Φ	(α, β)	$^3J(\beta, \gamma)$
2.45		$\overline{6.5}$	17.0	5.6 & 6.0	>60 or <−60	17.0	6.0 & 6.0	>60 or <−60	13.8	7.5	−80 or −150	6.3 & 8.5[f]	>7.5	−80 or −150		8.6,5.4, 7.2,7.8
3.65		$\overline{6.5}$	17.0	6.0 & 6.0	>60 or <−60	17.1	6.0 & 6.0	>60 or <−60	13.8	7.5	−80 or −150	5.4 & 9.2[g]	8.0			7.5
5.8	15.0	6 & 7.0[d]	17.0	<2	180	17.0	5.5 & 6.0	>60 or <−60	14.0	7.5	−80 or −150	5.0 & 9.0[h]	7.5	−80 or −150		$\overline{7.5}$
7.6		>7.0	17.0	NO			Ca. 6.0[k]	>60 or <−60	13.5	7.5	−80 or −150	5.0 & 9.0[h]	7.5	−80 or −150	6.4	$\overline{7.5}$
10.1	13.7	6.9 & 7.3[e]	17.0	NO			NO		14.0	NO		5.0 & 9.2[i]	NO			$\overline{7.5}$

[a] $\overline{J}$ values designate mean values as extracted from degenerated patterns; NO, not observed; estimated accuracy ±0.1 Hz. [b] pH were measured with a pD electrode, and the reported values are uncorrected. Calibration against buffer solutions in $(CD_3)_2SO$-d_6–H_2O indicates the following conversion: pH = pH_{unc} − 0.38. [c] Rotameric populations around χ_1 are a, b, and c, given respectively in footnotes *d*–*i*. [d] 0.310, 0.401, 0.289. [e] 0.392, 0.428, 0.180. [f] 0.337, 0.538, 0.124. [g] 0.255, 0.602, 0.143. [h] 0.218, 0.583, 0.199. [i] 0.218, 0.602, 0.180. The rotamers a, b, and c are defined as having the C^β–C_{ar} bond antiperiplanar with respectively (a) the C′=O, (b) the NH, and (c) the C^α–H bonds. Conformations a and b may be interchanged, and a correct choice is not possible because of degeneracy. *i* > and < correspond to range of about 5°. [k] Approximate values only, as the result of overlap with $H\alpha_{i+1}$ and some broadening.

TABLE II: The Predicted Coupling Constants (^3J) for β-CH_2 and γ-CH_2 Protons in Rotamers *x*, *y*, and *z* (Figure 4) and the Experimental Coupling Constants of Met in Met-E.[a]

Predicted	*x*	*y*	*z*	Experimental	
$J(\beta, \gamma)$	3.5/4.0	~12	<3.5/4[b]	$J(\gamma_A, \beta_B)$	8.6
$J(\beta', \gamma)$	<3.5/4.0[b]	3.5/4	~12	$J(\gamma_A, \beta_B)$	5.4
$J(\beta, \gamma')$	~12	3.5/4	<3.5/4[b]	$J(\gamma_B, \beta_B)$	7.2
$J(\beta', \gamma')$	<3.5/4.0[b]	~12	3.5/4	$J(\beta_A, \gamma_B)$	7.8

[a] It is noteworthy that an upfield shift is expected for a hydrogen atom which is flanked by a gauche substituent. Therefore the computed populations do take account of the observed signal locations of H-β and H-γ, as experimentally found. The partners that would be subjected to such an upfield shift are marked with an arrow in the representation in Figure 4, and, if rotamers *x* and *y* are the preponderent ones, indeed H-γ' and H-β, identified as respectively H-γ_B and H-β_B, corroborate these suggestions. [b] Lowered because of the presence of an antiperiplanar negative group.

effect is in operation, a proximity which is not necessarily constitutional in nature but can also be conformational. We can, however, try to rationalize the shift results with the sometimes pronounced changes in coupling constants. Thus, it has already been pointed out that some couplings are found to change around pK_1 (χ_1-Phe) and pK_2 (χ_1-Tyr). Further, upon going from the cationic to the anionic species there is a remarkable change for ϕ_{i+1} from +60° (or −60°) to an apparent value of 180°. It is noteworthy, therefore, that, although the major conformational alterations undoubtedly involve ϕ (and probably Ψ) of Gly_{i+1}, and although rotameric changes along C_α–C_β of Phe and Tyr occur at low and high pH, respectively, this is not revealed by shift changes of any hydrogen belonging to the Gly_{i+1} residue. Is there any fortuitous compensation for alterations in field gradient contributions (that also would change appreciably in the same region) or even concomitant shift compensations arising from changes in Ψ? We think this is the case. The sensitivity of Met-H_α to NH_2 titration and Tyr-H_β to COOH titration (at the other end of the backbone) discloses a head-to-tail proximity, e.g., a folded conformation. Furthermore, the change of ϕ_{i+1} in shifting from the cationic to the anionic species should be accompanied by changes in Ψ_{i+1} to preserve the folding.[4] A further choice of a specific conformation requires a knowledge of Ψ values. Without recourse to special methods (such as the measurements of $^3J(^{15}N_{i+1}, C^\alpha H_i)$ in ^{15}N-enriched samples), a good guess can be made from considerations of allowed minima conformations out of a Φ, Ψ steric map (Goodman et al., 1970). The Φ_{i+1} = 180° observed at pH 5.8 deserves special comments. This is a disallowed region of the Φ, Ψ conformational map and we interpret this as an apparent value, e.g., a weighted mean of the value observed at pH <5.8 (Φ_{i+1} = ±60°) and another of opposite sign, hence Φ_{i+1} = ∓90°. In principle therefore (Table I) only two Φ values around each of the four residues remain, together with the corresponding two Ψ values corresponding to nearby minima in the steric map, thus resulting in 2^4 = 16 possible forms left as possibilities for the conformation of Met-E. Some of these possibilities are easily ruled out. Any combination for which Φ_{i+4} value would have an opposite sign to Φ_{i+3} would bring the Phe_{i+3} side chain in a quasi-axial position. A sequence for Φ_{i+1} to Φ_{i+4} of −60, −60, −90, −90° (or the same set of reverse sign) would bring all the C=O dipoles in the same direction (a distorted α-helix-like structure with C=O more or less parallel to its axis) and can thus also be rejected. Together with model building it is very probable that a folded structure would have the following Φ, Ψ sets (values between brackets corresponding to the second form observed at pH 5.8 and above): $\Phi_{i+1} \rightarrow \Phi_{i+4}$ = −60° (+90°), < −60°, ca. −80°, and −140°, and $\Psi_{i+1} \rightarrow \Psi_{i+4}$ = ca. −30° (+40°), small, −60°, or 120 ± 40°. These are folded structures, almost identical with those observed in $(CD_3)_2SO$ (Roques et al., 1976b) and close to a β_I-bend type (Lewis et al., 1973). Further, the rotational behavior of the side chains of Met, Phe, and Tyr is interesting since they may be involved with different binding sites on the receptor. The Phe side chain occupies a somewhat more extreme rotameric distribution than does the side chain of Tyr (especially at pH

[4] It is noteworthy that the *J* values extracted for Phe and Met (~7.5 Hz) make the occurrence of a random coil conformation less likely, for which a lower value, e.g., 6.1 Hz, has been advanced (Tonelli and Bovey, 1970; Glickson et al., 1973).

<10). Therefore, the former seems to be more constrained, e.g., in pointing preferentially away from the folded backbone of Met-E (Table I). Also the side chain of Met seems to be pointing away from the backbone though there is freedom of rotation around χ_1. This interpretation is based on the observation that *S*-methyl does not show any change in its shift value on pH variation (Figure 1) which suggests that this group is not proximal to either ionizable sites.

The fact that similar conformations for the peptidic backbone of Met-E are found in $(CD_3)_2SO$ and in aqueous media at different pH led us to believe that the observed features in both media can serve as a safe model for the final conformation of the endogenous opiate at the receptor.

Thus the relative flexibility around Tyr-Gly_{i+1} and the rigidity around Gly_{i+2}-Phe-Met in Met-E suggests that, if the tyrosine portion is involved in the primary binding site on the receptor, then Phe and Met side chains would be involved in secondary binding sites. The much lower binding of Tyr-Gly-Gly-Phe compared with Met-E recently reported by Bradbury et al. (1976a,b) indeed reflects the importance of secondary binding sites, which is not possible for opiates.

We think that for the final conformation of Met-E at the receptor, the following features that were disclosed (see also Roques et al., 1976a,b) are of major importance: (a) there is a relatively stiff region in the Gly_{i+2}-Phe-Met fragment; (b) that Met-E possesses a possibility for tailoring its shape through a conformationally flexible moiety located around at least one of the Gly residues.

One might wonder if the succession of Gly residues in peptides in general is not aimed to give these an intrinsic flexibility, e.g., for tailoring purposes. This possibility comes also out of earlier investigations (Lewis et al., 1971) showing that Gly has a high probability of occurrence at more than one position of bends (e.g., $i + 1$, $i + 2$, and $i + 3$). One should also recognize that Gly residues are the strongest potential helix breakers known (Chou and Fasman, 1974b). Otherwise stated, a succession of two or more Gly residues gives peptides enough adaptability as to allow them to function as hand-to-glove inducers. We believe that the successful preparation (Goldstein et al., 1975) of the endorphinic peptide Tyr-$(Gly)_3$-Lys-Met-Gly illustrates some major features necessary for opiate reception as presented in this contribution. The heptapeptide contains the relatively small opioid region constituted by H_3N^+-Tyr and possesses moreover repeated Gly residues in order to tailor this substrate to the active site. The choice of a third Gly residue (allowing the easy adoption of the needed Φ, Ψ values for binding) may also be important for the action of the opiate, as it seems necessary to have a well-defined succession of Φ, Ψ values at the $i + 2$ to $i + 4$th position.

It would be a step forward in the understanding of acceptor–receptor interactions between peptide fragments to have spectral data of such peptidic substrates at our disposal and to relate their conformational behavior in *varying* media, as we have been able to do now with Met-E in H_2O and H_2O–$(CD_3)_2SO$ mixture. The assignments in the latter solvent mixture can be extrapolated to those in the aqueous solution (Wessels et al., 1973).

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